MOLECULAR CHARACTERIZATION AND TESTING HYBRIDITY OF INTERSPECIFIC CROSSES IN BLACK PEPPER (*Piper nigrum* L.)

By

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DECLARATION

       I, hereby declare that this thesis entitled **"Molecular characterization and testing hybridity of interspecific crosses in black pepper (*Piper nigrum L.*)"** is a bonafide record of research work done by me during the course of research and that it 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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To my

Beloved

parents

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ABBREVIATIONS

%	Percentage
~	Approximate
≈	Approximate
>	Greater than
μg	Microgram
A	Ampere
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
bp	Base pair
cc	*cubic centimetre*
cm	Centimetre
CMS	Cytoplsmic Male Sterile
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EEO	Electroendosmosis
g	Gram
GD	Genetic Distance
ha	Hectare
ISSR	Inter Simple Sequence Repeat
Kb	Kilo basepairs
KM	Karimunda
L	Litre
M	Molar
MAS	Marker Assisted Selection
mg	Milligram

ml	Millilitre
mM	Milli mole
NBPGR	National Bureau of Plant Genetic Resources
ng	Nanogram
ºC	Degree Celsius
OD	Optical Density
P1	Panniyur 1
P2	Panniyur 2
P3	Panniyur 3
P5	Panniyur 5
PC	*Piper colubrinum*
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
P^H	Hydrogen ion concentration
PIC	Polymorphism Information Content
pM	Pico molar
PRS	Pepper Research Station
PVP	Poly vinyl pyrolidine
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Rotations per minute
SAHN	Sequential Agglomerative Hierarchical Non-overlapping
SCAR	Sequence Characterized Amplified Region
SRAP	Sequence Related Amplified Polymorphism
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Microsatellite Sites
STR	Short Tandem Repeat
STS	Sequence Tagged Sites

TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UK	Uthirankotta
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volts
β	Beta
µl	Microlitre

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), renowned as "King of spices", is a major revenue earning spice crop of many countries. It is widely cultivated across various parts of the world apart from India. *P. nigrum* belonging to the family Piperaceae, is a perennial climber. Cultivated *P. nigrum* is monoecious, having hermaphrodite and protogynous flowers and predominantly self-pollinated. *P. nigrum* is a tetraploid species with chromosome number 2n=52 (*x*=13) (Jose and Sharma, 1984).

Black pepper occupies an area 2,36000 ha with production of 46,745 tonnes during 2010-2011 (Spices Board, 2011). However, in productivity, India occupies the last position among the leading black pepper producing countries in the world (310 kg/ha). One of the major reasons for this peculiar situation is the high incidence of disease especially *Phytophthora* foot rot, caused by *Phytophthora capsici*. It is prevalent in all pepper growing areas causing heavy crop loss every year.

As India is the primary centre of origin of black pepper, the indigenous genetic resources are reservoirs of useful genes for plant improvement programme. The development of improved varieties through hybridization has made a major contribution in increasing productivity and quality of plants in different crop plants (Dhillon *et al*., 2009).

All the released varieties and cultivars of black pepper in Kerala are susceptible to *Phytophthora* foot rot. Although only a narrow range of variability in *Phytophthora* tolerance is reported among the cultivated types, the exotic wild species, *Piper colubrinum*, is reported to have resistant genes (Ravindran, 2000a). Moreover, a partially fertile interspecific hybrid (*P. nigrum* x *P. colubrinum*) with partial resistance has been reported by Vanaja *et al*. (2008). But Varma *et al*. (2009) reported that interspecific hybridization using *P. colubrinum* for the

transfer of disease resistance genes to the cultivated pepper was unsuccessful due to incompatibility problems.

One of the major problems faced by pepper breeders is the difficulty in identifying true hybrids from the crossed progenies before planting. The traditional method of hybrid identification based on morphological characters is influenced by environmental factors and frequently lacks the resolving power to identify hybrids at the juvenile stage. Therefore, black pepper plants are to be grown to maturity (i.e. 3 to 4 years) to confirm hybridity (George *et al*., 2005). To overcome this disadvantage, biochemical markers are being used in many crops. However, level of polymorphism, repeatability and accuracy of results using biochemical markers to distinguish cultivars are subject to question (Pallavi *et al*., 2011). A reliable method for identification of hybrid pepper at the early stage of the plants is thus essential. Molecular markers used to detect DNA polymorphism are the most direct answer to the problem (Manigbas and Villegas, 2005).

The use of DNA markers for characterization and identification of genotypes is essential for the early detection of true inter and intra-specific hybrids, parentage of a cultivar and patent protection (Benedetti *et al*., 2000). DNA markers not only allow the easy and reliable identification of breeding lines, hybrids (Bastia *et al*., 2001) and cultivars (Mohanty *et al*., 2001) but also facilitate the assessment of genetic diversity (Pradeepkumar *et al*., 2003) and relatedness among germplasm (Milligan, 2003).

One of such techniques is the use of Random Amplified Polymorphic DNA (RAPD) markers (Williams *et al*., 1990; Welsh & McClelland, 1990) for the identification of genotype in crop plants (Yamagishi, 1995). This method gained importance due to its simplicity, efficiency and non requirement of sequence information (Karp *et al*., 1997). RAPD assay have been extensively applied to assess genetic diversity in the genus *Piper* (Sen *et al*., 2010) and also has been used for hybrid identification (George *et al.*, 2005), evaluation and characterization of germplasm (Bai *et al*., 2003), verification of hybridity in

interspecific crosses (Benedetti *et al*., 2000), for fingerprinting of genomes (Welsh and McClelland, 1990), tagging of genes (Martin *et al*., 1991) and marker-assisted selection (MAS) (Sixin and Anderson, 2003).
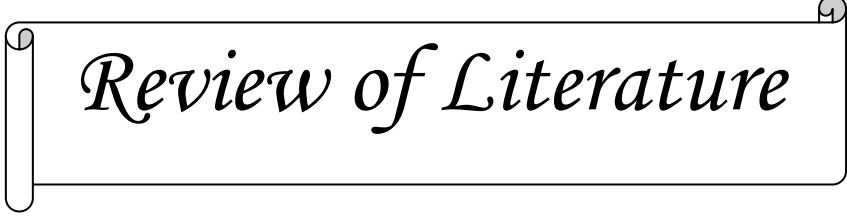
Other molecular markers, particularly the co-dominant markers such as Simple Sequence Repeats (SSR) are of great importance for rapid assessment of hybrid and parental line seed purity (Yashitola *et al.*, 2002 and Sundaram *et al.,* 2008). Microsatellite or SSRs are short tandem repeats of DNA sequences of only a few base pair (1-6bp) in length. They are relatively abundant and more evenly dispersed in the genome, and analysis requires only small amount of starting DNA (Powell *et al.,* 1996). Any primer or pair of primers when used to amplify a particular SSR locus in a number of genotypes will reveal SSR polymorphism (Gupta *et al*., 1996). This polymorphism is in the form of differences in length of amplified product, each length representing an allele at a particular locus. The length differences are attributed to the variation in the number of repeat units at a particular SSR locus. One of the distinguishing characteristics of these markers is that they can identify co-dominance, polymorphism and inheritance in a plant. Thus, SSR can be used as tool to assist in selecting specific, genetically diverse parents for use in a breeding program (Cordeiro and Henry, 1999) and to check the hybridity more efficiently.

In hybridity testing, true hybrids are detected by the presence of DNA sequences corresponding to both alleles contributed by the two parents. SSR markers are now widely accepted, replacing other molecular markers in plant DNA fingerprinting and cultivar identification. They are known to be reliable indicators. These markers allow the early identification of true interspecific hybrids for further evaluation and crossing and simultaneously enable early disposal of non hybrids, thus delivering substantial saving in time and resources (Cordeiro *et al*., 2000).

While acknowledging the handicap of high ploidy level in black pepper, the present study entitled 'Molecular characterization and testing hybridity of interspecific crosses in black pepper (*Piper nigrum* L.)' is an attempt to detect true interspecific hybrids from the cross *P. nigrum* x *P. colubrinum* using known RAPD and SSR molecular markers.

The present study has been undertaken with the following objectives:

1. To characterize the six interspecific hybrids from crosses using six varieties / cultivars of *P. nigrum* as female parent and *P. colubrinum* as the common male parent maintained at Pepper Research Station, Panniyur.
2. To test the hybridity of putative F_1 hybrids using Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) markers.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Black pepper: A major spice of Kerala

Black pepper (*Piper nigrum* L.), the king of spices originated in the Western Ghat of South India is a major revenue earning spice crop for many countries. It is a perennial climber and belongs to the family Piperaceae. Cultivated *P. nigrum* is monocious, having hermaphrodite and protogynous flower, and is predominantly a self pollinated crop. It is a tetraploid species with chromosome number 2n=52 (X=13) (Jose and Sharma, 1984).

India ranks 9th in the world pepper production with an area of 1.81 lakh ha and production of 46, 745 tonnes (Spice Board, 2011). India was the world's largest exporter of black pepper until a decade ago, providing 40 per cent of world's pepper demand. Now that position is occupied by Vietnam with an export of 1, 16, 670 tonnes followed by Brazil (42,190 tonnes), pushing India to the third position (26,380 tonnes) (International Pepper Community, 2011).

The drastic drop in the black pepper production in India has been attributed mainly to the pronounced mortality of vines by the dreaded disease, foot rot caused by *Phytophthora capsici*. The other major constraints for low production of black pepper are majority of old gardens being occupied with traditional cultivars having poor genetic potential, non-adoption of improved package of practices, poor management of gardens, drought *etc*.

2.2 Brief description of black pepper

2.2.1 Botanical description

Black pepper is a climbing perennial shrub. Main stem is stout, trailing/climbing and rooting at the node. The stout climbing stem is highly flexible with leathery dark green leaves. Leaves are entire, variable in length and breadth. Pepper exhibits dimorphic branching. The straight, upward growing,

orthotropic branches help in climbing on the standard and the laterally growing,

plagiotropic branches bear flower and fruits.

2.2.2 Floral biology

Ravindran *et al.* (1997) described the development of inflorescence and the sympodial mode of growth continue by the activity of the axillary bud. As axillary bud develope, the inflorescence (a catkin commonly called spike) is pushed out so that it became leaf opposed. Spike emergence is observed to be during June-July following the monsoon rains and take 2 to 4 weeks to complete in each vine.

In cultivated varieties flowers were reported to be bisexual, sessile and bracteate. Protogyny was also reported in most of the cultivars with the male and female phase separated by 1 to 14 days. Anthers bithecous with four microsporangium and emerged on either side of the ovary. Stigma is 3 to 4 lobed, style absent and the periods of receptivity of stigma vary from 3 to 9 days. Anther emergence to dehiscence take 2 to 4 days and stigma emergence in all the flower in a spike require 5 to 15 days to complete. Fruits are globose and bright red when ripe, seeds usually globose. Fruits botanically described as drupe.

Pepper vine in its wild state is mostly dioecious, but most of the cultivated types are monoecious, a condition which probably originated from the wild ones as a result of continuous selection and vegetative propagation by man through ages (Krishnamurthy, 1969).

2.3 Black pepper (*Piper nigrum* L.) and *Phytophthora* foot rot

One of the major reasons for the low productivity in India is the high incidence of disease especially the dreadful *Phytophthora* foot rot disease. It is prevalent in all pepper growing areas and causes heavy crop loss every year (Anandraj and Sharma, 1991; Sarma *et al*., 1994).

In India, this disease was first reported by Barber (1902) from Waynad region of Kerala. Butler (1906) coined the term 'wilt', due to the rapid death of the plant. Later, Muller (1936) reported a similar type of disease from Dutch East

Indies and coined the term 'foot rot'. Nambiar and Sarma (1977) referred the disease as 'quick wilt disease' of black pepper based on sudden death and wilting of vines. However, the terminologies of the disease have been changed to *Phytophthora* foot rot of black pepper and to *Phytophthora* rot of black pepper (Vijayaraghavan, 2003).

The destruction of vines in India due to *Phytophthora* foot rot disease has been recorded to be 40 to 50 per cent, which indicates the seriousness of the disease infestation (Dewaard, 1979). Harper (1974) has reported a yield loss of 50 per cent in Indonesia due to this disease. Crop losses due to 25-30 per cent vine deaths have been reported from Kerala (Nambiar and Sarma, 1977). Sastry (1982) and Dutta (1982) recorded heavy incidence of the disease in Uttara Kannada and Shimoga districts of Karnataka causing 100 per cent death of vines in some gardens.

There is no report regarding highly effective control measure to tackle this disease; all the cultivated types have been found susceptible to the disease and have to be chemically protected. The high cost incurred in this kind of disease control, harmful effect of extensive and frequent use of fungicide, and the demand for 'clean spices' free from chemical residues, have led to an intensive search for a host-plant resistance. Keeping view of the economic losses caused by fungal pathogen, conventional breeding programmes are in operation to develop disease resistant cultivars. However, this approach has not been very successful. Biotechnological approaches could be utilized to compliment conventional breeding as well as to develop transgenic lines of this crop by incorporating disease resistance genes.

2.3.1 Host plant resistance to *Phytophthora*

For majority of *Phytophthora* problems effective control practices have been much slower to evolve. Selection and breeding for resistance or tolerance have been advocated. In most instances, however, suitable germplasm has been difficult to find, particularly in perennials where 15-20 years of time frame is

needed to evaluate resistance. A research for root stock resistance or tolerance has been actively perused, especially in India since black pepper originated from Kerala. Techniques for mass screening of open pollinated seedling progenies and to assess relative degree of tolerance/resistance in rooted cuttings to *Phytophthora capsici* have been developed (Sarma *et al.,* 1990). Though it is quite hard task to identify high degree of resistance in open pollinated crops, even a medium level field tolerance is greatly appreciated. Kuch and Khew (1980) reported root inoculation and leaf inoculation with zoospores as effective screening techniques useful in selecting for resistance in black pepper to *Phytophthora palmivora.*

There is no evidence of absolute resistance to *Phytophthora* available in cultivars. But, low level of tolerance in certain cultivars like Kulluvalli, Palaulaut, Belantung and Bangka had been identified. Some wild relatives including *Piper colubrinum, P. hispidum* and *P. arifolium* are resistant (Sitepu and Prayitno, 1979).

Muller (1936) was the first to report black pepper variety Belantung from Indonesia as resistance to foot rot. Indian pepper cultivars, Uthirankotta and Indodnasian varieties Djambi and Belantung possess appreciable tolerance (Holliday and Mowat, 1963). Ruppel and Almeyda (1965) opined that out of five pepper species tested, *Piper adunum. Piper seabrum* sw. and *Piper treleasanum* Bitt. and Wils showed partial resistance. Albuquerque (1968) observed resistance in *Phytophthora colubrinum* Link., *Phytophthora obliqum* Ruiz and Pav. var. *eximum.*

Sarma *et al.* (1982) reported that out of 41 cultivars and 73 wild types of *Piper* spp. tested, cultivars, Narayakodi, Kalluvally, Uthirankotta and Balankottta were found tolerant. Hegde (1983) conducted mass screening of seven cultivars in wilt sick plot and could not get a single resistant plant. Dutta (1982) tested the seedlings raised from seeds and cuttings of healthy pepper vines survived in badly infected garden and reported that none of them were resistant.

Black pepper varieties Kutching, Bangka, Belantung, Jambi, Karimunda, Lampung, Daun kecil, Lampung daun lebar, Panniyur-1 and Uthirankotta were highly susceptible to *P. capsici.* While varieties, Cheriakanyakadan and Balankotta were less susceptible (Kueh and Liang, 1988). During 1979, about 1200 open pollinated high yielding varieties Kuching black pepper seedlings in Sarawak were inoculated with *P. palmivora* (Butler) Butler. By the end of 1980, nine seedlings appeared to be resistant to fungus (Mohd and Hussin, 1986). During 1987-88, 140 cultivars, 174 hybrids, 72 wild types, and a large number of seedlings raised from open pollinated seeds were screened for reaction to *Phytophthora capsici.* Among them tolerant reaction was found in 15 cultivars, 12 hybrids and 50 open pollinated seedlings (Ramachandran *et al.,* 1988).

Piper colubrinum was immune to *P. capsici* and its adaptability as root stock to black pepper was poor. Cultivar Karimunda was highly susceptible to *P. capsici*. (Kueh and Liang, 1988). Sarma *et al*. (2000) screened 137 hybrids for their reaction to *Phytophthora capsici* through stem inoculation techniques, three hybrids HP-423, HP-664 and HP-756 showed tolerant reaction.

Among 70 hybrids, 25 cultivars and 16 Kottandan selections screened for resistance against *P. capsici*, 7 hybrids, 3 cultivars and 9 accessions were found tolerant (Veena *et al*., 2003).

Screening of 343 black pepper hybrids, 7 cultivars and 9 wild accessions for their reaction to *Phytophthora capsici* indicated that 9 hybrids, 4 cultivars and 2 wild accessions were tolerant. Seedling progenies of P-24 (resistant line) and KS-27 (susceptible line) were screened against *P. capsici* and a higher percentage of seedlings of P-24 showed a tolerant reaction (Anon., 2003).

Recently, the roles of β-1, 3 glucanase and chitinase enzymes in the defense mechanism of black pepper against foot rot disease were analysed. Nazeem *et al*. (2008) evaluated the protein profiles of a relatively tolerant and a susceptible black pepper (*Piper nigrum*) variety along with that of a resistant wild species (*Piper colubrinum*) to detect variations in the defense related proteins/enzyme expression in response to *P. capsici* infection. The native protein profile indicated the expression of two additional proteins in *P. nigrum*. The over-expressed protein was characterized as β -1, 3 glucanase. The intensity of expression was directly related to the level of tolerance. The resistant genotype *P. colubrinum*, possessed higher β-1, 3 glucanase activity than the *P. nigrum* varieties tested.

2.4 Crop improvement in black pepper

Majority of the present-day Indian black pepper cultivars, numbering about 100, are land races representing direct introduction from the wild (Ibrahim *et al.,*1984) and most of the varieties released for cultivation are clonal selections from the existing land races while a few are of hybrid origin (George *et al*., 2005). As India is the primary centre of diversity of *P. nigrum* the indigenous resources are reservoirs of useful genes for plant improvement programmes (Pradeepkumar and Karihaloo, 2001).

One of the major research aim of different institutions is developing high yielding, good quality varieties of black pepper with tolerance to diseases and pest (Babu and Ravindran, 1994). The development of improved cultivars through hybridization has made a major contribution to increased productivity and quality of plants in different crop plants (George *et al*., 2005). In black pepper, combining yield and quality parameters has been a perennial goal for improvement programmes (Pradeepkumar *et al*., 2003). More emphasis is now given in improvement programmes for quality parameter like piperine, oleoresin and oil rather than whole black pepper as the export of value added products is now gaining importance.

More than 15 high yielding varieties have been released for cultivation among which two are hybrids *viz*., Panniyur 1 and Panniyur 3 developed at the Pepper Research Station, Panniyur, Kerala. These varieties have special distinguishing characters such as long spike, high fruit setting, bold berries and high yield. After the release of the popular variety Panniyur-1, the station has released Panniyur-2, Panniyur-3, Panniyur-4, Panniyur-5, Panniyur-6 and Panniyur-7. IISR Girimunda and IISR Malabar Excel are the two recent high yielding varieties from Indian Institute of Spices Research (IISR), Kozhikode (Ravindran and Johny, 2000).

Interspecific hybridization has contributed significantly to the genetic enhancement in many crops. In developing synthetic amphidiploids, interspecific

hybridization is a useful tool. This tool is useful for introducing alien variation and introgressing desirable genes across species (Roy, 1984), generating morpho-physiological variations (Prakash, 1973), and creating genetic diversity (Choudhary and Joshi, 2001). Successful interspecific hybridization, to varying extents, was reported in many crops. In some cases the hybrids were completely sterile while in other cases partially fertile.

Sasikumar *et al*. (1999) reported the first successful interspecific hybridization between *P. nigrum* x *P. attenuatum* and *P. nigrum* x *P. beriberi*. The hybrids has the same chromosome number (2n=52) as their parents.

Vanaja *et al*. (2008) reported an interspecific hybrid in black pepper to impart resistance to *Phytophthora* foot rot disease through hybridization between *P. nigrum* and *P. colubrinum.*

2.5 Morphological markers

Observation of phenotypes has been the classical approach to differentiate plant cultivars, morphological trait being the main target of such observations. The description of the plant morphology is an invaluable source of information of the genetic variability and has been the first criterion used to classify plant varieties.

According to Bretting and Widrlechner (1995) the data obtained using morphological, karyological and molecular markers were found useful in managing germplasm *ex situ.* Among these, morphological traits were the oldest and widely used genetic markers because of their simplicity and rapid, inexpensive assays. Even though molecular markers are more specific, morphological markers assisted with proper statistical analysis provide information in breeding of various crop such as maize (Desai and Singh, 2001) and *Brassica juncea* (Lakshmikant and Gulati, 2001), serve as single gene controlled marker in linkage studies (Gour and Gour, 2001) and help to assess genetic diversity in germplasm collections (Raje and Rao, 2001).

Moreover, the use of morphological and physiological traits for plant improvement program is the basis of evaluation for both the conventional and modern breeding approaches.

Kerala has records of more than hundred black pepper cultivars with highly variable characters (George *et al.,* 2005). Only a few of them have been identified and reported to be economically productive. Distinct variations in morphological traits among Indian landraces have been reported (Mathew *et al*., 1999).

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

2.5.1 Vegetative characters

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

Stem characters like internodal length, branching nature, direction of growth of branches *etc*. were reported to vary with cultivars (Kanakamany, 1982; Ibrahim *et al*., 1984; Sujatha and Namboothiri, 1995).

2.5.2 Reproductive characters

Variations in yield traits among Indian cultivars have been report based on spike length, floral composition, fruit number and size (Ibrahim *et al*., 1984; Babu and Ravindran 1994; Prasannakumari *et al*., 2001).

Inflorescence characters like number and length of spike, sex types of flower, stamen and stigmatic characters *etc*. were reported to be of economic

importance as they influence yield (Nambiar *et al*., 1978; Chandy *et al*., 1979; Kanakamany, 1982). Similarly the number of berries per spike, percentage of developed and undeveloped berries per spike, 100 berry weights, 100 berry volumes, drying percentage as well as chemical composition also manifests pronounced variation (Nambiar *et al*., 1978; Kanakamany, 1982; Raja *et al*., 1983; Sujatha and Namboothiri, 1995).

2.6 Molecular markers for varietal identification

Before the development of molecular markers, morphological markers were found to be a source in varietal identification and assessing genetic diversity, but they have certain limitations. Most of the vegetative characteristics are influenced by environmental factors, and show continuous variation and have a high degree of plasticity. In an attempt to overcome these problems, biochemical and molecular techniques have been used to monitor genetic variability, and to solve taxonomic and phylogenetic problems, markers based on protein differences were widely used. Iso-electric variants of proteins, referred to as isozymes, were found to be markers for specific chromosomes/chromosomal regions. Many studies have aimed at assessing the genetic diversity of different crops using allozyme markers (Hamrick and Godt, 1996). The study of phylogenetic and taxonomic relationships requires a flexible and reliable marker system to detect high levels of polymorphism.

Isozyme markers have been used for the diversity studies and genetic mapping of different crops (Trujillo *et al.,* 1995; Paull *et al.,* 1998) but their use remained limited as they revealed low level of polymorphism and the isozyme expression found highly influenced by the environmental conditions (Hernendez *et al.,* 2001). However, the DNA based marker techniques seem to provide the means for generating useful information on genetic relatedness and diversity.

2.6.1 Markers at DNA level

Plant molecular biology offers a great potential for plant breeding as it promises to provide several tools to reduce the time taken to produce crop

varieties with desirable characters. With the use of molecular techniques it would now be possible to hasten the transfer of desirable genes among varieties and to introgress novel genes from related wild species. Polygenic characters which were previously very difficult to analyse using classical genetic analysis, would now be easily tagged using molecular markers.

The DNA markers assumed importance since they can be used as genetic markers that are associated with economically important traits (Darvasi and Soller, 1994). DNA markers because of their heritable nature were found to act as versatile tools in the fields like taxonomy, physiology, embryology, genetic engineering *etc*. Major applications of these DNA markers in genetics and breeding are in i) Diversity analysis and phylogenetic studies, ii) Mapping genes and iii) Marker Assisted Selection (MAS).

Techniques which are particularly promising in assisting selection for desirable characters involve the use of two types of molecular markers such as hybridisation based molecular markers such as Restriction Fragment Length Polymorphisms (RFLP) (Botstein *et al*., 1980) and Polymerase Chain Reaction (PCR) based molecular markers such as Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Sequence Characterized Amplified Regions (SCAR) (Williams *et al*., 1991), Simple Sequence Repeats (SSR) (Hearne *et al*., 1992), Sequence-Tagged Sites (STS) (Fukuoka *et al*., 1994), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz *et al*., 1994) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995). The utility of molecular markers in crop breeding is reviewed by Mohan *et al*. (1997) and Gupta and Roy (2002).

