DEVELOPMENT AND VALIDATION OF NOVEL EST-SSR MARKERS IN BLACK PEPPER (*Piper nigrum* L.)

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"DEVELOPMENT AND VALIDATION OF NOVEL EST-SSR MARKERS IN BLACK PEPPER (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"DEVELOPMENT AND VALIDATION OF NOVEL EST-SSR MARKERS IN BLACK PEPPER (*Piper nigrum* L.)"** is a record of research work done independently by Ms. Sherin Jose under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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SHERIN JOSE

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LIST OF ABBREVIATIONS AND SYMBOLS USED

µl	Micro litre
µ*M*	Micro Molar
et al.	Co- workers/ Co-authors
°C	Degree Celsius
AFLP	Amplified Fragment Length Polymorphism
viz.,	Namely
APS	Ammonium persulphate
Bp	Base pair
COA	College of Agriculture
CTAB	Cetyl trimethyl ammonium bromide
DARwin	Dissimilarity Analysis and Representation for windows
DBT	Department of Biotechnology
DNA	Deoxyribo Nucleic Acid
EST	Expressed Sequence Tags
GMATo	Genome-wide Microsatellite Analyzing Tool
G	Gram
ICAR	Indian council of Agricultural Research
IISR	Indian Institute of Spice Research
ISSR	Inter Simple Sequence Repeats
KAU	Kerala Agricultural University
MIDH	Mission for Integrated development of Horticulture
MISA	MIcroSAtellite
Ml	milli litre
NCBI	National Center for Biotechnology Information

Ng	Nano Gram
Nm	Nano meter
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PIC	Polymorphism information content
PRS	Pepper Research Station
RAPD	Random Amplified Polymorphic DNA
RFLP	Random Fragment Length Polymorphism
SRAP	Sequence related amplified polymorphism
SSR	Simple Sequence Repeats
SSRIT	Simple Sequence Repeat Identification Tool
sp.	Species
spp.	Several species
TAE	Tris-Acetate EDTA
TBE	Tris-Borate EDTA
TEMED	Tetramethylethylenediamine
t ha^{-1}	Tonnes per hectare
TROL	Tandem Repeat Occurrence Locator
TRF	Tamdem Repeat Finder
U	Unit
UPGMA	Unweighted pair group method using arithmetic average

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), the king of spices belonging to the family Piperaceae is a perennial climbing vine and a native of the tropical forests of Western Ghats, South India. India ranks third in production and is a leading exporter of black pepper in the world (GOK, 2016). Black pepper is cultivated to a large extent in Kerala, Karnataka, Tamil Nadu and to a limited extent in Maharashtra, North eastern states and Andaman and Nicobar islands. In Kerala, production of black pepper has increased from 40.6 thousand tonnes (2014-2015) to 42.1 thousand tonnes (2015-2016) and shares 75 per cent of total production of the country (GOK, 2016). It is economically valuable because of the berries which are extensively used as a spice and has medicinal value. Piperine (1-piperoyl piperidine), the most prominent amide alkaloid found in fruits of black pepper, helps to stimulate metabolism and absorption, boost the efficacy of drugs and prevent tumour development.

The genus *Piper* is rich with more than 3000 species of which 115 species are of Indian origin (Saji, 2006). In black pepper itself more than 100 cultivars are available. Phenotype based characterization and diversity analysis has limitations as to the availability of sufficient number of distinguishing morphological traits, dependence on the developmental stages and influence by environmental factors. Biotechnological tools have supplemented the conventional breeding programmes of conservation, characterization, improvement and utilization of various genotypes in this genus.

Genetic variation can be assessed more precisely, quickly and cheaply through DNA markers. DNA markers are not influenced by the environment and their inheritance can be monitored. Diverse DNA markers are available based on genomic abundance, locus specificity, level of polymorphism, reproducibility, technical requirement and financial investment. Suitable marker system can be selected depending on the type of study to be undertaken and that would fulfill at

least few of the characteristics of an ideal marker. An ideal marker should have high polymorphism, co-dominant inheritance, high reproducibility, frequent occurrence in the genome, easy availability and selective neutral behaviour to environmental conditions.

Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR) are the various DNA markers used in molecular studies in this crop. Among these various classes of markers, SSR markers are the preferred marker system in many studies due to their hypervariability, co-dominance, mendelian inheritance, multiallelism, reproducibility, good genome coverage, chromosome specific location, amenability to high throughput genotyping and automation (Parida *et al*., 2009).

SSRs, referred to as microsatellites, are a class of DNA sequences consisting of simple motifs of one to six nucleotides that are repeated in tandem up to a few dozen times per site (Litt and Luty, 1989). The variation in the repeating units results in the polymorphic bands which are detected by Polymerase Chain Reaction (PCR) with the help of locus specific flanking region primers (Farooq and Azam, 2002). The major limitation of SSR markers is that its development depends on sequence information. However, after the advent of Next Generation Sequencing (NGS) methods, genomic or Expressed sequence tags (EST) data is available for many crops.

EST-SSR is the simple sequence repeat derived from expressed region of the genome. SSRs can be detected from EST sequences using different SSR search tools like, MIcroSAtellite (MISA), Genome-wide Microsatellite Analysis Tool (GMATo), SSRFinder, Sputnik, SSR Identification Tool (SSRIT), SSRSEARCH, Tandem Repeat Occurrence Locator (TROL), Tandem Repeat Finder (TRF) and RepeatMasker. EST-SSR markers are very informative markers which can be used

for population genetics studies ranging from individual level to that of closely related species (Varshney *et al*., 2005).

In black pepper, most of the genomic analysis reports are based on RAPD markers (Pradeepkumar *et al*., 2003; George *et al*., 2005; Sen *et al*., 2010), AFLP markers (Joy *et al*., 2007) and ISSR markers (Jiang and Liu, 2011b; Sheeja *et al*., 2013) whereas the use of SSR markers are limited. Nine genomic SSRs were used for characterizing twenty clonal varieties of black pepper and four species of *Piper* (Menezes *et al*., 2009). Genetic diversity among 40 genotypes of black pepper and four species of *Piper* was analyzed using seven genomic SSR markers (Joy *et al*., 2011). Eleven SSR markers were used to distinguish *P. colubrinum*, *P. nigrum* and their corresponding hybrids (Jagtap *et al*., 2016). The major reason for the limited use SSR marker in black pepper is the lack of sufficient sequence information and low transferability rate of genomic SSR in closely related species or related genera. However, this can be tackled by EST databases as it is easy to be obtained than the complete genome sequence.

EST databases are the source of SSRs which are associated with the gene of interest and transferable across related genera. The association of EST-SSR markers with phenotype can be studied through change in SSR repeats present within the gene having a known function. Such EST-SSR markers are powerful tools for marker assisted selection as they are tightly linked with the specific genes. Hence, it is necessary to mine the EST databases in order to access useful SSR markers. So far EST-SSR marker analysis has been carried out in 148 wild species and varieties of black pepper including 104 wild species from Asia, 10 wild pepper from America, 33 varieties from different countries and one accession of *Piperomia pellucida*. Those studies can be the stepping stone in the development of more EST-SSR markers in black pepper (Wu *et al*., 2016).

In an earlier experiment funded by Department of Biotechnology (DBT), ESTs during berry development stage of black pepper was developed using Ion Torrent (Ion Personal Genome Machine™, Life Technologies) single-end sequencing technology (Sujatha, unpublished). These ESTs can be mined for detecting SSRs using appropriate identification tool. Since SSR markers are ideal for detecting the differences between and within species, molecular profiling using EST-SSR markers in black pepper varieties, cultivars and different *Piper* species are very valuable in studying interrelationship among the genotypes.

Keeping all the points in view, the present study was undertaken with the following objectives

- To develop novel EST-SSR markers in black pepper.
- To validate the developed EST-SSR in varieties and cultivars of black pepper and related *Piper* species.
- To study the genetic relationship among the genotypes.

Review of Literature

2. REVIEW OF LITERATURE

2.1 BLACK PEPPER: KING OF SPICES

Black pepper is the oldest and most widely used spice which is having great economic importance. It is considered as black gold because it fetches higher price in the market. Black pepper is mainly cultivated in the sub mountainous tracts of Western Ghats which requires high rainfall and humidity. Among the cultivars of black pepper considerable variability is observed. Resistant or tolerant sources are available in the diversified germplasm. For genetic improvement through hybridization, selection of diverse parents and superior progenies play a crucial role. (Krishnamoorthy and Parthasarathy, 2010).

2.2 GENETIC RESOURCES OF *Piper* sp.

Hooker was the first who studied Indian *Piper* in his 'Flora of British India'. Forty five species of *Piper* were reported by him, of which twenty species belongs to India. Rao (1914) collected fourteen species of *Piper* from Western Ghats and listed in 'Flowering plants of Travancore'. Babu *et al.* (1993) have reported three taxa from silent valley biosphere reserve namely *P. silentvalleyensis, P. nigrum var. hirtellosum, P. pseudonigrum* and two taxa from sugandhagiri cardamom plantation, Wayanad, Kerala namely *P. sugandhi* and *P. sugandhi var brevipilis*. *P. galeatum, P. sugandhi* and *P. wighti* are the species most closely resemble cultivated *P. nigrum*.

2.3 VARIETIES AND CULTIVARS OF BLACK PEPPER

Superior lines of black pepper varieties were released through hybridization or open pollination or clonal selection from the popular cultivars by the systematic research efforts in the last three decades. Under All India Coordinated Research Programme (AICRP) on spices, Pepper Research Station, Panniyur has released eight varieties namely Panniyur-1, Panniyur-2, Panniyur-3, Panniyur-4, Panniyur-5, Panniyur-6, Panniyur-7 and Panniyur-8 yielding between 1.27 and 2.57 tonnes/ha.

Among these Panniyur-1, Panniyur-3 and Panniyur-8 are the hybrid varieties. Vijay is a hybrid black pepper released from College of Horticulture, Vellanikkara, Kerala Agricultural University (KAU), Thrissur. Sreekara, Subhakara, Panchami, Pournami, IISR Sakthi, IISR Thevam, IISR Girimunda and IISR Malabar excel are the varieties released from ICAR-Indian Institute of Spices Research (ICAR-IISR) with yield ranging from 2.3 to 2.8 t ha^{-1}. CPCRI regional station, Palode released a variety PLD-2 with a potential yield of 2.4 t ha^{-1} (Ravindran *et al.*, 2000). More than 100 cultivars of black pepper are being cultivated in India. Aimpiriyan, Arakulam munda, Balankotta, Cheriyakaniyakadan, Kalluvally, Karimunda, Kottanadan, Kuthiravally, Narayakodi and Vadakkan are some major cultivars grown in Kerala.

The requirement of easy tools is essential for identifying better genotypes from the diversified collection of germplasm. This selection can be achieved through different genetic markers. Breeding assisted with markers significantly shortens the development time of varieties. Hence markers can be applied to replace the evaluation of a trait that is difficult or expensive to evaluate.

2.4 GENETIC MARKERS: AS TAGS

Genetic markers are the genetic tags that are determined by allelic forms of genes or genetic loci. They can be transmitted from one generation to another and thus they can be used as experimental probes or tags to keep track of an individual, a tissue, a cell, a nucleus, a chromosome or a gene. Classical markers and DNA markers are the two branches of genetic markers. Classical markers include morphological markers, cytological markers and biochemical markers. DNA markers include Restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), Random amplified polymorphic DNA (RAPD), Simple sequence repeats (SSR) and Single nucleotide polymorphism (SNP) based on different polymorphism detecting techniques or methods (Nucleic acid hybridization, PCR – polymerase chain reaction and DNA sequencing) (Collard *et al.*, 2005).

2.4.1 Classical markers

2.4.1.1 Morphological markers

Morphological markers generally represent genetic polymorphism which can be easily identified and manipulated. Morphological markers included visible traits such as leaf shape, flower colour, pubescence colour, seed shape and seed colour. Ravindran *et al.* (1997) studied twenty two morphological characters of black pepper. They found that leaf and spike characters were the important diagnostic characters. Black pepper cultivars were categorized into three distinct groups namely cultivars with spike length less than leaf length, cultivars with spike length more or less equal to leaf length and cultivars with spike length more than leaf length.

Jagtap *et al.* (2016) utilized morphological characters for studying six putative hybrids obtained from separate interspecific crosses between *P. colubrinum* as male parent and six genotypes of *P. nigrum* as female parent. There was significant difference in the fruit characters among different interspecific crosses. Shoot tip colour was similar in female parents and their respective hybrids except in the case of cross between Panniyur-5 and *P. colubrinum*. However, morphological markers available are limited, and many of these markers are not associated with important economic traits such as yield and quality. Morphological markers exhibit undesirable effects on the development and growth of plants.

2.4.1.2 Biochemical/protein markers

Isozymes reflect the products of different alleles rather than different genes because the difference in electrophoretic mobility is caused by point mutation as a result of amino acid substitution. Therefore, isozyme markers can be genetically mapped onto chromosomes and then used as genetic markers to map other genes. They are also used in seed purity test and occasionally in plant breeding (Kumar *et al.*, 2009). Sasikumar *et al.* (1999) reported isozyme studies in black pepper and

found out phylogenetic relationship among *P. nigrum*, *P. barberi* and *P. attenuatum*. Sujatha (2001) studied genetic identity of clones and tissue culture derived black pepper varieties using isozyme marker. Very clear fingerprint characteristic of each tissue culture derived vines was studied based on peroxidase.

2.4.2 DNA markers

DNA marker is a small region of DNA sequence showing polymorphism (base deletion, insertion and substitution) between different individuals. Southern blotting, a nucleic acid hybridization technique (Southern, 1975), and PCR, a polymerase chain reaction technique (Mullis, 1990) are two basic methods to detect the polymorphism. The variation in DNA samples or polymorphism for a specific region of DNA sequence can be identified based on the product features, such as band size and mobility using PCR or molecular hybridization followed by electrophoresis. AFLP, RFLP, RAPD, ISSR, SSR and SNP are the commonly used DNA markers.

AFLPs are the fragments obtained through the amplification using directed primers from restriction of genomic DNA (Matthes *et al.*, 1998). This technique does not require sequence information. Joy *et al.* (2007) evaluated thirty black pepper cultivars using AFLP marker. Variation within the cultivars was shown by 158 polymorphic markers among the total 173. The accessions were grouped into three major clusters and four diverse cultivars by the Unweighted pair group method using arithmetic average (UPGMA) and based on this dendrogram was derived.

RFLP is a co-dominant marker which can easily determine whether the linked trait is present in a homozygous or heterozygous state. This is highly desirable for selecting recessive traits (Winter and Kahl, 1995).

RAPD marker detects nucleotide sequence polymorphism in DNA by using a single primer of arbitrary nucleotide sequence polymorphism mostly ten base pair

long (Black *et al.*, 1992). Sujatha (2001) studied genetic fidelity of clones of each variety and tissue culture derived black pepper using RAPD markers. Clones under each variety were found to be monomorphic in RAPD analysis where as within tissue culture plants variants could be detected. RAPD markers were successful in distinguishing four black pepper varieties (Panniyur-1, Panniyur-2, Panniyr-4 and Subhakara) and in assessing genetic distance between them.

Pradeepkumar *et al.* (2003) analyzed the genetic diversity in *P. nigrum* through RAPD analysis in twenty two cultivars of *P. nigrum* and one accession each of *P. longum* and *P. colubrinum*. RAPD markers in the range of 372 were generated from twenty four primers, of which 367 were polymorphic. Landraces showed more genetic divergence than the advanced cultivars. Among the landraces two distinct groups were obtained where one comprised of Uthiramkotta, Balankotta, Perumkodi and Karimunda which are predominantly cultivated in South and Central Kerala. The second group comprising of Aimpiriyan, Cholamundi, Sumandy, Dodiga, Malligeswara, Kalluvally and Karimunda are widely cultivated in North Kerala and South Karnataka. The distinctness of *P. colubrinum* among three species was revealed through dendrogram derived from UPGMA.

Sen *et al.* (2010) screened twenty two RAPD primers in eight species of *Piper* namely *P. nigrum*, *P. longum*, *P. betle*, *P. argyrophyllum*, *P. chaba*, *P. galeatum*, *P. trichostachyon* and *P. hymenophyllum*. Eleven primers were selected for the analysis of different species. Out of 149 RAPD fragments, 137 fragments were found to be polymorphic. In ten primers, 27 RAPD fragments were identified as species specific and showed fixed frequencies in that particular species.

Jiang and Liu (2011a) characterized seventy four plants of different *Piper* species using RAPD and Sequence related amplified polymorphism (SRAP). Out of 111 fragments, 110 RAPD fragments were polymorphic obtained using nine selected RAPD primer. One hundred and twenty seven reproducible fragments were

identified using nine SRAP primer combinations. Dendrogram created with the RAPD markers and SRAP markers were topologically dissimilar. *Piper* species were grouped into five clusters in the dendrogram based on the RAPD markers. Cluster I comprised of *P. hainanense* and *P. bonii*. *P. austrosinense*, *P. puberulum* and *P. boehmeriaefolium* were included in cluster II. Cluster III consisted of *P. nigrum P. sarmentosum and P. betle*. *P. laetispicum* and *P. curtipedunculum* was incorporated in cluster IV. *Piper kadsura* was included in cluster V. All the *Piper* species were distinguished from each other in the dendrogram based on the SRAP markers.

ISSR regions are amplified through PCR technique by taking the repeat region as primer. ISSR analysis can be applied in genetic identity, parentage, strain identification and taxonomic studies (Kumar *et al*., 2009). Jiang and Liu (2011b) used ISSR to evaluate 74 genotypes of *Piper* sp. in China. Out of 248 bands, 247 were found to be polymorphic which shows high level of diversity among the genotypes. Sheeja *et al*. (2013) analyzed genetic diversity among 27 species of *Piper* using ISSR markers. They have identified 35 species specific bands in different species.

SSRs are stretches of DNA consisting of tandemly arranged repeating units of one to six base pair (bp) (Gupta *et al*., 1996), characterized by their co-dominant inheritance, wide genomic distribution, hyper variable and multiallelic nature (Powell *et al*., 1996; Parida *et al*., 2009).

The genesis of these repeats occurs primarily due to slipped strand mispairing (Levinson and Gutman, 1987) and subsequent errors during DNA replication/ repair/ recombination (Schlotterer and Tautz, 1992), nucleotide composition of repeat motifs (Katti *et al*., 2001) or unequal crossing-over between sister chromatids. The frequency of repeats decreases exponentially with repeat length (Metzgar *et al*., 2000; Katti *et al*., 2001). This might be due to the fact that longer microsatellites exhibit higher mutation rates than shorter ones (McConnell *et al*., 2007).

The occurrence of SSRs in the promoter region influences transcriptional activity (Kashi *et al.*, 1997). In plants, SSRs are much more abundant and preferentially associated with untranslated regions (UTRs) of the transcribed regions (Morgante *et al.*, 2002). The presence of SSR in non coding regions influences gene regulation, transcription (Martin *et al.*, 2005; Lawson and Zhang, 2006) and recombination events (Bagshaw *et al.*, 2008). Multiallelic microsatellites are considered to be the best marker system for the detection of intervarietal polymorphisms (Stepien *et al.*, 2007).

Mogalayi (2011) characterized KAU released black pepper varieties (Panniyur-1 to Panniyur-7) using different molecular markers- RAPD, ISSR and SSR. Genomic DNA of each variety was amplified using 10 each of selected RAPD and ISSR primers and 8 SSR primers. The polymorphism information content (PIC) value varied from 0.86 to 0.90 for RAPD analysis and 0.80 to 0.89 for ISSR analysis. DNA fingerprint of black pepper varieties was developed based on distinct bands produced through three classes of markers. The variety Panniyur-1 and Panniyur-3, hybrids of same parents showed 76 per cent similarity.

2.5 BIOINFORMATICS IN SSR MARKER DEVELOPMENT

With the increase in availability of the sequence information, the need for automated methods also enhanced. Benson (1999) created a program Tandem Repeat Finder (TRF) in order to find out the repeat regions in DNA sequence. Two files of results were returned for input sequence file. The location and statistical properties of the tandem repeats were described in the summary table. Alignment of each repeat with its consenses sequence was expressed in the second file. Castelo *et al.* (2002) developed Tandem Repeat Occurrence Locator (TROL) based on the *Aho Corasick* Algorithm (ACA) which is fast and took 127 seconds to find all 20 bp length SSRs in complete *Arabidopsis* genome. SSR primers together with SSR detection, generic

primers, hybridization oligos can be designed using the Batchprimer3 program (You *et al.*, 2008).

Hong *et al.* (2011) synthesized SSR-Primer Generator for the discovery of SSR, designing the primers and homology based search of DNA sequences. DNA sequences in the FASTA format were processed in the Java Runtime Environment (JRE) platform and results were given in the HTML tabular format. Wang *et al.* (2013) released a novel tool named GMATo (Genome-wide Microsatellite Analysis Tool) for the identification and analysis of microsatellites in the large genome. Raw FASTA DNA sequences were given as the input file and the output was retrieved in the tabular format containing SSR loci information and statistical distribution. Ponyared *et al.* (2016) developed ESAP plus (EST Analysis Pipeline plus) which is comprehensive and convenient bioinformatic tool in which users can upload raw EST data. This tool mainly offers four main procedures namely preprocessing, clustering and assembly, SSR mining and SSR primer designing.

Wei *et al.* (2011) classified SSRs into genomic SSRs and EST-SSRs based on the original sequences used to identify microsatellite region. EST derived SSR markers show good transferability across taxonomic boundaries and can be used as anchor markers for comparative mapping and evolutionary studies.

