Development of microsatellite markers for black pepper (*Piper nigrum* L.) and related species

Thesis submitted to

University of Calicut

For the award of the degree of

Doctor of Philosophy

(Biotechnology)

By

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2015

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CERTIFICATE

This is to certify that the thesis entitled **"Development of microsatellite markers in black pepper (*Piper nigrum* L.) and related species"** submitted to the University of Calicut by Ms. K. Anupama at the ICAR-Indian Institute of Spices Research, Kozhikode for the award of the degree of **Doctor of Philosophy** in **Biotechnology** is a bonafide record of research work carried by her under my supervision and guidance. No part of the work has formed the basis for the award of any other degree or diploma previously. All sources of helps received by her during the course of the study have been duly acknowledged.

Date: 25th May 2015
Place: Kozhikode

(K. Nirmal Babu)

पी ए बी एक्स PABX: 0495-2731410/2731753/2731345 निदेशक का कार्यालय Director's Office: 0495-2730294 परियोजना समन्वयक Project Coordinator: 0495-2731794, एटिक ATIC: 0495 - 2730704. आई आई एस आर प्रयोगिक प्रक्षेत्र, पेरुवण्णामुषि ISSR Experimental Farm, Peruvannamuzhi: 0496 2249371 कृषि विज्ञान केन्द्र Krishi Vigyan Kendra, पेरुवण्णामुषि Peruvannamuzhi: 0496-2666041, तार Grams: RESEARCH CALICUT. फैक्स Fax: 0091-495-2731187 ई-मेल Email: mail@spices.res.in वेबसाईट Website: www.spices.res.in

DECLARATION

I hereby declare that the thesis entitled **"Development of microsatellite markers for black pepper (*Piper nigrum* L.) and related species"** submitted by me for the award of the degree of **Doctor of Philosophy** in **Biotechnology** at Calicut University, is a bonafide record of research work carried out at ICAR- Indian Institute of Spices Research, Kozhikode under the supervision and guidance of Dr. K. Nirmal Babu, Project coordinator, AICRP on Spices, ICAR- Indian Institute of Spices Research, Kozhikode, Kerala. This thesis or part of it has not been submitted to any other university for the award of other degree or diploma previously. All sources of help received by me during the course of this study have been duly acknowledged.

Kozhikode

25-05-2015

Anupama K.

ACKNOWLEDGEMENTS

Genesis of this thesis would not have been possible without the guidance and help of several individuals who in one way or another contributed and extended their valuable assistance. It is a pleasure to convey my gratitude to them in my humble acknowledgement.

I am grateful to Dr. M. Anandaraj, Director, ICAR-Indian Institute of Spices Research, Kozhikode, for his encouragement and support during the entire research work.

I am grateful to Dr. V. A. Parthasarathy, former Director, who gave the permission to conduct the research at IISR and his guidance.

It is with immense pleasure and gratitude, I thank my supervising teacher, Dr. K. Nirmal Babu, Project coordinator, All India Co-ordinated Research Project on Spices (AICRPS), Indian Institute of Spices Research, Kozhikode for his constant support and advices.

I express my sincere thanks to Dr. B. Sasikumar, Head, Division of Crop Improvement and Biotechnology, ICAR-Indian Institute of Spices Research, Kozhikode for his valuable suggestions and constructive criticisms.

I take this opportunity to thank Mr. B. Krishnamoorthy, then the Head (In charge),Division of Crop Improvement and Biotechnology, ICAR-Indian Institute of Spices Research, Kozhikode for all his encouraging words, affection and suggestions.

I express my profound gratefulness to Dr. K. V. Saji for his timely help, advices and support.

My heartfelt thanks to Dr. Santhosh J. Eapen and DISC, IISR, Kozhikode for their timely help rendered during the research tenure.

I am grateful to Dr. P. R. Manish Kumar, Head, Department of Biotechnology, University of Calicut, who has given insights in to the fundamentals of molecular biology.

I express my special thanks to Mr. K. Sudhakaran, Artist &Photographer, IISR, Kozhikode, for his artistic help in photography, cover page design and preparation of gel plates for my research work.

I place on record my sincere thanks to Scientists of the Crop Improvement and Biotechnology Division, IISR Dr. J. Rema, Dr. Johnson K. George, Dr. R. R. Nair, Dr. D. Prasath, Dr. P Rajeev, Dr. Lijo Thomas, Ms. P. Umadevi and Ms. S. Aarathi for their help and suggestions.

I would like to extend my sincere thanks to all the staff members at HRD, PME, library, Accounts & Administration for their help during my stay at IISR, Kozhikode.

I am grateful to Mr. K. P. Premachandran, Technical staff, Experimental farm, Peruvannamuzhi for his help in collecting samples.

I also express my sincere thanks to Director of Research, the staff at DOR and CHMK Library, University of Calicut for their help and cooperation.

I have been blessed with a cheerful group of fellow lab mates. I would like to express my heartiest thanks to Mrs. Cissin Jose, Ms. E. J. Suraby, Mr. Rahul P. Raj, Mrs. Anu Cyriac for being the strong pillars over the years. Their unselfish cooperation and much needed

involvements have helped me to complete this task. My thesis would not have been possible without their helps. Also Ms. Thankam, Mr. Venkitesh and Mrs. Prameela lab staffs for their daily helps and constant support.

Words fall me to express my deepest appreciation to Dr. Indu S. Nair for being a great friend, constant source of support and inspiration.

My heartfelt thanks to all other research scholars and friends at IISR, especially, Dr. Y. K. Bini, Ms. D. Sruthi, Ms. P. B. Krishna, Ms. Blessy M. Baby, Mr. M. Deepesh, Mrs. Neema Malik, Mr. K. P. Sayuj, Mr. Ritto Paul for their helps rendered during the course of this research.

I extend my sincere thanks to all my friends at Crop Improvement and Biotechnology and Biocontrol department, IISR for providing a good friendly working atmosphere during the tenure of my doctoral research.

I express my gratitude to all the members of Project Coordinator's cell, Mrs. Alice Thomas, Ms K. M Soumya., Ms. H. J Akshita., Mrs. C. N. Divya. and Mr. K. Chandran Nair for their love and cooperation.

I acknowledge my deep gratitude and indebtedness to my parents and siblings for their blessings, encouragement and unceasing love and affection throughout my career. Words cannot express how grateful I am to my in-laws for all their love, patience and understanding.

I am thankful to all other staff members of the Indian Institute of Spices Research, Kozhikode for their help and affection and to all my friends and well wishers whom I may not have mentioned here but have always been helpful at appropriate time.

I owe my sincere thanks to my dear husband Mr. Suresh Kumar K. K. for abiding love, support and understanding which underpin my diligence overcoming all the vicissitudes to complete this doctoral degree. I thank my sweetie pie Arpitha for being such a bundle of joy and light of my life.

My special thanks are due to ***Council of Scientific and Industrial Research (CSIR)****, New Delhi, for providing the much needed financial and administrative support during my doctoral research.*

Finally I thank the Almighty for seeing me, not only through years of relentless effort to acquire acknowledge, but also a frantic race against time and resources. Thus far the lord has brought me and I am grateful for the strength and blessings showered on me for the fruitful completion of this endeavor.

Anupama K.


To my family...

CONTENTS

Page No.

List of Tables

List of Figures

Abbreviations

AFLP	Amplified fragment length polymorphism
ARMS	Amplified refractory mutation system
Ta	Annealing temperature
~	Approximately
Bp	Base pair
BLAST	Basic local alignment search tool
CTAB	Cetyltrimethyl ammonium bromide
cDNA	Complementary deoxy ribonucleic acid
^{0}C	Degree Celsius
dNTP	Deoxy nucleotide triphosphate
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed sequence tag
GI	Gen Info Identifier
g	Gram
h	Hour
IISR	Indian Institute of Spices Research
ISSR	Inter simple sequence repeats
IPTG	Iso propyl β-D- thiogalactopyranoside
Kb	Kilobase
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
mm	Millimolar
min	Minute
M	Molar
ng	Nanogram
NCBI	National Centre for Biotechnology Information
OD	Optical density
ml^{-1}	Per milliliter
%	Percentage
pm	Picomoles
PAGE	Poly acrylamide gel electrophoresis
PVP	Poly vinyl pyrrolidone
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
rpm	Rotation per minute
sec	Seconds
SCAR	Sequence characterized amplified regions
SRAP	Sequence related amplified polymorphism
SAHN	Sequential, agglomerative, hierarchical and nested clustering
STR	Short tandem repeats

SSR	Simple sequence repeats
SNP	Single nucleotide polymorphism
SDS	Sodium dodecyl sulphate
spp	Species
TEMED	Tetramethyl-ethylene diamine
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
UV	Ultra violet
U	Unit
UPGMA	Unweighted pair group method of arithmetic mean
X-gal	5-bromo-4-chloro-3-beta-D-galactopyranoside

INTRODUCTION

Chapter 1

INTRODUCTION

Most agricultural and food production systems relied on the utilization of locally adapted plant and animal species. Such plant or animal genetic resources comprises species that are of cultural, agricultural and economic importance to man. A plant species of such importance as a spice in agriculture is black pepper.

Black pepper, the “King of Spices”, is one of the most important and widely used spice in the world. The black pepper of commerce is the dried ripened berries of *Piper nigrum* L. Black pepper is a woody climber, grown commercially in the South Western region of India, comprising of the states of Kerala, Karnataka, Tamil Nadu and Goa. The humid tropical evergreen forests bordering the Malabar Coast, the Western Ghats, is the centre of origin and diversity for the black pepper. The Malabar Coast was involved in the cultivation and trade of black pepper since ancient times and the plants were introduced to Indonesia, Malaysia and subsequently to other pepper growing countries from the region. Presently pepper is grown in more than twenty six countries.

Black pepper, with its innate pungency and flavor, is an essential and inevitable constituent in many cuisines, and the singular one which is served unfailingly at the dining table. It was being in use for different purposes in the past, and continues to be so currently and will remain so in future as well. It was an important spice and food ingredient for the west; an essential part of the embalming mixture for ancient Egyptians; a beneficial drug for the ancient Aryans, and for the

Indians it was a spice as well as a medicine which is a fundamental component of many traditional *Ayurvedic* drugs. Stories on pepper richly coloured with imagination were carried by ancient sailors to distant places and its fame reached both Western and Eastern lands.

Piper nigrum L. belongs to the family Piperaceae of the Series Microembryeae of monoclamidae (Bentham and Hooker, 1880).The genus *Piper* is broadly distributed in the tropical and sub tropical regions of the world. The main centers of distribution are Central and South America and South Asia (Trelease and Yuncker, 1950). There are other economically important species excluding black pepper *i.e.,* Indian long pepper (*Piper longum* L.), betel vine (*P. betle* L.), Java long pepper (*P. chaba* Hunter), Tailed pepper (*P. cubeba* L.), Kawa pepper (*P. methysticum* Forster), West African pepper (*P. clusim* C.D.C.), Benin pepper (*P. guineense* Schum. & Thonn.) and Japanese pepper (*P. kadzura* (Choisy) Ohwi.). Of this black pepper, long pepper and betel vine are the three economically important *Piper* species grown in India.

The genus *Piper*, established by Linnaeus (1753) in his "*Species plantarum"*, recognized seventeen *Piper* species. The genus *Piper* is characterized by small highly reduced flowers packed closely to form spikes. The male flowers contain 2-3 anthers subtended by a bract and the female flowers are represented by the naked ovary. More than 6000 *Piper* binomials are recorded by International Plant Name Index (IPNI) of which 119 are of Indian origin (Saji, 2006). Eighteen species are found to be present in sub mountainous tracts of Western Ghats and nearest peninsular and coastal regions (Hooker, 1886, de Candolle, 1912, Rama Rao, 1914, Gamble, 1925, Ravindran *et al*., 1987, Velayudhan and Amalraj, 1992, Nirmal Babu *et al*., 1993 and Tyagi *et al*., 2004).

The Indian *Piper* species are unisexual plants, but the Central and South American species are mainly bisexual. But the cultivated black pepper is of bisexual types. Over 100 cultivar of black pepper are in India, 20 of them are generally cultivated in the major pepper growing areas. Cytological studies has shown that the cultivated black pepper is having chromosome number of 2n (4X) = 52, X=13 (Mathew, 1958; 1972) while 2n=104 was noted in wild types. Black pepper and its related taxa are either tetraploids or polyploids and the polyploidy has played significant role in the speciation of South Indian *Piper* (Mathew, 1958; Rahiman, 1981).

Though black pepper is the most valuable economically important spice crop and the best known agricultural product in the genus *Piper*, little is known about its exact distribution, botany, taxonomy and evolution. The earlier works in this area are of Rheede (1688), Miquel (1843), de Condolle (1869), Hooker (1886), Rama Rao (1914), Gamble (1925), Fyson (1932), Kanjilal *et al*. (1940), Manilal (1988), Rahiman (1981), Ravindran (1991) and Mathew (1998).

India has one of the largest diversity of *Piper* and a world collection of 3500 accessions of black pepper cultivars and related species which are maintained at Indian Institute of Spices Research, Kozhikode. Appropriate identification and characterization of plant materials is essential for the successful conservation of plant genetic resources and to ensure their sustainable use. The improvement of crop genetic resources is dependent on continuous infusions of wild relatives, traditional varieties and the use of modern breeding techniques. All these processes require an assessment of diversity to select genotypes resistant to biotic and abiotic stresses, high in quality and productivity.

Genetic diversity may be gauged using morphological, biochemical and molecular characterization. In Black pepper the role of phylogeny is particularly significant, as major breakthroughs in varietal evolution have been achieved through the conventional breeding. However due to reduced floral structures morphological characterization is not fully effective in genetic diversity analysis of *Piper*. In the absence of discernable phenotypic characters molecular markers become a powerful tool for estimating genetic diversity within a species, and to determine genetic relationships between individuals and populations. Molecular markers are utilized in both basic (*i.e*. phylogenetic analysis and search for useful genes) and applied research (*i.e.* marker assisted selection). A vast array of DNA based molecular markers has been discovered since 1980 and newer marker types are developed every year.

Among the molecular markers, microsatellites or simple sequence repeats (SSRs) have emerged as the most widely used and versatile marker type employed for many applications including genome mapping, phylogenetics and population genetics studies (Goldstein and Schlotterer, 1999). Microsatellites are tandem repeats of one to six nucleotide long DNA motifs (Thiel *et al*., 2003), characterized by the relative abundance, hypervariable nature, locus specificity, codominance, and multiallelic nature (Powell *et al*., 1996). SSRs are ubiquitous in the coding and non-coding regions (Tautz and Renz., 1984; Toth *et al*., 2000) of prokaryotes and eukaryotes. High degree of allelic variation revealed by microsatellite markers results from variation in number of repeats at a locus caused by replication slippage or unequal crossing over during meiosis.

Methods have been developed for isolating microsatellites from organism with abundant microsatellite loci (Tautz, 1989, Weber and May, 1989) and organisms with

less abundant microsatellite loci (Ostrander *et al*., 1992, Armour *et al*., 1994, Kandpal *et al*., 1994, Hamilton *et al*., 1999, Glenn and Schable, 2005). Microsatellites can be developed directly from genomic DNA libraries or from libraries enriched for microsatellites. The development of SSR markers for a new species is time consuming and labour intensive (Zane *et al*., 2002; Squirrell *et al*., 2003). However, once developed, these markers provide endless high- throughput applications in molecular breeding by providing accurate, cost effective and reliable genotyping. Additionally, microsatellites can also be identified by searching public databases such as GenBank with the advent of bioinformatics tools. ESTs (Expressed Sequence Tag) can be obtained through partial random sequencing of cDNA libraries, are 300-500 nucleotide long single pass mRNA sequences (Adams *et al*., 1991), representing a snapshot of genes expressed in a given tissue. Expressed Sequence Tags of many plant species has been generated and a large number of microsatellites have been detected using bioinformatics tools leading to the development of EST-SSR or genic SSR Markers. These EST derived microsatellites are having several advantages; easy identification by electronic sorting, present in the expressed portion of the genome, cost effective and quick approach, and has easy transferability across a number of related species (Varshney *et al*., 2005).

The microsatellite markers developed in one species could effectively transfer among closely related taxa, which is commonly known as transferability or cross species amplification, has been successfully demonstrated in many crop species (Varshney *et al*., 2007; Oliveira *et al*., 2013). Both genic (EST SSRs) and genomic SSR markers can be transferred across species. However transferability depends on the extent of conservation in the nucleotide sequences flanking the microsatellite loci (Schlotterer and Pemberton, 1994; Zane *et al*., 2002) and the stability of these

sequences during evolution (Decroocq *et al*., 2003). Transfer of microsatellite primers can offer an alternative to the *de novo* development of SSR markers in plant species (Peakall *et al*., 1998), reducing the cost of development and opening new perspectives for population genetic studies.

There are quite a few reports on molecular characterization of *Piper* using molecular markers by Ajith *et al.* (1997), Banerjee *et al.* (1999), Lebot *et al.* (1999), Babu *et al.* (2003), Sen *et al.* (2010), Sheeja *et al.* (2013) and Chowdhury *et al.* (2014). A few reports are also available on the development of microsatellite markers in the genus *Piper*. Menezes *et al.* (2009) and Joy *et al.* (2011) developed and characterized a total of 16 microsatellite markers from black pepper and used them to study the genetic diversity of 20 varieties from Brazilian collections and 40 popular genotypes and four different species of *Piper* from India respectively. Liao *et al.* (2009) and Yoshida *et al.* (2014) reported isolation and characterization of 11 and 9 polymorphic microsatellite loci from two distant *Piper* species *Piper polysyphonum* and *Piper solmsianum* respectively. Development of highly reliable and reproducible molecular markers for assessing the genetic diversity assumes significance to facilitate the crop improvement programme through molecular breeding.

The present study was carried out with the objective of developing adequate (10-15) microsatellite markers to study the genetic architecture of black pepper and related species that would enable future crop improvement programmes in the genus *Piper*. The specific aims were to;

- Develop a set of genomic microsatellite markers by constructing small insert genomic libraries enriched for SSRs for isolation of microsatellites.
- Develop a set of genic microsatellite markers (EST SSRs) by exploiting the EST sequences deposited in the public domain.

- Characterize the SSRs and test their ability to identify polymorphism in diverse black pepper germplasm.
- Assess the cross- species amplification potential (transferability) of genic and genomic SSR markers so developed in wild *Piper* germplasm.
- To give a better understanding of inter-relationship between *Piper* species and among cultivars and varieties of black pepper.

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

2.1. The genus *Piper*

The genus name *Piper* was derived probably from the Greek name for black pepper, *Peperi* (Ravindran *et al*., 2000). The genus *Piper* is considered to be one of the largest genera of angiosperms (Kubitzki *et al*., 1993) and is widely distributed in the tropical and semi tropical regions of the world particularly Asia and Central America. The genus *Piper* comprises about 3000 species of which 119 are of Indian origin. The *Piper* species are mainly herbs, shrubs, creepers, climbers and trees. Black pepper, being the most valuable economically important spice crop and the best known agricultural product in the genus *Piper*. The phylogenetic position of the Piperaceae is very much confused and one view is that it is diverse assemblage of dicots termed 'paleoherbs' (Donoghue and Doyle, 1989; Loconte and Stevenson, 1991), plants that resemble monocots in certain vegetative characters.

The genus *Piper* is distributed both pantropicaly and neotropicaly. The neotropicaly distributed *Piper* species are mainly bisexual forms and are shrubs or small trees and the species from the Asian origin are unisexuals and woody climbers (Yunker, 1958). Two independent centres of distribution for the genus *Piper* in India are Trans Gangetic provinces and South Deccan (Hooker, 1886). However, according to Rahiman (1987) the *Piper* species are distributed in three centres; the sub Himalayan and North East Indian, the Western Ghats and the Eastern Ghats of Andhra Pradesh. The centre of origin of the black pepper is the sub mountainous tracts of Western Ghats. The patterns of distribution of the genus *Piper* vary from endemic to widespread. The greatest diversity of *Piper* species comes about in the

Tropical America (~700 species) followed by South Asia (~400 species) (Jaramillo and Manos, 2001). Some of the economically important species of pepper along with black pepper are *P. betle* L. (betel vine) cultivated extensively in India, where the leaves are used as mastictory: *P. longum* L. - Indian long pepper, *P. chaba* Hunter - Java long pepper, *P. mullesua*, the fruit and roots of which are used in indigenous medicine and *P. cubeba* - tailed pepper used in indigenous medicine.

2.1.1. Black pepper (*Piper nigrum* L.)

Black pepper, considered as '*King of Spices*' or '*Black Gold'*, is the most widely used spice of the world, and occupies a proud place in the cuisines of both the west and the east. Black pepper of commerce is the dried fruits of the perennial climbing vine *Piper nigrum* L. It contributes towards flavor, taste, antimicrobial and antioxidant properties. It is used in many forms of traditional medicine and is an ingredient in many medicinal preparations. It was originally collected as a forest produce and was later domesticated. Currently pepper is grown in about 26 countries. The major pepper growing countries are India, Indonesia, Brazil, Malaysia, Sri Lanka, Thailand, Vietnam, Guatemala, Mexico, Madagascar and Malavi. *Piper nigrum* L. is the native of India with maximum diversity in cultivated types occuring in the state of Kerala.

2.1.2. Taxonomy and Phylogeny

The family Piperaceae was classified under the sub class Monoclamidae in the series Microembryeae (Bentham and Hooker, 1880). Engler (1893) placed the genus *Piper* under the series Piperales.

Linnaeus (1753) in his "*Species Plantarum",* described 17 species of *Piper*. Eighteen species were reported by Roxburgh (1832) of which 7 were from Indian

Peninsula. Kunth (1822) described the species from South America and Blume (1826) on East Indian species. Miquel (1843) in his "*Systema Piperaceum"* subdivided the family into Piperaceae and Peperomeae with 15 genera and 304 species in Piperaceae and 5 genera and 209 species in Peperomeae. de Condolle (1923) considered three genera; *Piper*, *Peperomia* and *Verhuellia* in his "*Piperacearum Clavis Analytica*". Piperaceae is counted as one of the most primitive families of angiosperms derived from the herbaceous protangiosperm with minute and simple flowers. On account of some of the similar morphological characters, *Piper* shares characters with two other families; Chloranthaceae and Winteraceae (De Figueredo and Sazima, 2004). The genus *Piper* also shows resemblances to Saurarus and Chloranthus in view of naked flowers, one celled ovary, sessile stigma, orthotropous ovule and albuminous seed (Le Mount and Decarens, 1876; Heywood, 1978). But these two orders don't show the primitive vascular system present in *Piper* (Corner, 1976).

Datta and Dasgupta (1977) reported the evolution of *Piper* and *Peperomia* related to adaptation with two different conditions; *Piper* adapted with damper and lower lands due to the presence of vascular arrangements where as *Peperomia* evolved in drier and higher lands which shows the xerophytic adaptation due to the reduced bundle surface volume ratio. The most authoritative floristic study of Western Ghats was that of Gamble (1925) in his "Flora at Presidency of Madras", in which the following species together with taxonomic keys were given. *P. argyrophyllum, P. attenuatum, P. barberi, P. trichostachyon, P. galeatum, P. hapnium, P. hookeri, P. hymenophyllum, P. longum, P. nigrum, P. schmidtii* and *P. wightii.* In 1981, Rahiman described a new species, *P. bababudani*, from the Bababudan hills of Karnataka. Ravindran *et al*. (1987) reported a new species, *P. silentvalleyensis*, and the only bisexual wild species from Western Ghats. The other new reports of *Piper* are *P.*

pseudonigrum (Velayundhan and Amalraj, 1992) and *P. sugandhi* (Babu *et al*., 1993). Ravindran (1991) suggested a taxonomic key for the *Piper* species occurring in Western Ghats. He subdivided the genus into two sections - Pippalli and Maricha - based on the orientation of spikes, erect or pendent.

2.1.3. Economic importance of black pepper

The two major products of *P. nigrum* are black pepper and white pepper. Black pepper is used directly as a spice and also for preparation of its derivatives - pepper oleoresin and black pepper oil; white pepper is also used as direct spice. The major use of black and white pepper on a worldwide basis is for domestic culinary purpose while in the industrialized countries both forms find extensive use in flavoring of processed foods.

Pepper oleoresin obtained by the solvent extraction of pepper possesses the full organoleptic properties of the spice and is mainly used for flavoring of processed foods. Pepper oleoresin is also used in several pharmaceutical formulations. Black pepper oil through distillation posses the aroma and flavor of the spice, but lacks the pungency; it finds application in food and flavoring and in perfumery.

The various forms of whole preserved green and pink pepper are prepared in several of the spice producing countries for the export.

2.1.4. Economically important species of *Piper*

***P. nigrum* L.** (Black pepper)

Black pepper (*P. nigrum*) is the most widely used spice in the world. Most of the cultivated species are bisexual that is derived from wild ones, as a result of continuous selection and vegetative propagation by man through ages.

***P. betle* L.** (Betel vine)

Betel pepper leaves are chewed as masticator which is used in the paan industry. The extract of *P. betle* has been reported to have anti-hypertensive activity (Raj *et al*., 1998). In some areas leaf juice is used in eye afflictions. The plant is considered useful in treating madness, strangulation of intestine, venereal sore and syphilis and dysentery. in tribal medicine practices (Jain and Tarafder, 1970). Betel leaf is also used in certain indigenous medicinal preparations. Betel leaves also posses anti-oxidant action.

***P. longum* L.** (Indian long pepper)

Being one of the most important medicinal plants used in Indian system of medicine viz, Ayurveda, Sidha and Unani, long pepper forms one of the important ingredients in various compound preparations used for asthma, anorexia and also in snuffs used in coma (Chopra *et al*., 1956 and Nadkarni, 1954).

***P. chaba* Hunt.** (Java long pepper)

The fruits are pungent and aromatic, stimulant, carminative used in cough, cold and in hemorrhoidal affliction. It is also given in colic, timpanists and renal diseases.

***P. cubeba* L.** (Cubed or tailed pepper)

The essential oil of cubeba showed antibacterial and antifungal activity and for relief from throat affliction. It is also used as flavoring agent in certain liquors, cigarettes, sauces and also in perfumery to impart an exotic note.

***P. methysticum* Forster** (Kawa)

It is the source of narcotic drugs. The roots are used for making potent beverage Kawa-Kawa.

***P. sarmentosum* Roxb.**

This species is known for its culinary and medicinal properties. It is reported to have pharmacological properties *viz*. anti-tuberculosis (Hussain *et al*., 2008), anticancer (Ariffin *et al*., 2009) and antimalarial (Rukachaisirikul *et al*., 2004). It also possesses allellopathic effect (Pukclai and Kato- Noguchi, 2011).

2.1.5. Cytology

Darlington and Wylie (1961) reported the basic chromosome number of the genus *Piper* as X=12, 14, 16 and somatic number of *P. nigum* as 2n=128. In contrast, Mathew (1958) reported the chromosome number of *P. nigum* as 2n=52. Similarly, Mathew (1972) reported chromosomal number of 2n=52 for six cultivars and four wild types and 2n=104 for two other wild types.

2.2. Molecular diversity

2.2.1. Genetic diversity

Information on genetic diversity is essential in optimizing both conservation and utilization of plant genetic resources. Success of a crop breeding programme relied on the degree of variability present in the germplasm. Crop genetic diversity is important for crop adaptation to withstand pests and diseases and it is a significant prerequisite for plant breeders to augment the progress of traits of economic value such as yield. Various methods are available for estimating the genetic diversity of

crops, such as morphological, biochemical and molecular markers. Measurements of genetic diversity can be generated using conserved accessions in gene banks (Gilbert *et al.,* 1999; Parzies *et al.,* 2000). DNA-based molecular markers have several advantages over the conventional phenotypic markers since their presence is not dependent on the growth stage of the crop, climatic change and can be found in all tissues (Mondini *et al.,* 2009).

Morphological markers have been primarily applied for estimating diversity studies in a number of crops. Although morphological diversity being a good indication of genetic diversity, but sometimes it can be misleading. Morphological traits are often susceptible to phenotypic plasticity; conversely, this allows assessment of diversity in the presence of environmental variation.

Although isozyme marker based analysis has the advantage that an array of enzymatic loci can be assayed conventionally on one individual using small amount of materials, their application is limited since they are affected by extraction methodology, plant tissue, and plant stage, few isozyme assays per species and the enzymatic loci account for a non-random part of the entire genome.

In current scenario, the molecular markers become marker of choice than that of other markers for crop genetic diversity because these markers are generally unaffected by external impact. A vast array of DNA based molecular markers has been discovered since 1980 and new marker of choice are developed every year. The choice of benchmark could be based on cost, technical labour, level of polymorphism, reproducibility, locus specificity and genomic abundance (Garcia *et al.,* 2004). Molecular markers performed by highlighting differences (polymorphisms) within a nucleic acid sequence between different individuals. These differences may be because of insertions, deletions, translocations, duplications and point mutations.

2.3. Molecular markers

There are generally two categories of DNA markers: (A) Those based on DNA-DNA hybridization (B) Those based on polymerase chain reaction. The major hybridization based and PCR based molecular markers widely employed in genetic diversity analysis is as follows:

2.3.1. DNA-DNA hybridization:

The molecular marker system based on DNA-DNA hybridization was started in the 1970s. RFLP markers were the first DNA based molecular marker developed so far (Botstein *et al*., 1980).

2.3.2. Restriction Fragment Length Polymorphism (RFLP):

The polymorphism at the DNA sequence level is often measured as a variation in length of restriction fragments generated by cutting DNA with restriction enzymes that reveal a pattern difference between DNA fragment sizes among the individual organisms. (Botstein *et al.*, 1980). Such polymorphism is referred as RFLP.

A numbers of steps are involved in RFLP analysis. Restriction enzyme fractionated genomic DNA is separated by gel electrophoresis and then transferred to a hybridization membrane. A 'DNA probe', a short fragment of labeled DNA, is hybridized to the filter (Saiki *et al.*, 1985; Kumar *et al.*, 2009). These variations may be due to evolutionary processes, spontaneous mutations and unequal crossing over (Gonzalez-Chavira *et al.*, 2006) or from differences in DNA sequences (additions or deletions, or gross chromosomal changes such as inversions or translocations) and these changes of fragment sizes detectable as restriction fragment length polymorphisms (Michelmore and Hubert, 1987).

The advantages of RFLPs are that it gives highly reproducible banding patterns and are co-dominant markers, hence, heterozygotes are distinguishable. Nonetheless, RFLP has a number of disadvantages. The limitations of RFLPs are that, it is laborious, expensive, time-consuming, generally uses radioactive reagents, and requires large quantities of high quality genomic DNA (Mondini *et al*, 2009) and difficult to automation (Semagn *et al.,* 2006). The extent of polymorphism is limited for intra-species.

2.3.3. Polymerase chain reaction (PCR) based:

PCR is a molecular biology technique for enzymatically replicating small quantities of DNA reaction (Mullis, 1990). PCR is effective in estimating DNA sequence variation as it provides amplification of the DNA between two specific priming sites in the genome. PCR based markers require less DNA per assay than RFLP that require higher the concentration.

2.3.4. Random Amplified Polymorphic DNA (RAPD):

RAPD markers were developed in the early 1990s (William *et al*., 1990). It is based on PCR amplification of random DNA segments with short, arbitrary primers (William *et al*., 1990). The polymorphism generated by RAPD markers are due to the mismatches in the primer binding sites or insertion/deletion events.

An oligonucleotide primer used for RAPDs is single and short arbitrary primer usually ten base pairs long and amplifies many loci simultaneously and therefore a number of multiple markers can be assayed within a single PCR reaction. Each product is produced from a region of the genome which is complementary to the primer close to each other for amplification to perform. In RAPD analysis the

amplicons are visualized after ethidium bromide staining and there is no need for hybridization with labeled probes as in RFLPs (Kumar *et al.,* 2009).

The RAPD method is simple, quick, less expensive, very small amount of DNA is required and thus can be used in laboratories with limited resources. The main advantage of RAPD is that, DNA probes and sequence information for primer designing are not required. Disadvantages of RAPDs are its poor reproducibility and when used in linkage map production, the same loci may not be detectable in different populations. RAPDs are dominant markers and do not differentiate between homozygous and heterozygous markers.

2.3.5. Amplified Fragment Length Polymorphism (AFLP)

AFLP was developed to overwhelm some of the drawbacks of reproducibility of RAPDs as the technique combines the digestion of DNA with some specific restriction endonucleases with a PCR-based technique (Sandal *et al*., 2002). The first step in AFLP analysis involves the fragmentation of genomic DNA with a combination of rare cutting (*Eco* RI or *Ps* I) and frequent cutting (*Mse* I or *Taq* I) restriction enzymes (Vos *et al.*, 1995) then the oligonucleotide adapters are allowed to ligate to the restricted DNA fragment's ends. Either a pre-selection step is performed using magnetic beads followed by a round of selective PCR or two selective rounds of PCR amplification are applied (Vos *et al*., 1995). In the last step, the amplified products are separated by polyacrylamide gel electrophoresis and can be visualized using radioactive or fluorescent labeling.

When compared to RAPD, AFLP results are more efficient and reproducible. (Semagn *et al*., 2006). Compared with RFLPs, AFLPs is quick, less labour intensive and generate much more information (Powell *et al*., 1996). The technique is amenable to high-throughput analysis which is an additional benefit. AFLPs are

greatly efficient markers and could be useful in genetic resource exploitation and identification of novel traits (Crouch and Ortiz, 2004). However, AFLPs are dominant markers.

2.3.6. Inter simple sequence repeat (ISSR) markers

Inter simple sequence repeat (ISSR) technique is a PCR based technique, reported by Zietkiewicz *et al*. (1994), which comprise the amplification of DNA segments between two identical microsatellite repeat regions ‘oriented in opposite direction using primers designed from microsatellite core regions. The technique uses microsatellite primers, usually 16−25 bp long, of di-nucleotide, trinucleotide, tetra-nucleotide or penta-nucleotide repeats to target multiple genomic loci. The primers can be either unanchored (Meyer *et al*., 1993; Wu *et al*., 1994) or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al*., 1994). ISSR primers generate polymorphism whenever one genome misses the sequence repeat or has a deletion or insertion or translocation that modifies the distance between the repeats. The di-nucleotide repeats anchored either at 3' or 5' end reveal high polymorphism (Joshi *et al*., 2000). The primers anchored at 3' end give clearer banding pattern as compared to those anchored at 5' end (Blair *et al*., 1999). ISSR technique is simple, quick and less costly like RAPDs. But ISSR markers have high reproducibility than RAPD primers due to the longer primer length. The main disadvantage of ISSR markers is that they segregate as dominant markers and they showed segregation distortion (Wang *et al*., 1998 a).

2.3.7. Microsatellite or Simple Sequence Repeats (SSRs)

Currently, microsatellites are the most frequently used molecular markers in genetic diversity analysis. Microsatellites or simple sequence repeats (SSRs) are

tandem repeats of one to six nucleotide long DNA motifs found at high frequency in the nuclear genome of most taxa. (McCouch *et al.,* 1997; Zane *et al*., 2002; Thiel *et al*., 2003). The term microsatellite markers first coined by Litt and Luty (1989) are variously termed as Simple Sequence Repeats (SSRs) (Jacob *et al*., 1991) and Short Tandem Repeats (STRs) (Edward *et al*., 1991). The existence of microsatellites was first documented by Hamada *et al*., (1982) from various eukaryotes ranging from yeast to vertebrates. Further studies by Tautz and Renz (1984) confirmed the abundance of microsatellites in plants and many other eukaryotes. Microsatellites have gained considerable importance in molecular research owing to many desirable characters including multiallelic nature, hypervariability, codominant inheritance, reproducibility, amenability to automation, and high throughput genotyping (Powell *et al*., 1996a; Parida *et al*., 2009). The extraordinary high mutation rate (10^{-2} to 10^{-6} mutations per locus per generation) of SSRs has made them popular molecular markers for many applications including genetic mapping, population genetics, phylogenetics, pedigree analysis and also in DNA forensics (Goldstein and Schlotterer, 1999).

The microsatellites repeat sequences are abundant and distributed in the entire genome with variable frequency (Sharma *et al*., 2007; Guo *et al*., 2009). They are ubiquitous in both coding and noncoding regions (Tautz and Renz, 1984; Guptha *et al*., 1996; Katti *et al*., 2001) of prokaryotes and eukaryotes (Zane *et al*., 2002). Microsatellite locus exhibits between 5-40 repeats, but longer strings of repeats are also possible. Di, tri and tetra nucleotide repeats are the most common choices for molecular genetic studies. Dinucleotide repeats account for the majority of microsatellites for many species (Li *et al*., 2002). Trinucleotide and hexanucleotide repeats are the most likely repeat classes to be present in coding regions because they

do not cause frame shift (Toth *et al*., 2000). Mononucleotide repeats are less valid due to the problems with amplification. On comparing coding and non-coding regions in different plant species, it was observed that tri and tetra-nucleotide microsatellite motifs are more common within introns, whereas other types of motifs are found within exons (Toth *et al*., 2000).

Microsatellite sequences are especially useful to differentiate closely related genotypes; due to their high degree of variability, they are, therefore, preferred in population studies (Smith and Devey 1994) and for the identification of closely related cultivars (Vosman *et al*., 1992).

They are also present in chloroplastic (Provan *et al*., 2001; Chung and Staub, 2003) and mitochondrial genomes (Soranzo *et al*., 1999; Rajendrakumar *et al*., 2007). However, taxon specific variation in frequency distribution and abundance of different SSRs was detected (Hancock, 1999; Toth *et al*., 2000). The overall microsatellite content in the genome also correlates with the genome size of the organisms (Hancock, 1996) with a higher density of simple sequence motifs in the non-coding regions than in coding regions of eukaryotes (Hancock., 1995; Li *et al*., 2002). In plants SSRs are much more abundant and preferentially associated within untranslated regions (UTRs) of the transcribed regions than in the other genomic regions. (Morgante *et al*., 2002). AT repeat rich nature of plants and AC repeat rich nature in animals has appeared to be a general feature for differentiating plant and animal genomes (Powell *et al*., 1996).

The location of microsatellite in the genome determines its major role in genomics. They have potential enough to effect gene regulation, development and evolution. The presence of SSRs in the coding regions lead to the appearance of

repetitive patterns in the protein sequences (Katti *et al*., 2001) and several reports indicates the influence/association of microsatellites in regulating molecular functions/gene expression. The involvement of microsatellite markers in the regulation of transcription factors (Martin *et al*., 2004) and recombination events (Benet *et al*., 2000; Bagshaw *et al*., 2008) is well presented.

The polymorphic nature of microsatellites was identified by Litt and Lutty (1989) by amplifying $(TG)_n$ in the human cardiac actin gene and detecting 12 alleles in 37 unrelated individuals. Weber and May (1989) also reported the successful amplification and polymorphic nature of 10 dinucleotide loci. The genesis of these repeats occurs primarily due to slipped strand mispairing and subsequent errors during DNA replication/ repair/recombination (Levinson and Gutman, 1987; Scholtterer and Tautz, 1992; Katti *et al*., 2001), nucleotide composition of repeat motifs (Katti *et al*., 2001) or unequal crossing over between sister chromatids (Innan *et al*., 1997).

Though the microsatellite markers are considered to be versatile, they are credited with many advantages and disadvantages. Because of its high polymorphism, co dominant inheritance, ease of scoring and high genome coverage, microsatellites are generally considered to be one of the most powerful genetic markers for phylogenetic, DNA fingerprinting, mapping and evolutionary studies (Parida *et al*., 2009). Compared to other markers *viz*., RAPD, RFLP and AFLP techniques, microsatellites are more informative and variable (He *et al*., 2003; Lee *et al*., 2004). The technique is PCR based, hence requires less quantities of template DNA (10-100ng per reaction) (Kumar *et al*., 2009). Microsatellites also appeals scientific attention because they are linked to genes of agronomic importance (Yu *et al*., 2000). Moreover the reproducibility of microsatellites is high due to the lengthy primers and high annealing temperatures during genotyping. The simultaneous amplification of

several loci in a single reaction using compatible multiple primers (multiplexing) of microsatellite markers enhances the information per assay and reduces the unit costs involved in analysis (Kumar *et al*., 2009).

Despite the broad applicability of SSR markers in molecular research their development remains prime bottle neck in majority of the species. This is because of the fact that these ought to be isolated *de novo* for much of the species tested for the first time. Besides this, comparatively low frequency of microsatellites present in plant genome causes particular reason for their large scale development. Conventional genomic library construction for microsatellite isolation is laborious, cumbersome, time consuming, cost intensive, requires high level of expertise and are often complex with a wide range of steps (Powell *et al*., 1996; Zane *et al.*, 2002; Squirell *et al*., 2003). In addition, SSR markers developed for one species generally exhibit less transferability when applied to different species of the same taxa or different related taxa. This limited transferability of SSRs across taxonomic boundaries might be due to point mutations in the primer binding sites (Kumar *et al*., 2009), thus requires the development of specific markers for individual species (Roa *et al*., 2000; Kindiger, 2006). AT dinucleotide which is the most abundant repeats among plants are difficult to isolate from libraries because of their palindromic nature (Powell *et al*., 1996).

Another issue associated with microsatellites is the occurrence of null alleles (no PCR product generation). The cause of null alleles might be due to the poor primer annealing caused by nucleotide sequence divergence, inconsistent DNA template quality or low template quality (Ellegren, 2004) or mutations in the primer binding sites (Jones *et al*., 1998). This may give rise to intricacy in the determination of allelic and genotypic frequencies and an underestimation of heterozygosity (Kumar *et al*., 2009) which may lead to biased results. Homoplasy is the next problem using

microsatellites as a reliable tool for phylogenetic studies because alleles considered being identical in state is not necessarily identical by descent (Estoup *et al*., 2002). Another major problem during microsatellite analysis is the occurrence of stutter bands that are artifacts in the technique that appears by DNA slippage during PCR amplification. These can complicate the interpretation of the data because size determination of the fragments is more difficult and heterozygotes may be confused with homozygotes. However, the interpretation may be made clear by including appropriate reference genotypes of known band sizes in the experiment (Kumar *et al*., 2009).

2.4. Utilization of Microsatellites in Plant Breeding

2.4.1. Cultivar identification

A set of SSR markers has been detected for the identification of soybean cultivars (Song *et al.,* 1999). He *et al.* (2003) could successfully differentiate 19 diverse tomato cultivars using 65 SSR markers. Sixty apple cultivars were characterized with eight polymorphic SSR primers and cultivar identification diagram CID map was constructed which could clearly distinguish each cultivar studied (Liu *et al*., 2014). Pourabad *et al*. (2015) used 12 microsatellite markers to distinguish between 40 rice cultivars and the analysis showed complete discrimination of cultivars from each other except for two. They proposed SSR markers as a complementary tool for morphological characteristics in DUS tests. A specific set of SSR markers should be developed for each economically-important agricultural crop, which can be utilized to protect the intellectual property rights of plant commercial companies.

2.4.2. Marker-assisted selection

Marker-assisted selection (MAS) which involves the indirect selection of a trait of interest based on genetically associated molecular markers play an important role in enhancing the efficiency of plant breeding programmes. SSR based marker-assisted selections have been widely used in breeding programmes. For example, in wheat breeding programmes the gene for resistance to the Hessian fly (*H32*), the gene for adult-plant leaf rust resistance and yellow pigment content (*YP*) were successfully identified by flanking SSR markers of Xgwm3 and Xcfd223 (Sardesai *et al.,* 2005), GWM296 (Hiebert *et al.,* 2007), and Xwmc809 (He *et al.,* 2008), respectively. Two SSR markers have been successfully employed in marker assisted breeding for resistance to powdery mildew in field pea (Ek *et al.,* 2005). Neeraja *et al*. (2007) identified a major QTL for submergence stress (*Sub1*) on chromosome 9 by employing flanking SSR markers in backcross breeding program in rice. Tyrka *et al.* (2008) identified a polymorphic SSR marker QLB1 that co-segregated with the locus for resistance to the barley yellow mosaic virus which was used for resistance selection in barley breeding. One microsatellite marker, TA194 showed linkage to *Fusarium oxysporum* wilt resistant locus at 85% probability in chickpea for marker assisted selection (Ahmad *et al*., 2014).

2.4.3. Molecular mapping

Condit and Hubbell (1991) first reported genetic mapping in plants with microsatellite markers in tropical trees followed by Akkaya *et al.* (1992) in soybean and then in rice (Wu and Tanksley 1993; Zhao and Kochert 1993).

Microsatellite marker based comparative mapping revealed homologies between seven linkage groups between *Quercusrobur* (L.) and *Castaneasativa* (Mill.)

and orthologous loci were also identified (Barreneche *et al.,* 2004). Comparative mapping based on EST-SSR markers in wheat, barley, rye, and rice confirmed and identified the conservative chromosome regions between wheat and rice and the presence of orthologues of barley EST-SSRs in different species (Yu *et al.,* 2004b; Varshney *et al.,* 2005b). SSR-based comparative mapping between *Arabidipsis thaliana* and *Brassica rapa* detected the presence of small genomic fragments of *A. thaliana* that were scattered throughout the entire linkage map of *B. rapa*. The study also identified a synteny region between *B. rapa* and *A. thaliana* (Suwabe *et al.,* 2006).

SSR markers have been successfully employed for association mapping in crops such as potato, maize, wheat, and soybean. (Simko *et al.,* 2004) identified an association between a microsatellite marker and QTL for resistance to *Verticillium dahlia* in tetraploid potato cultivars and eventually the QTL was cloned. In wheat, an association between SSR markers and kernel size was detected on three chromosome regions by association mapping approach using elite germplasm (Breseghello and Sorrells, 2006a). Association mapping approach enabled the identification of the association between microsatellite marker loci and resistance to *Stagnosporanodorum* glume blotch (SNG) in wheat (Tommasini *et al.,* 2007). Two common SSR markers were detected in soybean in two separate populations that were associated with iron deficiency chlorosis (Wang *et al,.* 2008). Wei *et al.,* (2014) performed association analysis with 20 microsatellite markers to identify the marker loci linked to 13 morphological traits and 10 physiological traits in a wild *P. simonii* population which consisted of 528 individuals. A total of 18 SSR markers were found to be associated with five yield related traits in wild soybean (*Glycine soja* Sieb. and Zucc.).

2.4.4. Population and evolutionary studies

Microsatellite markers can be employed to analyze the population structure within and among natural populations and to determine the potential progenitors. Analysis of variation among barley populations including wild *Hordeum* species, wild progenitors of cultivated barley, barley landraces, and barley cultivars using chloroplast microsatellite markers revealed a decrease in cytoplasmic diversity between wild progenitors and barley cultivars as well as between barley landraces and barley cultivars (Provan *et al.*, 1999). Due to the inefficiency of morphological characters in defining most species of cultivated potato, genotyping of 750 potato accessions (742 potato landraces and 8 wild species) was performed using 50 SSR markers. The genotyping data confirmed the reclassification of the cultivated potato into four species: *S. tuberosum*, *S. ajanhuiri*, *S. juzepczukii*, and *S. curtilobum* (Spooner *et al.*, 2007).

2.5. Classification of microsatellites

Microsatellites can be classifieds based on the number of nucleotide per repeat unit, the nature of the repeated unit, and their position within the genome (Miah *et al*., 2013).

2.5.1. A. Based on number of nucleotide per repeat motif- with respect to the number of nucleotides per repeat unit microsatellites can be classified di-, tri-, tri-, tetra-, penta-, hexa- nucleotide repeats.

Mononucleotide	- (A) *n*
Dinuleotide	- (CA) *n*
Trinuceotide	-(CAT) *n*
Tetranucleotide	- (CAGA) *n*

Pentanucleotide - (AAAGG) *n*

Hexanucleotide - (GGGAAA) *n*

(*n*- number of variables)

2.5.2. B. Based on the arrangement of nucleotides in the repeat motifs- With respect to the arrangement of nucleotide within the motifs Weber (1990) used the term perfect, imperfect, composite or interrupted (Oliveira *et al*., 2006; Miah *et al*., 2013) to categorize microsatellites.

a. Perfect microsatellites - the repeat sequence is not interrupted by any base not belonging to the motif (e.g. CTCTCTCTCTCTCTCTCT)
b. Imperfect microsatellites - a pair of bases is present between the repeat motifs that does not match the motif sequence (e.g. CTCTCTCTCTCTACTCTCT)
c. Interrupted microsatellite - a small sequence within the repeated sequence that does not match the motif sequence (e.g. CTCTCTCTCTCTGGGGCTCTCT)
d. Compound/Composite microsatellites - two adjacent distinctive repeats present within the sequence (e.g. TATATATATAGTGTGTGT).

2.5.3. C. Based on the position within the genome - There are three types; genomic/ nuclear microsatellites, genic (EST) microsatellites and organellar microsatellites (chloroplast SSRs and mitochondrial SSRs) (Kalia *et al*., 2011).

a. Genomic/nuclear microsatellites - microsatellites isolated from the nuclear genome (genomic DNA of an organism with or without the construction of genomic library).
b. EST/genic microsatellites - microsatellites identified by data mining or exploiting EST sequences either from cDNA library or EST databases,

which are typically by products of the sequence data from genes or large EST sequencing projects.

c. Organellar microsatellites (chloroplast SSRs and mitochondrial SSRs) - microsatellites identified from the chloroplast/mitochondrial genome of an organism.

2.6. Strategies for microsatellite development

To develop robust set of polymorphic microsatellite marker for a new species, microsatellite loci should be isolated from the genome along with sufficient flanking nucleotide sequence region to ease primer designing. The PCR conditions are required to be optimized and the primers developed need to be screened in a limited number of related and non-related individuals for estimating their polymorphic potential. Thus, the *de novo* development of microsatellite markers is a tedious, time consuming and costly endeavor process (Rassmann *et al*., 1991; Zane *et al*., 2002; Squirrel *et al*., 2003; Thiel *et al*., 2003). The methods and strategies for microsatellite isolation has evolved substantially from traditional genomic library construction (both enriched and non-enriched) (Zane *et al*., 2002) to mining databases (genomic and EST databases) (Victoria *et al*., 2011) and high throughput isolation through next generation sequencing (Egan *et al*., 2012).

2.6.1. Genomic/Nuclear SSRs

2.6.1.1 Development of microsatellite markers from SSR enriched genomic DNA libraries

The different strategies used for the isolation of microsatellite loci from genomic libraries were critically reviewed by Zane *et al*. (2002).

The methods can be broadly categorized into two (Zane *et al*., 2002; Kalia *et al*., 2011)

1. **Selective hybridization method -** facilitating the selection of microsatellite containing DNA fragments by hybridization to probes (Karagyozov *et al*., 1993; Armour *et al*., 1994; Kandpal *et al*., 1994; Hamilton *et al*., 1999; Glenn and Schable, 2005).
2. **Primer extension method -** selective amplification of microsatellite containing DNA fragments by priming with repeat specific oligonucleotides (Ostrander *et al*., 1992; Paetkau, 1999).

1. Selective hybridization method:

The selective hybridization strategy based microsatellite enrichment technique is a relatively simple, robust, reproducible and cost effective approach for isolating large number of microsatellites from diverse plant species with higher efficiency (Kalia *et al*., 2011). This method involves the fragmentation of genomic DNA either by sonication or by restriction enzymes and ligation to known sequence (linker or adapter). The DNA fragments are then denatured and hybridized with

1) Biotinylated oligos in vectrex- avidin matrix (Kandpal *et al*., 1994).

   Or

2) Oligo nucleotides were subjected to bind to nylon membrane (Karagyozov *et al*., 1993; Edwards *et al*., 1996).

   Or

3) Biotynilated and captured on streptavidin coated magnetic beads (Brown *et al*., 1995; Hamilton *et al*., 1999; Glenn and Schable, 2005; Geng *et al*., 2010).

4) Biotinylated SSR probe- streptavidin coated magnetic bead complex (Triplex affinity capture protocol, White and Powell, 1997).

After the hybridization step and several washes with buffer for removing nonspecific binding, the probe bound DNA fragments were eluted. The fragments were recovered by PCR amplification, cloned and sequenced.

2. Primer extension method:

These methods (Ostrander *et al*., 1992; Paetkau, 1999) rely on the construction of primary genomic library, in which fragmented genomic DNA is inserted into a phagemid or a phage vector in order to obtain a single stranded DNA library. ssDNA is then used as a template for a primer extension reaction, primed with repeat specific oligonucleotides, which generates a double stranded product only from vectors containing the desired repeat. During the construction of primary library, only a limited portion of the investigated genome is cloned and sequenced. However it is unclear whether the primer extension approach is effective for tri and tetra nucleotide repeats too. The Ostrander protocol has not been tested for tri or tetra nucleotide repeat enrichment, whereas the Paetkau protocol produced only 0-25% positive clones when using a tetra nucleotide repeat primer in the extension step.

Hybridization capture is the predominantly used strategy, as it allows enrichment and selection prior to cloning thereby providing a faster and easier way to process multiple samples (Glenn and Schable, 2005). Using these protocols, a large number of microsatellite markers have been developed in economically important crops.

2.6.1.2. Development of microsatellite markers from non-enriched genomic DNA libraries

To construct non enriched genomic DNA library fragmented genomic DNA was ligated and transformed to suitable vectors. The clones were hybridized to nylon filters and screened using radio labelled microsatellite probes or enriched with biotinylated probes and captured with streptavidin coated magnetic beads. This method has been applied for microsatellite isolation in *Citrus limon* (Golien *et al*., 2006) and *Phaseolus vulgaris* (Blair *et al*., 2009).

2.6.2. Development of microsatellite markers from PCR based molecular markers

2.6.2.1. SSR development from RAPD markers

In this method, isolation of microsatellite region was done by southern hybridization of RAPD fingerprints with repeat probes followed by screening of positive clones (Ender *et al*., 1996) or cloning of all RAPD amplicons (Lunt *et al*., 1999).

2.6.2.2. SSR development from ISSR markers

Inter simple sequence repeat (ISSR) amplifications was used to analyze microsatellite motif frequency in many crop plants to evaluate the genetic diversity among the cultivars. Blair *et al.* (1999) studied the rice genome by developing SSR markers from ISSR amplification.

2.6.2.3. SSR development from AFLP markers

In this approach, AFLP markers with enrichment processes (Hakki and Akkaya, 2000) or in combination of randomly amplified microsatellite primer and a selective primer for amplifying restricted fragments containing microsatellite repeats were used to isolate SSR markers (Van Eijk *et al*., 2001). FIASCO (Fast Isolation by AFLP Sequences Containing Repeats) is another approach for microsatellite isolation in which AFLP bands were subjected to hybridization with biotinylated probes and captured using magnetic beads (Zane *et al*., 2002).

2.6.2.4. SSR development through Sequential Reverse Genome Walking (SRGW)

The 'Sequential Reverse Genome Walking' (SRGW) strategy was employed by Joy *et al*. (2011) which primarily involved the generation of a genomic walking (GW) library, which was enriched in two consecutive primary and nested secondary PCR steps (using SSR oligos as reverse primers in combination with two adaptor specific primers). The PCR products were cloned and sequenced. Two sets of flanking primers (F1 and nested secondary F2 primer) were designed based on the flanking sequence identified from one end of the microsatellite motif. A 'sequential reverse walk' was then initiated with the rest of GW libraries using the flanking primers (F1 and F2) and the adaptor specific primers (AP1 and AP2). The secondary nested PCR products were cloned and sequenced to generate SSR markers.

2.6.2.5. Development of microsatellite markers using high throughput sequencing or next generation sequencing

Recently, Next Generation Sequencing techniques (454 pyrosequencing) (Droege and Hill., 2008; Davey *et al*., 2011; Malausa *et al*., 2011, Mirimin, 2013) is becoming versatile which is a feasible way for screening entire genome using

bioinformatics tool for the identification of microsatellites even in case of non-model organisms. With the development of next generation DNA sequencing technologies, large nucleotide sequence databases of EST and genomic DNA have been generated for many plant species and are available for screening simple sequence repeats (Yang *et al*., 2012; Zalapa *et al*., 2012; Wu *et al*., 2013). SSR markers based on cDNA or EST have a number of additional advantages such as being tightly linked to traits of interest, having a high rate of transferability between related species, and providing genetic diversity and mapping analyses for the expressed regions (Varshney *et al*., 2005).

2.6.3. EST derived microsatellite markers

ESTs are sequenced portion of complementary DNA copies of mRNA and they represent part of the transcribed portion of the genome in given conditions (Poncet *et al*., 2006). With the advent of bioinformatics tools TROLL (Castelo *et al*., 2002), MISA (Thiel *et al*., 2003), Msat commander (Faircloth, 2008), Websat (Martins *et al*., 2009), QDD (Meglecz *et al*., 2010).

EST SSRs have more advantages and disadvantage when compared to genomic SSRs. Due to the public availability of large quantities of gene sequences data, the generation of genic/EST SSRs are simple and easy and are more readily transferable (Gupta *et al*., 2003) than those derived from untranslated regions (Pashley *et al*., 2006). As EST SSRs reside in the conserved portion of the genome, null alleles are less problematic when compared to genomic SSRs.

The generation of EST SSR is limited to the availability of EST sequences and thus could be applied only to economical and widely exploited crops whose nucleotide sequence information is shared in the public domain (Varshney *et al*.,

2005; Pashley *et al*., 2006). The relatively low abundance of SSRs within the transcribed region is also a limiting factor for the large scale development of genic SSR markers. Moreover the EST SSR markers exhibit lower polymorphism and are less efficient in distinguishing closely related individuals when compared to that of genomic SSR markers (Cho *et al*., 2000; Scott *et al*., 2000; Chabane *et al*., 2005). Using this effective approach, large numbers of SSR markers for expressed regions have been successfully developed for many plant species (Lu *et al*., 2013; Blair and Hurtado, 2013).

2.6.4. Organelle microsatellites (Chloroplast microsatellites; cp SSRs)

Chloroplast microsatellites are developed from the sequenced chloroplast genome of plants. The polymorphism is exhibited both in conserved and variable region of the cp SSRs genome. The microsatellite repeat motifs present in the chloroplast genome are mainly mononucleotides (A and T) (Brya n*et al*., 1999). Cp SSRs are uniparentally inherited which will be useful for studying cytoplasmic diversity, cyto-nuclear interactions (Vendramin *et al*., 1996). The most successful way for the identification of chloroplast microsatellites is *de novo* sequencing of non-coding chloroplast regions (Ebert and Peakall, 2009).

2.6.5. Cross species amplicability/transferability of microsatellite (SSR) markers

Comparative genetics and phylogenetic studies have exposed that the gene content and order are conserved among closely related species. Analysis on sequence data may derive to the conclusion that several plants hold much homology between genome of the closely related taxa. Thus the microsatellite markers developed for one species could be transferred in related species and even in genera to detect polymorphism. The potential to inventively transfer the SSR markers are generally

termed as transferability. Such cross amplicability may compensate with the traditional laboratory methods for the library construction to isolate microsatellite markers which is expensive, laborious and time consuming.

Transferability hold out potential for the low cost development of microsatellite markers for related species and thus open wider aspect for the application of these markers in diversified areas of plant molecular research. This method of microsatellite detection and its polymorphism is significant in such crops where neither sequence information nor genetic maps are available (Kalia *et al*., 2011). The cross transferability has been successfully described in many species (Ellis and Burke, 2007; Varshney *et al*., 2007). However, EST SSR markers showed greater cross species transferability than genomic SSRs, as they reside in the expressed regions and thus are more conserved than non-coding regions. (Varshney *et al.*, 2005).