Among the various molecular marker techniques, Restriction Fragment Length Polymorphism (RFLP) was the first DNA marker used for the construction of genetic maps of agronomically important species and for mapping of heritable traits (Tanksley *et al.,* 1989). However, their utility has been hampered due to the involvement of radioactive isotopes, labour intensive and

time consuming steps. Among the marker systems available SSRs have been proved to be more useful in marker assisted selection (Jena *et al*., 2008).

Molecular markers have several advantages over the phenotype based markers that were previously available to plant breeders. They offer greater scope for improving the efficiency of plant breeding by carrying out selection indirectly on the trait of interest by using a set of tightly linked markers to the trait of interest. The markers at DNA level are used to construct the linkage maps, locate the genetic loci on specific chromosomes, characterize the germplasm for its genetic diversity and finally to exercise marker assisted selection (MAS).

2.7 Types of DNA markers

2.7.1 Restriction Fragment Length Polymorphism (RFLP) markers

Variations in DNA sequences have been extensively exploited as genetic markers for genome mapping in the last 10 years. One of the most important achievements is the advent of RFLP (Botstein *et al.,* 1980). Restriction Fragment Length Polymorphism (RFLP) analysis is a powerful tool for developing precise high-density molecular genetic maps because it reveals reliable and stable polymorphism. Markers based upon DNA probes have introduced a new dimension to the development of genetic maps and the mapping of agronomically and physiologically important characters. The major strength of DNA probes is that they have the potential to reveal an almost unlimited number of polymorphism (Wyman and White, 1980).

2.7.2 Randomly Amplified Polymorphic DNA (RAPD) markers

In 1990, molecular markers generated using Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) was used to identify Randomly Amplified Polymorphic DNA (RAPD) in several organisms including plants (Welsh and McClelland, 1990; Williams *et al.,* 1990). RAPD are commonly inherited as dominant markers, where the presence of a particular band is dominant, and its absence is recessive (Tingley and Tufo, 1993). RAPD analysis has found applications in population studies (Welsh *et al*., 1991), biosystematics (Stiles *et*

al., 1993), gene tagging (Ranade *et al.*, 2001) and fingerprinting (Virk *et al.*, 1995; Mackill, 1996). RAPD has also been successfully applied in molecular ecology (Hadrys *et al.*, 1992), genetic analysis (Williams *et al.*, 1993), assessing variation in plants (Newbury and Ford-Lloyd, 1993) combining ability in rice (Radhidevi *et al.*, 2002) and to study the extent of diversity within plant germplasm (Virk *et al.*, 1995).

2.7.2.1 RAPD in black pepper

Pradeepkumar *et al.* (2003) reported molecular characterization of *Piper nigrum* L. cultivars using RAPD markers. Thirteen land races and nine advanced cultivars of *P. nigrum* were used. DNA was extracted according to the CTAB method with minor modifications. A total of 34 decamer primers (series OPA, OPB *etc.*) were screened using three representative genomic DNA samples of black pepper. Of these, 24 primers that yielded consistent and clear band patterns were selected for the final analysis of the 22 accessions. The 24 selected decamer primers generated 372 amplicons and the total number of markers per primer ranged from 4 (OPV05) to 21 (OPF09). The range of polymorphic markers per primer was 3 (OPV05) to 21 (OPF09), with a mean of 15.3.

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

Nazeem *et al.* (2005) studied the variability and relatedness among 49 accessions of black pepper (*Piper nigrum* L.) using molecular marker RAPD and AFLP. The similarity matrix was subjected to cluster analysis and dendrogram generated using the software NTSYS pc 2.1. The dendrogram revealed an average similarity of 63 per cent among accessions. Two selections from the variety Karimunda, namely Subhakara and Shreekara, together form a single cluster with

almost 90 per cent similarity. The dissimilarity observed between the varieties Panniyur-1 and Panniyur-3 the progenies of the same parentage, Uthirankotta and Cheriyakaniakkadan was only 18 per cent.

Keshavachandran *et al.* (2005) reported the DNA fingerprinting of *P. nigrum* and *P. longum* cultivars using RAPD. Fourteen land races and three advanced cultivars of *P. nigrum* as well as eleven land races and one advanced cultivar of *P. longum* were studied. Forty decamer primers were screened using two representative genomic DNA samples of black pepper. Of these, 10 primers that yielded clear and dominant band patterns were selected for final analysis of the 29 accessions and these generated 119 amplification products.

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

Budiguppe *et al*. (2007) used RAPD for the evaluation of genetic relatedness among 6 *Piper* species. Six *Piper* species were screened using RAPD with decamer primer of arbitrary sequence. Out of 100 primers screened, 12 were selected which gave clear and bright fragments. DNA banding patterns generated by RAPD were recorded as '1' for presence and '0' for absence. Genetic distance between these 6 species was calculated based on the RAPD data set as per Squared Euclidean Distances. Based on the number of bands all the species were grouped into 3 clusters and the dendrogram revealed maximum similarity between *P. betel* and *P. longum* and also between *P. nigrum* and *P. mullesua* species.

Kizhakkayil *et al.* (2008) reported molecular characterization of traded black pepper (*Piper nigrum* L.) from India, Indonesia, Vietnam and Malaysia. Molecular profiling (RAPD) and clustering of these samples revealed a comparatively high genetic similarity within the samples from a particular country than between any two countries. The UPGMA dendrogram constructed based on

the similarity coefficient revealed a total of four groups in two different clusters. The two Indonesian samples formed cluster I, while others formed cluster II. The genuine Indian varieties and the traded pepper from India formed separate group in cluster II. Similarly, black pepper from Malaysia and Vietnam also formed distinct groups in cluster II. The aspect of genetic similarity was discussed in relation to the origin and spread of black pepper.

Anandaraj *et al*. (2008) developed the SCAR marker for *Phytophthora* resistance in black pepper (*Piper nigrum* L.). About 3000 lines of black pepper germplasm were screened with decamer primers. The genomic DNA was extracted using modified CTAB method and subjected to PCR using 15 decamer random primers. Out of the 15 RAPD primers tested, primer OPA-01 amplified four regions out of which three were monomorphic and the remaining one was present in all the moderately resistant lines while absent in the susceptible control IISR-Subhakara. The specific band (~ 360 bp) that appeared in OPA-01 in the agarose gel was eluted, cloned, sequenced and converted into a SCAR marker. The SCAR primer was tested on selected black pepper seedlings. SCAR primers amplified DNA from moderately resistant seedlings only. The unique band was developed into a SCAR marker to differentiate moderately resistant and susceptible lines.

Jiang *et al*. (2009) reported the molecular characterization of Kava (*Piper methysticum*) to compare the genetic relationships among Kava and it's wild relatives by using RAPD markers. Twenty-eight accessions of Kava and it's wild relatives were studied. 20 random primers selected from 80 random primers for RAPD amplification. Total 40 bands were amplified by 20 random primers, in which 20 bands were polymorphic (12 per cent) for one or more species, 16 bands were monomorphic and rest 4 bands were Kava unique. The data analysis and cluster analysis were conducted by the software MVSP3.13f to create a dendrogram using UPGMA. Cluster analysis grouped the 28 accessions into six groups at similarity coefficient of 0.36, indicating that Kava was genetically distant from black pepper and its wild relatives. The similarity index values

ranged from 0.125 to 1 indicating the presence of enormous genetic diversity at molecular level. The similarity coefficients of pepper population ranged from 0.612 to 0.989. This showed that genetic difference within pepper population was relatively small.

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

Sen *et al*. (2010) analysed genetic diversity in *Piper* species using RAPD markers. Among 22 decamer RAPD primers screened, 11 were selected for comparative analysis of different species of *Piper*. High genetic variations were found among different species of *Piper.*

2.7.2.2 RAPD in testing hybrids

Magdalita *et al.* (1997) performed molecular analyses of *Carica papaya* x *C. cauliflora* interspecific hybrids. The 72 decamer primers screened for DNA amplification of *C. papaya*, *C. cauliflora* and the putative interspecific hybrids, generated a total of 207, 225 and 182 bands respectively. Overall, 64 per cent of bands were polymorphic between *C. papaya* and *C. cauliflora*. Seventeen (24 per cent) of these primers showed high resolution of the integrated parental bands into the putative interspecific hybrids. All the putative hybrids tested had at least one paternal RAPD band. However, any one of the 10-mer primers alone cannot identify all of the putative interspecific hybrids as the primers failed to assay other male bands when used individually.

Hulya (2003) studied the potential of Randomly Amplified Polymorphic DNA (RAPD) markers in varietal identification and genetic purity test of *Capsicum annuum* hybrid varieties. Five Jalepeno hybrid varieties and their corresponding parents were screened for polymorphic RAPD markers with 12

arbitrary 10-mer primers. Out of the 12 primers, six primers generated 11 useful RAPD markers to determine seed purity of all tested hybrid varieties. Among a total of 177 bands observed, 14 bands contributed by nine primers were polymorphic in the five pepper varieties and four RAPD markers were found to be cultivar-specific markers for three hybrid varieties.

Mehetre *et al*. (2004) analysed interspecific hybrid between *Gossypium arboreum* and *Gossypium stocksii* using RAPD markers. For this analysis, seven random primers were selected and seven types of markers identified of which Type IV markers were used in determining the hybrid nature of the offspring. 8.8 per cent of Type IV markers present in the offspring were shared with the male parent confirming the hybrid status. 19.3 per cent of bands were shared by male and female parents and offspring, while 15.8 per cent of markers were shared by the female parent and the offspring. Male and female parent had 15.8 per cent common bands, which were not shown in the offspring. 19.3 per cent of markers were unique each to male and female parents, while the hybrid showed 1.8 per cent novel bands. Also the studies on similarity matrix showed that the hybrid was more similar to female parent than male parent.

Seyal *et al.* (2008) used RAPD for hybrid authentication in upland cotton (*Gossypium hirsutum* L.). 16 decamer primers were selected and amplified a total of 518 fragments in the parents and hybrids and out which 76 loci were polymorphic. On an average, 7.13 bands per primer were observed with maximum of eight bands and minimum of six. The primer GLG-17 was found to produce 87.5 per cent polymorphic fragments. The lowest polymorphism (42.85%) was seen in primer GLH-2. Comparison of the RAPD banding pattern of the parents with the respective hybrids clearly identified genuine hybrids. The cluster dendrogram based on similarity matrix obtained by Unweighted Pair Group Method using Arithmetic average (UPGMA) also revealed the same results. This study suggested that RAPD analysis can be utilized for both reliable and less time consuming identification of hybrids.

Dongare *et al*. (2010) used RAPD for identification and genetic purity testing of cotton F_1 hybrid. Interspecific hybrid Phule-388 and it's parents RHCb-001 as male and RHC-006 as female were studied. Out of the 20 decamer primers, two primers OPA-08 and OPA-11 were found to be useful in differentiating parents and hybrid. OPA-08 primers resulted in amplification of 300bp and 500bp male specific amplicons as well as 1300bp female specific amplicons were also seen in hybrid Phule-388. Similarly, OPA-11 primer amplified 1000bp and 1800bp male specific amplicons and 1300bp female specific amplicon led strongly to support that RAPD markers can be conclusively used for hybridity testing.

Asif *et al.* (2006) reported genotyping analysis for hybrid identification in maize (*Zea mays* L.) using DNA fingerprinting technology. A total of 40 random decamer primers were surveyed. Of these, OPR-03, OPR-11 and OPT-06 were polymorphic which not only verified the purity among three lots of the same maize hybrid type, but also distinguished FM2 maize hybrid type from FM3. These three polymorphic primers produced unique banding patterns that clearly detect the purity of the hybrid lots. This result revealed that RAPD is a powerful tool for purity detection.

Liu *et al.* (2007a) reported genetic purity of a hybrid cabbage cultivar 'Zaoxia 16' using RAPD, ISSR, SRAP and SSR markers. Genetic relationships of the F_1 hybrids and their parents were analyzed with 157 RAPD primers, 54 ISSR primers, 84 SRAP primer combinations, and 44 SSR primers. Three RAPD primers (NAURP2006, NAURP2020, and NAURP2031), two ISSR primers (NAUISR1058 and NAUISR1062), one SRAP primer combination (NAUSR04/NAURS05) and two SSR primers (NAUSSR1011 and NAUSSR1031), which produced male and female parent-specific markers simultaneously, were selected for testing the genetic purity of the F_1 seeds. A total of 210 'Zaoxia 16' hybrid individuals were investigated with these eight selected primers. Of these, 12 appeared to be false hybrids. Nine of the 12 putative false hybrids, confirmed with all eight primers, exhibited similar banding patterns to

the female parent, suggesting that they could be derived from selfing of the female parent. This study showed that RAPD, ISSR, SRAP, and SSR markers are highly efficient and reproducible for genetic purity testing of commercial hybrid seeds.

Akhare *et al*. (2008) studied RAPD profile in Sorghum for identification of hybrids and their parents. Out of 15 decamer primers used for RAPD analysis, six primers yielded good and scorable amplified products for four sorghum hybrids namely CHS-14, CSH-9, CSH-19R and CSH-15R and their respective parental lines. The percentage of polymorphism over six primers varies from 37.49 to 54.44.

Identification of sugarcane interspecific F_1 hybrids "*Saccharum officinarum × Erianthus fulvus*" was performed with Random Amplified Polymorphic DNA (RAPD) analysis. Of 280 RAPD primers used, two primers, OPA-19 and OPN-11, were found to be the most suitable for identification of the hybrids and the hybrids facticity check out rate was 70.6 and 68.3 per cent, respectively (Zhang *et al.,* 2008).

2.7.3 Amplified Fragment Length Polymorphisms (AFLP) markers

Amplified Fragment Length Polymorphism (AFLP) is a powerful, reliable (Lu *et al*., 1996), stable and rapid assay with potential application in genome mapping (Thomas *et al*., 1995), DNA fingerprinting (Paul *et al*., 1997), gene tagging (Maksem *et al*., 1995), phylogenetic analysis of closely related plant species (Joy *et al*., 2007) and marker assisted breeding (Vos *et al*., 1995). This technique combines the reliability and robustness of RFLP and the power of PCR techniques. The reproducibility of AFLP is ensured by using restriction site specific adapters and adapter specific primers with a variable number of selective nucleotide under stringent amplification conditions (Vos *et al*., 1995). The large number of AFLP markers makes them an attractive choice for fine scale mapping (Thomas *et al*., 1995). Since polymorphism is detected as the presence or absence of amplified restriction fragments, AFLP are usually considered dominant markers (Mackill, 1996).

2.7.4 Simple Sequence Repeats (SSR) or Microsatellites markers

Simple Sequence Repeats (SSR) are tandem repeats of DNA sequence of only a few base pairs (1-6bp) in length. The most abundant being the dinucleotide repeat (McCouch *et al*., 1997). The term microsatellite was introduced to characterize the simple sequence stretches amplified by PCR (Hearne *et al*., 1992). These are also known as Short Tandem Repeats (STR) or Simple Sequence Repeats (SSR) and differ from minisatellite in which repeated sequences are having repeat units ranging from 11-60bp in length (Edwards *et al*., 1996). Microsatellite sequences are abundant, dispersed throughout the genome and are highly polymorphic in plant genomes, even among closely related cultivars, due to mutations causing variation in the number of repeating units in genomes (Condit and Hubbell, 1991).

A number of strategies have been designed to exploit microsatellite sequences for the study of DNA polymorphism in eukaryotes. They involve both hybridization and PCR based approaches. Oligonucleotide fingerprinting, a hybridization based approach represents polymorphism due to variation in the length of the restriction fragments that carry the microsatellites (Weising *et al*., 1998), while PCR based approaches detect variation in the length of microsaellites (Li and Quiros, 2001). Microsatellite generally display higher levels of polymorphism (Beckmann and Soller, 1990; Brown *et al*., 1996; Senior *et al*., 1998) and are amenable to automated genotyping strategies. It can be amplified by PCR and efficiently detect DNA polymorphism (Pejic *et al*., 1998).

SSR marker technology has been developed and used for genome mapping and DNA fingerprinting in different plant species such as black pepper (Mogalayi, 2011); rice (Wu and Tanksley, 1993; Yang *et al*., 1996); wheat (Roder *et al*., 1995); barley (Saghai *et al*., 1994) and sorghum (Dean *et al*., 1999).

2.7.4.1 Advantage of microsatellite markers

Microsatellites are considered as an important class of DNA markers because of their abundance and length hyper-variability. They occur frequently

and randomly in all eukaryotic DNA examined (Beckmann and Soller, 1990) and represent a vast source of highly informative markers (Weber, 1990). Microsatellites are highly polymorphic than RFLP and RAPD and easily assayed by PCR with small samples of genomic DNA. These markers are co-dominant and are inherited in Mendelian fashion and thus can be used for linkage analysis. They possess a selectively neutral behaviour (no pleiotriopic effects) and are highly reproducible, fast and easy to assay and can be developed at reasonable costs. These highly informative markers can be rapidly and reliably visualized using silver staining without the use of radio isotopes.

2.7.4.2 SSR in varietal identification

The first applications of microsatellite in plants were in cultivar identification, purity assessments and thus became the best choice in genotyping cultivars (Weising *et al*., 1991; Beyermann *et al*., 1992). Akkaya *et al.* (1992) reported that the polymorphism of SSR marker was a new source of PCR based molecular markers in soybean and also other plant genomes. In black pepper SSR markers reported are limited.

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

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

concerning population structure and demographic history of *P. polysyphonum* for conservation efforts.

Joy *et al.* (2011) used SSR markers to analyse genetic diversity among forty popular genotypes and four different species of black pepper in South India. A total of 62 alleles with an average of 15.5 alleles over 4 loci were identified. All the SSR primers showed an average Polymorphism Information Content (PIC) value of 0.85. The estimated average Shared Allele Frequency ranged between 1.57 and 20.12 per cent. The PCA plot revealed four closely related individual groups and identified Karimunda, wild pepper and a local landrace 'local b' as the most divergent genotypes. Cluster analysis exposed the genetic relatedness between hybrids and selections with other known cultivars.

In rice, more than 2740 Simple Sequence Repeats (SSR) markers have been developed and used to construct genetic maps (Wu and Tanksley, 1993; Chen *et al*., 1997). The microsatellite-derived DNA fingerprints are ideally suited for the identification of rice genotypes as reported by Ramakishana *et al*. (1994). Rice microsatellites have been demonstrated to be polymorphic between (Yang *et al*., 1994; Panaud *et al*., 1996; Akagi *et al*., 1997) and within rice varieties (Olufowote *et al*., 1997). Zhou and Gustafson (1995) were able to identify and fingerprint 57 rice cultivars released in USA using microsatellites. Pronvan *et al*. (1997) quantified intra and inter cultivar polymorphism between the cultivated and wild rice.

Gealy *et al.* (2002) used SSR marker to determine the genetic diversity represented by accessions of red rice and to identify DNA markers that useful in identifying hybrids between red and cultivated rice. Seventy-nine red rice accessions, 10 known or putative hybrid derivatives of red rice and cultivated rice (RC hybrids) and seven rice cultivars were analyzed using microsatellite DNA markers developed for cultivated rice. Microsatellite markers differentiated awned and awnless red rice accessions, six of the seven rice cultivars, and all 10 cultivated rice (RC hybrids) tested.

Yashitola *et al.* (2002) analysed purity of two CMS lines and six restorer lines of rice and their hybrids using microsatellite and STS markers. Thirteen microsatellite and five STS markers were used in the analysis of these lines. Of the 13 microsatellite loci analyzed, five were polymorphic and eight were monomorphic for the lines that were screened. Of the five STS loci analyzed, three were polymorphic and two were monomorphic for the lines that were screened. The frequency of heterozygosity for a hybrid ranged from 7.7 per cent to a maximum of 38.5 per cent with respect to microsatellite loci and 0 to 60 per cent for STS loci.

Nandakumar *et al*. (2004) used microsatellite markers for fingerprinting and assessing variation within parental lines and testing the genetic purity of hybrid of rice. Ten Sequence Tagged Microsatellite Sites (STMS) markers were used for fingerprinting of 11 rice hybrids and their parental lines. Nine STMS markers were found polymorphic across the hybrids and produced unique fingerprints for the 11 hybrids. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped the hybrids into three clusters. The genetic similarity between the hybrids ranged from 0.33 to 0.92 with an average similarity index of 0.63.

Sundaram *et al.* (2008) identified informative SSR markers capable of distinguishing hybrid rice parental lines. 10 cytoplasmic male sterile (CMS) and restorer (R) lines along with 10 popular Indian rice varieties analysed using a set of 48 hyperpolymorphic SSRs distributed uniformly across the rice genome. All the SSR markers were polymorphic, amplifying a total of 163 alleles, with an average of 3.36-3.85 allelic variants per locus. Twenty-seven SSR markers showed amplification of an allele, which was very specific and unique to a particular parental line and not amplified in any other rice genotype. With a set of 10 SSR markers, all the public bred Indian rice hybrids along with their parental lines were clearly distinguished.

Study was conducted on a series of hybrid rice varieties, first Iranian hybrid rice (IRH1), fingerprinting and genetic purity determination of hybrid seeds using

microsatellite (SSR) and Random Amplified Polymorphic DNA (RAPD) markers (Hashemi *et al*., 2009). Sixteen rice genotypes including 3 cytoplasmic male sterile (CMS) lines, 5 restorer lines and their 8 hybrid combinations were used, fifteen microsatellite and twenty nine RAPD markers were used for DNA profiling of hybrids and their parental lines. Ten SSR loci and 15 RAPD multi loci were chosen for further molecular analyses. The results revealed that the SSR loci RM 154, RM 206, RM 337 (with 6, 5 and 4 observed genotypes, respectively) and RAPD multi loci OPA04, OPA08, OPA17, OPB14, OPC04, OPG11 and OPG13 have the highest efficacy for DNA profiling and discrimination of rice hybrids and lines. The SSR markers amplified the unique fingerprints for the restorer lines, they could not differentiate between 2 CMS lines (Neda-A and Nemat-A) and their resultant hybrids. The rice CMS lines and restorer lines could be uniquely identified by RAPD multi locus amplified profile at 7 informative loci. Cluster analysis based on shared alleles and Jaccard's similarity coefficient using UPGMA algorithm, grouped the rice genotypes into 3 and 4 major clusters, according to their microsatellite and RAPD fragment similarities respectively.

Sayed *et al*. (2001) also studied on the variation and evolution of Simple Sequence Repeats (SSR) loci in the cotton diploid and polyploid genome. They found differences between dinucleotide microsatellite repeats in diploid and polyploid *Gossypium* genomes of natural and synthetic origion.

Kebede *et al*. (2007) analysed the genetic diversity in a diploid cotton species, *G. herbaceum* (A1) and *G. arboretum* (A2) by using microsatellite markers. Forty-one germplasm accessions were evaluated with 32 microsatellite loci. Sixty primer pairs were tested for amplification of microsatellite markers primarily in the A genome cottons. Of the 60 primer pairs, only 32 gave polymorphic patterns and were used to assess genetic diversity in twenty *G. herbaceum* (A1) and twenty one *G. arboretum* (A2) accessions. Genetic similarities based on Jaccard's coefficient were calculated using the SIMQUAL program of the Numeric Taxonomy Multivariate Analysis System (NTSYS-pc). Based on data from microsatellite analysis, genetic similarity values among *G.*

herbaceum accessions ranged from 0.77 to 0.97, with a mean of 0.89, whereas the values for *G. arboretum* ranged from 0.82 to 0.98, with a mean of 0.89, showing that intra-specific genetic variability in the two species is similar. Genetic similarities among accessions of the two A-genome species ranged from 0.62 to 0.86 with a mean of 0.70 shows a pair-wise genetic similarity matrix for selected accessions of the two A-genome species. An UPGMA tree and principal coordinate analysis based on genetic similarity matrices showed distinct clusters consistent with the genomic groups.

Asif *et al.* (2009) verified parentage of F_1 hybrids of cotton using random amplified polymorphic DNA (RAPD) and microsatellite (SSR) assays. Out of 500 primers surveyed, 3 decamer random primers (OPM-07, OPU-01 and OPV-01) and 3 EST based SSR primers (MGHES-06, MGHES-17 and MGHES-24) were found extremely polymorphic between two cotton parents (FH-883 and FH-631S). These highly informative primers differentiated cotton parents FH-883 and FH-631S and also confirmed the parentage of their F_1 hybrids.

Liu *et al*. (2007b) used RAPD, ISSR and SSR marker for assessment of genetic purity of tomato (*Lycopersicon esculentum* L.) hybrid cultivars 'Hezuo 903' and 'Sufen No. 8. Genomic DNA from the two F_1 hybrids and their corresponding parental lines were screened with 218 RAPD decamer primers, 54 ISSR primers and 49 SSR primers. Among the 321 primers, 4 primers for 'Hezuo 903' and 3 for 'Sufen No. 8', produced both female and male parent-specific markers, and were selected for testing the genetic purity. A total of 210 hybrid individuals of each cultivar were analyzed using the identified primers. The combined results of the marker analysis showed that eight of the 210 F_1 plants in 'Hezuo 903' and 13 of 210 in 'Sufen No. 8' were false hybrids, and the overall genetic purity of the two F_1 hybrid seed lots was 96.2 and 93.8 per cent, respectively. Study showed that RAPD and SSR markers provide a practical and efficient tool in quality control of the tomato commercial hybrid seeds.