2.6 GENOMIC SSR MARKERS

Genomic SSRs are isolated from the genomic DNA with or without the construction of genomic DNA library. Menezes *et al.* (2009) analyzed 20 varieties of *P. nigrum* from the germplasm collection of Brazilian Agricultural Research Corporation. They developed nine microsatellite markers from an enriched library of *P. nigrum*. Joy *et al.* (2011) developed seven SSR primers through sequential reverse genome walking strategy. Four loci with a total of 62 alleles with an average of 15.5 alleles were identified. An average PIC value of 0.85 was showed by SSR primers. Genetic diversity among 44 genotypes including cultivars and varieties was analyzed

using seven developed SSR markers. Anupama *et al.* (2015) used SSR primers developed by Menezes *et al.* (2009) and Joy *et al.* (2011) for analyzing cross transferability and genetic variability among twenty three *Piper* species. Out of the sixteen primers, seven primers were successfully amplified in all the genotypes.

2.7 EST-SSR MARKERS

The generation of SSR markers from EST resources is relatively fast and inexpensive (Thiel *et al.*, 2003) and could be achieved rapidly using bioinformatics softwares. EST-SSRs reveal variation in the expressed regions of the genome, thereby detecting perfect marker-trait associations. They exhibit high transferability across a much broader taxonomic range (Gupta *et al.*, 2001) and null alleles are less problematic (Leigh *et al.*, 2003; Rungis *et al.*, 2004) than those derived from untranslated regions (Pashley *et al.*, 2006). Senan *et al.* (2014) reported that EST derived markers have a prominent role when the study is concerned with the identification of functional polymorphisms in key genes.

2.7.1 EST-SSR markers in black pepper

Hu *et al.* (2015) detected SSR in black pepper using the MIcroSAtellite (MISA) software in which unigenes were used as reference data. SSRs with a length of more than 150 bp on both ends of the unigene were retained, and these sequences were used to design primers in Primer Premier 6.0. A total of 5509 SSR loci with dinucleotide repeat, trinucleotide repeat, tetranucleotide repeat, pentanucleotide repeat and hexanucleotide repeats were detected from 5252 unigenes by scanning the transcriptome data. Among these SSRs, the trinucleotide repeat motifs were the most abundant, accounting for 3557 SSRs (64.57 per cent), followed by 1607 (29.17 per cent) dinucleotide repeat motifs, 130 (2.36 per cent) hexanucleotide repeats, 116 (2.10 per cent) pentanucleotide repeat motifs and 99 (1.80 per cent) tetranucleotide repeat motifs. The main motifs were the dinucleotide, AG/CT repeat and AT/TA repeat and the trinucleotide, CCG/CGG repeat and AGG/CCT repeat.

Wu *et al.* (2016) analyzed genetic diversity among 148 accessions of black pepper using 13 EST-SSR markers. Transcriptome data of *P. nigrum* and *P. flaviflorum* were used for mining SSRs. Eight trinucleotide repeats and five dinucleotide repeats were selected for designing primers. The mean PIC value was 0.93.

2.7.2 EST-SSR in spices, plantation crops and medicinal plants

Xiao *et al.* (2013) developed 30 SSR markers from Illumina transcriptome sequence data in coconut. MSATCOMMANDER software was used for scanning 57304 unigenes in order to locate the repeat regions. Seventy six microsatellite loci comprising 27 dinucleotide repeat, 42 trinucleotide repeat and 7 tetranucleotide repeat were selected from a total of 4796 microsatellites. Out of 76 SSRs, 30 were selected based on scorability, polymorphism and amplification in thirty individuals. Ninety one alleles were detected from 30 SSRs with an average of 3.03 alleles per locus.

Anjali *et al.* (2015) identified 290 SSR from 5050 unique EST sequences using the software WEBSAT in cardamom. Only 206 SSR were used for designing the primer and the remaining eighty four SSR could not take for designing primer as its flanking sequences were too short. Twenty primers were selected after checking the quality using NETPRIMER. Among the twenty primers, twelve had produced expected amplicons in 18 cardamom accessions.

Ferrao *et al.* (2015) identified 24031 microsatellites from 130792 EST sequences of coffee using Gramene software. Total 146 primers were designed using Primer3 and Primer select software, of which 101 primer pair showed clear amplification in *Coffea arabica*. Twelve *Coffea arabica*, five *Coffea canephora* and three interspecific hybrid of *Coffea arabica and Coffea canephora* were used for the validation of markers. Two genotypes, *Coffea arabica* and Hibrido de Timor had the same number of duplicate loci (4) and contained 31 and 25 monomorphic loci.

Kumar *et al.* (2015) assembled 1316 ESTs of *Mentha piperita* into 155 contigs and 653 singletons. Of these, 110 sequences were found to contain 130 SSRs, with a frequency of 1 SSR per 3.4 kilo bases (kb). AG/CT (43.8 per cent) and AT/AT (16.2 per cent) were the most frequent dinucleotide repeat found. Primers were successfully designed for 68 SSR containing sequences (62 per cent). The 68 primers amplified in 13 accessions of *M. piperita* and 54 produced clear amplicons of the expected size. Thirty three primers (61 per cent) were found to be polymorphic among *M. piperita* accessions, showing 2 to 4 alleles with an average of 2.33 alleles per SSR, and the PIC value varied between 0.13 and 0.51 (average 0.25). All the amplified SSRs showed transferability among four different species of *Mentha*, with the maximum in *M. arvensis* (87.0 per cent) and minimum in *M. citrate* (37.0 per cent).

Mahajan *et al.* (2015) identified 471 SSR containing ESTs from a total of 23260 ESTs in *Ocimum basilicum*. Mononucleotide repeats were found most abundantly followed by trinucleotide repeat, dinucleotide repeat and hexanucleotide repeat. Primers were designed only for 257 EST sequences. Among the 257 primers, only 25 primers were utilized for the validation. Fourteen primers were shown amplification in different accessions of *Ocimum*. Three species of *Ocimum* and one species of related genus *Hyptis* were used for studying the cross transferability of these EST SSR markers.

Hou *et al.* (2017) identified 1134 SSR loci using MISA from 3519 sequences in rubber and designed 739 primer pairs using the software primer3. Mononucleotide repeats were high in number accounting for 38.89 per cent followed by dinucleotide (36.95 per cent) and trinucleotide (18.17 per cent). Polymorphism was shown by 180 pairs of primers out of 739 primer pairs and 386 pairs showed monomorphic bands.

Sahoo *et al.* (2017) screened 12593 EST sequences for detecting SSR using MISA software in turmeric. The criteria for selecting SSR motifs were twelve

repeated units for mononucleotide, seven units for dinucleotides and three units for trinucleotide, tetranucleotide and pentanucleotide repeats. Total 50 primers pairs were designed using primer3Plus software program. Fifty primers were validated in 96 samples and 11 SSR loci were found to be polymorphic.

2.7.3 EST-SSR in cereals and millets

Varshney *et al.* (2002) found out that trimeric repeats are the most abundant (54 per cent to 78 per cent) class of microsatellites followed by dimeric repeats (17 per cent to 40 per cent) in cereals. Among the trimeric repeats, the motifs CCG are the most common in all cases ranging from 32 per cent in wheat to 49 per cent in sorghum. When all these SSRs were analyzed for assessing their potential to develop new markers, unique primer pairs could be designed for 30 per cent to 70 per cent of the total non-redundant microsatellites which were up to 3 per cent of total ESTs in the studied species.

Thiel *et al.* (2003) screened a total of 24595 barley EST sequences corresponding to approximately 12.8 Mega bases (Mb) for microsatellites. The identification and localization of microsatellites were accomplished by PERL5 script (named as MISA) and identified both perfect microsatellites as well as compound microsatellites which are interrupted by a certain number of bases. The search for microsatellite revealed 2019 microsatellites from 1856 ESTs and 136 ESTs contained more than one SSR.

According to Chabane *et al.* (2005), the EST-SSR markers developed in cultivated barley were polymorphic in wild and cultivated varieties and produced high quality markers. Ten of these functional markers were polymorphic across the accessions studied. EST markers indicated a clear separation between wild and cultivated barley than genomic SSRs. The EST-SSRs are a valuable source of new polymorphic markers and should be highly applicable to barley genetic resources, providing a direct estimate of functional biodiversity.

Jia *et al.* (2007) obtained 30 SSRs from 1213 EST sequences and primers were designed for 26 SSRs in foxtail millet. Among them, four pairs of SSR primers amplified polymorphic products in 12 foxtail millet cultivars and one accession of *Setaria viridis*, a wild relative of foxtail millet, with 10 alleles detected for the four loci and 2.5 alleles per locus. In addition, ten SSR markers could be transferred to other nine Gramineae species. The putative functions of 11 ESTs containing polymorphic and transferable SSRs were also identified.

Yadav *et al.* (2007) identified 162 sequences containing SSRs from 6800 *Pennisetum* sp. EST sequences derived from National Center for Biotechnology Information (NCBI) database. Only 19 SSRs were selected for designing primer which consisted of three tetranucleotide repeat, eight trinucleotide repeat and eight dinucleotide repeat. The primers were validated on seven parental lines of four existing mapping population in pearl millet. Eight SSR primer pairs were failed to amplify. Four of the eleven primer pairs amplified were shown polymorphism in high resolution agarose gel (2.5 per cent). In the platforms having higher resolving power than agarose, monomorphic loci showed variation among the parents.

Ramu *et al.* (2013) selected forty five EST-SSR markers from the set of fifty five developed SSRs in sorghum. Total number of alleles yielded from the marker was 360 with an average of nine alleles per marker. Out of the 360 alleles, cultivated sorghum accessions contributed 320 alleles and 257 alleles were given by wild genotypes. The PIC value of the markers ranged from 0.1379 to 0.9483 with an average of 0.523.

Yang *et al.* (2016) screened 121210 unigenes using simple sequence repeat identification tool (SSRIT) and identified 8389 potential SSRs in wheat. Trinucleotide repeats (62.33 per cent) were the most abundant repeat motifs. Three hundred EST-SSRs were randomly selected and tested in five wheat cultivars. Allelic polymorphism was shown by 177 primer pairs and 401 alleles were detected.

Number of alleles per loci ranged from one to six.

2.7.4 EST-SSR in pulses and oilseed crops

Choudhary *et al.* (2008) used two sources for the development of EST-SSR markers in chickpea. They screened 1309 EST sequences available in the NCBI nucleotide database and identified 133 microsatellites. Simultaneously, 159 EST-SSRs were identified from 822 seed ESTs generated from the cDNA library from immature seeds. TROL program was used for identifying the repeat motifs. Trinucleotide repeats (51.2 per cent) were the most abundant followed by dinucleotide (37.3 per cent), tetranucleotide (6.9 per cent) and pentanucleotide (4.9 per cent). One hundred and eighty three primers were designed and 94 were validated in two chickpea genotypes. Thirty four primers were not amplified or produced anomalous sized fragments. Fourty nine primers out of the 60 functional EST-SSR markers produced single expected sized alleles. Two to four alleles were amplified by 11 primers.

Raju *et al.* (2010) identified a total of 5085 unigenes developed based on 9888 ESTs generated in the study and 908 public domain ESTs in pigeon pea. Microsatellites were searched with a Perl script program, MISA for identification and localization of SSRs. The SSR motifs, with repeat units more than five times in dinucleotide repeat, trinucleotide repeat, tetranucleotide repeat, pentanucleotide repeat and hexanucleotide repeats were considered as SSR search criteria in MISA script.

Kumar *et al.* (2015) used CAP3 program for assembling 16473 EST sequences downloaded from NCBI database in cluster bean. After removing the vector sequences and trimming of poly A and poly T tails, 16008 were given as high quality sequences. Using MISA program, 170 SSRs were identified from 2146 unique sequences, of which 151 containing one SSR and 51 had two or three SSR which was considered as compound SSR. Trinucleotide repeats were found to most

abundant (57 per cent) followed by dinucleotide repeat (38.2 per cent) and tetranucleotide repeat (3.5 per cent). Fifty primer pairs were synthesized to amplify and evaluate polymorphism among 32 genotypes. The amplicon size was ranged from 130 bp to 680 bp. Seventy three alleles were obtained from 45 polymorphic loci with an average of 1.87 alleles per primer. The mean PIC value was 0.13.

Sharma *et al.* (2015) retrieved 1025 EST sequences of horse gram from NCBI database and 33 sequences were tagged as contaminants. The remaining 992 sequences were assembled into unigenes or contigs using DNA Starlagergene software. Six hundred and seventeen unigenes were searched for SSR using SSRIT. PRIMER3 software was used for designing primers. Twenty accessions of horse gram were used for the validation of 245 primers pairs. Polymorphism was given by 115 primer pairs and those primers amplified 309 alleles with an average of 2.64 per locus. The cross transferability of the primers were studied in 12 related legume species.

2.7.5 EST-SSR in tuber crops

You *et al.* (2015) identified 5278 SSRs in taro transcriptome data. A total of 2858 primer pairs were designed for marker development. Hundred primers were randomly selected and synthesized. Among them, 72 primer pairs were successfully amplified and 62 were polymorphic in taro accessions. The number of alleles ranged from 2 to 14 for each different polymorphic locus and the PIC value ranged from 0.01 to 0.82. The phylogenetic tree was also constructed to analyze the genetic diversity in 68 taro accessions.

2.7.6 EST-SSR in vegetable crops

Yi *et al.* (2006) developed 10232 non-redundant ESTs in capsicum and 1201 SSRs were found, corresponding to one SSR in every 3.8 kb of the ESTs. Eighteen percent of the EST-SSR was dinucleotide repeats, 66.0 per cent were trinucleotide

repeats, 7.7 per cent tetranucleotide repeats, and 8.2 per cent pentanucleotide repeats. AAG (14 per cent) and AG (12.4 per cent) motifs were the most abundant repeat types. Based on the flanking sequences of these 1201 SSRs, 812 primer pairs that satisfied melting temperature conditions and PCR product sizes were designed. Dinucleotide SSRs and EST-SSR markers containing AC motifs were the most polymorphic.

Jiang *et al.* (2012) used Phred program for selecting ESTs containing quality value more than 20 and sequences containing more than 150 bp in radish. Low quality sequences, vector sequences, contaminated sequences, chimeric sequences were removed by Vector strip, Trimseq and Trimest. There were 5088 unigenes assembled from 10052 ESTs. From 176 unigenes 179 SSRs were identified having a range of length 121 bp to 1199 bp. They stated that 121 unigenes out of 176 unigenes in radish had significant similarity to proteins with the known or predicted functions in Genbank protein database. The unigenes without any predicted functions were considered as novel genes. Most of the unigenes showed similarity with *Arabidopsis* followed by *Brassica* and *Raphanus*. SSRLocator I v.1 software was used for detecting microsatellite. Primer3 was used for designing the primers following the parameters such as product size of 150 bp to 500 bp, 15 bp to 25 bp primer length, 40 per cent to 60 per cent GC content, 45°C to 55°C melting temperature and lack of hairpin structures, primer dimers or occurrence of mismatch.

Izzah *et al.* (2017) analyzed 202 EST sequences from *Brassica oleracea* SSH-cDNA library. These ESTs were assembled using CAP3 software into 12 unigenes consisting of 20 contigs and 152 singletons. They identified twelve EST containing SSR using a web based tool, RepeatMasker software. Trinucleotide repeats were most abundant (50 per cent of the identified SSR) followed by dinucleotide, hexanucleotide and pentanucleotide repeats. Eight primer sets were designed from twelve EST containing sequences. Primers from remaining four sequences were not

designed due to the short flanking sequence. A total of 30 alleles were produced from five polymorphic EST-SSR markers with an average of six alleles per locus.

2.7.7 EST-SSR in fruit crops

Dillon *et al.* (2014) detected 1000 Simple Sequence Repeat motifs from 24,840 ESTs from mango (*Mangifera indica* L.). The most common repeat found was trinucleotide repeats. Twenty five EST-SSR were selected which involved in fruit and flavor development pathways, stress response and plant development. Polymorphism was shown by 24 EST-SSR markers. Five EST-SSR markers failed to show polymorphism within *Mangifera indica* varieties. Eighty six alleles were identified with an average of 5.38 alleles per locus. The EST-SSR marker having the highest number of alleles shows polymorphic information content of 0.843 and the marker having lowest number of alleles show PIC value of 0.036.

Luo *et al.* (2015) developed EST derived marker systems using information from the mango fruit transcriptome. Using simple sequence repeats, 218 of 230 primer pairs showed stable amplification for seven mango genotypes with amplicons ranging from 84 bp to 160 bp. Polymorphic products were yielded by 93 primer pairs. The proportion of polymorphic bands ranged from 16.67 per cent to 100 per cent, with a mean of 55.64 per cent. In contrast, 86 primer pairs exhibited good amplification with clear bands for target region amplification polymorphism analysis, and a total of 66 primer combinations were polymorphic.

2.8 EST-SSR in diversity analysis

Ramu *et al.* (2013) clustered sorghum genotypes based on the geographical origin with the help of factorial analysis and neighbor joining method. Allele numbers, gene diversity, heterozygosity and PIC value were estimated with the help of PowerMarker v3.2 software. Genetic distance was assessed with the help of

DARwin v5 software. Cluster analysis of the genotypes gave eight main clusters with an average gene diversity of 0.5690.

Ferrao *et al.* (2015) analyzed the rate of cross taxa transferability of developed markers in four different species of genus *Coffea* (*C. canephora, C. eugenioides, C. congensis* and *C. racemosa*) and two natural interspecific hybrids (triploid) originated by natural cross between *C. arabica* and *C. racemosa* and Hibrido de Timor originated by natural cross between *C. arabica* and *C. canephora.* They had used two approaches for analyzing genetic diversity. StructureHarvester program was used for categorizing genetic groups based on the Δk values. Based on the value tetraploid and triploids were grouped into one and diploids were categorized into other. In the second approach, the genotypes were grouped based on the Jaccard similarity index and seven subsets were obtained. Four subsets corresponded to diploid species and three were formed by polyploids.

You *et al.* (2015) studied genetic diversity in taro using 69 samples including 63 cultivars and 5 wild wetland taros. Gel images were converted into two binary qualitative data based on the presence or absence of alleles. Data analyses were carried out by NTSYSpc version 2.1 computer program package. Jaccard similarity coefficient was used for pairwise comparisons. The dendrogram was constructed through UPGMA using similarity values.

Kumar *et al.* (2015) used allelic data for grouping cluster bean genotypes based on pair wise dissimilarity indices. The data was analyzed in DARwin v 6.0.7 for the phylogenetic tree and clustered the genotypes into three distinct groups. The relationship among the genotype was visualized using factorial analysis based on simple matching coefficient. Factorial analysis revealed that the genotypes belong to a particular cluster grouped into a single factorial plot.

Sharma *et al.* (2015) clustered horse gram accessions into two groups based on Jaccards similarity coefficient, UPGMA method and principal coordinate analysis using NTSYS pc2.02e. CERVUS version 3.0 was used to calculate diversity measures expected heterozygosity, observed heterozygosity and polymorphic information content.

Izzah *et al.* (2017) used five polymorphic markers for assessing genetic diversity of broccoli cultivars and its related species. UPGMA clustering dendrogram was developed based on genetic similarity values. Clustering analysis was carried out in the NYSYS-PC subprogram, SAHN (Sequential, agglomerative, hierarchical, and nested clustering method). Number of alleles, specific alleles, major allele frequency, expected heterozygosity and polymorphic information content were calculated using Powermarker version 3.25. The genotypes were divided into three major group.

Sahoo *et al.* (2017) grouped 96 genotypes of turmeric and constructed the phylogenetic tree based on dissimilarity matrix with 1000 bootstrap replicates using DARwin software package v 5. DNA profile of 11 SSR loci was scored based on the presence or absence of bands. Ninety six genotypes were grouped into two main clusters.

Materials and Methods

3. MATERIALS AND METHODS

The study on the development and validation of novel Expressed Sequence Tags- Simple Sequence Repeats (EST-SSR) markers in black pepper was carried out using the facilities available in Department of Plant Biotechnology, College of Agriculture (COA), Padannakkad. The present study included mainly three aspects, development of SSR primers from EST data, validation of developed EST-SSRs using different genotypes and studying the genetic relationship among the genotypes.

3.1 MATERIALS

3.1.1 Plant Materials

The plant materials were collected from Hi-Tech black pepper nursery under MIDH (Mission for Integrated development of Horticulture) programme in the Department of Plant Biotechnology, COA, Padannakkad; Indian Council of Agricultural Research-Indian Institute of Spice Research (ICAR-IISR), Kozhikode; ICAR-IISR-Experimental Farm, Peruvannamuzhi, Kozhikode; and Pepper Research Station (PRS), Panniyur. There were 19 varieties and 16 cultivars of black pepper included in the study. Along with *P. nigrum*, other 18 *Piper* species were incorporated in the study (Table 1). The plant materials collected as cuttings were propagated by planting in polybags and kept in the rooting chamber in the pepper nursery for one month. The plant materials collected as rooted cuttings were maintained in the nursery.

3.1.2 Chemicals, glassware and plasticware

The chemicals used in the present study were of good quality (AR grade) procured from HIMEDIA, Merck India Ltd and SRL laboratories. Glass ware was provided by Borosil Glass Works Ltd, India and plastic ware supplied by Tarsons India Ltd. EST-SSR primers were synthesised by Integrated DNA Technologies

(IDT). The reagents used for Polymerase Chain Reaction (PCR) like PCR buffer with $MgCl_2$, dNTP, Taq polymerase enzyme etc were procured from Genei.