2.7. Genetic diversity analysis of Black pepper germplasm using molecular markers

Kumar *et al*. (2001, 2003) characterized 24 black pepper (*Piper nigrum* L.) accessions (9 advanced cultivars and 13 landraces) using RAPD markers. Twenty two out of 34 primers tested were able to get clear banding pattern. Cultivar specific bands were obtained for all the cultivars tested except for Panniyur 3. Good variability was observed among the tested cultivars. Twenty two cultivars of black pepper exhibited the genetic proximity among landraces than advanced cultivars. Nirmal Babu *et al.* (2003) conducted studies on fourteen major cultivars and ten released varieties of black pepper using RAPD markers. Cluster analysis indicated that there are distinct differences between most of the cultivars and varieties. Sreedevi *et al*. (2005) characterized seven high yielding black pepper cultivars using RAPD markers. Out of

the 14 primers, 9 could produce unique bands in 6 of the tested lines. An attempt was also made by Nazeem *et al*. (2005) to analyze the variability and relatedness among 49 cultivars/accessions of black pepper using RAPD and AFLP markers. RAPD could give better results but AFLP profiling could not give distinct clustering among the tested lines. Joy *et al*. (2007) studied the genetic relationships among thirty popular and agronomically important cultivars of black pepper using AFLP analysis. The dendrogram constructed by unweighted pair group method analysis (UPGMA) grouped the 49 accessions into three major clusters and four diverse cultivars with only 30% similarity. Karimunda, the popular cultivar showed to be unique in the fingerprint profile.

Menezes *et al*. (2009) developed and characterized nine microsatellite markers from an enriched library of black pepper. They used these SSRs to study the genetic diversity of 20 varieties from Brazilian germplasm collection. Joy *et al*. (2011) developed seven microsatellite markers for black pepper of which four were found to be polymorphic. The four markers generated 62 alleles with an average of 15.5 alleles over 4 loci. SSR primers showed an average Polymorphism Information Content (PIC) value of 0.85. The estimated average Shared Allele Frequency ranged between 1.57 and 20.12%. The PCA plot disclosed four closely related individual groups and identified Karimunda, Wild pepper and a local landrace as the most divergent genotypes.

2.8. Molecular Phylogeny of Piperaceae

Jaramillo and Manos (2001) reported phylogenetic analysis of sequences of the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA based on a

worldwide sample of the genus *Piper*. Sequences from a 51 species of *Piper* were aligned to yield 257 phylogenetically informative sites.

Chaveerach *et al.* (2002) studied inter relationships between 3 species of *Piper* - *P. kadsura, P. retrofractum* and *P. chaba* using morphological characters and RAPD profiles and demonstrated a closer relation between *P. retrofractum* and *P. kudsura* than between *P. chaba* and *P. retrofractum* . Nirmal Babu *et al.* (2003) studied molecular interrelationships between 24 *Piper* species using RAPD profiles. The phylogenetic trees grouped *P. longum, P. hapnium* and *P. mullesua* in one group and *P. attenuatum* and *P. argyrophyllum* in another group. *P. pseudonigrum, P. nigrum* and *P. galeatum* are clustered together.

Wadt *et al.* (2004) studied 49 genotypes belonging to three species of *Piper viz: Piper hispidinervum*, *Piper aduncum*, and *Piper hispidum*, to assess inter and intra-specific relationships using RAPD markers. Cluster analysis indicated three distinct groupings of the genotypes corresponding to *Piper hispidinervum*, *Piper aduncum*, and *Piper hispidum* and it supported the existence of *P. hispidinervum* and *P. aduncum* as two separate species. Gaia *et al.* (2004) used RAPD to study the genetic diversity in 18 accessions from 4 natural populations of pimenta de macao (*P. aduncum*) from Brazil.

In order to compare the genetic relationships among Kava, Pepper and its wild relatives Jiang *et al*. (2009) conducted research on Kava by using RAPD and SCAR molecular markers. Total 170 bands were amplified by 20 random primers, in which 20 bands were polymorphic (12%). Cluster analysis grouped the 28 accessions into six groups at a similarity coefficient of 0.36, where 6 materials of Kava formed a group, representing that Kava was distantly related to Pepper and its wild relatives.

Two pairs of Kava specific SCAR markers designed could be used for molecular identification of Kava species.

The genetic diversity of eight *Piper* species *viz*., *P. nigrum*, *P. longum*, *P. betle*, *P. chaba*, *P. argyrophyllum*, *P. trichostachyon*, *P. galeatum*, and *P. hymenophyllum* was evaluated by RAPD markers (Sen *et al.*, 2010). High genetic variations were observed among the *Piper* species tested. Out of the 149 RAPD fragments amplified, 137 fragments were found to be polymorphic (91.95%). All eight species generated species-specific bands. The genetic distances ranged from 0.21 to 0.69. Jiang and Liu (2011) applied RAPD and SRAP (Sequence Related Amplified Polymorphism) analysis to study genetic diversity among 74 individual plants of *Piper* spp in Hainan Island. The dendrogram generated with the RAPD markers was topologically different from the dendrogram based on SRAP markers, but the SRAP technique clearly distinguished all *Piper* spp from each other. The SRAP technique was found to be more efficient for studying genetic diversity compared to RAPD technique.

Comparative study of RAPD and ISSR markers were used to analyze the genetic diversity among 15 cultivars of betel vine (Patra *et al.*, 2011). All the cultivars were related to each other with an average similarity of 0.2913. Sheeja *et al*. (2013) reported the genetic diversity analysis of 27 *Piper* species using ISSR markers. The UPGMA dendrogram grouped them into six clusters. Thirty five species specific bands were generated for 19 different *Piper* species. Four unique bands were achieved for *P. galeatum*. The genetic diversity analysis of six different species of *Piper* from Northeast India using RAPD marker was reported by Chowdhary *et al*. (2014) in which one fifty nine polymorphic bands generated showed high level of genetic variation among the species.

Liao *et al.* (2009) reported the isolation and characterization of eleven polymorphic microsatellite loci from an endemic species, *Piper polysyphonum* from China. Allele numbers ranged from two to ten, of these four loci exhibited a departure from Hardy- Weinberg equilibrium, possibly due to population mixture. Nine microsatellite loci were developed and characterized for natural populations of *Piper solmsianum* (Yoshida *et al.*, 2014), a potential source of bioactive secondary metabolites, and analyzed to estimate the levels of genetic diversity in this species. These microsatellite markers should provide a more reliable means to understand the population structure and inter relationships in the genus *Piper*.

2.9. Development and utilization of genomic SSR markers in major spice crops

Bory *et al.* (2008) developed fourteen microsatellite primers from di-nucleotide CT/GT enriched genomic library of *Vanilla planifolia* followed by Billotte *et al.* (1999) protocol. Though the markers were found to be monomorphic within cultivated accessions, eleven markers were shown to be polymorphic among two closely related species and some transferable and polymorphic across fifteen other wild American, African and Asian species.

Menezes *et al.* (2009) developed nine microsatellite markers for *Piper nigrum* L. from an enriched library constructed based on the protocol given by Billotte *et al.*, (1999). The markers were tested on twenty varieties of Brazilian germplasm collection. With another protocol 'Sequential Reverse Genome Walking (SRGW)' strategy, Joy *et al.* (2011) developed seven microsatellite markers for black pepper of which four were found to be polymorphic.

Liao *et al.* (2009) reported the isolation and characterization of eleven polymorphic microsatellites loci from *Piper polysyphonum* from the AFLP sequences

containing repeats (FIASCO) proposed by Zane *et al*. (2002) with modifications. The number of alleles per locus ranged from two to ten. Yoshida *et al*. (2014) constructed microsatellite-enriched library for *Piper solmsianum* according to the protocol described by Billotte *et al*. (1999) with modifications. Of the 19 SSR loci derived from the genomic enriched library, 14 were optimized (65%) and nine loci were highly polymorphic. The observed average number of alleles per locus was 3.3, with a maximum of six and a minimum of one. The mean of PIC values was 0.693, and the values ranged from 0.375 to 0.86. These microsatellite markers provide a reliable means to understand the population structure and its relationships in the genus *Piper*.

Eight polymorphic microsatellite markers were developed and characterized from a microsatellite enriched library with modified biotin-streptavidin capture method (Dixit *et al*., 2005) for ginger (*Zingiber officinale* Rosc.) (Lee *et al*., 2007). A total of 34 alleles were found across 20 accessions with an average of 4.3 alleles per locus.

Siju *et al.* (2010) reported 18 genomic microsatellite markers developed for turmeric (*Curcuma longa* L.) from genomic library enriched for microsatellite followed by Glenn and Schable protocol (2005). The estimation of these markers on 20 turmeric accessions generated 103 alleles with an average of 5.7 alleles per locus. The discriminating power of the markers ranged from 0.19 to 0.70.

A total of 16 SSR loci were developed and optimized in garlic (*Allium sativum* L.) from $(CT)_8$ - and $(GT)_8$ - enriched library (Cunha *et al.*, 2012). Ten loci were found to be polymorphic when tested on 75 accessions. A total of 44 alleles were identified, with an average of 4.4 alleles per locus. Earlier eight polymorphic SSR markers were also developed and characterized in garlic by Ma *et al*., (2009).

Lee *et al.* (2004) reported the development of 40 microsatellite markers that showed specific, scorable amplification products and polymorphisms in cultivated and wild *Capsicum* (*Capsicum annum* L.) genotypes. These markers have shown a high level of PIC value, 0.76.

2.10. Development and utilization of genomic SSR markers in other economically important crops

A total of thirty eight microsatellite markers have been isolated in coconut (*Cocos nucifera* L.) from an enriched library based on Edwards *et al*. (1996) protocol (Rivera *et al*., 1999). High levels of polymorphism were detected with an average of 5.2 alleles per locus and genetic diversity values (*D*) from 0.141 to 0.809. Fifteen microsatellite loci was developed from microsatellite enriched genomic library according to Edwards *et al*., (1996) protocol in Tea (*Camellia sinensis*). Average number of allele per locus ranged from 5-13.

Microsatellite markers were developed for castor (*Ricinus communis*L.) from a microsatellite enriched library following the protocol of Billotte *et al*. (2005). Twelve SSR primers were characterized on 30 accessions of castor. The number of alleles observed for each loci ranged from two to five, with an average of 3.3 alleles per locus. (Bajay *et al*., 2009).

Twelve polymorphic microsatellite markers were isolated and characterized for *Amaranthus hypochondriacus* and observed successful amplification in 18 other *Amaranthus* species (Lee *et al*., 2008). A total of 92 alleles were detected across the 20 accessions, with an average of 7.7 alleles per locus.

Golien *et al.* (2005) isolated and characterized seven polymorphic microsatellite loci from two libraries constructed from the genomic DNA non

enriched for repeats (TC and AC) and enriched for AC repeats in Citrus. Twenty three new SSR loci were characterized from $(GA)_n$ and $(GT)_n$ microsatellite enriched library of Gauva (*Psidium guajava* L.) (Risterucci *et al*., 2005). Nine newly developed microsatellite markers were developed and characterized in Grape (*Vitis vinifera* L.) by Goto-Yamamoto *et al*., (2006). A set of 104 microsatellite markers were developed from a microsatellite enriched library constructed from the genotype TMV2 for peanut (*Arachis hypogaea* L.) (Cuc *et al*., 2008). Odeny *et al*. (2009) reported the presence of 20 SSR markers from microsatellite enriched genomic libraries of pigeon pea (*Cajanus cajan* [L] Millsp.). The markers developed were analyzed among *Cajanus* germplasm.

Thirteen microsatellite markers were isolated from SSR-enriched library of Walnut (*Juglans regia* L) using the modified protocol of fast isolation by AFLP sequences containing repeats (FIASCO) (Najafi *et al*., 2014). Successful polymorphism of the 13 primer pairs was observed in various genotypes of *J. regia*. The number of polymorphic alleles ranged from 2 to 4 (with an average of 4.35). The polymorphic information content values ranged from 0.47 to 0.88 (with an average of 0.69). TC/AG and GAA/CTT class of repeats were the most abundant di-nucleotide and tri-nucleotide repeats, respectively.

Microsatellite markers for mango were developed from a genomic library enriched for $(GA)_n$ and $(GT)_n$ dinucleotide repeats (Duval *et al*., 2005). Twenty eight microsatellite primers were found to be polymorphic among 15 mango cultivars. Schnell *et al*. (2005) developed fifteen microsatellite loci based modified version of the method described by Edwards *et al.* (1996), 13 primers revealed polymorphism with 2-7 alleles per locus. Six microsatellite loci were isolated from an AC-enriched genomic library constructed from ‘Irwin’ mango according to the procedure of

Yamamoto *et al*. (2002) by Honsho *et al*. (2005). Twenty one polymorphic SSRs were developed from a non-enriched genomic library using an automated high-throughput system in cashew (*Anacardium occidentale* L.). These markers were transferable across three related tropical tree species (*A. microcarpum*, *A. pumilum* and *A. nanum*) (Croxford *et al*., 2006). Four hundred and sixty six EST SSR markers developed in sunflower (*Helianthus annus* L.) when tested on wild *Helianthus* species 413 markers (88.6%) were capable of producing amplicons in one or more species, whereas 69 markers (14.8%) amplified products from safflower (*Carthamus tinctorius*) and 67 markers (14.4%) generated amplicon products from lettuce (Heesacker *et al*., 2008).

Eleven polymorphic microsatellite markers were developed for pomegranate (*Punica granatum* L.) from enriched library following the protocol of Hamilton *et al.* (1999). Using the 11 markers 44 alleles amplified over 13 loci, with an average of 3.38 alleles per locus. The mean polymorphism information content (PIC) value was 0.433 over 13 loci, which shows that the majority of the microsatellite loci are highly informative (Ebrahimi *et al*., 2010). Pirseyedi *et al*. (2010) added 12 more polymorphic microsatellite markers to the *Punica granatum* germplasm. The microsatellite markers were developed from di- and trinucleotides repeat-enriched genomic library constructed using the fast isolation by AFLP sequences containing repeats (FIASCO) protocol (Zane *et al*., 2002) of *Punica granatum* L. The genetic diversity of these loci was assessed in 60 genotypes of pomegranate. The number of polymorphic alleles per locus ranged from two to five with an average of 2.9. The polymorphic information content ranged from 0.26 to 0.61 (average 0.43). Soriano *et al*. (2011) developed 117 microsatellite loci from a CT/AG enriched pomegranate library using a modified enrichment protocol (Aranzana *et al*., 2002). The

polymorphism information content (PIC) value across all loci ranged between 0.09 and 0.71, with an average of 0.37.

Genomic library was constructed for pineapple (*Ananas comosus* (L.) Merr.) in reference to the SAM method (Hayden and Sharp, 2001) by Feng *et al*. (2013). Out of the 36 primers, 24 of them produced clear and reproducible bands of expected size, and 13 markers showed polymorphism when checked on selected samples.

Nine polymorphic microsatellite loci were developed in cornelian cherry (*Cornus mas* L.) by constructing a microsatellite-enriched library. These markers were used to characterize cornelian cherries from China, central Europe and the United States which amplified perfect and imperfect repeats and 2 to 11 alleles were detected per locus (Wadl *et al*., 2013).

Yang *et al*. (2014) developed and characterized 10 polymorphic microsatellite markers in *Ficus sermentosa* var. *henryi* following the protocol Tong *et al*. (2012). The number of alleles per locus ranged from 2- 15. Sixty nine coffee genomic microsatellite markers were developed from microsatellite enriched genomic DNA library described by Hendre and Aggarwal, 2004; Hendre *et al.*, 2008; Hendre and Aggarwal, 2014). Twenty one microsatellite markers were found to be polymorphic among the tested genotypes.

Ohtsuk i*et al*. (2014) reported the development of microsatellite markers for *Vitex rotundifolia* (Verbenaceae) using an improved technique for isolating codominant compound microsatellite markers (Lian and Hogetsu, 2002; Lian *et al.*, 2006). Out of the 33 primer pairs designed 10 primers were found to be polymorphic among 25 genotypes of *V. rotundifolia*. The number of alleles per locus ranged from one to six.

Maurya *et al*. (2015) developed 1122 microsatellite primers in *Jatropha curcus* with the help of next generation 454 sequencing method. Out of these 1,122 SSRs, 447 (39.83 %) were found to be polymorphic among *J. curcas* accessions. A higher transferability of SSRs (76 %) to *J. integerrima* was also observed. 41 SSRs randomly chosen was amplified across 96 accessions of *J. curcas*, which detected a total of 152 alleles ranging from 2 to 9 with an average of 4.0 ± 1.9 alleles /SSR.

2.11. Genic/EST SSR markers in spice crops

Siju *et al*. (2010) reported the development of robust set of 17 polymorphic EST derived microsatellite markers in turmeric for evaluating 20 turmeric accessions. The number of alleles ranged from 3-8 per loci.

Twenty six EST-SSR markers amplified 130 polymorphic DNA fragments and the number of polymorphic alleles per SSR marker ranged from 2 to 13 with an average of 5 alleles. Observed heterozygosity and polymorphism information content (PIC) of the SSR markers were between 0.23 and 0.88, and 0.20 and 0.87, respectively (Ipek *et al*., 2015).

2.12. Genic/ EST SSR markers in other economically important crops

Scott *et al*. (2000) isolated 124 SSRs from 5000 *Vitis* Expressed Sequence Tags. Ten primer pairs were polymorphic at the level of cultivars, *Vitis* species, and between related genera. SSRs that were from the 3' untranslated region (3' UTR) were most polymorphic at the cultivar level, the 5' untranslated region (5' UTR) SSRs were most polymorphic between cultivars and species, and those SSRs within coding sequence were most polymorphic between species and genera.

Borrone *et al*. (2007) developed seventy informative loci from the expressed sequence tags of 24 *Persea Americana* var. Americana Mill. accessions. Genetic

diversity analysis of sorghum using 40 EST SSR markers revealed high genetic variance (71.7%) suggesting that the germplasm lines included in the set are more diverse (Ramu *et al*., 2013). A total of 360 alleles with an average of 9 alleles per markers were detected in the reference set. The PIC value ranged from 0.1379-0.9483 with an average of 0.5230.

One hundred and six EST based primers were designed from 477 sequences and tested on chickpea (*Cicer arietinum* L.) accessions. Forty four EST markers were polymorphic when screened across nine annual *Cicer* species and seven legume genera (Choudhary *et al*., 2009).

On screening 65000 EST sequences 27,656 non-redundant SSRs were identified, 1918 primer pairs were obtained from that, and 68 primer pairs were designed which could amplify all the *Citrus* accessions. But 22 of these primers revealed polymorphism (Palmieri *et al*., 2007). Kong *et al*. (2006) developed 20 polymorphic SSR markers from cucumber (*Cucumis sativus* L.) ESTs from National Center for Biotechnology Information (NCBI). The average number of allele was 3.3 per locus ranging from 2-6 alleles. Hu *et al*. (2010) developed novel 28 microsatellite markers from cucumber ESTs. The markers showed polymorphism among 21 cucumber accessions with 2-7 alleles per loci (mean- 3.77) with PIC range from 0.091 to 0.748.

A total of 586 microsatellites were identified from 18,552 peas (*Pisum sativum* L.) ESTs from the NCBI database. Out of the 49 primers designed, nine markers were found to be polymorphic that revealed two to three alleles per locus. The polymorphism information content value ranged from 0.18 to 0.58 with an average of 0.41 (Gong *et al*., 2010a). Eleven novel EST-SSR loci were generated and

characterized among 29 faba bean individuals from China and Europe. The number of alleles ranged from 1 to 3 in each population (Gong *et al*., 2010b).

Dong *et al*. (2006) reported unigene sequences from ESTs of sweet orange *Citrus sinensis* Osbeck), trifoliate orange and other *Citrus* species and cultivars were mined for SSRs. 25 primer pair revealed polymorphism within six *Citrus* cultivars. Palmieri *et al*. (2007) developed sixty eight EST SSRs from the unigene sequences of Cit EST database and validated in different *Citrus* spp. and *Poncirus trifoliate*.

One hundred and eighty seven out of 419 expressed sequence tag (EST)-SSR from cassava were polymorphic among the *J. curcas* accessions. The EST-SSR markers comprised 26.20%. dinucleotide repeats, 57.75% trinucleotide repeats, 7.49% tetranucleotide repeats, and 8.56% pentanucleotide repeats, whereas the majority of the G-SSR markers were dinucleotide repeats (62.96%). Thirty-six EST-SSRs were chosen to analyze the genetic diversity among 45 *J. curcas* accessions.

By exploiting 10,829 EST sequences from the databases, 184 primes were successfully designed of which 87 markers produced 272 alleles when tested on 12 *H. brasiliensis* cultivated varieties and four related species (Feng *et al*., 2009). Forty four EST SSR markers were developed for coffee (*Coffea canephora* Pierre Ex A. Froehner) by exploiting 13, 175 unigene ESTs, and all 44 gave amplification and revealed 65.9% to81.8% polymorphism for tetraploid and robusta genotypes.

Nishitani *et al*. (2009) developed 73 SSR sequences from 98 EST sequences which gave clear amplification in the entire pear cultivars tested. Eight hundred and eighty one EST SSRs were identified from 24000 peanut EST sequences, 251 from them could be successfully amplified. Most of these SSRs shown polymorphism in

the wild type peanut; however, there were only a small number of the SSRs showed polymorphism in cultivated peanut (Feng *et al*., 2012).

Kumari *et al*. (2013) identified 5,673 SSRs from 42,477 ESTs of *Jatropha curcas* that include 48.8 % simple and 52.2 % compound SSR motifs. Dinucleotide repeats were the most abundant (26.5 %), followed by trinucleotide (23.1 %) and tetranucleotide repeats (0.8 %). Of the 32 primers developed, 24 primer pairs exhibited polymorphism among 42 accessions giving an average of 2.33 alleles per polymorphic marker. The PIC value ranged from 0.02 to 0.5 with an average of 0.402 indicating moderate level of informativeness within these EST-SSRs markers. Feng *et al*. (2013) identified 22 polymorphic EST derived microsatellite markers for pineapple (*Ananas comosus* (L.) Merr.) by exploiting 5659 pineapple EST sequences obtained from NCBI which could detect 1110 SSR loci.

A total of 182 SSR primer pairs were developed in grape (*Vitis vinifera* L.) for the study on the parental polymorphism. Among the 182 SSR primers, 142 primer pairs (78%) could produce amplicons at expected size range, among which 52 primer pairs (36.62%) showed polymorphism between the two parents. These polymorphic bands were further analyzed among the 94 F1 lines, which generated 162 bands and 98 of them were polymorphic in both parents (60.86%) polymorphism, with an average of 1.88 polymorphic DNA bands for each primer pair. Upon evaluation of chi-square test, 33 of the clearly amplified polymorphic bands followed a 3:1 ratio, and 37 followed a 1:1 ratio which indicated distorted segregation ratios (Kayesh *et al*., 2013).

Kaur *et al*. (2015) developed 43 SSR primers from 2000 EST sequences of peach deposited in NCBI. Thirty-eight primers gave scorable amplification, 20 being

polymorphic. The coefficient values were found to range from 0.483 to 0.711 in six peach genotypes and 0.451 to 0.975 in all 22 genotypes studied.

2.13. Cross transferability of EST SSRs

Genic or EST SSRs can be transferred both to species within a genus and to less extent to multiple genera within a family (Ellis and Burke, 2007). Cross transferability of EST SSRs has been reported in many crop species.

Cross transferability of EST SSR generated from finger millet to pearl millet was reported by Arya *et al*., (2009). Out of the seventeen primers developed 11 were successfully transferred to pearl millet with an average transfer rate of 64.7%. Gasic *et al*., (2009) reported the transferability of 68 apple EST SSR markers tested in 50 individual members of the Rosaceae family, representing three genera and 14 species ranging from 25% in apricot to 59% in the closely related pear. Twenty EST SSRs checked in 17 wild *Fragaria* species displayed high cross transferability with an average of 95%. In sunflower 88.6% of transferability rate with 413 EST SSR markers was reported by Heesacker *et al.* (2008).

The 18 EST SSR markers developed by Siju *et al*. (2010) in turmeric (*Curcuma longa* L.) revealed 100 % cross species transferability when tested on 13 related species. The 14 SSR markers developed in cultivated Vanilla (*Vanilla planifolia* L.) were all transferable to *Vanilla tahitensis* but 11 loci were found to be polymorphic between these two species (Bory *et al*., 2009).

Yuan *et al*. (2013) developed 494 EST SSR primers in *Lilium regale* of which 172 exhibit polymorphism with successful transferability in 16 individual species with 537 alleles in 181 loci. The PIC value for 172 primers ranged from 0.111-0.830 with an average value of 0.493. Out of the 27 genic (EST SSR) markers developed in

Vigna radiate, 97% of them could be successfully transferred to eight important *Vigna* species like cow pea, azuki bean, black gram, rice bean, and moth bean (Gupta *et al*., 2014). High level of transferability was observed in Cassava to *Jatropha curcus* (Wen *et al*., 2010) where they found the level of transferability was higher for EST SSRs (44.63%) than for genomic SSRs (29.67%). This statement supports the previous reports for wheat and related species (Zhang *et al*., 2005), and also for coffee (Aggarwal, 2007) where genic SSR were found to be more efficient in transferability compared to genomic SSRs.

Mishra *et al*., (2011) developed 20 SSR primers in medicinally important plant *Catharanthus roseus*, which revealed high transferability (31-57%) in three medicinally important plants. Of the 44 EST SSR markers developed in Spruce, 25 were widely transferred across spruce species (57%) (Rungis *et al.*, 2004). Seventeen polymorphic EST SSR markers developed in *Curcuma longa* were completely transferred (100%) to 13 *Curcuma* species. (Siju *et al*., 2010 a).

Two hundred and twenty-seven EST-SSRs were evaluated in sugarcane, allied genera of sugarcane and cereals, and 134 of which revealed polymorphism and the cross transferability rate ranged from 87.0% to 93.4% in *Saccharum* complex, 80.0% to 87.0% in allied genera, and 76.0% to 80.0% in cereals (Singh *et al*., 2013).

Tang *et al*. (2006) tested 243 wheat EST-SSR markers, from which 216 (88.9%) produced amplicons in wheat, 211 (86.8%) in barley, 187 (77.0%) in rice and 166 (68.3%) in maize. Zhang *et al*. (2005) reported the transferability of bread wheat EST-SSRs to closely related *Triticeae* species, ranging from 76.7% for *A. tauschii* to 90.4% for *T. durum*. The rates were lower for more distant relatives such as barley (50.4%) or rice (28.3%). Similar results were obtained by Yu *et al*. (2004), who

observed that a total of 53% of the wheat EST-SSR markers produced amplicons in barley.

The polymorphic markers showed 75.56–85. 19% transferability among four species of *Jatropha* and 26.67% transferability across genera in *Ricinus communis*. Investigation of genetic relatedness showed that *J. curcas* and *J. integerrima* are closely related. The observed results indicated the high efficiency of EST-SSRs on cross species/genera amplification and are useful for the identification of genetic diversity of *Jatropha* and its close taxa and to select the desired related species for wide crossing to improve new varieties of *Jatropha* (Laosatit *et al*., 2013).

The cross species transferability rate of the markers ranges 50-100 % across eight species. The utility of these markers was assessed in eight *Lavandula* species (Adal *et al*., 2014). More than 80% of EST SSR markers developed in pear could be transferred to apple which belongs to the subfamily Maloideae and 30% of the pear EST SSRs was also transferable to almond and peach, in the subfamily, Prunoideae (Nishitani *et al*., 2009). Kaur *et al*. (2015) developed 43 SSR primers from 2000 EST sequences of peach. Thirty-eight primers showed scorable amplification, 20 primers being polymorphic which were then used to carry out transferability studies in apricot, apple, rose and strawberry revealing 50%, 95%, 95% and 45% transferability, respectively.

Cross-genera transferability of simple sequence repeat (SSR) markers among three economically important plants of family Euphorbiaceae has been proposed. A set of SSR loci generated from cassava (199), rubber tree (49) and physic nut (42) were used to determine transferability with five accessions each of cassava, rubber tree and physic nut. The highest transferability (59.18%) was observed from cassava to rubber tree, followed by rubber tree to cassava. Low transfer rates were found

between cassava and physic nut, and between rubber tree and physic nut. These identified transferable markers for cassava, rubber tree and physic nut (37, 61 and 46, respectively) will be useful for comparative mapping and genomic studies (Whankaew *et al*., 2013).

The 20 polymorphic microsatellite markers developed in peach (*Prunus persica* L.) (Kaur *et al*. (2015) were taken to carry out transferability studies in apricot, apple, rose and strawberry which displayed 50%, 95%, 95% and 45% transferability, respectively.

2.14. Cross transferability of genomic SSRs

Out of the six microsatellites, three polymorphic and three monomorphic, have been characterized for the first time in 18 bamboo species (Nayak and Route, 2005). Monomorphic simple sequence repeats (SSRs) have been found to be cross amplified in most of the tested species while polymorphic ones in only three to four species. The number of alleles per locus ranges from 2 to 13. Allelic diversity ranges from 0.041 to 0.870. Polymorphic information content (PIC) values for two were > 0.3.

Cross transferability of sugarcane genomic microsatellites in other *Saccharum* species and five cereal species showed a transferability rate of 93.2% (Parida *et al*., 2009). Twelve polymorphic microsatellite markers developed for *Amaranthus hypochondriacus* were successfully amplified in 18 other *Amaranthus* species representing cultivated grain and vegetable species, their putative progenitors and wild species (Lee *et al*., 2008).

Watcharawongpaiboon and Chunwongse (2008) developed fourty five SSR markers using library enrichment procedure in cucumber. Some of the markers were transferable to other *Cucurbit* species (melon, watermelon, pumpkin and bitter gaurd).

Twenty three new SSR loci were characterized from (GA)n and (GT)n microsatellite enriched library of *Psidium guajava* L. that also showed transferability to three other *Psidium* species.

Menezes *et al*. (2009) reported that out of the nine microsatellite markers developed in black pepper (*Piper nigrum* L.), five markers were successfully transferred to four distinct species *viz*., *P. attenuatum*, *P. hispidinervium*, *P. tuberculatum* and *P. colubrinum*. Cross transferability of 17 genomic SSR markers evaluated in fourteen *Phoenix* (date palm) species resulted in complete transferability in all the species tested, indicating the conservation of priming sites flanking the microsatellite loci (Akkak *et al*., 2009).

Out of the forty nine microsatellite markers developed in *Jatropha curcus* L., 391 markers showed cross species amplification in six sister taxa (*J. glandulifera, J. gossypifolia, J. integerrima, J. multifida, J. podagrica* and *J. tanjorensis* (Sudheer *et al*., 2011). Twenty EST SSR tested in 17 wild *Fragaria* species showed high cross species amplification (95%). Sixty microsatellite markers (30 genomic and 30 ESTSSRs) in wild *Allium* species revealed 10 % genomic SSRs and 53.3% EST SSRs cross transferability (Khar *et al*., 2011). Gao *et al*. (2005) reported the cross transferability of 60 rice SSR markers to three wild rice species *O. rufipogon* (100%), *O. officinalis* (90%), *O. granulate* (73.3). Garcia Moreno *et al*. (2010) developed 119 microsatellite markers from sunflower (*Helianthus annus* L.), 17.6% of these markers were transferable to 22 lines and germplasm accessions of safflower (*Carthamus tinctorius* L.). Kumar *et al*. (2011) studied the transferability of eight microsatellite loci in three mangrove species (*Rhizophora annamalayana, R. apiculata* and *R.mucronata*), all were successfully amplified and polymorphic. Sixteen alleles were generated in eight loci. The number of alleles for overall species was 0.3333 per

locus and for overall locus was 0.2619 per species. From the data it was observed that *Rhizophora annamalayana* and *R. mucronata* were found genetically much closer (0.006) than *R. apiculate* (0.008).

Twenty three simple sequence repeat (SSR) primer pairs developed for guava (*Psidium guajava* L.) were transferred to two species of eucalyptus (*Eucalyptus citriodora*, *Eucalyptus camaldulensis*), bottlebrush (*Callistemon lanceolatus*) and clove (*Syzygium aromaticum*), belonging to the family Myrtaceae and subfamily Myrtoideae. Of the 23 SSR loci assayed, 18 (78.2 %) gave cross-amplification in *E. citriodora*, 14 (60.8 %) in *E. camaldulensis* and 17-17 (73.9 %) in *C. lanceolatus* and *S. aromaticum*. Eight primer pairs were completely transferable to all four species (Rai *et al*., 2013).

Oliviera *et al*. (2013) analyzed the cross transferability of 41 SSR markers developed for yellow passion fruit (*Passiflora edulis* f. *flavicarpa* Sims) to 11 different *Passiflora* species. The mean transferability observed was 68.8%, the transferability was higher for the *Passiflora* sub genus compared to other tested sub genus. Sathya and Jayamani (2013) used a set of thirty-five microsatellite primer pairs derived from adzukibean (*Vigna angularis* (Willd.) Ohwi & Ohashi) to estimate the transferability in green gram and related *Vigna* species. Thirty-two were successfully amplified across the thirty six genotypes and twenty eight primers revealed polymorphism. A total of 83 microsatellite alleles were generated out of it with an average of 2.96 alleles per locus. Ohtsuki *et al*. (2014) reported that the thirty three microsatellite markers developed for *Vitex rotundifolia* were successfully transferable to *V. trifolia* and seven pairs showed polymorphism.

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

3. 1. Plant materials

3.1.1. Plant materials selected for microsatellite enriched genomic DNA library construction

The most popular black pepper variety Panniyur1, maintained at Indian Institute of Spices Research (IISR), Kozhikode, was selected for black pepper genomic DNA library construction enriched for microsatellites.

3.1.2. Plant materials selected for characterization and genotyping of microsatellite markers

3.1.2.1. Cultivated types of black pepper

Thirty nine cultivated types of black pepper including 16 released varieties and 23 important cultivars (Table1, Fig. 1-5) which represent maximum diversity in black pepper germplasm maintained at the conservatory of IISR were selected for the present study.

3.1.2.2. *Piper* species

Twenty one *Piper* species (Table 2, Fig. 6-8) collected from Western Ghats, North East region of the country and Exotic species were used to study the utility of microsatellite markers.

Table 1. Black pepper varieties and cultivars selected for the study and their salient features

Sl. No.	Variety name	Collection Number	Place of collection	Salient features
1	Panniyur 1	874	Kannur, Kerala, India	Hybrid of Uthirankotta x Cheriyakaniakadan. High yielding, Long spikes bearing large berries, early bearing in nature and performs well in open conditions. Adaptable to all pepper growing areas, except under heavy shade.
2	Panniyur 2	4127	Kannur, Kerala, India	Selection from cv. Balancotta. Found to be tolerant to shade. Widely adaptable to all pepper growing areas in Kerala.
3	Panniyur 3	4128	Kannur, Kerala, India	Hybrid of Uthirankotta x Cheriyakaniakadan; Late maturing in nature and performs well in open conditions. Vigorous in growth. Apt to all pepper growing areas in Kerala.
4	Panniyur 4	4129	Kannur, Kerala, India	Selection from cultivar Kuthiravally. Performs well under partial shade. Stable yielding in nature, Fit to grow in all pepper growing areas in Kerala.
5	Panniyur 5	4130	Kannur, Kerala, India	Open pollinated progeny of Perumkodi. Adaptable to all pepper growing areas. Found to be tolerant to shade and nursery diseases. Suitable as an intercrop for arecanut gardens.

6	Panniyur 6	-	Kannur, Kerala, India	Clonal selection from cv. Karimunda. Suited to all black pepper growing areas. Performs under partial shade and open cultivation.
7	Panniyur 7	-	Kannur, Kerala, India	Open pollinated progeny selection from Kuthiravally. Vigorous and hardy in nature with a regular fruit bearing capacity. Suitable for Kerala under open conditions as well as partial shade.
8	Subhakara	KS27	Palakkad, Kerala, India	Selection from cv. Karimunda (KS 27).Adaptable to all black pepper growing tracts in Kerala and southern Karnataka. Produces high quality berries.
9	Sreekara	KS14	Kozhikode, Kerala, India	Selection from cv. Karimunda (KS 14); Suitable for all black pepper growing areas of varying conditions.
10	Panchami	856	Wayanadu	Selection from cv. Aimpiriyan (coll 856). Late maturing in nature. Suitable for all areas of Kerala, except drought prone regions.
11	Pournami	812	Idukki, Kerala, India	Selection from Ottaplackal (coll 812). Exhibits root knot nematode tolerance. Adaptable to all pepper growing regions of Kerala.
12	PLD-2	5085	Thiruvanathapuram, Kerala, India	Clonal selection from cv. Kottanadan; Suitable for Thiruvananthapuram and Kollam districts of Kerala.

13	IISR Sakthi	5009	Kozhikode, Kerala, India	Open pollinated progeny of cv. Perambramundi. Tolerant to Phytophthora foot rot disease.
14	IISR Thevam	1041	Idukki, Kerala, India	Clonal selection of cv. Thevanmundi. Vigorous in growth with broad leaves. Field tolerant to Phytophthora foot rot disease.
15	IISR Girimunda	Hybrid	-	Hybrid of Narayakodi x Neelamundi; Suitable for high altitude areas.
16	IISR Malabar Excel	Hybrid	-	Hybrid of Cholamundi x Panniyur 1.Suitable for high altitude areas. Berries are enriched with oleoresins.

Sl. No.	**Cultivar**	**Collection Number**	**Place of collection**	**Salient features**
1	Kottanadan	959	Kannur, Kerala, India	Morphologically resembling Aimpirian, grows vigorously, having broad leaves with even margin. Performs well in plain and hilly regions up to an altitude of 700-800m. High yielding and widely adapted; produces high quality berries.
2	Neelamundi	809	Idukki, Kerala, India	Vines are vigorous in nature with medium/ large ovate shaped leaves with even margins. Tolerant to Phytophthora foot rot disease. Found suitable for high elevation areas with moderate yielding capacity with medium quality berries.

3	Kuthiravally	849	Kannur, Kerala, India	As the name resembles, spikes looks like long horse tail, leaves are ovate in shape and medium large with even margin. Stable yielder with high quality good fruit setting capacity.
4	Kalluvally	984	Kozhikode, Kerala, India	Plants are hardy medium sized and hairy in nature. Leaves looks round elliptical in shape and dark green in colour with even margins. Gives medium yield and quality.
5	Narayakodi	1601	Pathanamthitta, Kerala, India	Moderately vigorous vines. Leaves size ranges from small to medium with wavy margins and twisted leaf blade. Berries possess persistent stigma. Found to be field tolerant to Phytophthora foot rot. Fruits are small in size with medium yield and quality.
6	Perambramunda	1240	Wayanad, Kerala, India	Medium long spikes with bold berries; medium in yield and quality. Resembles Neelamundi in morphology.
7	Poonjaranmunda	832	Idukki, Kerala, India	Leaves are broadly ovate in shape with a cordate base; long spike with moderate yielding capacity.
8	Valiakaniakkadan	884	Kozhikode, Kerala, India	Medium to long spikes in length with bold berries which gives a medium yield.
9	Cheriakaniakkadan	924	Ernakulam,	Leaves are small and lanceolate in shapes with an acuminate tip.

			Kerala, India	Spikes are short and bearing small fruits. Shows early maturity with poor yielding and medium quality.
10	Uthirancotta	929	Kozhikode, Kerala, India	Predominately female; poor in yield.
11	Balankotta	1000	Kozhikode. Kerala, India	Vigorous growing nature. Leaves are very large and elliptical, slightly drooping and branches with even margins. Tolerant to Shade. Medium in yield with bold fruit of medium quality.
12	Arakkulam munda	894	Wayanad, Kerala, India	Large leaves with ovate shape and wavy margin. Early maturing variety with medium yield and quality.
13	Thommankodi	966	Kozhikode, Kerala, India	Ovate to widely ovate leaves seen in the main stem. Long spike with good fruit bearing. Fruits medium and globose nature. Good yielding with quality berries. Morphologically resembles Kuthiravally.
14	Thevanmundi	1041	Idukki, Kerala, India	Moderately large leaves and medium length spikes with good fruit bearing capacity. Berries looks medium oblong in shape gives good yield and medium quality.
15	Chumalakodi	5029	Wayanad, Kerala, India	Medium ovate shaped leaves and short to medium length spike. Medium sized fruits give medium yield and quality.

16	Nedumchola	1058	Wayanad, Kerala, India	Bearing the smallest leaves among the cultivars with round base. Very short spike (4-6 cm) sets very small berries. Poor in yielding.
17	Malamundi	805	Idukki, Kerala, India	Leaves ovate with round base. Medium length spikes with small peduncle. Bisexual flowers; medium sized fruits with good setting capacity.
18	Karimkotta	844	Kannur, Kerala, India	Hardy in nature. Common cultivar of Malabar with poor yielding capacity.
19	Perumkodi	803	Idukki, Kerala, India	Ovate to ovate elliptic shaped leaves. Medium length spikes exhibits poor fruit setting. Bold fruits with medium quality attribute.
20	Karimunda	815	Idukki, Kerala, India	Leaves ovate and small with even margins. Dark green leaves and spikes. High spiking intensity with good yielding capacity with medium quality berries. Very popular cultivar with wide adaptability. Shade tolerant in nature.
21	Kumbhachola	1114	Kozhikode, Kerala, India	A cultivar reported as pollu beetle resistant and drought tolerant
22	P24		Kozhikode,	Open pollinated progeny of cv.Perambramundi. Tolerant to

		Kerala, India	Phytophthora foot rot disease.
23	O4-P24-1	Kozhikode, Kerala, India	Open pollinated progeny of IISR Sakthi, moderately resistant to *Phytophthora capsici* infection

Table 2. *Piper* species selected for the study and their botanical description.

Sl. No:	***Piper* Species**	**Collection Number**	**Place of collection**	**Botanical description**
1	*P. betle* L.	5473	Thiruvananthapuram, Kerala, India	A perennial dioecious creeper; 5-20 cm long leaves, broadly ovate, slightly cordate and often unequal at the base, shortly acuminate, acute and entire, with often an undulated margin, glabrous, yellowish or bright green, shining on both sides; spikes pendent and filiform; fruits rarely produced, often sunk in the fleshy spike, forming a nodule like structure. Native of Malaysia, cultivated extensively in India. Wild forms reported from Nilambur forests of Kerala and Kolli hills of Tamil Nadu.
2	*P. nigrum* L.	6426	Palakkad, Kerala, India	A woody climber with swollen internodes found growing almost 1300 m MSL. Runner shots arise from the base. Leaves simple, thick, glabrous and petiolate, size ranges from 10-24 cm, lamina ovate, elliptic or elliptic lanceolate. Base acute to cordate. Wax glands are present on the lower leaf surface and on young shoots.

				Spike pendent, filiform with green, greenish white or purple in color and long glabrous. Unisexual in wild but bisexual in cultivated. Bracts copular with decurrent base. Stamens two, style absent, stigma three to five lobed. Ovary superior. Fruit is green when young and changes to red on ripening. Seeds spherical to ovate and bitter- pungent. Commonly used as spice besides in the indigenous medical system.
3	*P. sugandhi* Babu et. Naik.	6021	Wayanad, Kerala, India	A stout woody climber, dioecious and perennial, reaching to a height of 10m or more; branches terete, swollen at the nodes, glabrous, orthotropic shoot tips purple; leaves alternate, glabrous, ovate-lanceolate, acuminate, base round to acute and often oblique, margins slightly wavy, male spikes slender, fleshy, filiform and pendant or recurved, female spike slightly thicker than male spike flower held at right angles to the rachis, stipulate, stamen two, filaments short and thick. Aligned to *P. nigrum* but differ from it in having stipulate flowers and deeply copular bract, alinged to *P. galeatum*, but differs from it in having the pungent fruit as in black pepper, also aligned to *P. trichostachyon*, but differs from it in having stipitate flowers, nature of bract and in having pungent fruit (Ravindran *et al*., 1990).
4	*P. trichostachyon* C.D.C.	639	Wayanad,	A large woody dioecious climber very closely resembles to *P.*

			Kerala, India	*galeatum* but spikes are slightly hairy. Occurs at medium elevations upto 1000 m MSL. Leaves simple, alternate and petiolate, five nerved, glabrous and thick. Ovate leaf shape, round leaf base, glabrous and acuminate tip; Pendent, filiform, hairy spike; hirtellous decurrent, connate bracts forming a fleshy cup; unisexual flowers, two stamens, short and bilobed; superior ovary, three lobed stigma; very bold fruits, spherical to oblong and bitter in taste; on ripening color changes from green yellow, then to orange.
5	*P. galeatum* C.D.C.	4577	Tamil Nadu, India	A woody unisexual climber with smaller nodes found at medium elevations of 500-800m. MSL in the Western Ghats. Leaves simple, alternate and petiolate, glabrous and thick. Lamina ovate, lanceolate to ovate elliptic, leaf base round, veining acrodomous. Spike pendent, filiform with green to greenish yellow in color, 6-8 cm long with loosely arranged flowers. Spike glabrous, branches prominent and fleshy. Male spike long, sometimes reaching up to 25 cm, bracts prominent, connate, forming a fleshy cup or boat shaped structure, shortly stipulate and recurved, glabrous, style absent, stamens two, carpel single, ovary obovate, stigma 3-4 lobed. Fruits bold round green turn yellow on ripening and then orange red. (Ravindran *et al.*, 1990).
6	*P. barberi* Gamble.	613	Tamil Nadu,	A perennial dioecious climber and spike born on slender very long

			India	dangling peduncle. Runner shoots aphyllate. Leaves glabrous, lanceolate with pinnately reticulate venation, dimorphic and slightly serrate. Juvenile shoots slender with persistent scale leaves, orthotropic shoots have small leaves, 5-7 cm long and 2.0-5.0 cm broad. Tip acuminate. Male spikes narrow, 7-10 cm, female spikes 4-7 cm, cylindrical, pendulous, borne on long, slender peduncle, bractspeltate, orbicular. Flowers unisexual. Ovary 0.5-1.0 mm, sessile, one celled one ovuled, stigma 3 lobed papillate, style absent. An endangered species, not related to any other known South Indian species.
7	*P. attenuatum* Buch.Han.ex Wall.	4634	Kottayam, Kerala, India	A unisexual climber occurring almost all the forests of Western Ghats up to an elevation of 1000m MSL. Leaves simple, thin. Glabrous and small lamina shape ovate to ovate elliptic, veining acrodromous with seven nerves from the base, the outer pair reaching only half to two third of the leaves. Petiole long, tip acuminate and glabrous. 7-ribbed from the base, spikes thin, long, filiform, pendant, female 7-15 cm long, male 8-18 cm long, peduncle about 2 cm, glabrous (Rahiman and Nair, 1987). Fruits are oblong to round and bitter in taste.
8	*P. argyrophyllum* Miq.	5369	Kollam, Kerala, India	A slender, scandent, perennial climbing shrub, dioecious, main stem and branches glabrous, young shoot puberlous, leaves thin,

				ovate to elliptic, petiolate, lower side of the leaves slight silvery, base round to cordate, younger leaves often minutely hairy, especially along the veins on the lower side, petiole about 1-1.5 cm, grooved, glabrous or puberlous. Spikes thin, filiform, pendant and unisexual. Bracts sessile, oblong and adnate to the rachis. Female flowers are sessile and male flowers are slightly stipitae. Stamens three, stigma four lobed and style absent. Ovary superior, fruits oblong, deciduous, and bitter in taste, turns black on ripening. Morphologically similar to *P. attenuatum* but differ from it in having 5-nerved nature of leaf base and shorter, greenish white fruiting spikes and silvery scales on the underside of the leaves (Rahiman and Nair, 1987).
9	*P.hymenophyllum* Miq.	644	Wayanad, Kerala, India	Ascandent, slender climber, dioecious having prominently pubescent branchlets and leaves, hairs more prounced on the young shoots. Found in Western Ghats forests up to elevation of 750 m altitude. Leaves thin and broad, lamina shape ovate to ovate elliptic or elliptic to lanceolate with cordate or acute base, veining acrodomous, tip acuminate, hairy all over the leaf and young shoots, Seven nerved with 2-3 pairs arising from the leaf base or near to it. Spike thin long and pendent. Male spike 5-15 cm, female spike 6-18 cm in length, peduncle hairy, bracts sessile, oblong and adnate to the rachis, stamens three, style absent, stigma 3-4 lobed,

				recurved and pappillate. Fruits oblong or sometime round. Seeds are bitter, turns black when ripens. (Ravindran *et al*., 1990).
10	*P. bababudani* Rahiman.	5396	Karnataka, India	A hardy climbing vine with dark purple shoot tip color; erect lateral branch with around 42 cm length, ovate leaf lamina, round leaf base, acrodromous type of veining, glabrous, coriaceous leaf texture; spike filiform glabrous white in color, 10.8 spike length; pistillate flowers; deeply copular with decurrent bract; round fruit, initially bitter then pungent taste, large round fruit, green to yellow and orange while ripening; male spike 12.1 cm long, two stamen, short filament.
11	*P. longum* L.	5565	Kannur, Kerala, India	A slender, aromatic, creeping, dioecious, occurring in the lower elevations of WesternGhats and other parts of India. Vegetative branches creep and spread on the ground; fruiting branches erect, young branches puberlous; hairs minute and multicellular, older branches glabrous, leaves are distinctly dimorphic; petiolate upto 3 cm long, cordate with acute leaf base and acrodromous type of veining and elliptic, lanceolate leaves, base unequally cordate with pronounced auricle in fruiting branches. Leaves adnate or shortly petiolate, Spike cylindrical, erect creamy white to yellowish white when young. Peduncle 1-2 cm long, female spike2-3 cm long, male spikes 6-10 cm long, yellow on maturity. Bracts peltate, orbicular,

				glabrous. Flowers bracteates, laterally fused, stamens 3-4, carpel single, style absent, stigma four lobed, short pappilate. Fruits very small, laterally fused, spicy and pungent, fruits turn green to black when ripen, seeds very small. Dried spikes and fruits are extensively used in indigenous medical systems (Rahiman and Nair, 1987).
12	*P. hapnium* Buch.-Ham.	5501	Kollam, Kerala, India	A slender, dioecious climber occurring in low elevations of Western Ghats and other parts of India. Vegetative branches creep and spread on the ground, fruiting branches are erect. Leaves petiolate with prominent ribs from the base. Nerves on the lower side of young leaves are pubescent with minute deciduous. Mature leaves glabrous; acuminate leaf tip, seven nerved, shortly petiolate and campylodromous veining. Spike is cylindrical and thick. Male spikes are slender 4.5-5 cm long and sometimes sickle shaped and brown colored. Female spikes are about 5.0-5.5 cm long. Peduncle about 2-2.5 cm long. Flowers unisexual subtended by peltate orbicular bracts, laterally fused and sessile. Stamens 2, stigma 3-5 lobed. Fruits are very small, turn green to black when ripen. Dried spikes are used as long pepper in the indigenous system of medicine. An endangered species listed in the red data book.
13	*P. peepuloides* Roxb.	5526	West Bengal,	A slender climbing shrub with free spreading slender branches.

			India	Glabrous, branches rigid terete often warted. Leaves membraneous, shortly petioled, very uniform oblong linear- or ovate- oblong caudate- acuminate, base rounded 3-5 nerved nearly to the tip, 5-12.5 by 2.5-5 cm., variable in breadth, sometimes narrowed to a minutely cordate base; petiole of upper leaves 0.31-0.63 cm., of lower rarely 1.25 cm. Erect spike, male spikes slender, 5-7.5 cm long with peltate bracts; stamens 2-4; female 1.25-1.67 cm, cylindrical longer than their peduncle. Fruits minute and 0.21 cm in diameter.
14	*P. sarmentosum* Roxb.	5466	Andaman Nicobar Islands, India	Monoecious, normally small shrubs, 30 cm tall, sometimes climber, all parts glabrous. Petiole 1-2.5 cm long; leaf blade thin to thick chartaceous or papery, light to dark green, broadly ovate to elliptic, 4.5-6 cm wide, 7.5-9.5 cm long; apex acute; leaves on epiphytic branches base deeply equally cordate with rounded lobes, leaves on free branch base cuneate to subtruncate; veins 7, all basal. Erect spike. spike with male and female flowers together straight up, cylindrical, 1-1.5 cm long, 0.3-0.5 cm in diameter; peduncle ca. 1.5 cm long; bract rounded; stamen 1; stigmas 3- 4. Female spike white cylindrical, other characters are as above. Fruiting spike 1-2 cm long, 0.5-1 cm in diameter. Flowering on year round, many in rainy season.

15	*P. ribesioides* Wall.	5525	West Bengal,India	A stout climber, thick branches deeply furrowed when dry. 20-30 cm long leaves, very coriaceous linear or ovate to oblong acuminate base, deeply cordate, 5-9 nerved at the very base, 3 nerved higher up, petiole 2.5 – 5 cm, very strong nerves, slender nervules; very stout petiole; young leaves small, lanceolate, cordate, short, stout fruiting spike, glabrous pedicel; 2.5-7.5 cm long spikes, rachis of spike stout rigid; short coraceous bracts; fruits 1.25 cm in diameter.
16	*P. thomsoni* Hook.	5528	West Bengal, India	A scandent, bushy dioecious species; propagation is either through seeds or runners. Male spikes long, erect and filiform (5-11 cm), female spikes short, erect and sub globose (1.5- 2.0 cm) with 30-40 closely packed minute berries subtended by peltate bracts; endemic to North Eastern Himalaya.
17	*P. hamiltonii* C.D.C.	5536	Andaman Nicobar Islands, India	A climbing shrub often creeping on the ground, glabrous, branches subterete or angular, petioled, coraceous, elliptic or almost rounded leaves, 5-7 nerved from above the base, 5-705 by 2.5-12.5 cm long, pale when dry, strong nerves, nervules transverse, base nearly equal, petiole 1.25-2.5 cm long; orbicular, ciliate bracts; fruiting spikes slender, fruit ovoid, male spikes slender, 3.75-7.5 cm; fruiting spikes 10-15 cm, flexuous; 1.25 cm peduncle; fruits rachis glabrous except the ciliate pits.

18	*P. colubrinum* Link ex Kunth Link ex C.D.C	392	Kannur, Kerala, India	A distantly related species from South America which is resistant to foot rot disease caused by *Phytophthora capsici* and as well as *Radopholus similis*. Fused berries, elliptical, spike length 60 mm, conical and glabrous spikes, peltate bract.
19	*P. arboretum* Aubl.	3363	Kannur, Kerala, India	An evergreen shrub or a small tree native to South America; usually grows from 2 - 8 m tall, occasionally to 11 m; spike length 90 mm, erect filiform, glabrous spike, peltate orbicular bracts, fused and elliptical berries.
20	*P. ornatum* N.E.Br.	3362	Wayanad, Kerala, India	Climbing vine can grow to about 15 feet; heart-shaped leaves up to 13cm (5 inch) long and 10cm (4 inch) wide. The leaves have reddish, 2cm (0.8 inch) long leaf-stalks, which are attached not at the end of the leaf, but slightly toward the middle, upper leaf surface are mottled pattern of green, pink while the underside is purple red. *P. ornatum* is a vine endemic to Peru, South America.
21	*P. magnificum* Hort.ex.Gentil.	5816	Wayanad, Kerala, India	A small, erect shrub having winged stem, leaves ovate to broadly elliptic or suborbicular, apex broadly acute, base cordate to auriculate, deep green above, glossy, bright maroon beneath. Petiole broadly winged. Origin in Peru, now cultivated extensively as an ornamental foliage plant.