Benor *et al.* (2008) studied the genetic diversity of 39 determinate and indeterminate tomato inbred lines collected from China, Japan, S. Korea, and USA. Using 35 SSR polymorphic markers, a total of 150 alleles were found with moderate levels of diversity and a high number of unique alleles existing in these tomato lines. The mean number of alleles per locus was 4.3 and the average Polymorphism Information Content (PIC) was 0.31, which confirms that SSR markers are highly informative. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering at genetic similarity value of 0.85 grouped the inbred lines into four groups, where one USA cultivar formed a separate and more distant cluster. The average genetic similarity matrix result was also consistent with clustering of the inbred lines with their growth habit.

Roy *et al*. (1999) identified the DNA markers linked with tolerance to the PHS (preharvest harvest sprouting) in bread wheat. One hundred RILs (recombinant inbred lines) were developed. Two parents were analyzed with two hundred and thirty two STMS (Sequence Tagged Microsatellite Sites) and one hundred and thirty eight STS primer pairs. The total of three hundred primers proved functional by giving the scorable PCR products. Fifty seven STMS and thirty STS primers gave reproducible polymorphism between the parental lines.

Ganeva *et al*. (2010) assessed genetic diversity of Bulgarian durum wheat (*Triticum durum* Desf.) landraces and modern cultivars using 14 highly polymorphic microsatellite markers. A total of 100 alleles were identified, with an average of 7.14 alleles per marker. The gene diversity values (He) of the markers for the total samples ranged from 0.23 (WMS357 and WMS631) to 0.77 (WMS46), with an average of 0.52. The landraces that were collected from 18 sites in Southern Bulgaria showed 2-11 alleles per locus with an average of 6.07. The microsatellite analysis suggested that the genetic diversity among landraces was lower compared to the diversity levels for durum wheat in countries close to the main centre of wheat domestication.

Ahmed *et al.* (2011) reported authentication of wheat (*Triticum aestivum* L.) F$_1$ hybrids using SSR markers. A total of 15 SSR primers of Xgwm series and 5 of X series were used to find out the codominant loci in the hybrid and single dominant loci in parents. Three primers from X series, namely, X66-5b, X-135-1a, and X-129-2b, gave the polymorphic bands in hybrids. Out of 15 primers, Xgwm- 314 and Xgwm-311 gave the polymorphic banding pattern.

Odeny *et al*. (2009) reported the new microsatellite markers for pigeonpea (*cajanus cajan* (L.) millsp.) and tested the transferability of soybean microsatellites in pigeonpea. Primers were designed for 113 pigeonpea genomic SSRs, 73 of which amplified interpretable bands. Thirty-five of the primers revealed polymorphism among 24 pigeonpea breeding lines. The number of alleles detected ranged from 2 to 6 with a total of 110 alleles and an average of 3.1 alleles per locus. GT/CA and GAA class of repeats were the most abundant dinucleotide and tri-nucleotide repeats respectively. Additionally, 220 soybean primers were tested in pigeonpea, 39 of which amplified interpretable bands and showed little genetic diversity within cultivated pigeonpea.

Saxena *et al.* (2010) did molecular characterization of hybrid parents and purity assessment of pigeonpea (*Cajanus cajan* L.) hybrid using SSR marker. Total 148 SSR markers were used for diversity analysis. Marker profiles obtained and allelic data were used to prepare a dissimilarity matrix and to construct a two dimensional (2D) plot using the factorial analysis method with DARwin V5.0.128 software. In total, 41 (27.7%) markers showed polymorphism with 2 to 6 (average 2.6) alleles and 0.01 to 0.81 (average 0.34) Polymorphism Information Content (PIC) value. Two SSR markers, CCB4 and CCttc006, were found most suitable for purity assessment of hybrid seeds of the ICPH 2438 hybrid.

Cordeiro *et al*. (2000) reported characterisation of microsatellite markers from sugarcane varieties (*Saccharum* spp.) derived from complex interspecific hybridisation between the species *S. spontaneum* and *S. officinarum*. A set of microsatellite markers for genome analysis in cultivated sugarcane was identified

from an enriched genomic DNA library constructed from *Saccharum* sp. cv Q124. Sequencing of 798 sugarcane genomic DNA clones from an enriched microsatellite library, yielded 457 inserts containing microsatellite repeat motifs. Primer sets were designed and synthesised for over 100 microsatellite sequences and tested on a set of five sugarcane cultivars. The number of alleles recorded per marker across the five genotypes tested ranged between 3 and 12 alleles with an average of 8. Markers that show polymorphism had a PIC value between 0.48 and 0.8. The mean PIC value of the markers on the genotypes tested was 0.72.

Cordeiro *et al.* (2003) studied genetic diversity between members of the *Saccharum* genera (*S. officinarum*, *S. sponfaneum*, *S. sinense*), Old world *Erianthus* Michx. sect. *Ripidium*, North American *E. giganteus* (*S. giganteum*), Sorghum and Miscanthus using SSR marker. Six SSR markers were tested on 66 accessions and produced a total of 187 distinct alleles. Similarity coefficient calculations and clustering revealed a genetic structure for *Saccharum* and *Erianthus* sect. *Ripidium* that reflected the close relationship previously identified using other marker systems. The results indicated that SSRs will be an ideal means for the identification of the genetic constitution of modern sugarcane cultivars of interspecific origins.

Cai *et al*. (2005) reported the genetic relationship between a drought and cold tolerant wild relative of sugarcane *Erianthus rockii* and the *Saccharum* complex using microsatellite (SSR) and AFLP markers. Genotypes representing seventeen species from five genera in the *Saccharum* complex were evaluated with approximately 200 microsatellite (SSR) and Amplified Fragment Length Polymorphism (AFLP) markers and similarity matrices constructed. Principal Component Analysis (PCA) was undertaken separately with the AFLP and SSR data. The results from both data sets were very similar and suggested that *E. rockii* was distinct from other *Erianthus* and *Saccharum* species. *E. rockii* clustered with *E. fulvus* and two *Miscanthus* species, mid-way between the major *Saccharum* and *Erianthus* clusters.

Manigbas and Villegas (2005) used SSR to identify true hybrids of sugarcane. In this study, 918 progenies derived from four sugarcane crosses were tested. Fifty SSR primer pairs were screened for polymorphism using DNA samples from the parents and their progenies. One primer revealed highly polymorphic bands and was used to assess and evaluate the hybridity of the progenies in each of the populations. The true hybrids and non hybrids were determined by the comparison of the distinct banding patterns of the parents and progenies. The SSR banding profiles of each parents and progenies were scored manually for the presence (1) or absence (0). Molecular analysis showed that among progenies derived from the cross P80-13 x V86-550, 82 per cent were true hybrids and 18 per cent selfed or non hybrids. Similarly, 87 and 47 per cent true hybrids as well as 13 and 56 per cent non hybrids or selfed progenies obtained from the cross V88-354 x V88-550 and P80-13 x P87-27 respectively. This investigation has proved that SSR markers can be a powerful tool in sugarcane variety improvement program. With the technology, it is possible to efficiently select new varieties in a short period of time. The results imply that the existing 7 year breeding cycle for sugarcane can be reduced by 2 year.

Wang *et al.* (2002) reported identification of parents of maize F_1 hybrids through SSR profiling of maternal and hybrid tissue. Genotypes were examined using ten SSR loci for each hybrid and their parents. SSR loci were selected that distinguished between all parental genotypes for each hybrid. One hundred SSR profiles were generated using pericarp tissue (10 hybrids $\times$ 10 SSR loci). Ninety-four per cent of amplifications from pericarp tissue resulted in the SSR profile of the female inbred parent of the hybrid. Five per cent of the profiles showed both female and male contributions to the hybrid and one per cent showed only the contribution of the male parent, possibly due to a failure of SSRs to always report allheterozygote alleles.

Two sets of tropical maize inbred lines, one derived from the BR-105 population and another derived from the BR-106 population, were assayed for Amplified Fragment Length Polymorphism's (AFLP) and for Simple Sequence

Repeats (SSR) marker (Barbosa *et al*., 2003). Heterotic group and single cross yield performance assigned in order to investigate genetic distances among lines and their relationship. Cluster analysis was in agreement with the original assignment for heterotic groups. Inbred line 16, derived from BR-106, was assigned to the BR-105 set, in agreement with single cross yield performance from intra- and interpopulation crosses for SSR where another two lines from BR-106 were also assigned correlation coefficients. Genetic Distances (GD) with F_1 grain yield and heterosis were high for BR-106 × BR-106 crosses (0.91 and 0.82 for AFLP and SSR, respectively), moderate for BR-105 × BR-105 crosses (0.52 for AFLP and SSR) and low for BR-105 × BR-106 crosses (0.29 and 0.16 for AFLP and SSR, respectively). The AFLP and SSR-based GDs indicated that the different heterotic group lines are genetically more divergent than those of the same heterotic group.

Pongsai *et al*. (2009) used microsatellite markers to identify heterotic pattern of F_1 hybrid of two tropical maize populations. The fifty SSR markers revealed a total of 153 alleles, with 3.06 alleles per locus on average. The Genetic Distance (GD) between the two S_0 sub-populations of the YNP_0 and GXP_0 showed a low difference value of 0.03. The Genetic Distance (GD) of the S_1 selected lines from the two maize populations ranged from 0.14 to 0.94, with the average of 0.44, which manifested the high genetic diversity among the S_1 selected lines. The grain yield of the F_1 hybrids obtained from the crosses between the S_1 selected lines of both populations was evaluated.

Using SSR Markers, rapid and reliable purity identification of F_1 hybrids of maize (*Zea may* L.) reported by Wu *et al*. (2010). Out of the 10 pairs of primer used, four primer pair phi034, phi057, phi080 and nc030 gave polymorphism between the male and female parents of the two hybrids (Nongda 108 and Zhengdan 958). The purity of seed samples of the two F_1 hybrids was identified with these four pairs of primers.

Kaul *et al.* (2009) reported morphological and molecular analyses of *Rosa damascena* (cv. Jwala and Himroz) x *R. Bourboniana* interspecific hybrids. Twenty-two selected primer (Operon Technologies Inc.) of RAPD and 15 microsatellite primer pairs developed for *Rosa hybrida* were screened for parental polymorphism and three primer pairs for the loci Rh 48, Rh 78 and RhB510 were selected for verifying the hybridity of interspecific hybrids. According to presence or absence of bands RAPD and SSR markers were classified into seven types of markers. Cluster analysis, based on Jaccard's similarity coefficient using UPGMA, reliably discriminated the hybrids into 2 main clusters. The average similarity index across the hybrids was 0.63. The two parental lines Jwala and Himroz were 0.90 similar, whereas similarity with the third parental line (*R. bourboniana*) was 0.75 and 0.80 respectively.

Sehgal *et al.* (2009) assessed genetic diversity of the safflower (*Carthamus tinctorius* L.) world germplasm resources. Twenty-two RAPD primers from A, C, H, and K series, 18 SSR primers, and 10 AFLP primer combinations were used. The RAPD, SSR and AFLP primer combinations revealed 57.6, 68.0, and 71.2 per cent polymorphism, respectively, among 111, 72, and 330 genetic loci amplified from the accessions. The pairwise genetic similarities calculated between accessions for RAPD, ISSR, and AFLP markers ranged from 0.70 to 0.97, 0.44 to 0.95, and 0.50 to 0.97.

Eiadthong *et al.* (1999) reported use of SSR markers for identification of twenty-two mango cultivars (*Mangifera indica* Linn.) of Thailand and evaluation of their genetic variation. Out of 40 SSR primers screened, seven primers gave reproducible, polymorphic DNA amplification patterns, and were selected to construct a DNA fingerprint to distinguish the genotypes of mango. The number of bands generated ranged from 8 to 21 per primer. Using banding patterns obtained from these seven primers, each cultivar was distinguished from the others. Similarity coefficients were calculated based on 56 selected bands and UPGMA clustering analysis was performed. Two Thai mango cultivars (Nang Klangwan and Nong Saeng) were found to be very distant from the other

cultivars. The remaining 11 Thai cultivars were classified into three groups based on the dendrogram. Seven cultivars were placed in the same group as two Florida cultivars (Brooks and Edward), one Philippine cultivar (Carabao) and one Indonesian cultivar.

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

Chowdari *et al.* (1998) analysed genetic diversity in five cytoplasmic male sterile and seven restorer lines of pearl millet by using a $(GATA)_4$ microsatellite and Randomly Amplified Polymorphic DNA (RAPD). Twelve lines of pearl millet were surveyed for DNA polymorphism with 20 primers (OPA1-OPA20) from Operon Technologies and with a $(GATA)_4$ microsatellite probe. A total of 160 polymorphic loci were generated, and based on the polymorphism data, similarity index values ranged from 0.81 to 0.50. Cluster analysis was performed and relationships among these lines revealed that they were not in agreement with the available pedigree data.

Cappelle *et al*. (2007) used SSR marker to study regeneration and molecular characterization of a male sterile interspecific somatic hybrid between *Cichorium intybus* and *C. endivia*. Protoplast fusions between two fertile lines of *Cichorium* sp. have been made. Five pairs of primers for SSR markers (FDCA94, FDCB39, FDCB105, FDCB214, FDCD21) were used to analyse the hybrid nature of the ms protoplast fusion product. Among the 288 regenerated plants, only one was male sterile in spite of the relative high number of regenerated somatic hybrids.

Pallavi *et al.* (2011) used SSR markers for hybridity and seed genetic purity testing in sunflower (*Helianthus annuus* L.). 58 primer pairs were screened to identify specific markers associated with each hybrid and parental lines. Hybrid KBSH-44 could be clearly identified by using 'ORS 309 and ORS 170' based on

the banding pattern resolved on polyacrylamide gel (6%). The complementary banding pattern of both parents identified the hybrid. ORS 309 amplified allele size of 250bp specific to the female parent (CMS-17A) and 230bp specific to the male parent (RHA 95-C-1). These two bands of allele size 230bp and 250bp were found in hybrid KBSH-44 only. Another SSR primer 'ORS 170' was able to distinguish the hybrid KBSH-44 by amplifying allele of size 230bp a female specific (CMS-17A) allele and 200bp amplicon a male specific allele (RHA 95-C-1). SSR primer ORS 811 found specific to identify KBSH-53 and it amplified allele of size 270bp in its female parent (CMS-53A) and allele size of 230bp in its pollen parent (RHA 95-C-1). The hybrid has both the alleles from its parents at 270bp and 230bp.

A marker-assisted screening for resistance sources in soybean was also initiated by Yu *et al*. (1996) by using the Simple Sequence Repeats (microsatellite) markers, that were tightly linked to SMV (soybean mosaic virus) resistance gene (*Rsv1*). In order to screen the sixty seven (67) diverse soybean cultivars, breeding lines and plant introductions, the marker locus was used. Five variants were observed at the microsatellite locus.

Materials and Methods

3. MATERIALS AND METHODS

The study on "Molecular characterization and testing hybridity of interspecific crosses in black pepper (*Piper nigrum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2010-2012. The materials used and methodologies adopted are discussed in this chapter.

3.1 Materials

3.1.1 Plant Materials

Putative interspecific hybrids developed at Pepper Research Station (PRS), Panniyur by crossing six popular varieties/cultivars of *Piper nigrum* with *Piper colubrinum* were used for the present study (Plate 1). The hybrids were planted during 2004 at PRS, Panniyur and started spiking.

Rooted cuttings of the putative hybrids and their corresponding parents from six interspecific crosses were collected from Pepper Research Station (PRS), Panniyur and maintained as potted plants in the green house at CPBMB, College of Horticulture, Vellanikkara. The six interspecific hybrids used were:

1. Panniyur 5 x *Piper colubrinum* (P5PC)
2. Panniyur 3 x *Piper colubrinum* (P3PC)
3. Panniyur 2 x *Piper colubrinum* (P2PC)
4. Panniyur 1 x *Piper colubrinum* (P1PC)
5. Karimunda x *Piper colubrinum* (KMPC)
6. Uthirankotta x *Piper colubrinum* (UKPC)

3.1.2 Laboratory chemicals, Glass ware and Plastic ware

The chemicals used in the present study were of good quality (AR grade) procured from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq* DNA polymerase, dNTPs, *Taq* buffer and molecular marker (λDNA/*Hind*III+*Eco*RI double digest) were supplied by Bangalore Genei Ltd. All

Panniyur 5

Panniyur 5 x *P. colubrinum*

Panniyur 3

Panniyur 3 x *P. colubrinum*

Panniyur 2

Panniyur 2 x *P. colubrinum*

**Plate 1: Putative interspecific hybrids and their respective parents used
in the study**

Panniyur 1

Panniyur 1 x *P. colubrinum*

Karimunda

Karimunda x *P. colubrinum*

Plate 1: Putative interspecific hybrids and their respective parents used in the study

Uthirankotta

Uthirankotta x *P. colubrinum*

Male parent *Piper colubrinum*
**Plate 1: Putative interspecific hybrids and their respective parents used
in the study**

the plastic ware used were obtained from Axygen and Tarson India Ltd. The decamer primers were obtained from Operon Technologies Inc. (Alamedda, Calif.), and SSR primers were obtained from Sigma Aldrich Chemical Pvt. Ltd.

3.1.3 Equipment and Machinery

The present research work was carried out using molecular biology facilities and equipments available at CPBMB, College of Horticulture. Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500). NanoDrop^R ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Eppendorf Master Cycler (Eppendorf, USA) and Veriti Thermal Cycler (Applied Biosystem, USA). Horizontal gel electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc - BIO-RAD was used for imaging and documenting the agarose gel. The details are given in Annexure I.

3.2 Morphological analysis

Morphological observations of parents and putative hybrids maintained at Pepper Research Station (PRS) were recorded as per descriptor of black pepper (IPGRI- Biodiversity International, 1995). The characters included were as follows:

3.2.1 Stem characters

a. Internodal length (cm)

Average of ten randomly selected internodal lengths.

b. Angle of insertion of reproductive branch

Angle subtended by the spike bearing branch with the main stem was measured in degree with the help of protractors. Observation were recorded from ten spike bearing branches.

c. Thickness at node and internode (cm)

Circumference at node and internode was measured for ten randomly selected nodes and internodes and average computed.

3.2.2 Leaf characters

Leaf characters (from plagiotrope) like length, width, area, petiole length, colour, texture and shape were recorded for young and mature leaves. Ten leaves were chosen at random from all vines as per black pepper descriptor.

a. Length of leaf lamina (cm)

Average of ten randomly selected mature leaves measured from the base of the midrib to the tip.

b. Breadth of leaf lamina (cm)

Average of ten randomly selected mature leaves measured at the maximum width.

c. Leaf area (cm^2)

Leaf area was calculated from the ten randomly selected young and mature leaves of plagiotropes and the average computed.

Leaf area was calculated (Kandiannan *et al*., 2002) as follows:

Leaf area (cm^2) = k x leaf length (cm) x maximum width of the leaf (cm); where k= an empirical constant (0.84).

d. Length to breadth ratio

Length to breadth ratio of the leaves of reproductive branch was calculated from the length and width of lamina and mean arrived at.

e. Petiole length (cm)

Petiole length was measured from mature leaf in centimetre and mean arrived at.

f. Shape of lamina, lamina base, lamina margin

Lamina shape was recorded by visual observation as ovate, lanceolate, elliptic, cordate or other shapes.

Lamina base shape was recorded as oblique, round, cordate or other by visual observation.

The margin was recorded by visual observation as even or wavy.

g. Lamina texture

Texture was recorded by visual observation as membranous, coriaceous or others.

h. Lamina colour

The colour of lamina was recorded by visual observation.

3.2.3 Reproductive Characters

3.2.3.1 Spike characters

Spike length, spike orientation, spike shape, spike colour, number of spike, fresh and dry weight of spike, were recorded for 10 spikes selected at random.

a. Number of spike per branch

Average of ten randomly selected lateral branches.

b. Length of spikes (cm)

Average of ten randomly selected spikes.

c. Berries per spike

Number of berries was counted for ten randomly selected spikes and the average computed.

d. Well developed berries per spike (%)

Number of well developed berries was counted for ten randomly selected spikes and the average computed.

Percentage of well developed berries per spike was calculated as follows:

Well developed berries per spike (%) = No. of well developed berries / Berries per spike x 100

e. Fruit setting (%)

Fruit setting percentage was calculated for ten randomly selected spikes and the average computed.

Fruit setting percentage was calculated as follows

Fruit setting (%) = No. of well developed berries per spike / Total No. of berries per spike x 100

f. Thousand berry fresh weight (g)

Average of hundred well developed berries taken from randomly selected spikes and converted to thousand berries.

g. Thousand berry dry weight (g)

The harvested spikes were dried in partial shade for a period of four to five days and their weight was recorded.

h. Spike shape, orientation and colour

Spike shape was recorded as oblong, cylindrical or other by visual observation. Spike orientation was recorded by visual observation as erect or suberect. Spike colour was recorded as reddish brown, greyish brown or greenish black when spike are fully developed.

3.2.4 Seed germination of Culture P5PC

Fully ripe berries of P5PC were harvested and after removing the flesh, the seeds were germinated in green house. Seedlings obtained from viable seeds were used for molecular characterization.

3.3 Molecular analysis

Molecular analysis of the putative interspecific hybrids and their parents were carried out with two different marker systems- Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR).

3.3.1 Genomic DNA extraction

The plant genomic DNA was isolated from pale green tender leaves (1st or 2nd leaf from tip) of six interspecific hybrids as well as their parents using CTAB extraction method suggested by Rogers and Bendich (1994) with slight modifications.

3.3.1.1Reagents:

The procedure for preparation of reagents required for DNA isolation is given in Annexure II.

1. CTAB extraction buffer (4x)
2. CTAB (10%)
3. β- mercaptoethanol
4. Chloroform : isoamyl alcohol (24:1)
5. Chilled isopropanol
6. Ethanol (70 per cent)
7. RNase A (DNase free)
8. TE buffer

3.3.1.2 Procedure for DNA isolation

Young and tender leaf tissue (1g) was weighed and ground in liquid nitrogen using mortar and pestle along with 50 μl of β-mercaptoethanol and a pinch of Poly Vinyl Pyrrolidone (PVP). The sample was ground into fine powder using excess of liquid nitrogen, 4 ml of extraction buffer (4x) and the powder was transferred to a sterile 50 ml centrifuge tube containing 3 ml of pre-warmed extraction buffer (total 7 ml). The homogenate was incubated for 30 minutes at

65ºC with occasional mixing by gentle inversion. Equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 15 minutes at 4ºC. The contents got separated into three distinct phases. The aqueous phase containing DNA was pipetted out into a fresh 50 ml Oakridge tube and RNase A (10mg/ml) of about 3 μl was added to remove RNA contaminant and incubated in water bath at 37ºC for 30 minutes. After incubation, one tenth volume of 10 per cent CTAB solution was added followed by purification with equal volume of chloroform: isoamyl alcohol (24:1) mixture and centrifuged at 10,000 rpm for 15 minutes at 4ºC. The supernatant was collected, 1/3 volume of chilled isopropanol was added and incubated at -20ºC for 30 minutes. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 minutes at 4ºC. The pellet was collected and washed first with 70 per cent alcohol and then with 100 per cent alcohol. Pellet was air dried for 30 minutes at room temperature and dissolved in 100 μl TE buffer.

3.3.2 Purification of DNA

The DNA which had RNA as contaminant was purified by RNase treatment and further precipitation. The RNase treatment was carried out during the DNA isolation steps, before pelleting the DNA.

3.3.3 Assessing the quality of DNA by Electrophoresis

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

3.3.3.1 Reagents and Equipments

1. Agarose
2. 50X TAE buffer (P^H 8.0)
3. 6X Loading /Tracking dye
4. Ethidium bromide (0.5 μg/ml)
5. Electrophoresis unit, power pack (BIO-RAD), gel casting tray, comb
6. UV transilluminator (Wealtec)

7. Gel documentation and analysis system (BIO-RAD)

Chemical composition of buffers and dye are given in Annexure III. The procedure followed for agarose gel electrophoresis was as follows:

Agarose (0.8%) was weighed and dissolved in TAE buffer (1X) by boiling, added ethidium bromide (0.5μg/ml) and mixed well. The open end of the gel casting tray was sealed with cello tape and kept on a horizontal surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (5 μl) along with tracking dye (1 μl) was loaded into the wells using a micropipette carefully. λDNA/*Eco*RI+*Hind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

Then the gel was taken from the electrophoresis unit and viewed under UV transilluminator for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in gel documentation system (Gel DOC-It ^{TM} Imaging system UVP (USA). The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.3.4 Assessing the quality and quantity of DNA by NanoDrop method

The quality and quantity of genomic DNA was estimated using NanoDrop^{R} ND-1000 spectrophometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1μl autoclaved distilled water as blank. One micro litre of nucleic acid sample was measured at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} ratios were recorded to assess the purity of DNA. A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality of DNA. The quantity of DNA in the pure sample was calculated using the formula OD_{260}= 1 is equivalent to 50 μg double stranded DNA/ μl sample.

$$1\text{OD at } 260 \text{ nm} = 50 \text{ μg DNA/ml}$$

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

3.4 Molecular Markers used for the study

Two different types of markers viz., Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) were used for the study in order to confirm the result at molecular level.