Table 1: List of black pepper varieties, cultivars and *Piper* species for testing EST-SSR primers.

Sl no.	Varieties	Pedigree
Place of collection: COA, Padannakkad		
1.	Panniyur 1	Hybrid:Uthirankotta xCheriyakaniyakadan
2	Panniyur 2	Open pollinated progeny selection from Balankotta
3	Panniyur 3	Hybrid: Uthirankottax Cheriyakaniyakadan
4	Panniyur 4	Clonal selection from Kuthiravally Type II
5	Panniyur 5	Open pollinated progeny selection from Perumkodi
6	Panniyur 6	Clonal selection from Karimunda (PRS 22)
7	Panniyur 7	Open pollinated progeny selection from Kalluvally
8	Panniyur 8	Hybrid: Panniyur 6 × Panniyur 5
9	Vijay	Hybrid: Panniyur 2 x Neelamundi
Place of collection: ICAR-IISR, Kozhikode		
10	Panchami	Selection from Aimpiriyan (Coll.856)
11	Pournami	Selection from Ottaplackal (Coll. 812)
12	Sreekara	Clonal selection from Karimunda (KS 14)
13	Subhakara	Clonal selection from Karimunda (KS 27)
14	IISR Thevam	Clonal selection from Thevanmundi
15	IISR Sakthi	Open pollinated progeny from Perambramundi
16	IISR Girimunda	Narayakodi × Neelamundi
17	IISR Malabar excel	Cholamundi × Panniyur-1

Table 1 continued

18	Arka Coorg Excel	Seedling selection
19	PLD- 2	Clonal selection from Kottanadan (Coll.2559)
Sl no.	**Cultivars**	
Place of collection: COA, Padannakkad		
20	Karimunda	
Place of collection: PRS, Panniyur		
21	TMB-2	
22	TMB-4	
23	Palikodi	
Place of collection: ICAR-IISR, Experimental Farm , Peruvannamuzhi		
24	Aimpiriyan	
25	Narayakodi	
26	Vadakkan	
27	Thevanmundi	
28	Arakulam munda	
29	Kottanadan	
30	Arivally	
31	Balankotta	
32	Kalluvally	
33	Kuthiravally	
34	Malamundi	
35	Karivilanchi	
Sl no.	***Piper* species**	
Place of collection: COA, Padannakkad		
36	*P. longum*	
37	*P. colubrinum*	

Table 1 continued

Place of collection: PRS, Panniyur		
38	*P. attenuatum*	
39	*Piper* sp. (North East Fragrance)	
Place of collection: ICAR-IISR, Experimental Farm , Peruvannamuzhi		
40	*P. arboretum*	
41	*P. chaba*	
42	*P. argyrophyllum*	
43	*P. hymenophyllum*	
44	*P. betle*	
45	*P. peepuloides*	
46	*P. thomsoni* (male)	
47	*P. thomsoni*(female)	
48	*P. magnificum*	
49	*P. ornatum*	
50	*P. sylvaticum*	
51	*Piper* sp. (Anand)	
52	*Piper* sp. (Kottakal)	
53	*Piper* sp. (North East)	

3.1.3 Equipments and machinery

Centrifugation was carried out in high speed refrigerated centrifuge (Thermo scientific) and microspin centrifuge (Eltek). Eppendorf Biophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Eppendorf Mastercycler gradient. Molecular imager^{R}Gel Doc^{TM} XR+ (Biorad) with Image lab^{TM} software was used for imaging and documenting the gel.

3.2 METHODS

3.2.1 Detection of SSR in EST sequence

ESTs during berry development in black pepper were produced in an earlier experiment by using Ion Torrent (Ion Personal Genome Machine™, Life Technologies) single-end sequencing technology, which resulted in 1.29 million sequenced reads corresponding to 202 Mb total nucleotides. From these short reads 684 contigs (average length-375 bp) and 1048 unigenes were assembled (Sujatha, unpublished). These unigene sequences were utilized for the present study.

The EST data was searched for all mononucleotide, dinucleotide and trinucleotide repeats. SSRs were detected using microsatellite motif identification program, MIcroSAtellite (MISA) identification tool (http://pgrc.ipk-gatersleben.de/misa/) and Genome-wide Microsatellite Analyzing Tool (GMATo) (https://sourceforge.net/projects/gmato/files/). Specification given for selecting the microsatellite includes minimum repeat length of ten for mononucleotide, six for dinucleotide, five for trinucleotide, tetranuleotide, pentanucleotide and hexanucleotide. The maximum number of bases interrupting two SSRs in a compound microsatellite was given as 100.

MISA tool requires PERL language for the execution. Sequence in FASTA format was required and the criteria for selecting repeat units were given as default setting. The command for executing the files was given in command prompt or cmd.exe. The result was obtained in two files namely statistics file and misa file. GMATo was also used for detecting EST- SSR for confirmation. GMATo requires JAVA script for the execution of the files. This tool is more user-friendly in which the sequence file in FASTA format can be directly browsed. The parameters like repeat times, minimum length and maximum length of repeats were inserted into the

dialogue box. The output was retrieved in the tabular format containing SSR loci information and statistical distribution.

3.2.2 Designing of Primers

Sequence specific forward and reverse primers were designed from 5' end and 3' end of the flanking region of repeat region of eight unigene sequences. Primers were designed using OligoCalc: Oligonucleotide Properties Calculator (basic.northwestern.edu/biotools/OligoCalc.html) based on the following criteria: (1) primer length ranging from 18 bp- 23 bp with 20 bp as the optimum (2) product size ranging from 100 bp- 400 bp (3) melting temperature (Tm) between 53°C and 63°C; (4) a GC content between 40 per cent-70 per cent. Eight forward primers and nine reverse primers were designed for eight unigene sequences.

3.2.3 Genomic DNA extraction

The plant genomic DNA was isolated from young leaf tissue (first or second leaf from the tip) from all the genotypes using modified Cetyl trimethyl ammonium bromide (CTAB) extraction method (Doyle, 1987).

3.2.3.1 Reagents

1) CTAB extraction buffer (2 per cent)- (0.1 *M* Tris, 0.02 *M* EDTA, 4 *M* NaCl, 2 per cent CTAB)
2) Chloroform : isoamyl alcohol (24:1)
3) 5 *M* NaCl
4) Isopropanol
5) 70 per cent Ethanol
6) Ultrapure water or nuclease free water

3.2.3.2 Procedure for DNA isolation

- Ten milli litre of CTAB extraction buffer was preheated to 65° C.

- One gram of fresh young leaf sample of black pepper was weighed and ground to a fine powder in a chilled mortar and pestle using liquid nitrogen(-196°C).
- A pinch of polyvinyl pyrolidone (PVP) was added to the ground leaf tissue and mixed well.
- The sample was transferred into a 50 ml polypropylene centrifuge tube containing 10 ml of preheated CTAB buffer. Forty micro litre of Beta mercaptoethanol was added and mixed by inverting the tubes for 10-15 seconds.
- The tubes containing leaf homogenate was then incubated at 65°C for 30 minutes with occasional mixing in a water bath. The homogenate was later allowed to cool to room temperature and extracted with 4 ml of chloroform: isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 15 minutes at 4°C.
- After the centrifugation, two phases were evident with tissue debris at the bottom and aqueous phase on the top. The aqueous phase was transferred to a fresh 50 ml polypropylene centrifuge tube and extracted again using 4 ml of chloroform: isoamyl alcohol (24:1) followed by centrifugation for 15 minutes at 12000 rpm at 4°C.
- The supernatant was collected to a fresh 50 ml polypropylene centrifuge tube. One and a half volume of chilled isopropanol and half volume of 5 *M* NaCl added into the tube followed by incubation of tubes at -20°C for 30 minutes.
- After incubation, the tubes were centrifuged at 12000 rpm for 10 minutes at 4°C in order to settle the pellet of DNA at the bottom of the tube.
- DNA pellet was washed with 70 per cent ethanol by centrifuging at 10000 rpm for 5 minutes at 4°C.
- Ethanol was discarded and the pellet was air dried and dissolved in 100 µl nuclease free water.
- The dissolved DNA was stored at -20°C.

3.2.4 Assessment of quality and quantity of DNA using eppendorf biophotometer

The quantity and quality of DNA were estimated using Biophotometer. Before taking the sample readings, the blank was adjusted to zero by taking 50 µl distilled water in the cuvette. Five micro litre of genomic DNA was diluted to 50 µl by adding 45 µl nuclease free water. Fifty micro litre of diluted DNA sample was measured at a wavelength of 260 nm and 280 nm and A_{260}/ A_{280} ratio was recorded to assess the purity of the DNA. A_{260}/A_{280} value of 1.8-2.0 indicates good quality DNA.

3.2.5 Assessment of Quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through 0.7 per cent agarose gel electrophoresis.

3.2.5.1 Materials required

- Agarose
- 1X Tris-Acetate EDTA (TAE) buffer
- Ethidium bromide (0.5 µg/ml)
- 6 X gel loading / Tracking dye
- Casting tray
- Electrophoresis unit
- Gel documentation system

3.2.5.2 Procedure

- 0.7 g agarose was weighed and melted in 100 ml 1X TAE buffer by boiling. The agarose was heated until it is dissolved.
- Cooled the gel to room temperature. Five micro litre of ethidium bromide (0.5µg/ml) was added and thoroughly mixed by swirling before pouring into the gel apparatus.

- The gel casting tray and comb were washed with distilled water in order to remove dirt. The open end of the gel casting tray was sealed with cellophane tape and kept on a horizontal surface.
- The casting trays were fitted with properly cleaned combs. The gel was poured on to the casting trays without making any air bubble.
- The gel was allowed to solidify at room temperature for 30 minutes. After solidification, combs were removed gently by pouring a little amount of buffer.
- The gel was immersed slowly into the gel tank containing 1X TAE buffer with wells directed towards the cathode.
- One micro litre of each DNA sample was mixed with one micro litre gel loading dye and was loaded into the wells without poking the gel. An aliquot of a standard molecular weight of 100 bp fragment (Thermo scientific) was used to assess the size of the DNA fragment.
- The gel was run at a constant voltage (80 V) for one hour. The power was turned off when the tracking dye reached 2/3rd length of the gel.
- The gel was observed under ultraviolet rays and the image was documented in gel documentation system. The image was examined for intactness and clarity of DNA band.

3.2.6 Screening of EST-SSR primers

Primer screening was done using Panniyur-1 DNA. The melting temperatures of forward and reverse EST-SSR primers are given in Table 2.

Table 2: Estimates of melting temperature of EST-SSR primers

Sl no.	Forward primer	Melting temperature	Reverse primer	Melting temperature
1	PNSF1	59.1°C	PNSR1	53.9°C
2	PNSF2	56.2°C	PNSR2	51.5°C

3	PNSF3	53.5°C	PNSR3	54.1°C
4	PNSF4	59.4°C	PNSR4a	53.1°C
5	PNSF4	59.4°C	PNSR4b	50.5°C
6	PNSF5	58.7°C	PNSR5	55.4°C
7	PNSF6	54.1°C	PNSR6	53.0°C
8	PNSF7	56.5°C	PNSR7	50.8°C
9	PNSF8	58.7°C	PNSR8	54.5°C

Gradient PCR was carried out to fix the optimum annealing temperature for each primer set, based on the melting temperature of forward and reverse primers. The PCR was set to the temperature gradient of 5°C with a mean temperature of 53°C and the temperature profile was in the range of 48.2°C- 58.4°C.

PCR amplification using genomic DNA of Panniyur-1 was carried out with different EST-SSR primer sets. The PCR reaction mix was set up with the components: 1X PCR Buffer with $MgCl_2$, 200 μM dNTPs, 0.05 μM each forward and reverse primers, 1U Taq polymerase and 50 ng DNA template.

Thermal profile was programmed as follows

Initial denaturation	- 94°C	- 2 minutes	
Denaturation	- 92°C	- 1 minute	40 cycles
Annealing	- 48.2°C - 58.4°C	- 1 minute	
Extension	- 72°C	- 1 minute	
Final extension	- 72°C	- 10 minutes	
Hold	- 4°C		

Each EST-SSR primer set was screened at four different temperatures which are given in the Table 3. The optimum annealing temperature was decided based on the amplification pattern.

Table 3: Standardization of annealing temperature of selected EST-SSR primers using black pepper variety Panniyur-1

Primer set	T1	T 2	T 3	T 4	Selected temperatures
PNS1	53.4°C	54.8°C	56.1°C	57.1°C	**56.1°C**
PNS2	49.6°C	50.7°C	53.4°C	54.8°C	-
PNS3	52.0°C	53.4°C	54.8°C	56.1°C	**54.8°C**
PNS4a	52.0°C	53.4°C	54.8°C	56.1°C	**56.1°C**
PNS4b	49.6°C	50.7°C	53.4°C	-	-
PNS5	53.4°C	54.8°C	56.1°C	57.1°C	-
PNS6	52.0°C	53.4°C	54.8°C	-	**53.4°C**
PNSF7	49.6°C	50.7°C	52.0°C	54.8°C	-
PNS8	53.4°C	54.8°C	56.1°C	58.0°C	**56.1°C**

T1- Temperature 1, T2- Temperature 2, T3- Temperature 3, T4- Temperature 4

3.2.7 Validation of EST-SSR primers

The selected five EST-SSR primer sets were validated using fifty three genotypes including black pepper varieties, cultivars and different species of *Piper* (Table 1). PCR amplification using genomic DNA of fifty three genotypes was carried out with selected five EST-SSR primer sets at optimum annealing temperature. The PCR reaction mix was set up with the components: 1X PCR Buffer with $MgCl_2$, 200 μM dNTPs, 0.05 μM each forward and reverse primers, 1U Taq polymerase and 50 ng DNA template.

Thermal profile was programmed as follows

Initial denaturation	- 94°C	- 2 minutes	
Denaturation	- 92°C	- 1 minute	40 cycles
Annealing	- 52°C - 56°C	- 1 minute	
Extension	- 72°C	- 1 minute	

Final extension	- 72°C	- 10 minutes
Hold	- 4°C	

PCR products of each primer set were loaded into 4 per cent agarose gel. In order to assess the size of the amplified product, 50 bp ladder was used.

3.2.8 Polyacrylamide gel electrophoresis (PAGE)

PAGE is more advantageous than agarose gel since it is having greater resolving power. PAGE was carried out in order to confirm the result obtained by agarose gel electrophoresis.

3.2.8.1 Reagents

- 40 per cent Acrylamide mix (19:1- Acrylamide:Bisacrylamide)
- 10 per cent Ammonium persulphate (APS)
- Tetra methyl ethylene diamine (TEMED)
- 10X Tris-Borate EDTA (TBE)

3.2.8.2 Procedure

- Glass plates and spacers were cleaned thoroughly with distilled water and ethanol. Glass plates were assembled with spacers.
- The gel solution was prepared with a concentration of 6 per cent. The following components are essential for preparing for 10 ml of gel solution.

40 per cent Acrylamide	-	2 ml
10X TBE (pH-8)	-	0.5 ml
10% APS	-	100 µl
TEMED	-	10 µl
Distilled water	-	7.39 ml
		10 ml

- After pouring the gel, the comb was inserted carefully without making any bubbles.
- Acrylamide was allowed to polymerize for 30 minutes to 45 minutes at room temperature.
- The comb was removed after the polymerization of the gel. The wells were washed thoroughly with the buffer using syringe. Then the gel was allowed to pre run for 30 minutes at 80 V.
- The wells were washed after pre running. The samples were loaded into the wells after mixing it with an equal volume of 6 X loading buffer.
- The electrodes were connected to power pack, turn on the power and start the electrophoresis run.
- The gel was allowed to run in 0.5 X TBE for 4 hours at 80 V.
- The gel was run till the marking dye reaches desired distance.
- After electrophoresis, glass plates were detached and allowed for staining.
- Ethidium bromide was used for staining the gel. The gel was soaked in ethidium bromide solution (0.5µg/ml) for 15 minutes followed by soaking in distilled water for 15 minutes.

3.2.9 Data Analysis

Sizes of amplified fragments were estimated with the aid of Image lab software by gel documentation system using 50 bp as size standards. The amplicons obtained using five EST-SSR primer combinations were scored as binary matrix based on the presence (1) or absence (0) of the alleles. Allelic scoring was also done and analysed with the help of software, DARwin (Dissimilarity Analysis and Representation) version 6 (Perrier *et al.*, 2003). Analysis was done based on the dissimilarity between the individuals.

3.2.9.1 Polymorphism information content

Polymorphism information content (PIC) was calculated in order to measure the informativeness of the marker. PIC value was calculated based on the following equation (Weir, 1990)

$$PIC= 1-\sum (P_i)^2$$

where P_i is the frequency of the i^{th} allele in the genotypes examined

3.2.9.2 Cross species transferability

Cross species transferability of the EST-SSR marker was calculated as proportion of primers showing successful amplification for each species based on the following formula (Ferrao *et al.*, 2015)

$$T_{MAR}= SA_{esp}/NM_{esp}$$

where T_{MAR} is the transferability of EST-SSR markers, SA_{esp} is the successful amplification of primer sets in each species and NM_{esp} is the total number of primers used for the analysis of each species.

3.2.9.3 Analysis on genetic relationship

Genetic diversity among fifty three genotypes was determined by subjecting the binary data matrix as well as allelic data to cluster analysis. The 0/1 matrix was used to calculate dissimilarity value between two pairs of genotypes using dice coefficient.

3.2.10 Putative functions of EST sequences

Putative functions of the five unigene sequences containing polymorphic and transferable SSRs were identified by blastn (http://www.ncbi.nlm.nih.gov/).

Results

4. RESULTS

An investigation was carried out to develop EST-SSR markers in black pepper and to validate the developed EST-SSR markers in varieties and cultivars of black pepper. Genetic diversity among the genotypes belonging to *Piper* species was also done using the developed EST-SSR markers. The results of SSR detection, development of markers, amplification of genomic DNA and analysis of molecular data are presented below.

4.1 DETECTION OF EST-SSR

Totally 1048 unigene sequences having a total length of 518179 bp (approximately 0.5 Mb) were examined for detecting the SSRs. Sixty eight unigene sequences containing SSR, which accounts for 6.49 per cent of all the sequences, were identified using microsatellite detection tools, MISA and GMATo (Table 4). Seventy SSRs were identified from sixty eight sequences. Two sequences were identified with more than one SSR, referred as compound SSR.

4.1.1 Frequency of SSRs

Analysis of the nucleotide sequences containing SSR revealed that mononucleotides were most abundant, represented by 88.6 per cent among all the identified SSRs. Dinucleotide repeats and trinucleotide repeats accounted for 2.8 per cent and 8.6 per cent respectively (Table 5).

4.1.2 Distribution of microsatellite classes

Totally 12 types of repeat motifs were observed in EST-SSR sequences including four types of mononucleotide repeats, two types of dinucleotide repeats and six types of trinucleotide repeats. AGG, CAC, GCG, TCA, TCT and TGG were the trinucleotide repeats present in the EST sequences. AT and TA were dinucleotide repeats identified from the EST sequences. $(T)_{10}CCCGAGCC(AT)_7$ and $(C)_{10}G(C)_{10}$

were the two compound SSRs found in the sequence. A, C, G and T were mononucleotide repeats found in high frequency than dinucleotide and trinucleotide repeats. The number of SSR repeat motif was in the range of 5 to 12. The trinucleotide repeats AGG, CAC, GCG, TCA and TCT were repeated five times. TGG was repeated for eight times and dinucleotide repeats, AT and TA was repeated seven and nine times respectively. Highest repeat motif was for the mononucleotide, A_{12}. Frequencies of identified SSR motifs are summarized in Table 5.

4.2 PRIMER DESIGNING

Ten SSR motifs including dinucleotide repeat, trinucleotide repeat and compound SSR were selected for designing primers. One of the repeat motifs was omitted since it is located on the border of the unigene sequence. One more dinucleotide repeat was also avoided because it is a part of a selected compound SSR. Mononucleotide repeat motifs were not selected for designing primer. Nine sets of primers including forward and reverse primers with adequate length, GC content, melting temperature were designed using OligoCalc: Oligonucleotide Properties Calculator. The product size was in the range of 180 bp to 252 bp. Two reverse primers were designed for the fourth set (PNS4) since the product size was small (146 bp) for one reverse primer. The details of the EST-SSR primers are included in Table 6.