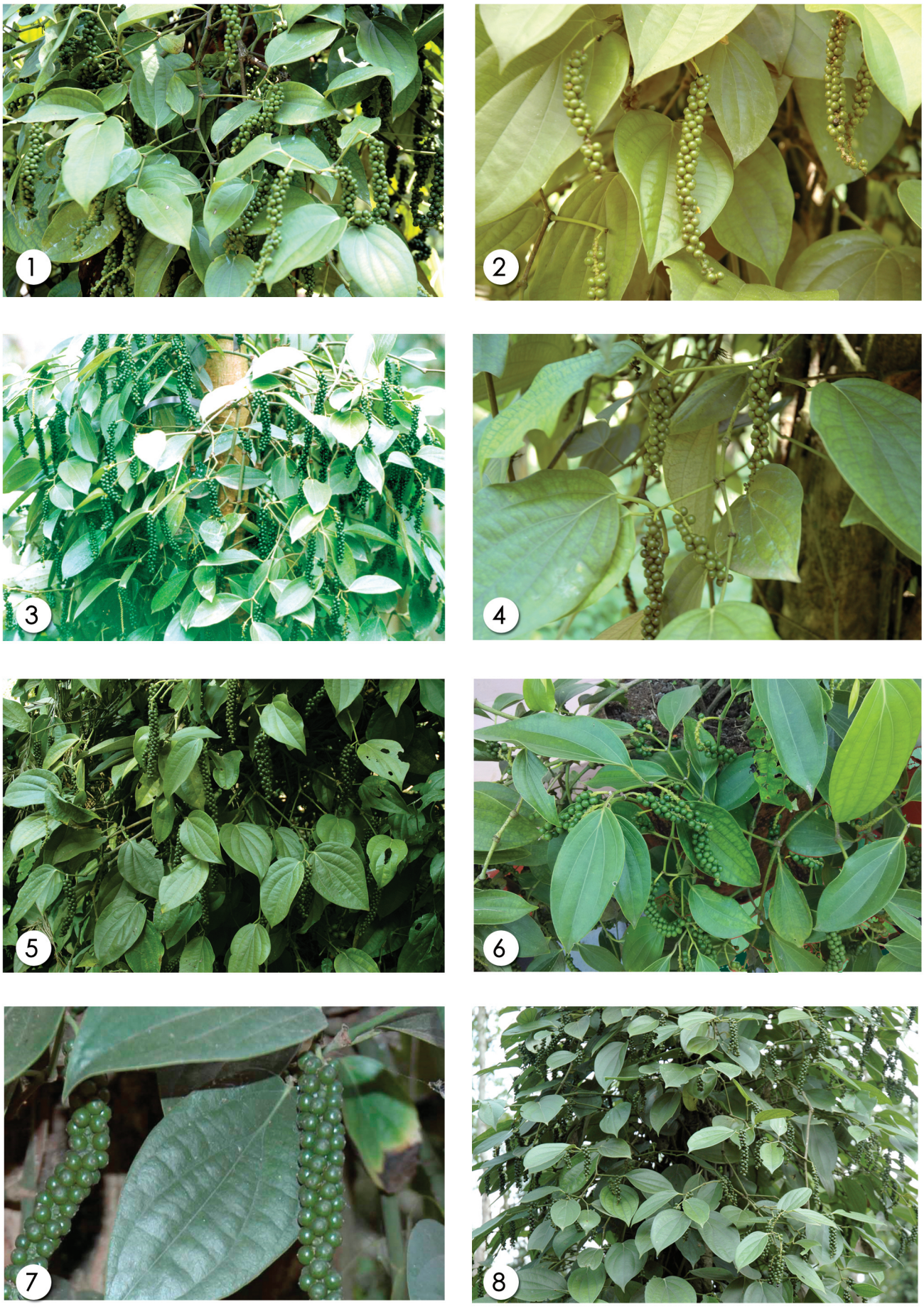

Fig. 1. Sixteen released varieties of black pepper. 1. Panniyur-1, 2. Panniyur-2, 3. Panniyur-3, 4. Panniyur-4, 5. Panniyur-5, 6. Panniyur-6, 7. Panniyur-7, 8. Subhakara

Fig. 2. 9. Sreekara, 10. Panchami, 11. Pournami, 12. PLD- 02, 13. IISR Sakthi, 14. IISR Thevam, 15. IISR Girimunda, 16. IISR Malabar Excel

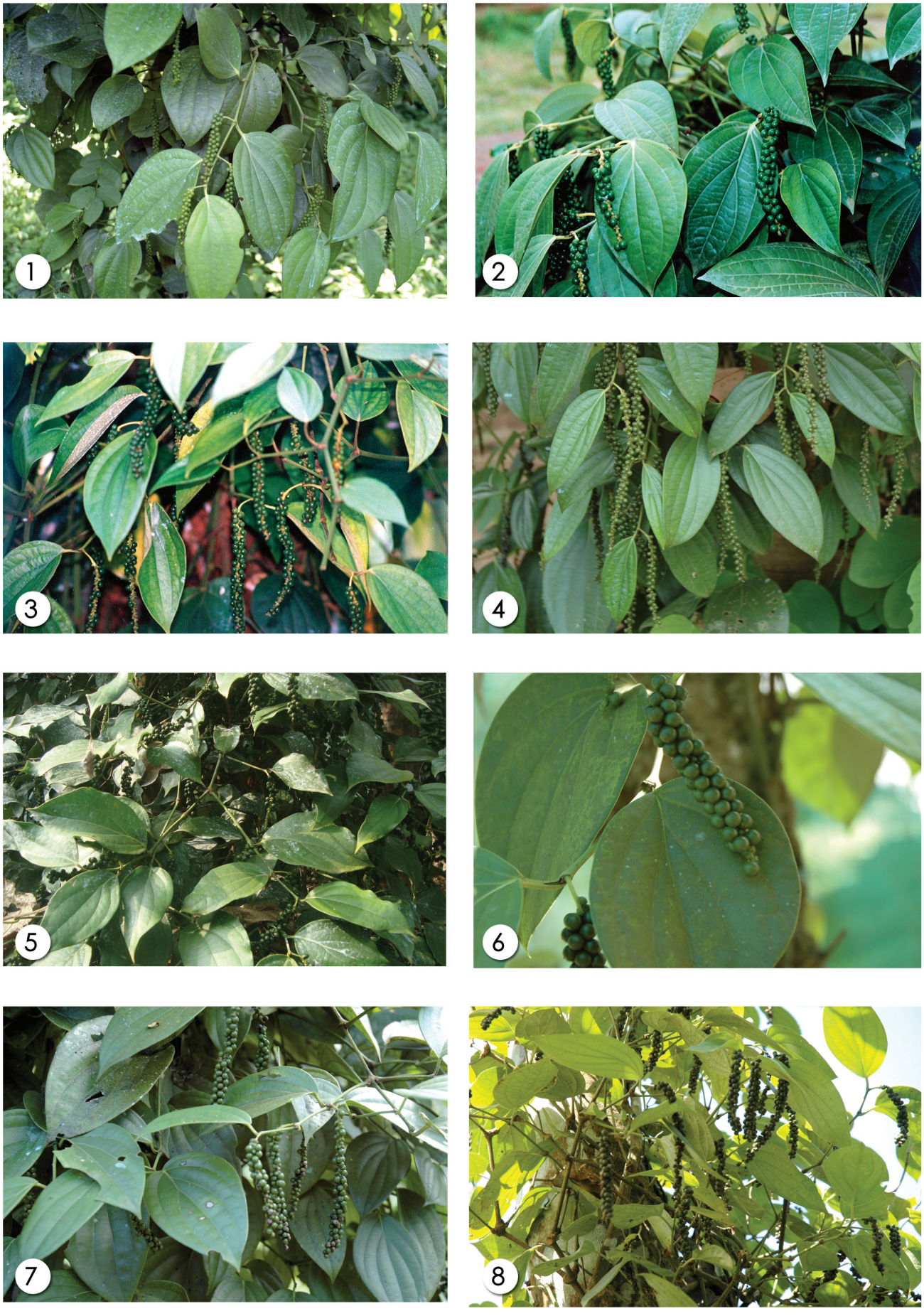

Fig. 3. 23 cultivars of black pepper. 1: Kottanadan, 2: Neelamundi, 3. Kuthiravally, 4: Kalluvally, 5: Narayakodi, 6: Perambramunda, 7: Poonjaranmunda, 8: Valiakaniakkadan

Fig. 4. 9: Cheriyakaniakkadan, 10: Uthirancotta, 11: Balancotta, 12: Arakulam munda, 13: Thommankodi, 14: Thevanmundi, 15: Chumalakodi,16: Nedumchola

Fig. 5. 17: Malamundi, 18: Karimkotta, 19: Perumkodi, 20: Karimunda, 21: Kumbhachola 22: P 24 (IISR Sakthi), 23: P 24 O4 (O4-P 24-1)

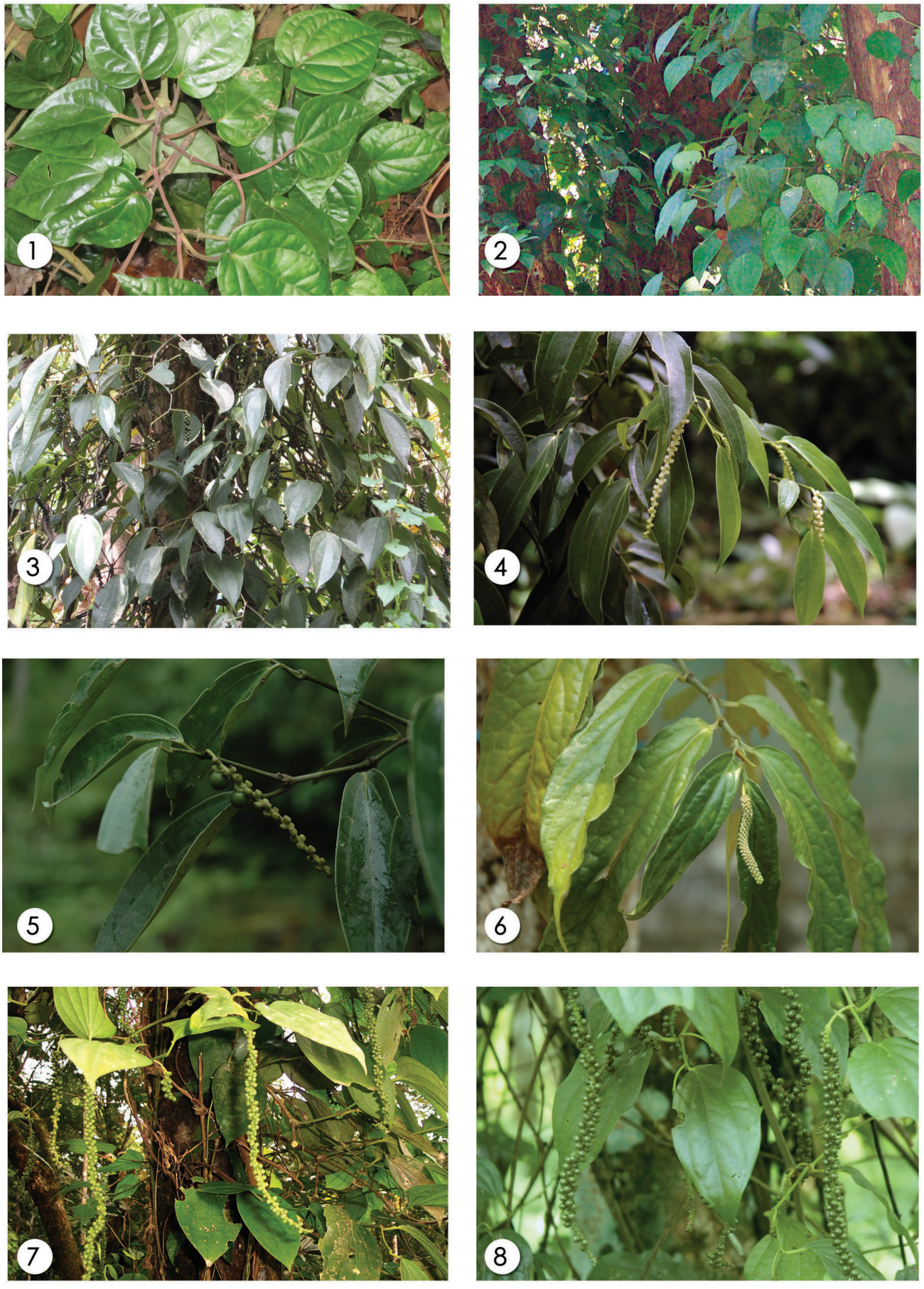

Fig. 6. 21 *Piper* species 1. *P. betle*, Lane 2: *P. nigrum*, 3. *P. sugandhi*, 4: *P. trichostachyon*, 5: *P. galeatum*, 6: *P. barberi*, 7: *P. attenuatum*, 8: *P. argyrophyllum*,

Fig. 7. 9: *P. hymenophyllum*, 10: *P. bababudani*, 11: *P. longum*, 12: *P. hapnium*, 13: *P. peepuloides*, 14: *P. sarmentosum*, 15: *P. ribesioides*, 16: *P. thomsoni*.

Fig. 8. 17: *P. hamiltonii*, 18: *P. colubrinum*, 19: *P. arboreum*, 20: *P. ornatum*, 21: *P. magnificum*.

3.2. DNA extraction and Quantification

3.2.1. Extraction of high quality genomic DNA

Presence of high concentrations of polyphenolic compounds and secondary metabolites are the major trouble associated with genomic DNA extraction of black pepper. High quality genomic DNA was extracted from leaf samples of 16 released black pepper varieties, 23 cultivars and 21 *Piper* species. DNA extraction protocol described by Doyle and Doyle (1990) with modifications was used. The reagents required for this protocol is described in Appendix- Ia.

The DNA extraction protocol is as follows:

- Two grams of fresh leaf tissue was ground in pre- chilled mortar and pestle using liquid nitrogen and the powdered tissue was transferred to a 50 ml oakridge tubes containing 10 ml preheated CTAB extraction buffer containing 0.5% β-mercaptoethanol.
- The homogenate was incubated at 65 ^{0}C for 60 min with occasional mixing.
- An equal volume of Phenol: chloroform: isoamyl alcohol (25: 24:1) was added to the homogenate and mixed by gentle inversion.
- The samples were centrifuged at 8000 rpm for 15 minutes at room temperature.
- The aqueous supernatant was transferred to a new tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 8000 rpm for 10 minutes.
- The aqueous phase was transferred to a fresh tube.
- The DNA was precipitated by adding 2/3 volume of ice cold isopropanol into the tube, mixed gently by inversion and incubated at -20 °C for 60 min.
- DNA was precipitated by centrifugation at 8000 rpm for 15 minutes at 4 °C.
- The supernatant was discarded, washed the pellet with 70% ethanol and the tubes were subjected to vacuum drying for 20-30 min.
- The DNA pellet was dissolved in 1 ml of TE buffer.

Further purification:

- The sample was then treated with 3 µl of RNAase (10 mg/ ml) and incubated at 37 °C for 30 min in water bath.
- An equal volume of chloroform: isoamyl alcohol (24:1) was added to the tube, mixed well and centrifuged at 10000 rpm for 10 minutes.
- To the aqueous phase, taken in a new tube, an equal volume of ice cold isopropanol was added and incubated at -20 °C for 30-60 min.
- The precipitated DNA was made into a pellet by centrifugation at 8000 rpm for 15 minutes at 4 °C.
- The supernatant was decanted carefully and the pellet was washed with 70% ethanol.
- Ethanol was decanted and the pellet was air- dried or vacuum dried and dissolved in 500 µl TE buffer.

3.2.2. Quantification of DNA

The quantity and integrity of the DNA was confirmed by resolving on a 0.8% (w/v) agarose gel stained with ethidium bromide. Electrophoresis was carried out at 80V in a maxi submarine electrophoresis system (Owl, USA) using a programmable power supply Thermo EC 105.

For spectrophotometric DNA quantification, 1 µl of DNA samples were diluted using TE buffer and the absorbance was taken at 260 nm using Biophotometer (Eppendorf, Hamburg, Germany). The concentration of DNA (µg/ml) in the sample was calculated manually. Quality of DNA was analyzed by measuring the absorbance ratio of DNA at 260 nm and 280 nm .

$$\text{Total quantity of DNA } (\mu g/\mu l) = \frac{\text{O.D. at 260nm x 50 x Dilution factor}}{1000}$$

$$\text{Dilution factor} = \frac{\text{Volume made}}{\text{Volume of the aliquot}}$$

3.3. Development of microsatellites (EST SSRs/Genic SSRs) by exploiting EST databases.

3.3.1. Retrieval of EST sequences

A total of 206 EST sequences were downloaded from dbEST database of NCBI (http://www.ncbi.nlm.nih.gov/dbEST) using the keyword "*Piper*".

3.3.2. EST preprocessing

3.3.2.1. Vector clipping, trimming and redundancy elimination

The vector contaminated sequences if any were removed using online tool Vecscreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and trimmest tool available at Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::trimest).

3.3.3. EST clustering and assembly

The non-redundant EST sequences were then clustered using CAP3 online tool of mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::cap3) to collect overlapping ESTs from the same transcript of a single gene into a unique cluster to reduce redundancy.

3.3.4. EST analysis for repeat motifs

The hypervariable repeats in the generated non redundant EST dataset were identified and measured using Websat software (http://wsmartins.net/websat/) (Martins *et al*., 2009) or MISA (http://pgrc.ipk-gatersleben.de/misa/) (Thiel *et al*., 2003). If two SSRs were present close to each other in one EST, they were counted as individual rather than

compound SSRs. The steps involved to identify EST SSRs are given as a flowchart (Fig. 9).

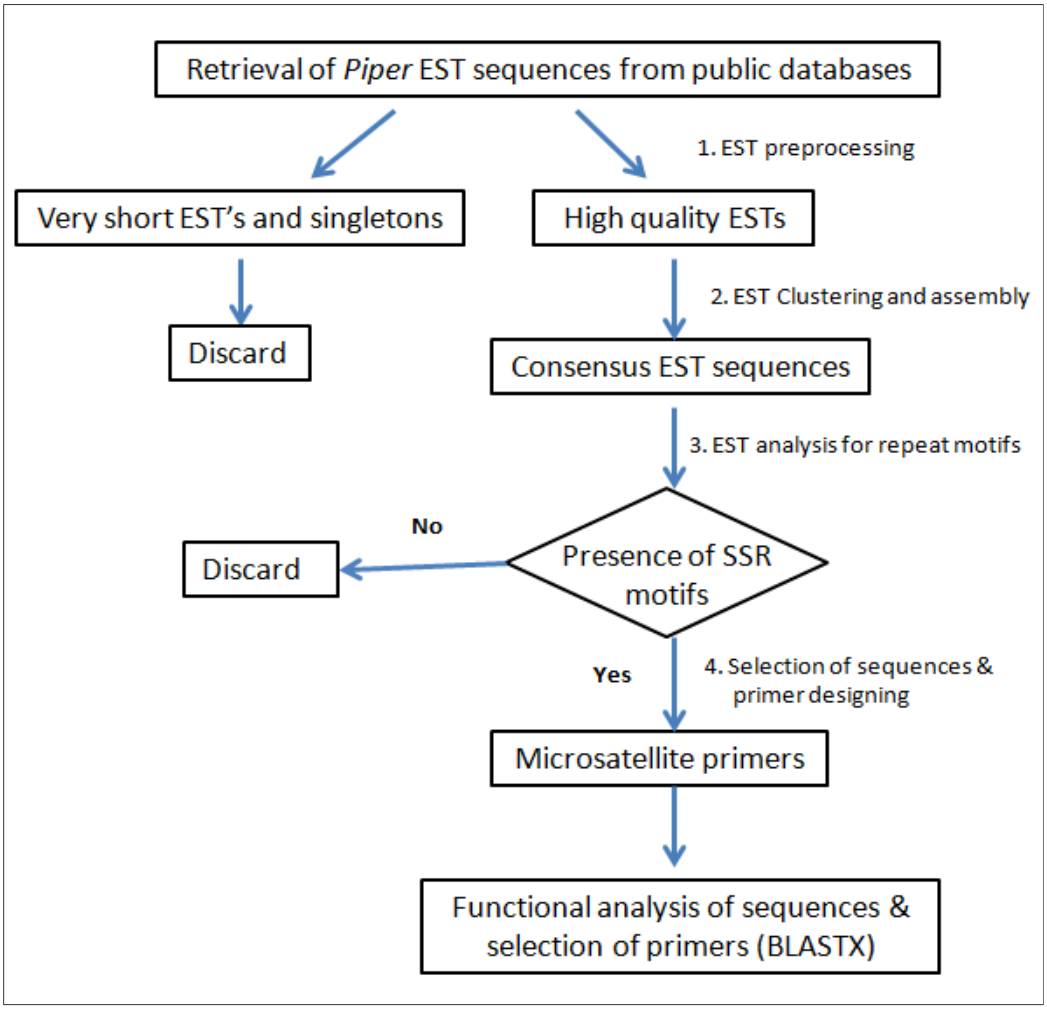

Fig.9: Generic steps involved in EST-SSR generation

3.3.5. Designing of primers flanking microsatellite repeats

Primers flanking microsatellite repeats were designed using online tool, Primer 3 (Rozen and Skaletsky, 2000). The parameters followed for primer designing are described in Table 14. The quality of the designed primers was validated using Northwest Oligocalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). Primers which

were devoid of primer dimer/cross dimer and hairpins were selected and synthesized (Sigma Genosys, Bangalore, India). The putative functions of sequences containing polymorphic EST SSRs were detected using BLAST X (Altschul *et al.*, 1997) by comparing against the non- redundant protein database.

Table 3. Parameters used for designing of microsatellite primers

Parameter	Values			Definition
	Min.	Opt.	Max.	
Primer Size	18	20	27	Length of the primer; specificity and the temperature depend on this parameter.
Primer T_m (^{0}C)	55	60	66	Melting temperature (T_m) depends on primer length and % of GC content
Max Tm difference	0	-	2	Maximum acceptable difference between the T_m of both primers
Primer GC (%)	35	40-55	70	Percentage of Gs and Cs in any primers
Salt conc. (mMol)	-	50	-	Total salt concentration, activation according to T_m
3' end dimmers	-	2	-	Complementary part between both primers from the 3' end of the primers
Max #N's (bases)	-	-	4	Maximum allowable length of a mononucleotide repeat
G/C clamping	-	1	-	G or C nucleotide at the 3' end of the primer

3.4. PCR profiling of EST- SSR markers

The primer pairs were screened and tested for amplification using the genomic DNA isolated from three *Piper* species; *Piper betle*, *Piper nigrum* and *Piper colubrinum*. Amplification of the SSR loci was carried out in a 25 µl PCR mixture with the following components.

PCR component	Volume
Nuclease free water	15.4 µl
10 X reaction buffer	2.5 µl
$MgCl_2$ (25 mM/µl)	2.0 µl
dNTP mix (10 mM/µl)	1.0 µl
Forward primer (10 pm/µl)	1.0 µl
Reverse primer (10 pm/µl)	1.0 µl
Template DNA (20 ng/µl)	2.0 µl
Taq DNA polymerase (5 U/µl)	0.1 µl

Amplification was performed with the following profile

Step	Temperature	Time	Cycle
Denaturation	94 °C	5 min	1X
Denaturation	94 °C	30 s	
Annealing	Ta °C*	45 s	35 X
Extension	72 °C	1.0 min	
Final extension	72 °C	20 min	1X
Hold	15 °C	∝	

*- Annealing temperature varied for different primers

3.4.1. Separation of alleles by agarose gel electrophoresis and denaturing polyacrylamide gel electrophoresis

The PCR products were resolved on 3% agarose gels stained with ethidium bromide to detect the level of polymorphism. (Appendix II a) 10-15% denaturing polyacrylamide gels were used to resolve amplified products and silver stained to analyze size variation of different alleles. The steps involved in setting up and casting the denaturing PAGE gel and silver staining is given in the Appendix (II b and II c).

3.5. Characterization of microsatellite markers

Those markers which produced polymorphic profiles in *P. betle, P. nigrum* and *P. colubrinum* were selected and used for genotyping in 16 released varieties, 23 cultivars of black pepper and 21 *Piper* species. The data obtained were then followed for cluster analysis. The PCR profiles were repeated twice in case of ambiguity.

3.6. Construction of small insert microsatellite enriched genomic DNA library in black pepper

An enriched genomic DNA library for black pepper was generated and screened for various microsatellite motifs. The procedure for library construction was adopted from Glenn and Schable (2005).Steps involved in the isolation of microsatellite loci is represented in Fig. 10.

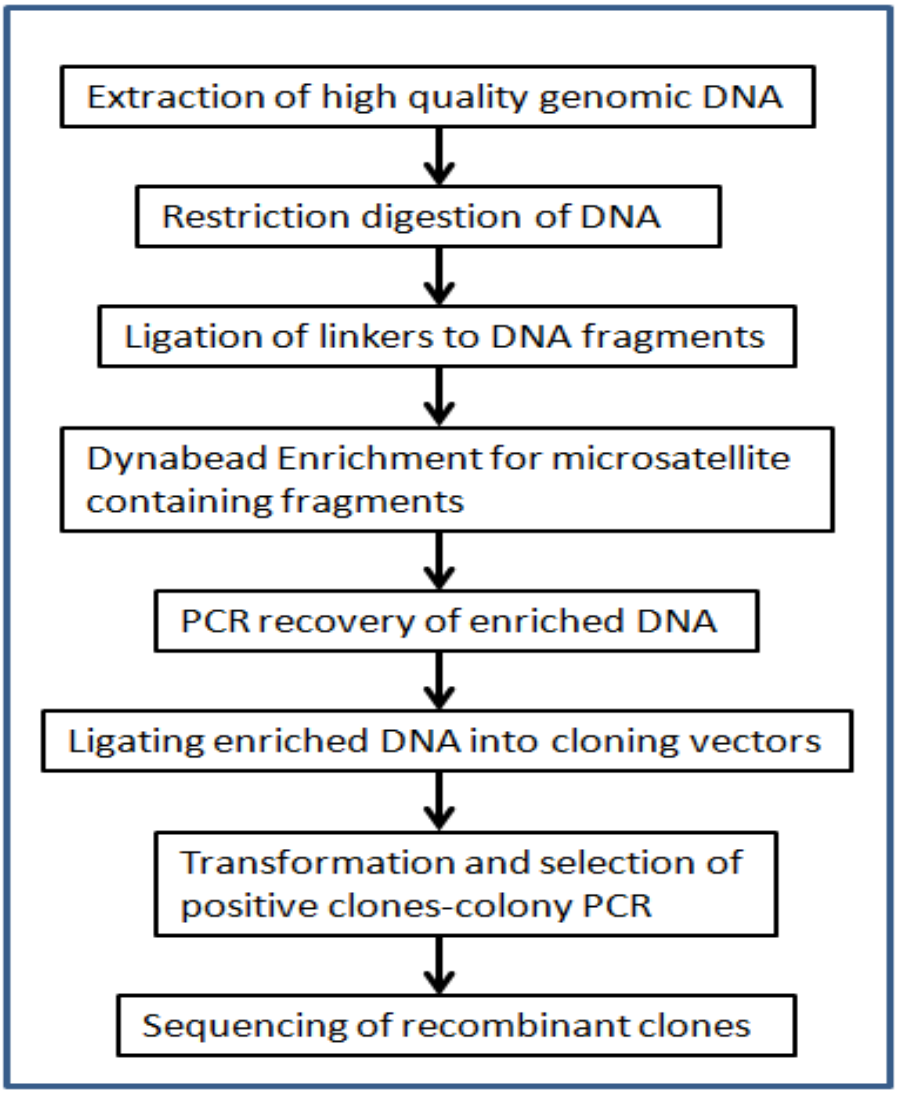

Fig. 10. Steps involved in selective hybridization protocol for constructing enriched genomic DNA library

3.6.1. Isolation of high quality genomic DNA

High quality genomic DNA was extracted from fresh leaf material of black pepper variety Panniyur 1 as described in section 3.2.1. was used for microsatellite library construction.

3.6.2. Selection and standardization of suitable restriction enzymes for size fractionation of genomic DNA

The first step in constructing a microsatellite library is to use restriction enzymes to cut genomic DNA into fragments ranging from 200 bp to 2 Kb. This sizes of DNA that are potentially large enough to contain a microsatellite and a flanking region, but small enough to insert into plasmid.

Optimized digestion conditions were determined by varying genomic DNA concentration, restriction enzymes, enzyme units per µg DNA and digestion period. Four restriction enzymes were randomly chosen for fragmenting the genomic DNA (Table 4). The genomic DNA was digested by using four restriction enzymes viz., *Alu* l, *Bst* U I, *Eco* R I and *Rsa* I. The digestion was carried out in 50 µl reaction volume containing 1X reaction buffer, 1-2 µg of purified genomic DNA, 20 Unit restriction enzyme at appropriate temperature for 8-12 h. After digestion the restriction enzymes were inactivated for 10 min incubation at 65- 70^0 C.

The size of the digested DNA fragments was monitored by resolving 5 µl aliquot of the restricted DNA on a 1.0% agarose gel along with 100 bp and 1 Kb DNA ladder (Thermoscientific, USA.). Among the four restriction enzymes *Alu* 1 and *Rsa* 1 were taken for further steps based on restriction pattern.

Table 4. Restriction enzymes screened for fragmenting black pepper genomic DNA

Sl. No.	Restriction enzymes	Recognition sequence	Temperature
1.	*Alu* I	AG/CT	37 ºC
2.	*Bst* U I	CG/CG	65 ºC
3.	*Eco* R I	G/AATTC	37 ºC
4.	*Rsa* I	GT/AC	37 ºC

3.6.3. Preparation of double stranded linker

The linkers will provide the primer-binding site for subsequent PCR steps. The double stranded linkers were prepared by mixing equal volumes of equal molar amounts of SuperSNX24 forward primer (SuperSNX24 Forward: 5'GTTTAAGGCCTAGCTAGCAGAATC) and SuperSNX24+4p reverse primer (Super SNX24+4P Reverse: 5'pGATTCTGCTAGCTAGGCCTTAAACAAAA) (e.g., 100 µl of 10 µM each). Salt concentration was made upto a final concentration of 100 mM (*i.e.*, 4 µl of 5M NaCl for 200 µl of primers). This mixture was heated to 95 °C, cooled slowly to room temperature to form the ds SuperSNX linkers and was stored at -20 °C for further reactions.

Reaction component	Volume
SuperSNX24 primer (10 µM)	100.0 µl
SuperSNX24+4p primer	100.0 µl
NaCl (5M)	4.0 µl

3.6.4. Ligation of linkers to DNA fragments

The double stranded SNX linkers were ligated to the restricted DNA fragments using T_4 DNA ligase enzyme. The linker ligation was done separately for both *Alu* 1 and

Rsa 1 restricted genomic DNA fragments. The ligation mixtures were incubated overnight for 16 ^{o}C. The reaction components and the volume taken were as follows:

Reaction component	Volume
Restricted DNA	25.0 µl
10x Ligase Buffer	2.0 µl
ds Super SNX linkers	10.0 µl
DNA ligase (400 U/µl	2.0 µl
Xmn 1	1.0 µl

3.6.5. Confirmation of linker ligation to restricted DNA

The ligation of linkers to digested DNA fragments was assured by performing a PCR using linker ligated DNA fragments as template. The 25 µl reaction contained the following components.

PCR Component	Volume
Nuclease free water	15.4 µl
10 X reaction buffer	2.5 µl
$MgCl_2$ (25 mM/µl)	2.0 µl
dNTP mix (10 mM/µl)	1.5 µl
SuperSNX24 primer (10 mM/µl)	1.3 µl
Linker ligated DNA	2.0 µl
BSA (250 µg/ml)	2.5 µl
Taq DNA polymerase (5 U/µl)	0.2 µl

The PCR was carried out with 30 cycles with the following regimes in thermal cycler (Eppendorf Master Cycler Gradient S) with the following temperature profile

Step	Temperature	Time	Cycle
Denaturation	94 °C	2 min	
Denaturation	94 °C	20 s	
Annealing	60 °C	20 s	30 X
Extension	72 °C	1.5 min	
Final extension	72 °C	5 min	
Hold	15 °C	∝	

To confirm the amplification 5 µl of the PCR product was viewed on 1.0 % agarose gel along with 100 bp and 1Kb DNA ladders (Thermoscientific, USA).

3.6.6. Hybridization of oligonucleotide probe

Six sets of biotinylated olgonucleotide probes [$(AG)_{12}$, $(TG)_{12}$, $(ACT)_{12}$, $(AAAC)_{6}$, $(ACCT)_{6}$, $(ACTG)_{6}$] (Sigma Genosys, Bangalore) were used for enrichment. The compositions of solutions and buffers used for the hybridization and enrichment process are described in Appendix- Ic.

The hybridization of biotinylated oligonucleotide probes to the linker ligated DNA fragments were carried out in 50 µl reaction volume with the following components.

Hybridization Component	Volume
2x Hybridization Solution (12x SSC, 0.2% SDS)	25.0 µl
Biotinylated microsatellite probe (1 µM each)	6.0 µl
Linker ligated DNA	7.5µl
Nuclease free water	11.5 µl

A PCR was performed to facilitate the hybridization reaction. The PCR regime is as follows.

Temperature	Time	Cycle
94 °C	5 min	
94 °C- 70 °C	Quick ramping	
70 °C- 50 °C	Slow ramping	99x
50 °C- 20 °C	Slow ramping	20 x
20 °C- 15 °C	Quick ramping	
15 °C	∝	

Immediately after the hybridization was over the enrichment was performed using the streptavidin coated magnetic beads (Dynabeads, Dynal, Oslo, Norway).

3.6.7. Dynabead enrichment, magnetic separation, and elution of microsatellite enriched DNA fragments

- 50 µl of Dynabeads was washed and transferred to a 1.5 ml eppendorf tube.
- 250 µl of TE buffer was added to the tube, shaken well and beads were captured using Magnetic particle Concentrator (MPC) (Dynabeads, Dynal, Oslo, Norway).
- The step was repeated twice with 250 µl TE, followed by two washes with 250 µl of 1X hybridization solution and captured using MPC.
- The beads were then re-suspended in 150 µl of 1X hybridization solution.
- The DNA+ probe mix (3.4.6.) was added to the 150 µl of washed, re-suspended Dynabeads.
- The mix was incubated at room temperature for 30+ min in an orbital shaker.
- The beads were then captured using MPC and the supernatant was removed by pipetting with a P200 pipetter.
- The Dynabeads were washed twice with 400 µl of washing solution 1 (2x SSC, 0.1% SDS) and captured each time with MPC.
- Two additional washing was done with 400 µl of washing solution 2 (1X SSC, 0.1% SDS).

- This was followed by two washes with 400 µl of washing solution 2 and by heating the solution to within 5-10 °C of the T_m of the oligo mix (45-50 °C).
- The beads were quickly captured using MPC and the supernatant was immediately transferred to a fresh 1.5 ml eppendorf tube.
- 200 µl of TLE was added to the tube, vortexed and incubated at 95 °C for 5 min.
- The beads were immediately captured using MPC and the supernatant was quickly removed by pipetting to a fresh eppendorf tube (This supernatant contains the enriched fragments- the "Gold").
- 22 µl of sodium acetate/ EDTA was added to the supernatant, mixed well by pipetting up and down.
- 444 µl of 95% ethanol was added to the mix and incubated at -20 °C for 30-60 min.
- The tubes were then centrifuged at 12000 rpm for 10 min at 4 °C.
- The supernatant was discarded and 500 µl of 70 % ethanol was added to the tube and centrifuged at 12000 rpm for 1 min at 4 °C.
- The pellet was air dried and was resuspended in 25 µl of TLE ("Pure Gold").

3.6.8. PCR recovery of microsatellite enriched DNA

A PCR was performed to recover the enriched DNA fragment using the supernatant (Pure Gold) as the template.

PCR Component	Volume
Nuclease free water	13.0 µl
10 X reaction buffer	2.5 µl
$MgCl_2$ (25 mM/µl)	2.0 µl
dNTP mix (10 mM/µl)	1.5 µl
SuperSNX24 primer (10 mM/µl)	1.3 µl
BSA (250 µg/ml)	2.5 µl
Enriched DNA fragment (pure gold)	2.0 µl
Taq DNA polymerase (5 U/µl)	0.2 µl

The cycling parameters used for PCR amplification is as follows:

Step	Temperature	Time	Cycle
Denaturation	94 °C	2 min	
Denaturation	94 °C	20 s	
Annealing	60 °C	20 s	20 X
Extension	72 °C	1.5 min	
Final extension	72 °C	10 min	
Hold	15 °C	∝	

5 µl of the PCR product was checked on 1.0% agarose gel along with 100 bp and 1Kb ladder (Thermoscientific, USA) to verify the success of DNA recovery.

3.6.9. Cloning of enriched DNA fragments

The microsatellite enriched DNA fragments were cloned using pCR® 2.1 TOPO (Invitrogen, Carlsbad, CA) vector which can accommodate more colonies with even less nucleotide base pair following manufacturer's instructions.

3.6.10. Topo cloning reaction

- 4 µl of PCR product was mixed with 1 µl of pCR® 2.1 TOPO cloning vector to get a final volume of 5 µl.
- Reaction mix was mixed gently and incubated for 5 min at room temperature
- After the incubation 1 µl of TOPO cloning stop solution was added to that and mixed for several seconds at room temperature.
- The tube was briefly centrifuged and placed on ice.

3.6.10.1. Transformation of competent cells/Bacterial transformation

The vector incorporated with DNA fragments were transformed into TOP10 cells following manufacturer's instructions described below.

- The SOC medium was thawed and brought to room temperature.
- One vial of one short TOP 10 chemically competent cell was thawed on ice.
- 2 µl of the TOPO cloning reaction was added to a vial of One Shot Top 10 cells and mixed gently.
- The vials were incubated on ice for 30 min.
- The cells were then subjected to heat shock at 42 °C for 30 sec in a water bath without shaking.
- The cells were removed from the 42 °C water bath and placed immediately on ice for 2 min.
- 250 µl of room temperature SOC medium was added aseptically to each vial and incubated at 37 °C for 60 min and placed on ice.
- 50-150 µl of each transformation mixture was spread on pre -incubated selective agar plates using a sterile L rod.
- The plates were incubated at 37 °C for overnight.

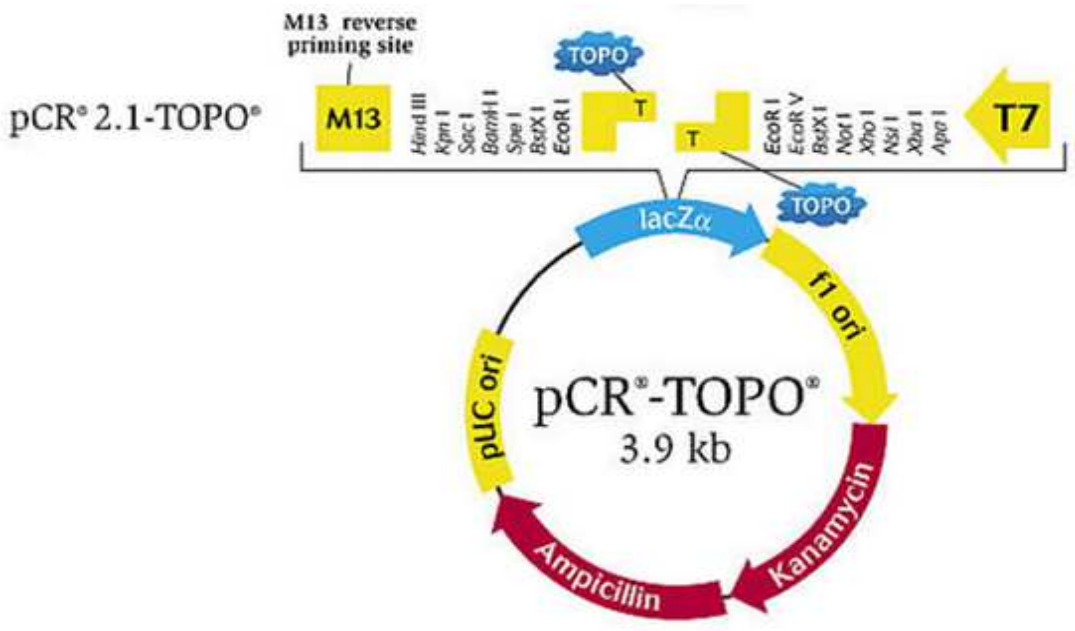

Fig. 11. Vector map of pCR 2.1-TOPO

3.6.10.2. Preparation of selective agar plates

- 50 ml of LB agar was melted and allowed to cool to ~40 °C.
- 50 µl kanamycin (20 mg/ml) (Appendix 1d) was added to the 50 ml molten LB agar, mixed and poured onto sterile petriplates.
- The agar was allowed to solidify for 20-30 min and incubated at 37 °C until plating.

3.6.11. Screening of positive clones

- Recombinant clones (clones with insert) were identified from the master plates
- The colonies obtained were streaked onto a new kanamycin+ LB agar plates and incubated overnight at 37 °C.
- The clones were confirmed by colony PCR with vector specific primers and stored in 20% glycerol at -80 °C.

3.6.11.1. Identification of positive clones by colony PCR

Colony PCR helps in determining the insert size and/or orientation of the insert in the vector directly. Bacterial colonies were used directly as templates in colony PCR using M 13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers. The PCR component and volume is given below.

PCR Component	Volume
Nuclease free water	12.9µl
10 X reaction buffer	2.5 µl
$MgCl_2$ (25 mM/µl)	2.0 µl
dNTP mix (10 mM/µl)	0.5 µl
M 13 forward primer (10 µM)	1.0 µl
M 13 reverse primer (10 µM)	1.0 µl
BSA (250 µg/ml)	2.5 µl
Taq DNA polymerase (5 U/µl)	0.2 µl
DNA template from bacterial colony grown	1.5 µl

Amplification was performed in a thermal cycler with the following amplification profile.

Step	Temperature	Time	Cycle
Denaturation	94 °C	5 min	
Denaturation	94 °C	30 s	
Annealing	58 °C	45 s	35 X
Extension	72 °C	1.0 min	
Final extension	72 °C	20 min	
Hold	15 °C	∝	

The insert size was confirmed by resolving the PCR product on a 1.0 % agarose gel.

3.6.12. Isolation of plasmid DNA

Positive clones from the enriched library were inoculated to 3 ml LB broth containing kanamycin (50mg/l) and incubated at 37 °C with shaking at 250 rpm. Plasmid DNA was isolated using QiagenQIA quick Plasmid Mini Kit (Qiagen, Germany) following the manufacturer's instructions as described below:

- 5 ml of overnight incubated bacterial culture was pelleted by centrifugation at 12000 rpm for 3 min at room temperature.
- The supernatant was removed without disturbing the pellet.
- Pelleted bacterial cells was re-suspended in 250 µl buffer P1 and transferred to a microcentrifuge tube.
- 250 µl buffer P2 was added and mixed by inverting the tubes 4-6 times till the solution becomes clear.
- 350 µl buffer N3 was added and immediately mixed by inverting the tubes 4-6times.
- Centrifuged at 13000 rpm for 10 min at room temperature.
- The supernatant was transferred to QIA prep Spin column by decanting and centrifuged at 12000rpm for 1min and the flow through was discarded.

- The column was washed by adding 750 µl of buffer PE and centrifuged at 12000 rpm for 1 min and the flow through was discarded.
- Centrifuged for 1min to remove residual wash buffer.
- The spin column was transferred into a new collection tube.
- 50 µl of elution buffer was added directly to the center of the spin column membrane and centrifuged at 12000 rpm for 1 min.
- The plasmids were stored at -20 °C

The presence of the plasmid DNA was checked by resolving it on a 1.0 % agarose gel and documented (Gel Logic 200 image analysis system, Kodak) and quantified spectrophotometrically (Biophotometer plus, Eppendorf, Germany).

3.6.13. Plasmid DNA restriction digestion

Plasmid DNA was restricted with the restriction enzyme *Eco* R1 to confirm the insert size.

3.6.14. Sequencing of plasmids

The plasmids were sequenced bidirectionally using ABI prism (Xcelris Labs Ltd, Ahmedabad) using M 13 forward and reverse primers. Contigs were assembled using DNA Baser version 3.5.4. Vector contamination was checked using Vecscreen software and Bioedit softwares were used to remove vector sequence as well as linker sequences.

3.6.15. Identification of duplicate sequences/ redundancy elimination and clustering

The sequences were assembled into contigs and singletons to avoid redundancy using the online tool CAP3.

3.6.16. Identification of microsatellites within the sequenced clones

Microsatellite repeats were detected from the generated non-redundant dataset using Websat (Martins *et al.*, 2009) and MISA (Thiel *et al.*, 2003).

3.7. Designing of primers flanking microsatellite repeats (genomic and EST SSR)

Primers flanking microsatellite repeats were designed using the web based computer program, Primer 3 (Rozen and Skaletsky, 2000). The parameters followed for primer designing are described in Table 5. The quality of the designed primers, their self-complementarity, possible secondary structure were validated using Northwest Oligocalc. Primers which are devoid of primer dimer, cross dimer and hairpin were selected and custom synthesized (Sigma Genosys, Bangalore).The putative functions of sequences containing polymorphic EST SSRs were detected using BLAST X (Altschul *et al.*, 1997) by comparing against the non- redundant protein database.

Table 5. Parameters used for primer designing

Parameter	Values			Definition
	Min.	Opt.	Max.	
Primer Size	18	20	27	Length of the primer; specificity and the temperature depend on this parameter.
Primer T_m (^{0}C)	55	60	66	Melting temperature (T_m) depends on primer length and % of GC content
Max Tm difference	0	-	2	Maximum acceptable difference between the T_m of both primers
Primer GC (%)	35	40-55	70	Percentage of Gs and Cs in any primers
Salt conc. (mMol)	-	50	-	Total salt concentration, activation according to T_m
3’ end dimers	-	2	-	Complementary part between both

			primers from the 3’ end of the primers
Max #N’s (bases)	-	4	Maximum allowable length of a mononucleotide repeat
G/C clamping	1	-	G or C nucleotide at the 3’ end of the primer

3.8. PCR assessment of microsatellite markers

The primer pairs were screened and tested for amplification using the genomic DNA isolated from three distinct genotypes of *Piper*; *Piper betle, Piper nigrum* and *Piper colubrinum*. Amplification of the SSR loci was carried out in a 25 µl PCR mixture with the following components.

PCR component	Volume
Nuclease free water	15.4 µl
10 X reaction buffer	2.5 µl
$MgCl_2$ (25 mM/µl)	2.0 µl
dNTP mix (10 mM/µl)	1.0 µl
Forward primer (10 pm/µl)	1.0 µl
Reverse primer (10 pm/µl)	1.0 µl
Template DNA (10 ng/µl)	2.0µl
Taq DNA polymerase (5 U/µl)	0.1 µl

Amplification was performed with the following profile.

Step	Temperature	Time	Cycle
Denaturation	94 °C	5 min	
Denaturation	94 °C	30 s	
Annealing	Ta °C*	45 s	35 X
Extension	72 °C	1.0 min	
Final extension	72 °C	20 min	
Hold	15 °C	∝	

*- Annealing temperature varied for different primers

3.9. Separation of alleles

The PCR products were resolved on 3% agarose gels stained with ethidium bromide to detect the level of polymorphism. 10-15% denaturing polyacrylamide gels were used to resolve amplified products and silver stained to analyze size variation of different alleles. The steps involved in setting up and casting the denaturing PAGE gel is given in the Appendix IIc.

3.10. Characterization of microsatellite markers

Those markers which produced polymorphic profiles were selected for genotyping in 16 released varieties, 23 cultivars of black pepper and 21 *Piper* species. The data obtained were then followed for cluster analysis. The PCR profiles were repeated twice in case of ambiguity.

3.11. Microsatellite nomenclature

All new genomic microsatellite primers developed from black pepper were assigned the name BPSSR (Black Pepper SSR) and BPM (Black Pepper Microsatellite).

3.12. Cross species amplification of microsatellite loci

The microsatellite markers developed in black pepper were tested to amplify in twenty three *Piper* species with the same PCR conditions (section 3.4).The PCR reaction was repeated twice in case of ambiguity.

3.13. Cluster analysis

Only clear and prominent bands were scored for assessing genetic diversity among the genotypes. The markers exhibiting 100% transferability were selected for cross species amplification in genus *Piper*. The presence (1) and absence (0) of bands were recorded for individual genotypes and the data were entered into binary matrix as discrete variable. This binary matrix was used to estimate the genetic similarity as Dice coefficient using SIMQUAL subroutine in the similarity routine of NTSYS- pc version 2.02i software package. The resultant similarity matrix was employed to construct dendrograms using Sequential Agglomerative Hierarchical Nesting (SAHN) based on Unweighted Pair-Group Method with Arithmetic Average (UPGMA).The similarity matrix that was obtained from Dice similarity coefficient was subjected to principal coordinates analysis (PCA) to investigate the structure of the population. The robustness of the dendrogram was evaluated with a bootstrap analysis performed on the binary data set using 1000 permutations in Past 3 software (Hammer *et al*., 2001).

3.14. Polymorphic Information Content value of polymorphic microsatellite markers

The Polymorphism Information Content (PIC) defines the discriminatory power of each polymorphic marker which depends on the number of alleles and relative frequency of an allele in the population. The PIC for each individual SSR allele was calculated following the formula described by Weir (1990) *i.e*. PIC= 1- $\sum P_i^2$, where P_i is the frequency of the i^{th} allele in the genotypes examined, which can be simplified to PIC=

2PiQi, where P_i is the frequency of presence and Q_i is the frequency of absence of a particular band (Tehrani *et al*., 2008).

3.15. Other sources of markers (previously reported microsatellite markers)

Previously reported sixteen microsatellite markers developed in Black pepper *viz*., nine SSR primers developed by Menezes *et al.* (2009) and seven SSR primers developed by Joy *et al.* (2011) were also used for characterization in 23 black pepper cultivars and 21 *Piper* species. Optimization procedure for all these primers was similar to that already described except annealing temperature which varied for individual primers (section 3.4). The sequence information of the primers used for the study is given in Table 6 and 7.

Table 6. List of nine black pepper SSR primers (Menezes *et al.*, 2009)

SI. No.	Locus Name	Forward Sequence	Reverse 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′

Table 7. List of seven black pepper SSR primers (Joy *et al*., 2011)

SI. No:	Locus Name	Forward Sequence	Reverse Sequence
1	PnCA25	F 5′ GTGTGTTATTTGTCTCTGGGTTTTTCC 3′	R 5′CTTTGGGGCACTCTAACCATCGTCTG 3′
2	PnCA88	F 5′ CACATATTTTTCTTACATTGCG3′	R 5′GATTATGGGCTGCCGGATTT3′
3	PnGT119	F 5′ CCCAACTTCAGAATGATTATACAGC3′	R 5′CTGGGCAGTAAGCAAACATA3′
4	PnAG30	F 5′ ACTAAGGCTAATGTGATAACCTGAGGA 3′	R 5′ATCCCTGGATGGAAATTTGAAGGCTTGC 3′
5	PnGT2	F 5′ CTAGAGAGTAACAGTTATCACTTCACAG 3′	R 5′CTAGCAAATTTGTTCTCTAATTCACATGT 3′
6	PnGATA10	F5′CTCCCAGTTATACAACATCACAACCTAGCAC3’	R 5′AGAGGCTTGTCTTAGTTGTGCTCGGGA 3′
7	PnCA9Fb	F 5′ TCATCAATCACACCTAAAAGAAGGCTATCC 3′	R 5′ATGTGGCTATGGGGAACGGTCAGGGGT 3′

PCR assessment of 16 microsatellite markers, separation of alleles on denaturing PAGE gels, characterization of markers, cluster analysis was carried out as described earlier (section 3.6, 3.7, 3.8, 3.9, 3.11, 3.13).

3.16. Sequencing of microsatellite carrying PCR product amplified by the primer BP SSR 11 and their multiple sequence alignment

In order to confirm the microsatellite repeat variation in terms of allelic difference, the alleles generated by the primer BP SSR 11 from 3 genotypes (Panniyur 2, Panniyur 3 and Pournami) were sequenced individually. The sequences obtained were aligned using CLUSTAL W multiple sequence alignment program (Thompson *et al*., 1994) of Bioedit software (Hall, 1999) and also with Mega 6 (Tamura *et al*., 2013) sequence alignment programme.

3.17. Sequencing of microsatellite carrying PCR product amplified by the primer PC EST SSR 22 and their multiple sequence alignment

To reveal the presence of repeat motif in the amplified loci the PCR product generated with the primer PC EST SSR 22 from the genotype Sp2 (*P. nigrum*) were sequenced. The obtained sequences were aligned with the original sequence using CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994) of Bioedit software (Hall, 1999) and also with Mega 6 (Tamura *et al.*, 2013) sequence alignment programme.

RESULTS

Chapter 4

RESULTS

4.1. Isolation of genomic DNA

Genomic DNA of good quality was isolated from thirty nine black pepper genotypes (16 released varieties and 23 cultivars of black pepper) 21 *Piper* species (Fig. 12, 13, 14). A260/A280 ratio of 1.7-1.8 indicated that extracted DNA is devoid of proteins and polysaccharides.

4.2. Identification of microsatellites by exploiting EST databases

To develop polymorphic genic (EST) SSR markers the EST sequences available in the public domain were retrieved and mined for SSRs using computational approaches.

4.2.1. EST Database and Sequence Retrieval

A total of 206 EST sequences were downloaded from genbank at NCBI website (http://ncbi.nlm.nih.gov) using the keyword "*Piper*". Out of the 206 sequences, 87 EST sequences were from *Piper nigrum*, 50 from *P. colubrinum*, 63 from *P. longum* and 6 from *P. tuberculatum*.

4.2.2. Sequence Analysis and clustering

Cluster analysis has employed to identify overlapping *Piper* EST sequences representing same native transcripts as putative non-redundant sequences using CAP3.91. The sequences were assembled to 6 contigs, 68 singletons in *P. nigrum*; 7 contigs and 35

singletons in *P. colubrinum*; 4 contigs and 6 singletons in *P. longum* and 6 singletons in *P. tuberculatum*.

4.2.3. Distribution and identification of EST-SSRs

Redundancy elimination and exclusion of poly A tail resulted in 74 non-redundant *Piper nigrum* ESTs with a total base pair of 39409 which was then used to for SSR detection.

Since the sequence availability was limited, microsatellites *viz*., penta and hexanucleotide) with ≥2 repeats were set as a parameter for the identification of SSR from the sequence. When ≥2 repeating microsatellites were excluded from the dataset di nucleotide repeats were the most common SSRs. Out of the 74 *Piper nigrum* EST sequences examined, 66 SSR were detected which clustered into 46 different types of motifs as represented in Table 8. Number of SSR containing sequences was 41, while 18 sequences had more than one SSR and 11 were SSR present in compound formation.

The most abundant microsatellite repeat motif detected in *P. nigrum* was pentanucleotides (51.52% of 66) followed by hexanucleotides (37.88% of 66) (both are repeated two times). The occurrence of mononucleotide repeat motif is only 6.06% of 66 microsatellites. Di and trinucleotides repeats were found but of least frequency. Tetranucleotide motif was completely absent in the available dataset of *P. nigrum*.

A total of 15 microsatellite SSRs were detected from *P. longum* ESTs comprising of 11525bp. There were 11 SSR containing sequences and 2 sequences possessed more than one SSR.4 SSRs were involved in compound SSR formation. Seven different types of motifs were present among identified *Piper longum* ESTs. Pentanucleotide repeat

motif was the most abundant (46.67%) among the microsatellites followed by hexanucleotides motif (26.67%). Mono and dinucleotide repeats were observed but is of less occurrence. Tri and tetranucleotide repeats were altogether absent in the scanned database of *P. longum* (Table 9).

Within the dataset of 42 non-redundant EST sequences of *P. colubrinum* comprising of 10904 bp, 13 SSRs were identified which clustered into 13 different types of motifs. Number of SSR containing sequences was 11 while 2 sequences had more than one SSR and 2 were involved in compound SSR formation. Pentanucleotide repeat motif was found to be the most abundant (38.46% of 13) and was followed by hexanucleotides (30.77% of 13) in the case of *P. colubrinum* database studied. All other types of nucleotide repeats were detected but of least occurrence (Table 10).

Only one microsatellite repeat was detected among six unigenes of *Piper tuberculatum* ESTs, representing 2459 bp and the motif identified was a pentanucleotide (AAGCG/CGCTT) (Table 11).

Pentanucleotide (50.53% of 95) and hexanucleotide (34.73% of 95) repeat motif constituted the major repeat motifs in *Piper* ESTs. All the penta and hexanucleotide motifs observed in the present study were of 2 repeats. Excluding two time repeats from the dataset the major part of the sequence contained are di nucleotide repeats. Trinucleotide and tetranucleotide repeats were of least (3.18% of 95) occurrence in all the four species of *Piper* analysed.

The details of microsatellite containing EST sequences retrieved from NCBI of *P. nigrum, P. longum, P. colubrinum* and *P. tuberculatum* were given in Table 5. The primer designing and genotyping of EST SSR markers are described in 4.4 and 4.5.

Table 8. Occurrence of microsatellite repeats in *P. nigrum* L. ESTs (accessed from NCBI database).

SSR motif	Number of repeats					Total Number of repeats
	2	3	4	5	>5	
A/T	-	-	-	-	4	4
AG/CT	-	-	-		1	1
AAG/CTT	-	-	-	-	1	1
AAAAC/GTTTT	1	-	-	-	-	1
AAAAG/CTTTT	2	-	-	-	-	2
AAACC/GGTTT	2	-	-	-	-	2
AAACG/CGTTT	1	-	-	-	-	1
AAAGG/CCTTT	1	-	-	-	-	1
AAATC/ATTTG	2	-			-	2
AAATT/AATTT	1	1	-	-	-	2
AACAC/GTGTT	2	-	-	-	-	2
AACAG/CTGTT	1	-	-	-	-	1
AACAT/ATGTT	1	-	-	-	-	1
AACTC/AGTTG	1	-	-	-	-	1
AACTT/AAGTT	2	-	-	-	-	2
AAGAC/CTTGT	1	-	-	-	-	1
AAGGT/ACCTT	1	-	-	-	-	1
AAGTC/ACTTG	1	-	-	-	-	1
AATCC/ATTGG	1	-	-	-	-	1
AATGG/ATTCC	3	-	-	-	-	3
AATGT/ACATT	1	-	-	-	-	1
ACAGC/CTGTG	2	-	-	-	-	2
ACATG/ATGTC	1	-	-	-	-	1

SSR Motif	Number of repeats					Total number of repeats
	2	3	4	5	>5	
ACTGC/AGTGC	1	-	-	-	-	1
AGAGC/CTCTG	2	-	-	-	-	2
AGATG/ATCTC	1	-	-	-	-	1
ATATC/ATATG	1	-	-	-	-	1
CCCCG/CGGGG	1	-	-	-	-	1
AAAAAC/GTTTTT	3	-	-	-	-	3
AAAAAG/CTTTTT	2	-	-	-	-	2
AAACTG/AGTTTC	2	-	-	-	-	2
AAAGAC/CTTTGT	1	-	-	-	-	1
AACCTG/AGGTTC	1	-	-	-	-	1
AACGAT/ATCGTT	1	-	-	-	-	1
AACTGT/ACAGTT	1	-	-	-	-	1
AAGCTC/AGCTTG	1	-	-	-	-	1
AAGTGC/ACTTGC	1	-	-	-	-	1
ACAGTC/ACTGTG	1	-	-	-	-	1
ACCTCC/AGGTGG	2	-	-	-	-	2
ACTGCT/AGCAGT	1	-	-	-	-	1
AGATGG/ATCTCC	1	-	-	-	-	1
AGCCCC/CTGGGG	1	-	-	-	-	1
AGGCCG/CCTCGG	2	-	-	-	-	2
ATATCC/ATATGG	1	-	-	-	-	1
ATCATG/ATCATG	1	-	-	-	-	1
CCCCCG/CGGGGG	2	-	-	-	-	2

Table 9. Occurrence of microsatellites in *P. longum* ESTs
ESTs (accessed from NCBI database).

SSR motif	Number of repeats					Total number of repeats
	2	3	4	5	>5	
A/T	-	-	-	-	2	2
AC/GT	-	-	-		2	2
AAAAT/ATTTT	2					2
AACTG/AGTTC	2					2
AGCTC/AGCTG	3					3
AAACAT/ATGTTT	2					2
ACTGCT/AGCAGT	2					2

Table 10. Occurrence of microsatellites in *P. colubrinumn*
(accessed from NCBI database).

SSR motif	Number of repeats					Total number of repeats
A/T	-	-	-	-	1	1
AT/AT	-	-	-	1		1
ACG/CGT	-	-		1		1
AAAT/ATTT	-	1				1
AAAGG/CCTTT	1					1
AAGGG/CCCTT	1					1
ACATG/ATGTC	1					1
ACGAG/CGTCT	1					1
ACTAG/AGTCT	1					1
AAAATG/ATTTTC	1					1
AAGTCG/ACTTCG	1					1
ACATAT/ATATGT	1					1
ACATCG/ATGTCG	1					1

Table 11. Occurrence of microsatellites in *P. tuberculatum* ESTs
(accessed from NCBI database).