3.4.1 DNA amplification conditions

The PCR condition required for effective amplifications in RAPD and SSR analysis include appropriate proportions of the component of the reaction mixture. The reaction mixture includes template DNA, assay buffer A or B, $MgCl_2$, *Taq* DNA polymerase, dNTPs and primers. The aliquot of this master mix was dispensed into 0.5 ml or 0.2 ml PCR tubes. The PCR was carried out in an Eppendorf Master Cycler (Eppendorf, USA)/Veriti Thermal Cycler (Applied Biosystem, USA).

3.4.2 RAPD (Random Amplified Polymorphic DNA) analysis

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

The amplification was carried out in an Eppendorf Master Cycler (Eppendorf, USA). PCR amplification was performed in a 20 μl reaction mixture as constituted below:

Composition of the reaction mixture for PCR

        a) Genomic DNA (40ng)   - 2.0 μl

        b) 10X *Taq* assay buffer B   - 2.0 μl

c) MgCl₂   - 1.5 μl

c) dNTP mix (10mM each)   - 1.0 μl

d) *Taq* DNA polymerase (3U)   - 0.3 μl

e) Decamer primer (10 pM)   - 1.5 μl

f) Autoclaved distilled water   - 11.7 μl

Total volume   - 20.0 μl

The thermocycler was programmed as follows:

94 $^{\circ}$C for 4 minutes   -   Initial denaturation

92 $^{\circ}$C for 1 minute   -   Denaturation

37 $^{\circ}$C for 1 minute   -   Primer annealing   } 40 cycles

72 $^{\circ}$C for 2 minutes   -   Primer extension

72 $^{\circ}$C for 8 minutes   -   Final extension

4 $^{\circ}$C for infinity to hold the sample

3.4.2.1 Screening of random primers for RAPD analysis

Thirty decamer primers (Operon Technologies) were screened for RAPD analysis (Annexure IV).

3.4.2.2 Random primers selected for RAPD Assay

Out of 30 decamer primers screened for RAPD analysis, 10 primers (Table 1) yielded the best amplification products.

The amplified products were run on 1.3 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA/*Eco*RI+*Hind*III double digest). The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-It ^{TM} Imaging system

Table 1: List of selected decamer primers

Sl. No.	Primer	Nucleotide Sequence
1	OPA 8	5'-GTGACGTAGG-3'
2	OPA 10	5'-GTGATCGCAG-3'
3	OPA 17	5'-GACCGCTTGT-3'
4	OPA 28	5'-GTGACGTAGG-3'
5	OPA 30	5'-AGGTGACCGT-3'
6	OPC 08	5'-TGGACCGGTG-3'
7	OPC 09	5'-CTCACCGTCC-3'
8	OPC 14	5'-TGCGTGCTTG-3'
9	OPP 08	5'-ACATCGCCCA-3'
10	OPAH 09	5'-AGAACCGAGG-3'

UVP (USA). The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic band in kb/bp of bases was recorded in comparison with marker.

3.4.3 SSR (Simple Sequence Repeats) analysis

The good quality genomic DNA (25 to 30ng/µl) isolated from black pepper leaf samples were subjected to SSR as per the procedure reported by Menezes *et al.* (2009). SSR primers supplied by Sigma Aldrich, USA with good resolving power were used for amplification of DNA. SSR primers for SSR assay were selected after an initial screening of primers.

The amplification was carried out in a Veriti Thermal Cycler (Applied Biosystem, USA). PCR amplification was performed in a 20 µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR

a) Genomic DNA (30ng)	-	1.5µl	
b) 10X *Taq* assay buffer A	-	2.0µl	
c) dNTP mix (10mM each)	-	1.0µl	
d) *Taq* DNA Polymerase (3U)	-	0.3µl	
e) Forward Primer (10pM)	-	0.75µl	
f) Reverse Primer (10pM)	-	0.75µl	
g) Autoclaved Distilled Water	-	13.7µl	
Total volume	-	20.0µl	

The thermalcycler was carried out with the following programme

94°C for 1 minute   -   Initial denaturation

94°C for 1 minute   -   Denaturation

Primer annealing temperature denoted in table 2 for 1 min   } 30 cycles

72°C for 1 minute   -   Primer extension

72°C for 5 minutes   -   Final extension

4°C for infinity to hold the sample

3.4.3.1 Screening of SSR Primers for SSR Analysis

Fifty four SSR primers were screened by PCR for SSR analysis (Annexure V).

3.4.3.2 SSR primers selected for SSR assay

Out of 54 primers screened for SSR analysis, 11 primers (Table 2) yielded the best amplification products.

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA/*Eco*RI+*Hind*III double digest and 100bp ladder). The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-It ^{TM} Imaging system UVP (USA). The documented SSR profiles were carefully examined for amplification of DNA as bands. The band which were considered as polymorphic among the varieties, were selected for further studies.

3.4.4 Scoring of bands and data analysis

Scoring of bands on agarose was done with the Quantity one software (Biorad) loaded in Gel Doc. 100bp ladder from Invitrogen was used as molecular weight size marker for each gel along with DNA samples. The bands were scored as 1 and 0 for the presence and absence respectively and their size recorded in

Table 2: List of selected SSR primers

Sl. No.	Name of Primers	Annealing temperature	Sequence
1	PN A5	58 ℃	**F** 5'-CTTCCAGACCAATAATCAACTT-3' **R** 5'-ATCCCAAAATACACAACAATTC-3'
2	PN B5	58 ℃	**F** 5'-GTTTTGAATGGGTCGGTGAT-3' **R** 5'-ATTGTTCTGATTTCTTCGTTATTG-3'
3	PN E3	58 ℃	**F** 5'-TTTGTGTCCTCTCCCTCTCC-3' **R** 5'-AAGACTAAATAGGCAAGGCAAA-3'
4	PN F1	58 ℃	**F** 5'-ACTTCAGTGCTATTTTTATCTTCC-3' **R** 5'-CCAACGCCCACTCTCAT-3'
5	PN G11	58 ℃	**F** 5'-TTACTAGTGTCCACCCCCACT-3' **R** 5'-TCGATGGAAAGTCACCCTCT-3'
6	PN H4	53 ℃	**F** 5'-CTTTTCCCACAATTCAGTCTCG-3' **R** 5'-ACCCATGCGTGTATCTTCTCAG-3'
7	PN D10	58 ℃	**F** 5'-GTGTTACCTTTGGGGCATTCA-3' **R** 5'-TGTGTCAGGGCATCAAACC-3'
8	PnAG30	53 ℃	**F** 5'-ACTAAGGCTAATGTGATAACCTGAGG-3' **R** 5'-ATCCCTGGATGGAAATTTGAAGGCTTGC-3'
9	PnCA9	53 ℃	**F** 5'-TCATCAATCACACCTAAAAGAAGGCTATCC-3' **R** 5'-ATGTGGCTATGGGGAACGGTCAGGGGT-3'
10	PnGATA10	53.7 ℃	**F** 5'-CTCCCAGTTATACAACATCACAACCTAGCAC-3' **R** 5'-AGAGGCTTGTCTTAGTTGTGCTCGGGA-3'
11	PnGT2	50 ℃	**F** 5'-CTAGAGAGTAACAGTTATCACTTCACAGC-3' **R** 5'-CTA GCAAATTTGTTCTCTAATTCACATGT-3'

relation to the molecular weight marker used. The result obtained from RAPD and SSR were transformed into data matrix as discrete variables. Jaccard's coefficient of similarity was used to derive the dissimilarity matrix and dendrogram was generated by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Only distinct and well resolved fragments were scored. The resulting data were analysed using the software package NTSYS pc version 2.02i (Rohlf, 2005) and DARwin (Version 5.0.158).

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

Polymorphic Information Content (PIC) was used for confirming the suitability of the primers selected for hybridity testing. The PIC value (Hollman *et al*., 2005) of a marker detects polymorphism within a population depending on the number of detectable alleles and their frequency.

PIC value of a primer is represented as $PIC = 1 - \Sigma\ pi^2$, where *pi* is the frequency of the ith allele.

Results

4. RESULTS

The study on "Molecular characterization and testing hybridity of interspecific crosses in black pepper (*Piper nigrum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2010-2012. The results of different experiments are described in this chapter.

4.1 Morphological analysis

The morphological markers recorded were of quantitative as well as qualitative in nature.

4.1.1 Quantitative characters

The biometric observations recorded include 15 vegetative and 12 reproductive characters (Tables 3 and 4). For all the 15 vegetative characters measured, the data recorded for the six hybrids were similar to the respective female parents and differed from that of the male parent. The male parent is common (*P. colubrinum*) and there are six female parents and six hybrids. Hence for effective comparison of hybrids with either of the parental characters based on statistical analysis, the data for each character was pooled over the six hybrids and average was worked out. Similarly the pooled average of six female parents was also calculated for each character (Table 5).

4.1.1.1 Vegetative characters

Measurements were made from the 15 vegetative characters relating to leaf and stem of the putative interspecific hybrids and respective parents of six interspecific crosses in black pepper.

(i) Leaf characters

The observations for various leaf characters *viz.,* length and width of lamina, L/W ratio, area and petiole length were recorded for young and mature leaf of

plagiotrope (Table 3). The result shows that, the leaf characters of hybrids are similar to that of the respective female parents and differ from the male parent *Piper colubrinum* (Fig. 1).

The characters recorded for young leaves of the hybrids were found similar to that of respective female parents while the male parent recorded a L/W ratio of 1.93 and area of 31.8cm^2. However the average value for these characters were on par with in hybrids, female and male parents.

Length and width of mature leaf of the hybrids and consequently the derived variables, L/W ratio and leaf area, were found similar to respective female parents. The leaf area of Karimunda, Panniyur 3, Panniyur 2 and respective hybrids were low while Panniyur 5, Panniyur 1 and Uthirankotta along with their hybrids showed higher values than *P. colubrinum* (94.16 cm^2). The area recorded was maximum for Uthirankotta (161.21 cm^2) and minimum in hybrid KMPC (70.15 cm^2) closely followed by hybrid P2PC (70.72 cm^2). However, the average value for hybrids compared to male parent showed significant difference only for leaf length and L/W ratio. Petiole length of mature leaf recorded was minimum (1.3 cm) in *P. colubrinum*, P3PC and Panniyur 3 (1.4 cm). All other *P. nigrum* genotypes used as female parents and the hybrids recorded higher values (1.6 cm - 5.5 cm).

(ii) Stem characters

For the stem characters recorded, *viz*. intermodal length and thickness as well as nodal thickness in both ortho and plagiotropes, the hybrids resembled the female parents (Fig. 1). All the orthotrope characters recorded higher values for *P. colubrinum* than the female parents and hybrids (Table 3). Comparison of the average values for hybrids with male parent showed significant difference, while that with female parent were on par (Table 5). For the fruiting branches, internodal length of hybrids ranged from 5.0 cm to 6.8 cm while in male parent it was 6.8 cm. Thickness of node (2.0 cm - 2.9 cm) and internode (1.0 cm - 1.7 cm) were generally higher in *P. nigrum* genotypes and the hybrids when compared to

Table 3: Mean of putative interspecific hybrids and their respective parents for various quantitative traits of leaf and stem

Genotypes*	Young leaf characters				Mature leaf characters					Stem characters					
										Orthotropes			Plagiotropes		
	Length [cm]	Width [cm]	L/W ratio	Leaf area [cm²]	Length [cm]	Width [cm]	L/W ratio	Leaf area [cm²]	Petiole length [cm]	Internode length [cm]	Thickness at node [cm]	Thickness at internode [cm]	Internode length [cm]	Thickness at node [cm]	Thickness at internode [cm]
P5	9.7	4.5	2.15	37.1	16.1	8.5	1.89	116.32	2.5	10	6	4	6.1	2.1	1.1
P5 x PC	9.5	4.3	1.93	39.5	15.2	8.0	1.90	103.36	2.4	11	6	3.5	6.8	2.6	1.5
P3	8.6	4.0	2.15	29.2	14.3	6.7	2.13	81.43	1.4	7.2	2.3	2	5.3	2.6	1.6
P3 x PC	8.4	4.2	2.00	29.9	13.9	6.2	2.24	73.25	1.3	7.5	5	3	5.1	2.3	1.4
P2	8.2	3.9	2.10	27.1	14.2	7.2	1.97	86.90	1.9	7.6	3	2.5	6.7	2.4	1.3
P2 x PC	8.0	3.9	2.05	26.5	12.8	6.5	1.96	70.72	1.7	7.8	5	4	6.4	2.7	1.3
P1	7.8	5.0	1.56	33.1	14.8	11.5	1.28	144.67	5.3	7.5	2.5	2	5.4	2.9	1.7
P1 x PC	7.5	4.9	1.53	31.2	13.5	11.2	1.20	128.52	5.5	7.3	2.5	2	5.0	2.5	1.5
KM	8.7	4.5	1.93	33.2	13.5	6.5	2.07	74.58	1.7	6.5	6	4	6.4	2.2	1.1
KM x PC	8.5	4.7	1.80	33.9	13.1	6.3	2.07	70.15	1.6	6.5	6	5	6.7	2.6	1.4
UK	8.7	3.4	2.55	25.1	17.4	10.9	1.59	161.21	1.6	7.7	2.9	2.4	6.2	2.2	1.0
UK x PC	8.2	3.2	2.56	22.3	14.4	9.1	1.58	111.38	1.4	7.5	2.7	2.4	5.5	2.0	1.2
PC	8.5	4.4	1.93	31.8	16.4	6.6	2.48	94.16	1.3	15	6.5	4.5	6.8	2.0	1.1

*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda; UK – Uthirankotta; PC – *Piper colubrinum*

a. Young leaf

b. Mature leaf

c. Stem (orthotrope)

d. Stem (plagiotrope)

d. Stem (plagiotrope)
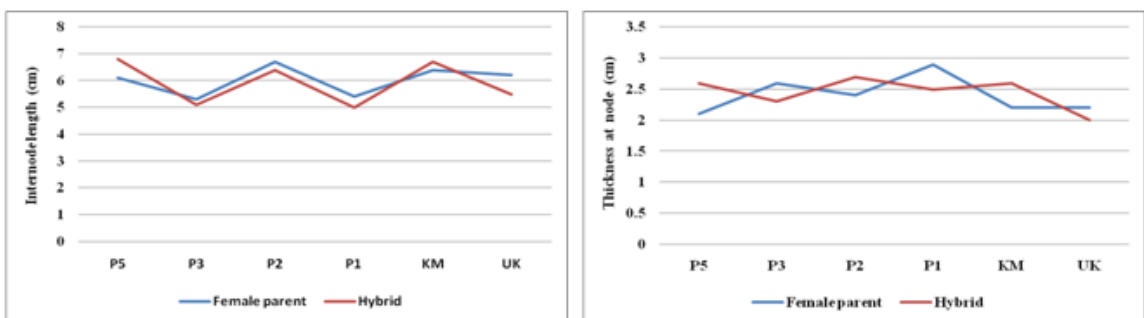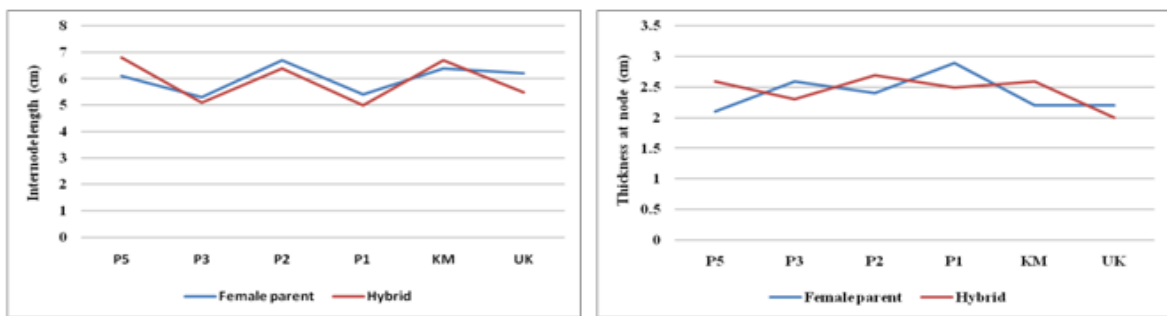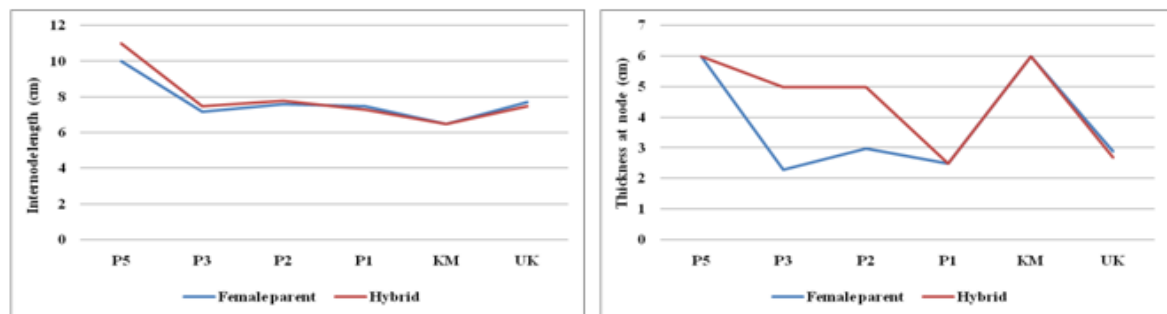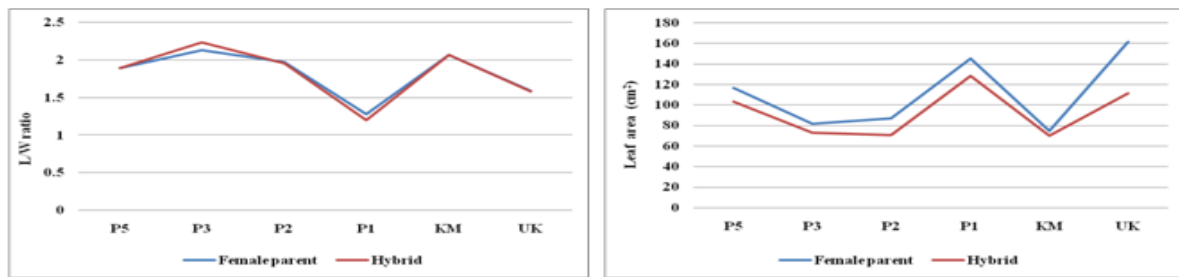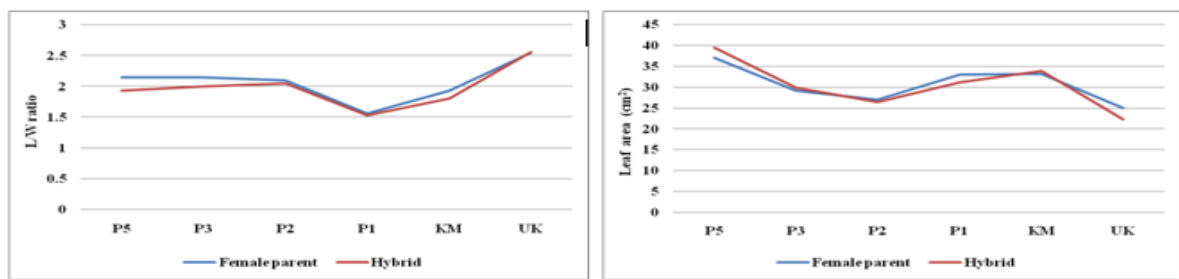
Fig. 1: Trend in vegetative characters of hybrids and their female parents

that in *P. colubrinum* (2.0 cm and 1.1 cm respectively). The average values for various characters of fruiting branches also showed significant difference from the male parent, but were on par with the average of female parents.

4.1.1.2 Reproductive characters

All the six hybrids started bearing and observations were recorded for five spike characters *viz.* number of spikes per branch, spike length, spike peduncle length, fresh and dry spike weight (Fig. 2a and Fig. 2b). Eight berry characters *viz.*, number of berries per spike, weight and volume of thousand fresh and dried berries, number and percentage of well developed and under developed berries per spike and fruit setting percentage for the putative hybrids and their respective parents were also recorded from all the vines (Table 4).

(i) Spike characters

The spike characters were compared between the hybrids and parents. In all the six crosses except Karimunda x *P. colubrinum,* with respect to all the five characters recorded, hybrids showed lower values than the respective female parents (Fig. 2a and Fig. 2b).

In KMPC, the average number of spikes per lateral branch was slightly higher (9nos) than the female parent Karimunda (8nos). The number of spikes per branch ranged from 6 to14. A maximum of 14 spikes per branch was recorded for Panniyur 1 followed by Panniyur 5 and Uthirankotta (12 spikes), while lowest value recorded for hybrid P3PC (6 spikes). The average value of this character for hybrids (9nos) was found significantly different from female parents (11.3nos) and male parent (11nos).

Spike length (7.1 cm - 17 cm) differed significantly between the six female parents and the common male parent (4.6 cm). For all the hybrids it was on par with the respective female parents (Fig. 2a and Fig. 2b; Table 5). Spike peduncle length did not show much variation between the female parents and hybrids with a range of 1.0 cm - 1.8 cm with an average of 1.5 cm in female parents and 1.4 cm

in hybrids. However the average value of hybrids (1.4 cm) was found significantly different from male parent (1.2 cm). Only in hybrid UKPC (1.0 cm) it was on par with both parents.

Fresh spike weight (7.1g - 17.8g) and dry spike weight (4.2g - 11g) also showed significant difference between female parents and the male parent (0.5g and 0.2g respectively). With respect to both characters the hybrids differed significantly from both parents (Fig 2a and Fig. 2b; Table 5).

ii) Berry characters

These characters could be recorded only for the female parents and the hybrids as there was no berry setting in *P. colubrinum*.

The number of berries per spike was found to differ among the six female genotypes, lowest in Karimunda (62nos) and highest in Panniyur 1(125nos). The hybrids showed lower values for this character compared to respective female parents (Table 4), the lowest and highest value in KMPC (50nos) and P1PC (101nos) respectively. However, the average value for hybrids (75.3nos) was on par with that of female parents (88nos) (Table 5).

The weight as well as volume of 1000 fresh and dry berries of hybrid and their female parents were recorded (Table 4). 1000 fresh berry weight ranged between 120-170g in parental genotypes while in hybrids it was 100-140g only. After drying, the range was 44-72g in parents while in hybrids, it was 30-42g. Among the parents, fresh and dry berry weight was highest in Panniyur 1 while among hybrids, fresh weight was highest in P1PC but dry weight was highest in P2PC. For these characters, the average value also differed significantly between hybrids and female parents (Table 5). However, for 1000 fresh and dry berry volume, the individual hybrids did not show much variation from the respective parents and the average values also did not differ significantly. Fresh and dry berry volume was highest in Uthirankotta (160cc and 70cc) and correspondingly in hybrid UKPC (136cc and 65cc).