Table 4. Sequence characteristics of black pepper unigenes carrying repeat motifs

Sl no	Name of the unigene	Length of the unigene (bp)	Repeat motif of SSR	Start region of SSR in unigene	End region of SSR in unigene
1	Pnc 230	830	$(A)_{12}$	203	214
2	Pnc 31	636	$(A)_{11}$	609	619
3	Pnc 90	1023	$(A)_{11}$	981	991
4	Pnc 254	351	$(A)_{11}$	320	330
5	Pnc 329	244	$(A)_{11}$	195	205
6	Pnc 522	346	$(A)_{11}$	249	259
7	Pnc 30	668	$(A)_{11}$	50	60
8	Pnc c9	1006	$(A)_{10}$	923	932
9	Pnc 52	671	$(A)_{10}$	636	645
10	Pnc 113	775	$(A)_{10}$	744	753
11	Pnc 127	522	$(A)_{10}$	72	81
12	Pnc 165	660	$(A)_{10}$	645	654
13	Pnc 186	1488	$(A)_{10}$	1134	1143
14	Pnc 199	470	$(A)_{10}$	343	352
15	Pnc 201	765	$(A)_{10}$	750	756
16	Pnc 207	842	$(A)_{10}$	813	822
17	Pnc 219	283	$(A)_{10}$	28	37
18	Pnc 240	309	$(A)_{10}$	238	247
19	Pnc 296	454	$(A)_{10}$	438	447
20	Pnc 382	273	$(A)_{10}$	237	246
21	Pnc 387	579	$(A)_{10}$	311	320
22	Pnc 461	195	$(A)_{10}$	151	160
23	Pnc 508	133	$(A)_{10}$	97	106
24	Pnc 544	364	$(A)_{10}$	94	103
25	Pnc 625	278	$(A)_{10}$	246	255
26	Pnc 690	213	$(A)_{10}$	177	186
27	Pnc 308	409	$(A)_{10}$	77	86
28	Pnc 553	225	$(A)_{10}$	42	51
29	Pnc 303	529	$(C)_{11}$	64	74
30	Pnc 458	241	$(C)_{11}$	12	22
31	Pnc 629	402	$(C)_{11}$	4	14
32	Pnc 599	377	$(C)_{10}$	12	21
33	Pnc 583	188	$(C)_{10}$	10	19
34	Pnc 560	237	$(C)_{10}G(C)_{10}$	8	28
35	Pnc 454	840	$(G)_{10}$	158	167
36	Pnc 51	1002	$(T)_{11}$	28	38
37	Pnc 109	685	$(T)_{11}$	23	33
38	Pnc 297	1472	$(T)_{11}$	37	47
39	Pnc 316	630	$(T)_{11}$	22	32
40	Pnc 75	734	$(T)_{11}$	28	38
41	Pnc 105	756	$(T)_{11}$	32	42
42	Pnc 7	926	$(T)_{10}$	31	40
43	Pnc 28	833	$(T)_{10}$	29	38
44	Pnc 55	1224	$(T)_{10}$	37	46
45	Pnc 100	541	$(T)_{10}$	6	15

Table 4 continued

46	Pnc 137	622	$(T)_{10}$	33	42
47	Pnc 185	1044	$(T)_{10}$	7	16
48	Pnc 193	745	$(T)_{10}$	175	184
49	Pnc 251	379	$(T)_{10}$	212	221
50	Pnc 354	388	$(T)_{10}$	13	22
51	Pnc 367	532	$(T)_{10}$	201	210
52	Pnc 432	585	$(T)_{10}$	16	25
53	Pnc 481	451	$(T)_{10}$	29	38
54	Pnc 500	471	$(T)_{10}$	7	16
55	Pnc 507	320	$(T)_{10}$	8	17
56	Pnc 533	319	$(T)_{10}$	8	17
57	Pnc 536	303	$(T)_{10}$	26	35
58	Pnc 13	676	$(T)_{10}$	25	34
59	Pnc 17	475	$(T)_{10}$	127	136
60	Pnc 45	711	$(T)_{10}$	36	45
61	Pnc 59	1332	$(TA)_9$	1278	1295
62	Pnc 554	362	$(T)_{10}$ CCCGAGCC$(AT)_7$	108	139
63	Pnc 57	784	$(TGG)_8$	252	275
64	Pnc 68	495	$(TCA)_5$	189	203
65	Pnc 80	813	$(AGG)_6$	643	660
66	Pnc 105	756	$(GCG)_5$	32	46
67	Pnc 116	836	$(CAC)_5$	79	93
68	Pnc 302	738	$(TCT)_5$	332	346

Table 5. Features and frequencies of identified SSR motifs

Type of SSR	**Repeat motif**	**Number of repeats**	**Total**	**Grand total**	**Frequency (per cent)**
Mononucleotide	$(A)_{10}$	21	28	62	88.6
	$(A)_{11}$	6			
	$(A)_{12}$	1			
	$(C)_{11}$	3	7		
	$(C)_{10}$	4			
	$(G)_{10}$	1	1		
	$(T)_{11}$	6	26		
	$(T)_{10}$	20			
Dinucleotide	$(TA)_9$	1	1	2	2.8
	$(AT)_7$	1	1		
Trinucleotide	$(TGG)_8$	1	1	6	8.6
	$(TCA)_5$	1	1		
	$(AGG)_6$	1	1		
	$(GCG)_5$	1	1		
	$(CAC)_5$	1	1		
	$(TCT)_5$	1	1		

Table 6. EST-SSR primer combinations designed from selected unigene sequences of black pepper

Primer name	Forward/ Reverse	Primer sequence (5'---3')	Number of base pair	GC %	Tm	Amplicon size (bp)	Sequence details
PNS1	F1	TATTTGCATCCCGGAGCGCAT	21	52	61.2	212	$(TGG)_8$
	R1	GAGCTTCAAGAGACAACAATGG	22	45	60.1		
PNS2	F2	CCATCCAAGGTCAATGCAGAATC	23	48	62.9	200	(TCA)5
	R2	CCACATCCAACTTTATTCTTCC	22	41	58.4		
PNS3	F3	GGTGAAGAAGGAGGAAGTAGT	21	48	59.5	201	(AGG)6
	R3	GATCATGCGTTTTACACAGGG	21	48	59.5		
PNS4a	F4	GGGCACGGTACCAGAGGA	18	67	60.8	146	(GCG)5
	R4a	GCAGACACAACATAGATCCC	20	50	58.4		
PNS4b	R4b	CAGATCACTATTCCACAACC	20	45	56.4	252	
PNS5	F5	TCGCGGCCAGTACACTACAA	20	55	60.5	188	(CAC)5
	R5	CATCGTTTAGGGAAGCTAGGC	21	52	61.2		
PNS6	F6	CGACAATGCGACGAAACAAATA	22	41	58.4	224	$(T)_{10}$CCCGAGCC $(AT)_7$
	R6	CAAATGGCTTGATCGAGATGA	21	43	57.5		
PNS7	F7	ATTCCTTCAGGCCAATCCTTCA	22	45	60.1	182	(TA)9
	R7	GTATAAACGGTCCAATGTAGTC	22	41	58.4		
PNS8	F8	AACTGACTGTCCACGGCTTTCT	22	50	62.1	201	(TCT)5
	R8	TCAGTCCTATGTGATCGCAAC	21	48	59.5		

Tm- melting temperature

4.3 ISOLATION AND QUANTIFICATION OF GENOMIC DNA

DNA was isolated from fifty three genotypes including black pepper varieties, cultivars and different species of *Piper* (Table 7) for amplification by selected EST-SSR primer sets. The extracted DNA was quantified using Biophotometer. The concentration of the extracted DNA was checked by 0.7 per cent agarose gel electrophoresis. Electrophoresis revealed single high molecular weight band without any degradation of the sample (Plate 1). Ratio of Absorbance at 260 nm and Absorbance at 280 nm for the various samples is provided in Table 7. A slight reduction in quality of DNA was due to polyphenols present in leaf tissues. Negligible amount of RNA contamination was observed in 13 genotypes. Working concentration of the DNA samples was adjusted to 100 ng/µl.

Table 7. Quantity and quality of genomic DNA isolated from different *Piper* genotypes

Sl No	Samples	Concentration (µg/µl)	A_{260} /A_{280}
1	Panniyur-1	129.1	1.79
2	Panniyur-2	89.7	1.85
3	Panniyur-3	123.3	1.92
4	Panniyur-4	136.5	1.86
5	Panniyur-5	95.0	1.39
6	Panniyur-6	120.1	1.79
7	Panniyur-7	39.8	1.97
8	Panniyur-8	67.0	1.80
9	Vijay	63.8	1.99
10	Panchami	18.8	2.02
11	Pournami	20.8	1.95

Table 7 continued

12	Sreekara	9.8	1.93
13	Subhakara	5.0	2.14
14	IISR Thevam	52.4	2.00
15	IISR Sakthi	22.3	1.99
16	IISR Girimunda	26.3	1.89
17	Malabar excel	20.5	1.82
18	Arka coorg excel	14.6	2.03
19	Palode-2	22.5	1.79
20	Karimunda	120.7	1.92
21	Palikodi	21.9	1.98
22	Aimpipriyan	31.4	1.91
23	Narayakodi	121.4	1.81
24	Vadakkan	57.8	1.90
25	Thevanmundi	34.8	1.96
26	Arakulam munda	20.8	1.81
27	Kottanadan	34.8	2.03
28	Arivally	23.5	1.80
29	Balankotta	46.1	1.89
30	Kalluvally	44.5	1.85
31	Kuthiravally	25.6	1.72
32	Malamundi	17.0	1.78
33	Karivilanchi	57.9	1.89
34	TMB-2	16.9	2.01
35	TMB-4	8.8	2.14
36	*P. longum*	44.5	1.72
37	*P. attenuatum*	29.5	1.98
38	*P. colubrinum*	14.8	1.84

Table 7 continued

39	*P. arboretum*	107.7	1.94
40	*P. chaba*	49.4	1.93
41	*P. argyrophyllum*	40.6	1.90
42	*P. hymenophyllum*	100.5	1.96
43	*P. betle*	68.4	1.97
44	*P. peepuloides*	40.8	2.03
45	*P. thomsoni* (male)	99.0	1.89
46	*P. thomsoni* (female)	93.2	1.97
47	*P. magnificum*	13.6	1.95
48	*P. ornatum*	37.3	1.94
49	*P. sylvaticum*	105.6	1.94
50	*Piper* sp. (Anand)	37.6	1.94
51	*Piper* sp. (Kottakkal)	49.0	1.84
52	*Piper* sp. (North East)	55.7	1.87
53	*Piper* sp. (North East Fragrance)	30.2	1.93

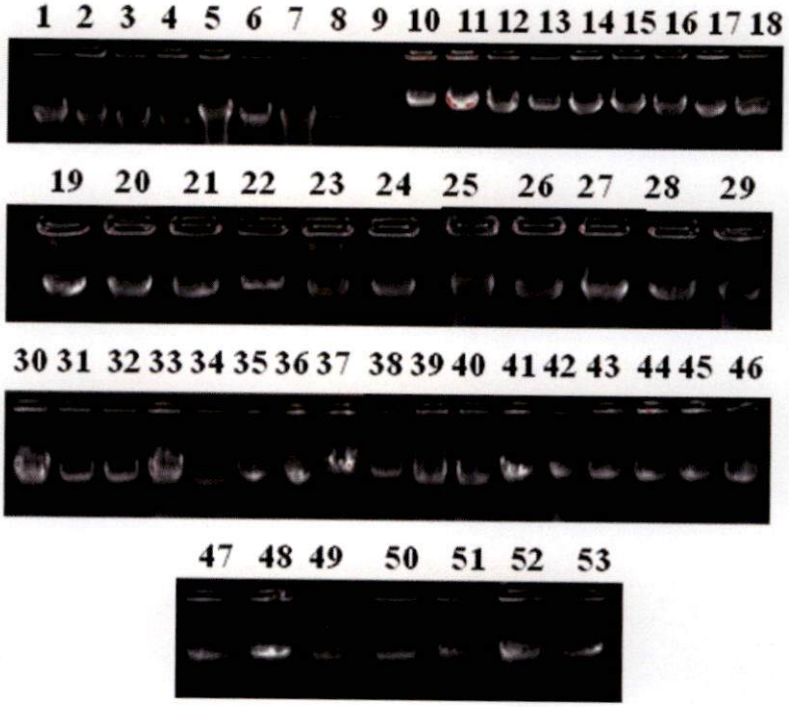


Plate 1. Agarose gel electrophoresis of genomic DNA

1- Panniyur-1, **2**- Panniyur-2, **3**- Panniyur-3, **4**- Panniyur-4, **5**- Panniyur-5, **6**-Panniyur-6, **7**- Panniyur-7, **8**- Panniyur-8, **9**- Vijay, **10**- Panchami, **11**- Pournami, **12**- Sreekara, **13**-Subhakara, **14**- IISR Thevam, **15**- IISR Sakthi, **16**- IISR Girimunda, **17**- IISR Malabar Excel, **18**- Arka Coorg Excel, **19**- PLD-2, **20**- Karimunda, **21**- Paalikodi, **22**- Aimpiriyan, **23**-Narayakodi, **24**- Vadakkan, **25**- Thevanmundi, **26**- Arakulam munda, **27**- Kottanadan, **28**-Arivally, **29**- Balankotta, **30**- Kalluvally, **31**- Kuthiravally, **32**- Malamundi, **33**- Karivilanchi, **34**- TMB-2, **35**- TMB-4, **36**- *P. longum*, **37**- *P. attenuatum*, **38**- *P. colubrinum*, **39**- *P. arboreum*, **40**- *P. chaba*, **41**- *P. argyrophyllum*, **42**- *P. hymenophyllum*, **43**- *P. betle*, **44**- *P. peepuloides*, **45**- *P. thomsoni*(male), **46**- *P. thomsoni*(female), **47**- *P. magnificum*, **48**- *P. ornatum*, **49**- *P. sylvaticum*, **50**- *Piper* sp. (Anand), **51**- *Piper* sp. (Kottakkal), **52**- *Piper* sp. (North East), **53**- *Piper* sp. (North East Fragrance)

4.4 SCREENING OF EST-SSR PRIMERS

Nine sets of primers designed from eight unigene sequences that contain dinucleotide repeat, trinucleotide repeat and compound repeat were screened using DNA of Panniyur-1 (Plate 2) at different annealing temperature. Amplification was strong at 56.1°C for PNS1, PNS4a and PNS8, 54.8°C for PNS3 and 53.4°C for PNS6. Based on the clarity and specificity of amplification, five primers *viz*., PNS1, PNS3, PNS4a, PNS6 and PNS8 were selected for further analysis (Table 8). The amplicons produced by PNS2 and PNS5 were faint and not of the expected size in PNS2. Amplification was not observed in the primer PNS7. PNS4a and PNS4b are the primer sets for the same region and good amplification was given by PNS4a. The annealing temperature of each primer was standardized based on the intensity of bands.

Table 8. Expected and observed product size of different primers

Sl No	Primer sets	Expected product size (bp)	Observed product size	Selected primers	Selected annealing temperature
1	PNS1	212	350 bp	✓	56.1°C
2	PNS2	200	No clear amplification	-	-
3	PNS3	201	250 bp	✓	54.8°C
4	PNS4a	146	200 bp	✓	56.1°C
5	PNS4b	252	Multiple bands	-	-
6	PNS5	188	No clear amplification	-	-
7	PNS6	224	225 bp	✓	53.4°C
8	PNS7	182	No clear amplification	-	-
9	PNS8	201	250 bp	✓	56.1°C

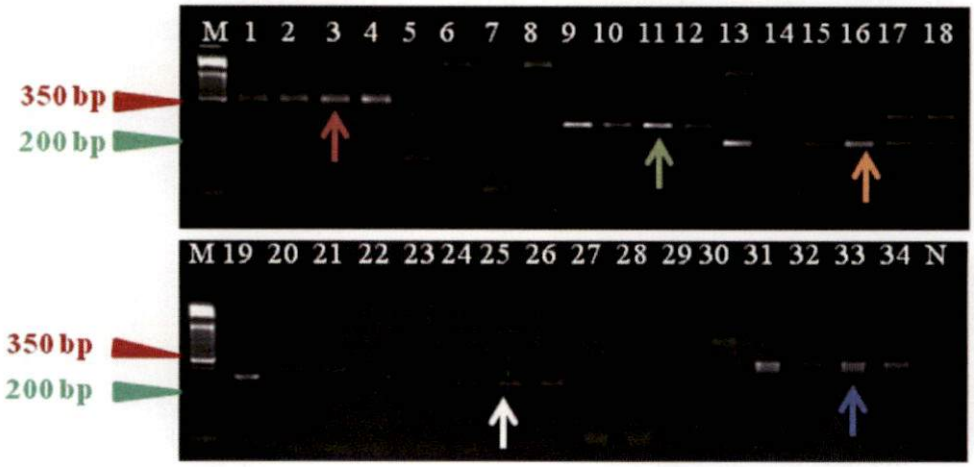


Plate 2. EST-SSR primer screening at different temperature using Panniyur-1 DNA :

M- 50 bp ladder

PNS1 at **(1)** 53.4°C **(2)** 54.8°C **(3)** 56.1°C **(4)** 57.1°C

PNS2 at **(5)** 49.6°C **(6)** 50.7°C **(7)** 53.4°C **(8)** 54.8°C

PNS3 at **(9)** 52.0°C **(10)** 53.4°C **(11)** 54.8°C **(12)** 56.1°C

PNS4a at **(13)** 52.0°C **(14)** 53.4°C **(15)** 54.8°C **(16)** 56.1°C

PNS4b at **(17)** 49.6°C **(18)** 50.7°C **(19)** 53.4°C

PNS5 at **(20)** 53.4°C **(21)** 54.8°C **(22)** 56.1°C **(23)** 57.1°C

PNS6 at **(24)** 52.0°C **(25)** 53.4°C **(26)** 54.8°C

PNS7 at **(27)** 49.6°C **(28)** 50.7°C **(29)** 52.0°C **(30)** 54.8°C

PNS8 at **(31)** 53.4°C **(32)** 54.8°C **(33)** 56.1°C **(34)** 58°C

N- Negative control

4.5 VALIDATION OF EST-SSR PRIMERS

Five primers among nine designed primers (Table 6) were used for validation in fifty three genotypes comprising of black pepper varieties, black pepper cultivars and *Piper* species. The PCR products were run on 4 per cent agarose gel to view the amplification and to detect polymorphism, if any. All the five primers successfully amplified the target region in different genotypes. Polymorphic as well as monomorphic bands were obtained and in most of varieties and cultivars, monomorphism was observed. Totally, 34 alleles were obtained from five EST-SSR markers. The average number of alleles per locus was 6.8.

4.5.1 EST-SSR marker PNS1

Among the fifty three genotypes, forty nine genotypes were amplified efficiently. Amplification was not observed in Panniyur-4, Sreekara, *P. colubrinum*, *P. magnificum*. The number of alleles observed per PNS1 was eight. Specific bands observed in this primer were in the size of 400 bp, 370 bp, 360 bp, 350 bp, 340 bp, 330 bp and 320 bp. The expected size of the amplicon is 212 bp. Varieties and cultivars of black pepper had shown band size in the range 320 bp to 370 bp. Longest amplicon (400 bp) was obtained in *Piper* sp. (North East) (Plate 3, lane 53). Faint bands amplified in *Piper* species were omitted and bands with good intensity were considered for scoring. The alleles observed in each genotype are given in Table 9.

4.5.2 EST-SSR primer PNS3

The primer PNS3 gave a good amplification pattern in all varieties and cultivars except in Pournami. Very faint amplification was observed in *P. magnificum* and *Piper* sp. (Kottakkal). There were seven alleles obtained using the primer PNS3. Specific bands obtained in this primer were 230 bp, 220 bp, 210 bp, 200 bp, 190 bp, 180 bp and 110 bp (Plate 4). The expected size of the amplicon was 201 bp which is congruent with the observed bands except for the allele 110 bp found

only in *P. colubrinum* and *P. arboreum*. Polymorphism was observed within black pepper varieties, cultivars and *Piper* spp. A unique band of 180 bp was observed in *Piper* sp. (Anand) (Plate 4, lane 50). Two alleles having a size of 230 bp and 210 bp were present in *Piper* sp. (North East). The alleles present in each genotype are listed in Table 9.

4.5.3 EST-SSR marker PNS4a

The primer PNS4a gave good amplification in all the varieties, cultivars and *Piper* species except in Panniyur-3 and *Piper* sp. (Kottakkal) (Plate 5). A faint band was observed in *P. sylvaticum*. The expected size of the amplicon is 146 bp which is in harmony with the observed bands. There were four alleles with size 160 bp, 150 bp, 140 bp and 130 bp. The monomorphic band having a size of 150 bp was present in all the cultivars of black pepper. There were two bands having a size of 150 bp and 130 bp present in TMB-4. *P. peepuloides* and *P. thomsoni* (male) are the species having additional band having a size of 130 bp other than 150 bp. An allele of size 160 bp was present in *P. ornatum*, *Piper* sp. (Anand), *Piper* sp. (North East). The alleles present in each genotype are given in Table 9.

4.5.4 EST-SSR marker PNS6

The primer PNS6 gave amplification in forty six genotypes. The expected size of the amplicon is 224 bp and the alleles obtained were 400 bp, 350 bp, 320 bp, 310 bp, 300 bp, 280 bp, 270 bp, 250 bp, 240 bp and 230 bp. In varieties and cultivars alleles were in the size of 250 bp, 240 bp and 230 bp. Polymorphic bands were present in the *Piper* species with a band size of 400 bp, 350 bp, 320 bp, 310 bp, 300 bp, 280 bp, 270 bp and 250 bp (Plate 6). The cultivar, Kalluvally and the species *viz*., *P. attenuatum*, *P. colubrinum*, *P. thomsoni* (male), *P. magnificum*, *Piper* sp. (North East) and *Piper* sp. (North East Fragrance) were the genotypes not amplified using PNS6. The alleles found in each genotype are listed in Table 9.

4.5.5 EST-SSR marker PNS8

The primer PNS8 gave good amplification in all genotypes except in Subhakara. The expected size of the amplicon was 201 bp and alleles obtained were in the size range of 340 bp, 330 bp, 320 bp, 310 bp, 300 bp and 290 bp. Two alleles were present in all the genotypes. Monomorphism was observed in some varieties and cultivars of black pepper (Plate 7). The alleles present in each genotype are given in Table 9.