SSR motif	Number of repeats					Total number of repeats
	2	3	4	5	>5	
AAGCG/CGCTT	1					1

Table 12. Details of the microsatellites regions containing sequences obtained from *P. nigrum*, *P. longum*, *P. colubrinum* and *P. tuberculatum* EST databases

SSR containing sequences in Piper nigrum L.	
Locus name	**Sequence**
GI190335106	TTGACGTGGTCGCCCGCGGGAGGTCAGGTTGCCGGCGATCTTTCTTGGGTGGTGAGAGGTAGTATTCTTGAGCCATGATCTCCAAATAGAGCT GATCCTTGAGCTTCCTAGGATGGTGAAAGCTCGCAGAGACCCAGAGAGGC**AGAAGAAGAAGAAGAAGA**TCAGACAGCTTATTGCCTAGA ATGGCTATTCTTACTCAAGCCAAAAGAGTTTCAATTTCTCTCCCACAGCTTAGCATCGTGCTCAGCACGTCTTCACACAAAAGCAGCGACGGCG GAAACCACGGCAGTGCCCACCGGGGAAGCCCTCTGTCTGGCAGACACTGGCGCAGTTGCTGTCCCTCACACACGGACCTTTGAACCTGTGGCT CTGTGACTCGCAGATCCTTGCCTCGGCAAAGCTCGGCCCAACATTTCCAAGTGGACATGATCAGCAAAACCATGATAAAAATGGCTGGGAACA GACGTGATCACCATTAGTCCTGAAAGCTAGAATCAGAAGCCGAGGATTTGAAGACCTGCCCGGG
GI189502757	ACTACCGTTTCGATGGCACCCCTGTTTACAAGGCCGCTTGGAGGGAGGCGAAGGAGTGCGTCTACGTCGAGCCCACGAGGGGTCGCCGGAGA AGGGCGTCTACTGGTACGGACACAAGTTTTGAGACAAGATCTTGCGTCGGGAATCGATGGTTGGGAAGTGGGTGGTCGATTATCTTGCCGTTC TTGTCTTTGTTATAATTTAGTATTGTTATGGTTATTTAGTCCGTCATTGCCGGCGACATGGTCG**TAATTTAATTTAATT**GAGGGATACTATTG TTGGAAGCCGCCCATCTTTTTTGGGTTTATAGTCGGTATCT
GI190335102	TTTCGAGCGGCGCCCGGGGCGGTAAGTGGCCCGGGTGGAGAGGAGCAGCGGCAGGTTCTGAGGAGGTTTAGGTTGCCGGAGAATGCTAAGGT GGAGGAGGTGAAGGCGGGGGTGGAGAACGGGGTGCTC**ACTGTGACTGTG**CCCAAGGTACTGCTCTATTATTCCAAATAAAGAAGTATTCA CATTATACAAATTTATCCGAAAAGTTGTGATCAACTAAAACGAATAATGGACTTAACCTACCTGCAAAAAACAAAGACTACCAAACCGCGCAA ATGCCTTTGTCATGACAGCTTAACTCATGCAAATTACGACTACTAAACAATAATTAGTAATAACTTCACTTTGTTAAACTAGGAGGTGAAGAAG TCTGAGGTTAAGGCCATTGAAATCTCTGGAAACTAAGAGTTTCGAAAAAGATAATATTGTGTCAAGGCCATGTGTGTCACCACGCTTTTCGTGA ATGTGTTTGGTCTCGGTTGGAGCTTGTTTTCTTTATCTTCGTCTTGTAATAAGGTGATGAATGGGTCTATGCGAGGATCGGTGTTGATGTCAGTT AGTATGAATGGGACATTCAGTTTAAAACTGTTATAATTTGATGTCTGATTACCGCTCAACAAATTCCAACACAACCATAACGAAGCCGGAAAA CCATAAGGGTTAAAGCCCTGGGGTGCCCTAATGAATGAACAAACCCA
GI190335104	CCCAACCATTTACTTTAACAGCAGAAAAATGTGGATTTCAAGAACTTGGAGCTGAAACTACTACTGGTGGGACCAAAATTGTGGGCACTAATG TGCAGTCTCCAACAGTAAAGAAGGCTCAGCCAATGGAACCTTCACAACCAGATAGCACCAAGCATGGTGATGAAGAGGATGCTTGCTCTGTTG CTTCTTCATAAATTCATGCTACCTTTTTGCAGCCAATGAAACCTTCACAACCAGATAACACCAAGCATGGTGATGAAGAGGATGCTTACTCTGT TGCTTCTTTCACTGCTGCATCAATTCAAGC**AATCAAATCA**AGGATTATTGCCGCAAGTGCTCGAGTATTTAGGGTCACTGAACGTGCAGAGA

	AGCGGAAAGAATTTTACCGAAAGCTAGAAGAGAAATACCAAGCTCTAGAAGCTGAAAAAAACCAGTCTGAAGCAACGACCAAGATTTTTGAT TTTTTCAGATTTGTTTTGTATGGGTAAATGTGTGTCTCTGTAGTCGTATCTGGTCATTAGCTTGTTTCCTGGTGTTGAAAATTTGGTTATTCCGGT CAGAAGTGATTAATCCAAATTTATTGGNTTGGGCNCCATGGCGGTTTCAGTGGGGAAACTGTCGGGCCTTGCTNAGGAATCGGCA
GI189502767	ACTTTCAATCAGGAGCAGAAAAAGATGGGTAACTTTTTCCGAGTGACCTGCATTTTGAATAATGCAGCTTGC**AAACTGAAACTG**AATGAAT TCCGGGAAGCTGCGAAGCTCTGTGCAAAGGCTTTGCAATTAGAGCCTGACAACGTGAAGGCTCTCTACCGTAGAGCTCAGTCTTACATTGAGA CAAGTGATCTGGACTTGGCAGAAATAGACTTATTGGCTGCAAAGGAAATTGAACCAGACAATGAGCCATTAAATCAACTACTGGAGAGAGTG AAAAAACTAAAGACACGAGCCAGTAAAATGGATATGAAATTTTACGCAAACATGCTGGCAAAGGTTAACATTGCACACTGTAAATAACTCAG AAATATCTTTATACTGCTCATTTTTGTTGAGTTAAAACAGATCTGATTAGGGTTGGAAAAATAAGCATTGAAGAGATTGTTTGCCTCAGCGGCT CTGTATCATTCTATAGGCGAGAGCTTTTAATTTCTTTTGTGATTAGTGATAAAAGAAGGGACGAGATAAATAATTTGGTGGGCATATGTTTGGA ATGTATTCTGCATGATGAGCTGTTGTTGATAAATGCTTGAAAGGGATGATGATATGTATAATAATACACAGGCTGCTGGGAGTATATATATGTA ACTTGTACCTGCCCGGGCGGCGCTCGAATCAAAAGGGGCC
GI189502766	ACTTACATCACAACAGTTTGCCACTACAGAAAACCGCGGATGTAGATCTCAGAAGTTTCCTACTGCAGTAGCGGCACCCTACGTTCCTGTGCG GCACTACCAGAATGTTTATCATGGGATGGCCCCAC**CTGTAACTGTAA**GAACGGCAGTTCCTGTGTTTTCAGCACCTCCTCATCCACCAACAC CTGGCTGCTGTGCTCCTCCAGGAATGGGGCCGGGAGTGCGAATGGCTCCAGCTGTGAATATAAGATCTGTTGTGCCAGTATTTGCAGCACCTCC ATCCGTGGTCAGAATTGAGGACCCCCCATCAGTTTTTGCTGCTCCTACAACACTGGCAAGGTCATCCATAAAGATCGAGGAGAAGGGTAATCC CACTTCATCAACAGCAGTTATAGCTCCACCTCCGCCCACACAGACTTCAGTGAAGACGGATGACATTACCAATTTGCCAGGGCCTTCCGTTTCC GCAGCTCCTCACTCTGTCCAGCCATCGGTTATAGTAGAAGAAACTAGCATCCCTGCTCCAAAGAACATGCAAAACCCAGTCATACAGAACCTA CAACAGCTAAAGATATGATGAGGAGGTTGTAAGTTGGAAAATGATTCTCTTTCTCCGTGCTGTTCCTTAACGTATTTGTTTGGTTTTTCCGATGT ATGTCGTAAATCATCCTGCGTAGTTAGCTTATGGTAGTAC
GI189502765	ACAGGAGAAAAAGTGATAGAATTTTATCTTCTGGAAAGCAACTCCTTCATGGGCTTACTTCTGTTCACTCTAAAAATGCTGGGGTGGACGTTGA AAAGAAGTCCAAGGCTAGAAAGGACAAAGCTGGAATTTTGTGTCATGCATCTTATGCTGCTATAAAGCAATATGGTTCACTCCCGTCTACTTG AATTCTTCACATTGAGACTTTGCAGAAAGAAATGATTATGACATCCGATCAGGCTTCTAACCAAAATCAATGGTTACCTAAAGGTTTTGCGTAT GTTCCAATAGGTTGCTTATCGAAGAGAAGCAAGAACCTGATGGATAATGTCTCCCCTTCAATGGGCGACTTGTTTGCACTTTCATCATGACTCC CAATTTTGCTCAAGCATATTGTTTGCCGCAATGGTGGAGCTTCAATGCTGTGGTAACTGTTTGATTAAAAGCAAAAAAAAAAAAAACCAGATC AATTGGTCAGCTGAGTTTTTGATGGAGCTCCTCAAGCTCCTGTTACTGCAGAGAAATGACAAGGCCAGGCTATCCTGCAGGATTTCTGGGGGT GGCTTTGCAAAATGTTCATTGCCAGCGGAAAGAGAGACCAGGGTGGTGTCATGATTTTTAACAGAACGTTAGTTGAATAGCTATGTTTGGCCT GTCCAATG**GAGATGAGAT**TTTACTCTGATACTTGTATATTAGGAAAGCTGGTGGCTTTCTTTTCTGTAGAATCTGTAAATTAGACCGTTGTAT TGTGTCG
GI189502763	ACAAATTCAGCACCGACATTCCCCAATATATGAAATTGTAACAGAAGCACCATCTTGCGTAGAGCAGGAGGATGAGCTCAGTTCCACTGGAAA

	ACCAGTCCACATCTGCTATATCCTTGAACATCTCTGAATCTATACACTTCGTTGCAGAATTGCGCAACAAACTGATTGCGAGAAAGGCTATCCA CCCCTTGCGAAAGATAAGGAAAACATATCTAAAAAGACTCCTATCAACCGATTCTTAAGCATATTGCAATCAACCACTCTATGCTAAACCCTG TCAGAATCAAAAAGAAGTTTCACCTTACAGTTCTGCAGTCCACTAATACCCCACAACAGAAATTCAATAGTCCATATGATACTTGTCATAAAA AGGAAGCTAGTAAAACTCTTGCCCTCTTAAGCCAGTTCCTAGATGAGATTCCATATGT**TGATCATGATCA**AACTTCATTGTCCGTCTGTTAC CACTAAAAAGTGCACACAAGACTTGGAGGTAACTGCGTGTACCTGCCCG
GI189502759	ACAAACCATTATTCGCTTCATATCATGAAACAAATAAAGAGGAAATTTATTCTTCTTATTCTAATTCCACCTTACTAACATTATTAGCTAAGGC ACCAAATATGGTCATATCCATGATCACACTTGTGACATAATATTAAAGCTATCCATTTGATGGCTAGCATCGATCACTGGGCAACCACCATTTC GATGGGGTTGTCCTCCACCTCCAAAGTGTCGGTGCTATTTGGCAGCACATCTAGCTTGACAGACGCCGGACAGGTCGAGCTCCGCGGCGTCGG GCAGGTGATGAATTTGGTGGCGGATGTCCACACACAACCTCACATTCATGATCCTCTCCAACATGACCGAGCCGCAGTGAAGCACCGCCGTGT TCACTCCCAATCCTTGGTTGATAGCGTTCACCACATCGACGGTCAGGTATTTGTTGGTGTGAGATGGCCTGATACCTCTTCTAGAGAGGAGATA GAGGAGGTTCGCCTTATCGCG**AAGTGCAAGTGC**TCTGGAGAAGTAGTCGCTCACCGTCAACCAGGTGCAGGGGCCGTTGTCTTTCCAAGTC TGCTCCCACGCCACCATGTTGTTGTCGTTGAGCATGCAGCCGAGCTCGGCCAATTCTGGTTGAGACTTCTATGATATTTTGGAGAGCCTTGGGA TCGAAGGAATTTAGAGCATTGATGCTGCGAAATTCCTTGCCAAAACCGGACGACAAGTCGTCTTTTTGGTCCAGGTGGAGGGAGCAGCATCGC CTTGCTGGGGGCAGA

SSR containing sequences in Piper colubrinum	
Locus name	**Sequence**
GI90988886	CTCTCCAACTGCAAAGTATGGTTCGGTAGCGTCCAAATAATCTTTGACATAACCTCCCCAGAATCCTCTAACAA**AGTCTAGTCT**CCATCCATCAT ATCCAATCTCCTCCCTAAGCCAGCGAAGCCATTCCTTGATATCATTCCTCACAAAAGTCTTGTGNAGTGATCAATATTTGGAGCCGCGTGGAAATT ATCACCACTACCCTCTATTGCCCCCTTCCCTGA
GI90988877	CTTTTTCGGGGGAGCGAAGGAGAGGCGCAGA**TCGTCGTCGTCGTCG**GCGGGGGGGAGGAGAGATGAAGATCTTCGTGAAGACCCTCAAGGG CTCCCAATTCGACGTCGAAGTCGACGCCGAAGCCACGGTTGCTGAAGTGAAAAAGCATATAGAAACTATGCAGGGTGCGGATGTTTACC
GI90988875	CATGCACCGTGTTTAAGACTGACCAGTATTGTTGCAACTCCGGCAGCTGTTCGCCGACGGACTACTCGAGGTTCTTCAAAACTAGGTGCCCGGATG CCTATAGCTACCCCAAGGATGATCCTACCAGCACTTTCACTTGCCCCGGCGGCACAAATTACAGAGTTGTCTTCTGCCCATGAATGGTTGATCCAA TTAACCATAAACCATGCATGCG**TATATATATA**AATAAATAATATATGTGATGAATAAGGTCTCTGAGCATTCCGGGATGAAGCTCGGGAGATTG GAGATCAGTTGGTAAAATAAGGAGTTGT

GI90988874	ACTCTCTTATGTGGTGCCTGTATTCATAGCAGAGATAACACCCAAAAATCTCAGAGGAGGGCTAACAACTGTAAATCAGTTGATGATTTGCACAGG TGTATCTGTCGCATACATCTTAGGCAACC**TCATGTCATG**GCGCATGCTAGCTCTAACGGGACTCATCCCATGTGTAATTTTACTGGTGGGGTTAT ATTTCATTCCANAATCCCCCAGATGGCTGGCAAAGGTCAATCGTCAGAAAGAATTTGTAGTAGCCCTTAGGAGGCTTAGAGGTAAGAATGCTGAC ATTTCCATTGAGGCATCTGAAATCCAGGATTACATAGAATCG
GI90988864	CCACCACATAATTATTACATATATATCTGTTATATTTACCATACGTCACGCAAATATCGCCCGTCCATCTGCAATTTTTTTACGAAGCTCTCAACCT GGGAGGCATCCATTGAACCCTGTTTTTGAACTATAGTATGGAGAACCCGATGCACATCCTTGGCCATGCCTTTGGCATCACCGC**ATACATATAC** **AT**AACCCCCTTGGGTGATCACATTCCATATCTTCTGAGGCCTTTTCAATCATCTTGTGCTGCACATACTCTTTACTGGTCCCTTCACGAGAGAAGGC AACAATCAGCTCATTCTGTTCCATTTTTAGGTNTTTGTTTGGGGATGCAGATAGCTGTCATAGAGTATACAAGGTCCATTCTTGGCCAC

Microsatellite containing sequences in *Piper tuberculatum*	
Locus name	**Sequence**
GI292494868	GGGCTCTCCACTAGGGACATGGTTGCCTTGTCCGGGTCACACACCATAGGTCAAGCCCGGTGCACAACCTTTAGGGCCACATATACAACGAGAGC AAATTTGAAGGTTCTTTGGCCAAGGACAAGACAGGCTAGGTGCCCAAGAAGATCGGGCTCTGGCGACAATAATTTGGCACCGTTAGATCTGCAGT CGCCGACCTTTCAACAATCACTATTACAAGAACCTCATCAGTATGAAAGGGCTATTGCACTCCGATCAAGAGCTGTTCAACAGGGGCTCGGTGGAT TCTCTTGCCGGACCTATAGTAGTAGCGAGACCACCTTCTTCAACGA**CTTCGCTTCG**GCCATGATCAAGATGGGAGACATTAGCCCGCTCACAGG CTCGAACGGAGAGATTAGGAAGAATTGCAGGAGGATAAAT

4.3. Isolation and identification of SSRs from genomic DNA of black pepper

To isolate more SSR from black pepper, genomic DNA libraries were constructed enriched for microsatellite repeats [$(AG)_{12}$, $(TG)_{12}$, $(ACT)_{12}$, $(AAAC)_{6}$, $(ACCT)_{6}$, $(ACTG)_{6}$] following the selective hybridization method (Glenn and Schable, 2005).

4.3.1. Restriction digestion

Among the four restriction enzymes *viz. Eco* R1, *Alu* 1, *Bst* U1 and *Rsa* 1 (Fig. 15) used for digesting genomic DNA, two restriction enzymes (*Rsa* I and *Alu* I) gave good restriction digestion pattern (Fig. 16a. and Fig. 16b.). The restriction with both the enzymes was carried out separately. They produced a continuous smear of fragments ranging from 200 bp to 2 Kb. Both the enzymes were selected for further steps based on the size range of fragments generated. The 50 µl restriction digestion mix contained 2 µg DNA with 1X reaction buffer and 20 U of restriction enzyme (*Rsa* I and *Alu* I) incubated for 8 h at 37 ºC.

4.3.2. Ligation of linkers to the DNA fragments

The double stranded linkers were prepared by mixing equal volumes of equal molar amounts of SuperSNX24 forward primer (SuperSNX24 Forward: 5'GTTTAAGGCCTAGCTAGCAGAATC) and SuperSNX24+4p reverse primer (SuperSNX24+4P Reverse: 5'pGATTCTGCTAGCTAGGCCTTAAACAAAA) (e.g., 100 µl of 10 µM each). Salt was added to a final concentration of 100 mM (*i.e.*, 4 µL of 5M NaCl for 200 µl of primers). This mixture was heated to 95 °C, cooled slowly to room temperature to form the ds SuperSNX linkers and stored at -20 ºC.

The *Rsa* I and *Alu* I restricted DNA fragments were then ligated by T4 DNA ligase separately. The reaction mixture (50 µl) contains 10 µl ds Super SNX linkers(5 µm), 3 µl 10X ligase buffer, 2 µl DNA ligase (New England Biolabs, 400 units/µl), 1 µl *Xmn*1 (NEB), 34 µl restricted DNA fragments, were mixed properly and incubated at 16 ºC for overnight in a thermal cycler (Eppendorf Master Cycler S).

4.3.3. Confirmation of linker ligation to restricted DNA fragments

The ligation of linkers to the restricted DNA fragments was confirmed by performing a PCR with linker ligated mix as template and SNX forward primer. The PCR products when separated on 1% agarose gel, a smear of fragments (200 bp - 1 Kb) centered at 500 bp was observed, indicating successful linker ligation (Fig.17a. and 17b.)

4.3.4. Hybridization of oligonucleotide probes/ PCR recovery of enriched DNA

After enrichment a light coloured pellet was formed on precipitation. The enriched fragments on PCR amplification with SNX forward primer produced a smear of fragments (200 bp - 1 Kb) centered at 500 bp, confirming successful enrichment of microsatellite containing fragments. (Fig. 18).

4.3.5. Ligation of enriched DNA to the plasmid vector

The microsatellite enriched DNA fragments were ligated to pCR® 2.1 TOPO from Invitrogen (Carlsbad, CA) and transformed into competent cells. A total of 185 white colonies obtained were transferred to master plates, incubated overnight at 37 ºC (Fig 19).

4.3.6. Screening, identification and selection of positive clones

One eighty five transformants were subjected to colony PCR using M 13 forward and reverse primers (vector specific primers) (Fig. 20.). One hundred and six clones having insert size of 300 bp to 1 Kb were identified and selected through colony PCR.

4.3.7. Isolation of plasmid DNA

Plasmid DNA was isolated from 106 clones with an insert size of 300 bp to 1 Kb. The plasmid DNA isolated from positive clones was represented in Fig. 21.

4.3.8. Restriction digestion of plasmid DNA

Plasmid DNA was restricted using *Eco* R1 restriction enzyme to confirm the insert size (Fig. 22.).

4.3.9. Sequencing of plasmids, redundancy elimination and clustering

A total of 106 plasmid DNA were sequenced at Xcelris Labs Ltd, Ahmedabad. The sequences obtained were subjected for contig assembly and microsatellite identification.

4.3.10. Identification of microsatellites from black pepper genome

Scanning of microsatellites in the non-redundant dataset revealed a total of 144 unique SSRs in the case of *P. nigrum*. Here also the two times repeats were set as parameters in case of hexa or penta nucleotides search from the dataset. From such analysis, pentanucleotides were the most aboundant (38.19% of 144) microsatellite repeats detected, followed by dinucleotide (18.10% of 144) and hexanucleotide repeats (13.90% 0f 144). Di nucleotide repeats were the major portion if two time repeats were

excluded from the dataset. Only a single type of mononucleotide repeat (A/T) was noticed. The other most abundant repeat motifs were (AC/GT) in dinucleotides, (AAG/CTT) in trinucleotides, (AAAC/GTTT) in tetranucleotides, (AATAT/ATATT) in pentanucleotides and (AAAAAG/CTTTTT) in the hexanucleotide.

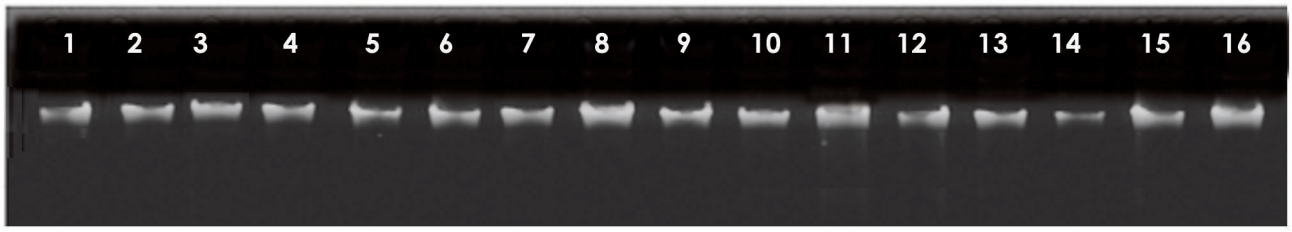

Fig.12.Genomic DNA isolated from 16 released varieties of black pepper. Lane 1: Panniyur-1, Lane 2: Panniyur-2, Lane 3: Panniyur 3, Lane 4: Panniyur-4, Lane 5: Panniyur-5, Lane 6: Panniyur-6, Lane 7: Panniyur-7, Lane 8: Subhakara, Lane 9: Sreekara, Lane 10: Panchami, Lane 11: Pournami, Lane 12: PLD-02, Lane 13: IISR Sakthi, Lane 14: IISR Thevam, Lane 15: IISR Girimunda, Lane 16: IISR Malabar Excel.

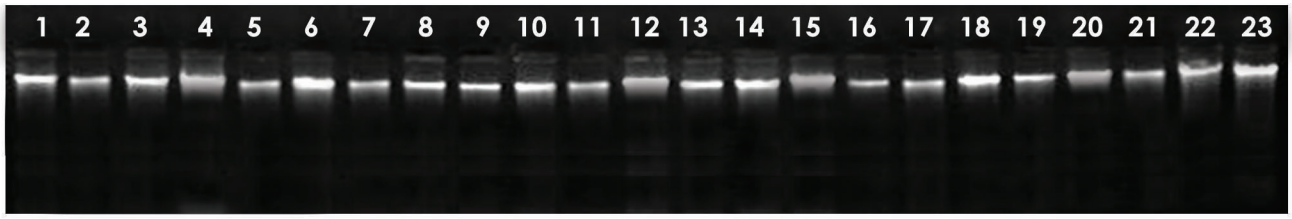

Fig.13. Genomic DNA isolated from 23 cultivars of black pepper. Lane 1: Kottanadan, Lane 2: Neelamundi, Lane 3: Kuthiravally, Lane 4: Kalluvally, Lane 5: Narayakodi, Lane 6: Perambramunda, Lane 7: Poonjaranmunda, Lane 8: Valiakaniakkadan, Lane 9: Cheriyakaniakkadan, Lane 10: Uthirancotta, Lane 11: Balancotta, Lane 12: Arakkulam munda, Lane 13: Thommankodi, Lane 14: Thevanmundi, Lane 15: Chumalakodi, Lane 16: Nedumchola, Lane 17: Malamundi, Lane 18: Karimkotta, Lane 19: Perumkodi, Lane 20: Karimunda, Lane 21: Kumbhachola , Lane 22: P 24 (IISR Sakthi), Lane 23: P 24 O4 (O4-P 24-1)

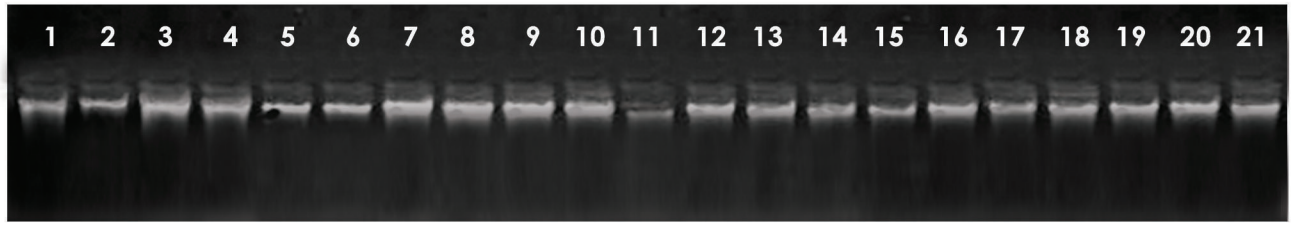

Fig.14. Genomic DNA isolated from 21 *Piper* species. Lane 1: *P. betle*, Lane 2: *P. nigrum*, Lane 3: *P. sugandhi*, Lane 4: *P. trichostachyon*, Lane 5: *P. galeatum*, Lane 6: *P. barberi*, Lane 7: *P. attenuatum*, Lane 8: *P. argyrophyllum*, Lane 9: *P. hymenophyllum*, Lane 10: *P. bababudani*, Lane 11: *P. longum*, Lane 12: *P. hapnium*, Lane 13: *P. peepuloides*, Lane 14: *P. sarmentosum*, Lane 15: *P. ribesioides*, Lane 16: *P. thomsoni*, Lane 17: *P. hamiltonii*, Lane 18: *P. colubrinum*, Lane 19: *P. arboreum*, Lane 20: *P. ornatum*, Lane 21: *P. magnificum*.

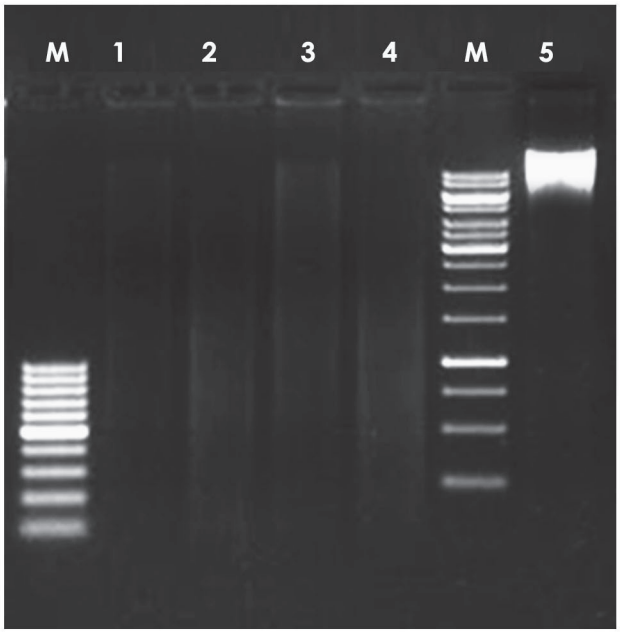

Fig. 15. Agarose gel showing the restriction digestion profile of Black pepper DNA using 4 different restriction enzymes. Lane M- 100 bp DNA ladder, Lane 1- *Eco* RI, Lane 2- *Alu* I, Lane 3- *Bst* UI, Lane 4- *Rsa* I, Lane M- 1Kb DNA ladder, Lane 5- Black pepper var panniyur 1 genomic DNA

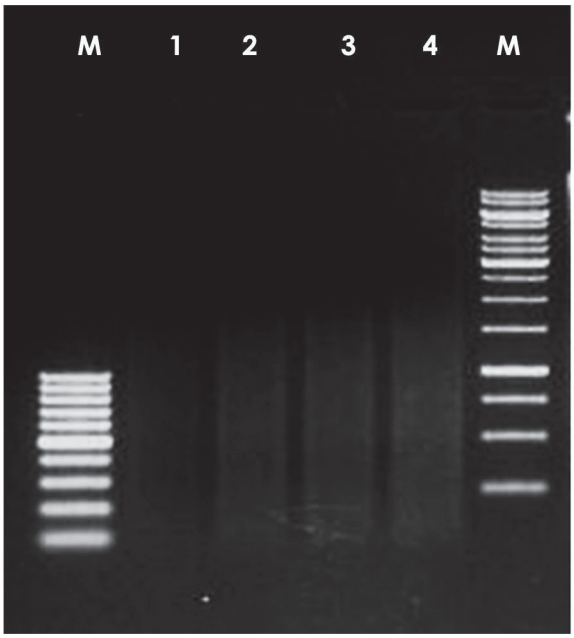

Fig. **16.** a). Gel image showing successful restriction digestion with *Alu* I. Lane M- 100 bp DNA ladder, Lane 1- 0.5 µg of genomic DNA, Lane 2- 1.0 µg of genomic DNA, Lane 3- 1.5 µg of genomic DNA, Lane 4- 2.0 µg of genomic DNA, Lane M- 1 Kb DNA ladder

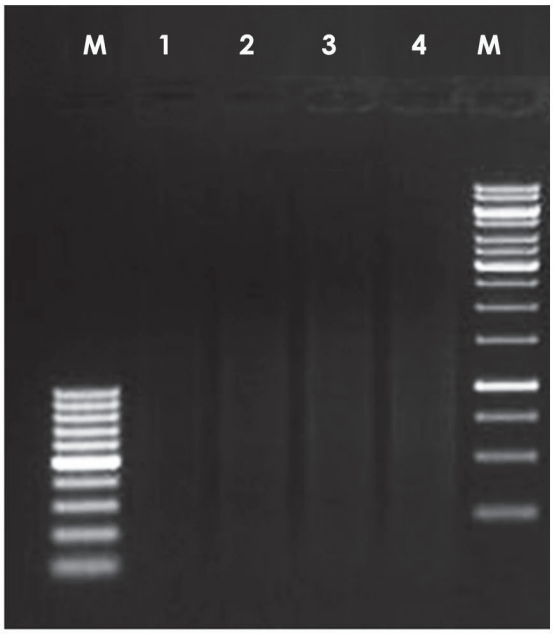

Fig. **16.** b). Gel image showing successful restriction digestion with *Rsa* I. Lane M- 100 bp DNA ladder, Lane 1- 0.5 µg of genomic DNA, Lane 2- 1.0 µg of genomic DNA, Lane 3- 1.5 µg of genomic DNA, Lane 4- 2.0 µg of genomic DNA, Lane M- 1 Kb DNA ladder

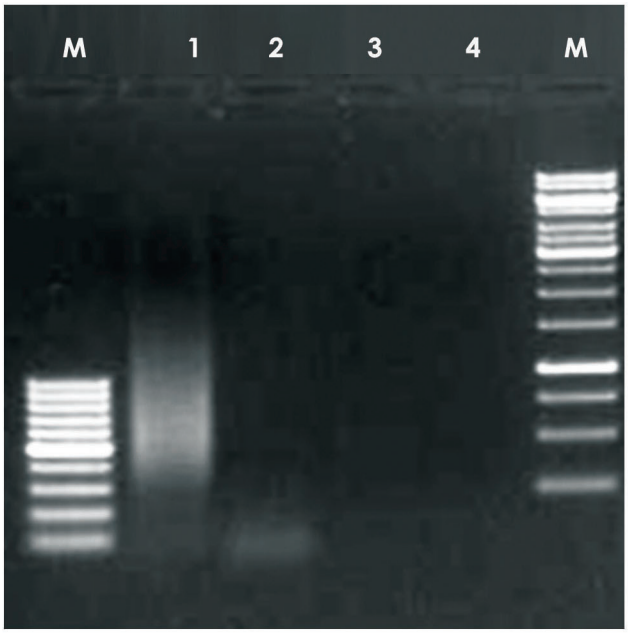

Fig. 17 a). Gel image showing successful linker ligation. Lane M- 100 bp DNA ladder, Lane 1- linker ligated DNA fragment digested by *Alu* I, Lane 2- control 1 (double stranded linker), Lane 3- control 2 (*Alu* I digested DNA), Lane 4- control 3 (sterile water), M- 1 Kb DNA ladder.

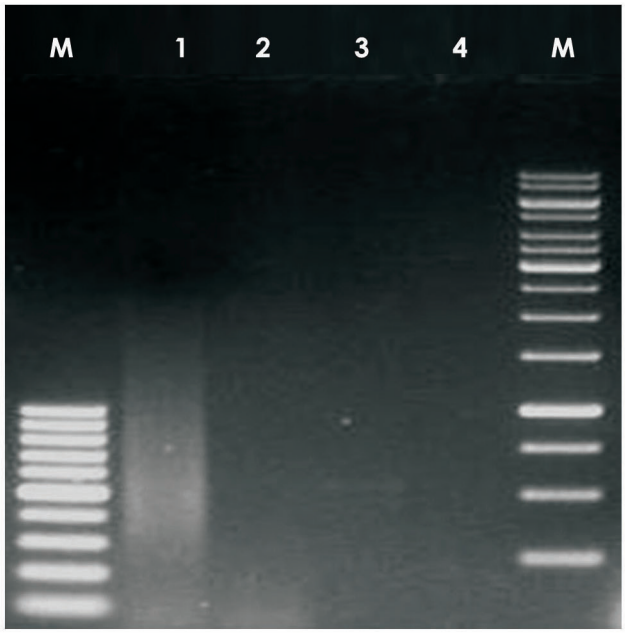

Fig. 17 b). Gel image showing successful linker ligation. Lane M- 100 bp DNA ladder, Lane 1- linker ligated DNA fragment digested by *Rsa* I, Lane 2- control 1 (double stranded linker), Lane 3- control 2 (Rsa I digested DNA), Lane 4- control 3 (sterile water), M- 1 Kb DNA ladder.

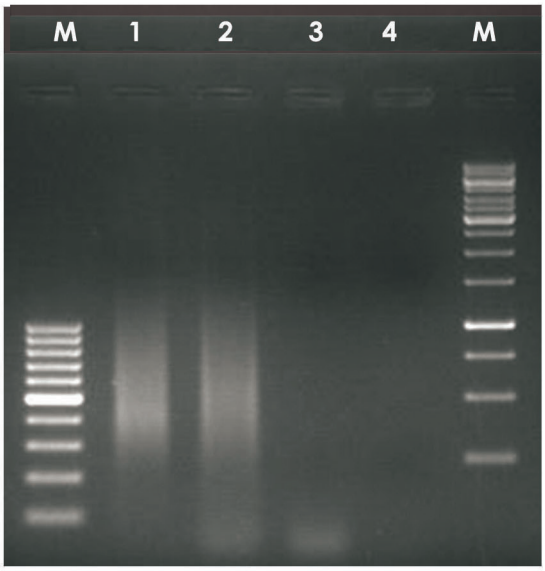

Fig. 18. Gel image showing successful enrichment using 5' biotinylated oligos. Lane M- 100 bp DNA ladder, Lane 1- enriched fragment, Lane 2- control 1 (linker ligated DNA fragment), Lane 3- control 2 (ds linker), Lane 4- control 3 (sterile water), M- 1 Kb DNA ladder.

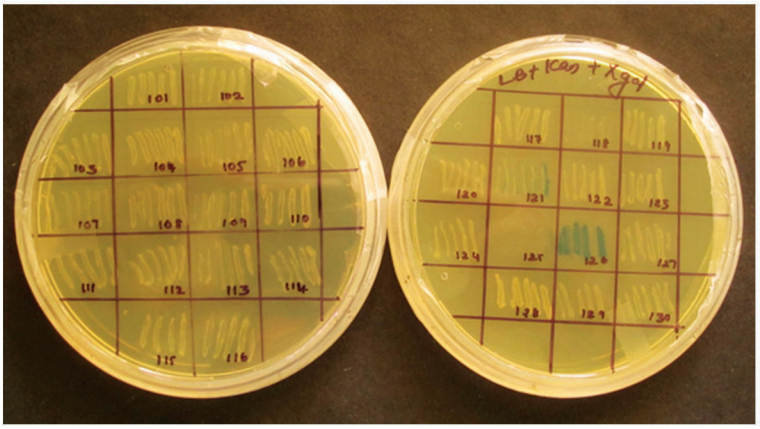

Fig. 19. Genomic DNA library enriched for microsatellite repeats

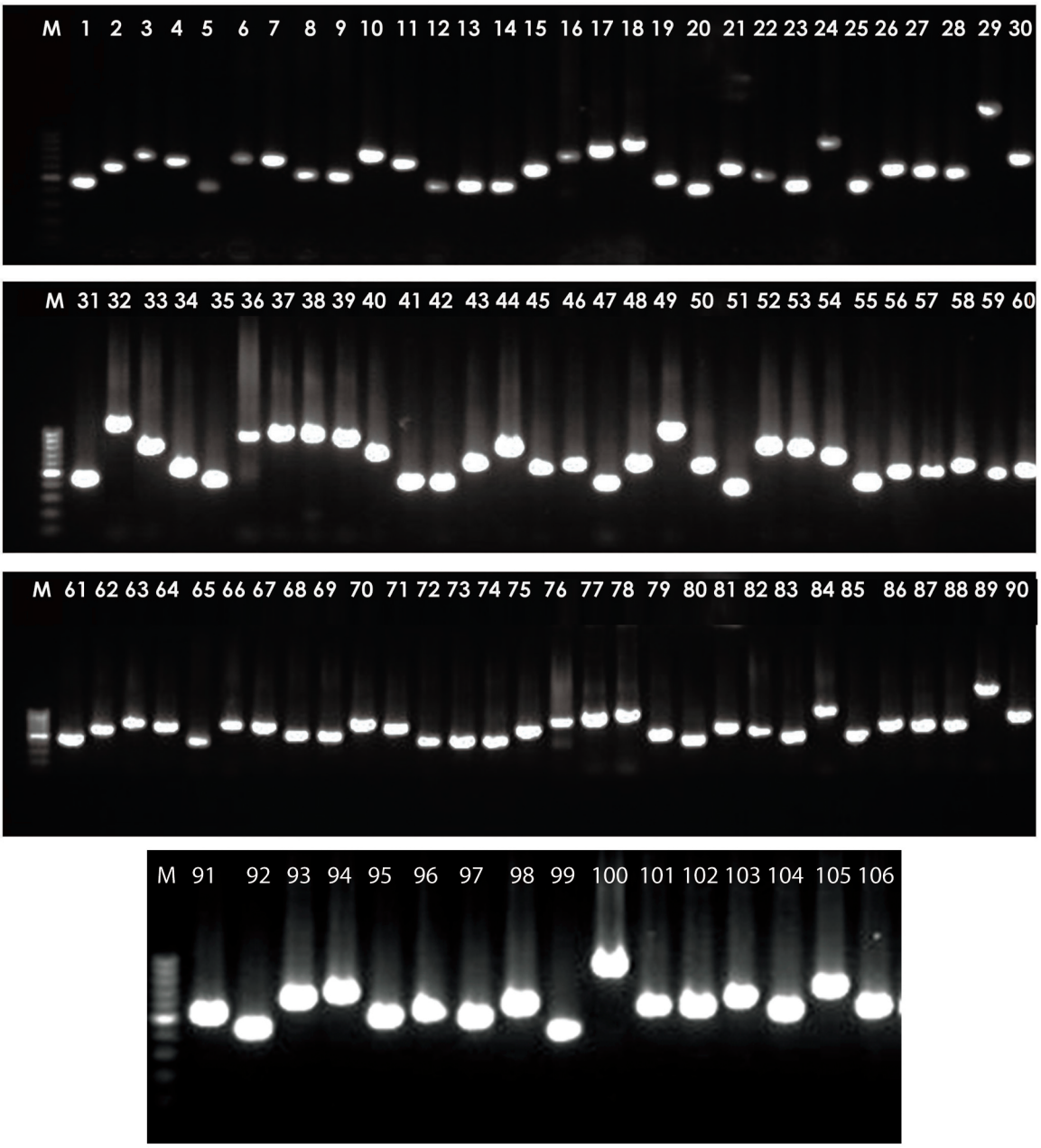

Fig. 20. Lane M: 100 bp DNA ladder; 1-106: Screening of putative recombinant clones from genomic DNA library enriched for microsatellite repeat probes [$(AG)_{12}$, $(TG)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(ACCT)_6$, $(ACTG)_6$] using colony PCR.

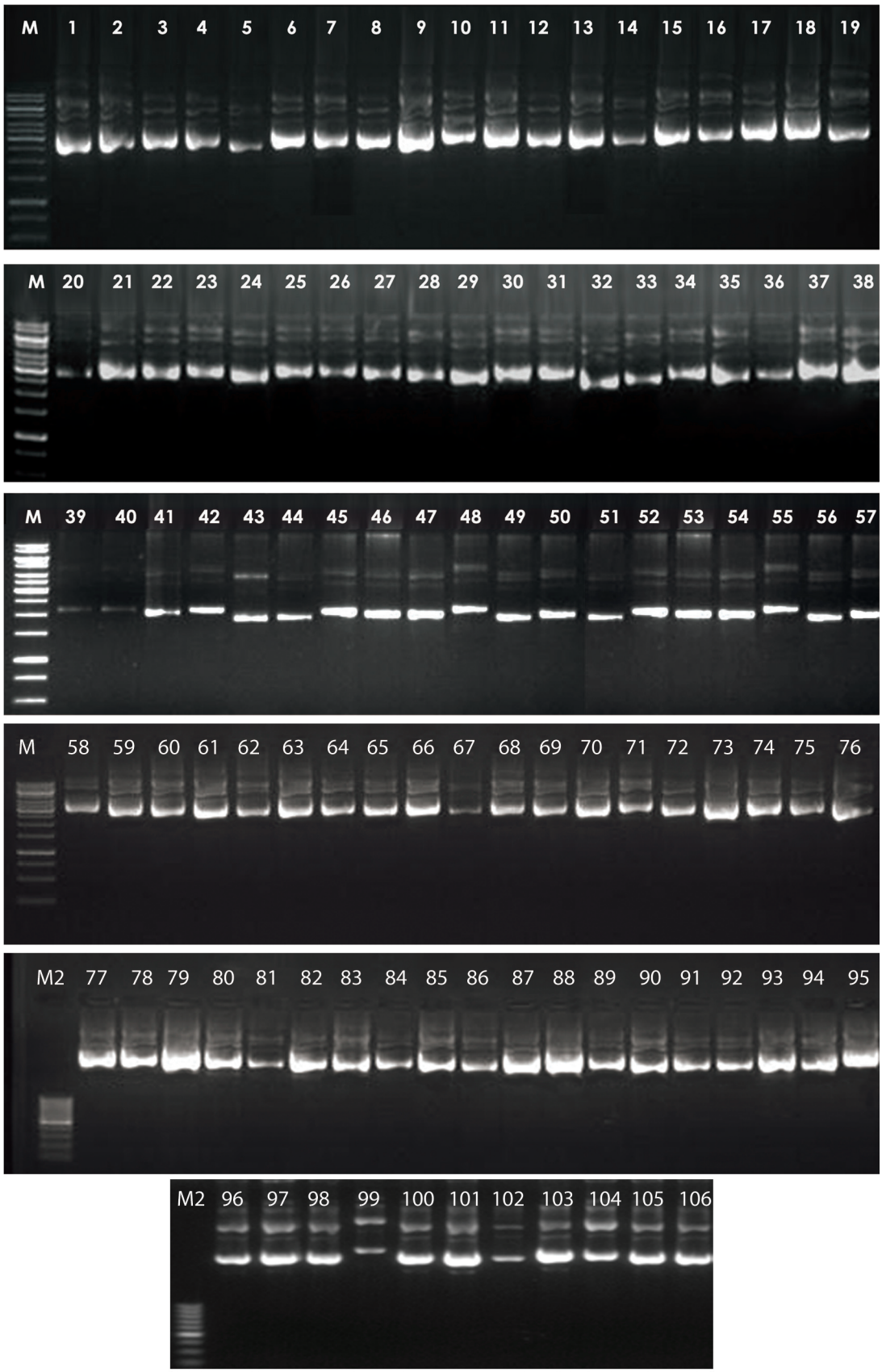

Fig. 21. Lane M: 1 Kb DNA ladder; 1-106: Plasmid DNA isolated from the selected putative recombinant clones, Lane M2: 100 bp DNA ladder

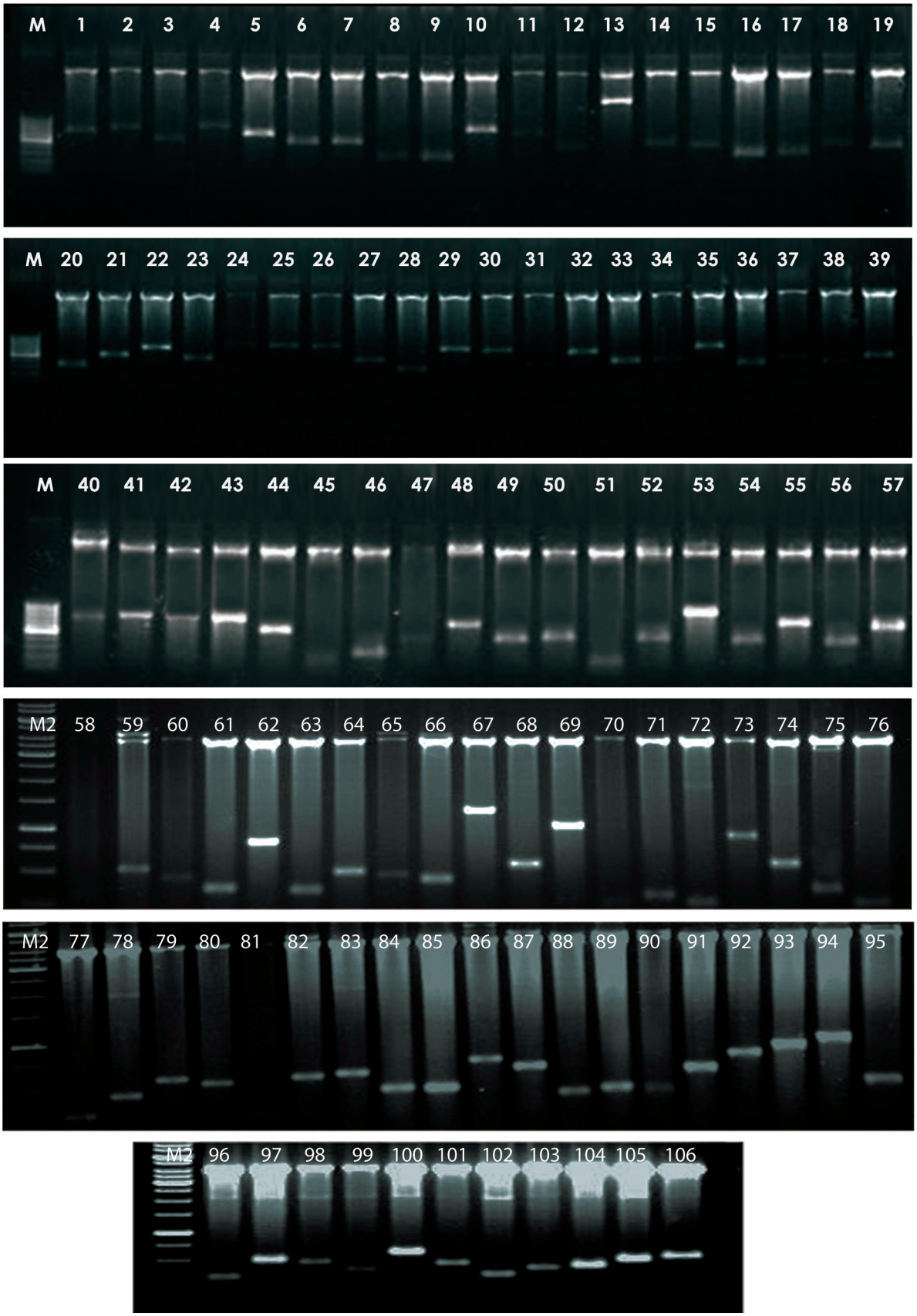

Fig. 22. Lane M: 100 bp ladder; lane M2: 1Kb DNA ladder; 1-106: plasmid DNA restricted with restriction enzyme *Eco R1*

Table 13. Details of some cloned genomic DNA sequences with the microsatellites regions obtained from black pepper genome.

Locus name	GenBank accession no.	Sequence
BPM CNTG 4	KC 347528	ACATGTATATGGGTGCATGTATATGTTTGTGTGTATGTATGTTTGTGTATGTATGC**ATGTATGTATGTA**TGA ATGATTTTGTTGGATTGACTGATTATGTGTGGTTATGATTATTGCTTCCGCTAAGTAGTTTGTGTGGTTATGAT TATTACTTCCGCTAAGTTGTAGTTAGGTAGAACTACATCGAGCTTGATCCCAACATGGGTATGTAGGCCAATC AAAATTGTTTGATTTCAGCTCATCTAAGATAGAATAAAAGAAGGTTAATACCATGTTAGTAGTAGTTAAATTA TGTTTAACTACTATTTAGATTTTTGGGGTGTTACAAATGGTATCAGAGCGAACCCGGTTCCGTCTTGGTTGATA GGAATCCAAAACCTACTGGGGT
BPM CNTG 5	KC 347529	ACTTTTTATGGTTAATACTGGTGATTAGATTCAGCTTATGTTTAATGTTGGTTTTAATTGTATATATTGTGAAAT TGAAAATTTGGATTTGTGGGATTAGTTGTGGTGATAAGAGATTGCTGTTTTTTTTTGGATTGTTTAATTCAACC GTGACAGAAGATCCTTGCTATAGTTGTGTTTGCTTGAAATTTTGTTCAATTTATTGCTTTTGGTTGACATTTGTA ATTGAATTTAGCTTGTATTCATTTTTTGTTTCTAATTGTATGGTGGGATTAATTTAGGTGATTAGAGATTGAAG ATTTTTGGACCTTAGTGATCGGATTTAGTGTAGTTTAATTTTTGGTTCTAATTGTATATTGAAATTAAA**GATT TGGATTTG**TGGGATTAATTGTGGTGATTAGAGATTGCTGTTTGTTTGGATTGTTTGGGGGTAATTCAATCGT GAAAGAAAGATCCTTTCTATATTTTGTTGGTTTGAACTTGTGTTCGATTGCCTTAGGTTGATATTTGTGGTTGA GTTTATCTTATGT
BPM CNTG 6	KC 347530	ACGAACCCTCCCTAGGGAACTAAAAAAAAAAGCCTTTAACGCTGGCAAGCGCTTCTTACTGGTCGGGTAAGA AGACCGATAGCCCCACCCAAGCTATCAACAACGAGCCCAGACCTTCATTTACGCTATTTCAGAAACGAGATTC TTATATAATATATATAATTGCATCTCTCTTTTTCTAATAGACTTGTGATGT**TGTGTGTGTGTGTGTG**AGAGAC CGAGCCTGATCAAAGTTCGGTCTTTTCTTTCTCTTTCTTCTGTGCTCATTTAGGCCGAGCATTCAAAGTTCGGC CCTTTTTTTTATTCCTACACTGCACATCATTGATTTTTTTGCCGAAGGCTAAATTCTCGATTCAAGTAAGTGGG ATGGGAGCCTGCTGTGGTTCAAAAAGCGAACTAATGATATAGAGTCTTATTCTCTCGGATAAGCTTTAAAAGG TTAGCTAGACCCTTTGCCATCGAACACTGTAAACAGAGTAAGAGAGCAAAACAGCAACACATAGTCACTGTC TAAATCAAAACCCCGTCGATAGCGTAGAGTCACACCTTGGATTGTTCCCAATGCCAAAGGAGGCTCACACGC CCTGTATAGATCATAGGCCTGAAAGAGGATACCGTAGACTTCAGATTTCATTTCTTCTTTATCAAAAGCCTTTC GGCGCCAATCTAGACAGACCATTCTCTGAGCTAGTAGGGCGTCTATAGGAGTATTGAGATCAAGGGCTACAA GGCTGT
BPM CNTG 9	KC 347535	ACAAGTTTTAACATGGGTATATATCTAGCTTACAGCTATTTTCAGTTTTTTTTTTTTTAAATGACGAACCCAGT

		AATCAAGAGTTTGTTAAAAAAAATTAATACCTGCAACTACATCAGCACAACCTTAATCTTGATGGGAGGAGG TTCATAGCAAAACATTTTTTTTTAAAAAAAAAGGA**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGA** **GAGAGAGAGAGA**GGGAGGAGGAATATTAATACATTTTAGTGATGATTTTTTGTGAAAGATTGTTAACTTC TTGAGAGGTGTAACCTTTAGTAAATAAAAAAAGAAATTAATTAATACCATATACACACACTATATGGAGACA CCGAGCCCTTTTATAGACCTCTTATGTTATAGATTTTTTGTGACTTTGTAGCTTCTATACATGGAATGAATGTG ATTGT
BPM CNTG 10	KC 347536	CTAGCAGAATCCTTGTGTGTTGGCTGAGTAGTTTAGTTTATGGCAATGTTTTGGATAGTGTTGAACATGGGTTA TGTTCCATGATTCTAATAATGACTCCGAAATGTGTGCTTTTTGTGTTTGGTTGTGATCTCAGATTGTGCAATTTT TTCGGTCAGGATTTTGGTTCTGAACTAAAAACAAATGAAAACAAAATACATATGCTATTTCTTGATTGATGGT TCTTTAATTAGTATTGTTGTTTGAAAGAGATTGTTTTGACCTTTATTATTTTGTGTATATGTGTCTGTTGCAGTT GGATCGAAGGTTTATGTGTTGGCTGAATAGTTTAGTTTATTGCAATGTTTTTGGATAGTATTGAACATGGATTA TTTTCTTGCATTCAAACGTGCTTCCAAATGTGGGTTTGCTTTTTGTGTTTGATTGTTATCTCAAATTGTGCATTT GGATGAAC**TTAGGTTAGG**ACGTTGGTTCTTGAATGTCTAGCAATCAATTTAAAGATCACTCAAACTTTAAA CTCACTTGATATGGTTACTGAAACTTTCTATTTTCCCCATTCCCTTCTTGAAAGCCTTGTTTAAGAAATTGATTA CTGTTTTTTGAGTATTTTTCATAGTTTGTTAAAAGATTTATATTGTTATATGTTTCATTTACAGAAGGAATAG
BPM CNTG 12	KC 347537	ACACAAATGACCTCTTCCTTTTTCAAATGTTGAAGAAAAAAATAAGATAAAATGTACCAGATGTTTATAAGGG AACTAGTCTCGTATATGAAAATTAGTGTATATCCCATTAAAGAGCTTTCATTGCATGGTGGGCTGTGAAGAAG AGAAAAGGGGGATGACTCAATA**AACAAAAACAAAAACAAAA**AAAAGAGAGAGGGGGATGAGCTGCTTC CTTCGTGGAGTTATCGAATGTCATATACATATAAATATATCATATATATGTTGCAGAGAGACAGGTAGGTGAT ATGCTTCATCTAAACTTGCACCCCCCTTAAATCTATACGGTCCCCCAGCTCCAATGTTTATGGTTATTTGTTGC TTGTTTACTGACAAGAATGGGTGAGCTACTAATGGACAAGGCACGCAACTACGGGTGGTCCTTTGTTTTCTGT GTGTGTCTGTGTTTTTTGTTTTCACCAGCTTCTCTGATTTTATATATGTGACATTGCATGCCAGTAAAATCTATC GGCCAACAGGAATGTAGTTGGTGTTCACTGCAAACACATCAATGCTATATGGTGGT
BPM CNTG 15	KU 189217	TAGAATTAAGGCCTAGCTAGCAGAATCACAAACTACACGGAAATAAAAAAAAAATGAAAACATAGGCTAGAA ACATTCAACGACCAACTAAAAT**CATACATACATACATA**ATCATACATGCTATTCCAAAACACACAATGAC ACCACGTGCAACCCGAACAACTCAACGGAGCAACAACATGTCATAAACATATACATGTATTGGAATCTAGTC GATGGTAACATGTTCATATCATGCATAAACATATACATGT
BPM 4	KC 347538	CTTCAATTTATATTGCTATAGTCTGTTTGTGATTTTTATTTCTTGTTTGTGTATAATTCTTTAACTGTGGATTAG GTTTGTTACATGATAATTTGATGTCTGTTGTTTGATTGCTTCCTTTATATTATTATAGTTTGTTTTTTACAT**TGT**

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BPM 6	KC 347539	AGGTGTGAGTCAGTTTCACTATCTCTCGGCTCACGAAAAACTCGATAAAAATCTTGTATAGGCTTGGCAGGGG ATTTAATAATTTAGGAATGAAGGCAACTTGCGGTTTATCTTGTTTTTGAAGTCAGACTTGAGGGATCACAACC TCGAGGATTGTTAATTTAAGGAAACTAGTTATATTTATTTATATAAAATAAAGTAACATGGTGGCAGGATAGC TCAGTTGGTCGAGCATTAGAATGAAAACTTAAAGGCTGTGTGTTCAAACCACGCTCTCAACATTGATATCATT TTTTATTAAAAACTAGCTCGTGACTTTATAATCGAGCTGGTTTAGAACGAGCTAGTTTTGAGTGTCTGTGTCTG TGTCTATGTCTGCGCCCTGTTTGCGTGAT**GTGTCTGTGTCTGT**GACTAGGTATGTCTCTGTGTCTGTCTCTT TGTGTGACTCTATTTGTGTTTTATGTCTTTCTCTGTCTATGT
BPM 8	KU 189202	ACGTCCTCTTTTAGTTTTTTGTTGTTCTTTGCGCTTGAAATTTGAGTTATTATCTTGGTTTTGTGTTGTGATGGA GGTGGTTGATGGTGTTGGTAGAGGATGAAATTTGATTGGGATTTTTAGAATTAGAAAGTTTGAAGTGGTGATT GATTGGCGTAAAGATGCGTTCTTTCTTTTGGTTTGTTGCTGTTGTGTGGAATCTCCAGTTGGGTTATTATTATAT A**TTTGTTTGTTTG**ATCTTCTGCTTTTTAAGTTCATGAATTGTATATGAGGGTTTGCTGCGGGTCGTCTACTGTT GTGGTGGAGGTGTTTAATTTGCTTAACAACATTTATTTGGCACTTAAGCTGGT
BPM 10	KC 347540	ACTTGTTTGTGACAGCTTACATCTTTCACGATTAGTAACTTTGAAAAACAATTACCAACACAAGGAACGTTTG AGAAACAAATATTACTAAGCATCAATGATAATTATGATATAAATCATTTATGAATCTCGATTAACTCTACCTG CCAACGTTCAAATAAATTACCAACAATTTACACATGTAACTGCTGTGAAAAAAGACACAAAAAAGAAGATAT GCTCCGAGCCAAAAAATCGACAACAAATTATCACAGTTAATGGATGGATTTGGAACGTTTTCCATCAATGTCA ACTCTTTTTTCAGAAGTAGAACGTCGAGCAGACAACTCCATGTATCTGAACCTCTTTATAAGCCGGAGGCAGT TTTCATCTGTAGCAGAATTAGAATGTCGAGGAGACATCCATGAGTCTGAACCTCTTTAAAAGCTGCTGGTAGC TTTCATCTTTGTCGTCTTTATCAGTTTCACATGTCCAGTCAGCAACTAAATCACGCCACTGCTTCCAATCAATG ATTGAATGCCGACGGTCTATAGAA**GTTTGTTTGTTT**GTTGAGCATTTCTTGTTTGTTTCATGTTTATCACGG CCATAGGAGTTGAAG
BPM 18	KC 347541	CTGGCTACTCTTAGAAAAAAACCAAAAATAAATGTTTACATTAGTGGAGATGAAGGCAAATGGCAAACATAT TTGTACATGAGCCTGAGTGTTATTTTCTTGTTGCAAATGAGAAGGATAAGGGAAAGGACTATCTT**ACACACA** **CACACACACACAC**ATGGAGTGCCTTCTTTTGGGTGTGATTGATGAAAGAGAATTTCTGATTTGATTGACAT ATTATTGCCTTTCGATTGTTTGATTAAGGATCATATGAGAACATAATGGGACTAAGTATTTTTGTGTAATAAA