Table 4: Berry characters of putative interspecific hybrids and their respective parents

Genotypes *	No. of berries per spike	1000 berries weight		1000 berries volume		Developed berries [%]	Undeveloped berries [%]	Fruit setting [%]
		fresh wt. [g]	dry wt. [g]	fresh volume [cc]	dry volume [cc]			
P5	102	140	47	120	60	79	21	84.59
P5 x PC	97	110	39	105	45	23	77	22.98
P3	92	120	44	115	50	83	17	89.25
P3xPC	84	110	33	110	50	11	89	11.52
P2	71	130	46	120	60	75	25	74.24
P2xPC	65	100	45	105	60	32	68	32.70
P1	125	170	72	149	70	93	7	96.0
P1xPC	101	140	39	135	60	21	79	26.58
KM	62	120	47	105	60	81	19	80.72
KM x PC	50	110	30	105	50	33	67	33.60
UK	76	160	51	160	70	72	28	82.43
UK x PC	55	120	34	136	65	17	83	21.54
PC	-	-	-	-	-	-	-	-

*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda; UK – Uthirankotta; PC – *Piper colubrinum*
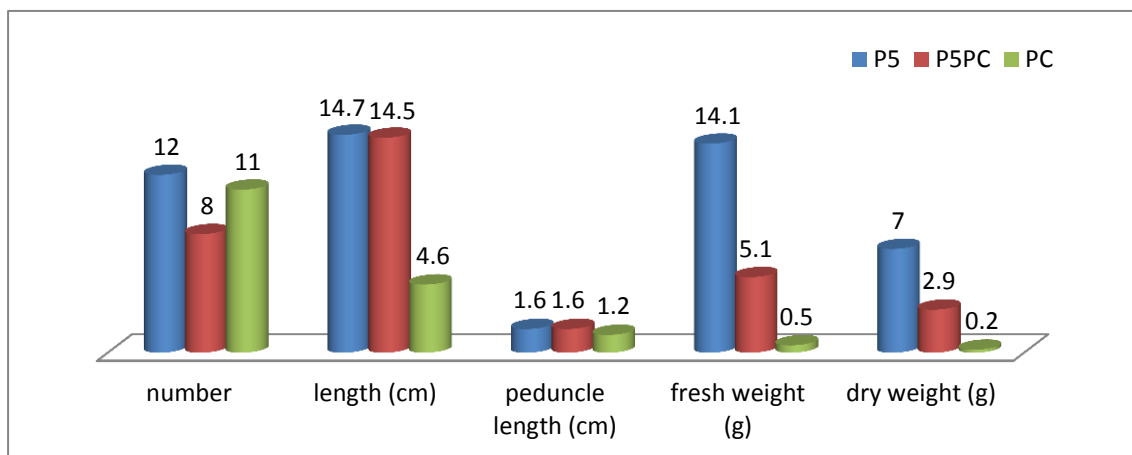
a. Hybrid P5PC and parents
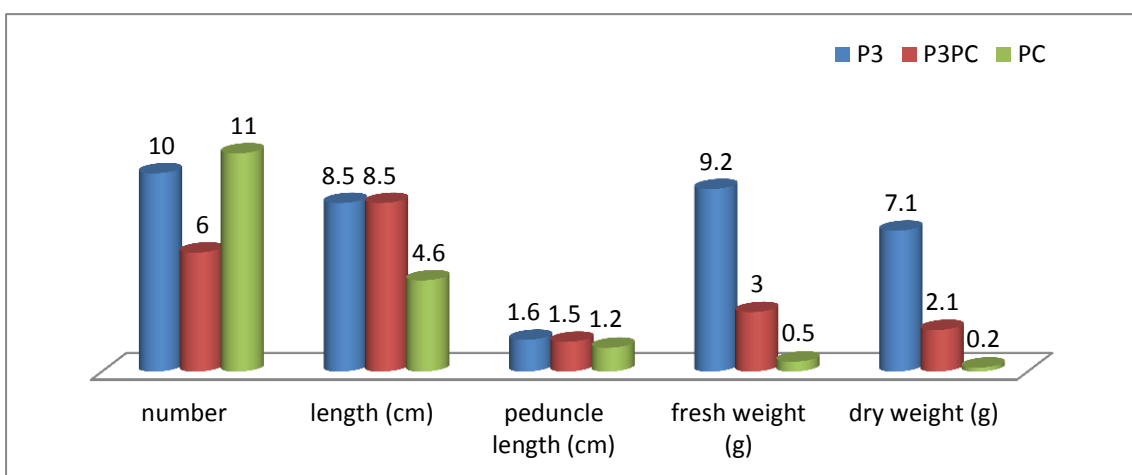
b. Hybrid P3PC and parents
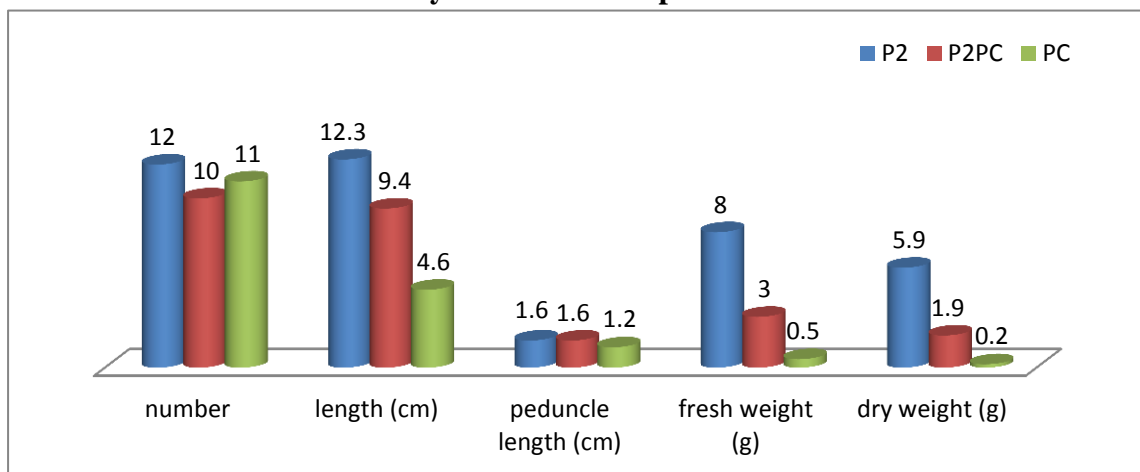
c. Hybrid P2PC and parents

Fig. 2a: Comparison of spike characters of parents and hybrids
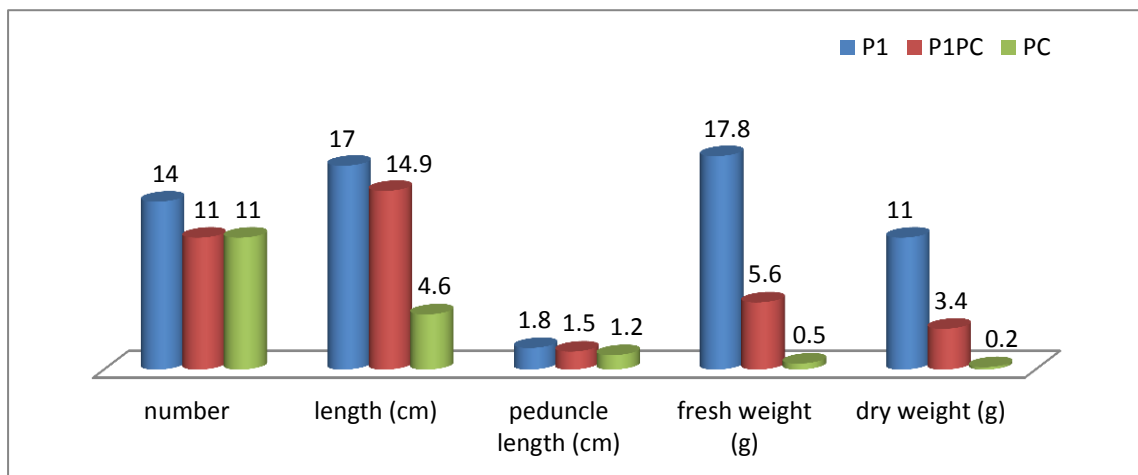
d. Hybrid P1PC and parents
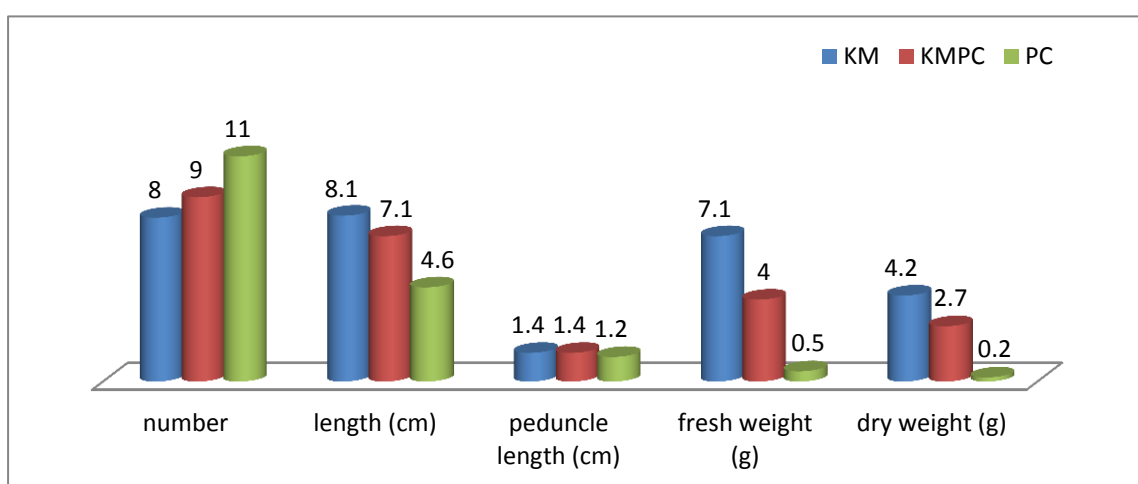
d. Hybrid KMPC and parents
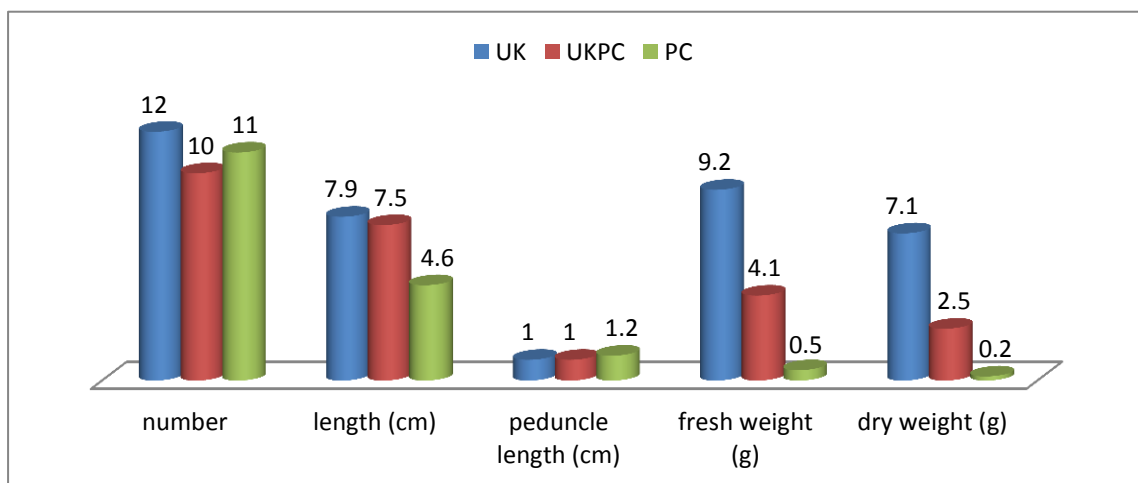
d. Hybrid UKPC and parents

Fig. 2b: Comparison of spike characters of parents and hybrids

The per cent of developed and undeveloped berries and correspondingly the fruit setting per cent in spikes showed significant difference in all six hybrids compared to their parents (Fig. 3). The parents showed a range of 74.2 - 96 per cent for fruit setting, with the lowest value in Panniyur 2 and highest in Panniyur 1. But for hybrids the range was 11.5 - 33.6 per cent only, lowest in P3PC and highest being in KMPC. The average value also showed significant difference (Table 5).

Analysis of pooled data for the six hybrids showed significant difference from that of *P. colubrinum* with respect to 38 per cent of the morphological characters studied. Spike weight of the hybrid was significantly different from both parents (8 per cent of the characters). Berry weight and setting was significantly lower in hybrids compared to *P. nigrum* (12%). 42 per cent of morphological characters did not show any significant difference between the hybrids and their respective parents (Fig. 4).

4.1.2 Qualitative characters

Measurements were made from the 28 qualitative characters relating to leaf, spike and fruit of the putative interspecific hybrids and respective parents of six interspecific crosses in black pepper. The characters, which showed similarity and variation between hybrids and their parents, are described below.

The plant growth habits differed significantly in hybrids and their parents of six interspecific crosses. The climbing growth habit was observed in all the six hybrids and their respective female parents, while trailing growth habit was observed in the male parent *P. colubrinum* (Plate 1).

Colour of shoot tip were similar in female parents and respective hybrids except for the hybrid P5PC (Plate 2a and Plate 2b). Panniyur 5 is having light purple shoot tip while its hybrid has pale green shoot tip. Panniyur 2, Karimunda, Uthirankotta and their hybrids showed a purple tinge. The petioles of the tender leaves at the shoot tip as well as leaf sheath were intensely purple coloured. Panniyur 1, Panniyur 3 and their hybrids showed pale green colour. However the

Table 5: Comparison of hybrid characters with *P. nigrum* and *P. coubrinum*

Characters	*P. nigrum* (pooled average of six female parents)	Hybrid (pooled average of six hybrids)	*P. colubrinum* (Male parent)	CD value (female parents v/s hybrids)	CD value (male parents v/s hybrids)
Young leaf characters					
Length (cm)	8.6	8.4	8.5	0.577	0.369
Width (cm)	4.2	4.2	4.4	0.049	0.808
L/W ratio	2.0	1.9	1.93	0.495	0.348
Leaf area (cm^2)	30.8	30.5	31.8	0.082	0.515
Mature leaf characters					
Length (cm)	15.0	13.8	16.4	1.787	2.955**
Width (cm)	8.5	7.8	6.6	0.554	1.582
L/W ratio	1.8	1.8	2.4	0.016	4.267**
Leaf area(cm)	110.8	92.8	94.1	1.00	0.124
Petiole length (cm)	2.4	2.8	1.3	0.465	2.152
Orthotrope					
Internode length (cm)	7.7	7.9	15	0.316	11.057**
Thickness at node (cm)	3.7	4.5	6.5	0.786	3.080**
Thickness at internode (cm)	2.8	3.3	4.5	0.8485	4.391**
Plagiotrope					
Internode length (cm)	6.0	5.9	6.8	0.249	2.660**
Thickness at node (cm)	2.4	2.4	2	0.307	4.258**
Thickness at internode (cm)	1.3	1.3	1.1	0.653	5.937**

Table 5 continued:

Characters	*P. nigrum* (pooled average of six female parents)	Hybrid (pooled average of six hybrids)	*P. colubrinum* (Male parent)	CD value (female parents v/s hybrids)	CD value (male parents v/s hybrids)
Spike characters					
No. of spikes per branch	11.3	9	11	2.092**	2.739**
Spike length (cm)	1.5	1.4	1.2	0.459	2.539**
Spike peduncle length (cm)	1.5	1.4	1.2	0.459	2.539**
Fresh Spike weight (g)	10.9	4.1	0.5	3.861**	8.194**
dry Spike weight (g)	7.0	2.5	0.3	4.749**	10.251**
Total No. of berries per spike	88	75.3	-	0.976	-
1000 fresh berries wt. (g)	140	115.0	-	2.440**	-
1000 dry berries wt. (g)	51.1	36.6	-	3.019**	-
1000 fresh berries volume (cc)	128.1	116.0	-	1.134	-
1000 dry berries volume (cc)	61.6	55	-	1.512	-
Fruit setting (%)	84.5	55.0	-	13.222**	-
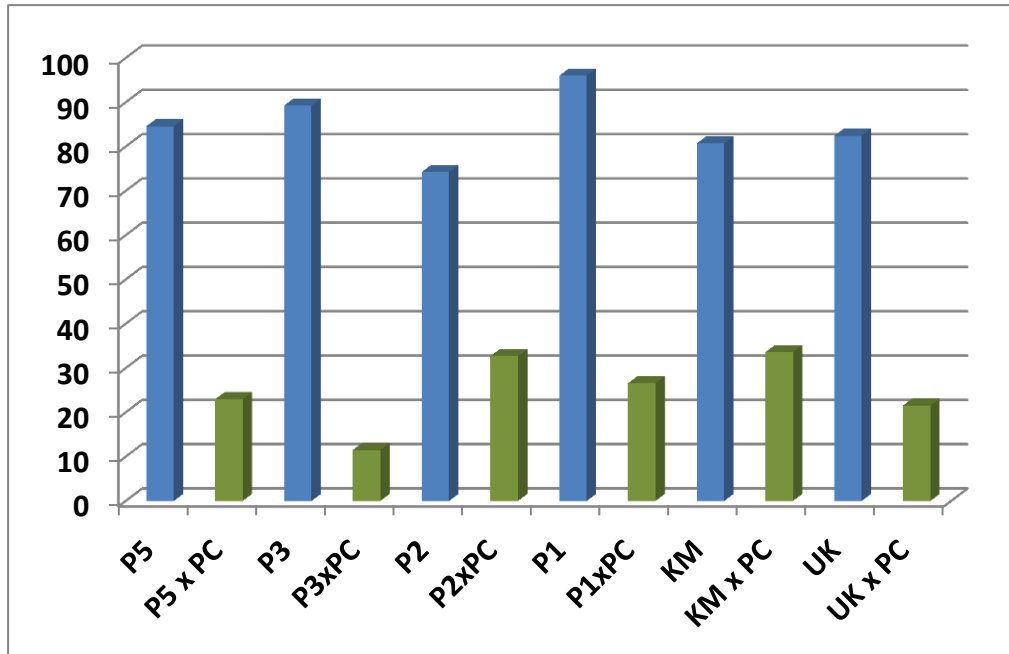
Fig. 3: Berry setting percentage in hybrids and female parents
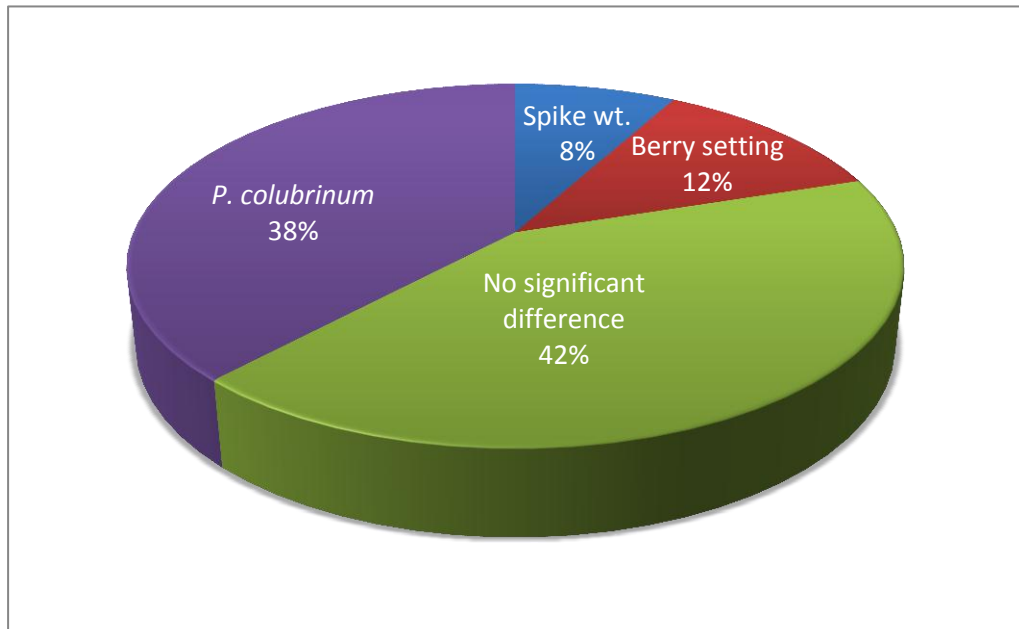
Fig. 4: Difference between hybrids and parents respective to total morphological characters studied

male parent *P. colubrinum* showed yellowish green shoot tip (Plate 2a and Plate 2b).

The shape, tip and base of young as well as mature leaves showed marked differences among parental genotypes. The hybrids resembled the female parents for these characters (Plate 3a and Plate 3b; Table 6 and 7). In *P. colubrinum* ovate elliptic leaves with acute tips were observed. The leaf base was characteristically oblique which was absent in all the hybrids.

The leaf texture, arrangement and venation showed marked differences between the male parent *P. colubrinum* and hybrids. In *P. colubrinum*, leaves were glabrous membranous, slightly wavy and eucamptodromous while evenly arranged glabrous coriaceous and campylodromous leaves were observed in all the hybrids and their female parents (Plate 3a and Plate 3b). The observations for various qualitative characters of leaf on the vegetative and reproductive branches are given in Table 6 and Table 7.

Stilt roots were observed in the male parent *P. colubrinum*, while absent in all the six hybrids and their respective female parents (Plate 4). The stem of *Piper colubrinum* was shiny greenish in colour, with erect lateral branches and no runner shoots whereas in hybrids and female parents, the main stem was greyish and thick with semi erect - horizontal laterals and several runner (climbing) shoots from the base (Plate 5 and Table 8)

The spike colour, orientation and shape differ significantly in the male parent *P. colubrinum* compared to hybrids and their female parents. Yellow, erect and cylindrical shaped spikes were observed in *P. colubrinum*, while green, prostrate and filiform spikes were observed in all the six hybrids and their respective female parents (Plate 6 and Table 8).

The observations for various qualitative characters of spike and berry are given in Table 8. Fruit is bold and spherical in Panniyur 1 and Uthirankotta as well as in the hybrids P1PC and UKPC whereas in Panniyur 5 and hybrid P5PC,

Light purple
(Panniyur 5)

Pale green
(Panniyur 5 x *P. colubrinum*)

Purple
(Panniyur 2)

Purple
(Panniyur 2 x *P. colubrinum*)

Pale green
(*P. colubrinum*)

**Plate 2a: Variation in anthocyanin pigmentation of parents and
interspecific hybrids**

Light purple
(Karimunda)

Light purple
(Karimunda x *P. colubrinum*)

Purple
(Uthirankotta)

Purple
(Uthirankotta x *P. colubrinum*)

**Plate 2b: Variation in anthocyanin pigmentation of parents and interspecific
hybrids**

Panniyur 5

Panniyur 5 x *P. colubrinum*

Panniyur 3

Panniyur 3 x *P. colubrinum*

Male parent *P. colubrinum*

Plate 3a: Variation in leaf shape of parents and interspecific hybrids

Panniyur 2

Panniyur 2 x *P. colubrinum*

Panniyur 1

Panniyur 1 x *P. colubrinum*

Karimunda

Karimunda x *P. colubrinum*

Uthirankotta

Uthirankotta x *P. colubrinum*

Plate 3b: Variation in leaf shape of parents and interspecific hybrids
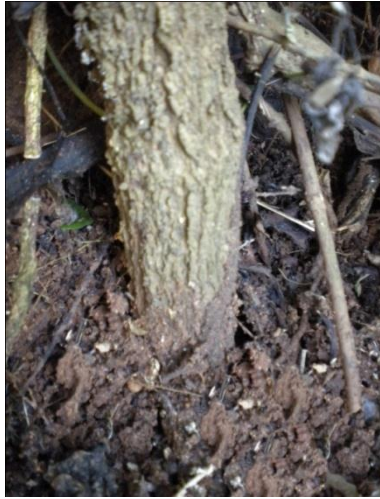
Absent in female parents and hybrids   Present in male parent (*P. colubrinum*)

Plate 4: Stilt root production in parents and interspecific hybrids

Present in female parents and hybrids   Absent in male parent (*P. colubrinum*)

Plate 5: Climbing roots production in parents and interspecific hybrids

Female parents and hybrids   Male parent (*P. colubrinum*)

Plate 6: Variation in spike colour, orientation and shape of parents and interspecific hybrids

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Table 6: Qualitative characters of young leaf of putative interspecific hybrids and their respective parents

Genotypes*	Young leaf characters					
				Shape		
	Shoot tip colour	Colour on upper side	Colour on lower side	Tip	Base	Leaf shape
P5	Light Purple	Pale green	Creamy green	Acuminate	Round	Ovate
P5 x PC	Pale green	Pale green	Creamy green	Acuminate	Round	Ovate lanceolate
P3	Pale green	Pale green	Creamy green	Acuminate	Acute	Ovate lanceolate
P3 x PC	Pale green	Pale green	Creamy green	Acuminate	Acute	Ovate lanceolate
P2	Purple	Pale green	Creamy green	Acuminate	Round	Ovate
P2 x PC	Purple	Pale green	Creamy green	Acuminate	Round	Ovate
P1	Pale green	Pale green	Creamy green	Acuminate	Round	Ovate
P1 x PC	Pale green	Pale green	Creamy green	Acuminate	Round	Ovate
KM	Pale purple	Pale green	Creamy green	Acuminate	Acute	Ovate elliptic
KM x PC	Pale purple	Pale green	Creamy green	Acuminate	Acute	Ovate elliptic
UK	Light Purple	Pale green	Creamy green	Acuminate	Cordate	Ovate elliptic
UK x PC	Light Purple	Pale green	Creamy green	Acuminate	Cordate	Ovate elliptic
PC	Yellowish green	Shiny pale green	Creamy green	Acute	Oblique	Ovate elliptic

*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda; UK – Uthirankotta; PC – *Piper colubrinum*

Table 7: Qualitative characters for mature leafs of putative interspecific hybrids and their respective parents

Genotypes*	Mature leaf characters									
	Colour on upper side	Colour on lower side	Shape			Leaf arrangement	Leaf texture	Leaf venation	Leaf margin	Hairiness
			Tip	Base	Lamina shape					
P5	Dark green	Dull green	Acuminate	Round	Ovate lanceolate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
P5 x PC	Dark green	Dull green	Acuminate	Round	Ovate lanceolate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
P3	Dark green	Dull green	Acuminate	Round	Ovate lanceolate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
P3xPC	Dark green	Dull green	Acuminate	Round	Ovate lanceolate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
P2	Dark green	Dull green	Acuminate	Round	Ovate lanceolate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
P2xPC	Dark green	Dull green	Acuminate	Round	Ovate lanceolate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
P1	Dark green	Dull green	Acuminate	Cordate	Cordate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
P1xPC	Dark green	Dull green	Acuminate	Cordate	Cordate	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
KM	Dark green	Dull green	Acuminate	Round	Ovate elliptic	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
KM x PC	Dark green	Dull green	Acuminate	Round	Ovate elliptic	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
UK	Dark green	Dull green	Acuminate	Cordate	Ovate elliptic	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
UK x PC	Dark green	Dull green	Acuminate	Cordate	Ovate elliptic	Alternate	Glabrous coriaceous	Campylodromous	Even	Absent
PC	Shiny dark green	Dull light green	Acute	Oblique	Ovate elliptic	Alternate	Glabrous membranous	Eucamptodromous	Slightly wavy	Absent

*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda; UK – Uthirankotta; PC – *Piper colubrinum*

Table 8: Qualitative characters for root, branch, spike and fruit of putative interspecific hybrids and their respective parents

Genotypes*	Root characters		Branch characters			Spike characters			Fruit characters			
	Stilt root production	Climbing root production	Angle of laterals	Rate of lateral production	Rate of runner production	Spike colour	Spike orientation	Spike shape	Colour of young berries	Colour of mature berries	Berry size	Fruit shape
P5	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green- turning orange red	Bold	Round
P5 x PC	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green- turning orange red	Medium	Round
P3	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green	Bold	Round
P3x PC	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green	Medium	Round
P2	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green turning Brownish red	Bold	Round
P2xPC	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green turning Brownish red	Medium	Round
P1	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green with reddish tinch	Bold	Round
P1x PC	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green with reddish tinch	Medium	Round
KM	Nil	Many	Semi-erect	Many	Many	Green	Prostrate	Filiform	Light green	Dark green- turning yellowish red	Medium	Oblong-Round
KM x PC	Nil	Many	Semi-erect	Many	Many	Green	Prostrate	Filiform	Light green	Dark green- turning yellowish red	Medium	Oblong-Round
UK	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green turning red	Bold	Round
UK x PC	Nil	Many	Horizontal	Many	Many	Green	Prostrate	Filiform	Light green	Dark green turning red	Medium	Round
PC	Many	Nil	Erect	Few	Nil	Light yellow	Erect	Cylindri-cal	Nil	Nil	Nil	Nil

*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda; UK – Uthirankotta; PC – *Piper colubrinum*

the berry was medium sized. In Panniyur 2, Panniyur 3, Karimunda and the respective hybrids also the berry was medium sized.