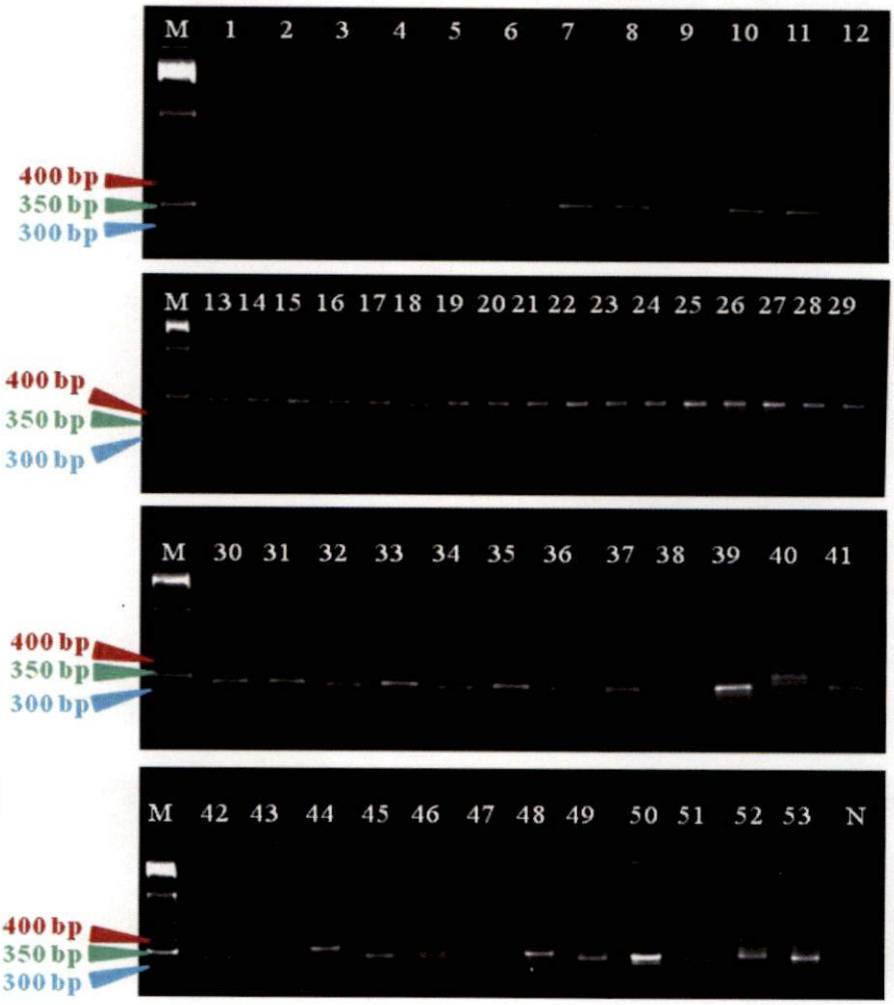


Plate 3. Validation of PNS1 EST-SSR marker in fifty three genotypes

1- Panniyur-1, **2**- Panniyur-2, **3**- Panniyur-3, **4**- Panniyur-4, **5**- Panniyur-5, **6**-Panniyur-6, **7**- Panniyur-7, **8**- Panniyur-8, **9**- Vijay, **10**- Panchami, **11**- Pournami, **12**- Sreekara, **13**- Subhakara, **14**- IISR Thevam, **15**- IISR Sakthi, **16**- IISR Girimunda, **17**- IISR Malabar Excel, **18**- Arka Coorg Excel, **19**- PLD-2, **20**- Karimunda, **21**- Paalikodi, **22**- Aimpiriyan, **23**- Narayakodi, **24**- Vadakkan, **25**- Thevanmundi, **26**- Arakulam munda, **27**- Kottanadan, **28**- Arivally, **29**- Balankotta, **30**- Kalluvally, **31**- Kuthiravally, **32**- Malamundi, **33**- Karivilanchi, **34**- TMB-2, **35**- TMB-4, **36**- *P. longum*, **37**- *P. attenuatum*, **38**- *P. colubrinum*, **39**- *P. arboreum*, **40**- *P. chaba*, **41**- *P. argyrophyllum*, **42**- *P. hymenophyllum*, **43**- *P. betle*, **44**- *P. peepuloides*, **45**- *P. thomsoni*(male), **46**- *P. thomsoni*(female), **47**- *P. magnificum*, **48**- *P. ornatum*, **49**- *P. sylvaticum*, **50**- *Piper* sp. (Anand), **51**- *Piper* sp. (Kottakkal), **52**- *Piper* sp. (North East), **53**- *Piper* sp. (North East Fragrance), **N**- Negative control

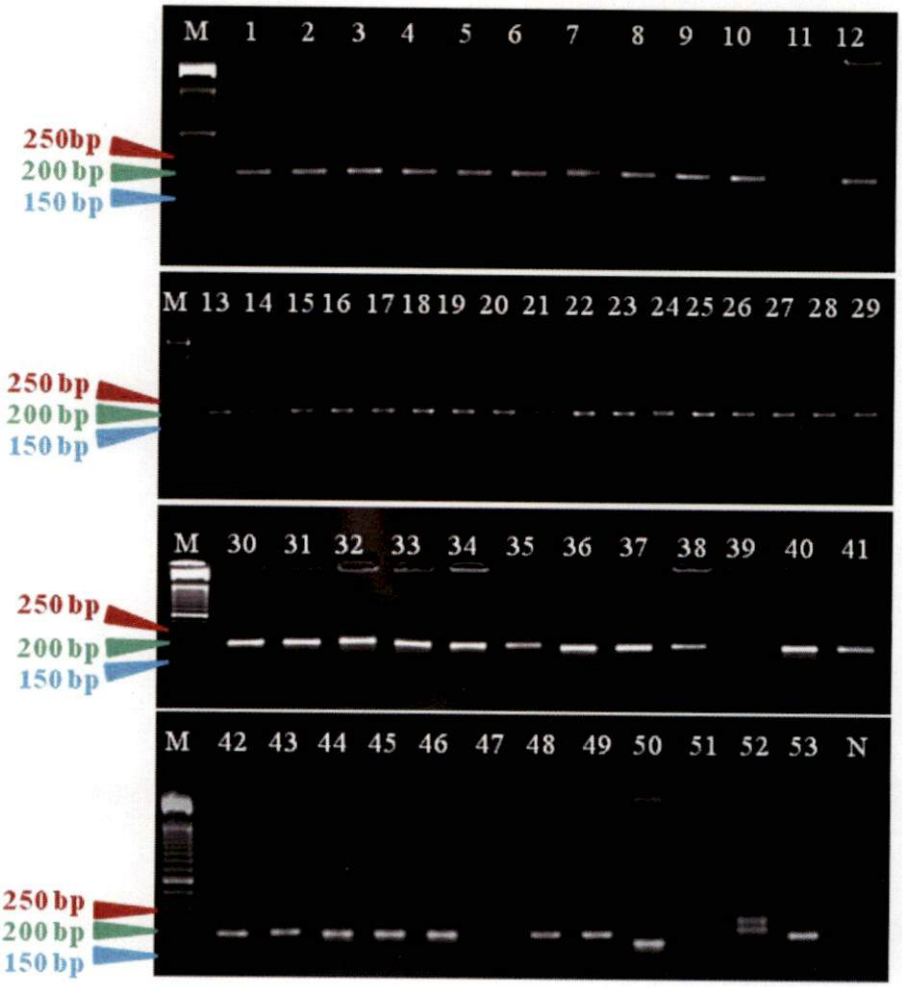


Plate 4. Validation of PNS3 EST-SSR marker in fifty three genotypes

1- Panniyur-1, **2**- Panniyur-2, **3**- Panniyur-3, **4**- Panniyur-4, **5**- Panniyur-5, **6**-Panniyur-6, **7**- Panniyur-7, **8**- Panniyur-8, **9**- Vijay, **10**- Panchami, **11**- Pournami, **12**- Sreekara, **13**- Subhakara, **14**- IISR Thevam, **15**- IISR Sakthi, **16**- IISR Girimunda, **17**- IISR Malabar Excel, **18**- Arka Coorg Excel, **19**- PLD-2, **20**- Karimunda, **21**- Paalikodi, **22**- Aimpiriyan, **23**- Narayakodi, **24**- Vadakkan, **25**- Thevanmundi, **26**- Arakulam munda, **27**- Kottanadan, **28**- Arivally, **29**- Balankotta, **30**- Kalluvally, **31**- Kuthiravally, **32**- Malamundi, **33**- Karivilanchi, **34**- TMB-2, **35**- TMB-4, **36**- *P. longum*, **37**- *P. attenuatum*, **38**- *P. colubrinum*, **39**- *P. arboreum*, **40**- *P. chaba*, **41**- *P. argyrophyllum*, **42**- *P. hymenophyllum*, **43**- *P. betle*, **44**- *P. peepuloides*, **45**- *P. thomsoni*(male), **46**- *P. thomsoni*(female), **47**- *P. magnificum*, **48**- *P. ornatum*, **49**- *P. sylvaticum*, **50**- *Piper* sp. (Anand), **51**- *Piper* sp. (Kottakkal), **52**- *Piper* sp. (North East), **53**- *Piper* sp. (North East Fragrance), **N**- Negative control

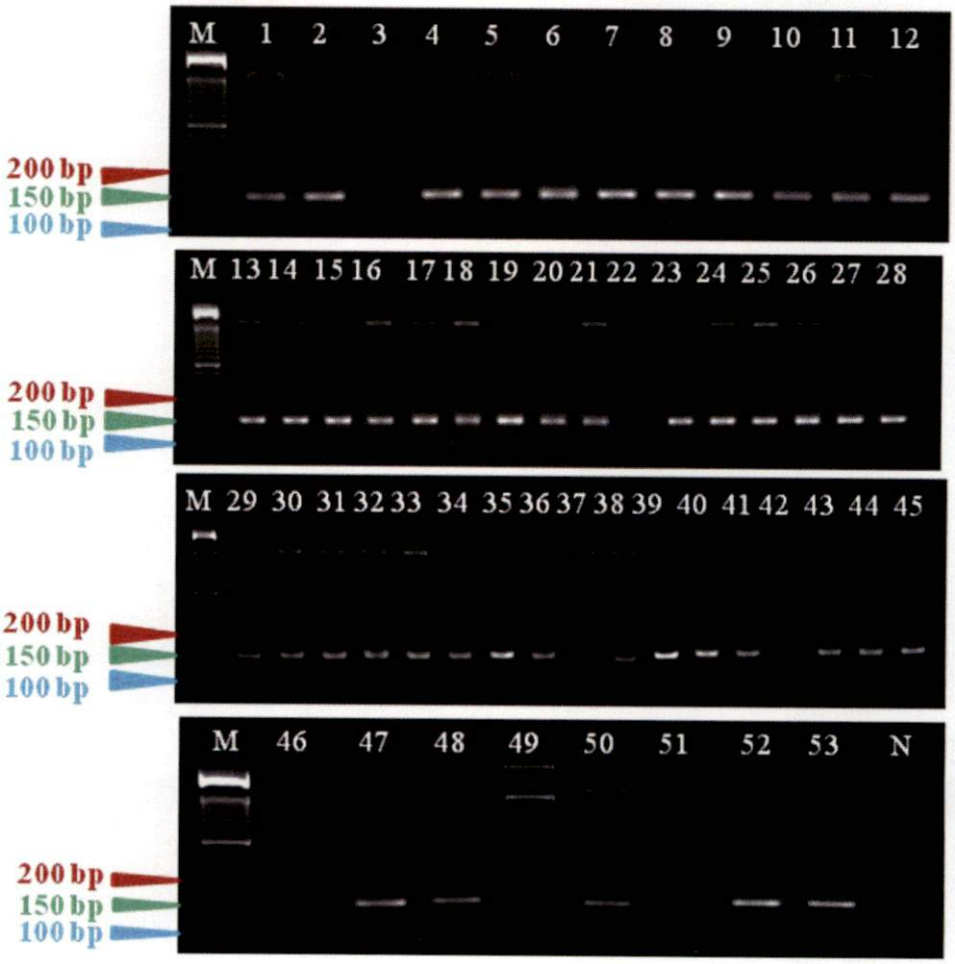


Plate 5. Validation of PNS4a EST-SSR marker in fifty three genotypes

1- Panniyur-1, **2**- Panniyur-2, **3**- Panniyur-3, **4**- Panniyur-4, **5**- Panniyur-5, **6**-Panniyur-6, **7**- Panniyur-7, **8**- Panniyur-8, **9**- Vijay, **10**- Panchami, **11**- Pournami, **12**- Sreekara, **13**- Subhakara, **14**- IISR Thevam, **15**- IISR Sakthi, **16**- IISR Girimunda, **17**- IISR Malabar Excel, **18**- Arka Coorg Excel, **19**- PLD-2, **20**- Karimunda, **21**- Paalikodi, **22**- Aimpiriyan, **23**- Narayakodi, **24**- Vadakkan, **25**- Thevanmundi, **26**- Arakulam munda, **27**- Kottanadan, **28**- Arivally, **29**- Balankotta, **30**- Kalluvally, **31**- Kuthiravally, **32**- Malamundi, **33**- Karivilanchi, **34**- TMB-2, **35**- TMB-4, **36**- *P. longum*, **37**- *P. attenuatum*, **38**- *P. colubrinum*, **39**- *P. arboreum*, **40**- *P. chaba*, **41**- *P. argyrophyllum*, **42**- *P. hymenophyllum*, **43**- *P. betle*, **44**- *P. peepuloides*, **45**- *P. thomsoni*(male), **46**- *P. thomsoni*(female), **47**- *P. magnificum*, **48**- *P. ornatum*, **49**- *P. sylvaticum*, **50**- *Piper* sp. (Anand), **51**- *Piper* sp. (Kottakkal), **52**- *Piper* sp (North East), **53**- *Piper* sp. (North East Fragrance), **N**- Negative control

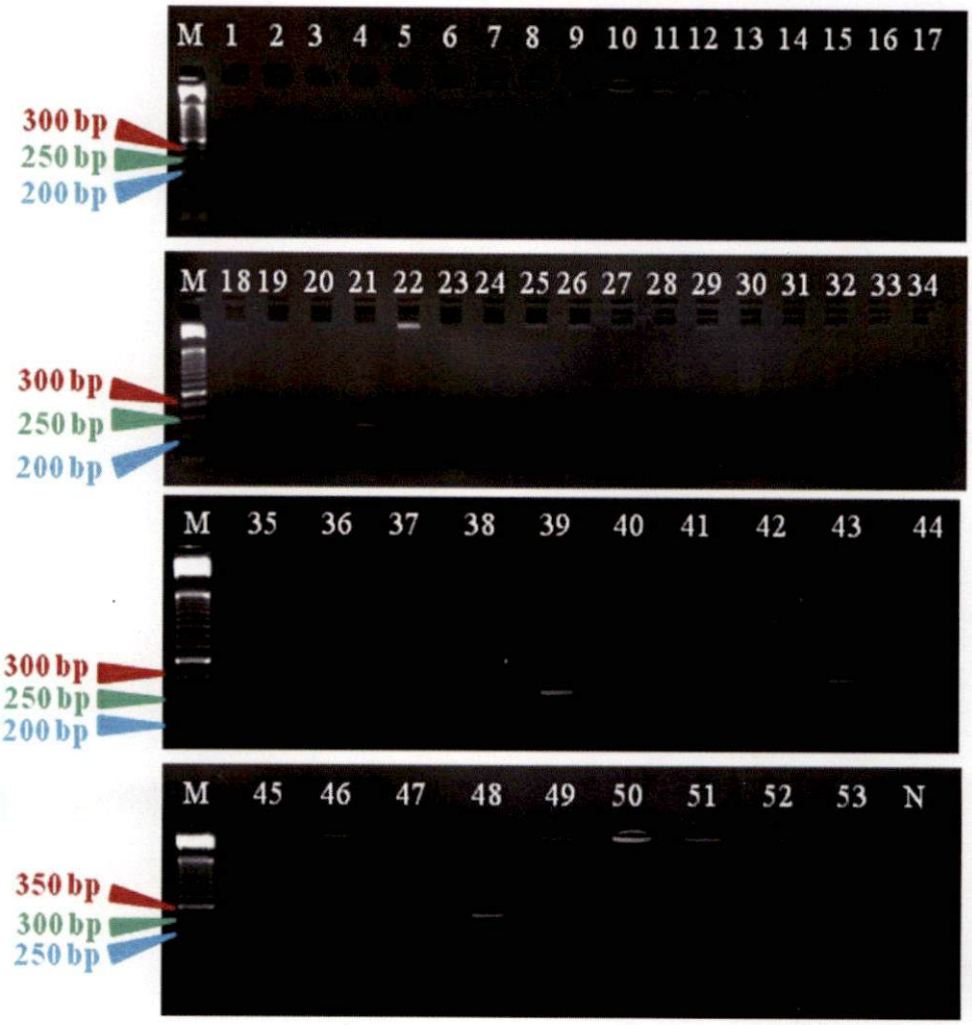


Plate 6. Validation of PNS6 EST-SSR marker in fifty three genotypes

1- Panniyur-1, **2**- Panniyur-2, **3**- Panniyur-3, **4**- Panniyur-4, **5**- Panniyur-5, **6**-Panniyur-6, **7**- Panniyur-7, **8**- Panniyur-8, **9**- Vijay, **10**- Panchami, **11**- Pournami, **12**- Sreekara, **13**- Subhakara, **14**- IISR Thevam, **15**- IISR Sakthi, **16**- IISR Girimunda, **17**- IISR Malabar Excel, **18**- Arka Coorg Excel, **19**- PLD-2, **20**- Karimunda, **21**- Paalikodi, **22**- Aimpiriyan, **23**- Narayakodi, **24**- Vadakkan, **25**- Thevanmundi, **26**- Arakulam munda, **27**- Kottanadan, **28**- Arivally, **29**- Balankotta, **30**- Kalluvally, **31**- Kuthiravally, **32**- Malamundi, **33**- Karivilanchi, **34**- TMB-2, **35**- TMB-4, **36**- *P. longum*, **37**- *P. attenuatum*, **38**- *P. colubrinum*, **39**- *P. arboreum*, **40**- *P. chaba*, **41**- *P. argyrophyllum*, **42**- *P. hymenophyllum*, **43**- *P. betle*, **44**- *P. peepuloides*, **45**- *P. thomsoni*(male), **46**- *P. thomsoni*(female), **47**- *P. magnificum*, **48**- *P. ornatum*, **49**- *P. sylvaticum*, **50**- *Piper* sp. (Anand), **51**- *Piper* sp. (Kottakkal), **52**- *Piper* sp. (North East), **53**- *Piper* sp. (North East Fragrance), **N**- Negative control

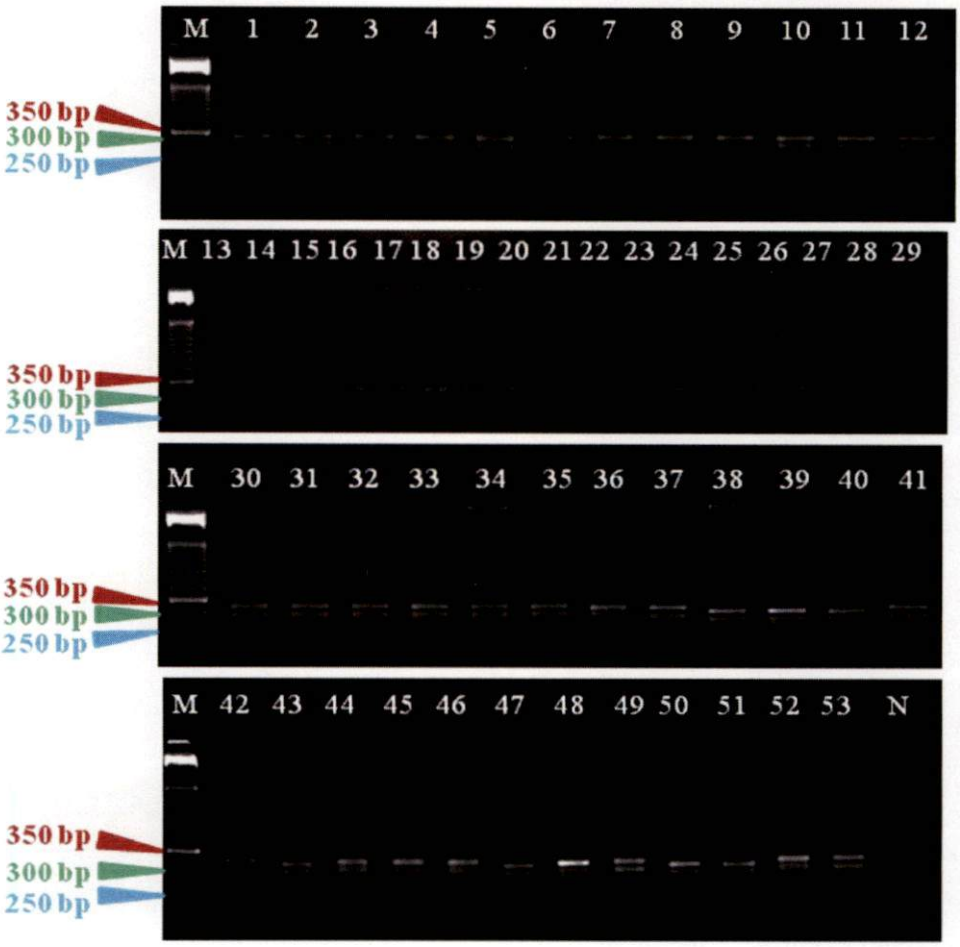


Plate 7. Validation of PNS8 EST-SSR marker in fifty three genotypes

1- Panniyur-1, **2**- Panniyur-2, **3**- Panniyur-3, **4**- Panniyur-4, **5**- Panniyur-5, **6**- Panniyur-6, **7**- Panniyur-7, **8**- Panniyur-8, **9**- Vijay, **10**- Panchami, **11**- Pournami, **12**- Sreekara, **13**- Subhakara, **14**- IISR Thevam, **15**- IISR Sakthi, **16**- IISR Girimunda, **17**- IISR Malabar Excel, **18**- Arka Coorg Excel, **19**- PLD-2, **20**- Karimunda, **21**- Paalikodi, **22**- Aimpiriyan, **23**- Narayakodi, **24**- Vadakkan, **25**- Thevanmundi, **26**- Arakulam munda, **27**- Kottanadan, **28**- Arivally, **29**- Balankotta, **30**- Kalluvally, **31**- Kuthiravally, **32**- Malamundi, **33**- Karivilanchi, **34**- TMB-2, **35**- TMB-4, **36**- *P. longum*, **37**- *P. attenuatum*, **38**- *P. colubrinum*, **39**- *P. arboreum*, **40**- *P. chaba*, **41**- *P. argyrophyllum*, **42**- *P. hymenophyllum*, **43**- *P. betle*, **44**- *P. peepuloides*, **45**- *P. thomsoni*(male), **46**- *P. thomsoni*(female), **47**- *P. magnificum*, **48**- *P. ornatum*, **49**- *P. sylvaticum*, **50**- *Piper* sp. (Anand), **51**- *Piper* sp. (Kottakkal), **52**- *Piper* sp. (North East), **53**- *Piper* sp. (North East Fragrance)