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BPM 19	KF 05540	GCCCGAGGTTGAT**CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTT** **CTCT**ATGCCTCTTTATCTATGATTAATATAGAACATAAGCAATTTAAGATGTTAGCTATTTCACAAAGAAAA GCGTTTGTTTTATTTCTTGTTGATCTAATTTTTGTTATCACTGCAGATGTTAGTGGATCCTCTAGGTTCCATAAG TAACAAGAACAAGAGCTTGAAGAGCTCACATCCAATTCCCGTTATCGATGATGTTTTGGAACCAGAAGACTA CCCACCACCTGGCCCCAATCGTCCATTACCTCCTGGTTGTTGCTAATTAAATACAGTGT
BPM 23	KF 111689	CTAAATACTAACGCCCGCTCATGATGCAATCATACATCCTATGTCACACGTGCATCCATCACACAT**CAGTCA** **CAGTCA**CATCAACCATGCATCAATCACACACATCAATAATAATCAATATACATTTTCCTACTCTACGTAGCA TTAGATTACTATACCTATCTAATCAACCTAATTAATCCCTTAGTATTTATTTTATTATCACACAAACACGCATC TTCAACATAAAAATATATATAGCACACCAATCATTCAATCATAGCATCCTAGCAGGTT
BPM 24	KF 147543	ACAACCACATCACCCGATTGCAAGCTCGACATGTCATCACTAACAATAGTCTAGATGATTTAAAAAATTTTAG GGGGGTGAAGTGATGATTTAAAAAATTTCAGGGGCATTAAGTGACATTTGCCCTGAATTATAATTAAGAAAA TTTCACGCATTTTCCAATTTTTTTCATACCAACCAAATAAAATCTAAGTTAAAAAAAGGCAATCTCCACTTTTA CATAT**TTATAATTATAA**AGTTCTAATCTACACTAGAAAAATATTTGCAAAAATAAACTTTTGCTACACCTG AAACAATAATAAACATCAAATAATGCATGCTTATGTAAAGATAGTTAAGCTCAACATGTCATCACTAACAAA AGTCTAGGGTGTGAAGTGATGATTTAAAAAATTTCAGGGGGTTAAGTGACATTTGCCGTGAATTATAACTAAA AAATTTTACGCATTTTCCAATTTTTTTCATACCAACCAAATAAAATCTAAGCTACAAAAAGGCAATCTCCACTT TTACATATTTATAATTACAAAGTTCTAATCTACACTAGAAAAAACATTTGCGAAAATAAACTTTTGCTACACC TGAATACATTAATAAACATCAAATAATGCATGCTTATGTAAAGATCGTTAAGCTCTAAATTCACAAACATCAA AATCAAACTACAAGTTAGGTCATATCAAACATCTGAATACAACTCAAGCTAAAACAAGCCAAACAAACTCAA TCAATTAGGAACCTTCGAAACCAGGGCACTCGT
BPM 26	KF 147545	ACTCGCTAACAAACCTAAGTAATAATCGAAAGAAAGATTTTAGCATAACACTAGTATGGGACGGTGTGCCAC TTATGTGTCATACTAGGTGCACGTGACACCTAAACCAATGAGCGTATATGGGACGCCTTTAGAATCAGCAGTG

		TTATCTATGTAGTATGGGTGGTATGGGACGCCTGTGCGATATGGATAACAGTGTCATGGGATAGTGTGCCCCA CTTTAGCTATGCATATAAGTGACAATGCTTAAATCATAATAATTAA**AATAATAATAATAAT**AACTGGACT GCATGGCTCCAACGGAACTCAAAGAGTTAAAGAGTCAACAAGAAGTGTTACGGGCAGGTTTCATTCAACCAA TACTTCACCCTGGGGAGCCCCTGTTCTTTTTGAAAAGAAGAGGATGGCTCACTTCGATTGTGT
BPM 29	KF 483424	ACGTAAGGTTGA**TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT CTCTC**TTTTTTTCAAAGACAAAAAAAAAAAAGGTGGAGAAAGAACATAAGCAATTAAAGATGTGAGCGTTTT TACAAATAAAAGTGTTTGTTTTATTTCTTGTTGATCTATTTTTTGTTGTCCCTGCAGATGTTAATGGATCCTCTA AGTTCCATAAGTAACACCAACAACACCTTGATGAGTTTTTTTGGAATTCCCGAAATCAATGATGCTTTGGAAC CCGAACACTACCCCCCACCCGGGTGTGATTGTATAT
BPM 30	KF 483425	CTCATAAAGTAGCAAATCTTGCAACCAGATAAATATTTTGGATATGTAATATTTTGATCTTAATAAAAATATT GTTTTCAAAAAAATTAATGATTTATTAATTATATATGAGAACTTCTTGGCTGTTTTTCTTTTGTAAAAAGAAAT TCTATAAAATCATTATTCTTGTGATCTAGATTTAGAAAAAAATCAAAAAGAATCCTAAAGCACATAAAAATA AGGAATTTGTGATTTAATTGTGATATAATGTTTTGCCACTTTATTAACAATTCCAGAAAAATAAAAAATAAAA AAACAATAAACAAACAAAGCGAACATTCAAACCCAACAACTACAAGTTACAACCGTGCCCAAACACATATAT GACCATTTCAAATAAATGTGGCCAAGAGTCAATCTTTATCGATATTTTTCTTTTTACAGCGTCAATATCCAATA ATACTTTTTATTTGAAATCTGAACAACCCATGCTGGCTGTCTTGGTTGGGGGCTGTCCAACCAATTTCAATGGG CAGGTTGTCCCGGCCAAGAGTGAACAAATTTAGTGCCCCTCCCACATA**ATTTTATTTT**TTATTTTCATTTTA ACTTAACGGCTTCCCGAATAGATCCTGCTCATGGCTTTCGGAGTCGAACTCTCTGTAAATGACTCTCTATCAAT ATATTTAATGATCGACTCCCCGGTCAAATAATAAAAGTTATTGATATAGTTTACTTTTTTTTACTTAGTTAAAT TAATATTTTAAAGTAATATAAATAATACATTTATCAATTTTAAGTGTCAGGAGAATAATAGATACTAATTTCA TATAATATTTTTTTATTCGATTAAAAAAATAATATGAATGACTATAAACTGGTGTAAACTCAAATCTGAGTTG GTTTTAGAGTCGTATCTAAGCCAAATCCATTCCAGGTCTAGTCCAAGGGACGAGACGGGGCTAGGTTTTGGCG GTTATTCCTAGTGATCCCGTGTTCCTGACGGATAGTCGGTACTATTCGAGTGGTGGAAACAATTAATCAAAGT GGATTCGTGTTATGGTTGCCGGCAAAGATCAAACTGTGGTGCATAGAGTATGTCGGGCGAGGGACCGACAGC GAGCAGTGTAGCAGTGAAGTAGTCGGTAACAAGTATCGAGGATGCCGCTACATCGGCATCGAGCATGGTGGG TTCCAGCCTGTACAAGGTCAAAGCTGGTCGGGAGGCCGCTACCGAGACGAGTATCAGCAGTGATTGAGAGTA ACAAGAG
BPM 31	KF 483426	ACAACATATCACAAAATATGGTGCATAGATTAAACTCAACAACCCAGCATGTTTCATGAGTTTGCACGTTCCA TTTGCCTAAGTTGGCCACCTCCAAGGCTGGT**CACACACACACACACACACACACACATACACACACAC A**TATATAAAGGGATAATATCAATAATAGGGCGACTACTGGTCCCAAACAACTAATAGCGAAAAGTCAAACTG

		GCATATGGACAAACATATTCACGTCCATGGGAAAATACTAAAACACCATGAAATCCCAAACAAGGGCTGCTA AATAAAAGGAGAGCCATCGTAAACCATCCCAAAACATACATAGAGAGGAAGGTTGGCACAATAACCATGGC CTCTCAACATACATAAACATATCATACAGCAATGCCCTTACACAATATCTATATAATACCTGCCACGGTCCTT GCATGGCACAGAAGGAAATATCACAAATTTCAAAAATCTGATGTGTTACGCAGT
BPM 32	KF 483427	CTGGTGCATGTGCCAGTCAAAGTGGGCTCTTGATTGGTGAGTGGGTTGTTCCATTCTTGAGGGATCCTGATAA CTCCACATTTTGAGTTCCTCAAATCTAGAGAAGTGTGGAGGATTATGGGTACTGTAATGCAGCACATGTCTAG ACATTTAGATTCAAATGATATTGAAGGCGCTTGCTTGAGAGTACTCGGCCTTAGTGCCTAGGGCCAGGAATTA TACAGAGGATGCAGAGGGCTTTAGTATTCTAGATTTCCCAAGCATGGGATAGTATGAAAATGCTGAGAGAGG CATATGACTAGTCAGAATGCGACGCTATTCAGGCCAAGGCTAACGATTAGTGCCCTTATGAGTGATTCTAATG TAGAAGAAAATATGATTGATTATATTTAACATGAAAGTCATCTCTCATGTGCCAGTTCGATTATTTAGTTATTT CTGGATTAGTAACACTCATTATTGACTTTTTCGTATTATATATGTAAGTGAGGCCTAAATGACTGAGAACTATT AGGACCATAGAGGAGTAGCAATGCAACATGCACACATATACCTGCCGAGATTTAATGAGAATGCAATGTGAT GAATTACTATGGATTATTGTGGATAACTTCTATAGTGTCTTATTGCAAGAAATGTTTGTGTGAAATAATAAAA TGATCTGTGTGTGTTGGTTATTATGAATAGAAGTGAATTGGATGAGCAAAGGGGTTCAACACCCGACTCAAA AAGATAAAATGTATTATGATGTGAATGTTGTGTGAAAAGTACAATGCTTTGGCAATGTTTTATATTTTTATCAT TACATATATATACTAT**TTGATATTGATA**AGGTAATCCCTATTGTACTTTTAAGGGATATTTTGTCCGTTTTG TTTTAAATATCGTTGCTTTTTGAATTTTTAATGGGTTTGTTTTGTTTTGCACATGGATTGCATATCATATCATTC ACTACCACAACGTTAGAG
BPM 52	KF 488561	ACTAGGGCTTGAAGCATATGACAACGGAACTGCATGGACAGAAGAACTTGCTTGCACAGAAGAGGATGATCC AGAGCCAAAGTGAGAGTCAATTAAGCTGCCCACATTAGACACACGAGAAAATGCGGGTCTGCTACCCTGAGG TTACATAATATCAGTTCACACATAGAAAAAACACACCAAAAACAAAGAGCCAGACAACTACAACCTC**AACA** **AACAAACA**CGTCAAATAAGGTCCCACAACTTTCAGCAAGTTAAACTACAAAAGTGGATACCAAAATGCCAA ATGTAGCTTGAGAAGAAACTCAACCCTATATCTAGCAACCATACCATACTGGATGGCTTCAACCTATCCGTTT GAAAATTAGGTTCCAAACTGACAATACCATTGGT
BPM 54	KU 189203	ACTTGCGTTGCTTTTATTATATTTACAAAAATGGTCAAAAATATTATTTAATTATGGGTCTTGTTATAATTTAG TTAGTTGAAAAGATCTTATAATTGGATGGTATTATAAGTTTATTTCAATATTTTTTGGATAAAGCAACTTATAC AATGCTAATGCTATTGCATTGCGCTAAAATCTTAGCTAGTTGTAGTTTGAAGTGTCTAACAGTGCAATGGTTC ATTTGTTGAGGCCACTAATTTTGTATAAGAATTTTAGCTTATTTTAGCAAGTTTACCCATGTTTTTATGTAATTG TTCTTTTTTCATTATGTTGTTACTGTAATCTTCCGCTCTTGTTTCTAGTTTGTAAGAAGAATTGAATCAATCAAA TAGACAAGAAGTTGAAGAGTTTTACCTTGGTAAGTATGTCAATAAATGTAAGTATTCTTCTGTTTCAGTTCAT

		ACCATGAACTTGTTATTACTCTTATATTGCTTTGGAAAAAAATATTAAATCTTC**TATATATA**TAAACCAATA ATAAAATCCACCCAAAATGATAGGGAAAGAATGCTTGAAGAAAAAAAATCACTTTGCCTGTTTCTGAATTGT CAAAGTCAGTTTTTCCCACACACTGGATATCTTCCACAGGTTGAGCAACCTGAATCGTATACAGAGCCATTCC TTTTTCTTGTGGATTCCAAGTCTCTCTCTCTTTAATCTCTGATGCCATGACCATGCTCGTTATTAAGATGCAGTC CATGGGTTCTTCCTACGCTCTAACAGTCCATCTCAGAGGGACTAGGATGGCACCAATTTGGAAAAAGAAGCA ATACATACCTCTACCACCTTACAACAAATATGTAAGGT
BPM 132	KF 488564	GCAGTCGTGTGTGGTAGATGGAGGACATGGTAGCTGTTATTCTTTTGGGGATTGTTGGGGTTTTTAGATAGG**G TGTGTGTGT**GGGTTGGATATTTTGATGTTACGTGGCTTTGTTTGGTTGTAGAAATTGGGGTGGGAATTGCAC TGGACTGCTTCTGATGTTGTGCTTCCCACTTCATATTTTATGTGTCGATTGCGATGAGGCAGAAGAGTGAGTA GAACTCATTGTCCGATTTGTTGTTATTCTGT
BPM 133	KF 488565	ACCTGATATGTTTCGTGGGGTCTTTGATTTATTAATGTATTATTGTTATCGTCTTGTAGGGTGTTACCTTTGGGG CATTCAAACAATTGCTTTCAAGGTGTATAAGCCTTAAAGTCCTATAATATAAATAATTAAGGTATGCAAGATG TATTTAAATACAGATGTGTGTGTGTGTGTGCGTGTGTGTGTGAA**AGAGAGAGAGAGAGAGAGAGAGA GAGAG**ATAAAAGAACATAGACTGGATGAGCTTATGGTTCATGCTGCTTGAAGGGTCCACGTGAAGACTAAC GGTTAGGGTTTGATGCCCTGACACACAATGATGCCTCTATCTATTTAATTTCTTTTTGTATGGCATATGGAACA AATAATTATGACTTAATGTTTACTTTACTGCTAGATCATTATCGTCTAAGAATAAGTTATCTAATATAAAGGTT AAAACTAAGT
BPM 154	KF488566	ACGGTCGGAGCGAGGTGGAGGAGCCTCGTCGGTATGGAGAATCGGAAGAATATGGGCGTGACGAGGACGAA GGATATGGTCGCCGTCAATATGTGAGTGAGTATTGCCTTTTA**GATTGATTGATT**GTTTTGAATGCTTTGTTT TCGTTGTTGTTGTTATTGTCATGCAATTGATCTATTGCTAATGAGTGTGATCCGTTCATTTTCTTTTTCTTTCTA GCTAGAGGTGATAGGATCAATTTATCCTTGGGTTCATTTTGCAGCAAGTTTACATTGTTTTATATTGTTTATTT ATTTGTCTCCTCTCTGAGATTTGAATTAAATTTTATTTTTGT
BPM 156	KU 189204	ACTCAGAGGTATGTGGATAAATACGCCAATATGCGAAAAATTGTTCGTCTCATATTTATTTCACATTAAAAAA AAAACATAAATAAAAAGGAAGGGGGAGTATTTGTTGCATGTGTTCATGTGATTTTATTCATATTCTTAACAGC GTTGAATGTCTAATCAGCTAAATTGTTTTCTTTTTTTTGTTGTTGCAGGCAAACAGTTATGGCGCTGGCGTTAT TTATTTCTGAGCTTCATCTAGGTGTCCTTACTTTTTATTGTTCATATTTTTGTTTGCAATTTTTATTGTGTATAAA TCATTCTGTAATACTCCTCGTCCTTTTTGCTTCTTTTAAAGTTTGTGCCTTCATTTCCTGTGCATCCTCATCAATC TATAGTATCTTTTACCTCACAATTTGTTAACGACGCAGAGCTCATTGCCATGCCAATCATACAT**ACACACAC**

		ACACACACACTCTCTCTCTCTCTCTCTCTGTTATTATCTTTTTTTTTTGCCCCCCCTTTCTTGACTGTTCTTCT ATCAACTCCTCCACATTCTGTTTCGCACTCTTCACCGAGTTAGCTGAGAAATGTTATTCACTTCTGTATGCTTC TTGTTACAATT
BPM 157	KU 189205	ACTCACTATTTATCTAATTTTCTATGAATACATTTTCTTTGTTTATTTATTAATTTTTTATTTATTATTATTATGT CTCGATTTACATGCCATATGTTTAGTTATAGGTTTCTTGGTATTATTTTCTTTGTTGTGATGTATTAAAGATATG AGCACACACCACGAACATGAATCATATAGAGATATTAGTATTTTTCTTAATAGTGGGTTCCGATGTAGGTTTC GAAGTCCGAGCACGTCGTAGATTTGCTGATATACTTGGAGCTTGGACCGCCGGTCTGGAGGA**CTCTCTCTC TCTCTCTCTCTCTCTCTCT**CCCCCAATTTATTGACAGATTACCTTCTATAACAGCAACTTCAATGGGCTCCAA CACTGGTTTAGAGCATTCTATTATATTAACTTCACCTGTATCCTGCTTTAACAAGTGTTCGGCTATTGATTTAT ATCCTCTCCGTCTGACACGATTTGCAAGGTCTTTTCTGCTAACATTAAACAAAAGAGTTCAATTGCATGGAAT AATAAACCCAAGATATCTACATCATGTATGGACATCATCTATTGTTCAAATAGCTTGTAGGCAGAAGAAAGA ATTTCCATAAACCCTTGCGCATCCTGCCTTCATTTTGATTAGCAGGTAATTAGAAAGTTGACAACTGTCCATAG CTTGAGT
BPM 159	KU 189206	ACTATTATGATTAGAATTGAACAGGATAAGCTAATGGGTCAAGACCCAATTCAAGATGATAAAAAATATTGA TGATGGAAAATTTGTGAGATAAGCAAGACGTTCAACATTAAGGATGATTTCACTCCCGAAGAGGAGGAAGAG GCCCTCGAGTGAGGAAGAATCGTTATAGGAATCTTTAGTTATGAAGACTACTGCGGTTTTTTTATTCTTGGTCG GTGGCGTAATGCTTATCATTGTTTACATTCGTGTTCTAGTTGGGTTATATGGTCGCCGCCTGTCGTTTGGATGT TTAGCGGTTATATCACTGTATATATACACCTCTGAGTGTCCAACCTCCCTCTATAATATTGTTTTACGTGTTTG CCTCTAAACGTTGGATGGGAGGAGTTATTTTATATATCAAATATTGGATGCATAGTTAAACATGTGCACACGT TGTCCTCAGTTTATGGAGGA**TTGTTTGTTTGT**GGGGGGTTTTCTTTGTGTTTGGTATTGTTTGTGTTTGGAC TGTGTTATTGTTTTAACTCATTCTGAACTGCTATTGTTTGAGGTCTTTTTATATATGCATGAGATTAGAATACAT ACCGGTGTAATTTTTTGTTCATGGTTCTCGCTTAAAACATGCACTAGTGGCATGAATCAAGATTATGTAGCTTG TTATATAGTATCAGCCATCCCCGTAGCTTTTTTTAAAAAGGGAACAAGTTGAGTTTAACAAGTGTTCGAACTC GGCTTGAATAGTTCAAAATTTGTTTGAATTTCCGTTTTAATCGAACAAATTTTGT
BPM 161	KU 189207	ACCGTGACGGGTGAAATGGAAGATGTGAGGACATAAGCATTGTATGTATGAATATGATTATGAGCCGTGCAT GCGAGCCGACACTTGCTTAAGTATGTTTGCTTATCGCCAATGTGAGGCAATCTTGAAGCTTCATAAGTTTTCGT CGGTAATGGAATACCCATTAGCTAGTTTGTATTAGTTATTTTTCGAACTAGTAACTCATTATTGACATTTTTCA TATAATATAGTAGGTCTTACAATCTTGAGTCAACTTTGGTAGGAGGATAGATAAACGCT**ACACACACACA CACAC**TTAATCTCAACGTAGTTATTTATTTTCTTACTTTTATTTATGTTTGATGTGTTTTAACATGTATTTAAT AATGTCTTCCATTCTTATGACACCAAGAAGGTTGTTTCGATCGTTTTTATTGATCGAGTCGT

BPM 172	KU 189208	AGGCCTAGCTAGCAGAATCACAAATCTAGGCTAAGATTTTTTGCAAGAAAGTTTAAGTATGGGTGGTTTTGAC CTTACGTTGTGATTCATGTATTTTCACATGGAGCTATAACAATAAGGAATGAGAAAAATGGAAACTAATTCAA TTCATCCATCTCACCAACTTTGGAATGTTAATCAAGCTAAGAACCATAAACAAGTGCTTCATGGGATGTAACC CAACTTGTTATCTTCATATCTTAAAAAAAATAAAAAAACAA**AGAGAGAGAGAGAGAGAGAGAGAGAGA GAGAGAGAGAGAGAGAGAGAGAGAGAG**ACAATAAAGCCTACATGATGAAGGCATGGTGACCCAAA AGGAAGAAACCAACCCTACCTAGGATCAAAAAGATAAATTGTGAAAGGAAAGTGAAAGGGAAAG
BP SSR 1	KU 189209	AGAATCACACAAGCATGGCAAACACACTCATGCAACCACACTTACACCCTCACATCTTCCATATGACGCTGGC CTTGGTACTCTTTATCCAGCTAGCCACTAAGAAAGTTAACAGACCAAATTATTAAATGTGTAAGCAATTTTAT AGAAAACTTTTATATCTATGTATTTATTAGAATAAACATATATGGATGCAGGGGAGGACGCCTTCTTATGTGC AGAACAAATGTTTATTTGCGAACGTATTTGATCCAATTCATCACGATTCACGAACAATAAGGTGGAATGACCG CCTTGCTAAACATCACAATAACACATCTTTAAAATCTCTCCGTGGTGCCTGTCTCTTCTTTTTACTGTTCTTTCT AATAAAAAGATAATTAGGGGCAATATAACCGGTAGAATTTTGACATTCAGGGATGCAAAGTGTTGA**ATATA TATAT**AGCTCTAATAATGATGAATATGTGGTTCAGCAGTGAAATGATGCTCCACCTTAATCTGTTTAGCTTGA CATAATTGAAATAGTGTTGCAGCACTATTTAGTGGCTTCCCTCTGTACATCTTGAAGTTCACATTTGTGGCCAA AAGAGAGGAATCAAT
BP SSR 2	KU 189210	GCCCTTGTTTAAGGCGTAGCTAGCAGAATCACAGACCAGCCAAAATCAGCAATTTAAAAGAGCTCGCCCTTTT GAAAGACAAACTAAAATAAGCTCTCAACTTTGGATACAAATTATCTCAATTGACAATGTTTGAGTGATAACTT TTACCTGAGCACCGATCAAAAGGTTTTCAGGTTTAATATCTCTGTGTATCACATGTTTTTCATGGCAATAGATG AGAGCTCTTGCCAAAGAAGCAATATACTGTCATGAACAACACATAGGAAACAAATTACAACATAATATTATT ATTTACATCGTCACATAAAGTCTAATAATCACCACGCAACATGCACAACAATTTAAACCCATAAAAGATCCA AATAAAACACCACCAATTACCGTGGCAACACGTTTCTCTCTGAAGTATTTGCATCTTTGCAGTTCTTTGTAGAG TTCACCTCTAGCTGCATATTCCACTACCAGATAAACACACGACTGCATTTGAAAACTTATTTAATGTTTTATAA ATACATTGTTACAACACAATCAACTAATAGATAGAAATAATCAGATGAAAAT**AATAAAAATAAA**TATAAA CAAGATAAACAAGTGCATGATATTAAAAAGACCACCATAAAAAATCATTACAAAGCAGGCCAACTACGATCA GAAAAATGGATCAATGGGCATATACTTAGATTCAATGCTTAACAATTTTAATTCCGATACACCAACTTTTCCT TCCAGTGATTCTGCAAACTAGGCC
BP SSR 3	KU 189211	ACTAGTTAATTTTTTTATTGGGGATTAAGGTAAAATTTTAGCTTGTTACATTTCTACGACCCTAAAAATTATAT AATCTAGACAAAATATTACGCCATGCACGGCCTATGTAAGAGATGATATTCAAATTTTATACGTAGGTCGTCG TTAAATGATGGCTATAATGATTTATTATTTTAGAGTCTAATTTTAATTGAATGAGGGAGGAGATTAATAATTT

		AGTTGATTTATTTAATTTTTTTTTTGAATTATTTATTGGCTATTAAAGAGAGCAGTAAGAAATTGCTTATTTAA AAAAAAATTAAATATAGTTAGTTTGCTTATATTGTGCAACAGAACAAGAGCAATTGATGTCCTCAAATTATAG TGAAAAATTATGATTCACAACTTAATTCCTTTGTTTCTTATACTAAACAATTATTCTTATCGGATCGAGTCAAA AATTGAATTTGGTTTGATGCTCCCAAATATTGGATGTATCTAATAATTACGTCTTAACATTCATGGTATTGAAT TTAACAATCTAGAACATATATAGGAAGCATACCTTGCAGCAGAATCAGATCGAACTGGAACGACAAACATAG TAATAATCACCTCACAAATCCGGTTAACCTAAATCTCTAACTCTACTTCACCTTGATTGATTCGA**GTGTGTG** **TGTGTGTGTGT**CGGCAAACCCTTTATTGTCTTGTTTATAAACATCATAAGTATTAGGAGTCTAAAATGAGT TCTAGCTCTCTTACTTATTTCAATTAATAAGTTGAGTGTTAGGAATTGAGTTTGTGACTCTCATACTTATATCTC AATTAATTAGATAAGCCTTAAGATTCCTTATTTGGGTGGGGCTCACCCTTTACATGCGTGATTCTACTAGCTAG GCCTTAAAC
BP SSR 4	KU 189212	ACCCATTATAGTCGGCAGGCCTTTTCTTGCCACTGGGAGGACGTTGATTGATGTTGCTGCTGGAGAATAAATA ATGAGAGTGGACAATGAAGAAGTGGTATTTAATGTTTTTAAGGCTATGCAATATCCGGAACCAGCAGATGAT TGTTTTTATGTTGATGTAATTCATCTCCTTCGTCAACTATTTTTGAGGAAATGTATCCTATGGACCCCTTAGCA GCCACATTAGTTT**TTGATGTTGATG**ATTCTAGTGATAAGGAGAGAGGGGAGTATTTGCATTCGCTAAATTC TACTCCAACATTTCCTCGAGGTCAAGATAAATTTGAATCTTTGGAGTTGCAATCTTCAATATCGAGGCGAATC AAGCCTTCCATTGAAGTCCCACCGACCTTAGAGTTGAAACAGCTTCCATCTCATTTGAGGTATGCATTTCTAG GTCCTTGTAACACTTTACCAGTTATTATTTCGTCAGCTTTAACTAGTATGCAGGAAGACAAGCTATTGAGAAT ATTGCGGGAGCATAAGCTGGCTATAGGTTGGACTATTGGTGACATTCGTGGTATTAGTCCTTCTTTTTGTATGC ATAAAATCATTCTTGAAGATGGATATAAACCTTCAATAGAACATCAGCGCCGGTTAAATCCAATCATGAAGG ATGTTGTCAAGAAAGAAATCATTAAGTGGCTTGATGCAGGTATAATTTTACCTATTTCTGATAGTTCTTGGGT AAGTCCTGTACAATGTGT
BP SSR 5	KU 189213	GAATCACCATTTTAATAGTTGTAGCCCGCACTACTGGACAGAATGTGTCGTAATAATTCAGCCCCTCCTTTTG ATTACAACCAAGGCTGATGGCTTTGTTGATCACTA**CAAGGCAAGG**CTGGTTGCTAAGGGCTTGAAGTTCAG CATTCATGGCATGACTCCATTTTGGAGATTTAGAAGCTTGACGGTAGTGGTGGGTTCAAAGGGGGGGAAGCA AGAAACCTGTAGGTAGAGGGTATAGGGAAACAAAGGCTTCTTTGAATGTGCTTACATGGGATGAGTAAGGGT GGTCGAGTAGATGAAAATGATAAGAGAGGTGGAGATGGAGGCGAAGTGGGAGGAAGAGGAATAGGCAAGG ATGGAGAAGAATAGGGCTGAAATTGGTGTGTTGCAGTGTGGTCGAAGGCATTTATTATTTCCATCTCCCATTT NCCCGACCATTGACCATTATCGTATTGCTTAACGCTTTCTTGACCATTTGCCGCTACTGGATTTTGAACATTCT GACTGACTATGAATGATTAAAAATTGGTAAATTTTGATTTGAAACACAAAGCTTTGGTAATGCTAAACTTTTG ATGTGTGTGGGGGTGTGTTTAGGTTGAGTAGATGTAGGAGGCAGACATTGAGCATGAGCTGGAGAAGACATC AAGGAGAGCATAAGGATGGAAGCTGTTGGAGTTGAGATGCGTTAGGAGAACTTGACGTATGAAGAGGGATA

		GTGGAAAGACATTGAGGATGAGCTGGAGAAGACATCAAGGGAGATTATGGAAAGAGACAAGGACTTCGGTA GAGATGTGTTAGAGTTAGGTGGAGAGATATGAGGAATCTTAAGAGACAAAATGGGAAGAAGAAAAGGAGGC GACACACTTGAACTTGAGGATTTGGTGGATTGATCTCTTTGAAGGGAATCTCGTTATCATTGACCATGACATG GTGGCTTATGGTAACTTTGTTTATAAGTGGATTATAGCAAGAATAACCATGGGGTGAGGGGCTATATCTGAGA AAAAACACACCAGGTTGTTTGGTACTTCGCTCGTCATTCTGAACTTACTTAAAGCGA
BP SSR 9	KU 189214	CCCTTGTTTAAGGCGTAGCTAGCAGAATCGCGAACTATANACATTTTCTTTGGAAAGGCAAATCAATTGACAA CCATTTGAAGGAGATGATCTTACACTGTTATATATTATGTCCAACTCATTGGAGAAGCTGTGAAGTTGATAAA CTGTAAAAACACACTAGTAAAACTGTAAAAACACACTAGTAAAACACACTAGCAGAA**TTCATTTCAT**ATC TCAGAATTGTTAAGATGGAGAGGAAGTTTGAAGTGTGTGGGTGGGTGGGAGTCGGTTAGTCGGCATTGAAGG AGACATTGTCTATAATCATCC
BP SSR 11	KU 189215	AGAATCACCTACAGAGTGCAATAACAGTCAATACAATAGCACGTGCATCACCAATCAGAAATCAGTCATGAA ACGCACACAAGATGATAACTTACACATAGGACAATGAATGAACCAGCTCCTGCATATCATCTGCTGTAAAGC CAACTTGATCCAAAAGAACATGGTAATGGGTTGGCCTTGTTGTTCCCTGACGCGAAGTGGCAAGGAAATTTTT GCATCAAGAAAAGTAACAATTTTCAGATATGTGCAACCGTTAATAACTACTTATTATCATTCCCGCATGGGAG CAGATGTAAAAGTCATAATTTTTTGGGTGGCATATTTTGTTGTCAATCACGGTTCCTGCATAAAGAAGAGTTT ATTCAGATAAAACTGATACACCCCTTTCCAGTTAATAACCACATGGATAGGTCAAAACGCTAATCCTACTTTT TTTACTGCTATATCATGCTTACCAGGCGGCACATTATCGGGAGAATTAGGCTGAAAAAATTTTGTATGGTGGT TCTTCTGGGCAATAATAACAGTAAACTTAGGAGACCACTTCTCATCCAGAAACTTGCATGCCTAAACAAGGGC AACGAACAAGGACAACAACTAGTCTTGTCAATTACAAGTTTTCATTCGCTCCAACCATCAAGTCCCAATCATT CTAAAAGAGT**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**TGTAGCAAACAAACCTCCATGATTTGATCTAGCTCAATA
BP SSR 13	KU 189216	AGAATCTTTTCTGCCCTTGGCTTAGGTAATCGTCTCTCATACAT**ACACACACACACAC**ATACATATAGACAAA CATATTCACACTCACACTTGCTCTTATAAATTATTGTATAAAATTCAATGGTAGCTGTTTGCTTTGACAATGGC ATGATTTAGTAGCAGTATATAGTATCATGTTCATCAACGATATGATTCAGTAGCAGTATGCTTCTATGGTTGTA TGTTTCATTAACGGTATGTTACACGAACTCTCTACAGTTTGTATGCTTCACCCCCAGCATGGTATGATAGGAGC TCATAGTCATTGTGTATGTGATTCTGCTAGATAGGCC

4.4. Designing primers flanking microsatellite repeats

The primers targeting amplification for microsatellite makers were designed using primer 3 software following the parameters described in Section 3.7.

4.4.1. EST SSR

Out of the 23 primer pairs designed, synthesized and tested for amplification in black pepper, 17 primers yielded good amplification products within the expected size range. Annealing temperature (Ta) for each primer was standardized by setting gradient PCR at different temperatures (Table 14.)

4.4.2. Genomic SSR

Out of the 37 primers designed 28 primers generated amplicons with the expected base pairs. Annealing temperature was standardized using gradient PCR at varying temperatures (Table 15.)

Table 14. Details of EST SSR primers developed from the genus *Piper*.

Sl.No.	Locus name	SSR Motif	Forward primer (5' - 3 ') Reverse primer (5' - 3 ')	Product size	GenBank number	BLAST X description (species, accession no.)
			P. nigrum			
1	PN EST SSR 1	$(AGA)_6$	TTCTTGGGTGGTGAGAGGTAGT GTTCAAAGGTCCGTGTGTGAG	320	GI190335106	Putative gamma-thionin [*Castanea sativa*](AAL15885.1)
2	PN EST SSR 2	$(TAATT)_3$	ATGGCACCCCTGTTTACAA GGCGGCTTCCAACAATAG	279	GI189502757	Microsomal oleate desaturase [*Arachishypogaea*],(AAF8229.1)
3	PN EST SSR 3	$(GAGCA)_2$	ACGAATAATGGACTTAACCTACCTG CTGCATGTGGAAGGAATTAGTG	210	CONTIG1	Predicted hypothetical protein [*Vitisvinifera*](XP002284360.1)
4	PN EST SSR 4	$(AATCA)_2$	ATGCTTGCTCTGTTGCTTCTTC AAAATTCTTTCCGCTTCTCTGC	216	GI190335104	Predicted hypothetical protein [*Vitisvinifera*](XP002284360.1)
5	PN EST SSR 5	$(TTTGT)_2$	CCGAAAGCTAGAAGAGAAATACCA CGACAGTTTCCCCACTGAAA	236	GI190335104	Predicted: hypothetical protein [*Vitisvinifera*](XP002284360.1)
6	PN EST SSR 6	$(ACTGTG)_2$	GCAGGTTCTTGAGGAGGTTTAG ACCGAGACCAAACACATTCAC	224	GI190335102	Heat shock protein 16.5 [*Agrostis stolonifera* var. *palustris*](AAC01560.1)
7	PN EST SSR 7	$(AAACTG)_2$	TTCCGAGTGACCTGCATTTT ATGGCTCATTGTCTGGTTCAAT	217	GI189502767	Peptidylprolyl isomerase; FK506-binding protein [*Arabidopsis thaliana*) (BAB02082.1)
8	PN EST SSR 8	$(CTGTAA)_2$	CGGCACTACCAGAATGTTTATC AGTGTTGTAGGAGCAGCAAAA	242	GI189502766	Predicted hypothetical protein [*Vitisvinifera*] (XP002285171.1)
9	PN EST SSR 9	$(GAGAT)_2$	CTCCTCAAGCTCCTGTTACTGC TACAGAAAAGAAAGCCACCAGC	227	GI189502765	Unnamed protein product [*Vitisvinifera*] (CBI19085.1)
10	PN EST SSR 10	$(TGATCA)_2$	TCCACTAATACCCCACAACAGA TACACGCAGTTACCTCCAAGTC	189	GI189502763	Predicted protein [*Physcomitrella patens* subsp. *patens*] (XP001786305.1)
11	PN EST SSR 11	$(CTTCCA)_2$	ACTGGGCAACCACCATTTC GTGAACGCTATCAACCAAGGAT	238	GI189502759	Hypothetical protein SORBIDRAFT_02g040170 [*Sorghum bicolor*] (XP002461070.1)

12	PN EST SSR 12	$(AAGTGC)_2$	ACGGTCAGGTATTTGTTGGTGT AGAAGTCTCAACCAGAATTGGC	217	GI189502759	Hypothetical protein SORBIDRAFT_02g040170 [*Sorghum bicolor*] (XP002461070.1)
13	PN EST SSR 13	$(CAAGCT)_2$	CGGTCAACAGAAGCTGGG ATGAGAGGGAGATGGTTGCAT	223	GI18950256	Predicted hypothetical protein [*Vitisvinifera*) (NP175372.1)
14	PN EST SSR 14	$(GCCCC)_2$	GGAAGGTTTCTGAGGAGAGACA AAGCGGAGGTGGATGTGTT	225	GI161318464	Leucine-rich repeat family protein / extensin family protein [*Arabidopsis thaliana*] (NP175372.1)
			P. colubrinum			
15	PC EST SSR 15	$(GACGA)_2$	TATATTACCACCCGCAACGAC AAACCACCAAGAGAACGAAGAA	233	CONTIG1	Rop guanine nucleotide exchange factor, putative [*Ricinuscommunis*] (XP0050622.1)
16	PC EST SSR 16	$(AGTCT)_2$	GTTCGGTAGCGTCCAAATAATC GGCAATAGAGGGTAGTGGTGATA	193	GI90988886	Predicted hypothetical protein [*Vitisvinifera*) (002270049.1)
17	PC EST SSR 17	$(TCG)_5$	GGGGAGCGAAGGAGAGGC ATTTGTTGTGCGCTTGGGTAAA	188	GI90988877	Hypothetical protein [*Cleome spinosa*] (ABD96879.1)
18	PC EST SSR 18	$(TA)_5$	TGATCCTACCAGCACTTTCACT TTACCAACTGATCTCCAATCTCC	187	GI90988875	Osmotin isoform precursor [*Piper colubrinum*] (ACH54108.1)
19	PC EST SSR 19	$(ATAA)_2$	TGATCCTACCAGCACTTTCACT TTACCAACTGATCTCCAATCTCC	187	GI90988875	Osmotin isoform precursor [*Piper colubrinum*](ACH54108.1)
20	PC EST SSR 20	$(TCATG)_2$	TCAGAGGAGGGCTAACAACTGTA GGAATGAAATATAACCCCACCA	151	GI90988874	Predicted hypothetical protein [*Vitisvinifera*] (XP002270927.1)
21	PC EST SSR 21	$(ATACAT)_2$	TAGTATGGAGAACCCGATGCAC TCGTGAAGGGACCAGTAAAGAG	150	GI90988864	Hypothetical protein OsJ_16444 [*Oryza sativa* Japonica Group](EEE61817.1)
22	PC EST SSR 22	$(CGACAT)_2$	GGAGGAACCATGCGTCTTG AAGGCCAGCAGTTTCAAGTTC	320	GI90988854	Hypothetical protein OsJ_02616 [*Oryza sativa* Japonica Group](FAZ12698.1)
			P. tuberculatum			
23	PT EST SSR 23	$(CTTCG)_2$	GCCGACCTTTCAACAATCACTA GGGCTAATGTCTCCCATCTTG	180	GI|92494868	Predicted: hypothetical protein [*Vitisvinifera*] (XP002278996.1)

Table 15. Details of genomic microsatellite primer pairs developed from *Piper nigrum* L.

Sl.No.	Primer Name	SSR Motif	Forward primer (5' – 3 ') Reverse primer (5' - 3 ')	Product size	GenBank accession number
1	BPSSR 01	$(AT)_5$	ACGAACAATAAGGTGGAATGAC AAACAGATTAAGGTGGAGCATC	236	KU 189209
2	BPSSR 02	$(AATAAA)_2$	TACCAGATAAACACACGACTGC TAGTTTGCAGAATCACTGGAAG	285	KU 189210
3	BPSSR 03	$(GT)_9$	GAACTGGAACGACAAACATAGT AGTAGAATCACGCATGTAAAGG	308	KU 189211
4	BPSSR 04	$(TTGATG)_2$	GAGAGTGGACAATGAAGAAGTG CGAGGAAATGTTGGAGTAGAAT	233	KU 189212
5	BPSSR 05	$(CAAGG)_2$	CCATTTTAATAGTTGTAGCCCG CTCCATCTCCACCTCTCTTATC	322	KU 189213
6	BPSSR 06	$(GAGGAA)_2$	AGGCTTCTTTGAATGTGCTTAC TCTGCCTCCTACATCTACTCAA	374	KU 189213
7	BPSSR 07	$(TTTGA)_2$	GAGAAGAATAGGGCTGAAATTG CTACTCAACCTAAACACACCCC	248	KU 189213
8	BPSSR 08	$(ACTTGA)_2$	AAGGACTTCGGTAGAGATGTGT AACAAAGTTACCATAAGCCACC	178	KU 189213
9	BPSSR 09	$(TTCAT)_2$	ATTGGAGAAGCTGTGAAGTTG CAATGCCGACTAACCGAC	164	KU 189214
10	BPSSR 10	$(CAATA)_2$	TCACCTACAGAGTGCAATAACAG GGAAAGGGGTGTATCAGTTTTA	389	1905462 (BankIt Number)
11	BPSSR 11	$(GA)_{28}$	TAAAACTGATACACCCCTTTCC AGCTAGATCAAATCATGGAGGT	385	KU 189215
12	BPSSR 12	$(TCAGA)_2$	CAGCTTTGTGGAACTAATGAGA CTACATAAGAATGCGAGCAGAT	261	1905464 (BankIt Number)
13	BPSSR 13	$(AC)_6$	CTTGGCTTAGGTAATCGTCTCT GGCCTATCTAGCAGAATCACAT	315	KU 189216
14	BPM CNTG 4	$(ATGT)_3$	TGTTTGTGTGTATGTATGTTTGTG CTACTTAGCGGAAGCAATAATC	220	KC 347528
15	BPM CNTG 5	$(GATTTG)_2$	ATTCAACCGTGACAGAAGATCC TCGAACACAAGTTCAAACCAAC	320	KC 347529
16	BPM CNTG 6	$(TG)_7$	CCCAGACCTTCATTTACGCTAT TGTGTTGCTGTTTTGCTCTCTT	300	KC 347530
17	BPM CNTG 9	$(GA)_{22}$	TAATCTTGATGGGAGGAGGTTC TCTATAAAAGGGCTCGGTGTCT	350	KC 347535
18	BPM CNTG 10	$(TTAGG)_2$	ATCGAAGGTTTATGTGTTGGCT AGAAGGGAATGGGGAAAATAGA	215	KC 347536
19	BPM CNTG 12	$(AACAAA)_3$	CACAAATGACCTCTTCCTTTT AAATAACCATAAACATTGGAGC	315	KC 347537
20	BPM CNTG 15	$(CATA)_4$	GCAGAATCACAAACTACACGGA	150	KU 189217

			TGAGTTGTTCGGGTTGCAC		
21	BPM 4 a	$(TG)_9$	GTCTGTTGTTTGATTGCTTCCT CGAAAGACACGGATAAAGTCAT	280	KC 347538
22	BPM 4 b	$(ATT)_4$	TCTGTTGTTTGATTGCTTCCTT CACGAAAGACACGGATAAAGTC	350	KC 347538
23	BPM 6	$(GTGTCT)_3$	ACTATCTCTCGGCTCACGAAAA ACTATCTCTCGGCTCACGAAAA	315	KC 347539
24	BPM 8	$(TTTG)_3$	ATGGTGTTGGTAGAGGATGAAA GCAGCAAACCCTCATATACAAT	260	KU 189202
25	BPM 10	$(GTTT)_3$	CAGAAGTAGAACGTCGAGCAGA CTATGGCCGTGATAAACATGAA	260	KC 347540
26	BPM 18	$(AC)_{10}$	GCAAATGAGAAGGATAAGGGAA AATCGAAAGGCAACAAGATGTC	220	KC 347541
27	BPM 26	$(AAT)_5$	AACCAATGAGCGTATATGGGAC GTGAGCCATCCTCTTCTTTTCA	280	KF 147545
28	BPM 31	$(CA)_{12}$	TAAACTCAACAACCCAGCATGT TATTGTGCCAACCTTCCTCTCT	300	KF 483426
29	BPM 52	$(AACA)_3$	GATCCAGAGCCAAAGTGAGAGT GCTGAAAGTTGTGGGACCTTAT	155	KF 488561
30	BPM 132	$(GT)_5$	GCTGTTATTCTTTTGGGGATTG TACTCACTCTTCTGCCTCATCG	260	KF 488564
31	BPM 133	$(AG)_{14}$	CTTGTAGGGTGTTACCTTTGGG AAGCAGCATGAACCATAAGCTC	180	KF 488565
32	BPM 154	$(GATT)_3$	ATGGAGAATCGGAAGAATATGG GAACGGATCACACTCATTAGCA	170	KF488566
33	BPM 156 a	$(AC)_9$	AGTTTGTGCCTTCATTTCCTGT AACATTTCTCAGCTAACTCGGTG	165	KU 189204
34	BPM 156 b	$(CT)_9$	GCCATGCCAATCATACATACAC GTGAAGAGTGCGAAACAGAATG	200	KU 189204
35	BPM 157	$(CT)_{13}$	TAGTGGGTTCCGATGTAGGTTT AGCCCATTGAAGTTGCTGTTAT	315	KU 189205
36	BPM 161	$(AC)_8$	TGAATATGATTATGAGCCGTGC ATCGAAACAACCTTCTTGGTGT	215	KU 189207
37	BPM 172	$(AG)_{26}$	GGTGGTTTTGACCTTACGTTGT TTCCCTTTCACTTTCCTTTCAC	295	KU 189208

4.5. PCR assessment of microsatellite markers in black pepper genotypes

4.5.1. EST SSR

Out of the 17 primer pairs amplified, 7 primers revealed polymorphism among the 21 *Piper* species studied. All the EST SSR primers produced monomorphic banding pattern on PAGE when tested on 39 black pepper genotypes.

4.5.2. Genomic SSR

Out of the 28 primers that generated amplicons, 13 primers were identified as polymorphic markers when tested on *Piper* species. Out of the 13 polymorphic markers 2 markers produced smearing amplicons and were not considered for further studies. Those primers which produced multiple bands or gave poor amplification were also excluded from the study. Out of which seven SSR primers have produced 100% transferability were used for genetic diversity analysis in *Piper* species. Five markers had generated polymorphism among 39 black pepper genotypes.

4.5.2.1. Utilization of genomic microsatellite markers in black pepper varieties

Among the 28 primer pairs which produced prominent bands, 5 primers exhibited polymorphism in 16 released varieties of black pepper (Fig 23, 24.). All the five polymorphic markers (BPM CNTG 15, BPSSR 11, BPM CNTG 5, BPM 154 and BPM 156 B) could generate a total of 26 alleles and the average number of alleles per loci was found to be 5.2. The maximum number of alleles was noticed with the markers, BPSSR 11 (10 of 26) followed by BPM CNTG 15 (5 of 26) and the least number of alleles was noticed with BPM 156 B (3 of 26). The maximum value of PIC was noticed with BPM CNTG 5 (0.30) and minimum value by BPM 154 (0.21) (Table 16).

Table 16. Locus name, repeat motif, sequence information, no. of alleles and PIC of five genomic microsatellite markers

Locus name	Repeat motif	Primer sequence Forward primer(5′-3′) Reverse primer (5′-3′)	No. of alleles	PIC
BPM CNTG 15	$(CATA)_4$	GCAGAATCACAAACTACACGGA TGAGTTGTTCGGGTTGCAC	5	0.27
BPSSR 11	$(GA)_{28}$	TAAAACTGATACACCCCTTTCC AGCTAGATCAAATCATGGAGGT	10	0.24
BPM CNTG 5	$(GATTTG)_2$	ATTCAACCGTGACAGAAGATCC TCGAACACAAGTTCAAACCAAC	4	0.30
BPM 154	$(GATT)_3$	ATGGAGAATCGGAAGAATATGG GAACGGATCACACTCATTAGCA	4	0.21
BPM 156 B	$(CT)_9$	GCCATGCCAATCATACATACAC GTGAAGAGTGCGAAACAGAATG	3	0.25

4.5.2.2. Genomic SSR markers: genetic diversity analysis in released varieties of black pepper

The sixteen released varieties were divided into five clusters with the aid of five genomic microsatellite markers (Fig. 25). The similarity coefficient of various clusters ranged from 0.69 to 0.96. Panniyur 1 and Panniyur 3 which are the hybrids of same parents shared 84% similarity. The percent similarity exhibited by Panniyur 4 and Panniyur 5 was 92%. These four varieties formed the first cluster. The second cluster consisted of Panniyur 2 and Panniyur 7 sharing 94% similarity along with Panniyur 6 which was linked to them at 86% similarity. Sreekara and Subhakara with 96% similarity to each other together with IISR Sakthi and IISR Thevam which were 92% similar formed third cluster. Panchami, Pournami, PLD-2 and IISR Girimunda were included in the fourth cluster. IISR Malabar Excel stood as an outgroup forming the fifth cluster with 69% similarity with other clusters. The SSR profiles clearly separated all the released varieties of black pepper.

Clusters	Released varieties of black pepper
Cluster I	Panniyur 1,Panniyur 3, Panniyur 4 and Panniyur 5
Cluster II	Panniyur 2,Panniyur 7 and Panniyur 6
Cluster III	Sreekara, Subhakara, IISR Sakthi and IISR Thevam
Cluster IV	Panchami, Pournami, PLD-2 and IISR Girimunda
Cluster V	IISR Malabar Excel

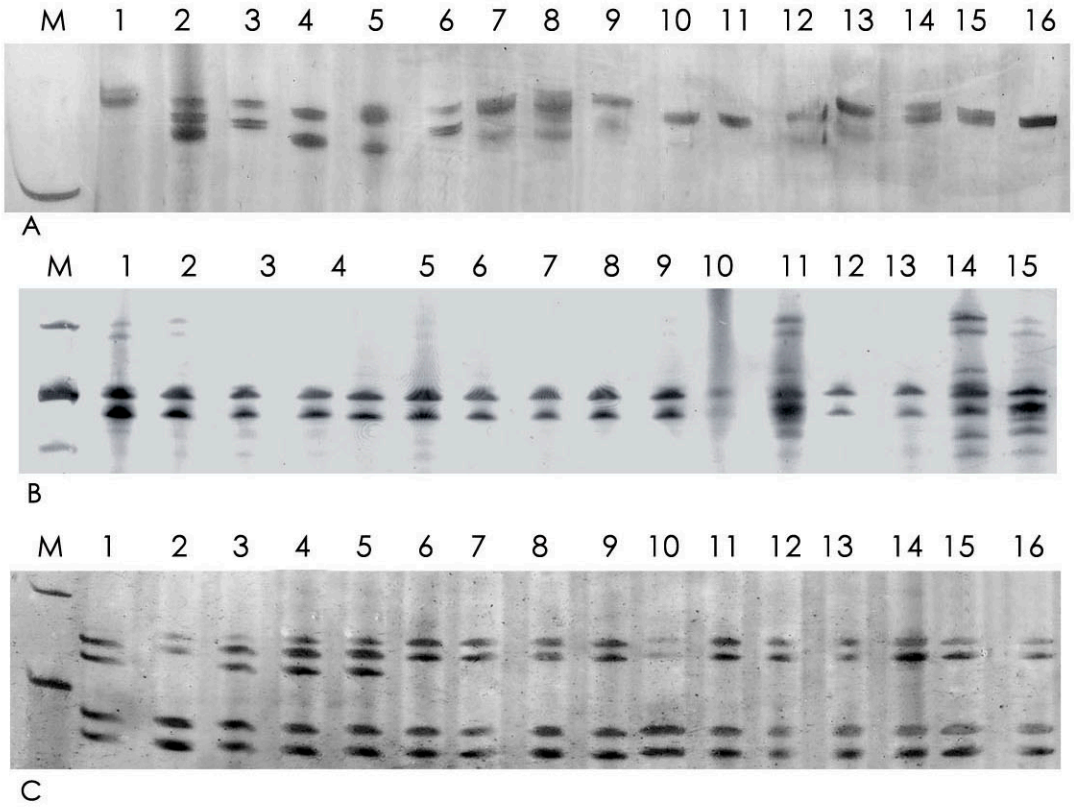


Fig. 23. Amplification profile of polymorphic genomic microsatellite markers in 16 released varieties of black pepper. Lane M-ULR (10 bp DNA ladder), A) BP SSR 11; B) BPM CNTG 15; C) BPM 154; Lane 1: Panniyur-1, Lane2: Panniyur-2, Lane 3: Panniyur-3, Lane 4: Panniyur-4, Lane 5: Panniyur-5, Lane 6: Panniyur-6, Lane 7: Panniyur-7, Lane 8: Subhakra, Lane 9: Sreekara, Lane 10: Panchami, Lane 11: Pournami, Lane 12: PLD- 02, Lane 13: IISR Sakthi, Lane 14: IISR Thevam, Lane 15: IISR Girimunda, Lane 16: IISR Malabar Excel

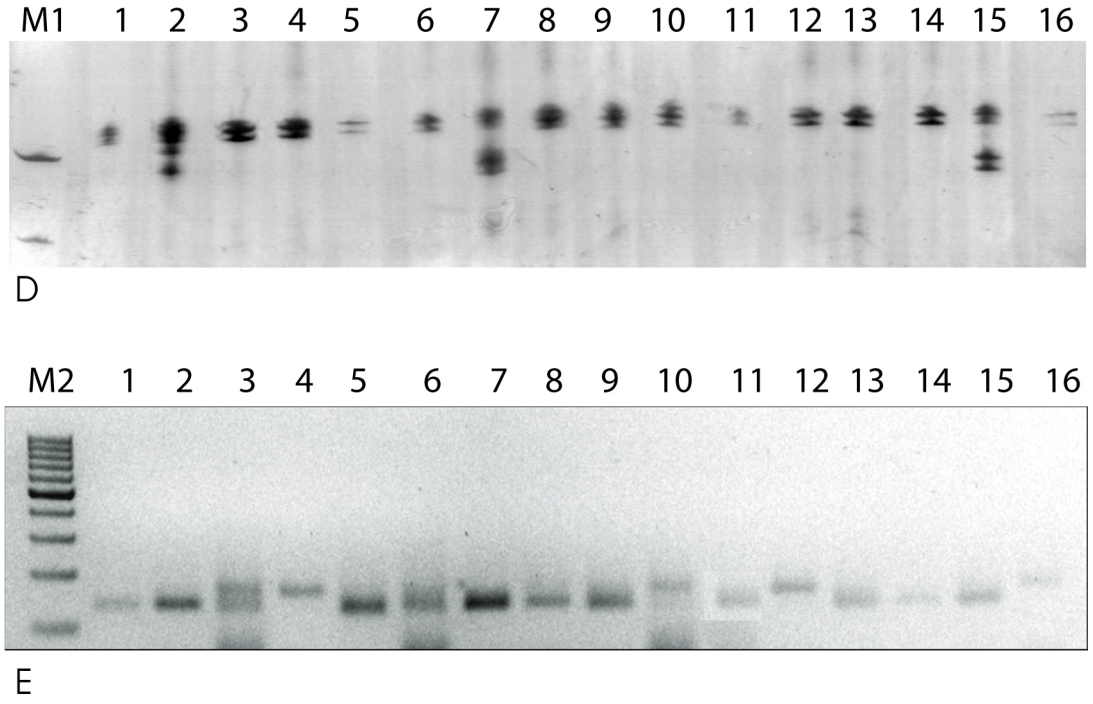


Fig. 24. Amplification profile of polymorphic genomic microsatellite markers in 16 released varieties of black pepper. Lane M1: ULR, Lane M2: 100 bp DNA ladder, D) BPM CNTG 5 (15% PAGE gel); E) BPM 156 B (3% agarose gel); Lane 1: Panniyur -1; Lane 2: Panniyur -2; Lane 3: Panniyur -3; Lane 4: Panniyur -4; Lane 5: Panniyur -5; Lane 6: Panniyur-6; Lane 7: Panniyur 7; Lane 8: Subhakara; Lane 9; Sreekara; Lane 10: Panchami; Lane 11: Pournami; Lane 12: PLD-02; Lane 13: IISR Sakthi; Lane 14: IISR Thevam; Lane 15: IISR Girimunda, Lane 16: IISR Malabar Excel.

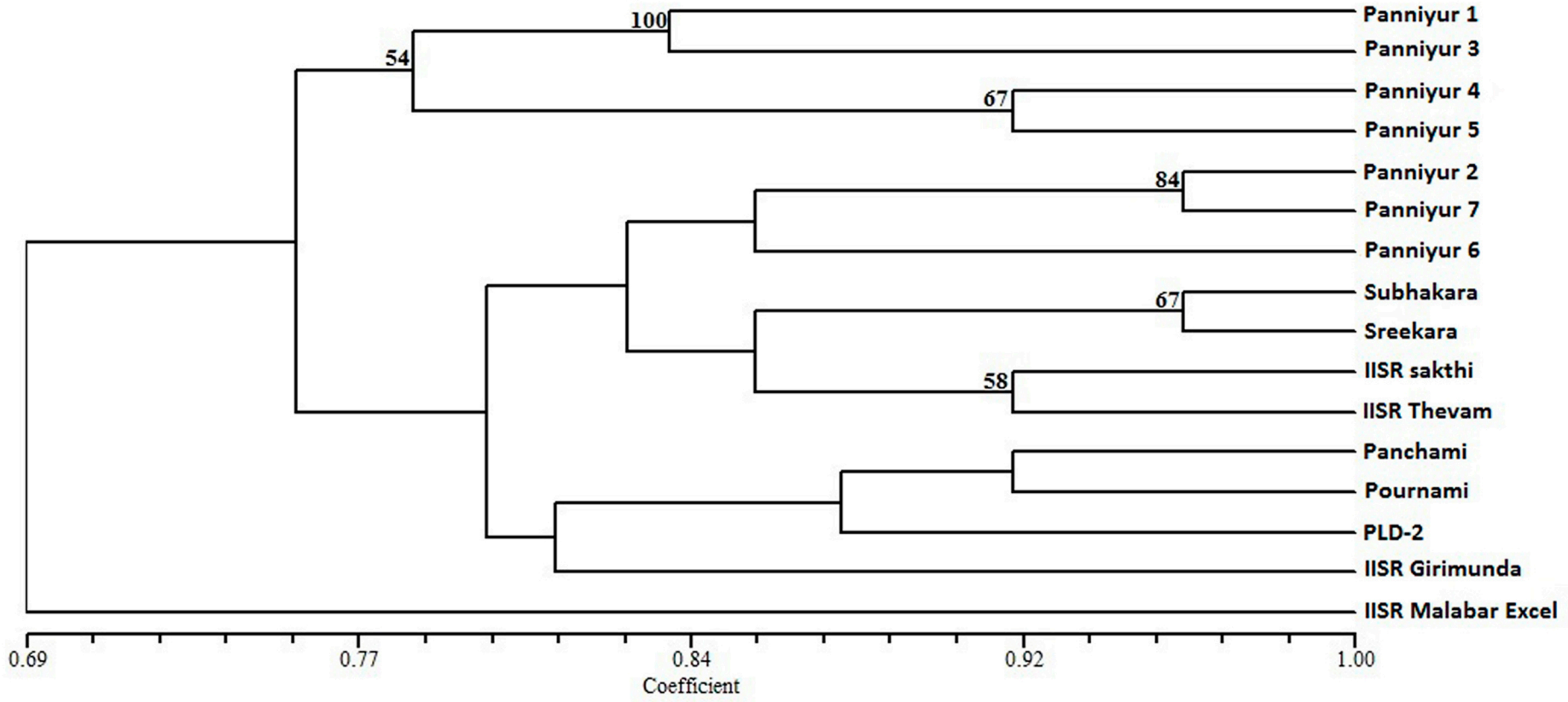


Fig. 25. UPGMA dendrogram showing genetic similarity among 16 released varieties of black pepper based on Dice similarity coefficient using 5 genomic microsatellite markers. Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the dendrogram.