4.1.3 Seed germination of the reported hybrid Culture P5PC

Fruits produced by the open pollinated partially fertile hybrid (Culture P5PC) were harvested at the fully mature stage, and after removing the flesh, the seeds were germinated using sand in trays and were kept in green house.

4.1.3.1 Germination of seeds under green house conditions

The fully matured seeds (96nos) were sown in sand and they started germinating after 40 to 45 days. In this method 12 per cent germination was obtained (Plate 7). The seedlings were transplanted at two leaf stages and used for further molecular analysis.

4.2 Molecular characterization

The results of the molecular charactrerisation of the interspecific hybrids and parents of black pepper were carried out using two different marker systems. The results of the experiment are detailed below.

4.2.1 Source of DNA

Pale green, tender leaves were collected from all the experimental vines for extraction of total genomic DNA. In black pepper, very tender, fresh, pale green leaves (0.5 to 1g) yielded good quality DNA in sufficient quantity. There was no browning of the extract if suitable antioxidants were added.

4.2.1.1 Isolation, purification and quantification of DNA

Genomic DNA isolated through the CTAB method reported by Roger and Bendich (1994) was not pure and had protein and RNA contamination (Plate 8a). However, the genomic DNA isolated with modified CTAB buffer as described earlier gave good quality DNA (Plate 8b). The agarose gel electrophoresis indicated clear discrete band without RNA contamination and spectophotometric

Plate 7: One month old seedling of open pollinated progeny of hybrid P5PC
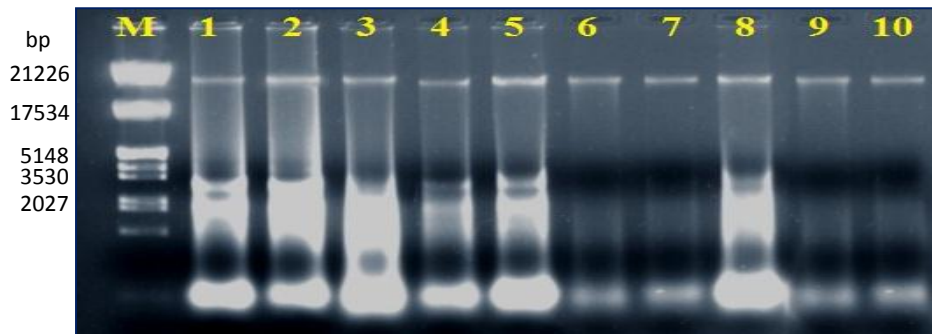
Lanes M: Molecular weight marker λ DNA/*Eco* RI+ *Hind* III double digest
1-10: Black pepper samples

a. Rogers and Bendich method
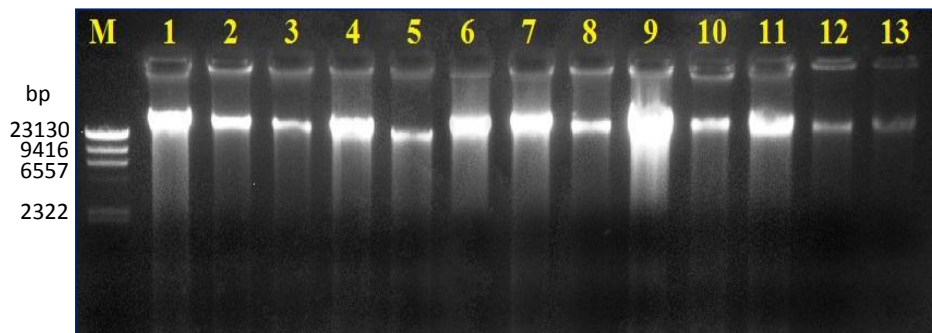
*Lanes M: Molecular weight marker λ DNA/ *Hind* III digest
1: Panniyur 5, 2: Panniyur 3, 3: Panniyur 2, 4: Panniyur 1, 5: Karimunda,
6: Uthirankotta, 7: *P. colubrinum*, 8: P5PC, 9: P3PC, 10: P2PC,
11: P1PC, 12: KMPC, 13: UKPC

b. Rogers and Bendich modified method

(***P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)**

**Plate 8: DNA isolated through different methods from tender leaves of black
pepper**

analysis gave ratio of UV absorbance ($A_{260}/_{280}$) between 1.8 and 2.0. Quality of DNA isolated through the modified method for selected hybrids and their parents are indicated in Table 9.

4.3 Molecular Marker Analysis

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

4.3.1 Random Amplified Polymorphic DNA (RAPD) analysis

Good quality genomic DNA from the putative interspecific hybrids and their corresponding parents were subjected to RAPD assay. The various experiments carried out under this included screening of random primers and RAPD with selected primers.

4.3.1.1 Screening of primers for RAPD analysis

Thirty decamer primers belonging to series OPA, OPAH, OPC, OPF, OPP and OPU were tested for amplification of genomic DNA of black pepper *viz.* Panniyur 5 and Panniyur 3.

RAPD analysis with the thermal settings identified gave good amplification. The amplification pattern was different corresponding to the decamer primer used (Plate 9).

Based on the amplification pattern, 10 out of 30 decamer primers were selected for RAPD assay of six interspecific black pepper hybrids and their parents (Table 10).

4.3.1.2 Amplification with selected primers

RAPD banding patterns produced by the 10 selected decamer primers for the parents and their hybrid populations was compared to assess hybridity at the DNA level.
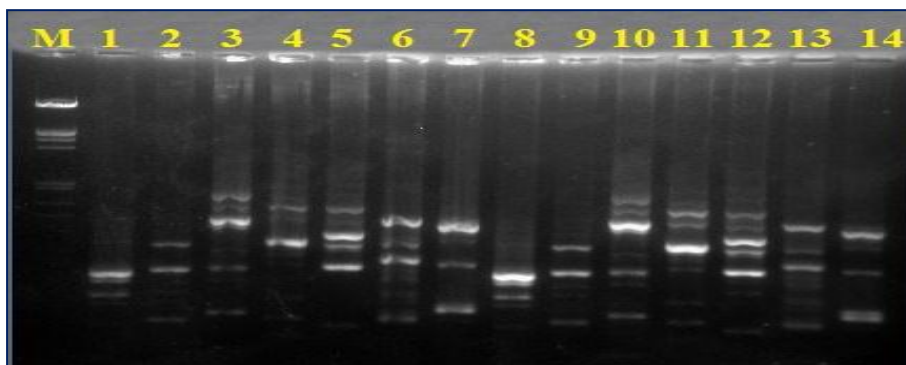
M: Molecular weight marker λ DNA/*Hind* III digest
Lane 1-7: Amplification pattern of Panniyur 5 with primers OPU 1, OPU 2, OPU 3, OPU 13, OPA10, OPP 08, OPF 9
Lane8-14: Amplification pattern of Panniyur 3 with primers OPU 1, OPU 2, OPU 3, OPU 13, OPA10, OPP 08, OPF 9
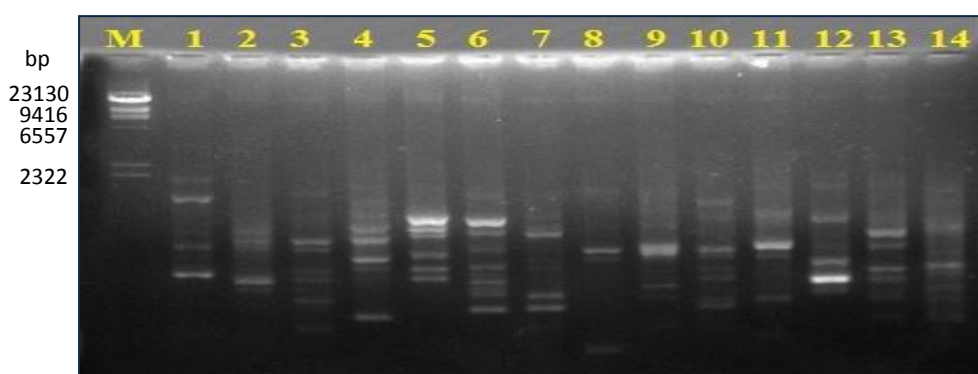
M: Molecular weight marker λ DNA/*Hind* III digest
Lane 1-14: Amplification pattern of Panniyur 5 with primers OPF 16, OPA 38, OPA 12, OPA30, OPA 28, OPAH 9, OPA 39, OPA 29, OPA 36, OPA 22, OPA 26, OPA 24
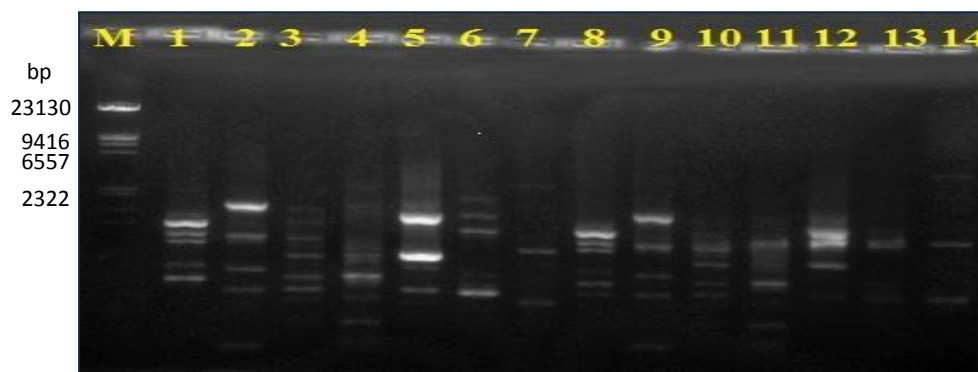
M: Molecular weight marker λ DNA/*Hind* III digest
Lane 1-7: Amplification pattern of Panniyur 5 with primers OPA 6, OPA 8, OP17, OPC 01, OPC 08, OPC 09, OPC 14
Lane 8-14: Amplification pattern of Panniyur 3 with primers OPA 6, OPA 8, OP17, OPC01, OPC 08, OPC 09, OPC 14

Plate 9: Screening of RAPD primers for amplification of black pepper DNA

Table 9: Quality and quantity of DNA isolated from Black pepper genotypes as assessed by Nano Drop spectrophotometer method

Genotype	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260/280}$	Quantity (ng/µl)
Panniyur 5	44.478	24.707	1.80	2223.92
Panniyur 3	30.818	16.622	1.85	1540.88
Panniyur 2	60.69	33.191	1.82	3034.52
Panniyur 1	45.416	24.004	1.89	2270.80
Karimunda	32.278	17.449	1.84	1613.88
Uthirankotta	20.503	10.951	1.87	1025.14
P. colubrinum	60.063	31.904	1.88	3003.17
Panniyur 5 x *P. colubrinum*	29.111	15.611	1.86	1455.57
Panniyur 3 x *P. colubrinum*	25.843	14.248	1.81	1292.13
Panniyur 2 x *P. colubrinum*	44.91	24.036	1.86	2245.50
Panniyur 1 x *P. colubrinum*	19.629	10.520	1.86	1167.10
Karimunda x *P. colubrinum*	23.342	12.792	1.82	936.25
Uthirankotta x *P. colubrinum*	22.878	12.434	1.84	1027.7

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

Sr. No.	Primers	Amplification pattern			Remark
		No. of bands	Types of bands		
			Distinct	Faint	
1	OPA 6	5	4	1	- - -
2	OPA 8	6	5	1	Selected
3	OPA 9	4	3	2	- - -
4	OPA 10	5	4	1	Selected
5	OPA 11	3	2	1	- - -
6	OPA 12	5	3	2	- - -
7	OPA 17	7	4	3	Selected
8	OPA 21	3	1	2	- - -
9	OPA 22	4	1	3	- - -
10	OPA 24	4	0	4	- - -
11	OPA 26	5	2	3	- - -
12	OPA 28	8	5	3	Selected
13	OPA 29	7	1	6	- - -
14	OPA 30	6	6	0	Selected
15	OPA 36	3	2	1	- - -
16	OPA 38	3	1	2	- - -
17	OPA 39	2	1	1	- - -
18	OPC 01	4	1	3	- - -
19	OPC 08	4	3	1	Selected
20	OPC 09	4	4	0	Selected
21	OPC 14	3	3	0	Selected
22	OPF 09	3	3	0	- - -
23	OPF 16	4	3	1	- - -
24	OPP 08	6	5	1	Selected
25	OPU 01	4	3	1	- - -
26	OPU 02	3	2	1	- - -
27	OPU 03	5	4	1	- - -
28	OPU 13	4	1	3	- - -
29	OPAH 06	3	1	2	- - -
30	OPAH 09	4	4	0	Selected

(i) Hybrid P5PC

The amplification pattern observed for the genomic DNA of hybrid P5PC and respective parents with 10 selected RAPD primers are shown in Plate 10 to Plate 14 and the comparison given in Table 11.

Total eighty clear distinct amplicons were observed with the 10 RAPD primers. The amplicons ranged in size from 250bp to 1400bp. Twenty four amplicons were monomorphic in hybrid P5PC and both the parents. Twenty seven monomorphic bands generated in hybrid P5PC and female parent Panniyur 5. Two polymorphic bands of size 1200bp and 1100bp were generated in female parent Panniyur 5 with primers OPA 28 and OPC 08 respectively but that were absent in hybrid P5PC. Twenty seven amplicons were polymorphic in the male parent *P. colubrinum*. The primer OPA 30 amplified one unique band of 800bp in the female parent Panniyur 5 and hybrid P5PC.

The amplification pattern with the 10 selected decamer primers shows higher degree of similarity between hybrid P5PC and female parent Panniyur 5 and none of the bands specific to male parent *P. colubrinum* was shared by hybrid P5PC.

(ii) Hybrid P3PC

The amplification pattern observed for the genomic DNA of hybrid P3PC and respective parents with 10 selected RAPD primers are shown in Plate 10 to Plate 14 and the comparison given in Table 12.

Total seventy nine clear distinct amplicons were observed with the 10 RAPD primers. The amplicons ranged in size from 250bp to 1400bp. Twenty two amplicons were monomorphic in hybrid P3PC and both the parents. Twenty seven monomorphic bands generated in hybrid P3PC and female parent Panniyur 3. Two polymorphic bands of size 950bp and 1400bp were generated in female parent Panniyur 3 with primers OPA 10 and OPAH 09 respectively but that were

Table 11: Comparison of RAPD banding pattern of interspecific hybrid between black pepper variety Panniyur 5 and *P. colubrinum*

Sl. No.	Primer	Total number of amplicons	Monomorphic in parents and hybrids	Polymorphic between parents			
				P5PC vs. Panniyur 5		P5PC vs. *P. colubrinum*	
				Monomorphic	Polymorphic	Monomorphic	Polymorphic (male specific)
1	OPA 08	6	2	2	-	-	2
2	OPA 10	6	2	2	-	-	2
3	OPA 17	10	3	4	-	-	3
4	OPA 28	6	3	1	1	-	1
5	OPA 30	12	1	5	-	-	6
6	OPC 08	7	3	1	1	-	2
7	OPC 09	11	3	2	-	-	6
8	OPC 14	6	-	4	-	-	2
9	OPP 08	8	3	3	-	-	2
10	OPAH 09	8	4	3	-	-	1
	Total	80	24	27	2	0	27

Table 12: Comparison of RAPD banding pattern of interspecific hybrid between black pepper variety Panniyur 3 and *P. colubrinum*

Sl. No.	Primer	Total number of amplicons	Monomorphic in parents and hybrids	Polymorphic between parents			
				P3PC vs. Panniyur 3		P3PC vs. *P. colubrinum*	
				Monomorphic	Polymorphic	Monomorphic	Polymorphic (male specific)
1	OPA 08	5	2	1	-	-	2
2	OPA 10	5	-	2	1	-	2
3	OPA 17	10	3	4	-	-	3
4	OPA 28	6	3	2	-	-	1
5	OPA 30	11	1	4	-	-	6
6	OPC 08	7	3	2	-	-	2
7	OPC 09	11	3	2	-	-	6
8	OPC 14	6	-	4	-	-	2
9	OPP 08	8	3	3	-	-	2
10	OPAH 09	10	4	3	1	-	2
	Total	79	22	27	2	0	28

absent in hybrid P3PC. Twenty eight amplicons were polymorphic in the male parent *P. colubrinum*.

The amplification pattern with the 10 selected decamer primers shows higher degree of similarity between hybrid P3PC and female parent Panniyur 3 and none of the bands specific to male parent *P. colubrinum* was shared by hybrid P3PC.

(iii) Hybrid P2PC

The amplification pattern observed for the genomic DNA of hybrid P2PC and respective parents with 10 selected RAPD primers are shown in Plate 10 to Plate 14 and the comparison given in Table 13.

Total eighty clear distinct amplicons were observed with the 10 RAPD primers. The amplicons ranged in size from 250bp to 1400bp. Twenty five amplicons were monomorphic in hybrid P2PC and both the parents. Twenty six monomorphic bands generated in hybrid P2PC and female parent Panniyur 2. One polymorphic band of size 1100bp was generated in hybrid P2PC with primer OPA 28 but that was absent in female parent Panniyur 2. But all other hybrids and respective parents were having this band. Twenty eight amplicons were polymorphic in the male parent *P. colubrinum*.

The amplification pattern with the 10 selected decamer primers shows higher degree of similarity between hybrid P2PC and female parent Panniyur 2 and none of the bands specific to male parent *P. colubrinum* was shared by hybrid P2PC.

(iv) Hybrid P1PC

The amplification pattern observed for the genomic DNA of hybrid P1PC and respective parents with 10 selected RAPD primers are shown in Plate 10 to Plate 14 and the comparison given in Table 14.

Table 13: Comparison of RAPD banding pattern of interspecific hybrid between black pepper variety Panniyur 2 and *P. colubrinum*

Sl. No.	Primer	Total number of amplicons	Monomorphic in parents and hybrids	Polymorphic between parents			
				P2PC vs. Panniyur 2		P2PC vs. *P. colubrinum*	
				Monomorphic	Polymorphic	Monomorphic	Polymorphic (male specific)
1	OPA 08	5	2	1	-	-	2
2	OPA 10	6	2	2	-	-	2
3	OPA 17	10	3	4	-	-	3
4	OPA 28	7	4	1	1	-	1
5	OPA 30	11	1	4	-	-	6
6	OPC 08	7	3	2	-	-	2
7	OPC 09	11	3	2	-	-	6
8	OPC 14	6	-	4	-	-	2
9	OPP 08	9	4	3	-	-	2
10	OPAH 09	8	3	3	-	-	2
	Total	80	25	26	1	0	28

Table 14: Comparison of RAPD banding pattern of interspecific hybrid between black pepper variety Panniyur 1 and *P. colubrinum*

Sl. No.	Primer	Total number of amplicons	Monomorphic in parents and hybrids	Polymorphic between parents			
				P1PC vs. Panniyur 1		P1PC vs. *P. colubrinum*	
				Monomorphic	Polymorphic	Monomorphic	Polymorphic (male specific)
1	OPA 08	5	2	-	1	-	2
2	OPA 10	4	1	1	-	-	2
3	OPA 17	10	3	4	-	-	3
4	OPA 28	7	4	2	-	-	1
5	OPA 30	11	1	4	-	-	6
6	OPC 08	7	3	2	-	-	2
7	OPC 09	11	3	2	-	-	6
8	OPC 14	4	-	2	-	-	2
9	OPP 08	8	3	3	-	-	2
10	OPAH 09	9	4	4	-	-	1
	Total	76	24	24	1	0	27

Total seventy six clear distinct amplicons were observed with the 10 RAPD primers. The amplicons ranged in size from 250bp to 1400bp. Twenty four amplicons were monomorphic in hybrid P1PC and both the parents. Twenty four monomorphic bands generated in hybrid P1PC and female parent Panniyur 1. One polymorphic band of size 1050bp was generated in female parent Panniyur 1 with primer OPA 8 but that was absent in hybrid P1PC. Twenty seven amplicons were polymorphic in the male parent *P. colubrinum*.

The amplification pattern with the 10 selected decamer primers shows higher degree of similarity between hybrid P1PC and female parent Panniyur 1 and none of the bands specific to male parent *P. colubrinum* was shared by hybrid P1PC.

(v) Hybrid KMPC

The amplification pattern observed for the genomic DNA of hybrid KMPC and respective parents with 10 selected RAPD primers are shown in Plate 10 to Plate 14 and the comparison given in Table 15.

Total seventy two clear distinct amplicons were observed with the 10 RAPD primers. The amplicons ranged in size from 250bp to 1400bp. Twenty six amplicons were monomorphic in hybrid KMPC and both the parents. Twenty two monomorphic bands generated in hybrid KMPC and female parent Karimunda. Twenty four amplicons were polymorphic in the male parent *P. colubrinum*. Two unique bands each of size 900bp and 1200bp in the female parent Karimunda and hybrid KMPC were amplified by the primers OPA 17 and OPC 09 respectively.

The amplification pattern with the 10 selected decamer primers shows higher degree of similarity between hybrid KMPC and female parent Karimunda and none of the bands specific to male parent *P. colubrinum* was shared by hybrid KMPC.

**Table 15: Comparison of RAPD banding pattern of interspecific hybrid
between black pepper cultivar Karimunda and *P. colubrinum***

Sl. No.	Primer	Total number of amplicons	Monomorphic in parents and hybrids	Polymorphic between parents			
				KMPC vs. Karimunda		KMPC vs. *P. colubrinum*	
				Monomorphic	Polymorphic	Monomorphic	Polymorphic (male specific)
1	OPA 08	5	2	1	-	-	2
2	OPA 10	5	2	1	-	-	2
3	OPA 17	10	3	4	-	-	3
4	OPA 28	3	2	-	-	-	1
5	OPA 30	11	1	4	-	-	6
6	OPC 08	7	3	2	-	-	2
7	OPC 09	11	6	2	-	-	3
8	OPC 14	4	-	2	-	-	2
9	OPP 08	8	3	3	-	-	2
10	OPAH 09	8	4	3	-	-	1
	Total	72	26	22	0	0	24

(v) Hybrid UKPC

The amplification pattern observed for the genomic DNA of hybrid UKPC and respective parents with 10 selected RAPD primers are shown in Plate 10 to Plate 14 and the comparison given in Table 16.

Total seventy three clear distinct amplicons were observed with the 10 RAPD primers. The amplicons ranged in size from 250bp to 1400bp. Twenty seven amplicons were monomorphic in hybrid UKPC and both the parents. Twenty four monomorphic bands generated in hybrid UKPC and female parent Uthirankotta. Twenty two amplicons were polymorphic in the male parent *P. colubrinum*. Four unique bands each of size 900bp, 600bp, 1100bp and 300bp in the female parent Uthirankotta and hybrid UKPC were amplified by the primers OPA 17, OPA 28, OPC 09 and OPAH 09 respectively.

The amplification pattern with the 10 selected decamer primers shows higher degree of similarity between hybrid UKPC and female parent Uthirankotta and none of the bands specific to male parent *P. colubrinum* was shared by hybrid UKPC.

4. 4 Simple Sequences Repeats (SSR) analysis

Good quality genomic DNA from the putative interspecific hybrids and their corresponding parents were subjected to SSR assay. The various experiments carried out under this included screening of SSR primers and SSR analysis with selected primers.