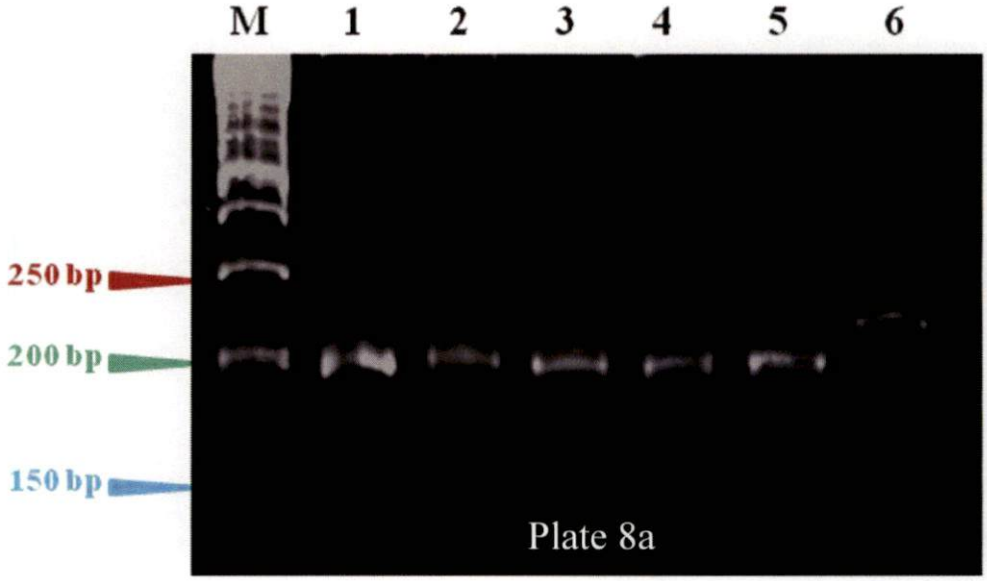
Table 9. Allelic representation of amplification of genomic DNA from different genotypes of black pepper using five EST-SSR markers

Sl no	Genotypes	Size of alleles (bp) obtained from different EST-SSR markers				
		PNS1	PNS3	PNS4a	PNS6	PNS8
1	Panniyur-1	370, 340	200, 200	150, 150	230, 230	330, 300
2	Panniyur-2	370, 340	210, 210	150, 150	230, 230	330, 300
3	Panniyur-3	370, 340	210, 210	-	230, 230	330, 300
4	Panniyur-4	-	210, 210	150, 150	230, 230	330, 300
5	Panniyur-5	370, 350	210, 210	150, 150	230, 230	330, 300
6	Panniyur-6	370, 350	210, 210	150, 150	230, 230	330, 300
7	Panniyur-7	350, 350	210, 210	150, 150	230, 230	340, 310
8	Panniyur-8	350, 350	200, 200	150, 150	230, 230	340, 310
9	Vijay	370, 340	200, 200	150, 150	230, 230	340, 310
10	Panchami	340, 340	200, 200	150, 150	230, 230	340, 310
11	Pournami	340, 340	-	150, 150	230, 230	340, 310
12	Sreekara	-	200, 200	150, 150	230, 230	340, 310
13	Subhakara	340, 340	200, 200	150, 150	230, 230	-
14	IISR Thevam	340, 340	220, 220	150, 150	230, 230	330, 300
15	IISR Sakthi	340, 340	220, 220	150, 150	240, 240	330, 300
16	IISR Girimunda	340, 340	220, 220	150, 150	240, 240	330, 300
17	IISR Malabar Excel	340, 340	220, 220	150, 150	240, 240	330, 300
18	Arka Coorg Excel	360, 340	220, 220	150, 150	240, 240	330, 300
19	PLD-2	350, 350	220, 220	150, 150	240, 240	330, 300
20	Karimunda	350, 350	220, 220	150, 150	240, 240	340, 310
21	Paalikodi	360, 360	220, 220	150, 150	240, 240	340, 310
22	Aimpipriyan	360, 360	220, 220	150, 150	250, 250	340, 310
23	Narayakodi	360, 360	220, 220	150, 150	250, 250	340, 310
24	Vadakkan	370, 370	220, 220	150, 150	250, 250	340, 310
25	Thevanmundi	370, 370	220, 220	150, 150	250, 250	340, 310
26	Arakulam munda	370, 370	220, 220	150, 150	250, 250	340, 310
27	Kottanadan	370, 370	220, 220	150, 150	250, 250	340, 310
28	Arivally	370, 370	220, 220	150, 150	250, 250	340, 310
29	Balankotta	370, 370	220, 220	150, 150	250, 250	330, 300
30	Kalluvally	330, 330	200, 200	150, 150	-	330, 300
31	Kuthiravally	330, 330	200, 200	150, 150	250, 250	330, 300
32	Malamundi	350, 320	200, 200	150, 150	240, 240	330, 300
33	Karivilanchi	330, 330	200, 200	150, 150	240, 240	330, 300
34	TMB-2	350, 320	200, 200	150, 150	240, 240	330, 300

35	TMB-4	320, 320	200, 200	150, 130	250, 250	330, 300
36	*P. longum*	350, 320	190, 190	150, 150	310, 280	330, 300
37	*P. attenuatum*	350, 320	190, 190	140, 140	-	330, 300
38	*P. colubrinum*	-	200, 110	140, 140	-	320, 290
39	*P. arboreum*	320, 320	200, 110	150, 150	270, 270	320, 290
40	*P. chaba*	360, 350	190, 190	150, 150	400, 310	320, 290
41	*P. argyrophyllum*	350, 320	190, 190	150, 150	270, 270	330, 300
42	*P. hymenophyllum*	330, 330	190, 190	150, 150	280, 280	330, 300
43	*P. betle*	340, 340	200, 200	150, 150	300, 300	320, 290
44	*P. peepuloides*	360, 360	190, 190	150, 130	250, 250	330, 300
45	*P. thomsoni* (male)	340, 340	190, 190	150, 130	-	330, 300
46	*P. thomsoni* (female)	340, 340	190, 190	150, 150	350, 280	330, 300
47	*P. magnificum*	-	-	150, 150	-	320, 290
48	*P. ornatum*	340, 340	190, 190	160, 160	320, 320	330, 300
49	*P. sylvaticum*	330, 330	190, 190	-	250, 250	330, 300
50	*Piper* sp. (Anand)	330, 330	180, 180	160, 160	280, 280	320, 290
51	*Piper* sp. (Kottakkal)	330, 330	-	-	350, 350	320, 290
52	*Piper* sp. (North East)	400, 340	230, 210	160, 160	-	330, 300
53	*Piper* sp. (North East fragrance)	330, 330	200, 200	150, 150	-	330, 300

4.6 POLYACRYLAMIDE GEL EECTROPHORESIS

The size of the PCR products obtained in agarose gel electrophoresis was confirmed using polyacrylamide gel electrophoresis (PAGE) using some of the random samples. The electrophoresis was carried out with 6 per cent polyacrylamide gel and visualized using ethidium bromide staining. In PAGE, PNS3 gave amplified product of size 200 bp in Panniyur-1, Panniyur-8, Panchami, Subhakara and Malamundi which was synchronous with the product obtained through 4 per cent agarose gel electrophoresis. Two bands (230 bp and 210 bp) present in *Piper* sp. (North East) was obtained by both agarose and polyacrylamide gel electrophores (Plate 8a). A band size of 150 bp was present in Panniyur-2, Sreekara and *Piper* sp. (North East Fragrance) using the primer, PNS4a which was in congruence with bands produced in agarose gel electrophoresis (Plate 8b). In TMB-4 two bands having a size of 150 bp and 130 bp were present (Table 9).



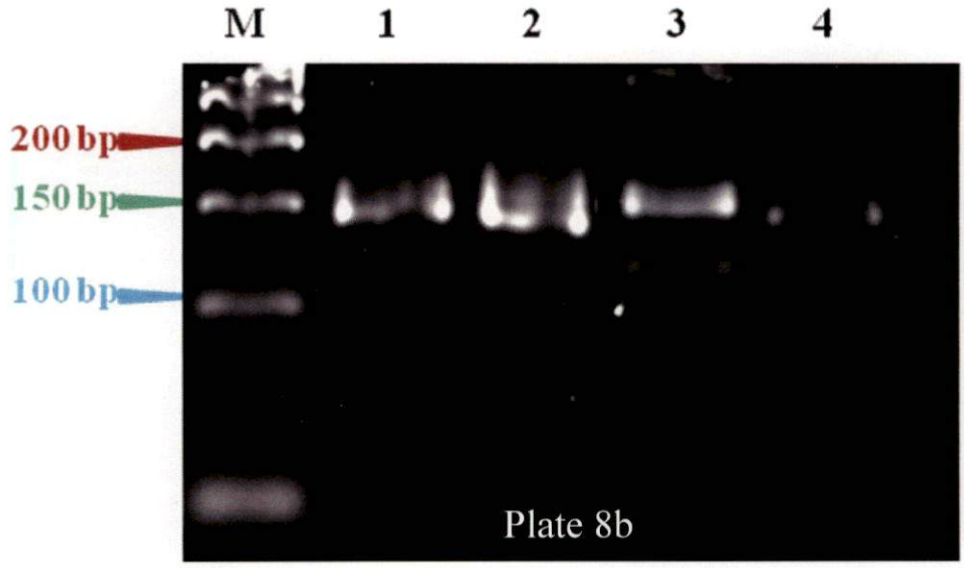


Plate 8: Poly acrylamide gel image of PNS3 and PNS4a primers

Plate 8a: PNS3 - **1**- Panniyur-1, **2**- Panniyur-8, **3**- Panchami, **4**- Subhakara, **5**- Malamundi, **6**- *Piper* sp. (North East)

Plate 8b:PNS4a - **1**-Panniyur-2, **2**-Sreekara, **3**- TMB-4, **4**-*Piper* sp. (North East Fragrance)

4.7 POLYMORPHISM INFORMATION CONTENT

The polymorphism information content (PIC) was in the range of 0.16 to 0.83 with an average of 0.49. PIC value was high with the primer PNS6. A total of 34 alleles were detected and the number of alleles per marker ranges from four to ten with an average of 6.8. All the five markers showed positive PIC value and were used for diversity analysis. PIC values of three markers (PNS1, PNS3 and PNS6) were higher than 0.5 which indicates a high level of polymorphism. The number of unique alleles generated and PIC value of the five markers are given in Table 10.

Table 10. List of EST-SSR markers with number of alleles and PIC value

Sl No	EST-SSR markers	Number of alleles per marker	PIC value
1	PNS1	7	0.75
2	PNS3	7	0.77
3	PNS4a	4	0.21
4	PNS6	10	0.83
5	PNS8	6	0.16

4.8 TRANSFERABILITY OF EST-SSR MARKERS IN DIFFERENT *Piper* SPECIES

Transferability of five EST-SSR markers specific to *P. nigrum* was tested in 18 different species of *Piper*. Polymorphism was observed among *Piper* species using five sets of primers (Plate 3, Plate 4, Plate 5, Plate 6, Plate 7). The rate of transferability was 100 per cent in 10 species. *P. magnificum* had shown transferability rate of less than 50 percent. Successful rate of amplification by five EST-SSR markers in different species and percentage of transferability are given in Table11.

Table 11. Transferability of five *P. nigrum* EST-SSR markers in different *Piper* species

Sl no	Genotypes	PNS1	PNS3	PNS4a	PNS6	PNS8	Number of successful amplification	Percentage of transferability
1	*P. longum*	✓	✓	✓	✓	✓	5	100
2	*P. attenuatum*	✓	✓	✓	×	✓	4	80
3	*P. colubrinum*	×	✓	✓	×	✓	3	60
4	*P. arboreum*	✓	✓	✓	✓	✓	5	100
5	*P. chaba*	✓	✓	✓	✓	✓	5	100
6	*P. argyrophyllum*	✓	✓	✓	✓	✓	5	100
7	*P. hymenophyllum*	✓	✓	✓	✓	✓	5	100
8	*P. betle*	✓	✓	✓	✓	✓	5	100
9	*P. peepuloides*	✓	✓	✓	✓	✓	5	100
10	*P. thomsoni (male)*	✓	✓	✓	×	✓	4	80
11	*P. thomsoni (female)*	✓	✓	✓	✓	✓	5	100
12	*P. magnificum*	×	×	✓	×	✓	2	40
13	*P. ornatum*	✓	✓	✓	✓	✓	5	100
14	*P. sylvaticum*	✓	✓	×	✓	✓	4	80
15	*Piper* sp. (Anand)	✓	✓	✓	✓	✓	5	100
16	*Piper* sp. (Kottakkal)	✓	×	×	✓	✓	3	60
17	*Piper* sp. (North East)	✓	✓	✓	×	✓	4	80
18	*Piper* sp. (North East Fragrance)	✓	✓	✓	×	✓	4	80

4.9 ANALYSIS ON GENETIC RELATIONSHIP

Genetic diversity analysis was carried with available five EST-SSR markers even though the number of markers were insufficient to study genetic relationship. Relationship between genotypes was studied based on the presence or absence of the alleles in each genotype. Dissimilarity was calculated based on the single data with the help of DARwin version 6 (Perrier *et al.*, 2003). Allelic data was also used for the analysis. The dissimilarity value was in the range of 0.08 to 0.87. The lowest dissimilarity value (0.08) was observed between the genotypes Panniyur-2 and Panniyur3, Vijay and Panchami, Arka Coorg Excel and IISR Sakthi, Arka Coorg Excel and IISR Girimunda, Arka Coorg Excel and IISR Malabar Excel. The highest dissimilarity (0.87) value was shown between the genotypes *P. longum* and Vijay, *P. longum* and *P. arboreum*, *Piper* sp. (North East) and *P. chaba* and *P. chaba* with the genotypes, TMB-2, Malamundi, Arka Coorg Excel, Panniyur-5 and Panniyur-6.

Fifty three genotypes were grouped into clusters based on the dice dissimilarity index (Table 12). Three main clusters (cluster I, cluster II and cluster III) were obtained and all the three clusters were bifurcated into two sub clusters. Cluster II-A was the largest sub cluster with 16 genotypes and cluster I-B was the smallest with six genotype.

There were 13, 23 and 17 genotypes were included in cluster I, cluster II, and cluster III respectively. Twelve varieties and one cultivar of black pepper were present in cluster I. Three KAU varieties, four IISR varieties and nine cultivars were present in cluster II-A. There were seven species of *Piper* included in cluster II-B. Cluster III comprised of eleven *Piper* species and six cultivars of black pepper and *Piper* species were in one group except *Piper* sp (North East Fragrance).

Table 12. Clustering pattern of 53 genotypes based on five EST-SSR markers

Clusters	Sub cluster	Number of genotypes	Genotypes
Cluster I	Cluster I-A	7	IISR Girimunda, IISR Sakthi, IISR Malabar Excel, Arka Coorg Excel, PLD-2, IISR Thevam, Balankotta
	Cluster I-B	6	Panniyur-1, Panniyur-2, Panniyur-3, Panniyur-4, Panniyur-5, Panniyur-6
Cluster II	Cluster II-A	16	Sreekara, Pournami, Panchami, Vijay, Panniyur-7, Panniyur-8, Subhakara, Thevanmundi, Vadakkan, Arakulam munda, Arivally, Kottanadan, Narayakodi, Aimpiriyan, Paalikodi, Karimunda
	Cluster II-B	7	*Piper* sp. (Kottakkal), *Piper* sp. (Anand), *P. magnificum, P. arboreum, P. colubrinum, P. betle, P. chaba*
Cluster III	Cluster III-A	10	*P. argyrophyllum, P. attenuatum, P. peepuloides, P. longum, P. ornatum, P. thomsoni* (male), *P. thomsoni* (female), *Piper* sp (North East), *P. sylvaticum, P. hymenophyllum*
	Cluster III-B	7	Karivilanchi, Kalluvally, Kuthiravally, TMB-2, TMB-4, Malamundi, *Piper* sp. (North East Fragrance)

4.10 SPECIES SPECIFIC ALLELES

Allelic size of 400 bp obtained using EST-SSR marker, PNS 1 was unique to *Piper* sp. (North East) (Table13). The marker PNS 3 also showed a unique allele in this species with two alleles having a size of 230 bp and 210 bp. Allele with a size of 180 bp was given by *Piper* sp. (Anand) using PNS 3 EST-SSR marker. Allelic size of 400 bp, 320 bp and 300 bp were unique to *Piper chaba, Piper ornatum and Piper betle* using PNS 6 marker.

Table 13. Unique SSR marker alleles for species identification in *Piper*

Sl no.	EST-SSR Primer	Unique allele	Genotype
1	PNS 1	400 bp	*Piper* sp. (North East)
2	PNS 3	230 bp, 210 bp	*Piper* sp. (North East)
3	PNS 3	180 bp	*Piper* sp. (Anand)
4	PNS 6	400 bp	*Piper chaba*
5	PNS 6	320 bp	*Piper ornatum*
6	PNS 6	300 bp	*Piper betle*

4.11 PUTATIVE FUNCTION OF EST-SSR MARKERS

Homology searches were performed in order to find the function of five polymorphic EST-SSR markers using BLASTN algorithm against other plant ESTs. The unigene sequence of PNS1 showed 76 per cent identity with MLO-like protein 1 of *Zizipus jujube*. PNS3 showed 76 per cent identity with *Nelumbo nucifera*, Plasma membrane associated cation binding protein 1. The EST sequence of PNS8 was 68

per cent identical to *Cucumis melo*, 60S ribosomal protein L6-1 (Table 14). Functions of two EST sequences (PNS4a and PNS6) were not available from NCBI BLAST *ie*, homology search.

Table 14. Putative proteins identified by BLASTN homology search for unigene sequences used to develop the five SSR markers

EST-SSR Primer	Putative protein	Plants	Query Cover	E-value	Identity
PNS1	MLO-like protein 1	*Ziziphus jujube* XM_016040193.1	70%	3e-54	76%
PNS3	Plasma membrane-associated cation-binding protein 1	*Nelumbo nucifera* XM_010262536.2	49%	3e-49	76%
PNS8	60S ribosomal protein L6-1	*Cucumis melo* XM_008463039.2	68%	6e-85	75%

Discussion

5. DISCUSSION

Molecular marker technology, such as RAPD and ISSR were widely used in germplasm analysis in black pepper. However the use of markers based on the sequence information is limited in this crop due to the unavailability of the genome sequence. Moreover, markers such as RAPD and ISSR are dominant and less reliable as they are sensitive to reaction condition and interfere with reproducibility of the results in different experiments. These limitations can be traversed by developing more SSR markers. Use of SSR marker in black pepper is limited as the number of SSR marker identified specifically for black pepper are fewer. Sequence databases are the sources of SSR but the volume of black pepper sequence data available for the analysis of SSR is limited. Roa *et al.* (2000) and Kindiger (2006) stated that SSR markers developed for one species generally exhibit less transferability across same or different taxa which necessitate the development of species specific SSR markers.

In black pepper, nine genomic SSR markers were developed by Menezes *et al.* (2009) and 20 varieties of black pepper and four species were used for validation of developed markers. Using these markers, they could characterize black pepper germplasm available in Brazil. Aforesaid markers were used by Anupama *et al.* (2015) and found that the microsatellite markers developed in black pepper were effective to study genetic diversity of genus *Piper*.

EST-SSR markers are preferred by many of the scientists since it is a fast and cost effective approach to marker development than genomic SSR. They are present in the gene rich region of the genome and are present abundantly (Scott, 2001). EST-SSR markers can be used in related species in which little information is available on ESTs or SSRs (Varshney *et al.*, 2005). In black pepper also ESTs have been obtained in a previous study and the data were utilized in the present investigation to mine the SSRs available in the sequences. This strategy was applied to several other crops like wheat (Gao *et al.*, 2004), capsicum (Yi *et al.*, 2006), cucumber (Hu *et al.*, 2010),

mango (Dillon *et al.*, 2014), cardamom (Anjali *et al.*, 2015), and broccoli (Izzah *et al.*, 2016). The results of the present study on 'Development and validation of novel EST-SSR markers in black pepper' are discussed in this chapter.