4.5.2.3. Utilization of genomic microsatellite markers in black pepper cultivars

The four polymorphic markers showed a considerable level of polymorphism among the 23 black pepper cultivars (Fig. 26, 27). A total of 17 alleles were generated with the 4 polymorphic markers (BPSSR 11, BPM 31, BPM 154 and BPM 156 B) and the average number of alleles per loci is 4.25 The maximum number of alleles was noticed with the marker BPSSR 11 and BPM 154 (5 of 17) followed by BPM 31 and the least number of alleles was noticed in BPM 156 B (3 of 17). The PIC values of each genomic microsatellite markers also showed variations. The maximum value of PIC was noticed with BPM 31 (0.39) and minimum value by BPM 156 B (0.23) (Table 17.).

Table 17. Locus name, repeat motif, sequence information, no. of alleles and PIC of four genomic microsatellite markers used in the present study

Locus name	Repeat motif	Primer sequence Forward primer(5′-3′) Reverse primer (5′-3′)	No. of alleles	PIC
BPSSR 11	$(GA)_{28}$	TAAAACTGATACACCCCTTTCC AGCTAGATCAAATCATGGAGGT	5	0.30
BPM 31	$(CA)_{12}$	TAAACTCAACAACCCAGCATGT TATTGTGCCAACCTTCCTCTCT	4	0.39
BPM 154	$(GATT)_3$	ATGGAGAATCGGAAGAATATGG GAACGGATCACACTCATTAGCA	5	0.29
BPM 156 B	$(CT)_9$	GCCATGCCAATCATACATACAC GTGAAGAGTGCGAAACAGAATG	3	0.23

4.6. Genomic SSR markers: genetic diversity analysis in black pepper cultivars

The twenty three cultivars in the present study were divided into seven clusters with the aid of four genomic microsatellite markers (Fig. 28). The similarity coefficient ranged from 0.64 to 1.00. Kottanadan and Karimkotta with 94% similarity to each other were identified as the first cluster. The second cluster included Perumkodi and Karimunda and IISR Sakthi and 04-P24-1 along with Nedumchola and Kumbhachola. Malamundi stood distinct but linked to the second cluster with

76% similarity. Neelamundi and Perambramunda which shared 88% similarity formed the fourth cluster. Kuthiravally and Balancotta as well as Arakkulam munda and Chumalakodi could not be separated. All the four along with Valiakaniakkadan and Thevanmundi were identified as the fifth cluster. The sixth cluster comprised of Kalluvally and Thommankodi with 94% similarity along with Poonjaramunda. Uthirankotta which showed 82% similarity to Narayakodi and Cheriyakkaniakadan formed the seventh cluster. Most of the clusters were supported by bootstrap values greater than 50.

The 4 SSR markers separated many of the cultivars. However IISR Sakthi and and its open pollinated progeny (P 24-O4-1) could not be separated with the SSR profile. Similarly Kuthiravally, Balancotta, Arakkulam munda and Chumalakodi were could not be separated with the SSR data. The clustering pattern was as follows:

Clusters	**Black pepper cultivars**
Cluster I	Kottanadan and Karimkotta
Cluster II	Nedumchola, Perumkodi, Karimunda, IISR Sakthi and O4-P24 and Kumbachola.
Cluster III	Malamundi
Cluster IV	Neelamundi, Perambramunda,
Cluster V	Kuthiravally, Balancotta, Arakkulam munda, Chumalakkodi and Valiakaniakkadan
Cluster VI	Kalluvally, Thommankodi and Poonjaranmunda
Cluster VII	Narayakodi, Cheriyakaniakkadan and Uthirankotta

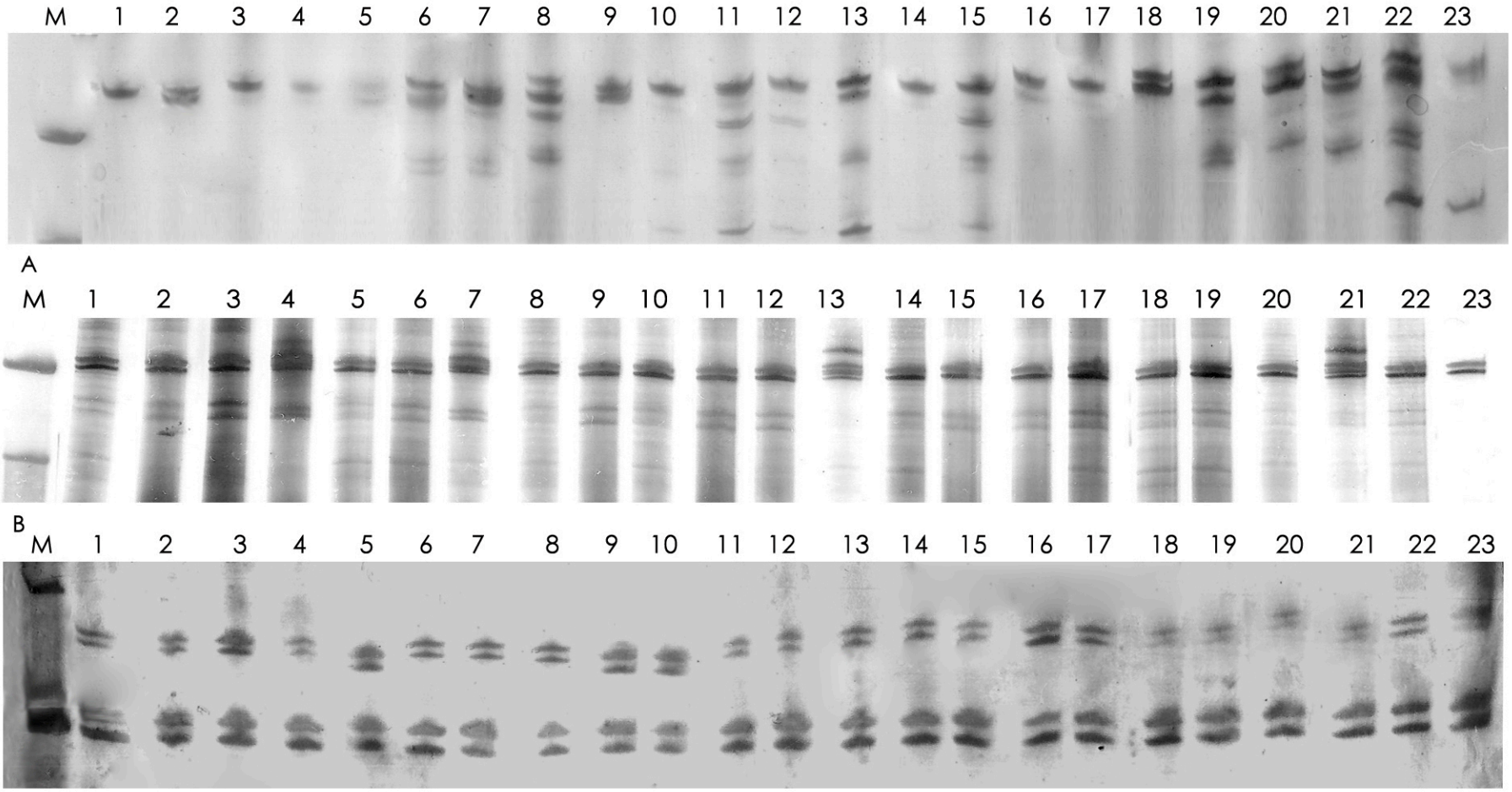


Fig 26. Amplification profile of polymorphic SSR markers in 23 black pepper cultivars. A) BP SSR 11 B) BPM 31 C) BPM 154. Lane M- ULR (10 bp DNA ladder), Lane 1: Kottanadan, Lane 2: Neelamundi, Lane 3. Kuthiravally, Lane 4: Kalluvally, Lane 5: Narayakodi, Lane 6: Perambramunda, Lane 7: Poonjaranmunda, Lane 8: Valiakaniakkadan, Lane 9: Cheriyakaniakkadan, Lane 10: Uthirancotta, Lane 11: Balancotta, Lane 12: Arakkulam munda, Lane 13: Thommankodi, Lane 14: Thevanmundi, Lane 15: Chumalakodi, Lane 16: Nedumchola, Lane 17: Malamundi, Lane 18: Karimkotta, Lane 19: Perumkodi, Lane 20: Karimunda, Lane 21: Kumbhachola , Lane 22: P 24 (IISR Sakthi), Lane 23: P 24 O4 (O4-P 24-1)

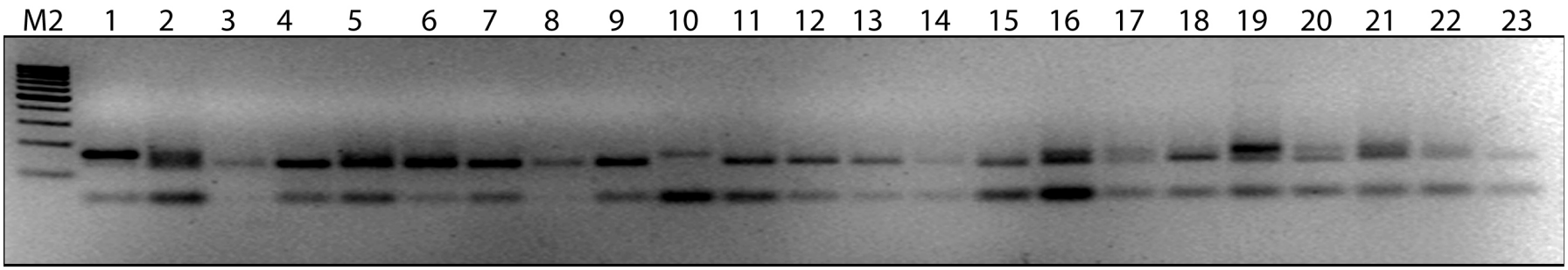


Fig. 27. Amplification profile of polymorphic SSR markers BPM 156 B in 23 black pepper cultivars (run on 3% agarose gel). Lane M: 100 bp DNA ladder, Lane 1: Kottanadan, Lane 2; Neelamundi, Lane 3: Kuthiravally, Lane 4: Kalluvally, Lane 5: Narayakodi, lane 6: Permbramunda, Lane 7: Poonjaranmunda, Lane 8: Valiakaniakkadan, Lane 9; Cheriyakaniakkadan, Lane 10: Uthirancotta, Lane 11: Balancotta, Lane 12: Arakkulam munda, Lane 13: Thommankodi, Lane 14: Thevanmundi, Lane 15: Chumalakodi, Lane 16: Nedumchola, Lane 17: Malamundi, Lane 18: Karimkotta, Lane 19: Perumkodi, Lane 20: Karimunda, Lane 21: Kumbhachola, Lane 22: P 24 (IISR Sakthi), Lane 23: P24 O4

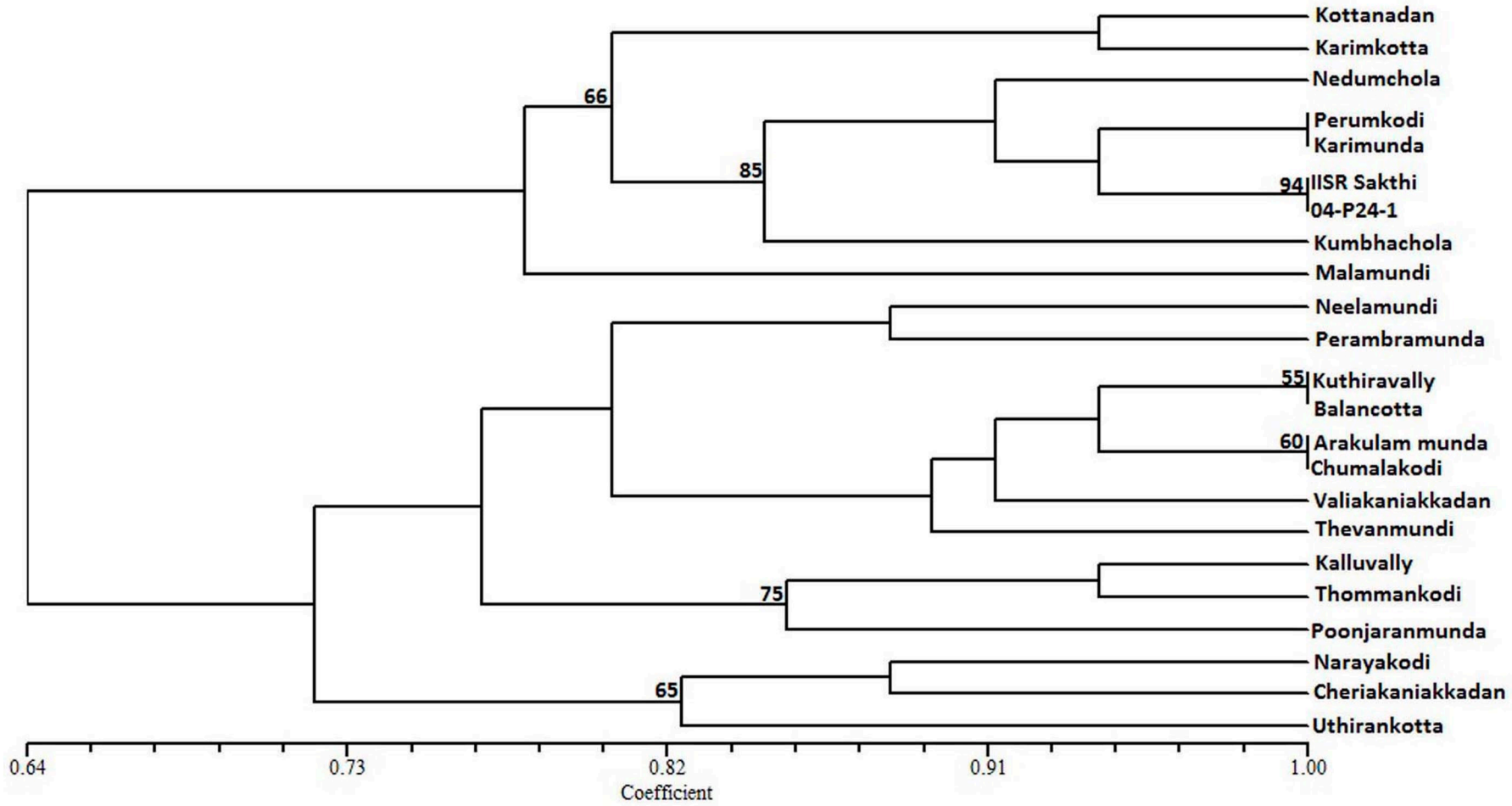


Fig. 28. UPGMA dendrogram showing genetic similarity among 23 black pepper cultivars based on Dice similarity coefficient using 4 genomic microsatellite markers. Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the dendrogram.

4.7. Transferability potential of microsatellite markers

A set of 14 polymorphic microsatellite markers (7 genomic and 7 EST-SSR) developed in the present study were tested for its effective transferability and polymorphism in 21 *Piper* species. All the 7 EST-SSR markers from *P. nigrum* and *P. colubrinum* were completely transferable to all the 21 *Piper* species. Among the 13 polymorphic genomic SSR markers, 7 markers were completely transferable to all the *Piper* species tested.

4.7.1. EST- SSR markers: its polymorphism and cross species amplification.

Seven microsatellite markers were employed to test the cross species amplification profiling of twenty one *Piper* species including the exotic species. All the 7 EST-SSR markers were considerably good in banding pattern and polymorphism (Fig 29, 30).

A total of 36 alleles were generated with seven EST derived microsatellite markers. The average number of allele per locus is 5.14. The maximum number of alleles was noticed with the marker, PN EST SSR 6 (8 of 36) followed by PN EST SSR 3 and PC EST SSR 15 with 7 alleles. The least number of alleles were observed with the microsatellite PN EST SSR 4 and PC EST SSR 22 with two alleles each. The PIC values of each EST derived microsatellite markers also showed variation. The maximum PIC value was noticed with PN EST SSR 4 (0.36) and minimum PIC was observed with PC EST SSR 22 (0.09) (Table 18.).

Table: 18. Locus name, repeat motif, sequence information, no. of alleles and PIC of seven EST SSR markers used for cross species ampification.

Locus name	Repeat motif	Primer sequence Forward primer(5′-3′) Reverse primer (5′-3′)	No. of alleles	PIC
PN EST SSR 3	$(GAGCA)_2$	ACGAATAATGGACTTAACCTACCTG CTGCATGTGGAAGGAATTAGTG	7	0.10
PN EST SSR 4	$(AATCA)_2$	ATGCTTGCTCTGTTGCTTCTTC AAAATTCTTTCCGCTTCTCTGC	2	0.36
PN EST SSR 6	$(ACTGTG)_2$	GCAGGTTCTTGAGGAGGTTTAG ACCGAGACCAAACACATTCAC	8	0.23
PN EST SSR 9	$(GAGAT)_2$	CTCCTCAAGCTCCTGTTACTGC TACAGAAAAGAAAGCCACCAGC	4	0.31
PC EST SSR 15	$(GACGA)_2$	TATATTACCACCCGCAACGAC AAACCACCAAGAGAACGAAGAA	7	0.15
PC EST SSR 19	$(ATAA)_2$	TGATCCTACCAGCACTTTCACT TTACCAACTGATCTCCAATCTCC	6	0.30
PC EST SSR 22	$(CGACAT)_2$	GGAGGAACCATGCGTCTTG AAGGCCAGCAGTTTCAAGTTC	2	0.09

4.7.2. EST SSR markers: genetic diversity analysis in *Piper* species

Seven microsatellite markers derived from the EST database could resolve 21 *Piper* species into nine clusters (Fig. 31). The similarity coefficient value ranged from 0.64 to 1.00. *P. betle* alone formed the first cluster. The second cluster consisted of *P. nigrum* and *P. sugandhi* as sub-cluster with 98% similarity along with the sub-cluster comprising of *P. trichostachyon* and *P. galeatum* sharing 98% similarity. The two sub-clusters showed 89% similarity with each other. *P. bababudani* was linked to the second cluster with 84% similarity forming the third cluster. *P. barberi* stood distinct in the EST based phylogram. *P. attenuatum* and *P. hymenophyllum* which shared 95% similarity along with *P. argyrophyllum* formed the fifth cluster. The *Piper* species from North- East region *viz*, *P. peepuloides*, *P. ribesioides*, *P. thomsoni* and *P. hamiltonii* were grouped together in the sixth cluster. *P. longum* and *P. hapnium* which showed 100% similarity with each other along with *P. sarmentosum* comprised the seventh cluster. The exotic species were assembled together in the eighth cluster but *P. colubrinum* stood apart from rest of the exotic species. The 3-D plot based on

principal coordinate analysis was in agreement with the phylogram generated (Fig. 32). The EST SSR markers separated all the 19 *Piper* species except *P. longum and P. hapnium*.

Cluster	***Piper* species**
I	*P. betle*
II	*P. nigrum, P. sugandhi, P. trichostachyon* and *P.galeatum*
III	*P. bababudani*
IV	*P. barberi*
V	*P. attenuatum, P. hymenophyllum* and *P. argyrophyllum,*
VI	*P. peepuloides, P. ribesioides, P. thomsoni* and *P. hamiltonii,*
VII	*P. longum, P. hapnium* and *P. sarmentosum*
VIII	*P. arboreum, P. ornatum* and *P. magnificum*
1X	*P. colubrinum*

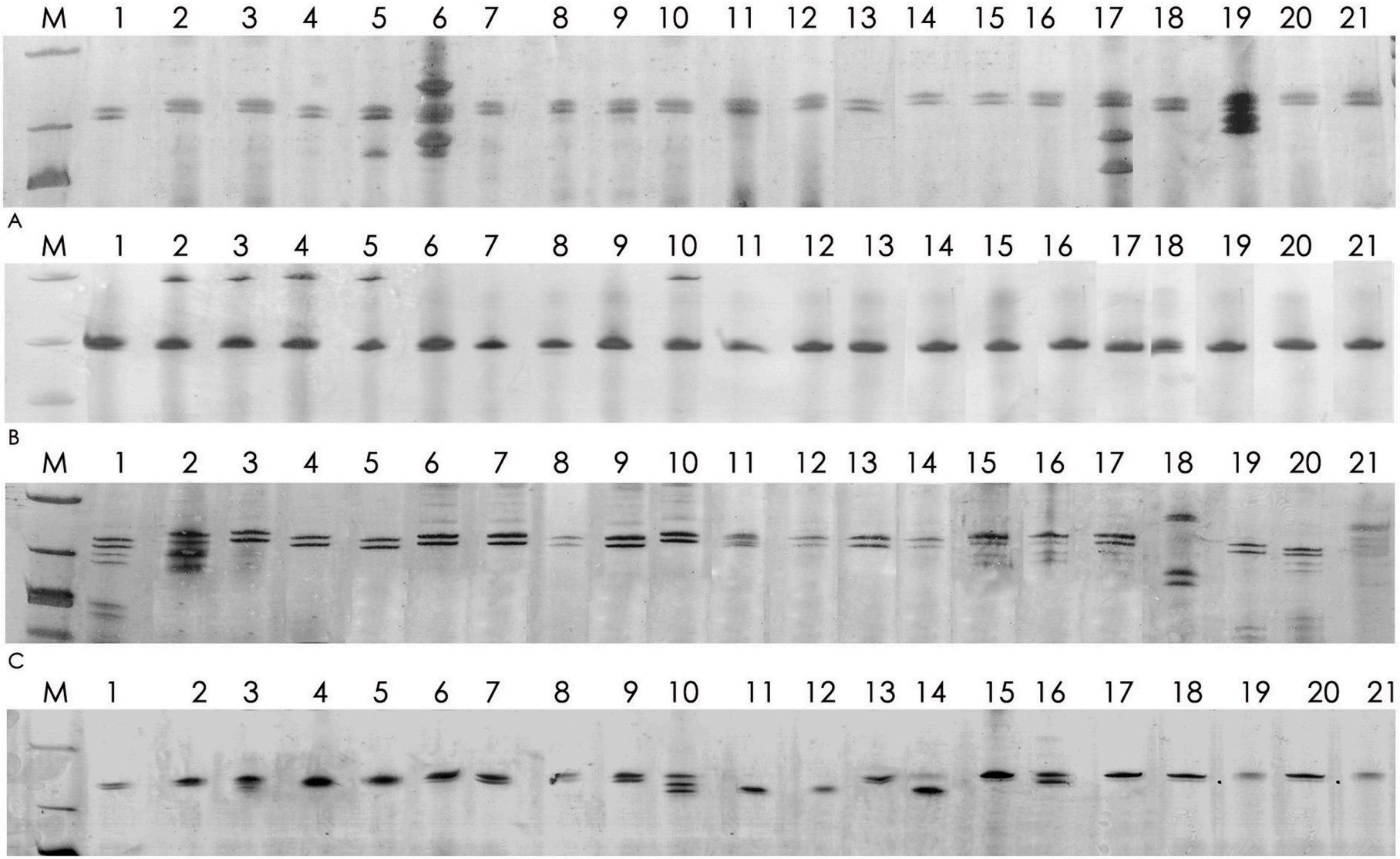


Fig. 29. Amplification profile of polymorphic EST -SSR markers in 21 *Piper* species. A) PN EST SSR 03 B) PN EST SSR 04 C) PN EST SR 06 D) PN EST SR 09. Lane M- ULR (10 bp DNA ladder), Lane 1: *P. betle*, Lane 2: *P. nigrum,* lane 3: *P. sugandhi,* Lane 4: *P. trichostachyon*, Lane 5: *P. galeatum*, Lane 6: *P. barberi* Lane 7: *P. attenuatum*, Lane 8: *P. argyrophyllum*, Lane 9: *P. hymenophyllum*, Lane 10: *P. bababudani,* Lane 11: *P. longum*, Lane 12: *P. hapnium*, Lane 13: *P. peepuloides* , Lane 14: *P. sarmentosum*, Lane15: *P. ribesioides*, Lane 16: *P. thomsoni*, Lane 17: *P. hamiltonii* ,Lane 18: *P. colubrinum*, Lane 19: *P. arboreum*, Lane 20: *P. ornatum*, Lane 21: *P. magnificum*

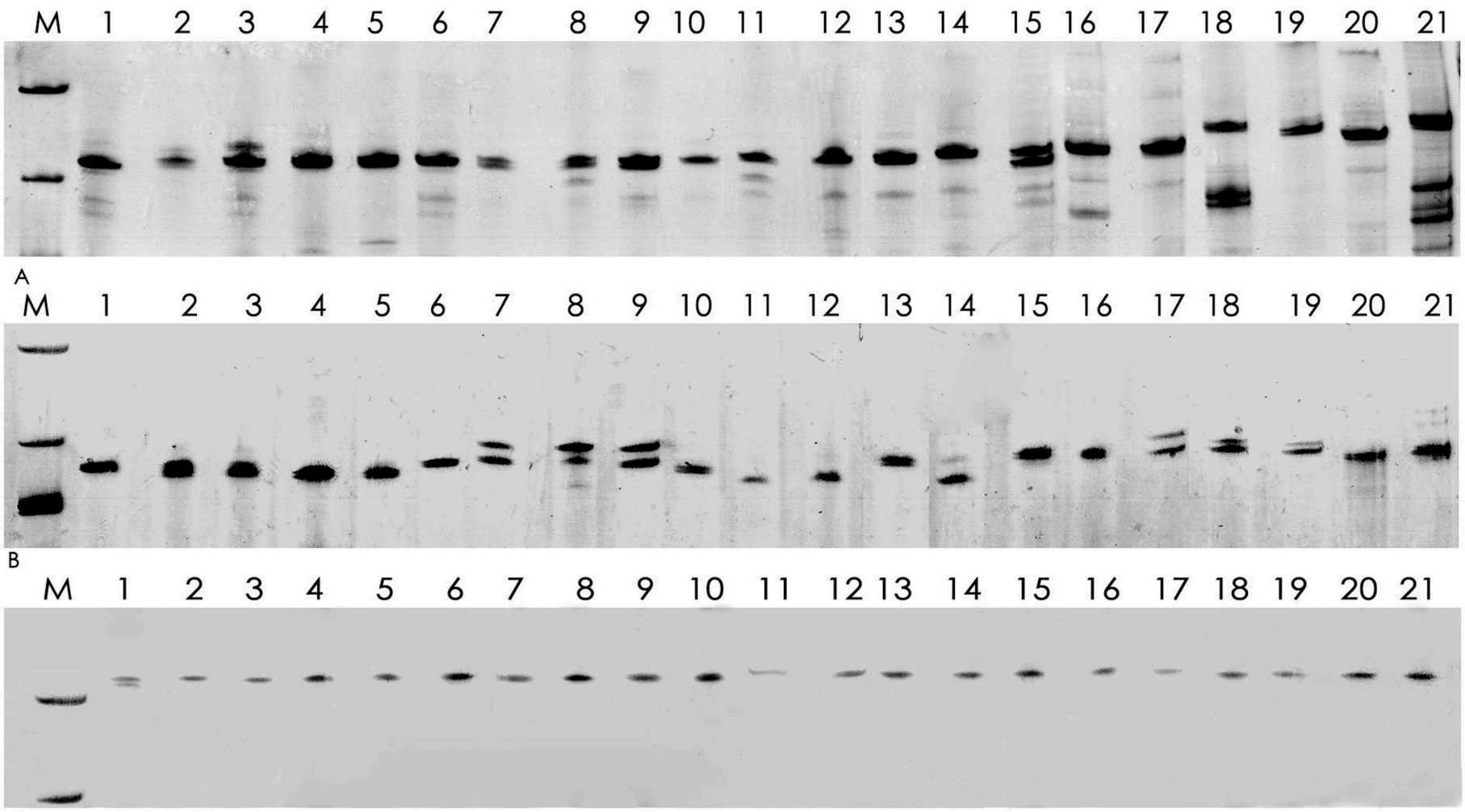


Fig. 30. Amplification profile of polymorphic EST -SSR markers in 21 *Piper* species. A) PC EST SSR 15 B) PC EST SSR 19 C) PC EST SR 22. Lane M- ULR (10 bp DNA ladder), Lane 1: *P. betle*, Lane 2: *P. nigrum,* Lane 3: *P. sugandhi,* Lane 4: *P. trichostachyon*, Lane 5: *P. galeatum*, Lane 6: *P. barberi* Lane 7: *P. attenuatum*, Lane 8: *P. argyrophyllum*, Lane 9: *P. hymenophyllum*, Lane 10: *P. baba-budani,* Lane 11: *P. longum*, Lane 12: *P. hapnium*, Lane 13: *P. peepuloides* , Lane 14: *P. sarmentosum*, Lane15: *P. ribesioides*, Lane 16: *P. thomsoni*, Lane 17: *P. hamiltonii*, Lane 18: *P. colubrinum*, Lane 19: *P. arboreum*, Lane 20: *P. ornatum*, Lane 21: *P. magnificum*

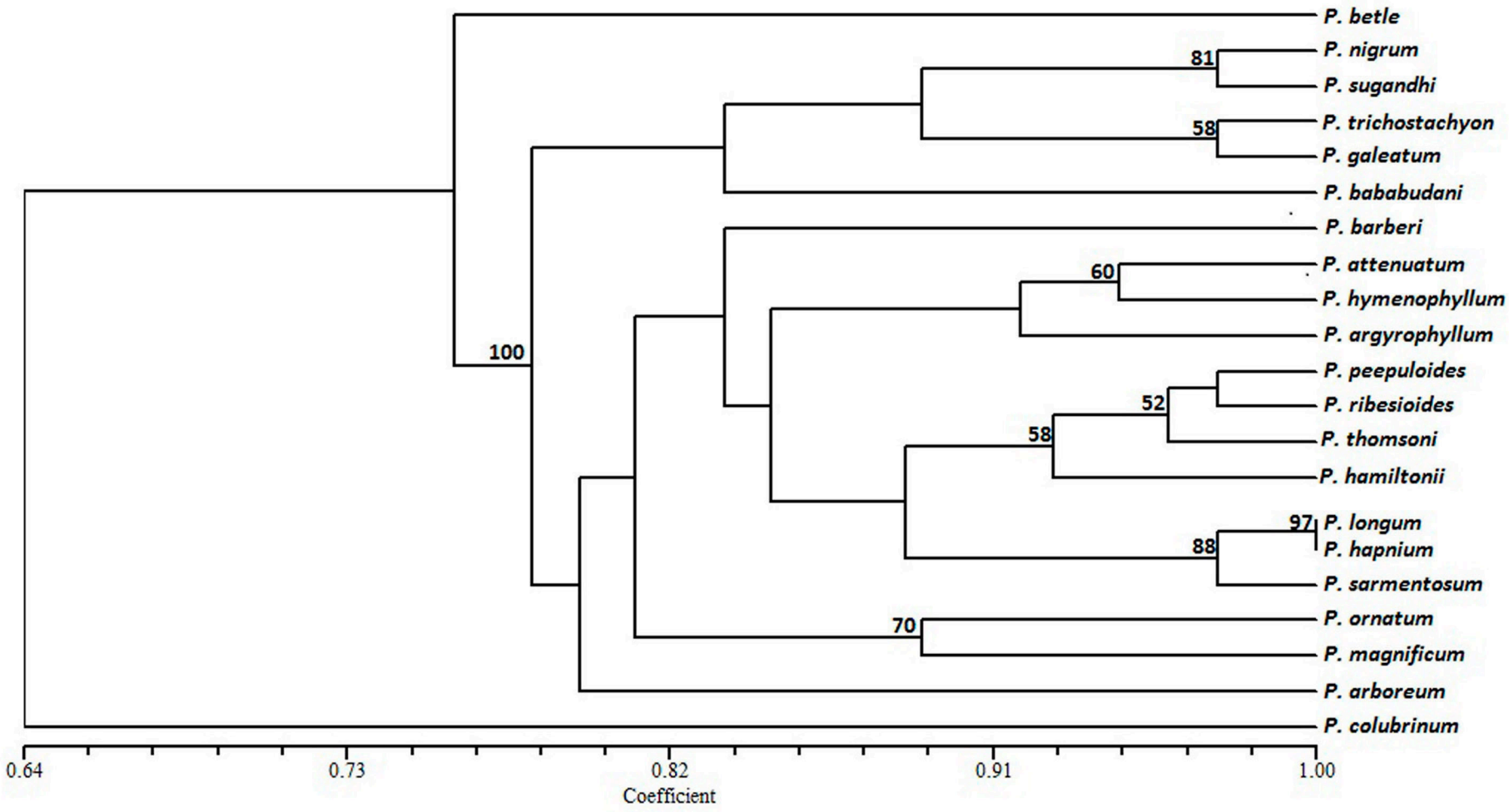


Fig. 31.UPGMA phylogram showing genetic similarity among 21 *Piper* species based on Dice similarity coefficient using seven EST SSR markers. Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the phylogram.

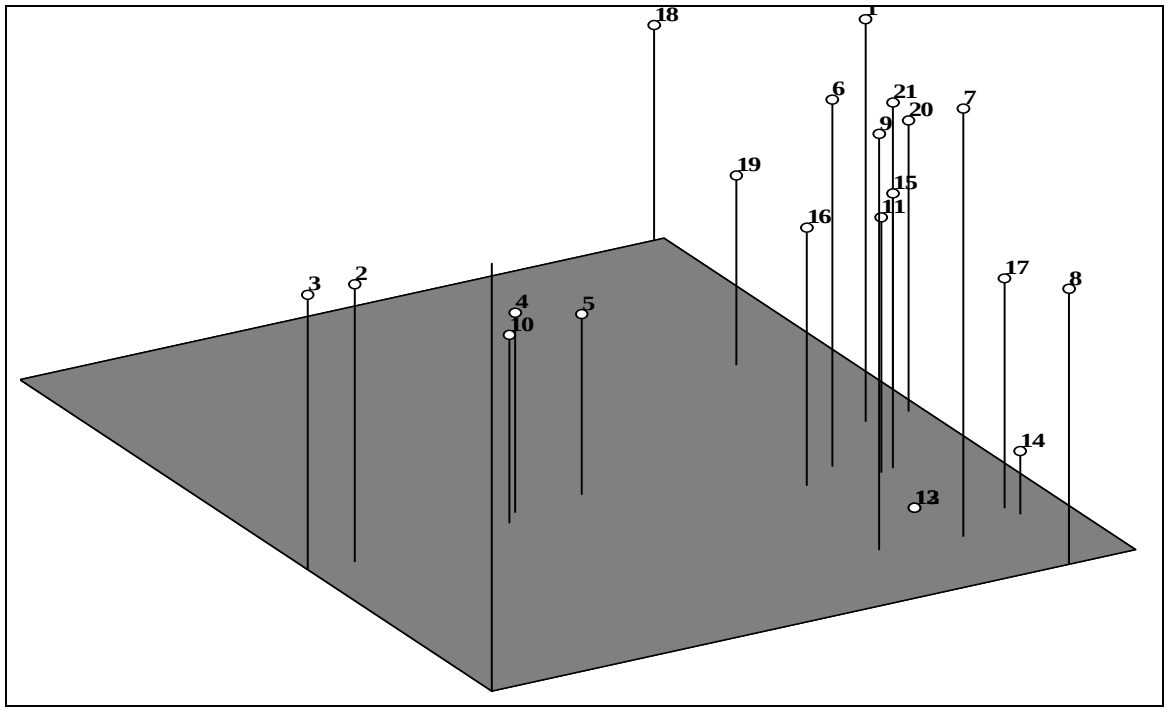


Fig. 32. The 3-D plot diagram showing the relationship among 21 *Piper* species based on principal coordinate analysis (PCA) using 7 EST based microsatellite markers. The number represents the genotypes. (1. *P. betle*, 2. *P. nigrum*, 3. *P. sugandhi*, 4. *P. trichostachyon*, 5. *P. galeatum*, 6. *P. barberi*, 7. *P. attenuatum*, 8. *P. argyrophyllum*, 9. *P. hymenophyllum*, 10. *P. bababudani*, 11. *P. peepuloides*, 12. *P. longum*, 13. *P. hapnium*, 14. *P. sarmentosum*, 15. *P. ribesioides*, 16. *P. thomsoni*, 17. *P. hamiltonii*, 18. *P. colubrinum*, 19. *P. arboretum*, 20. *P. ornatum*, 21. *P. magnificum*).

4.7.3. Genomic SSR markers: its polymorphism and cross species amplification.

Seven genomic microsatellite markers developed were used to analyze the cross species amplification profiling of twenty one *Piper* species including the exotic species (Fig 33, 34). A total of 43 alleles were generated with seven genomic microsatellite markers. Average number of allele per loci is 6.14. The maximum number of alleles were noticed with the marker, BPM 52 (10 of 43) followed by BP SSR 11 with 8 alleles. The least number of alleles were observed with the SSR marker BP SSR 12 and BPM CNTG 5 with only 4 alleles. The PIC values of each genomic derived microsatellite also showed variations. The maximum value of PIC was noticed with BP SSR 12 (0.40) and minimum value by BPM CNTG 5 (0.09).

Table: 19. Locus name, repeat motif, sequence information, no. of alleles, PIC of seven genomic microsatellite markers used for cross species amplification.

Locus name	Repeat motif	Primer sequence Forward primer(5′-3′) Reverse primer (5′-3′)	No. of alleles	PIC
BPM CNTG 5	$(ATGT)_3$	TTCAACCGTGACAGAAGATCC TCGAACACAAGTTCAAACCAAC	4	0.09
BPSSR 11	$(GA)_{28}$	TAAAACTGATACACCCCTTTCC AGCTAGATCAAATCATGGAGGT	8	0.29
BPSSR 12	(TCAGA)2	CAGCTTTGTGGAACTAATGAGA CTACATAAGAATGCGAGCAGAT	4	0.40
BPM 52	$(AACA)_3$	GATCCAGAGCCAAAGTGAGAGT GCTGAAAGTTGTGGGACCTTAT	10	0. 27
BPM 132	$(GT)_5$	GCTGTTATTCTTTTGGGGATTG TACTCACTCTTCTGCCTCATCG	5	0.25
BPM 133	$(AG)_{14}$	CTTGTAGGGTGTTACCTTTGGG AAGCAGCATGAACCATAAGCTC	5	0.18
BPM 154	$(GATT)_3$	ATGGAGAATCGGAAGAATATGG GAACGGATCACACTCATTAGCA	7	0.31

4.7.4. Genomic microsatellite markers: genetic diversity analysis in *Piper* species

Nine clusters were attained with the UPGMA dendrogram created with genomic SSR markers. (Fig. 35). The similarity coefficient ranged from 0.61 to 1.00. *P. betle* was the only species present in the first cluster. *P. attenuatum*, *P. hymenophyllum* and *P. argyrophyllum* are with 98% similarity and formed the second

cluster. Among the second cluster, *P. attenuatum* and *P. hymenophyllum* exhibited 100% similarity of coefficient. The third cluster was occupied by four *Piper* species and is *P. nigrum, P. galeatum, P. trichostachyon* and *P. sugandhi.* Among these, *P. nigrum* and *P. galeatum* showed more similarity with a 95% coefficient of similarity. Cluster IV consists of *P. longum* and *P. hapnium* and their similarity of coefficient was 93%.

P. barberi was the sole member of the fifth cluster. *P. bababudani* and *P. peepuloides* are also placed individually in cluster VI and VII respectively. *P. sarmentosum*, *P. ribesioides*, *P. thomsoni* and *P. hamiltonii* together formed the eighth cluster. Of these, *P. thomsoni* and *P. hamiltonii* showed 97% similarity. *P. arboretum, P. ornatum, P. magnificum* and *P. colubrinum* are occupied in the cluster IX with two sub groups. *P. colubrinum* and *P. ornatum* formed the first subgroup with 92% of similarity.The pattern of distribution of the 21 *Piper* species with principal coordinate 3-D plot matched with the dendrogram (Fig. 36).

Cluster	***Piper* species**
I	*P. betle*
II	*P. attenuatum, P. hymenophyllum* and *P. argyrophyllum*
III	*P. nigrum, P.galeatum, P. trichostachyon,* and *P. sugandhi*
IV	*P. longum* and *P. hapnium*
V	*P.barberi*
VI	*P. bababudani*
VII	*P. peepuloides*
VIII	*P. sarmentosum, P. ribesioides, P. thomsoni* and *P. hamiltonii*
IX	*P. colubrinum P. ornatum, P. arboreum, P. magnificum.*

The exotic species were clearly separated from Indian species and were together grouped in cluster IX. The grouping is clear and is in agreement with the present understanding of interrelationships in *Piper* species. However, these markers could not separate *P. attenuatum* and *P. hymenophyllum*.

4.7.5. Unique bands observed with microsatellite markers

Unique bands could be identified for most of the species with the aid of both genome and EST derived microsatellite markers developed in the present study.

Table 20. Discriminatory primers (EST SSR and genomic SSR) and unique bands specific to different *Piper* species.

Piper species	Unique band	Discriminatory primer
P. betle	2	PC EST SSR 22 $_{325}$
		BPM 133 $_{200}$
P. barberi	3	PN EST SSR 3 $_{220}$
		BPM 52 $_{180}$
		BPM 52 $_{220}$
P. colubrinum	2	PN EST SSR6 $_{180}$
		PN EST SSR6 $_{190}$
		PN EST SSR6 $_{220}$
		PC EST SSR 15 $_{230}$
P. arboreum	2	PN EST SSR 3 $_{190}$
		PC EST SSR15 $_{225}$
P. ornatum	1	PC EST SSR15 $_{220}$
P. magnificum	1	PC EST SSR15 $_{240}$
P. ribesioides	1	BPM CNTG 5 $_{300}$
		BPM CNTG 5 $_{310}$
P. hamiltonii	1	PN EST SSR 3 $_{190}$

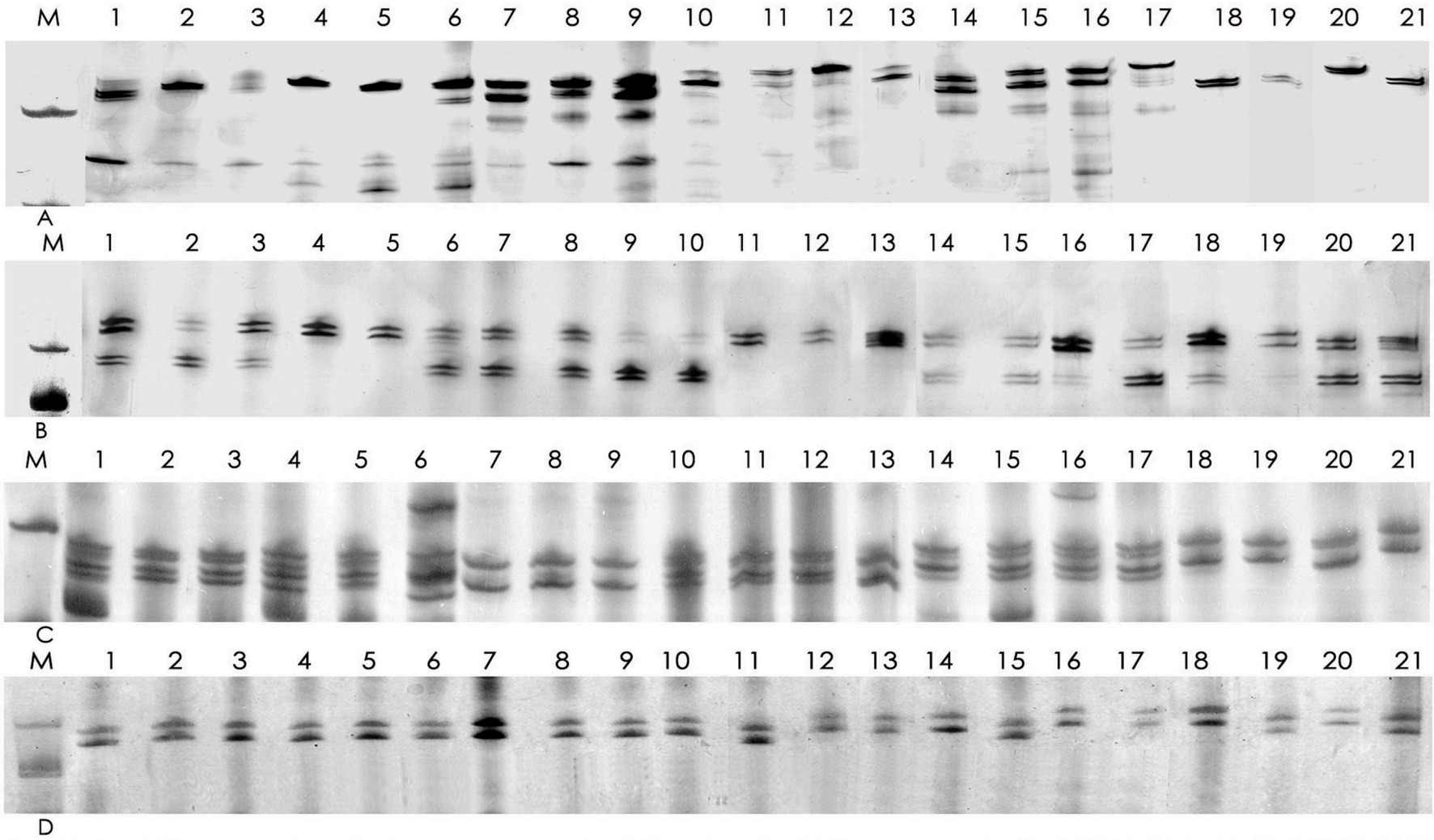


Fig. 33 . Amplification profile of polymorphic genomic SSR markers in 21 *Piper* species. A) BP SSR 11 03 B) BP SSR 12 C) BPM 52, D) BPM 132 . Lane M- ULR (10 bp DNA ladder), Lane 1: *P. betle*, Lane 2: *P. nigrum*, lane *3: P. sugandhi*, Lane 4: *P. trichostachyon*, Lane 5: *P. galeatum*, Lane 6: *P. barberi* Lane 7: *P. attenuatum*, Lane 8: *P. argyrophyllum*, Lane 9: *P. hymenophyllum*, Lane 10: *P. bababudan*i, lane 11: *P. longum*, Lane 12: *P. hapnium*, Lane 13: *P. peepuloides* Lane 14: *P. sarmentosum*, Lane15: *P. ribesioides*, Lane 16: *P. thomsoni*, Lane 17: *P. hamiltonii* ,Lane 18: *P. colubrinum*, Lane 19: *P. arboreum*, Lane 20: *P. ornatum*, Lane 21: *P. magnificum*

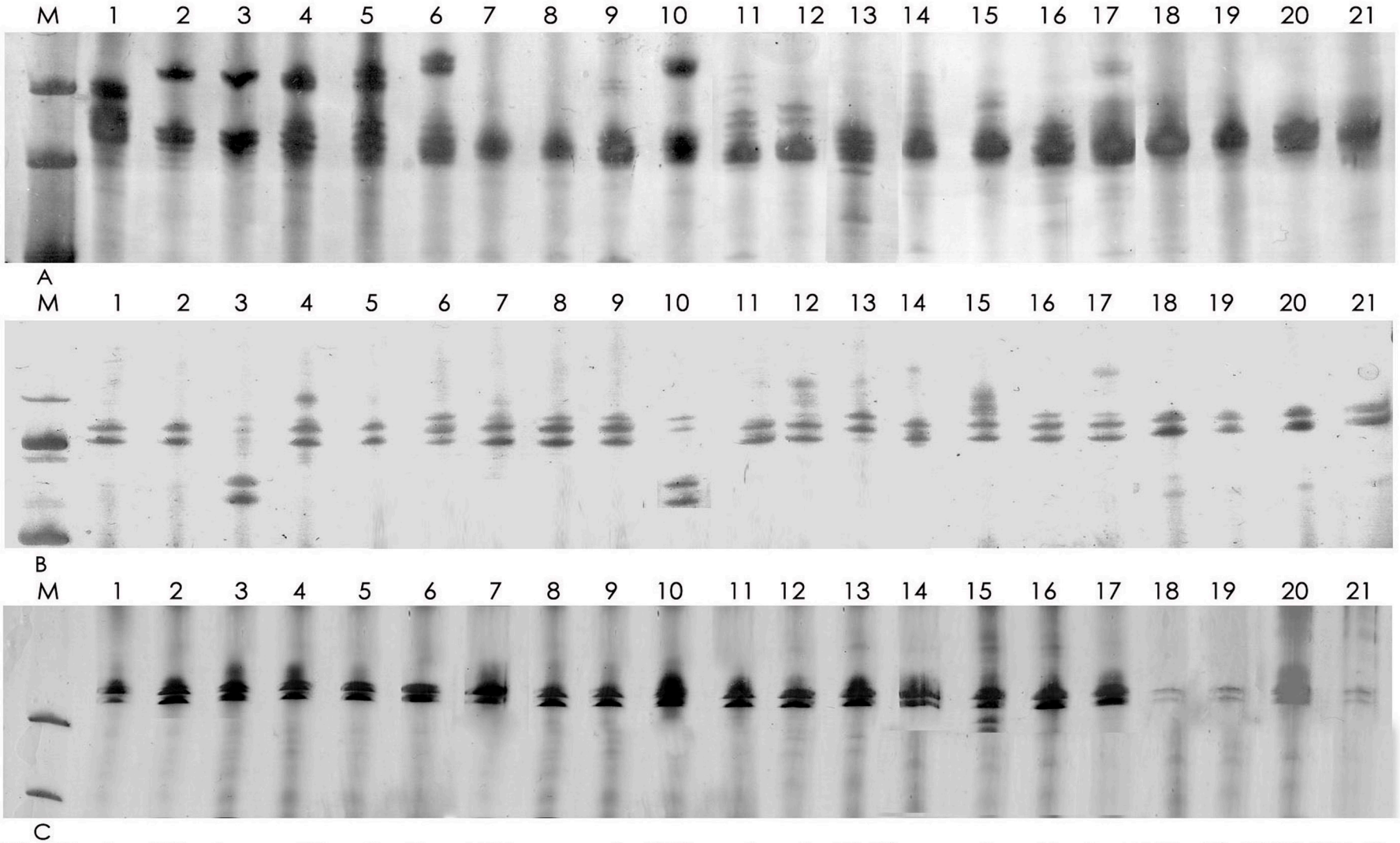


Fig. 34. Amplification profile of polymorphic genomic SSR markers in 21 *Piper* species. A) BPM 133 B) BPM 154 C) BPM 132 . Lane M- ULR (10 bp DNA ladder), Lane 1: *P. betle*, Lane 2: *P. nigrum,* lane *3: P. sugandhi*, Lane 4: *P. trichostachyon*, Lane 5: *P. galeatum,* Lane 6: *P. barberi* Lane 7: *P. attenuatum*, Lane 8: *P. argyrophyllum*, Lane 9: *P. hymenophyllum*, Lane 10: *P. bababudan*i, lane 11: *P. longum,* Lane 12: *P. hapnium,* Lane 13: *P. peepuloides* Lane 14: *P. sarmentosum*, Lane 15: *P. ribesioides,* Lane 16: *P. thomsoni*, Lane 17: *P. hamiltonii* , Lane 18: *P. colubrinum*, Lane 19: *P. arboreum*, Lane 20: *P. ornatum*, Lane 21: *P. magnificum*

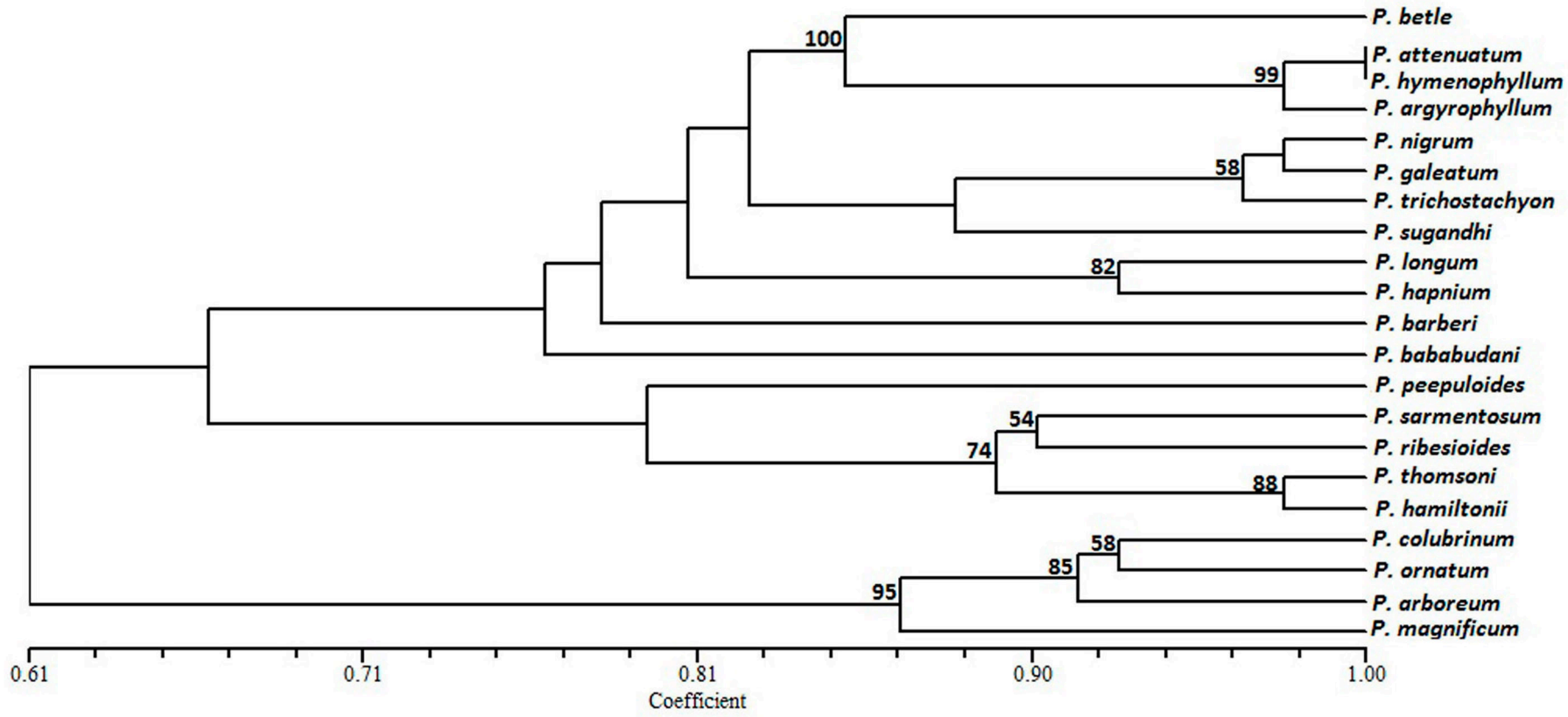


Fig. 35. UPGMA phylogram showing genetic similarity among 21 *Piper* Species based on Dice similarity coefficient using 7 genomic SSR markers. Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the phylogram

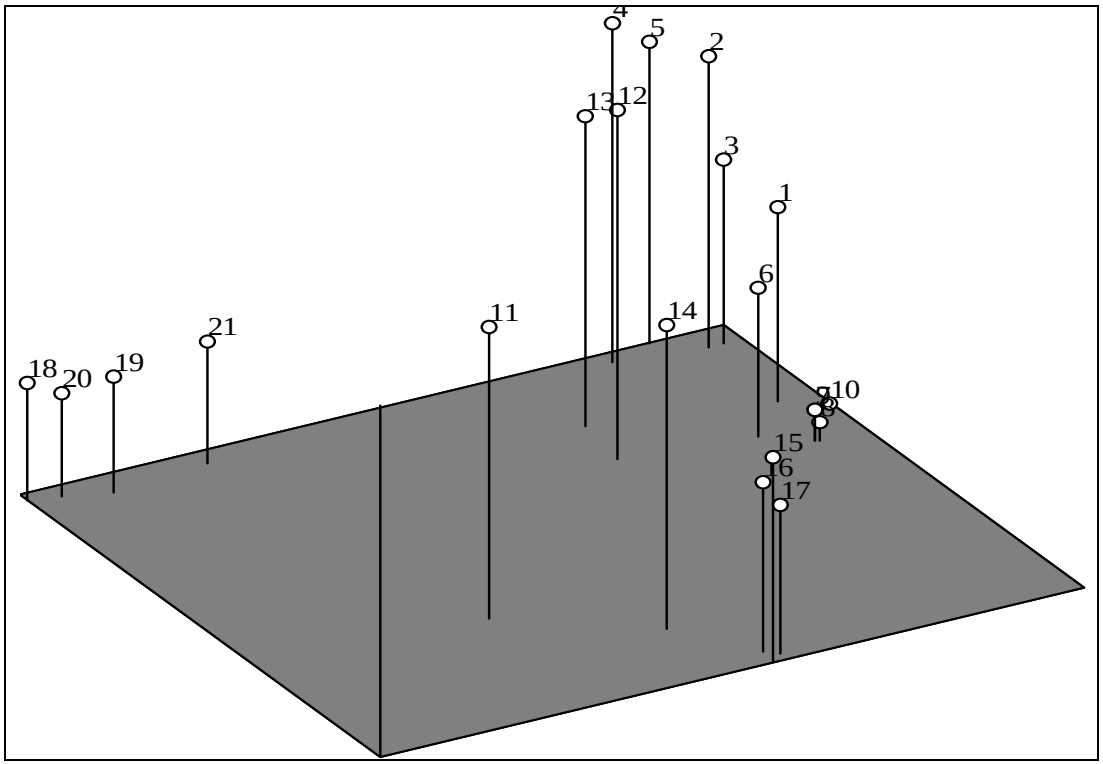


Fig. 36. The 3-D plot diagram showing the relationship among 21 *Piper* species based on principal coordinate analysis (PCA) using 7 genomic microsatellite markers. The number represents the genotypes. (1. *P. betle*, 2. *P. nigrum***,** 3. *P. sugandhi***,** 4**.** *P. trichostachyon*, 5. *P. galeatum*, 6. *P. barberi*, 7. *P. attenuatum*, 8. *P. argyrophyllum*, 9. *P. hymenophyllum*, 10. *P. bababudani*, 11. *P. peepuloides*, 12. *P. longum*, 13. *P. hapnium*, 14. *P. sarmentosum*, 15. *P. ribesioides*, 16. *P. thomsoni*, 17. *P. hamiltonii*, 18. *P. colubrinum*, 19. *P. arboretum*, 20. *P. ornatum*, 21. *P. magnificum*).

4.7.6. Combined SSR markers (Genomic SSR + EST SSR): genetic diversity analysis in *Piper* species

The matrix generated with genomic SSR and EST-SSRs were pooled together and used for constructing a consensus phylogram.