4.4.1 Screening of primers for SSR analysis

Fifty four primer sets were screened for amplification of SSR region in the genomic DNA of black pepper with thermal settings mentioned earlier (3.4.3). The amplification observed is presented in Plate 15.

Table 16: Comparison of RAPD banding pattern of interspecific hybrid between black pepper cultivar Uthirankotta and *P. colubrinum*

Sl. No.	Primer	Total number of amplicons	Monomorphic in parents and hybrids	Polymorphic between parents			
				UKPC vs. Uthirankotta		UKPC vs. *P. colubrinum*	
				Monomorphic	Polymorphic	Monomorphic	Polymorphic (male specific)
1	OPA 08	4	1	1	-	-	2
2	OPA 10	4	1	1	-	-	2
3	OPA 17	10	3	4	-	-	3
4	OPA 28	6	3	2	-	-	1
5	OPA 30	10	1	3	-	-	6
6	OPC 08	7	3	2	-	-	2
7	OPC 09	11	8	2	-	-	1
8	OPC 14	6	-	4	-	-	2
9	OPP 08	8	3	3	-	-	2
10	OPAH 09	7	4	2	-	-	1
	Total	73	27	24	0	0	22
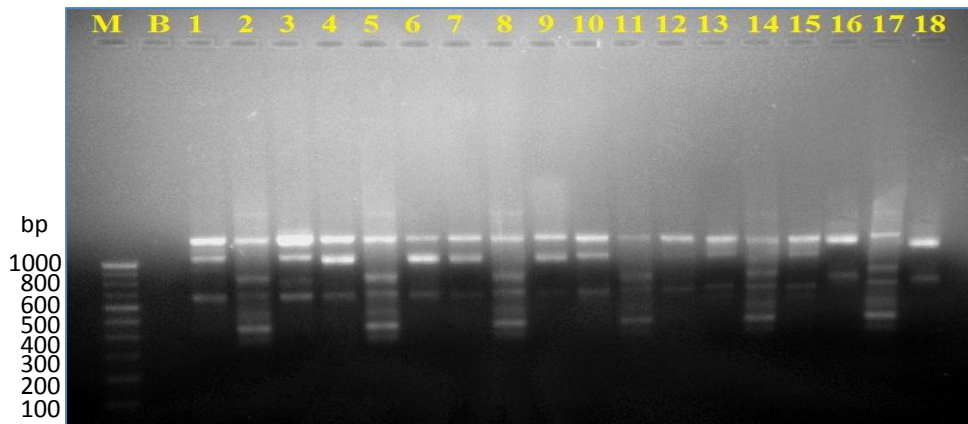
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UK PC

a. Amplification with primer OPA 8
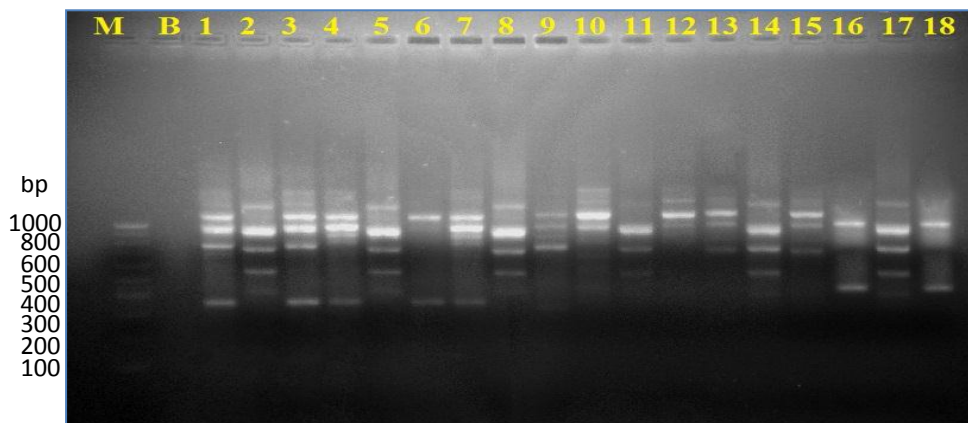
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer OPA 10

(***P5** – Panniyur 5; **P3** – Panniyur 3; **P2** – Panniyur 2; **P1** – Panniyur 1; **KM** – Karimunda;
UK – Uthirankotta; **PC** – *Piper colubrinum*)

**Plate 10: Amplification pattern of the six black pepper putative interspecific
hybrids and parents with RAPD primers OPA 8 and OPA 10**
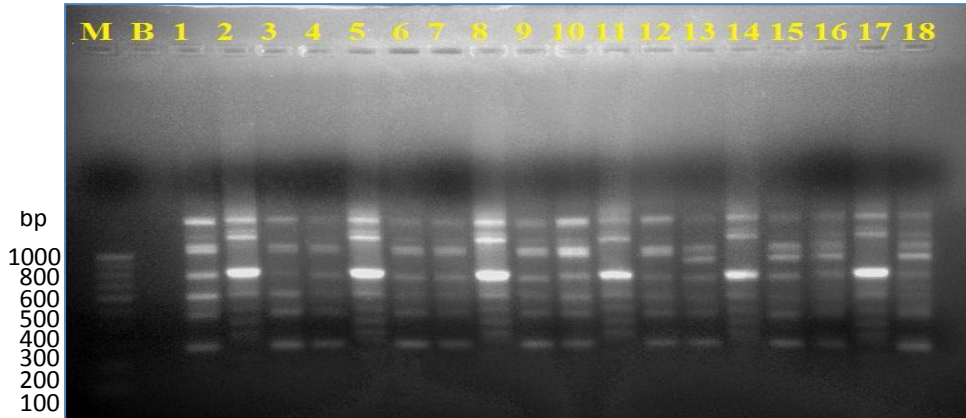
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer OPA 17
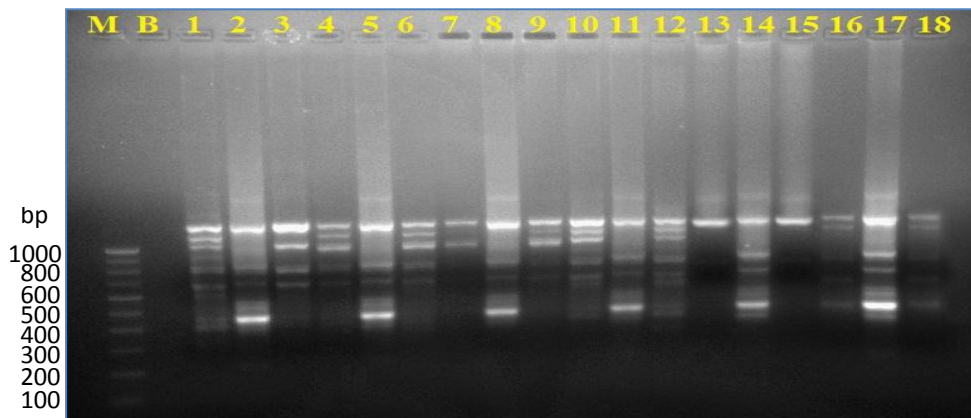
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer OPA 28

(*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

**Plate 11: Amplification pattern of the six black pepper putative interspecific
hybrids and parents with RAPD primers OPA 17 and OPC 28**
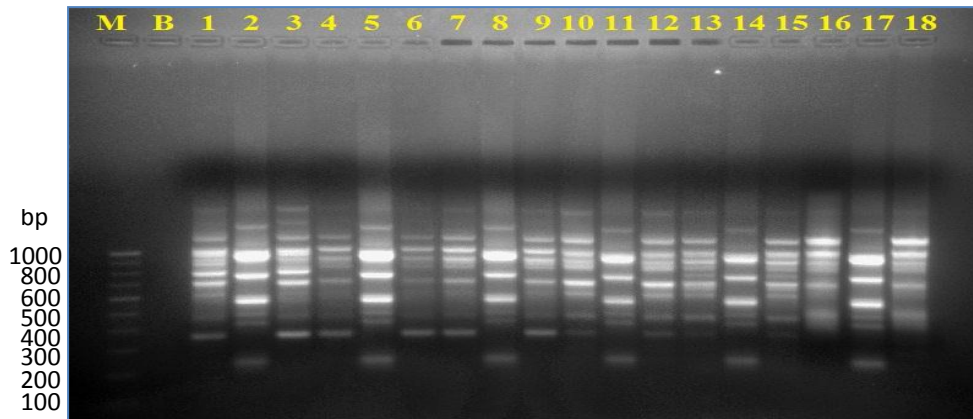
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer OPA 30
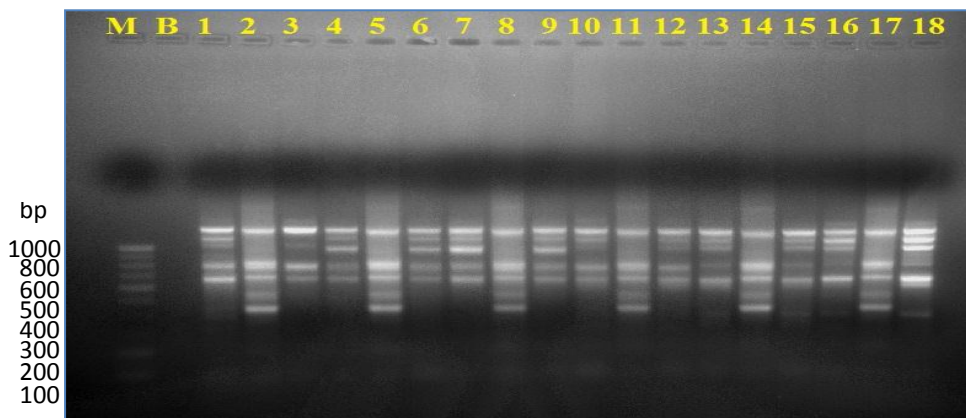
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer OPC 08

(*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

Plate 12: Amplification pattern of the six black pepper putative interspecific hybrids and parents with RAPD primers OPA 30 and OPC 08
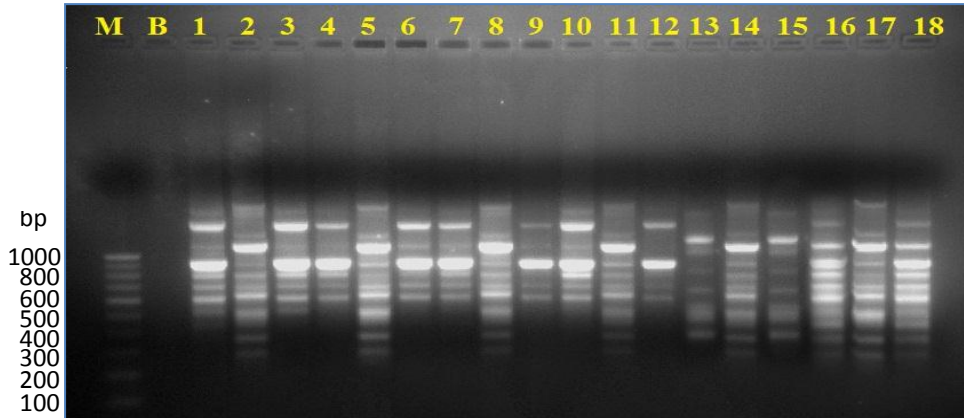
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer OPC 09
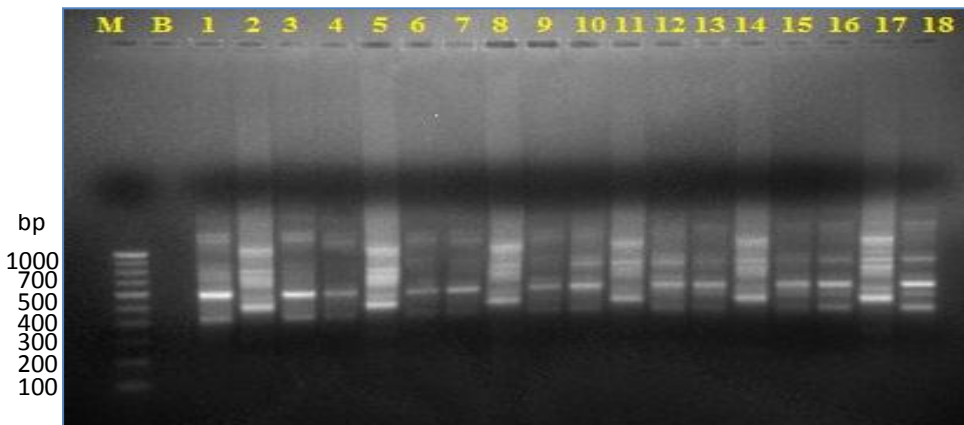
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer OPC 14

(*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

**Plate 13: Amplification pattern of the six black pepper putative interspecific
hybrids and parents with RAPD primers OPC 09 and OPC 14**
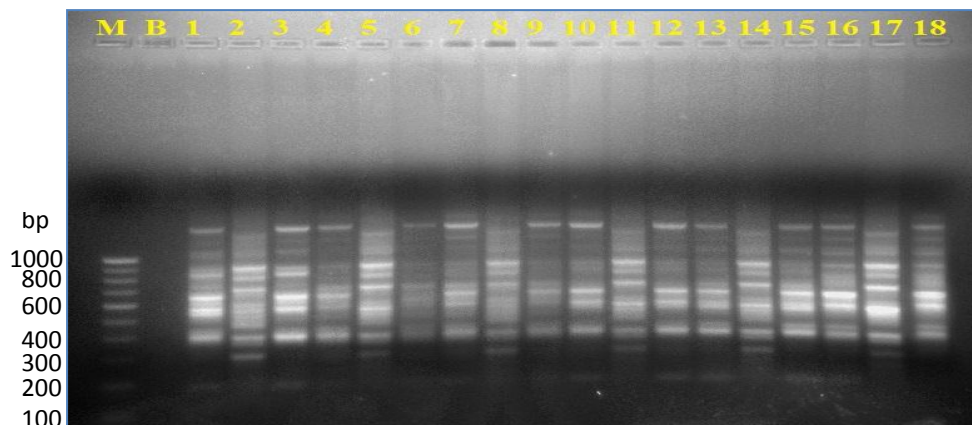
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer OPP 08
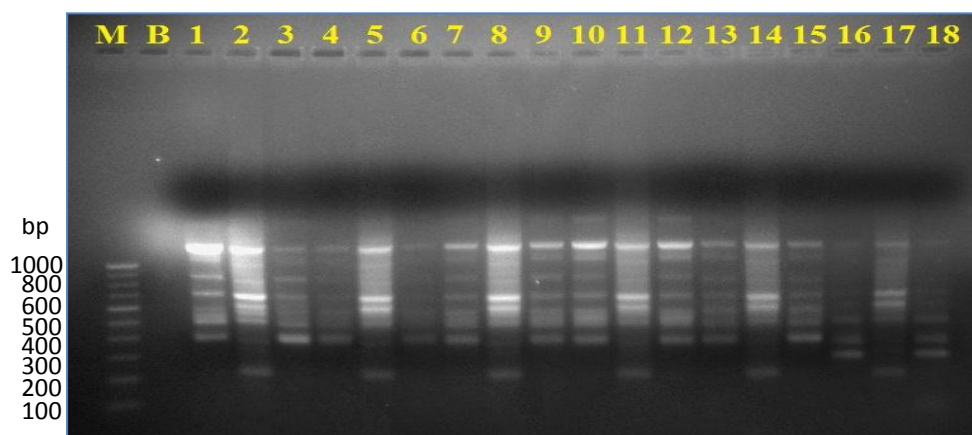
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer OPAH 09

(*****P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

**Plate 14: Amplification pattern of the six black pepper putative interspecific
hybrids and parents with RAPD primers OPP 08 and OPAH 09**

M: 100bp ladder/Marker; B: Control
1-15: Amplification pattern with SSR primers

Plate 15: Screening of SSR primers for amplification of black pepper DNA

Based on the amplification pattern eleven primer sets were selected for SSR assay and the details are provided in Table 17. The sequence data of the selected primers are provided in Annexure IV.

4.4.2 Amplification with selected primers

Out of fifty four screened SSR primers, eleven primers (listed in Table 17) were selected for further analysis. They were used for amplifying DNA of six putative hybrid and their parents. SSR banding pattern among parents and their hybrid populations was compared to assess hybridity at the DNA level.

(i) PN A5

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PN A5 are shown in Plate 16a. A total of 15 clear distinct loci were observed and the amplicons ranged in size from 50bp to 100bp.

Thirteen out of fifteen loci amplified monomorphic bands, present in both the parents and their hybrids of all six crosses. One unique loci of 100bp generated in the female parent Karimunda and in corresponding hybrid KMPC.

(ii) PN B5

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PN B5 are shown in Plate 16b. A total of 14 clear distinct loci were observed and the amplicons ranged in size from 250bp to 750bp.

Twelve out of fourteen loci amplified monomorphic bands, present in the female parents and their hybrids of all six crosses. Two polymorphic loci were generated in the male parent *P. colubrinum*. The amplification pattern with primer PN B5 shows the all interspecific hybrids were found to be homozygous for their female parent allele and absence of male parent specific allele.

Table 17: Details of amplification with 54 SSR primers screened for

SSR assay in black pepper

Sr. No.	Primers	Amplification pattern			Remark
		No. of bands	Types of bands		
			Distinct	Faint	
1	PN A5	1	1	0	Selected
2	PN B5	1	1	0	Selected
3	PN B9	0	0	0	- - -
4	PN E3	2	2	0	Selected
5	PN F1	1	1	0	Selected
6	PN G11	1	1	0	Selected
7	PN H4	1	1	0	Selected
8	PN H8a	0	0	0	- - -
9	PN D10	2	2	0	Selected
10	PC-b8	0	0	0	- - -
11	PC-b12	0	0	0	- - -
12	PC-b13	0	0	0	- - -
13	PnCA25	0	0	0	- - -
14	PnCA88	0	0	0	- - -
15	PnGT119	0	0	0	- - -
16	PnAG30	1	1	0	Selected
17	PnGT2	2	2	0	Selected
18	PnGATA10	1	1	0	Selected
19	PnCA9	2	2	0	Selected
20	LE aat001	0	0	0	- - -
21	LE aat002	0	0	0	- - -
22	LE ac001	0	0	0	- - -
23	LE at001	0	0	0	- - -
24	LE at002	0	0	0	- - -
25	LE at003	0	0	0	- - -
26	LE at004	0	0	0	- - -
27	LE at005	0	0	0	- - -
28	LE at006	0	0	0	- - -

Table 17 continued:

Sl. No	Primers	Amplification pattern			Remark
		No. of bands	Types of bands		
			Distinct	Faint	
29	LE at007	0	0	0	- - -
30	LE at008	0	0	0	- - -
31	LE at009	0	0	0	- - -
32	LE at010	0	0	0	- - -
33	LE at011	0	0	0	- - -
34	LE at012	0	0	0	- - -
35	LE at013	0	0	0	- - -
36	LE at014	0	0	0	- - -
37	LE at015	0	0	0	- - -
38	LE at016	0	0	0	- - -
39	LE at017	0	0	0	- - -
40	LE at018	0	0	0	- - -
41	LE at019	0	0	0	- - -
42	LE at020	0	0	0	- - -
43	LE at021	0	0	0	- - -
44	LE at022	0	0	0	- - -
45	LE at023	0	0	0	- - -
46	LE at024	0	0	0	- - -
47	LE at025	0	0	0	- - -
48	LE aat001	0	0	0	- - -
49	LE aat002	0	0	0	- - -
50	LE ac001	0	0	0	- - -
51	LE at001	0	0	0	- - -
52	LE at002	0	0	0	- - -
53	LE at003	0	0	0	- - -
54	LE at004	0	0	0	- - -

(iii) PN E3

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PN E3 are shown in Plate 16c. A total of 24 clear distinct loci were observed and the amplicons ranged in size from 50bp to 300bp.

Twenty one out of twenty four loci amplified monomorphic bands, present in the female parents and their hybrids of all six crosses and one loci monomorphic to all the hybrids and their female parents except hybrid KMPC and their female parent Karimunda. Two polymorphic loci were generated in the male parent *P. colubrinum*. The amplification pattern with primer PN E3 shows the all interspecific hybrids were found to be homozygous for their female parent allele and absence of male parent specific allele.

(iv) PN F1

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PN F1 are shown in Plate 17a. A total of 26 clear distinct loci were observed and the amplicons ranged in size from 150bp to 800bp.

Twenty three out of twenty six loci amplified monomorphic bands, present in the female parents and their hybrids of all six crosses. One unique loci of 75bp generated in the female parent Uthirankotta and in corresponding hybrid UKPC. Two polymorphic loci were generated in the male parent *P. colubrinum*. The amplification pattern with primer PN F1 shows the all interspecific hybrids were found to be homozygous for their female parent allele and absence of male parent specific allele.

(v) PN G11

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PN G11 are
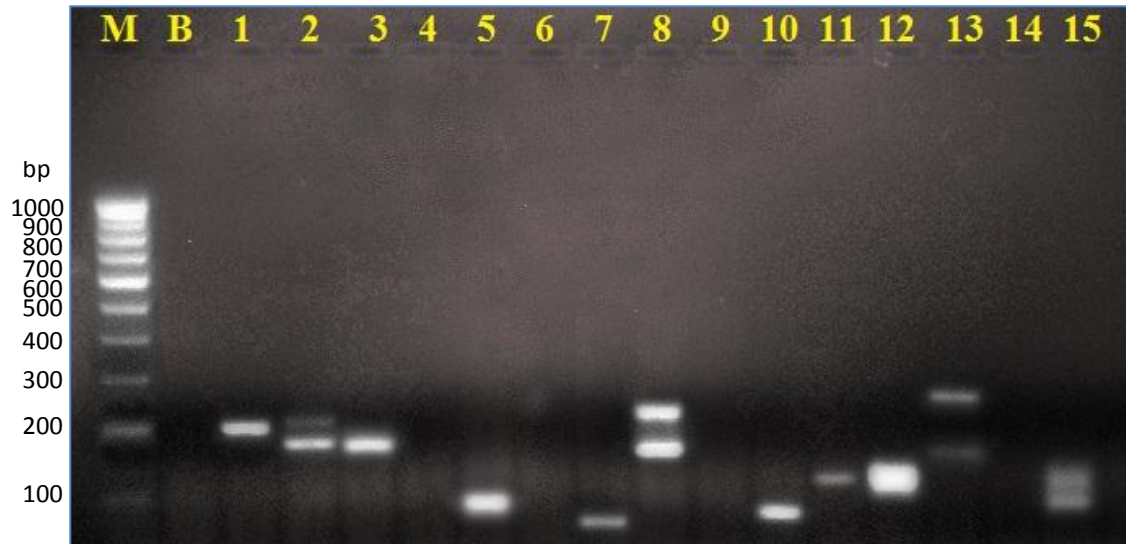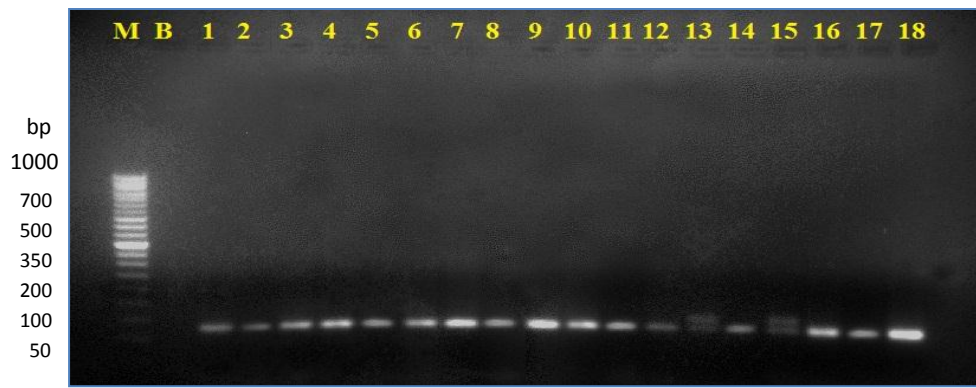
*Lanes M: 50bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer PN A5

*Lanes M: 50bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer PN B5

*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

c. Amplification with primer PN E3

(*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

**Plate 16: Amplification pattern of the six black pepper putative
interspecific hybrids and parents with SSR primers PN A5, PN B5 and
PN E3**

shown in Plate 17b. A total of 14 clear distinct loci were observed and the amplicons ranged in size from 200bp to 1200bp.

Twelve out of fourteen loci amplified monomorphic bands, present in the female parents and their hybrids of all six crosses. Two polymorphic loci were generated in the male parent *P. colubrinum*. The amplification pattern with primer PN G11 shows the all interspecific hybrids were found to be homozygous for their female parent allele and absence of male parent specific allele.

(vi) PnCA9

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PnCA9 are shown in Plate 17c. A total of 24 clear distinct loci were observed and the amplicons ranged in size from 125bp to 300bp.

Twenty two out of twenty four loci amplified monomorphic bands, present in the female parents and their hybrids of all six crosses. Two polymorphic loci were generated in the male parent *P. colubrinum*. The amplification pattern with primer PnCA9 shows the all interspecific hybrids were found to be homozygous for their female parent allele and absence of male parent specific allele.