5.1 EST-SSR DETECTION

In the present study, EST sequences during berry development stage of black pepper were used for detecting and mining SSRs. Seventy SSRs were detected from 1048 unigene sequence having a size of 518179 bp using MIcroSAtellite (MISA) analysis tool. The main criteria followed for detecting SSR by fixing a minimum number of repeating units; ten for mononucleotide repeat, six for dinucleotide repeat and five for trinucleotide repeat, tetranucleotide repeat, pentanucleotide repeat and hexanucleotide repeat. The maximum number of bases interrupting two SSRs in a compound microsatellite was given as 100. MISA software was used by Wu *et al.* (2016) in black pepper and number of repeat units was given as five for dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide repeats. Detection of mononucleotide repeat was not reported by them. Hou *et al.* (2017) also used MISA tool for identifying SSR in rubber with a slight difference in the number of repeating units of four for tetranucleotide repeat, pentanucleotide repeat and hexanucleotide repeat. Sahoo *et al.* (2017) used MISA tool in turmeric for identifying mononucleotide repeat having 12 repeating units, dinucleotide repeat having seven repeating units and tetranucleotide repeat, pentanucleotide repeat and hexanucleotide repeat having three repeating units.

The result of microsatellite search was confirmed by another microsatellite detection tool, GMATo which is a fast and efficient tool that can be adopted by any user even without the knowledge of computer programming languages (Wang *et al.*, 2013).

5.1.1 Frequency of SSR in EST sequence

Varshney *et al.* (2005) reported that frequency of SSR depends on the mining criteria, tools, genome composition and EST dataset size. In the current study, seventy EST-SSRs were identified from 518179 bp (approximately 518 kb) suggesting that one SSR occurs for every 7.4 kb. Two SSRs were separated by 7.4 kb which implies that frequency of SSR in black pepper EST sequence was more compared to crops like maize, tomato, poplar, *Arabidopsis* and cotton in which one SSR is present in 8.1 Kb, 11.1 Kb, 14.0 Kb, 13.8 Kb and 20.0 Kb respectively (Cardle *et al.*, 2000).

5.1.2 Distribution of SSR in EST sequence

In the present study, the occurrence of mononucleotide repeats (88.6 per cent) was found maximum followed by trinucleotide repeats (8.6 per cent) and dinucleotide repeats (2.8 per cent). Higher repeat motifs like tetra, penta and hexanucleotide repeats were absent. These results are on par with SSR mining of 55 dicotyledonous species carried out by Kumpatla and Mukhopadhyay (2005) and they found out that mononucleotide repeats were abundant in *Allium cepa, Capsicum annum, Gossypium arboreum, Gossypium hirsutum, Hevea brasiliensis, Linum usitatissimum, Medicago truncatula* and *Prunus armeniaca.* Similar results were also found in castor bean in which occurrence rate of mononucleotides was high (37.51 per cent) followed by trinucleotide repeat (34.63 per cent) and dinucleotide repeat (25.61 per cent) (Zhou *et al.*, 2012). In a recent study on rubber also, mononucleotides were the dominant (38.89 per cent) repeat found, among the entire repeat types (Hou *et al.*, 2017).

There were six trinucleotide repeats and two dinucleotide repeats with number of repeating unit ranging from five to nine. In a recent study on black pepper, Wu *et al.* (2016) reported eight trinucleotide repeats and five dinucleotide repeats from EST sequence, with the number of repeating unit ranging from five to nine. Among cereal species, trinucleotides repeats were frequently present (54 per cent- 78 per cent)

followed by dinucleotides repeats (17.1 per cent-40.4 per cent) and tetranucleotides (3 per cent- 6 per cent) (Varshney *et al.*, 2002). In coconut also, trinucleotide repeats (55.2 per cent) were most frequently observed followed by dinucleotide repeats (35.5 per cent) and tetranucleotide repeats (9.2 per cent) (Xiao *et al.*, 2013).

Trinucleotide repeats were most abundant in coding region as reported by Toth *et al.* (2000) based on survey and analysis of microsatellites in the different eukaryotic genome. Cloutier *et al.* (2009) stated that the presence of trinucleotide repeat in the coding region would not cause frame shift mutation. The reason is that addition or deletion of the trinucleotide repeat in the translated region would not disturb the open reading frame and thus can be tolerated. Li *et al.* (2002) claimed that dinucleotide repeats are more frequent in non coding region.

5.2 EST-SSR PRIMERS

In this study, 70 SSRs were detected from 68 unigene sequences and out of this ten SSR motifs were selected for designing primers. The remaining sequences contain mononucleotide repeats and hence were not selected for designing primers. It is because of the less reliability of mononucleotide repeat and problems with amplification (Li *et al.*, 2002). Among the ten selected repeats, one was excluded due to inadequate length of flanking region. Sufficient flanking region is required since the length of primer for SSR should be more than 20 bp. One of the dinucleotide repeats was a part of compound microsatellite and therefore it was also omitted. Finally, nine primers (forward and reverse primer) for eight repeats including six trinucleotide repeats and two dinucleotide repeats were designed. Primer designing from selected EST-SSR sequence was reported in other crops also. In the case of cardamom out of 290 SSRs identified from 5050 ESTs, only 206 primer pairs were synthesized and eighty four SSRs were excluded due to the short flanking region (Anjali *et al.*, 2015).

5.3 ISOLATION AND QUANTIFICATION OF GENOMIC DNA

The modified CTAB method was successful in isolating good quality DNA with sufficient quantity in all the genotypes except in Panniyur-5 in which the A_{260}/A_{280} ratio was 1.39. Negligible amount of RNA contamination was observed in 13 genotypes.

5.4 SCREENING OF EST-SSR PRIMERS

Among the nine primers screened for amplification using Panniyur-1 DNA as template, five primers gave specific amplification (62 per cent), which was further used for validation in 53 selected genotypes. Similar results were obtained in sugarcane (Cordiero *et al.*, 2001) and cardamom (Anjali *et al.*, 2015). Amplification was not successful with 40% of the primers designed.

In the present study four primer sets, PNS2, PNS4b, PNS5 and PNS7 were excluded because of non specific or no amplification. The amplification of PNS2 and PNS5 was faint while amplification was not observed in PNS7. The location of primer sets in splice sites might be the reason for the failure of amplification of PNS7. In all the five selected primers, the amplified product size was larger than expected size. This may be due to the amplification of small introns or duplication of SSR in the genome creating polymorphism between the genotypes. A similar type of result was reported in wheat (Sehgal *et al.*, 2012) and *Anthurium* (Wang and Chuang, 2013) in which size of expected and amplified products were different.

5.5. VALIDATION OF EST-SSR PRIMERS

SSR regions of fifty three genotypes were amplified using five primer sets. The PCR products were separated and visualized on 4 per cent agarose gel which was stained using ethidium bromide. The concentration of agarose was increased in order to resolve small linear DNA fragments. The size of PCR products obtained was ranged from 146 bp to 350 bp for different primers. The total number of alleles

produced in the present study was 34 and average allele per locus was 6.8. In sorghum, Kumar *et al.* (2017) used 3 per cent agarose gel for separation of PCR products of EST-SSR markers and reported that clear and unambiguous bands were obtained using 3 per cent agarose. All the five markers developed were efficient in amplifying the different black pepper varieties, cultivars and species.

5.5.1 EST-SSR marker PNS1

Within *P. nigrum* (varieties and cultivars) the size of amplicons varied from 320 bp -370 bp using the primer PNS1 while in other *Piper* sp. size varied from 320 bp to 400 bp (unique to *Piper* sp. (North East) - heterozygous with allelic combination 400/340). Among the 18 *Piper* sp., only five were heterozygous while in the 33 genotypes of *P. nigrum* where amplification was there, only nine showed hetrozygosity at this locus. The expected product size was 212 bp where as the obtained products was in the range of 320 bp to 370 bp. This might be due to the presence on small introns.

5.5.2 EST-SSR primer PNS3

There were only three alleles in the locus PNS3 (200, 210 and 220 bp) for all the *P. nigrum* genotypes and these were homozygous. The varieties Panniyur-1, Panniyur-8, Vijay, Panchami, Sreekara and Subhakara shared the allele of 200 bp in homozygous condition with cultivars Kalluvally, Kuthiravally, Malamundi, Karivilanchi, TMB-2 and TMB-4. Panniyur-2 to Panniyur-7 were homozygous for allele 210 bp while the rest of the varieties and cultivars were homozygous for allele 220 bp.

Among the other *Piper* species, *P. betle* and *Piper* sp. (North East Fragrance) were also homozygous for allele 200 bp. *P. colubrinum* and *P. arboreum* were heterozygous for this locus, sharing the allele 200 bp with *P. nigrum* while having a new allele of 110 bp, which is unique to these two species. Similarly *Piper* sp.

(Anand) is unique with 180 bp allele in homozygous condition. *Piper* sp. (North East) was heterozygous for this loci with alleles 230 bp and 210 bp. All other *Piper* species were different from the above genotypes wherein they shared an allele 190 bp in homozygous condition.

5.5.3 EST-SSR marker PNS4a

For the locus PNS4a, all the genotypes of *P. nigrum* were monomorphic with the allele size of 150 bp in homozygous condition, except cultivar, TMB-4 which was heterozygous having two alleles 150 bp and 130 bp. At this locus, TMB-4 resembles the species *P. peepuloides* and *P. thomsoni* (male), while *P. thomsoni* (female) resembles *P. nigrum* genotypes with homozygous 150 bp allele. *P. attenuatum* and *P. colubrinum* are unique with 140 bp allele in homozygous condition while *P. ornatum*, *Piper* sp. (Anand) and *Piper* sp. (North East) are unique with 160 bp allele in homozygous condition. Rest of the *Piper* species were monomorphic with *P. nigrum* sharing the allele 150 bp.

5.5.4 EST-SSR marker PNS6

For *P. nigrum* genotypes, locus PNS6 showed homozygous condition for three alleles, *viz*., 250 bp, 240 bp and 230 bp. Among the other *Piper* spp. only *P. peepuloides* and *P. sylvaticum* shared one allele (250 bp - homozygous) with *P. nigrum* genotypes. Rest of the species showed different alleles in homozygous state (270 bp, 280 bp, 300 bp, 350 bp) except *P. longum* (310 bp, 280 bp) and *P. chaba* (400 bp, 310 bp) in heterozygous state for two unique alleles which were not present in any other *Piper* species.

5.5.5 EST-SSR marker PNS8

Locus PNS8 is unique as all the 52 genotypes in which there was amplification, showed heterozygosity at this locus (Plate 7, Table 9). In Subhakara

there was no amplification. The combination of alleles 330/300 was the most predominant (found in 19 *P. nigrum* genotypes and 11 species of *Piper*). The allelic combination 340/310 was unique for rest of the 15 *P. nigrum* genotypes while the allelic combination 320/290 was unique for rest of the seven *Piper* sp.

5.6 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

In order to resolve fragments with negligible differences, PAGE was used in several EST-SSR studies. In the present study, the result obtained for some selected genotypes and primer sets in 4 per cent agarose gel was confirmed using 6 per cent native PAGE. PAGE was carried out with Panniyur-1, Panniyur-8, Panchami, Subhakara, Malamundi, and *Piper* sp. (North East) using the marker PNS3 and Panniyur-2, Sreekara, TMB-4, and *Piper* sp. (North East Fragrance) using the marker PNS4a. Gel staining was carried out using ethidium bromide. The band size produced in PAGE was similar to band produced in the agarose gel. In most of the studies, silver staining was followed in PAGE. In the present study ethidium bromide staining of polyacrylamide gel was used as reported by Sahoo *et al.* (2017) in turmeric. Bands in the expected sizes were visualized better using ethidium bromide staining in turmeric. Fu *et al.* (2016) also used native PAGE in tall fescue. It was found that PAGE was effective in the separation of PCR products in tall fascue (*Festuca arundinacea*) and this supports the present study.

5.7 POLYMORPHISM INFORMATION CONTENT

Polymorphism information content (PIC) indicates the informativeness of a marker. Botstein *et al.* (1980) defined any locus as highly polymorphic, if the PIC value is greater than 0.5. In the present study, PIC of the five primer sets was in the range of 0.16 to 0.83. PIC value was highest (0.83) for the marker PNS6 having ten alleles. Lowest PIC value (0.16) was observed in PNS8. Using genomic SSR markers, Anupama *et al.* (2015) reported PIC value ranging from 0.15 to 0.32 which indicates lower polymorphism among different species of *Piper*. This might be due

to the reason that genomic SSR are less transferable across the species. In the present study, remarkable polymorphism was exhibited by different *Piper* spp. PIC value in this study not only reveals the usefulness of marker but also the efficiency of the marker to differentiate genotypes for diversity analysis.

5.8 TRANSFERABILITY OF EST-SSR MARKERS

As the EST- SSR markers are derived from expressed region, these are transferable across the species. In the current study, five selected EST-SSR markers which were developed from *P. nigrum* used in different *Piper* species. *P. longum*, *P. arboreum*, *P. chaba*, *P. hymenophyllum*, *P. argyrophyllum*, *P. betle*, *P. peepuloides*, *P. thomsoni* (female), *P. ornatum* and *Piper* sp. (Anand) are the genotypes that were successfully amplified by all the five primers. Transferability at the rate of 40 per cent was observed in *P. magnificum*. The marker PNS8 was transferable in all the *Piper* species. Menezes *et al*. (2009) reported that five genomic SSR markers among the nine developed markers in black pepper were transferable across *P. attenuatum*, *P. colubrinum*, *P. hispidinervium* and *P. tuberculatum*. The higher level of transferability among different related species is due to the conserved flanking sequences of SSR (Chen *et al*., 2015).

5.9 ANALYSIS OF GENETIC RELATIONSHIP

Only five EST-SSR markers were identified in the present study as the number of di- and tri- nucleotide SSR available were few. However diversity analysis was carried out with the available markers inorder to have a preliminary information about the possible genetic relationship between different genotypes. Izzah *et al*., (2016) also reported the use of five EST-SSR markers for assessing genetic diversity of broccoli cultivars and its related species. Relationship between 53 genotypes was assessed based on dissimilarity indices using dice matrix using DARwin version 6 software. Dissimilarity value ranged from 0.08 to 0.87. Based on the dissimilarity index, 53 genotypes were grouped into three clusters. All the main

clusters were again divided into two sub clusters namely A and B. *Piper* species were present in cluster II and cluster III. In cluster II-A, *Piper* species except for *Piper* sp. (North East Fragrance) were present. *Piper* sp. (North East Fragrance) was present along with cultivars of black pepper in cluster III-B. This shows the genetic similarity between *P. nigrum* and *Piper* sp. (North East Fragrance).

Low dissimilarity value between *P. attenuatum* and *P. argyrophyllum* (0.23) and also between *P. hymenophyllum* and *P. argyrophyllum* (0.38) indicates their genetic similarity. Based on the morphological characters, taxonomists have grouped the above three species in a single group (Gamble 1925; Mathew and Mathew 2002; Ravindran 2000; Nirmal-Babu *et al.*, 2003). Molecular characterization using ISSR markers in black pepper also supported the similarity of these three species (Sheeja *et al.*, 2013).

P. thomsoni and *P. longum* were included in the same cluster (cluster I) and this was supported by the results obtained using ISSR markers in black pepper by Sheeja *et al.* (2013). In the present study, *P. arboreum* and *P. colubrinum* showed a dissimilarity value of 0.33. Dissimilarity value of 0.50 was observed between *P. magnificum* and *P. colubrinum* and these genotypes were present in cluster II-B. In the diversity analysis reported by Anupama *et al.* (2015) using genomic SSR, *P. magnificum*, *P. colubrinum* and *P. arboreum* were present in a single cluster.

Panniyur-1 and Panniyur-3 are the hybrids of common parents, Uthirankotta and Cheriyakaniyakadan. Both F_1 hybrids were included in cluster I-B with a dissimilarity value of 0.23 which shows the high genetic similarity between Panniyur-1 and Panniyur-3. Sreekara and Subhakara are two varieties obtained through clonal selection from two accessions of Karimunda namely, KS-14 and KS-27. But the dissimilarity value between Sreekara and Karimunda was 0.45 and value between Subhakara and Karimunda was 0.80. Dissimilarity value between Sreekara and Subhakara was 0.30 which indicates more similarity between them and they were

included in cluster II-A. The high dissimilarity value between Karimunda and its derivative might be due to the difference in characters between Karimunda accession used in the present study and Karimunda accession used for generating Sreekara and Subhakara. A similar result was reported by Pradeepkumar *et al.* (2003) using RAPD markers in which Subhakara and Sreekara had shown similarity and less similarity with parentage, Karimunda.

Panchami and its lineage, Aimpiriyan were included in the same cluster (cluster IIA) and dissimilarity value was 0.50. Balankotta and its derivative, Panniyur-2 was in the same cluster (cluster I) with a dissimilarity value of 0.38. This shows the genetic similarity between the parents and their progeny.

EST-SSRs are preferable over genomic SSR even though EST-SSRs are less in number. This is due to their transferability across taxa and their functional nature in defining genes for particular traits. The markers developed will be linked with a gene, as they are derived from the expressed region of the genome. In the present study also the limited number markers could bring out preliminary information about the existing genetic diversity between certain groups which was reported earlier using other molecular markers such as ISSR and RAPD. However this cannot be generalized as further information is needed based on more number of SSR markers.

5.10 ALLELES FOR SPECIES IDENTIFICATION

Alleles which are unique to a particular genotype were obtained using the EST-SSR markers, PNS 1, PNS 3 and PNS 6. Using PNS 1 marker, an allele of 400 bp sizes was observed in *Piper* sp. (North East) which is not present in other genotypes. Alleles of size, 230 bp and 210 bp were present in heterozygous condition using PNS 3 marker in *Piper* sp. (North East) which was unique to that genotype. PNS 3 also gave a unique allele of size 180 bp in *Piper* sp. (Anand). Using PNS 6 marker, unique alleles 400 bp, 320 bp and 300 bp were present in *P. chaba*, *P.*

ornatum and *P. betle*. These alleles act as fingerprint of the genotypes which are unique to those genotypes.

5.11 FUNCTIONAL CHARACTERIZATION OF EST-SSR MARKERS

The putative function of the developed EST-SSR markers was found out with the help of BLASTN. Functions of three markers, PNS1, PNS3 and PNS8 were obtained. Sequence of the EST-SSR marker, PNS1 showed homology with MLO like protein 1 which is involved in the modulation of pathogen defense mechanism. However since the ESTs used were those from berry development stage of black pepper, this needs further investigation. Putative functions of the EST sequence of PNS4a and PNS6 were not available in the NCBI database. Functions of those sequences have to be found and it may be a part of desirable gene for a trait of interest.

The present study was undertaken with the objective of developing EST-SSR markers in black pepper and validation of those markers in different genotypes. These markers were also used for studying the genetic relationship among the genotypes, though limited in number, as preliminary information. Also the study could identify seven SSR alleles unique to five different species of *Piper* which can be used for species identification.

Summary

6. SUMMARY

The present investigation was carried out at College of Agriculture, Padannakkad, Kasaragod, Kerala during the time period of 2015-2017. The study envisaged the development of EST (Expressed Sequence Tags) derived SSR (Simple Sequence Repeats) markers and validation of the developed EST-SSR markers in different genotypes and to test transferability of markers in different species of *Piper*. Extent of diversity among different genotypes and their genetic relationship was also studied.

1. Development of SSR primers from EST data

- EST sequences developed in an earlier experiment during berry development stage of black pepper were used for SSR mining.
- SSRs were detected from EST sequences with the aid of MIcroSAtellite (MISA) identification tool and confirmation of the result was carried out by Genome-wide Microsatellite Analysis Tool (GMATo).
- Seventy microsatellites were detected in 68 unigenes by examining 1048 unigene sequences having a total size of 518179 bp. Mononucleotides, dinucleotides and trinucleotides repeats were identified with frequencies of 88.6 per cent, 2.8 per cent and 8.6 percent respectively. Among these, two compound SSR was also identified. Ten SSR motifs including compound SSR, trinucleotide repeats and dinucleotide repeats were selected for designing primers. Two SSRs were excluded from designing primer since one of them was a part of compound SSR and the other with insufficient flanking region.
- In total, nine primers including forward and reverse primers were designed for the flanking region of SSR. The primer sets designed for the flanking region of SSR were PNS1, PNS2, PNS3, PNS4a, PNS4b (Two reverse primers were designed, R4a and R4b), PNS5, PNS6, PNS7 and PNS8.