Cluster	*Piper* species
I	*P. betle*
II	*P. barberi*
III	*P. attenuatum, P. hymenophyllum* and *P. argyrophyllum*
IV	*P. ribesioides*
V	*P. thomsoni* and *P. hamiltonii*
VI	*P. peepuloides*
VII	*P. longum, P. sarmentosum* and *P. hapnium*
VIII	*P. nigrum, P.galeatum, P. sugandhi* and *P. trichostachyon*
IX	*P. bababudani*
X	*P. arboreum, P. ornatum, P. magnificum* and *P. colubrinum*

The combined phylogram showed similarity to the phylogram constructed with genomic SSR markers (Fig. 37). The 79 alleles generated with combined phylogram clearly separated all the 21 *Piper* species. *P. longum* and *P. hapnium* were separated in the combined phylogram, eventhough they were placed in the same cluster with 100% similarity in EST SSR markers. Similarly *P. attenuatum* and *P. hymenophyllum* which could not be separated with genomic SSR markers were discriminated in combined phylogram. The PCA generated from pooled similarity matrix corresponded well with the UPGMA clustering. (Fig. 38).

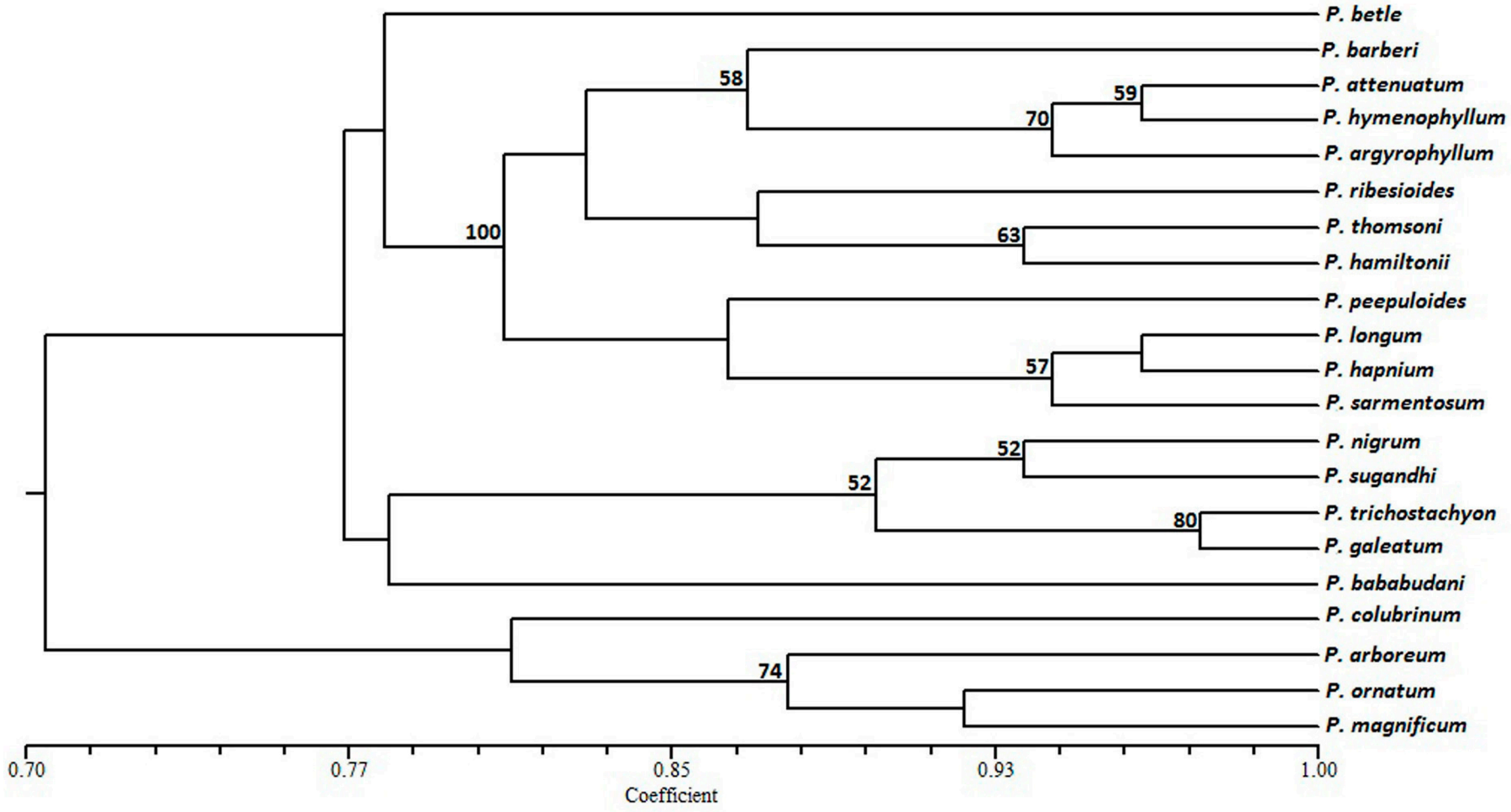


Fig. 37. UPGMA phylogram showing genetic similarity among 21 *Piper* species based on Dice similarity coefficient using 14 micro-satellite markers (7 Genomic SSR+ 7 EST-SSR). Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the phylogram.

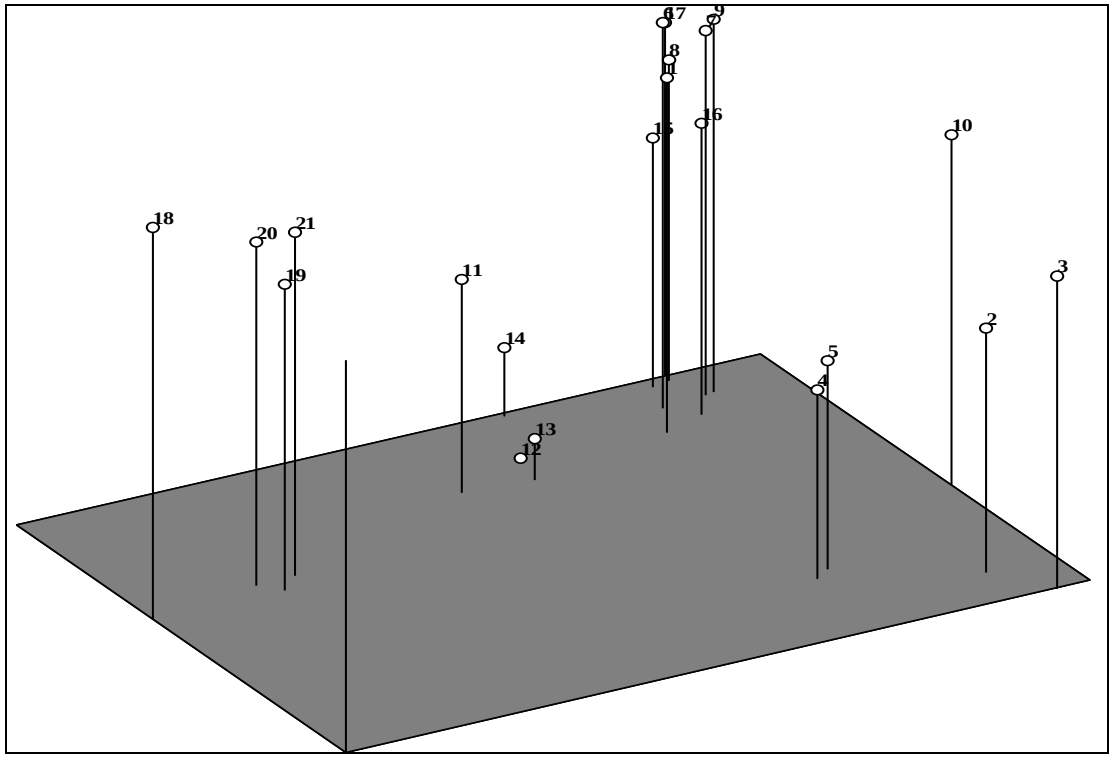


Fig. 38. The 3-D plot diagram showing the relationship among 21 *Piper* species based on principal coordinate analysis (PCA) using 14 (7 EST+ 7 genomic) microsatellite markers. The number represents the genotypes. (1. *P. betle*, 2. *P. nigrum***,** 3. *P. sugandhi***,** 4**.** *P. trichostachyon*, 5. *P. galeatum*, 6. *P. barberi*, 7. *P. attenuatum*, 8. *P. argyrophyllum*, 9. *P. hymenophyllum*, 10. *P. bababudani*, 11. *P. peepuloides*, 12. *P. longum*, 13. *P. hapnium*, 14. *P. sarmentosum*, 15. *P. ribesioides*, 16. *P. thomsoni*, 17. *P. hamiltonii*, 18. *P. colubrinum*, 19. *P. arboretum*, 20. *P. ornatum*, 21. *P. magnificum*).

4.8. Genetic diversity analysis using previously reported microsatellite markers

Previously reported 16 microsatellite markers were used for genetic diversity study among 23 black pepper cultivars and 21 *Piper* species.

4.8.1.Utilization of previously reported microsatellite markers in black pepper cultivars

From the previously reported SSR primers developed in black pepper (9 primers by Menezes *et al*., 2009 and 6 primers by Joy *et al*., 2011), six microsatellite markers gave polymorphism among 23 black pepper cultivars (Fig. 39, 40).

A total of 25 alleles produced with six microsatellite markers (PN H8a, PN E3, PN A5, PND10, PN F1 and Pn GT2). The average number of alleles per loci is 4.1. The maximum number of alleles was found with the primers PN D 10 (7 of 25) and the least number of alleles were noticed with the primer Pn GT2 (2 of 25). The maximum PIC value was obtained with the primer PN E3 (0.45) followed by PN F1 (0.43) and the least value noticed was 0.34 for the marker PnGT2.

Table 21. Locus name, repeat motif, sequence information, No. of alleles, PIC of six previously reported microsatellite markers used in the present study.

Locus	Repeat motif	Primer sequence Forward primer(5′-3′) Reverse primer (5′-3′)	No. of alleles	PIC
PN A5	$(AC)_{19}$	F 5′ CTTCCAGACCAATAATCAACTT 3′ R 5′ ATCCCAAAATACACAACAATTC 3′	5	0.35
PN E3	$(CA)_{13}$	F 5′ TTTGTGTCCTCTCCCTCTCC 3′ R 5′ AAGACTAAATAGGCAAGGCAAA 3′	3	0.45
PN D10	$(GT)_{13}$	F 5′ GTGTTACCTTTGGGGCATTCA 3′ R 5′ TGTGTCAGGGCATCAAACC 3′	7	0.35
PN F1	$(TG)_{11}$	F 5′ ACTTCAGTGCTATTTTTATCTTCC 3′ R 5′ CCAACGCCCACTCTCAT 3′	3	0.43
PN H8a	$(TG)_{16}$	F 5′ TGTGTCTTTTATATTTTTGATG 3′ R 5′ TATTAGTAGTTCTCCCTTTTGA 3′	5	0.37
PnGT2	$(GT)_5$AT (GT) AT $(GT)_{17}$GG $(GT)_{19}$	F 5′ CTAGAGAGTAACAGTTATCACTTCACAG 3′ F 5′ CTAGCAAATTTGTTCTCTAATTCACATGT 3′	2	0.34

4.8.2. Cluster analysis of black pepper cultivars using six microsatellite markers

Cluster	Black pepper cultivars
I	Kottanadan, Kalluvally, Neelamundi and Kuthiravally
II	Narayakodi, Poonjaranmunda and Thommankodi
III	Perambramunda, Valiakaniakkadan, Balancotta and Arakkulammunda
IV	Cheriyakaniakkadan and Uthirancotta
V	Thevanmundi, Malamundi, Nedumchola, Karimkotta and Perumkodi
VI	Karimunda, Kumbachola, IISR Sakthi and O4-P24-1
VII	Chumalakodi

The similarity coefficient of the dendrogram ranged from 0.43 to 0.96 (Fig 41). Kottanadan and Kalluvally showed 87% of similarity with each other and occupied the Cluster I along with Neelamundi and Kuthiravally which were 84% similar to each other. The coefficient of similarity between Narayakodi and Poonjaranmunda was 78% and Thommankodi was linked to them with 71% similarity and they formed the second cluster. Perambramunda and Valiakaniakkadan showed 75% similarity to each other. Balancotta and Arakulammunda shared 87% similarity. The third cluster comprised these four cultivars. Cheriakaniakkadan and Uthirancotta with 70% similarity were placed in the fourth cluster.

Thevanmundi, Malamundi and Nedumchola, Karimkotta and Perumkodi were accommodated in the fifth cluster. Karimkotta and Perumkodi together formed a subgroup with 75% affinity to each other. IISR Sakthi and O4P24-1 are 95% similar and had affinity towards Kumbhachola together formed the sixth cluster along with Karimunda. Chumalakodi was the only distinct member of the cluster VII.

The present study could identify one microsatellite marker (PN E3) which could differentiate IISR Sakthi (P24) from its open pollinated progeny O4P24-1.

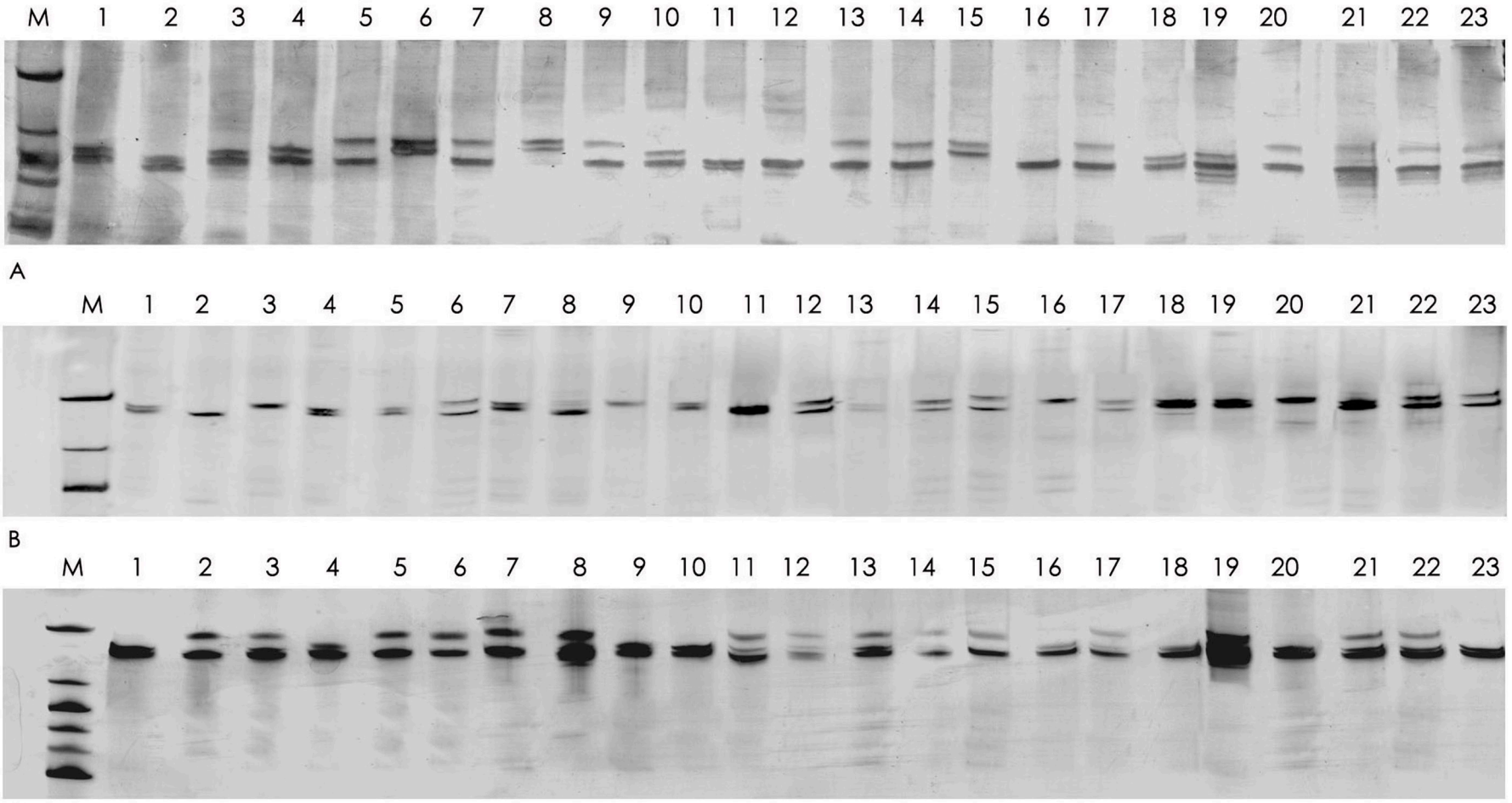


Fig. 39. Amplification profile of polymorphic SSR markers in 23 black pepper cultivars. a) PN A5 b) PN H8a c) PN E3 . Lane M-ULR (10 bp DNA ladder), Lane 1: Kottanadan, Lane 2: Neelamundi, Lane 3. Kuthiravally, Lane 4: Kalluvally, Lane 5: Narayakodi, Lane 6: Perambramunda, Lane 7: Poonjaranmunda, Lane 8: Valiakaniakkadan, Lane 9: Cheriyakaniakkadan, Lane 10: Uthirancotta, Lane 11: Balancotta, Lane 12: Arakkulam munda, Lane 13: Thommankodi, Lane 14: Thevanmundi, Lane 15: Chumalakodi, Lane 16: Nedumchola, Lane 17: Malamundi, Lane 18: Karimkotta, Lane 19: Perumkodi, Lane 20: Karimunda, Lane 21: Kumbhachola , Lane 22: P 24 (IISR Sakthi), Lane 23: P 24 O4 (O4-P 24-1)

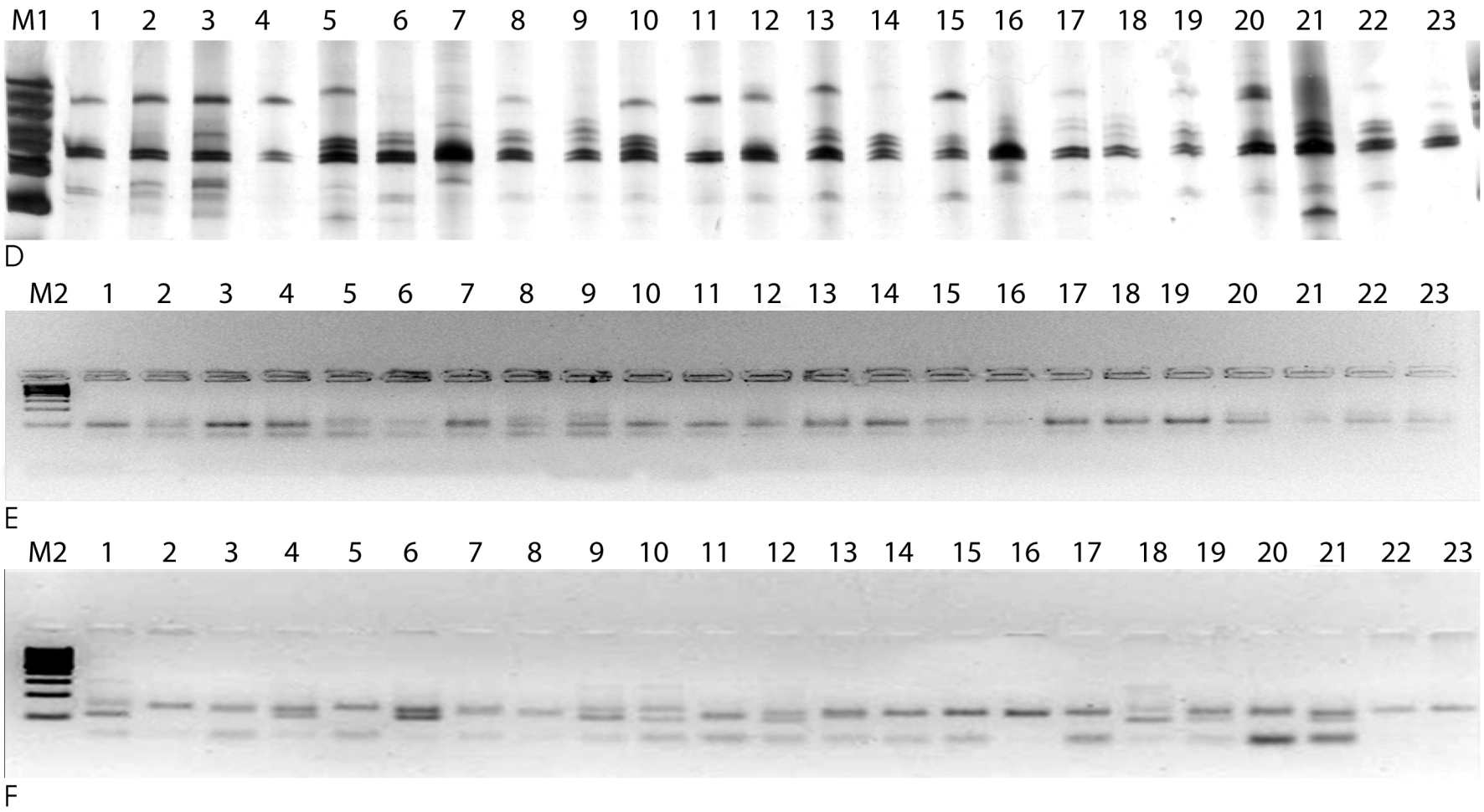


Fig. 40. Amplification profile of polymorphic SSR markers in 23 black pepper cultivars. D) PN D10 (15% PAGE gel) E) Pn GT2 F) PN F1 (run on 3% agarose gel). Lane M1: ULR, Lane M2; 100 bp DNA ladder. Lane 1: Kottanadan, Lane 2; Neelamundi, Lane 3: Kuthiravally, Lane 4: Kalluvally, Lane 5: Narayakodi, lane 6: Permbramunda, Lane 7: Poonjaranmunda, Lane 8: Valiakaniakkadan, Lane 9; Cheriyakaniakkadan, Lane 10: Uthirancotta, Lane 11: Balancotta, Lane 12: Arakkulam munda, Lane 13: Thommankodi, Lane 14: Thevanmundi, Lane 15: Chumalakodi, Lane 16: Nedumchola, Lane 17: Malamundi, Lane 18: Karimkotta, Lane 19: Perumkodi, Lane 20: Karimunda, Lane 21: Kumbhachola, Lane 22: P 24 (IISR Sakthi), Lane 23: P 24 O4 (O4-P 24-1)

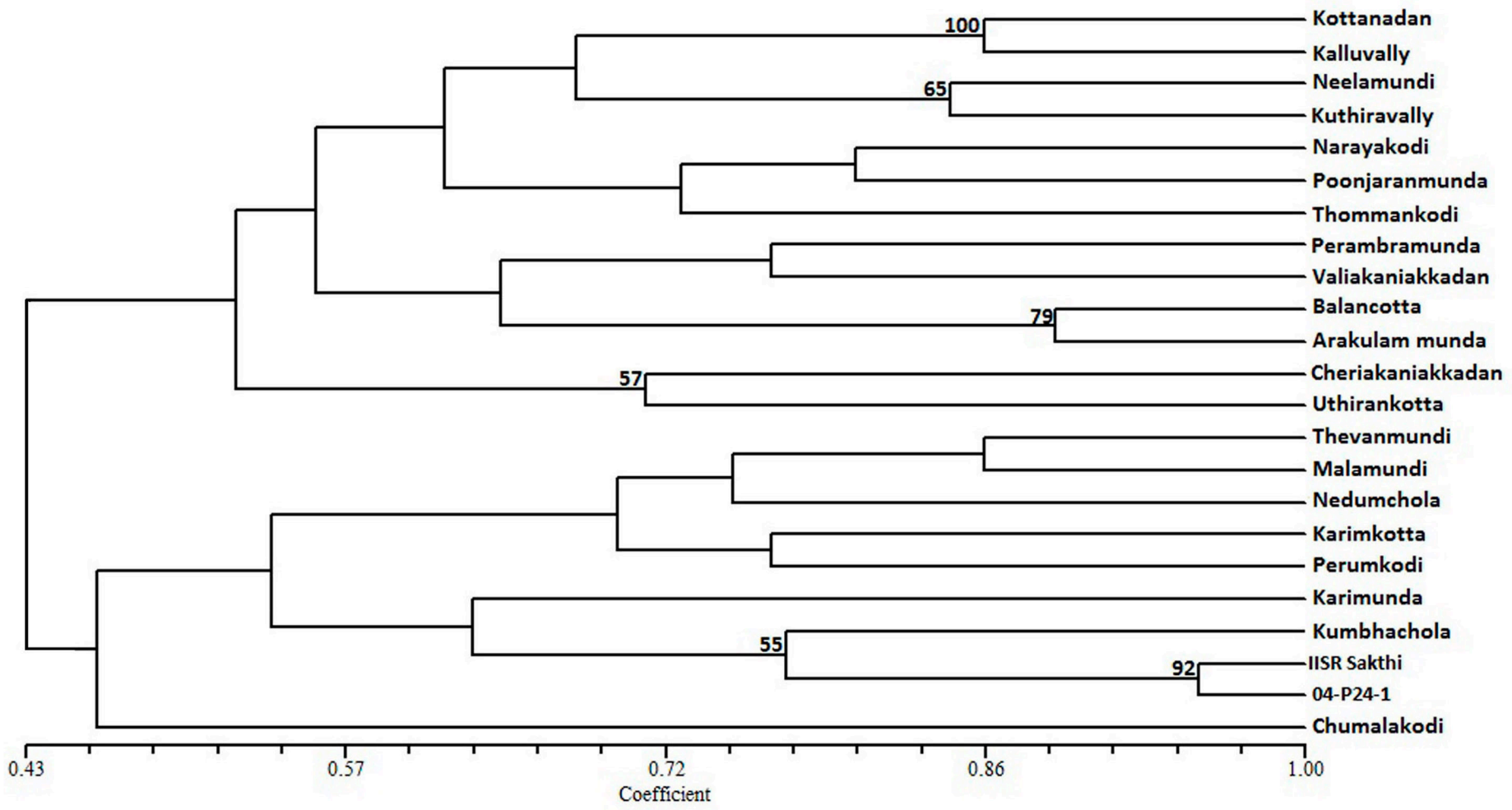


Fig. 41. UPGMA dendrogram showing genetic similarity among 23 cultivars of black pepper based on Dice similarity coefficient using 6 previously reported microsatellite markers. Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the dendrogram.

4.8.3. Utilization of previously reported microsatellite markers in *Piper* species

Six polymorphic microsatellite markers were used to study the cross species amplification profiling of 21 *Piper* species including the exotic genotypes (Fig. 43, 44). A total of 45 alleles were observed with six polymorphic microsatellite markers. The maximum number of alleles was noticed with the marker, PN D10 (11 of 45). The least number of alleles were observed with the microsatellites PnGT2 with 4 alleles. Variations are noticed in the PIC values ranged from 0.18 to 0.48. The maximum value of PIC was noticed with PN G11 (0.48) and minimum value by PN D10 (0.18) (Table 22.).

Table: 22. Locus name, repeat motif, sequence information, No. of alleles, PIC of six six previously reported microsatellite markers used for cross species amplification.

Locus	Repeat motif	Primer sequence Forward primer(5′-3′) Reverse primer (5′-3′)	No. of alleles	PIC
PN B5	$(TG)_{14}$	F 5′ GTTTTGAATGGGTCGGTGAT 3′ R 5′ ATTGTTCTGATTTCTTCGTTATTG 3′	5	0. 32
PN E3	$(CA)_{13}$	F 5′ TTTGTGTCCTCTCCCTCTCC 3′ R 5′ AAGACTAAATAGGCAAGGCAAA 3′	10	0. 20
PN G11	$(AC)_5$	F 5′ TTACTAGTGTCCACCCCCACT 3′ R 5′ TCGATGGAAAGTCACCCTCT 3′	6	0.48
PN D10	$(GT)_{13}$	F 5′ GTGTTACCTTTGGGGCATTCA 3′ R 5′ TGTGTCAGGGCATCAAACC 3′	11	0. 18
PnAG30	$(CT)_4TT\ (CT)_{16}$	F 5′ ACTAAGGCTAATGTGATAACCTGAGGA 3′ R 5′ ATCCCTGGATGGAAATTTGAAGGCTTGC 3′	9	0. 26
PnGT2	$(GT)_5AT\ (GT)_4$ AT $(GT)_{17}$ GG $(GT)_{19}$	F 5′ CTAGAGAGTAACAGTTATCACTTCACAG 3′ F 5′ CTAGCAAATTTGTTCTCTAATTCACATGT 3′	4	0. 28

4.8.4. Cluster analysis of *Piper* species with 6 polymorphic markers

The similarity coefficient of the phylogram ranged from 0.34 to 100%. Most of the *Piper* species were clearly separated with these markers except *P. argyrophyllum* and *P. hymenophyllum* which showed 100% similarity to each other (Fig. 45). The PCA generated from the similarity matrix is represented in Fig. 46.

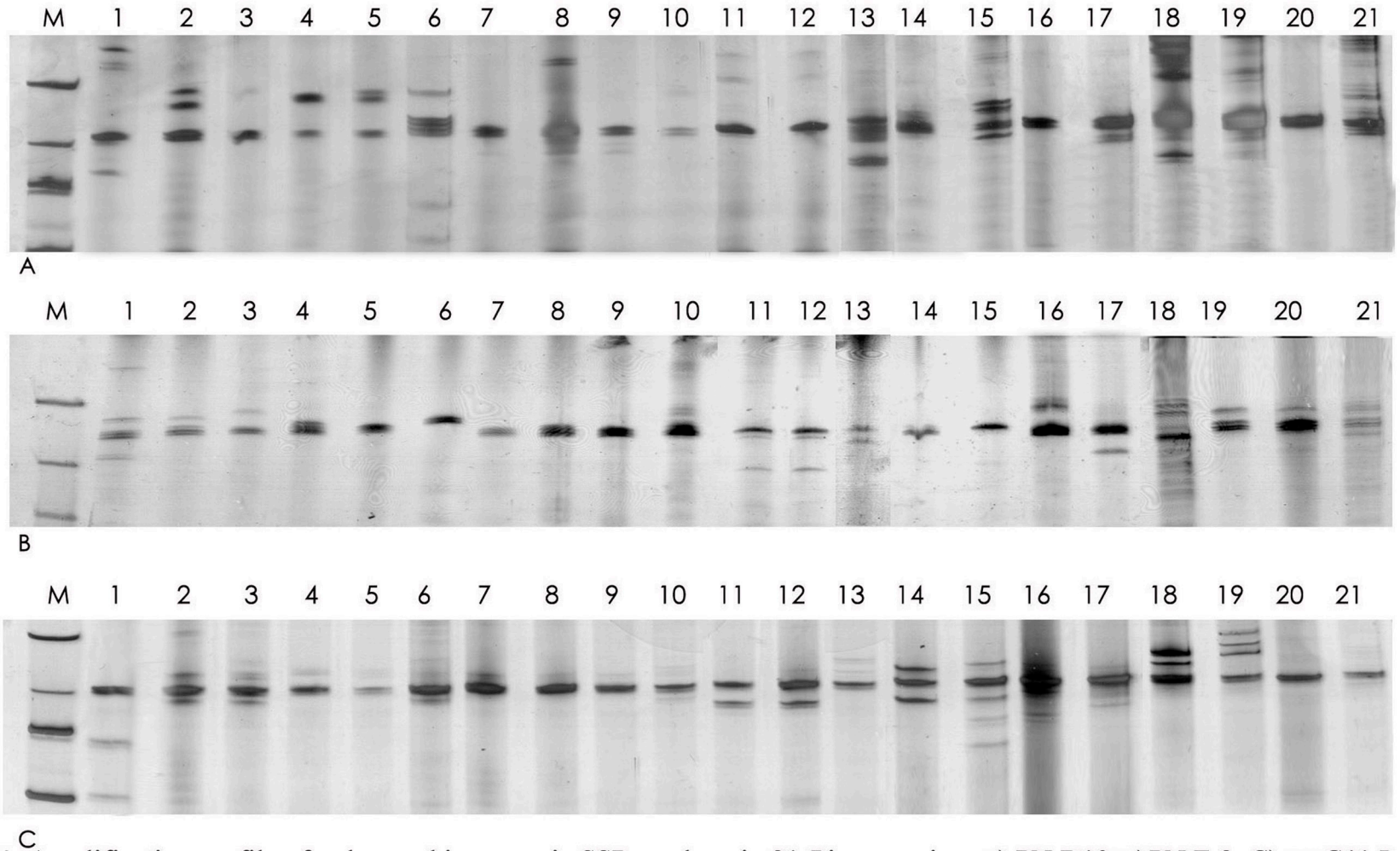


Fig. 43. Amplification profile of polymorphic genomic SSR markers in 21 *Piper* species. A) PN D10, B) PN E 3, C) PN G11 Lane M- ULR (10 bp DNA ladder), Lane 1: *P. betle*, Lane 2: *P. nigrum*, lane 3: *P. sugandhi*, Lane 4: *P. trichostachyon*, Lane 5: *P. galeatum*, Lane 6: *P. barberi* Lane 7: *P. attenuatum*, Lane 8: *P. argyrophyllum*, Lane 9: *P. hymenophyllum*, Lane 10: *P. bababudani*, Lane 11: *P. longum*, lane 12, *P. hapnium* Lane 13: *P. peepuloides*, Lane 14: *P. sarmentosum*, Lane 15: *P. ribesioides*, Lane 16: *P. thomsoni*, Lane 17: *P. hamiltonii* ,Lane 18: *P. colubrinum*, Lane 19: *P. arboreum*, Lane 20: *P. ornatum*, Lane 21: *P. magnificum*

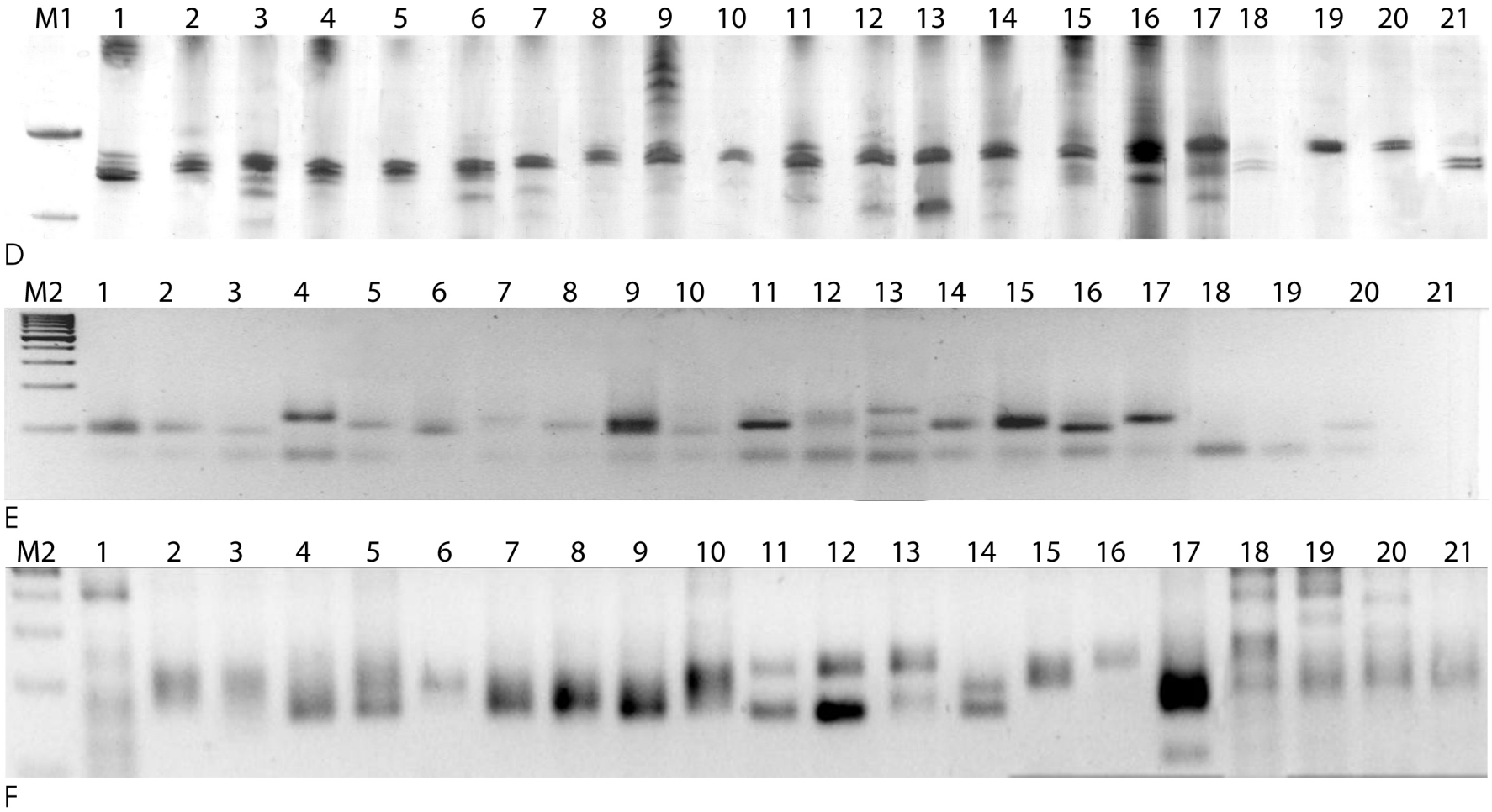


Fig. 44. Amplification profile of polymorphic SSR markers in 21 *Piper* species. D) PN B5 (15% PAGE gel) E) Pn GT2 F) Pn AG30 (run on 3% agarose gel). Lane M1: ULR, Lane M2; 100 bp DNA ladder. Lane 1: *P. betle*, Lane 2: *P. nigrum*, Lane 3: *P. sugandhi*, Lane 4: *P. trichostachyon*, Lane 5: *P. galeatum*, Lane 6: *P. barberi*, Lane 7: *P. attenuatum*, Lane 8: *P. argyrophyllum,* Lane 9*: P. hymenophyllum,* Lane 10: *P. bababudani,* Lane 11*: P. longum,* Lane 12*: P. hapnium,* Lane 13*: P. peepuloides,* Lane 14*: P. sarmentosum,* Lane 15*: P. ribesiodes,* Lane 16*: P. thomsoni,* Lane 17*: P. hamiltonii,* Lane 18: *P. colubrinum,* Lane 19*: P. arboreum,* Lane 20*: P. ornatum,* Lane 21*: P. magnificum*

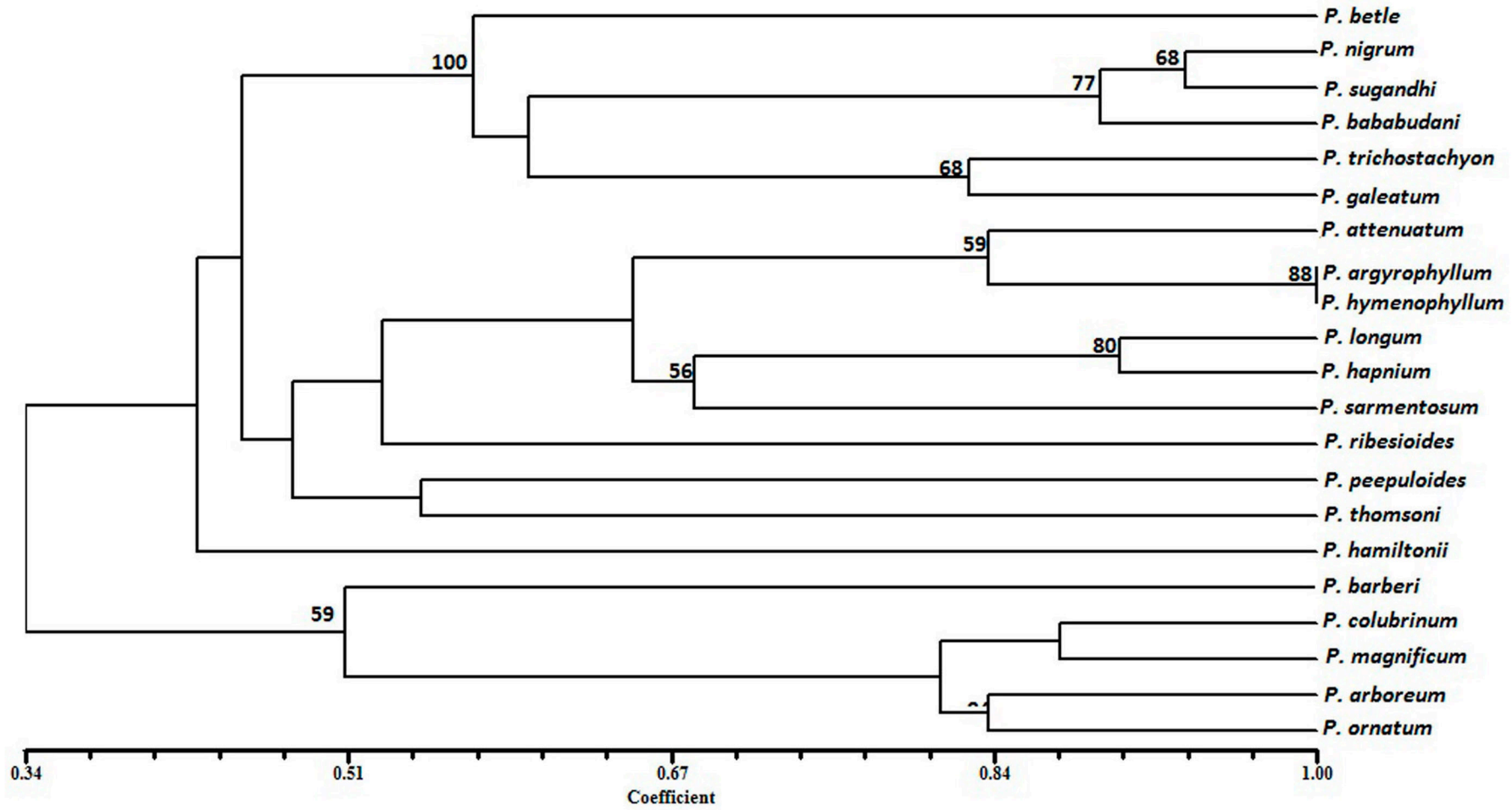


Fig. 45. UPGMA phylogram showing genetic similarity among 21 *Piper* species based on Dice similarity coefficient using 6 previously reported microsatellite markers. Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the phylogram.

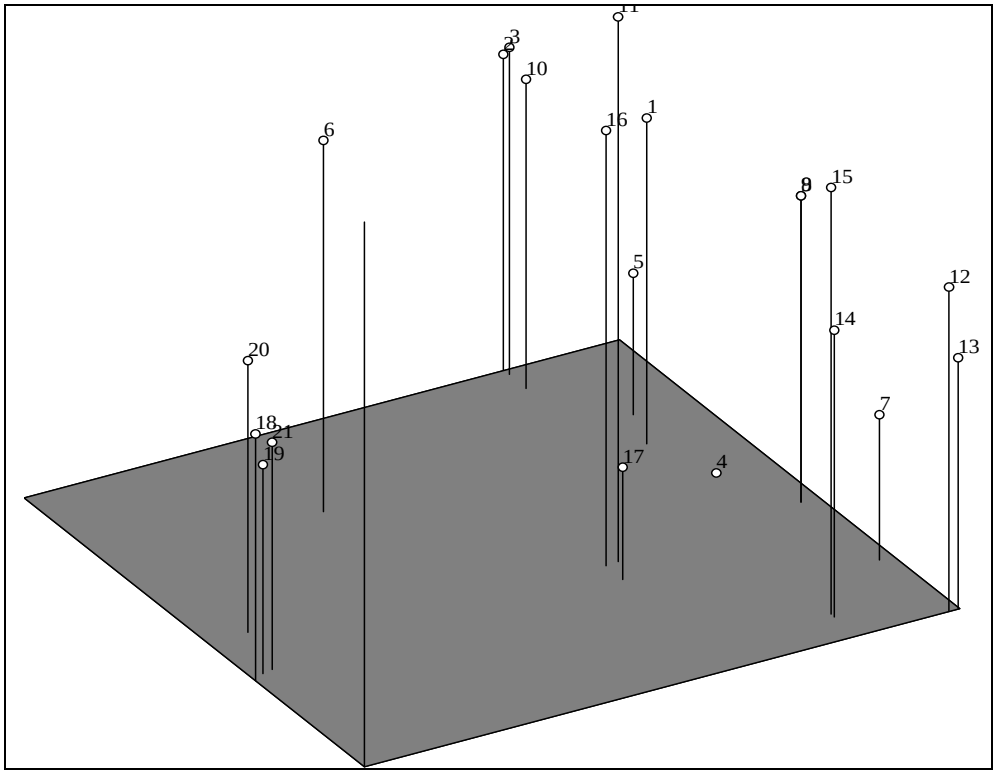


Fig. 46. The 3-D plot diagram showing the relationship among 21 *Piper* species based on principal coordinate analysis (PCA) using 6 previously reported microsatellite markers. The number represents the genotypes. (1. *P. betle*, 2. *P. nigrum***,** 3. *P. sugandhi***,** 4**.** *P. trichostachyon*, 5. *P. galeatum*, 6. *P. barberi*, 7. *P. attenuatum*, 8. *P. argyrophyllum*, 9. *P. hymenophyllum*, 10. *P. bababudani*, 11. *P. peepuloides*, 12. *P. longum*, 13. *P. hapnium*, 14. *P. sarmentosum*, 15. *P. ribesioides*, 16. *P. thomsoni*, 17. *P. hamiltonii*, 18. *P. colubrinum*, 19. *P. arboretum*, 20. *P. ornatum*, 21. *P. magnificum*).

4.9. Combined microsatellite markers (Genomic SSR + previously reported SSR markers): Genetic diversity analysis in black pepper cultivars

The 4 genomic and 6 previously reported microsatellite markers were combined to discriminate 23 black pepper cultivars. A total of 42 alleles generated were clearly separated 23 black pepper cultivars with a similarity co efficient ranged from 62 to 100%. (Fig. 42).

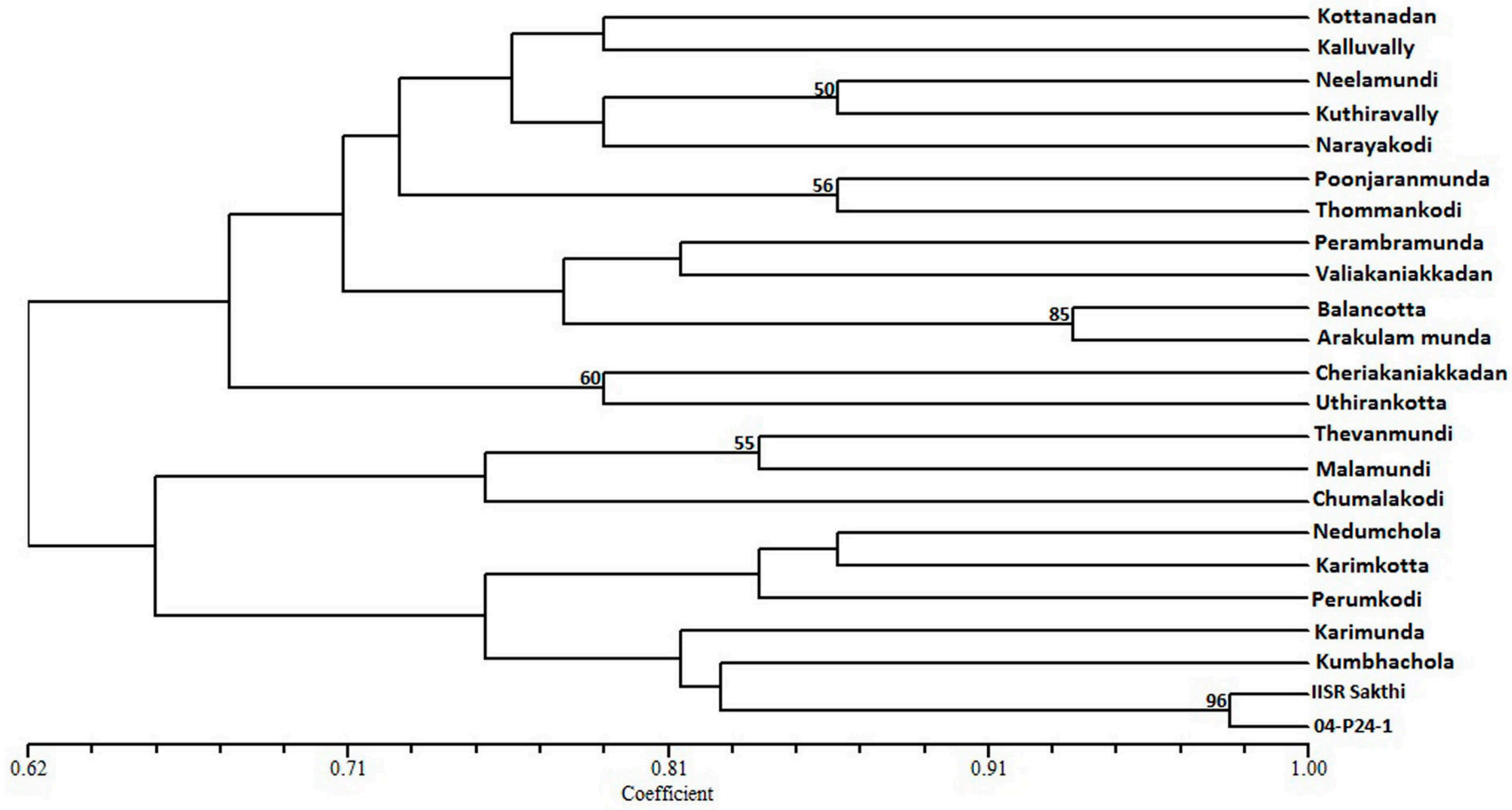


Fig. 42. UPGMA dendrogram showing genetic similarity among 23 black pepper cultivars based on Dice similarity coefficient using 10 microsatellite markers (Genomic SSR+available markers). Bootstrap values above 50% (based on 1000 permutations) are indicated in each node of the dendrogram.

4.10. Combined microsatellite markers (Genomic SSR + EST SSR + previously reported SSR markers): Genetic diversity analysis in *Piper* species

The matrix generated with genomic, EST-SSRs and previously reported markers were pooled together and used for constructing a phylogram. The similarity coefficient of the phylogram ranged from 0.48 to 1.00. The 124 alleles generated with 20 polymorphic microsatellite markers clearly separated all the 21 *Piper* species (Fig. 47). The PCA plot also matched with the phylogram exhibiting similar pattern of distribution of *Piper* (Fig. 48).

Cluster	***Piper* species**
I	*P. betle*
II	*P. nigrum* and *P. sugandhi*
III	*P. galeatum* and *P. trichostachyon*
IV	*P. bababudani*
V	*P. attenuatum P. hymenophyllum* and *P. argyrophyllum*
VI	*P. thomsoni* and *P. hamiltonii,*
VII	*P. longum, P. Sarmentosum* and *P. hapnium*
VIII	*P. peepuloides*
IX	*P. ribesioides*
X	*P. barberi*
XI	*P. arboreum, P. ornatum, P. magnificum* and *P. colubrinum*

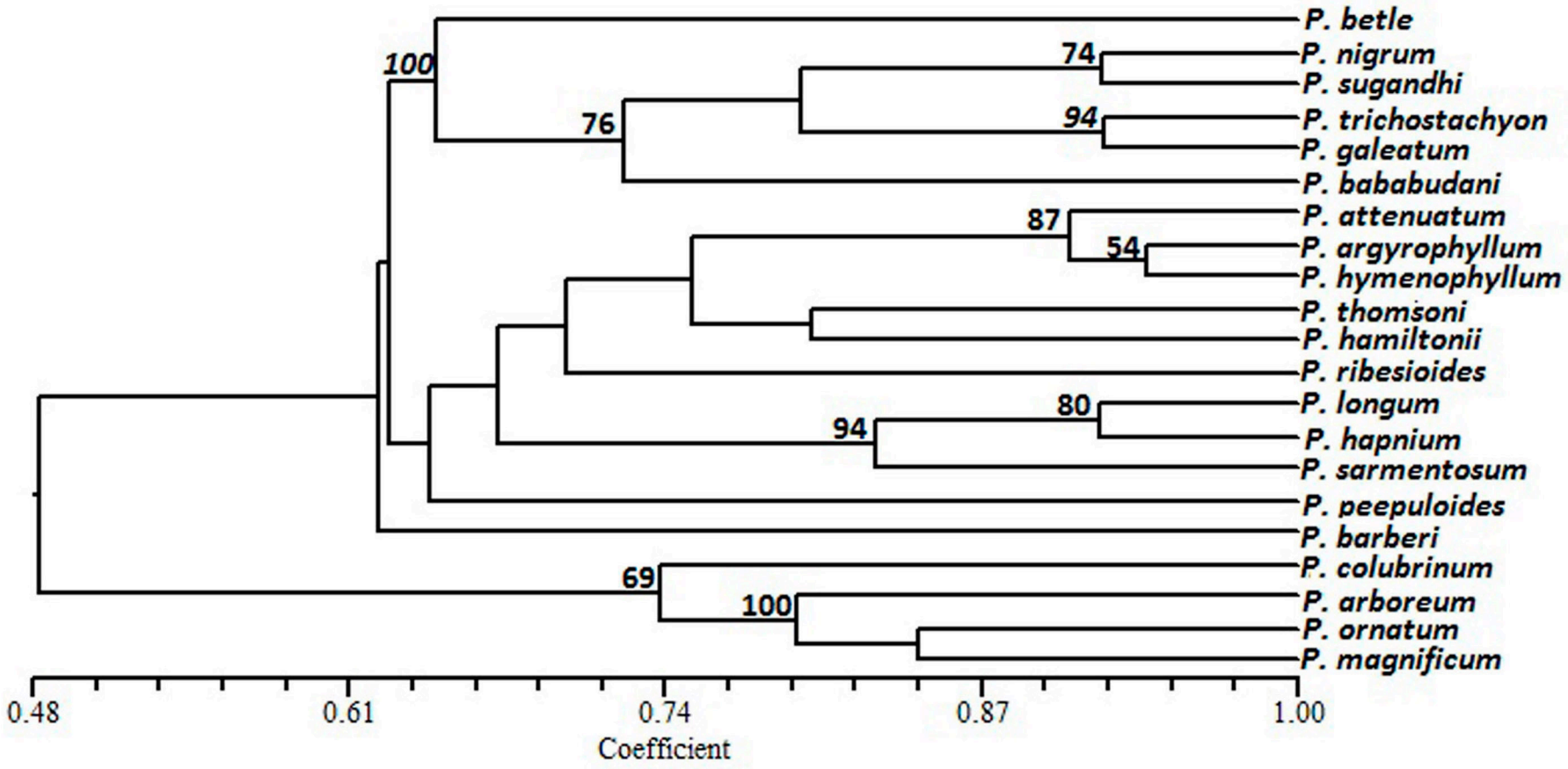


Fig. 47. UPGMA phylogram showing genetic similarity among 21 *Piper* species based on DICE similarity coefficient using 20 micro-satellite markers. Bootstrap values above 50% (based on 1000 permutaions) are represented in each node of the phylogram.

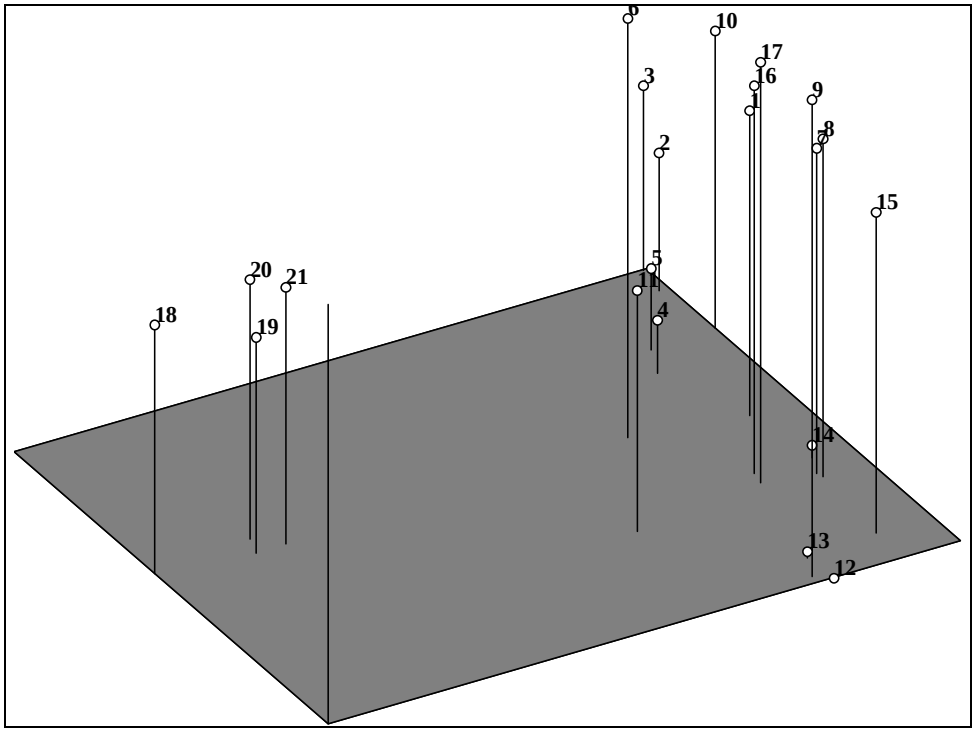


Fig. 48. The 3-D plot diagram showing the relationship among 21 *Piper* species based on principal coordinate analysis (PCA) using 20 (7 EST+ 7 genomic+ 6 previously reported) microsatellite markers. The number represents the genotypes. (1. *P. betle*, 2. *P. nigrum***,** 3. *P. sugandhi***,** 4**.** *P. trichostachyon*, 5. *P. galeatum*, 6. *P. barberi*, 7. *P. attenuatum*, 8. *P. argyrophyllum*, 9. *P. hymenophyllum*, 10. *P. bababudani*, 11. *P. peepuloides*, 12. *P. longum*, 13. *P. hapnium*, 14. *P. sarmentosum*, 15. *P. ribesioides*, 16. *P. thomsoni*, 17. *P. hamiltonii*, 18. *P. colubrinum*, 19. *P. arboretum*, 20. *P. ornatum*, 21. *P. magnificum*).

4.11. Multiple sequence alignment of different alleles from SSR loci generated with BP SSR 11 primer

The aligned sequence obtained from the three PCR products of the genotypes (Panniyur 2, Panniyur 3 and Pournami) amplified with BP SSR 11 showed the presence of microsatellite repeat variation. The multiple sequence alignment showed variation in the microsatellite repeat $(GA)_n$ in different alleles. The allelic variations were due to the length polymorphism of microsatellite repeats. The three genotypes showed microsatellite repeat variation for $(GA)_n$ (Panniyur 2- $(GA)_{20}$; Panniyur 3- $(GA)_{19}$; Pournami- $(GA)_{22}$) when compared with the original sequence $(GA)_{28}$. (Fig.49). The result obtained strengthened the reliability of the microsatellite primers developed.