2) Validation of developed EST-SSR primers

- Nineteen varieties, sixteen cultivars of black pepper and eighteen different species of *Piper* were collected from Hi-Tech black pepper nursery, College of Agriculture, Padannakkad, ICAR-IISR, Kozhikode, ICAR-IISR, Experimental farm, Peruvannamuzhi and Pepper Research Station, Panniyur. The plant materials were maintained in Hi-Tech black pepper nursery, College of Agriculture, Padannakkad.
- Genomic DNA was isolated using modified CTAB method and the quality of DNA was pure.
- Nine designed EST-SSR primers were screened using DNA of Panniyur-1. Gradient PCR was carried out for standardizing annealing temperature. EST-SSR primers were screened at different annealing temperatures and the most appropriate temperature was selected.
- Five primers, PNS1, PNS3, PNS4a, PNS6 and PNS8 were selected for further validation using 53 genotypes of *Piper* sp. Three primers, PNS2, PNS5 and PNS8 were avoided due to the non specific amplification or no amplification.
- Selected primers were validated in 35 black pepper genotypes and 18 different species of *Piper*. Polymerase chain reaction (PCR) was carried out using the selected primers and electrophoresis of PCR products was carried out on 4 per cent agarose gel.
- Based on the review on earlier studies, the size of the PCR products and their differences were confirmed using 6 per cent non-denaturing PAGE. PAGE was carried out using the primers, PNS3 and PNS4a. The genotypes Panniyur-1, Panniyur-8, Panchami, Subhakara, Malamundi and *Piper* sp. (North East) were assayed on PAGE using PNS3 primer set. Using PNS4a primer set, PAGE was performed in Panniyur-2, Sreekara, TMB- 4 and *Piper* sp. (North East Fragrance).

3) Informativeness of developed EST-SSR markers

- In total, 34 alleles were obtained from five primer sets. The average number of alleles per locus was 6.8.
- PIC value of different primers was calculated based on polymorphic bands obtained using each primer sets. Polymorphism was mainly observed in different *Piper* sp. where as in black pepper genotypes polymorphism was less. PIC value was in the range of 0.16 to 0.83. The maximum PIC value was given by PNS6 and minimum was shown by PNS8.

4) Transferability of EST-SSR markers

- Transferability of EST-SSR markers developed from *P. nigrum* was tested in eighteen different species of *Piper*.
- All the five EST-SSR markers were transferable in *P. longum, P. arboreum, P. chaba, P. argyrophyllum, P. hymenophyllum, P. betle, P. peepuloides, P. thomsoni* (female), *P. ornatum, Piper* sp. (Anand).
- Transferability rate of EST-SSR primers was less in *P. magnificum* (40 per cent).

5) Alleles for species identification

- Allelic size of 400 bp obtained using EST-SSR marker, PNS 1 was unique to *Piper* sp. (North East).
- The marker PNS 3 showed a unique allele in *Piper* sp. (North East) with two alleles having a size of 230 bp and 210 bp. Allele with a size of 180 bp was given by *Piper* sp. (Anand) using PNS 3 EST-SSR marker.
- Allelic size of 400 bp, 320 bp and 300 bp was unique to *Piper chaba, Piper ornatum and Piper betle* using PNS 6 marker.

6) Analysis on genetic relationship

- Analysis was carried out with available five EST-SSR markers.
- Scoring was done based on the presence (1) or absence (0) of the alleles in each genotype. Scored data were analyzed using the software, DARwin version 6 and dissimilarity between the genotypes was found out using dice matrix.
- Dissimilarity value was in the range of 0.08 to 0.87.
- Different *Piper* species were present in two clusters (cluster II and cluster III). In cluster III, ten *Piper* species were grouped into one (cluster III-A) and one species, *Piper* sp. (North East Fragrance) was present along with some black pepper cultivars. Seven *Piper* species were present in cluster II.
- Some of the parents and their derivative (Aimpiriyan and Panchami, Balankotta and Panniyur-2) were present in a single cluster which indicates the genetic similarity between the genotypes.

. The present study was undertaken with the objective of developing EST-SSR markers in black pepper and validation of those markers in different genotypes. These markers were also used for studying the genetic relationship among the genotype, though limited in number, as preliminary information. Also the study could identify seven SSR alleles unique to five different species of *Piper* which can be used for species identification. The markers developed will be linked with a gene, as they are derived from the expressed region of the genome. In the present study also the limited number markers could bring out preliminary information about the existing genetic diversity between certain groups which was reported earlier using other molecular markers such as ISSR and RAPD. However this cannot be generalized as further information is needed based on more number of SSR markers.

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Appendix

APPENDIX-I

DICE DISSIMILARITY INDICES OF 53 GENOTYPES

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2	0.14																		
3	0.23	0.08																	
4	0.33	0.17	0.27																
5	0.29	0.14	0.23	0.17															
6	0.29	0.14	0.23	0.17	0.00														
7	0.69	0.54	0.67	0.45	0.38	0.38													
8	0.54	0.69	0.83	0.64	0.54	0.54	0.17												
9	0.29	0.43	0.54	0.67	0.57	0.57	0.38	0.23											
10	0.38	0.54	0.67	0.64	0.69	0.69	0.33	0.17	0.08										
11	0.50	0.50	0.64	0.60	0.67	0.67	0.27	0.27	0.17	0.09									
12	0.50	0.67	0.82	0.60	0.67	0.67	0.27	0.09	0.17	0.09	0.20								
13	0.27	0.45	0.60	0.56	0.64	0.64	0.60	0.40	0.27	0.20	0.33	0.33							
14	0.23	0.23	0.33	0.27	0.38	0.38	0.67	0.67	0.54	0.50	0.45	0.64	0.40						
15	0.38	0.38	0.50	0.45	0.54	0.54	0.83	0.83	0.69	0.67	0.64	0.82	0.60	0.17					
16	0.38	0.38	0.50	0.45	0.54	0.54	0.83	0.83	0.69	0.67	0.64	0.82	0.60	0.17	0.00				
17	0.38	0.38	0.50	0.45	0.54	0.54	0.83	0.83	0.69	0.67	0.64	0.82	0.60	0.17	0.00	0.00			
18	0.43	0.43	0.54	0.50	0.57	0.57	0.85	0.85	0.71	0.69	0.67	0.83	0.64	0.23	0.08	0.08	0.08		
19	0.54	0.54	0.67	0.45	0.38	0.38	0.67	0.67	0.85	0.83	0.82	0.82	0.80	0.33	0.17	0.17	0.17	0.23	
20	0.85	0.85	1.00	0.82	0.69	0.69	0.33	0.33	0.54	0.50	0.45	0.45	0.80	0.67	0.50	0.50	0.50	0.54	0.33
21	0.85	0.85	1.00	0.82	0.85	0.85	0.50	0.50	0.54	0.50	0.45	0.45	0.80	0.67	0.50	0.50	0.50	0.38	0.50
22	0.85	0.85	1.00	0.82	0.85	0.85	0.50	0.50	0.54	0.50	0.45	0.45	0.80	0.67	0.67	0.67	0.67	0.54	0.67
23	0.85	0.85	1.00	0.82	0.85	0.85	0.50	0.50	0.54	0.50	0.45	0.45	0.80	0.67	0.67	0.67	0.67	0.54	0.67

24	0.69	0.69	0.83	0.82	0.69	0.69	0.50	0.50	0.38	0.50	0.45	0.45	0.80	0.67	0.67	0.67	0.67	0.69	0.67
25	0.69	0.69	0.83	0.82	0.69	0.69	0.50	0.50	0.38	0.50	0.45	0.45	0.80	0.67	0.67	0.67	0.67	0.69	0.67
26	0.69	0.69	0.83	0.82	0.69	0.69	0.50	0.50	0.38	0.50	0.45	0.45	0.80	0.67	0.67	0.67	0.67	0.69	0.67
27	0.69	0.69	0.83	0.82	0.69	0.69	0.50	0.50	0.38	0.50	0.45	0.45	0.80	0.67	0.67	0.67	0.67	0.69	0.67
28	0.69	0.69	0.83	0.82	0.69	0.69	0.50	0.50	0.38	0.50	0.45	0.45	0.80	0.67	0.67	0.67	0.67	0.69	0.67
29	0.38	0.38	0.50	0.45	0.38	0.38	0.83	0.83	0.69	0.83	0.82	0.82	0.80	0.33	0.33	0.33	0.33	0.38	0.33
30	0.33	0.50	0.64	0.40	0.50	0.50	0.82	0.64	0.67	0.64	0.80	0.60	0.56	0.45	0.45	0.45	0.45	0.50	0.45
31	0.38	0.54	0.67	0.45	0.54	0.54	0.83	0.67	0.69	0.67	0.82	0.64	0.60	0.50	0.50	0.50	0.50	0.54	0.50
32	0.43	0.57	0.69	0.50	0.43	0.43	0.69	0.54	0.71	0.69	0.83	0.67	0.64	0.54	0.38	0.38	0.38	0.43	0.23
33	0.38	0.54	0.67	0.45	0.54	0.54	0.83	0.67	0.69	0.67	0.82	0.64	0.60	0.50	0.33	0.33	0.33	0.38	0.33
34	0.43	0.57	0.69	0.50	0.43	0.43	0.69	0.54	0.71	0.69	0.83	0.67	0.64	0.54	0.38	0.38	0.38	0.43	0.23
35	0.43	0.57	0.69	0.50	0.57	0.57	0.85	0.69	0.71	0.69	0.83	0.67	0.64	0.54	0.54	0.54	0.54	0.57	0.54
36	0.60	0.60	0.71	0.54	0.47	0.47	0.71	0.71	0.87	0.86	0.85	0.85	0.83	0.57	0.57	0.57	0.57	0.60	0.43
37	0.69	0.69	0.67	0.64	0.54	0.54	0.83	0.83	1.00	1.00	1.00	1.00	1.00	0.67	0.67	0.67	0.67	0.69	0.50
38	0.83	1.00	1.00	1.00	1.00	1.00	1.00	0.82	0.83	0.82	1.00	0.80	0.78	1.00	1.00	1.00	1.00	1.00	1.00
39	0.86	1.00	1.00	1.00	1.00	1.00	1.00	0.85	0.86	0.85	1.00	0.83	0.82	1.00	1.00	1.00	1.00	1.00	1.00
40	1.00	1.00	1.00	1.00	0.87	0.87	0.86	0.86	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.87	0.86
41	0.71	0.71	0.69	0.67	0.57	0.57	0.85	0.85	1.00	1.00	1.00	1.00	1.00	0.69	0.69	0.69	0.69	0.71	0.54
42	0.69	0.69	0.67	0.64	0.69	0.69	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	0.67	0.67	0.67	0.69	0.67
43	0.69	0.85	0.83	1.00	1.00	1.00	1.00	0.83	0.69	0.67	0.82	0.82	0.60	0.83	0.83	0.83	0.83	0.85	1.00
44	0.71	0.71	0.69	0.67	0.57	0.57	0.85	0.85	1.00	1.00	1.00	1.00	1.00	0.69	0.69	0.69	0.69	0.71	0.54
45	0.54	0.54	0.50	0.64	0.69	0.69	1.00	1.00	0.85	0.83	0.82	1.00	0.80	0.50	0.50	0.50	0.50	0.54	0.67
46	0.57	0.57	0.54	0.67	0.71	0.71	1.00	1.00	0.86	0.85	0.83	1.00	0.82	0.54	0.54	0.54	0.54	0.57	0.69
47	0.80	0.80	1.00	0.75	0.80	0.80	0.78	0.78	0.80	0.78	0.75	0.75	0.71	0.78	0.78	0.78	0.78	0.80	0.78
48	0.54	0.54	0.50	0.64	0.69	0.69	1.00	1.00	0.85	0.83	0.82	1.00	0.80	0.50	0.50	0.50	0.50	0.54	0.67
49	0.67	0.67	0.64	0.60	0.67	0.67	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.64	0.64	0.64	0.64	0.67	0.64
50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
51	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
52	0.57	0.43	0.38	0.50	0.57	0.57	0.85	1.00	0.86	0.85	0.83	1.00	0.82	0.54	0.54	0.54	0.54	0.57	0.69
53	0.33	0.50	0.64	0.40	0.50	0.50	0.82	0.64	0.67	0.64	0.80	0.60	0.56	0.45	0.45	0.45	0.45	0.50	0.45

	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
21	0.17															
22	0.33	0.17														
23	0.33	0.17	0.00													
24	0.33	0.33	0.17	0.17												
25	0.33	0.33	0.17	0.17	0.00											
26	0.33	0.33	0.17	0.17	0.00	0.00										
27	0.33	0.33	0.17	0.17	0.00	0.00	0.00									
28	0.33	0.33	0.17	0.17	0.00	0.00	0.00	0.00								
29	0.67	0.67	0.50	0.50	0.33	0.33	0.33	0.33	0.33							
30	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.45						
31	0.83	0.83	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.33	0.09					
32	0.54	0.69	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.54	0.33	0.38				
33	0.67	0.67	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.50	0.09	0.17	0.23			
34	0.54	0.69	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.54	0.33	0.38	0.00	0.23		
35	0.85	0.85	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.38	0.33	0.23	0.29	0.38	0.29	
36	0.71	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.57	0.54	0.57	0.33	0.57	0.33	0.47
37	0.83	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	0.64	0.67	0.38	0.67	0.38	0.54
38	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.80	0.82	0.83	0.82	0.83	0.83
39	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	0.85	0.71	0.85	0.71	0.71
40	0.86	0.86	0.86	0.86	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.87	1.00	0.87	1.00
41	0.85	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.69	0.67	0.69	0.43	0.69	0.43	0.57
42	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	0.45	0.50	0.69	0.50	0.69	0.69
43	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.82	0.83	0.85	0.83	0.85	0.85
44	0.85	1.00	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.54	0.67	0.54	0.57	0.69	0.57	0.43
45	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	0.64	0.67	0.69	0.67	0.69	0.54
46	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.69	0.67	0.69	0.71	0.69	0.71	0.71
47	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.75	0.78	0.80	0.78	0.80	0.80
48	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	0.64	0.67	0.69	0.67	0.69	0.69
49	1.00	1.00	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.45	0.40	0.27	0.67	0.45	0.67	0.50

50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.82	0.83	1.00	0.83	1.00	1.00
51	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.78	0.80	1.00	0.80	1.00	1.00
52	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.69	0.67	0.69	0.71	0.69	0.71	0.71
53	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.45	0.00	0.09	0.33	0.09	0.33	0.33

	36	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**
37	0.29																
38	1.00	0.82															
39	0.87	0.85	0.33														
40	0.63	0.71	0.69	0.60													
41	0.33	0.23	1.00	0.57	0.60												
42	0.43	0.50	1.00	0.85	0.71	0.38											
43	1.00	1.00	0.45	0.38	0.57	0.85	0.83										
44	0.47	0.38	1.00	0.86	0.60	0.29	0.38	0.85									
45	0.57	0.50	1.00	0.85	0.71	0.38	0.33	0.67	0.23								
46	0.47	0.54	1.00	0.86	0.73	0.43	0.23	0.69	0.43	0.23							
47	0.82	1.00	0.50	0.60	0.64	1.00	1.00	0.56	1.00	1.00	1.00						
48	0.57	0.50	1.00	0.85	0.71	0.38	0.33	0.67	0.38	0.17	0.23	1.00					
49	0.54	0.45	1.00	1.00	0.85	0.50	0.27	1.00	0.33	0.45	0.50	1.00	0.45				
50	0.86	1.00	0.64	0.54	0.57	0.85	0.50	0.50	0.85	0.83	0.69	0.56	0.83	0.82			
51	1.00	1.00	0.56	0.64	0.67	1.00	0.80	0.60	1.00	1.00	0.82	0.43	1.00	0.78	0.40		
52	0.73	0.69	1.00	0.86	0.87	0.57	0.54	0.69	0.57	0.38	0.43	1.00	0.38	0.67	0.85	1.00	
53	0.54	0.64	0.80	0.83	1.00	0.67	0.45	0.82	0.67	0.64	0.67	0.75	0.64	0.40	0.82	0.78	0.67

1- Panniyur-1, **2**- Panniyur-2, **3**- Panniyur-3, **4**- Panniyur-4, **5**- Panniyur-5, **6**-Panniyur-6, **7**- Panniyur-7, **8**- Panniyur-8, **9**- Vijay, **10**- Panchami, **11**- Pournami, **12**- Sreekara, **13**- Subhakara, **14**- IISR Thevam, **15**- IISR Sakthi, **16**- IISR Girimunda, **17**- IISR Malabar Excel, **18**- Arka Coorg Excel, **19**- PLD-2, **20**- Karimunda, **21**- Paalikodi, **22**- Aimpiriyan, **23**- Narayakodi, **24**- Vadakkan, **25**- Thevanmundi, **26**- Arakulam munda, **27**- Kottanadan, **28**- Arivally, **29**- Balankotta, **30**- Kalluvally, **31**- Kuthiravally, **32**- Malamundi, **33**- Karivilanchi, **34**- TMB-2, **35**- TMB-4, **36**- *P. longum*, **37**- *P. attenuatum*, **38**- *P. colubrinum*, **39**- *P. arboreum*, **40**- *P. chaba*, **41**- *P. argyrophyllum*, **42**- *P. hymenophyllum*, **43**- *P. betle*, **44**- *P. peepuloides*, **45**- *P. thomsoni*(male), **46**- *P. thomsoni*(female), **47**- *P. magnificum*, **48**- *P. ornatum*, **49**- *P. sylvaticum*, **50**- *Piper* sp. (Anand), **51**- *Piper* sp. (Kottakkal), **52**- *Piper* sp. (North East), **53**- *Piper* sp. (North East Fragrance)

DEVELOPMENT AND VALIDATION OF NOVEL EST-SSR MARKERS IN BLACK PEPPER (*Piper nigrum* L.)

by

SHERIN JOSE

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ABSTRACT

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DEPARTMENT OF PLANT BREEDING AND GENETICS
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Abstract

ABSTRACT

The study entitled "Development and validation of novel EST-SSR markers in black pepper (*Piper nigrum* L.)" was carried out at College of Agriculture, Padannakkad, Kasaragod, Kerala during 2015-2017. The main objectives of the study were to develop EST-SSR markers in black pepper, validation of the developed EST-SSR markers in different genotypes of black pepper and to study the genetic relationship among different species of *Piper*.

Different genotypes (53 numbers) including nineteen varieties and sixteen cultivars of black pepper and eighteen different species of *Piper* were included in the study. The plant materials were collected from Hi-Tech black pepper nursery in College of Agriculture, Padannakad; Indian Institute of Spices Research (ICAR- IISR), Kozhikode; the Experimental farm of IISR, Peruvannamuzhi and Pepper Research Station, Panniyur.

Simple sequence repeats (SSR) were mined from the expressed sequence tags (EST) obtained in a previous study about the genes expressed during berry development stage of black pepper. The microsatellite identification tool, MISA was used for detecting SSRs from 1048 unigenes having a total size of 518179 bp and the results were confirmed using another microsatellite identification tool, GMATo. Seventy SSRs were detected from 68 unigene sequences which accounts for 6.49 per cent of total sequences. There were 62 mononucleotides (88.6 per cent), two dinucleotides (2.8 per cent) and six trinucleotides (8.6 per cent) identified from the sequences. Ten microsatellites including six trinucleotides, two dinucleotides and two compound SSRs were selected and primers were designed based on the corresponding unigene sequences to amplify these regions in the genomic DNA. Nine primers (which consist of forward and reverse primer) were designed, *viz.,* PNS1, PNS2, PNS3, PNS4a, PNS4b, PNS5, PNS6, PNS7 and PNS8. Primers were designed for eight repeat motifs including six trinucleotide repeats, one dinucleotide repeat and one compound repeat.

The developed primers were screened using genomic DNA isolated from black pepper variety Panniyur-1. Annealing temperature of the primers was standardized through gradient PCR. Based on the specificity of the amplification, five primers (PNS1, PNS3, PNS4a, PNS6 and PNS8) were selected for further validation.

Genomic DNA was isolated from the 53 genotypes of *Piper* spp. and amplified with the selected primers for validation. In total, 34 alleles were obtained for the five loci amplified by the five primer sets. The average number of alleles per locus was 6.8. Polymorphism Information Content (PIC) value of different primers was calculated based on the number of polymorphic bands obtained using each primer set.

All the five primers were successful in amplifying the corresponding locus in *P. nigrum* genotypes as well as in other *Piper* spp. Among the 34 alleles, seven were found only in *P. nigrum*, 16 were specific to other *Piper* spp. and 11 were shared by both groups. PIC value was in the range of 0.16 to 0.83. The maximum PIC value was given by PNS6 with 10 alleles bringing out the difference between all the species and minimum was shown by PNS8.

For cluster analysis using the software DARwin version 6, scoring was carried out based on the presence (1) or absence (0) of the alleles in each genotype using each primer. Dissimilarity between the genotypes was found out using dice matrix. Dissimilarity values were in the range of 0.08 to 0.87. The genotypes were grouped into different clusters based on the similarity between genotypes. Different *Piper* species were present in two clusters, Cluster II with seven species and Cluster III with eleven species. In Cluster III-B, one species, *Piper* sp. (North East Fragrance) was present along with six black pepper cultivars indicating that this may be a closely related species of *P. nigrum*. Some of the parental type and corresponding varieties derived from them (Aimpiriyan and Panchami, Balankotta and Panniyur-2) were present in single cluster which indicates the genetic similarity between these genotypes.

Allelic size of 400 bp obtained using EST-SSR marker, PNS 1 was unique to *Piper* sp. (North East). The marker PNS 3 also showed a unique allele in *Piper* sp. (North East) with two alleles having a size of 230 bp and 210 bp. Allele with a size of 180 bp was given by *Piper* sp. (Anand) using PNS 3 EST-SSR marker. Allelic size of 400 bp, 320 bp and 300 bp was unique to *Piper chaba, Piper ornatum and Piper betle* using PNS 6 marker.

The markers developed will be linked with a gene, as they are derived from the expressed region of the genome. In the present study also the limited number markers could bring out preliminary information about the existing genetic diversity between certain groups which was reported earlier using other molecular markers such as ISSR and RAPD. However this cannot be generalized, as further information is needed based on more number of SSR markers.