4. 12. Aligned sequence of the PCR product of the microasatellite marker PC ESTSSR 22

The presence of the repeat region $(CGACAT)_2$ in the amplified product of microsatellite marker PC ESTSSR 22 was confirmed by aligning the sequence (obtained by sequencing the PCR product of Sp2 (*P. nigrum*) with the original sequence (SSR containing EST sequence of *P. colubrinum* retrieved from NCBI). The Fig. 50 represents the presence of microsatellite repeats in both original sequence and PCESTSSR Sp2.

Fig 49. Multiple sequence alignement of the PCR products of BP SSR 11 primer. Each sequence obtained showed the presence of GA microsatellite motif with variable number of repeat motifs with conserved flanking regions

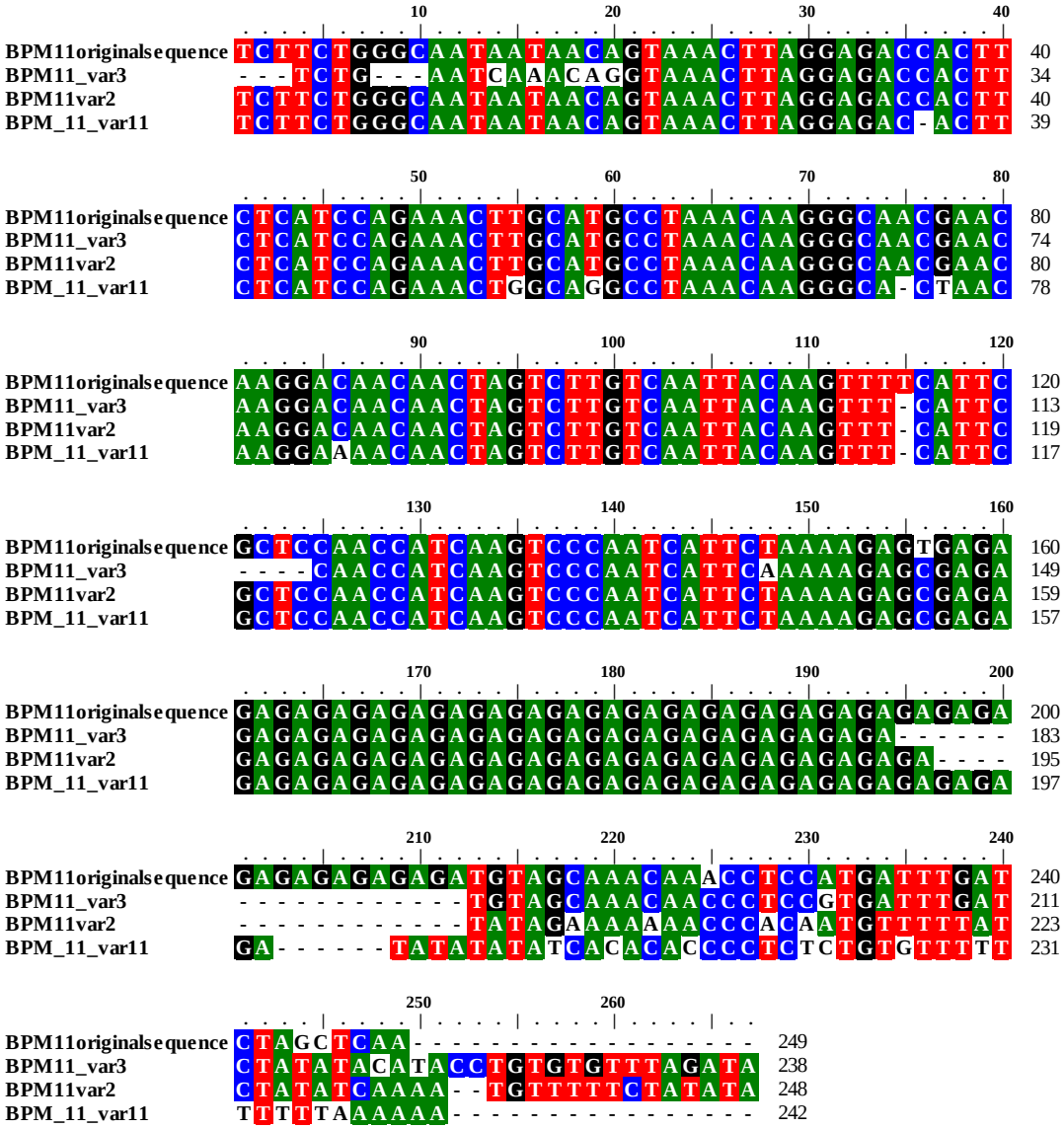
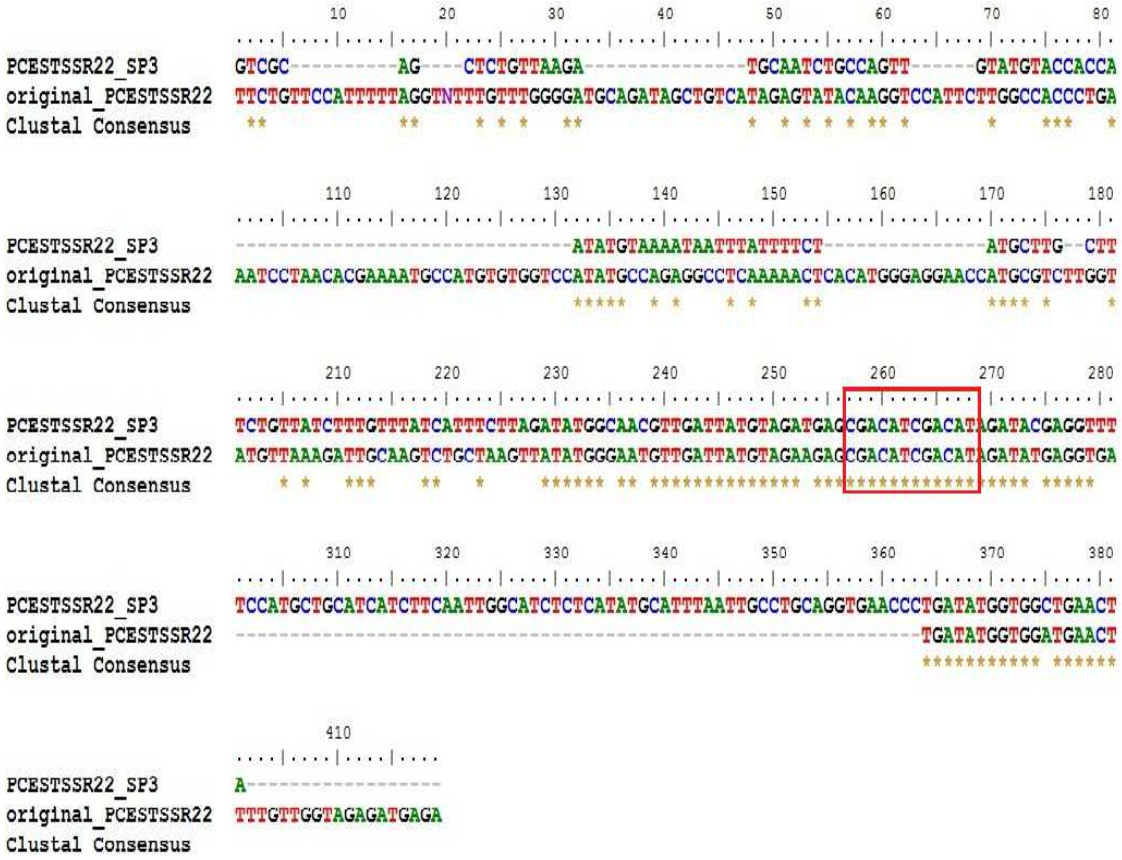


Fig 50. Aligned sequences of the PCR products of PCESTSSR 22 primer. The original sequence and the sequenced sample (PCESTSSR 22 Sp2) showed the presence of CGACAT di repeats.



DISCUSSION

Chapter 5

DISCUSSION

The genus *Piper*, the largest in the family Piperaceae, comprising over 3000 species of which about one hundred and nineteen are of Indian origin. Of these black pepper (*Piper nigrum* L.) is the most valuable economically important spice crop, which is consumed throughout the world. Apart from Asian countries such as Malaysia, India, Indonesia, Thailand, Vietnam, China and Sri Lanka, it is also cultivated in Brazil and Madagascar. The humid climatic conditions and the daily consumption in the diet made this spice crop a synonym of the Asian continent. The Western Ghats of Indian peninsula is the primary centre of origin of *Piper nigrum* L., the source of medicinally and commercially important black pepper (Ravindran *et al.*, 2000). 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 centre for the production of most of the black pepper in India.

Black pepper is ranked as the most important export oriented spice commodity. Improvement programmes on this crop have been predominantly based on selection from the available genetic variability in cultivated pepper plants. Currently the different centres are focusing on the primary gene pool for developing hybrid suitable for cultivar release.

Studies on genetic variability of any economically important plant has paramount importance as it help to make the best utilization of genetic potential of

genotypes for improvement of traits and for adaptations to various conditions. In addition, the genus *Piper* exhibits extreme reduction of floral characters which are the key factors for phylogenetic analysis. Hence molecular characterization of *Piper* species is needed for the development, utilization and conservation of the pepper resources. In genetic diversity studies, the most frequently used molecular markers are microsatellites or simple sequence repeats due to their high mutation rate, high reproducibility, codominant nature and high genome coverage. There have been few studies on genetic diversity of Indian black pepper using molecular markers (Pradeepkumar *et al.*, 2003; Joy *et al.*, 2007; Joy *et al*., 2011).

The present study was carried out with the objective of developing more number of microsatellite markers to study the genetic architecture of black pepper and related species that would enable future crop improvement programs in the genus *Piper*.

5.1. Exploiting EST databases for microsatellite detection

ESTs are sequenced portion of complementary DNA copies of mRNA and they represent part of the transcribed portion of the genome in given conditions (Poncet *et al*., 2006). Development and identification of microsatellite from ESTs can be achieved through electronic sorting that will reduce time and steps required for the genomic DNA library preparation and screening (Thiel *et al*., 2003; Varshney *et al*., 2005). During the mining of SSRs from EST databases, the retrieved sequences from the database were refined by trimming out the poly A/T tail followed by its clustering and conservation to unique sequences as reported by Siju *et al*. (2011) in *Curcuma longa*. Refining the original dataset reduces the size of the data and redundancy for frequency analysis (Kantety *et al*., 2002). The frequency of the presence of SSRs in

non-redundant ESTs accurately reflects the density of SSRs in the transcribed region of the genome (Varshney *et al*., 2005).

In the present study a total of 206 EST sequences were retrieved from genbank at NCBI website (http://ncbi.nlm.nih.gov) using the keyword "*Piper*". Out of the 206 sequences 87 EST sequences were from *Piper nigrum*, 50 from *P. colubrinum*, 63 from *P. longum* and 6 from *P. tuberculatum*. The sequences were assembled to 6 contigs, 68 singletons in *P. nigrum*; 7 contigs and 35 singletons in *P. colubrinum*; 4 contigs and 6 singletons in *P. longum* and 6 singletons in *P. tuberculatum* were obtained.

Since the availability of EST sequence in the genus *Piper* is very less (206 EST sequences), penta and hexanucleotide repeats of = 2 were also considered as a parameter for SSR detection. In all the studies it was observed that the parameters/limits used for SSR detection in frequency calculations were different. Again, "the estimation of distribution and frequency of EST SSRs are related to the search criteria and SSR mining tools, which is also observed for other crops (Varshney *et al*., 2005; Feng *et al*., 2009).

Including the penta and hexanucleotide repeats = 2, the present study identified pentanucleotide repeats followed by hexanucleotide comprising the major portion of the dataset from *Piper* EST sequences. All the penta and hexanucleotides repeats obtained in the present study were two time repeats only. Ince (2012) noticed abundance of hexanucleotide repeats even more than dinucleotide repeat motifs in *Chichorium* ESTs. Higher occurrence of hexanucleotide repeats may be due to the multiple repeats of the trinucleotides that are present in the coding region. The abundance of the two time pentanucleotide repeats may be due to its occurrence in the coding region of genome. Dinucleotide repeat motif form the most abundant repeat

motifs in genus *Piper* if penta and hexanucleotide two repeats are excluded from the analysis which is reported to be the major repeat motif in EST sequences of many crops (Rungis *et al*., 2004; Aggarwal *et al*., 2007). Trinucleotide and tetranucleotide repeats were of least (3.18% of 95) occurrence in all the four species of *Piper* studied.

The findings of earlier workers supports the current observation that the microsatellite repeat number present in ESTs are very less (Morgant *et al*., 2002; Li *et al*., 2004) and di nucleotide repeats are much less frequent in coding regions than in noncoding regions (Wang *et al.,* 1994; Li *et al*., 2004). When compared with the earlier reports on the frequency analysis of microsatellites from EST databases (Varshney *et al*., 2002; Thiel *et al*., 2003; Aggarval *et al.,* 2007) the low level of tri and tetranucleoide repeats observed in the present study could be due to the application of stringent limits for SSR availability and also identification in the quantity of sequence data analyzed. Comparing coding and non-coding regions in different plant species, it was observed that tri and tetranucleotide microsatellite motifs are more common within introns, whereas other types of motives are found within exons (Toth *et al*., 2000).

Microsatellite sequences are especially useful to differentiate closely related genotypes; due to their high degree of variability, they are, therefore, preferred in population studies (Smith and Devey 1994) and for the identification of closely related cultivars (Vosman *et al*., 1992).

5.2. Isolation and identification of microsatellites from enriched genomic DNA library

Microsatellite isolation from genomic DNA has been done earlier by screening of clones by colony hybridization with probes containing repeats (Rassman *et al*., 1991), but the method is not efficient for those crops which contain less

microsatellites. Hence this method was replaced by microsatellite enrichment protocols (Zane *et al*., 2002).

In the present study, seven microsatellite markers were developed for black pepper from genomic DNA library enriched for six microsatellite repeats [$(AG)_{12}$, $(TG)_{12}$, $(ACT)_{12}$, $(AAAC)_{6}$, $(ACCT)_{6}$, $(ACTG)_{6}$] following Glenn and Schable protocol (2005). This protocol is preferred for microsatellite isolation as it was modified to ensure efficient A-tailing of each PCR products to yields good results and it is a combination of best approaches from many other protocols to reduce the time and steps required.

Scanning of microsatellites in the non-redundant dataset revealed a total of 144 unique SSRs in the case of *P. nigrum*. Here also the two time repeats were set as parameters in case of hexa or penta nucleotides search from the dataset. From such analysis, pentanucleotides were the most abundant microsatellite repeats detected, followed by dinucleotide and hexanucleotide repeats. Dinucleotide repeats were the major portion if two time repeats were excluded from the dataset. Only a single type of mononucleotide repeat (A/T) was noticed. The other most abundant repeat motifs were (AC/GT) in dinucleotides, (AAG/CTT) in trinucleotides, (AAAC/GTTT) in tetranucleotides, (AATAT/ATATT) in pentanucleotides and (AAAAAG/CTTTTT) in the hexanucleotide. As in black pepper (AAAC/ GTTT) is reported to be the most abundant tetranucleotide repeats in *Doritis* (Jantasuriyarat *et al*., 2012).

Excluding mono, penta and hexa nucleotide repeats, the total number of repeats (Di- 26, Tri- 18, Tetra- 9) present in 70 sequences analyzed included 53 SSRs. The enrichment efficiency of these repeats in black pepper was found to be 77%. The enrichment efficiency of microsatellites in the present study was higher when compared to Menezes *et al*. (2009) where the enrichment efficiency was around 52%

in black pepper. A high percentage of enrichment efficiency of 84% was reported by Sigrist *et al*. (2010) in turmeric. But in the same crop 36% of enrichment efficiency was also reported by Siju *et al*. (2010). The enrichment rate was found to be in less range from 10-22% for many crops *viz*., in graminaceous species, namely sugarcane (Parida *et al*., 2009) wheat (Pestsova *et al*., 2000).

5.3. Isolation of EST SSR markers

This is the first report on the development of EST SSR markers from black pepper and related species. Seven polymorphic microsatellite markers were generated from a total of 23 primers tested (30%). Out of the 23 primer pairs synthesized and tested for amplification 17 primers yielded good amplification products (73%) within the expected size range. Except for 4 primers all other primers contained penta and hexanucleiotide of 2 repeats. It was also observed that the 7 polymorphic EST SSR markers were containing penta and hexa two repeats. The remaining 10 primers were found to be monomorphic. The lack of amplification of the remaining primers may be due to 1) the primer pair encompassing a long intron producing a PCR product that could not be visualized on the electrophoretic profile, 2) sequence errors or problems during primer synthesis, 3) usage of consensus sequences obtained from the compilation of several ESTs. (Nicot *et al*., 2004). Blast X resulted in the observation of one marker developed from *P. colubrinum* (PCEST 19) with a hit for osmotin isoform precursor (*Piper colubrinum*) with accession number ACH54108.1.

5.4. Isolation of Genomic SSR markers

During the development of genomic SSR markers, though 70 sequences were available containing microsatellite repeats. Both Class I (≥20bp size) and Class II (≥12bp ≤ 19 bp size) (Mun *et al.* 2006) motifs are discovered in genomic SSRs. Those

sequences that contained dinucleotide repeats were given priority for primer designing. Designing of primers failed for some sequences and this may be due to the lack of sufficient flanking sequences or repeat motifs located at the end of the sequences. Such failures in designing primers were also reported in many studies including sugarcane (Parida *et al*., 2009) garlic (Ma *et al*., 2009).

Among the 37 primers designed and synthesized, 28 primers generated amplicons with expected size (75%). The lack of amplification of the other primers may be due to divergence in the nucleotide sequence flanking the SSRs producing null alleles (Smulders *et al*., 1997). Out of the 28 primers that produced amplicons with expected size range, 13 primers were found to be polymorphic among the species tested. Two primers generated multiple bands and hence were excluded. Seven polymorphic markers were found to be 100% transferable (63%) among 21 *Piper* species. Out of the 28 primers which gave expected size ranged amplification products, 5 primers were found to be polymorphic among the black pepper genotypes. Success rate of more than 70% amplification for both genomic and EST SSRs have been reported in different crops (Varshney, 2005).

5.5. Characteristics of microsatellite markers in black pepper germplasm

During the validation of the markers, the scorable darker bands were considered to be alleles and the lighter bands produced may be stutters arising due to slippage of *Taq* DNA polymerase during PCR (Rao *et al*., 2000). The polymorphism information content (PIC) for each individual SSR allele was calculated following the formula described by Weir (1990) *i.e.* PIC= 1- $\sum P_i^2$, where P_i is the frequency of the i^{th} allele in the genotypes examined, which can be simplified to PIC= 2PiQi, where P_i

is the frequency of presence and Q_i is the frequency of absence of a particular band (Tehrani *et al.*, 2008).

5.5.1. EST SSR markers

EST SSR primers developed in *Piper* species could not reveal polymorphism among the black pepper genotypes. They were found to be polymorphic among *Piper* species only. This result is in agreement with the statement of other workers that EST SSR markers exhibit lower polymorphism and is less efficient in distinguishing closely related individuals when compared to that of genomic SSR markers (Cho *et al.*, 2000; Scott *et al.*, 2000; Chabane *et al.*, 2005). Greater DNA sequence conservation in transcribed region can lead to lower polymorphism in genic SSRs (EST SSRs) making them less efficient compared to genomic SSRs for fingerprinting or varietal identification studies (Varshney,

2005a). Moreover, the development of genic SSRs is restricted to those species for which there are sufficient sequence data available (Varshney, 2005).

5.5.2. Genomic SSR makers

In the present study 5 polymorphic microsatellite markers could detect a total of 26 alleles with an average of 3-10 allele per locus. The average number of alleles observed per loci was 5.2. However, Menezes *et al*. (2009) reported a total of 53 alleles with 9 markers in black pepper genotypes with number of alleles in the range 3-10 and the average allele detected per loci was found to be 5.8.

The sixteen released varieties were divided into five clusters with the aid of five genomic microsatellite markers. The similarity coefficient of various clusters ranged from 0.69 to 0.96. The I^{st} cluster consisted of two sub clusters; 1) Panniyur 1 and Panniyur 3 which are the hybrids of same parents and shared 84% similarity.

Though these two varieties are distinct in morphological characters they are the progenies of the same parents. The grouping of these two in one cluster may be due to their lineage. This is in agreement with the observation by Nazeem *et al*., 2005 who reported close similarity between Panniyur 1 and Panniyur 3 based on RAPD profiling. 2) Panniyur 4 and Panniyur 5 which exhibited 92% similarity. This grouping was also reported by Saji (2006) based on the morphological similarities. The second cluster consisted of Panniyur 2 and Panniyur 7 sharing 94% similarity along with Panniyur 6 which was linked to them at 86% similarity. The marker BPSSR 11 generated a different banding profile for Sreekara and Subhakara. But in general, both the varieties which are clonal selections from Karimunda showed 96% similarity to each other. Pradeepkumar *et al.* (2003) differentiated Sreekara and Subhakara with RAPD markers. Panchami, Pournami, PLD-2 and IISR Girimunda were included in the fourth cluster. IISR Malabar Excel stood as an outgroup forming the fifth cluster with 69% similarity with other clusters.

5.6. Genetic diversity analysis in black pepper cultivars using genomic SSR markers

The 23 cultivars of black pepper were characterized by four polymorphic genomic microsatellite markers. These cultivars were grouped into seven clusters with a similarity coeffecient ranged from 0.64 to 1.0. The four polymorphic markers discriminated all other cultivars except Perumkodi and Karimunda, IISR Sakthi from O4 P24- 1, Kuthiravally- Balancotta and Arakkulam munda - Chumalakodi. Perumkodi and Karimunda which were the part of second cluster showed 100% similarity to each other. Mathew *et al.* (2001) also reported similar results grouping Karimunda and Perumkodi in the same cluster based on 27 morphological characters. RAPD profiling of black pepper cultivars also grouped Karimunda and Perumkodi in

the same cluster with 50% similarity (Pradeepkumar *et al*., 2003). Most of the grouping was in agreement with the previous understanding with a few exceptions. Joy *et al.* (2011) discriminated majority of the black pepper cultivars with four de novo developed polymorphic microsatellite markers.

5.7. Polymorphism and cross species amplification (Transferability) of SSR markers

Because of the high polymorphism microsatellite markers are considered as the ideal choice of markers for phylogenetic studies. But the applications of these markers are limited because of the need for developing specific primers for individual plant species. The SSR primer pairs developed in one species could be used to detect the diversity in other species or genera. The potentiality to successful transfer of microsatellite markers across species is called cross species amplification or transferability. This offer an alternative to *de novo* development of SSRs in plants (Peakall *et al*., 1998).When the evolutionary distance between species increases the transferability success rate decreases (Steinkellner *et al*., 1997; Barbará *et al*., 2007) *i e*., the transfer rate is in agreement with the phylogenetic distance and sequence conservation among the species (Kalia *et al*., 2011).

5.8. EST SSR markers

Seven microsatellite markers developed in *P. nigrum* and *P. colubrinum* were used to analyze the cross transferability and genetic variability among twenty one *Piper* species. A total of 36 alleles were generated with seven EST derived microsatellite markers. The maximum number of alleles was noticed with the marker, EST-6 (8 of 36) and the least number of alleles were observed with the microsatellite EST-4 and EST-22 with two alleles each. The maximum value of PIC was noticed

with EST-4 (0.36) and minimum value by EST-22 (0.09). Guo *et al*. (2006) studied the cross species transferability of *Gossypium arboreum* derived EST-SSR in the diploid species of *Gossypium* and reported high level of transferability among the species. But on the contrary, Pashley *et al.* (2006) reported that EST derived microsatellites may be conserved over larger evolutionary distances and their transfer beyond genus level often appears to be limited.

5.8.1. Cluster analysis of 21 *Piper* species based on EST SSR data

Seven microsatellite markers derived from the EST database could resolve 21 *Piper* species into nine clusters. The similarity coefficient value ranged from 0.64 to 1.00. *P. betle* alone formed the first cluster. The togetherness of *P. nigrum*, *P. sugandhi*, *P. trichostachyon* and *P. galeatum* in a major cluster is in accordance with the findings of earlier workers (Ravindran, 2000; Babu *et al*., 2003; Saji 2006 and Sheeja *et al*., 2013). All the species in this cluster are unisexual climbers with pendent filiform spikes. *P. sugandhi* shows close resemblance with *P. nigrum*, but differs for the slightly stalked flowers (Ravindran, 2000). Hence these two species are placed in one cluster with 98% similarity. *P. trichostachyon* and *P. galeatum* placed in sub clusters sharing 98% similarity. These two though similar to *P. nigrum* other characters like flower and leaf characters differ from it (Ravindran *et al*., 2000). These two species are very much similar in several morphological characters expect the pubescent spike in *P. trichostachyon*. This is the main discriminatory character for separating these two species. Though *P. bababudani* is placed as a 3rd cluster it shares many morphological characters with these 4 species, hence linked to the 2nd cluster. *P. bababudani* is similar to *P. sugandhi* in general morphology but differs with the presence of fleshy peduncle.

P. barberi was alone placed as a 3rd cluster in between 1st cluster which includes *P. nigrum, P. sugandhi, P. trichstachyon, P. galeatum* and *P. bababudanui* and 4th cluster which included *P. argyrophyllum, P. attenuatum, P. hymenophyllum*and *P. barberi* is a unique South Indian species with reticulate venation (Ravindran *et al*., 2000). This species shares some of the characters with species in both the clusters. *P. barberi, P. attenuatum, P. argyrophyllum* and *P. hymenophyllum* have similar spike characters *viz*., spike orientation (pendulous), spike texture (glabrous) and bract type (peltate) except for *P. argyrophyllum* which has adnate bract.

P. attenuatum and *P. hymenophyllum* which shared 95% similarity along with *P. argyrophyllum* formed the fifth cluster. The three species shares common characters. *P. argyrophyllum* is morphologically similar to *P. attenuatum* but differs in leaf venation. *P. argyrophyllum* is 5 nerved and *P. attenuatum* is 7 nerved. *P. hymenophyllum* is also very much similar to *P. attenuatum* except hirsute nature on the stem and leaves (Ravindran *et al*., 2000; Saji, 2006).

The *Piper* species from North- East region *viz, P. peepuloides, P. ribesioides, P. thomsoni* and *P. hamiltonii* were grouped together in the sixth cluster.

P. longum and *P. hapnium* which showed 100% similarity with each other along with *P. sarmentosum* comprised the seventh cluster. Both the species possess erect and cylindrical spikes (Ravindran, 1996). *P. sarmentosum* was also placed in this cluster which showed morphological similarities with *P. longum* (Mathew *et al*., 2004). In fruit characters *P. sarmentosum* shows resemblances to *P. hapnium* (Mathew *et al*., 2004). Sheeja *et al*. (2013) also reported that *P. longum* and *P. sarmentosum* shares many morphological and reproductive characters and the molecular profiling

data with ISSR markers placed *P. sarmentosum* along with *P. longum* and *P. hapnium*. EST SSR markers could not differentiate *P. longum* and *P. hapnium.* These two are the only species having fused fruits. Both morphological and molecular studies clearly exhibited morphological and genetic similarities of *P. longum* and *P. hapnium* (Saji, 2006). The exotic species were assembled together in the eighth cluster but *P. colubrinum* stood apart from rest of the exotic species.

The 7 EST SSR markers developed in the present study separated all the *Piper* species except *P. longum* and *P. hapnium* which exhibited 100% similarity.

5.9. Genomic SSR markers

Seven genomic microsatellite markers were developed to analyze the cross species amplification profiling of twenty one *Piper* species including the exotic species. A total of 43 alleles were generated with seven genomic microsatellite markers. Average number of allele per loci is 6.14. The maximum number of alleles was noticed with the marker, BPM 52 (10 of 42). The least number of alleles were observed with the SSR marker BP SSR 12 and BPM CNTG 5 with only 4 alleles. The maximum value of PIC was noticed with BP SSR 12 (0.40) and minimum value by BPM CNTG 5 (0.09).

Saha *et al.* (2006) developed genomic SSRs from tall fescue and confirmed their transferability across multiple grass species and he also noticed that the polymorphism rate is higher in fescue than the other species studied.

5.9.1. Cluster analysis based on genomic SSR markers

Nine clusters were attained with the UPGMA dendrogram created with genomic SSR markers. *P. betle* was associated with *P. attenuatum, P. hymenophyllum*

and *P. argyrophyllum* with 88% similarity that formed the first cluster. *P. barberi* and *P. bababudani* were distinct as noticed by Saji (2006). Two species of *Piper* were grouped together into the second cluster, *P. thomsoni* and *P. hamiltonii* shared 97% of similarity between them.

P. attenuatum, P. hymenophyllum and *P. argyrophyllum* formed the third cluster. Of this, *P. attenuatum and P. hymenophyllum* were more similar with a coefficient of 100% similarity. *P. argyrophyllum* showed 97% similarity and have more affinity towards *P. hymenophyllum* and *P. attenuatum.* But according to Saji (2006), *P. attenuatum and P. argyrophyllum* are more similar than *P. hymenophyllum.* The fourth cluster was occupied by four *Piper* species and is *P. nigrum, P. galeatum, P. trichostachyon* and *P. sugandhi.* Among these, *P. nigrum* and *P. galeatum* showed more similar with a 95% coefficient of similarity. Babu *et al*., (1993) indicated the possible origin of *P. sugandhi* as a hybrid involving *P. nigrum* as one of the parents and *P. trichostachyon* or *P. galeatum* as the other parent.

P. peepuloides, was the sole member of the fifth cluster and showed 83% of similarity. Cluster VI consists of *P. longum, P. sarmentosum* and *P. hapnium.* Of this, *P. longum* and *P. sarmentosum* showed 95% of similarity with each other. *P. hapnium* showed 85% similarity with the group members. According to Ravindran *et al.* (1996), *P. hapnium* was closely related to *P. longum* but differ only in the climbing habit of the former. *P. bababudani* and *P. ribesioides* were placed individually in cluster VII and VIII respectively. This is in agreement with Saji (2006) who placed *P. bababudani* separately from the rest of the species. Both the species have a similarity coefficient below 75%. *P. colubrinum* and *P. ornatum* formed the first sub group 95% of similarity. *P. arboretum* and *P. magnificum* were more similar than the other group of the cluster and the coefficient of similarity is 97%.

The 7 genomic SSR markers differentiated all the 21 *Piper* species except *P. attenuatum* and *P. hymenophyllum* were more similar with a coefficient of 100% similarity, which share similar morphological characters (Saji, 2006).

Nine clusters were attained with 124 alleles generated with 20 microsatellite markers in which the similarity coefficient ranged from 0.61 to 1.00. *P. betle* was the only species present in the first cluster. *P. attenuatum, P. hymenophyllum* and *P. argyrophyllum* are with 98% similarity and formed the second cluster. Among the second cluster, *P. attenuatum* and *P. hymenophyllum* exhibited 100% similarity of coefficient. But according to Saji (2006), *P. attenuatum and P. argyrophyllum* are more similar than *P. hymenophyllum.* The third cluster was occupied by four *Piper* species and is *P. nigrum, P. galeatum, P. trichostachyon* and *P. sugandhi.* Among these, *P. nigrum* and *P. galeatum* showed more similarity with a 95% coefficient of similarity. Babu *et al*., (1993) indicated the possible origin of *P. sugandhi* as a hybrid involving *P. nigrum* as one of the parents and *P. trichostachyon* or *P. galeatum* as the other parent. Cluster IV consists of *P. longum* and *P. hapnium* and their similarity of coefficient was 93%. According to Ravindran *et al.* (1996), *P. hapnium* was closely related to *P. longum* but differ only in the climbing habit of the former.

P. barberi is placed alone in fifth cluster. *P. bababudani* and *P. peepuloides* are also placed individually in cluster VI and VII respectively. *P. sarmentosum, P. ribesioides, P. thomsoni* and *P. hamiltonii* together formed the eighth cluster. Of these, *P. thomsoni* and *P. hamiltonii* showed 97% similarity. All the exotic species *viz., P. arboretum, P. ornatum, P. magnificum* and *P. colubrinum* are occupied in the cluster IX with two sub groups. *P. colubrinum* and *P. ornatum* formed the first subgroup with 92% of similarity.The pattern of distribution of the 21 *Piper* species with principal coordinate 3-D plot matched with the dendrogram.

5.9.2. Cluster analysis based on combined SSR markers (Genomic-SSR + EST-SSR)

The 14 polymorphic microsatellite markers (7 EST SSR+ 7 genomic SSRs) discriminated the entire 21 *Piper* species with a similarity co efficient ranged from 0.70 to 1.0.

5.10. Unique bands observed with microsatellite markers

Unique bands were obtained for some of the *Piper* species with the aid of both genome and EST derived microsatellite markers. Unique bands were observed for *P. betle, P. barberi, P. colubrinum, P. arboretum, P. ornatum, P. magnificum, P. ribesioides,* and *P. hamiltonii*. Sen *et al*. (2010) and Sheeja *et al* (2013) also identified species specific bands obtained with RAPD and ISSR markers.

5.11. Genetic diversity analysis using previously reported microsatellite markers

5.11.1. Black pepper cultivars

From the previously reported 16 SSR primers developed in black pepper (Menezes *et al.,* 2009 and Joy *et al*., 2011) six microsatellite markers gave polymorphism among 23 black pepper cultivars.

Kottanadan and Kalluvally showed 87% of similarity with each other and occupied the first sub group in Cluster I Ravindran (1991) and Ravindran *et al.* (1997 a, b) reported cluster analysis of 44 major cultivars and grouped Kottanadan and Kalluvally together in a group as in the present study. Neelamundi and Kuthiravally are 84% similar to each other. This grouping matched with available report by Mathew *et al.* (2001). The coefficient of similarity between Narayakodi and Poonjaranmunda was 78% and Thommankodi was linked to them with 71%

similarity. Perambramunda and Valiakaniakkadan showed 75% similarity to each other. Ravindran (1997) has carried out a comparative study on pepper cultivars based on flavonoid profiles and clustered Narayakodi, Thommankodi and Perambramunda in the same cluster as reported in the present study.

Thevanmundi, Malamundi and Nedumchola accommodated in the second cluster and the first two cultivars were more similar and exhibited a coefficient of similarity of 85%. Karimkotta and Perumkodi together formed a subgroup with 75% affinity to each other and are similar to the findings of Ravindran *et al.* (1997). Chumalakodi was the only distinct member of the cluster. IISR Sakthi could be separated from its open pollinated progeny 04-P24-1 with primer PNE3 which was the most significant achievement. The dendrograms based on UPGMA supported good variability in many of the cultivars studied and these cultivars can be utilised in the convergent crosses for maximum hybrid vigour in black pepper (Ibrahim *et al*., 1985b).

The dendrogram generated with the data are in agreement with the findings of earlier workers to an extent (Ravindran *et al*., 2000; Saji, 2006).

5.11.2. *Piper* species

Six polymorphic microsatellite markers were used to study the cross species amplification profiling of twenty one *Piper* species including the exotic genotypes.

A total of 45 alleles were observed at the loci of the six polymorphic microsatellite markers. The maximum number of alleles were noticed with the marker, PND10 (11 of 45). The least number of alleles were observed with the

microsatellites PnGT2 and PnG11 each with 4 alleles each. The maximum value of PIC was noticed with PNG11 (0.48) and minimum value by PND10 (0.18).

The similarity coefficient of the phylogram ranged from 0.34 to 100%. Most of the *Piper* species were clearly separated with these markers except *P. argyrophyllum* and *P. hymenophyllum* which showed 100% similarity to each other, which is in agreement with the finding of Saji (2006).

5.12. Combined cluster analysis of genomic SSR pooled with EST SSRs and other available markers.

The 124 alleles generated with 20 polymorphic microsatellite markers clearly separated all the 21 *Piper* species with a similarity coefficient ranged from 0.43 to 0.96.

Though the SSR markers could not separate all the *Piper* species individually, these markers coupled with other microsatellite markers formed sufficient resources together to understand the genetic architecture of black pepper and *Pipe*r germplasm.

SUMMARYAND CONCLUSION

Chapter 6

SUMMARY & CONCLUSION

Black pepper, the supreme among the spices is the most widely used spice in the world. The black pepper of commerce is the dried mature fruits of the tropical perennial climbing vine *Piper nigrum* L. In genus *Piper*, the role of phylogeny is particularly significant, as major break throughs in varietal evolution have been achieved through the conventional breeding. However, due to reduced floral structures, morphological characterization is only partially effective in estimations of diversity in *Piper*. Molecular markers being independent of environment conditions have come up as an effective tool for characterization of genetic material. DNA markers can augment phenotypic evaluation in estimation of the genetic variability between species more efficiently and they can be particularly useful in resolving complex phylogenetic problems. Among the DNA markers, microsatellites are the most frequently used molecular markers in genetic diversity analysis due to its multiallelic, codominant and high genome coverage.

In this study three approaches were used for analysing inter relationships among *Piper* species, varieties and cultivars. They include SSR developed from genomic libraries enriched for SSRs (genomic SSRs), from the EST sequences deposited in the public domain (EST-SSRs) and SSRs reported earlier. Thirty nine black pepper genotypes including 23 cultivars and 16 released varieties from diverse geographical locations of Kerala and 21 *Piper* species including Western Ghats, North East and Exotic species maintained at IISR Experimental Farm, Peruvannamuzhi

were used for genetic diversity analysis and cross-species amplification studies, respectively.

Use of computational approaches to mine sequences such as expressed sequence tags (ESTs) in public databases permits rapid and economical discovery of SSRs. Out of the 206 sequences retrieved from genbank at NCBI website, 87 EST sequences were from *Piper nigrum*, 50 from *P. colubrinum*, 63 from *P. longum* and 6 from *P. tuberculatum*. After redundancy elimination a total of 66 microsatellite repeats were detected and the most abundant microsatellite repeat motif detected in *P. nigrum* was pentanucleotides (51.52%) followed by hexanucleotides (37.88%) when the parameters set as penta and hexanucleiotide repeats $\geq$ 2. A total of 15 microsatellite SSRs were detected from the non redundant database of *P. longum*. Pentanucleotide repeats motif was the most abundant (46.67%) among the microsatellites followed by hexanucleotide motif (26.67%). Only one microsatellite repeat was detected from the dataset of *P. tuberculatum* and the motif identified was a pentanucleotide (AAGCG/CGCTT).

Out of the 23 primer pairs synthesized and tested for amplification 17 primers yielded good amplification products (73%) and seven primers were found to be polymorphic markers. All the EST SSR primers produced monomorphic bands on PAGE when tested on 39 black pepper genotypes. Since the DNA sequence conservation is greater in coding region can lead to lower polymorphism in genic SSRs (EST SSRs) making them less efficient for fingerprinting closely related genotypes and also the nucleotide substitution rate is more in non coding region than coding region, a small insert genomic DNA library was constructed in black pepper.

Good quality genomic DNA was isolated from the fresh leaves of black pepper genotypes and *Piper* species. The selective hybridization protocol was used for the construction of microsatellite enriched genomic DNA libraries. Black pepper variety Panniyur 1 was used for library construction. Among the four restriction enzymes tested for digesting genomic DNA, *Rsa* 1 and *Alu* 1 were selected based on the optimum size of the restricted DNA fragment (<2Kb) and compatibility with the linkers used in the protocol. Suitable linkers (Super SNX) were ligated to the restricted fragments and the successful ligation was confirmed in a PCR using linker ligated DNA as template. The amplified fragments were used for enrichment with the aid of six types of 3' biotinylated oligos- [$(AG)_{12}$, $(TG)_{12}$, $(ACT)_{12}$, $(AAAC)_{6}$, $(ACCT)_{6}$, $(ACTG)_{6}$] and strepavidin coated magnetic beads. These enriched DNA fragments were amplified, cloned into pCR® 2.1 TOPO vector and transformed into competent cells. One eighty five transformants were subjected to colony PCR using M 13 forward and reverse primers (vector specific primers) and the insert size was confirmed by restriction digestion with *Eco* RI. One hundred and six clones having insert size of 300 bp to 1 Kb were identified and selected through colony PCR.

A total of 106 plasmid DNA were sequenced. Scanning the Microsatellites in the non-redundant dataset revealed a total of 144 unique SSRs in the case of *P. nigrum*. Among the microsatellite repeats detected, pentanucleotides were the most abundant followed by dinucleotide and hexanucleotide repeats when the parameters were set as penta and hexanucleiotide repeats ≥ 2. Out of the 37 primers designed, 28 primers generated amplicons of which 13 primers were identified as polymorphic markers. Seven SSR primers that have produced 100% transferability were used for genetic diversity analysis in *Piper* species. Five markers generated polymorphism among 39 black pepper genotypes.

The five microsatellite markers (CNTG 15, BP SSR 11, BPM CNTG 5, BPM 154 and BPM 156 B) developed from black pepper genomic library produced a total of 26 alleles. The average number of alleles per loci was found to be 5.2. The BP SSR 11 exhibited maximum number of alleles (10 of 26) followed by BPM CNTG 15 (5 of 26) and the least number of alleles was noticed in BPM 156 B (3 of 26). The maximum value of PIC was noticed with BPM CNTG 5 (0.30) and minimum value with BPM 154 (0.21).

The five SSR markers placed the 16 released varieties of black pepper to five clusters. The similarity coefficient of various clusters ranged from 0.69 to 0.96. Panniyur 1 and Panniyur 3 were clustered together though they exhibit different morphological characters shared 84% similarity. Panniyur 4 and Panniyur 5 exhibited 92% similarity. These four varieties formed the first cluster. The second cluster consisted of Panniyur 2 and Panniyur 7 sharing 94% similarity along with Panniyur 6 which was linked to them at 86% similarity. Sreekara and Subhakara which are clonal selections from Karimunda could separated from each other with BP SSR 11 marker. IISR Sakthi and IISR Thevam which were 92% similar formed third cluster. Panchami, Pournami, PLD-2 and IISR Girimunda were included in the fourth cluster. IISR Malabar Excel stood distinct forming the fifth cluster with 69% similarity with other clusters.

The four genomic microsatellite markers (BP SSR 11, BPM 31, BPM 154 and BPM 156 B) produced a total of 17 alleles with the average number of alleles per loci 4.25. The four polymorphic markers showed a considerable level of polymorphism among the 23 black pepper cultivars.The maximum number of alleles was noticed with the markers, BP SSR 11 and BPM 154 (5 of 17) followed by BPM 31 and the least number of alleles was noticed in BPM 156 B (3 of 17). The maximum PIC value

was noticed with BPM 31 (0.39) and minimum value with BPM 156 (0.23). The genomic microsatellite markers discriminated many of the cultivars. However, they failed to differentiate IISR Sakthi from its open pollinated progeny P24 O4 (O4 P24-1). Similarly Kuthiravally, Balancotta and Arakkulammunda could not be separated.

The EST SSR primers produced monomorphic bands on PAGE when tested on 39 black pepper genotypes. But 5 genomic SSR markers found to be polymorphic among black pepper genotypes were subjected for genetic diversity analysis. The five polymorphic markers (CNTG 15, BP SSR 11, BPM CNTG 5, BPM 154 and BPM 156 B) generated a total of 26 alleles and the average number of alleles per loci was found to be 5.2. The maximum number of alleles was noticed with the markers, BPM 11 (10 of 26) and the least number of alleles was noticed with primer BPM 156 B (3 of 26). The maximum value of PIC was noticed with BPM 31 (0.30) and minimum value by BPM 154 (0.21). The dendrogram based on these data clearly separated all the 16 released varieties with a similarity coefficient ranged from 0.69 to 0.96.

The four polymorphic genomic microsatellite markers (BP SSR 11, BPM 31, BPM 154 and BPM 156 B) generated 17 alleles and exhibited the average number of alleles per loci 4.25. The maximum number of alleles was noticed with the markers, BP SSR 11 and BPM 154 (5 of 17) and the least number of alleles was noticed in BPM 156 B (3 of 17). The maximum value of PIC was noticed with BPM 31 (0.39) and minimum value by BPM 156 (0.23). The dendrogram drawn with these markers could not separate IISR Sakthi from its open pollinated progeny. Similarly Kuthiravally, Balancotta and Perumkodi, Karimunda and Arakkulam munda, Chumalakodi also could not be separated.

In order to determine the microsatellite repeat variation in terms of allelic difference, the alleles generated by the primer BP SSR 11 from 3 genotypes (Panniyur 2, Panniyur 3 and Pournami) were sequenced individually. The sequences obtained from 3 genotypes (Panniyur 2, Panniyur 3 and Pournami) amplified with BP SSR 11 when aligned using CLUSTAL W and Mega 6 multiple sequence alignment program, they showed microsatellite repeat variation for $(GA)_n$ (Panniyur 2- $(GA)_{20}$; Panniyur 3- $(GA)_{19}$; Pournami- $(GA)_{23}$) (Fig 45).

The presence of the repeat region $(CGACAT)_2$ in the amplified product of microsatellite marker PC ESTSSR 22 was also confirmed by aligning the sequence (obtained by sequencing the PCR product of Sp2 (*P. nigrum*) with the original sequence (SSR containing EST sequence of *P. colubrinum* retrieved from NCBI).

A set of 14 polymorphic microsatellite markers (7 genomic and 7 EST-SSR) generated in the present study were tested for its effective transferability and polymorphism in 21 *Piper* species. All the 7 EST-SSR markers from *Piper nigrum* and *P. colubrinum* were completely transferable to all the 21 *Piper* species. Among the 13 polymorphic SSR markers generated, 7 markers were completely transferable to all the *Piper* species tested. The grouping was done using EST SSR markers alone, genomic SSR markers alone and also in combinations. The previously reported microsatellite markers were also used for cross transferability and are also added along with EST derived and genomic microsatellite markers to assess genetic diversity.

The seven polymorphic EST SSR markers used to analyze cross species amplification profiling of twenty one *Piper* species produced a total of 36 alleles. The average number of allele per loci was 5.14. The maximum number of alleles was

noticed with the marker, PN EST SSR-6 (8 of 36) and the least number of alleles were observed with the microsatellite PN EST SSR-4 and PC EST SSR-22 with two alleles each. The maximum value of PIC was noticed with PN EST SSR-4 (0.36) and minimum value by PC EST SSR-22 (0.09). These seven polymorphic markers separated all the species except *P. longum* and *P. hapnium* which exhibited 100% similarity.

Seven genomic microsatellite markers generated a total of 43 alleles with a maximum number of allele noticed for the marker BPM 52 (10 of 43). The least number of alleles were observed with the SSR marker BP SSR 12 and BPM CNTG 5 with 4 alleles. Average number of allele per loci was 6.14. The maximum value of PIC was noticed with BP SSR 12 (0.40) and minimum value by BPM CNTG 5 (0.09). The grouping generated with these markers are clear with the earlier understanding of inter relationship in *Piper* species. But the marker could not separate *P. attenuatum* and *P. hymenophyllum*.

The grouping was also done with 14 SSR markers (7 EST SSR+ 7 genomic SSR) for characterizing the genus *Piper*. The markers in combination clearly separated all the 21 *Piper* species which could not separated with these markers individually.

Previously reported 16 SSR primers developed in black pepper (Menezes *et al*., 2009 and Joy *et al*., 2011) were also used to study genetic diversity in black pepper cultivars and *Piper* species. A total of 25 alleles were produced with six microsatellite markers (PN H8a, PN E3, PN A5, PN D10, PN F1 and Pn GT2) on genotyping 23 black pepper cultivars. The average number of alleles per loci is 4.16. The maximum number of alleles was found with the primers PN D10 (7 of 25) and

the least number of alleles were found with the primer Pn GT2 (2 of 25). The maximum PIC value was observed with the primer PN E3 (0.45) followed by PN F1 (0.43) and the least value noticed was 0.34 for the marker PnGT2.These six markers discriminated all the black pepper cultivars with 43% similarity. The genomic SSR markers when pooled with previously reported markers has given much better grouping when analyzed individually.

In 21 *Piper* species, the six markers generated a total of 45 alleles were observed with 6 polymorphic SSR markers (PN B5, PN E3, PN G11, PN D10, Pn AG30, Pn GT2). The maximum number of alleles was detected with the primer PN D10 (11of 45) and least number of alleles (4 of 45) were produced with PnGT2. The maximum PIC was observed for the primer PN G11 (0.48) and minimum value by PN D10 (0.18). These markers separated all the *Piper* species except *P. argyrophyllum* and *P. hymenophyllum*. However, The microsatellite markers developed in the present study (EST SSR+ genomic SSR) along with other previouslyreported marker resources exhibited a clear separation between 21 *Piper* species which could not been done with the marker of choices individually.

Unique bands were also obtained with both genomic and EST SSR markers for *P. betle, P. barberi, P. arboreum, P. ornatum, P. magnificum* and *P. colubrinum, P. ribesioides* and *P. hamiltonii*.

In this study three approaches were followed for studying the interrelationships among *Piper* species, varieties and cultivars of black pepper. They were SSRs developed from ESTs, genomic libraryand SSRs reported earlier. These primers in combination have clearly separated all the species, varieties and cultivars though some of them failed to separate when they used individually. The genomic

resources developed and tested can be effectively utilized for studying genetic architecture and inter relationship in the genus, *Piper*. They can also be utilized in future for tagging important traits for varietal identification and marker assisted breeding.

The present study gave us 14 new (additional) microsatellite markers for genus *Piper* and 5 markers for black pepper to dissect the *Piper* genome and to have a better understanding about the genetic diversity in black pepper and related species. This helps us in wide hybridizations between most divergent cultivars for exploiting hybrid vigour in black pepper which was proved earlier by the development of Panniyur -1. These additional molecular markers will also help in supplementing the efforts made earlier (Nirmal Babu *et al*., 2003) in preparing molecular maps, tagging genes of important agronomic characters. This further helps in marker assisted breeding in black pepper and reducing breeding time.

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Chapter 7

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APPENDICES

APPENDIX- I

STOCK SOLUTIONS/ REAGENTS/ BUFFERS/ CULTURE MEDIA

Ia) DNA Isolation

1M Tris HCl (pH 8.0)

121.1 g of Trizma base (Sigma, USA) was dissolved in 800 ml of double distilled water. The pH of the solution was adjusted to 8.0 by adding concentrated HCl. The volume of the solution was made upto 1000 ml with distilled waterand sterilized by autoclaving.

0.5 M EDTA (pH 8.0)

186.1 g of ethylene diamine tetra acetic acid disodium salt dihydrate(Sigma, USA) was added to 800 ml of distilled water and stirred vigorously on a magnetic stirrer. The pH of the solution was adjusted to 8.0 with 10N NaOH and made up the final volume to 1000 ml. The solution was sterilized by autoclaving.

5M NaCl

292.2 g of NaCl (USB) was dissolved in 800 ml of distilled water. The final volume was made up to 1 L with distilled water and sterilized by autoclaving.

Chloroform: Isoamylalcohol (24:1)

96 ml of chloroform (Sigma, USA) was mixed with four ml of isoamyl alcohol (Sigma, USA) and stored in ambour coloured reagent 6 bottles at room temperature.

Phenol: Chloroform: Isoamylalcohol (25: 24: 1)

Equal volumes of Tris saturated phenol (pH > 6.8) (Sigma, USA) and chloroform: isoamylalcohol (24: 1) were mixed and stored in ambour coloured bottles at 4 ^{o}C.

CTAB extraction Buffer

Components used

100 mM Tris-Cl (pH 8.0)

20 mM EDTA

2 M NaCl

4 % CTAB

0.5 % β- mercaptoethanol (freshly added prior to preheating)

1 % PVP (w/v) (freshly added while grinding leaf tissue)

TE buffer (pH 8.0)

10 mM Tris-Cl (pH 8.0)

1 M EDTA (pH 8.0)

Ib) Electrophoresis of Nucleic Acids

50X TAE buffer

Trizma Base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100.0 ml

The final volume was made upto 1 L with distilled water.

50X TBE buffer

Tris base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20.0 ml

The final volume was made upto 1 L with distilled water.

Gel loading dye

Bromophenol blue	0.25 %
Xylene cyanol	0.25 %
Glycerol	30.0 %

Ethidium bromide (10 mg ml^{-1})

1 g Ethidium bromide was dissolved in 100 ml of distilled water, stirred for several hours on a magnetic stirrer to dissolve the dye. The solution was aliquotted, transferred to dark bottles and stored at room temperature. Wear gloves while using ethidium bromide solutions as it is carcinogenic.

PAGE gel loading buffer (Sambrook *et al*. 1989)

98 % formamide

10 mM EDTA (pH 8.0)

0.1 % xylene cyanol

0.1% bromophenol blue

Urea-Acrylamide 15 % mix – 60 ml

40 % acrylamide solution (29: 1)	22.5 ml
10 X TBE	6ml
Urea (7M)	25.2. g

All the above components were mixed, dissolved and made upto 60 ml using distilled water.

10 % APS

To 1 g of ammonium per sulphate, 10 ml of distilled water was added, mixed and stored at 4 ^{0}C.

Ic) Enrichment of Microsatellites

20X SSC

175.3 gof Sodium chloride (NaCl) and 88.2 g Sodium citrate (Himedia, USA) was dissolved in 800 ml of distilled water. The pH of the solution was adjusted to 7.0 with few drops of 10 N NaOH. The volume was adjusted to 1 L with water and sterilized by autoclaving.

2X Hybridization Solution

12X SSC, 0.2 % SDS (pH 7.0)

1X Hybridization Solution

6X SSC, 0.1 % SDS (warmed for proper dissolving)

Washing Solution - I

2X SSC, 0.1 % SDS (warmed for proper dissolving)

Washing Solution - II

1X SSC, 0.1 % SDS (warmed for proper dissolving)

Id) TRANSFORMATION/ RECOMBINANT SELECTION

Luria-Bertani (LB) broth

25 g LB broth powder (Himedia, India) was dissolved in 950 ml sterile distilled water. The volume was adjusted to 1000 ml using double distilled water and sterilized by autoclaving.

Luria-Bertani (LB) agar

40 g LB agar readymade powder (Himedia, India) was added to 950 ml double distilled water, melted by heating in a microwave oven. The volume was made upto 1000 ml and sterilized by autoclaving.

IPTG – 200 mg/ml

2 g of IPTG (Isopropyl thiogalactoside)(Fermentas, Germany) was dissolved in 8 ml of distilled water. The volume of the solution was adjusted to 10 ml with distilled water and filters sterilized. The solution was dispensed into 1 ml aliquots and stored at -20 ^{0}C.

X - Gal

20 mg of X-gal (5-bromo -4-chloro-3- indolyl-β-D- galactoside) (Fermentas, Germany) was dissolved in one ml of N, N- dimethyl formamide (Himedia, India) and aliquoted into 2ml microcentrifuge tubes wrapped in aluminium foil and stored at -20 ^{0}C.

Kanamycin

250 mg of Kanamycin (Himedia, India) was dissolved in 5 ml of sterile double distilled water. The solution was filter sterilized using a 0.22 micronmembrane filter (Himedia, India) and stored at -20 ^{0}C.

Ie) Silver Staining

Fixing solution (18-25 ºC)

Glacial acetic acid (CH_3COOH) (Himedia, India) was dissolved to 7.5% (v/v) with deionized water.

Formaldehyde solution (18-25 ºC)

15 ml of formaldehyde (Sigma, USA) was dissolved in 85 ml deionized water

Impregnating solution (18-25 ºC)

0.1 g silver nitrate ($AgNO_3$) (Sigma, USA) dissolved in 100 ml deionized water

Sodium thiosulphate stock solution (18-25 ºC)

0.2 g sodium thiosulphate was dissolved in 50 ml deionized water.

Developer solution (8 ºC)

3 g of Sodium carbonate (Na_2CO_3) dissolved in 100 ml deionized water.

Developer stop solution (4 ºC)

Glacial acetic acid was diluted to 7.5% with deionized water.

APPENDIX- II

IIa) Protocol followed for Agarose gel electrophoresis

- Cleaned gel casting tray and combs were wiped with alcohol
- The gel casting tray was placed horizontally and their open ends were sealed with tapes.
- The combs were placed in the gel tray and aligned properly.
- For preparing a 0.8% (w/v) agarose gel, 2.4 g agarose is weighed and transferred to a 300ml TAE buffer (1X) in a 500 ml conical flask.
- The agarose was dissolved completely in the bufferby heating to 80-85 ^{o}C in a microwave oven.
- The solution was allowed to cool to approximately 50-60 ^{o}C and 6 µl of ethidium bromide (10 mg/ml)was added to the molten agarose, mixed and poured to the gel tray. The tray was left undisturbed for 30 min.
- The combs and the sealed tapes were removed carefully and the gel tray was placed in the electrophoresis chamber immersed with enough TAE buffer (1X).
- The DNA sample was mixed with gel loading dye (6X) (Appendix 1b) and loaded onto the wells of agarose gel along with standard DNA marker.
- The lid of the electrophoresis chamber was carefully closed and was connected to the power supply (50-60 V).
- After enough separation of the gel loading dye, the DNA bands were documented with Kodak Gel Logic 200 image analysis system (Eastman Kodak Company, Rochester, NY).

IIb) Protocol followed for setting up and casting the denaturing PAGE gel

- Wash both the glass plates thoroughly with water and liquid detergent. Rinse the glass plates thoroughly with deionized water to remove detergent residues and wipe with tissue paper soaked in 70 % alcohol and air dry the glass plates.
- 15% PAGE gels (15% polyacrylamide; 7M urea) were prepared by mixing 60 ml urea; acrylamide mix (Appendix 1b) with 200 µl ammonium persulfate (10%) and 30 µl of TEMED (Sigma Aldrich, USA).
- The polyacrylamide mix was then poured onto the assembled gel plates of the sequencing electrophoresis apparatus (BioRad, USA) and allowed to polymerize for 20-30 min.
- The combs were carefully removed and all the wells were washed with buffer.
- The tank was filled with 1 X TBE and the gel was subjected to pre run at 200 V for 30-60 min. The wells were again washed with 1X TBE.
- The 5.0 µl PCR product were mixed with an equal volume of denaturing buffer (98% formamide, 10mM EDTA (pH 8.0) 0.05% xylene cyanol, 0.05% bromophenol blue), denatured at 94 °C for 5 min and immediately placed the samples on ice.
- The samples were resolved at a constant power of 220 V for 4-6 h (Run time depends as per the expected size of PCR products).

IIc) Protocol followed for silver staining PAGE gels

- The plates were carefully removed from the apparatus and kept on blotting paper. The spacers were also removed and separated the plates.
- The gel was then placed in a gel tray containing 600 ml fixing solution for 10 min.

- Followed by fixation, the fixer solution was decanted and washed twice with deionized water for 2-3 min.
- Washed gels were then soaked for 10 min at room temperature (22-24 ^{o}C) in 600 ml formaldehyde solution.
- The formaldehyde solution was decanted and 600 ml of silver nitrate solution was added and covered the tray and incubated for 15-20 min.
- Following silver impregnation, the solution was decanted and the gel was rinsed twice with 200 ml of deionized water for 5-10 sec.
- 600 ml of developer solution was added to the gel and let it until the bands appear with sufficient intensity (3-5 min).
- When the desired intensity was achieved, the developer solution was decanted and further development was stopped by impregnating the gels in 600 ml stop solution for 5-10 min.
- The stop solution was then poured off and the gel was washed two times with deionized water and scanned the image.

Publications:

Research Paper

Anupama K., Anu Cyriac, Saji K. V. and Nirmal Babu, K. (2015). Microsatellite markers based cross species amplification and genetic diversity analysis in the genus *Piper*. *International Journal of Advanced Research,* 3(5) 184-191.

Abstracts

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