(vii) PN H4

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PN H4 are shown in Plate 18a. A total of 23 clear distinct loci were observed and the amplicons ranged in size from 45bp to 200bp.

Twenty three out of twenty three loci amplified monomorphic bands, thirteen monomorphic bands present in both the parents and their hybrids of all six crosses whereas one monomorphic loci present in the hybrids and female parents of five interspecific crosses except hybrid P5PC and their female parent Panniyur 5. The amplification pattern of the all six hybrids with primer PN H4
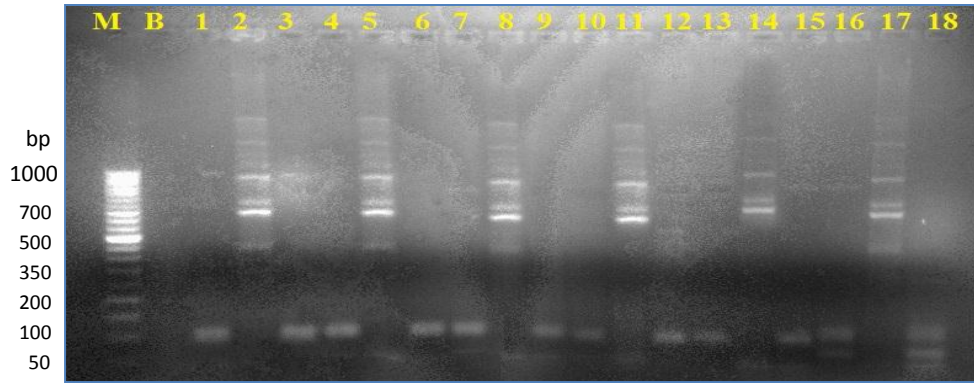
*Lanes M: 50bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer PN F1
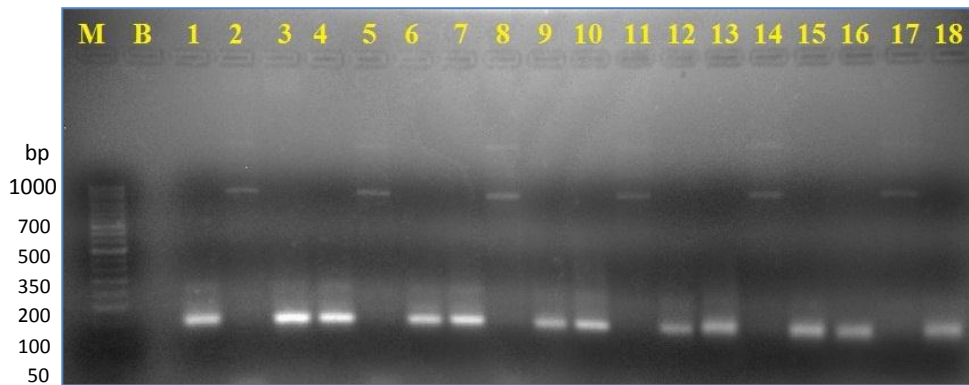
*Lanes M: 50bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer PN G11

*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

c. Amplification with primer PnCA9

(*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

**Plate 17: Amplification pattern of the six black pepper putative interspecific
hybrids and parents with SSR primers PN F1, PN G11 and PnCA9**

shows higher degree of similarity with respective female parents compared to the male parent.

(viii) PN D10

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PN D10 are shown in Plate 18b. A total of 24 clear distinct loci were observed and the amplicons ranged in size from 200bp to 650bp.

Twenty one out of twenty four loci amplified monomorphic bands, thirteen monomorphic bands present in both the parents and their hybrids of all six crosses whereas eight monomorphic loci present in the hybrids and female parents of five interspecific crosses except hybrid P5PC and their female parent Panniyur 5. Two polymorphic loci found in the hybrid P5PC and their female parent as well as one polymorphic loci generated in the male parent *P. colubrinum*. The amplification pattern of the all six hybrids with primer PN D10 shows higher degree of similarity with respective female parents compared to the male parent.

(ix) PnAG30

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PnAG30 are shown in Plate 18c. A total of 14 clear distinct loci were observed and the amplicons ranged in size from 100bp to 900bp.

Twelve out of fourteen loci amplified monomorphic bands, present in the female parents and their hybrids of all six crosses. Two polymorphic loci were generated in the male parent *P. colubrinum*. The amplification pattern with primer PnAG30 shows the all interspecific hybrids were found to be homozygous for their female parent allele and absence of male parent specific allele.
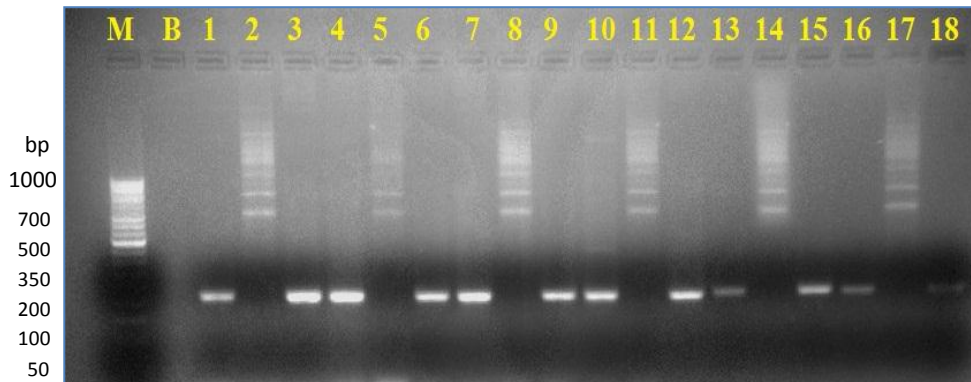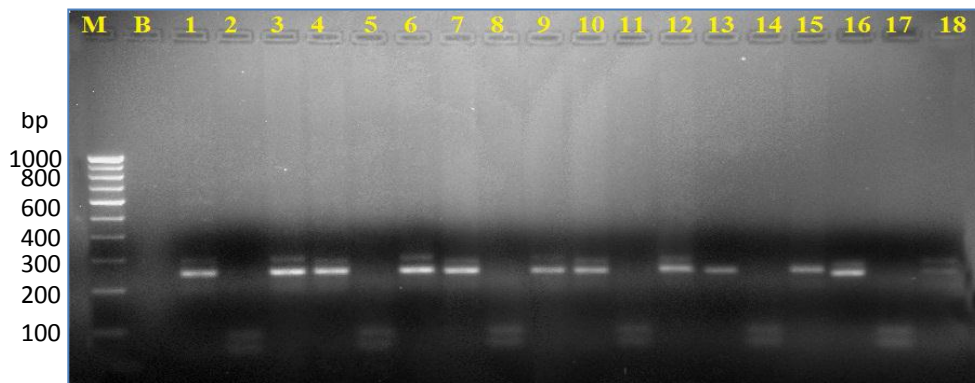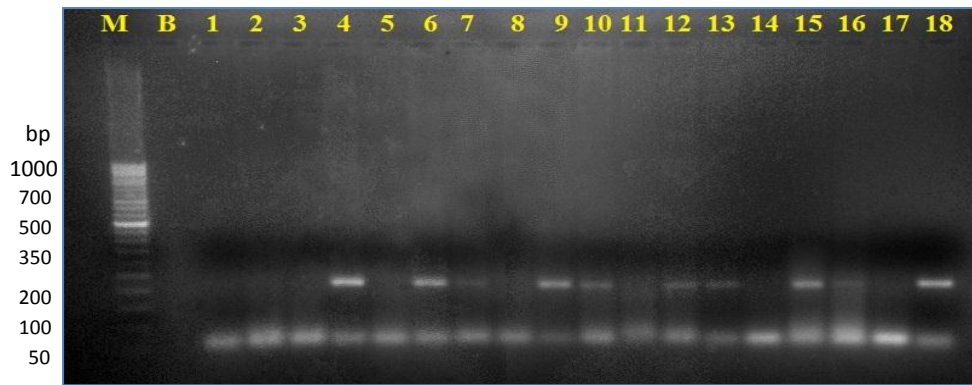
*Lanes M: 50bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer PN H4
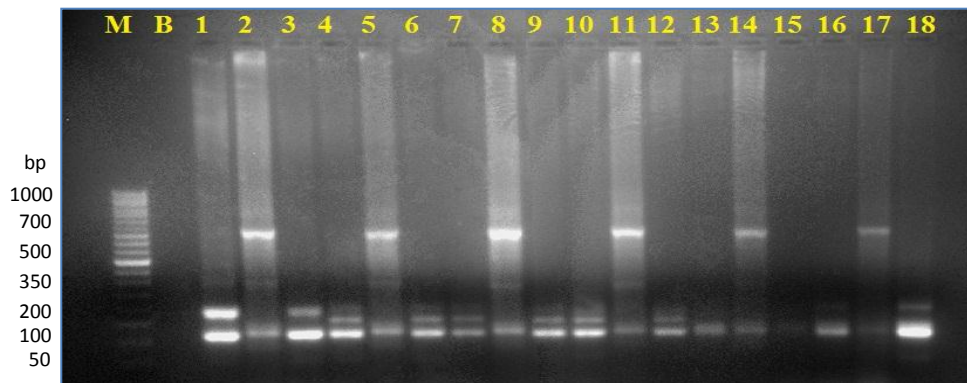
*Lanes M: 50bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer PN D10
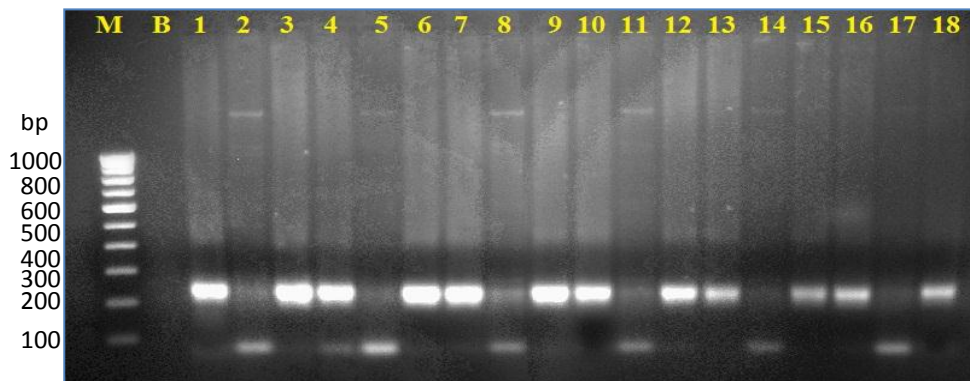
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

c. Amplification with primer PnAG30

(*P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

**Plate 18: Amplification pattern of the six black pepper putative interspecific
hybrids and parents with SSR primers PN H4, PN D10 and PnAG30**

(x) PnGT2

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PnGT2 are shown in Plate 19a. A total of 26 clear distinct loci were observed and the amplicons ranged in size from 100bp to 450bp.

Twenty four out of twenty six loci amplified monomorphic bands, present in the female parents and their hybrids of all six crosses. Two polymorphic loci were generated in the male parent *P. colubrinum*. The amplification pattern with primer PnGT2 shows the all interspecific hybrids were found to be homozygous for their female parent allele and absence of male parent specific allele.

(xi) PnGATA10

The agarose gel profile for the amplification pattern observed in the hybrids and respective parents of all six interspecific crosses with primer PnGATA10 are shown in Plate 19b. A total of thirteen clear distinct loci of size 80bp were observed. All observed loci amplified monomorphic bands, present in both the parents and their hybrids of all six crosses.

4.5.1 Resolving power of selected RAPD and SSR markers

The Resolving power (Rp) calculated for the random primer is presented in Fig. 5a. It ranged between 5.6 (OPA 08) and 13.8 (OPA 17) with an average of 9.99 for RAPD primers. SSR primers (Fig. 5b) recorded values ranging between 2.0 (PnGATA10) and 3.69 (PN E3 and PN D10) with an average 2.92.

4.5.2 Polymorphic Information Content (PIC) value for the selected RAPD and SSR primers

The Polymorphic Information Content (PIC) value calculated for the 10 selected RAPD primers (Fig. 6a) varied from 0.72 (OPA 08) to 0.87 (OPA 30 and OPP 08) with a mean of 0.82 (Table 18). The 11 selected SSR primers (Fig. 6b) recorded values ranging from 0 (PnGATA10) to 0.57 (PN E3, PnCA9 and
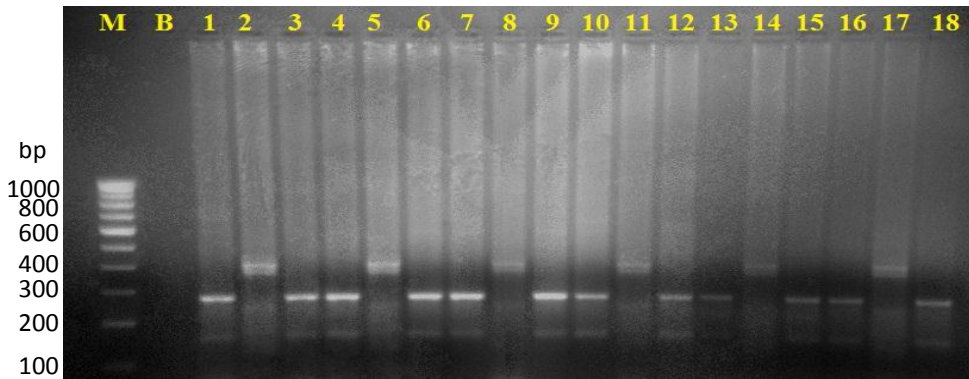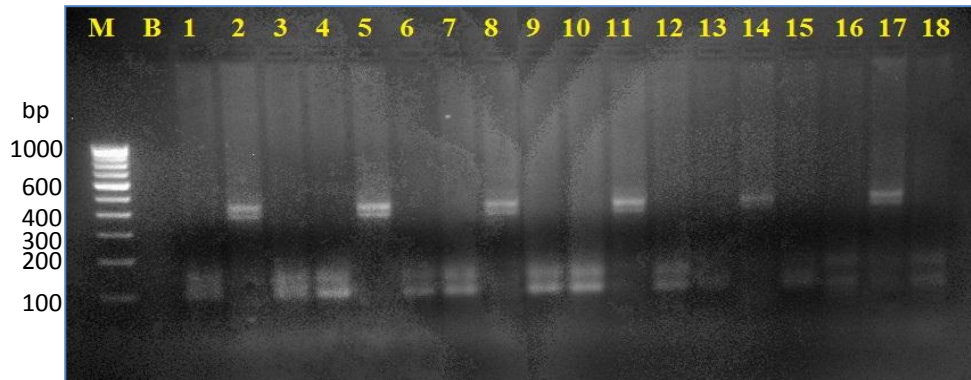
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

a. Amplification with primer PnGT2
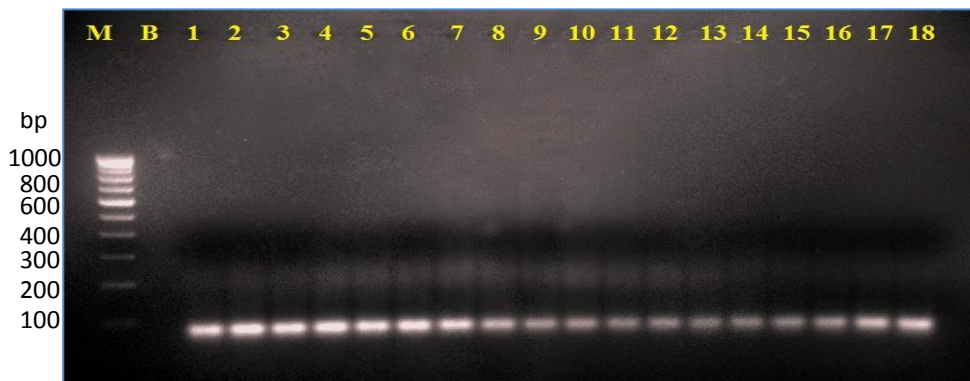
*Lanes M: 100bp ladder/Marker, B: Control
1: P5, 2: PC, 3: P5PC, 4: P3, 5: PC, 6: P3PC, 7: P2, 8: PC, 9: P2PC 10: P1,
11: PC, 12: P1PC, 13: KM, 14: PC, 15: KMPC, 16: UK, 17: PC, 18: UKPC

b. Amplification with primer PnGATA10

(*****P5 – Panniyur 5; P3 – Panniyur 3; P2 – Panniyur 2; P1 – Panniyur 1; KM – Karimunda;
UK – Uthirankotta; PC – *Piper colubrinum*)

**Plate 19: Amplification pattern of the six black pepper putative
interspecific hybrids and parents with SSR primers PnGT2 and
PnGATA10**

Table 18: PIC values for RAPD markers identified in hybrids and their respective parents

Sl. No.	Name of Primers	PIC values	Remark
1	OPA 8	0.7246	- - -
2	OPA 10	0.7797	- - -
3	OPA 17	0.8438	- - -
4	OPA 28	0.8325	- - -
5	OPA 30	0.8716	Selected
6	OPC 08	0.8113	- - -
7	OPC 09	0.8824	- - -
8	OPC 14	0.763	- - -
9	OPP 08	0.8712	Selected
10	OPAH 09	0.84	- - -
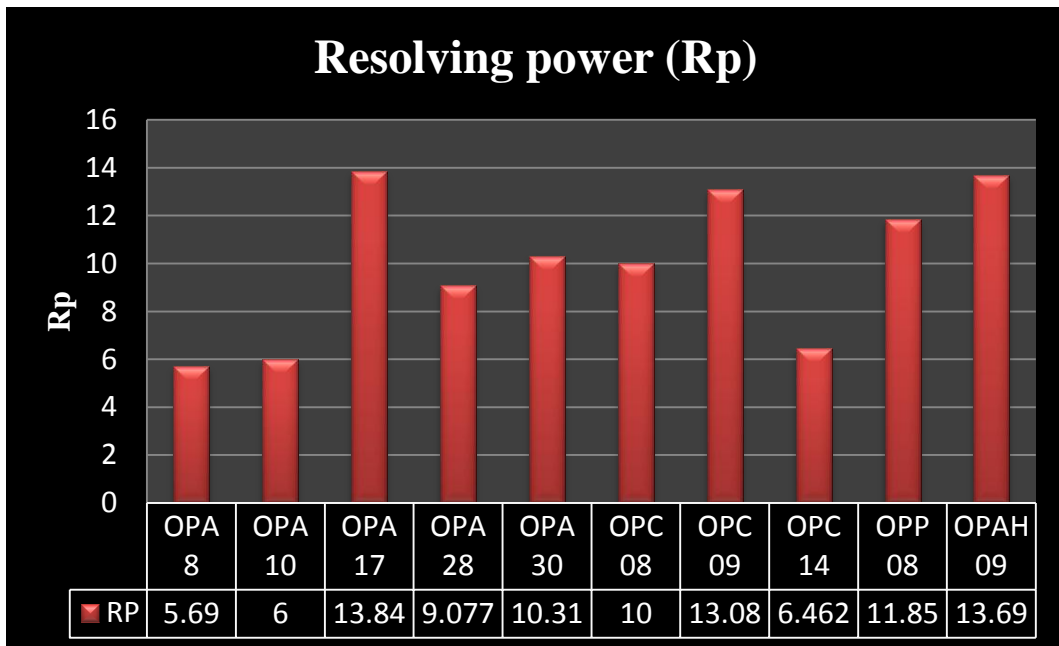
a. 10 selected RAPD primers

b. 11 selected SSR primers

Fig. 5: Resolving power (Rp) of selected RAPD and SSR primer

a. 10 selected RAPD primers

	OPA 8	OPA 10	OPA 17	OPA 28	OPA 30	OPC 08	OPC 09	OPC 14	OPP 08	OPAH 09
PIC Values	0.7246	0.7797	0.8438	0.8325	0.8716	0.8113	0.8824	0.763	0.8712	0.84

b. 11 selected SSR primers

	PN A5	PN B5	PN E3	PN F1	PN G11	PN H4	PN D10	PnAG 30	PnCA 09	PnGA TA10	PnGT 2
PIC Values	0.231	0.502	0.573	0.328	0.255	0.491	0.531	0.255	0.571	0	0.571
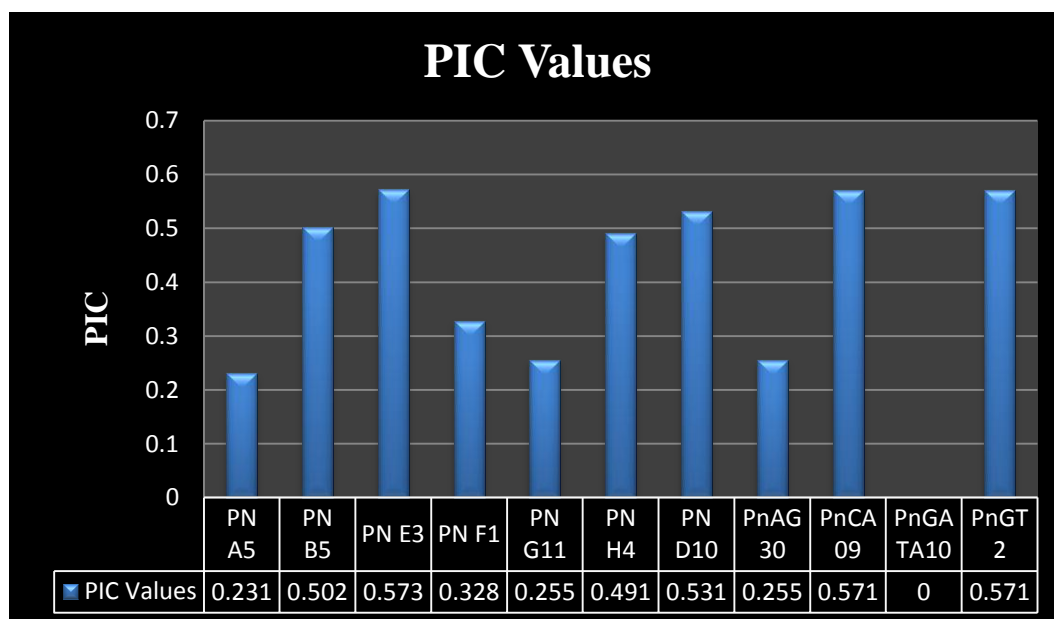
Fig. 6: Polymorphic Information Content (PIC) value for selected RAPD and SSR primer

pnGT2 with an average of 0.39 (Table 19). RAPD and SSR primers with PIC values >0.5 selected for molecular charaterization of open pollinated progeny of reported hybrid culture P5PC.

4.6 Molecular characterization of open pollinated progenies of the reported hybrid culture P5PC

Good quality genomic DNA from the seedlings of the reported hybrid culture P5PC and their corresponding parents were subjected to RAPD and SSR assay using primers selected based on PIC.

4.6.1 Molecular characterization

Genomic DNA of open pollinated progenies (6nos) of hybrid P5PC was isolated using CTAB method described in 3.3. The isolated DNA of progenies, its female parent hybrid P5PC, grandparents Panniyur 5 and *P. colubrinum* was subjected to molecular characterization using RAPD and SSR markers.

Two decamer and five SSR primers (listed in Table 18 and Table 19) with high PIC values were selected from earlier screened primers and used for amplifying DNA of open pollinated progenies of hybrid P5PC and their female parent and grandparents. RAPD and SSR banding pattern among progenies and their parents was compared to assess variability at the DNA level.

4.6.2 Amplification with selected decamer primers

The decamer primers OPA 30 and OPP 08 were selected for amplifying DNA of open pollinated progenies, their female parent (Culture P5PC) and grandparents Panniyur 5 and *P. colubrinum*.

(i) OPA 30

The agarose gel profile for the amplification pattern observed in open pollinated progenies, their female parent (Culture P5PC) and grandparents Panniyur 5 and *P. colubrinum* with the primer OPA 30 is shown in Plate 20a. A

Table 19: PIC values for SSR markers identified in hybrids and their respective parents

Sl. No.	Name of Primers	PIC values	Remark
1	PN A5	0.2311	- - -
2	PN B5	0.5015	Selected
3	PN E3	0.5729	Selected
4	PN F1	0.3281	- - -
5	PN G11	0.2551	- - -
6	PN H4	0.4914	- - -
7	PN D10	0.5312	Selected
8	PnAG30	0.2551	- - -
9	PnCA9	0.571	Selected
10	PnGATA10	0	- - -
11	PnGT2	0.571	Selected

total of eight clear distinct amplicons were observed and the amplicons ranged in size from 250bp to 1200bp.

One loci of size 900bp was found commonly shared by all the six open pollinated progenies, female parent hybrid P5PC as well as grandparents Panniyur 5 and *P. colubrinum*. Two loci of size 1200bp and 1300bp were found polymorphic to two progenies whereas found monomorphic in four progenies and their female parent as well as grandparents. Three loci of size 340bp, 650bp and 1100bp were found monomorphic in progenies and female parent as well as in grandparent Panniyur 5 whereas found polymorphic to the *P. colubrinum*. One amplicon of size 750bp was found polymorphic to three progenies whereas found monomorphic in their parents. One unique amplicon of size 550bp generated in *P. colubrinum*. The amplification pattern with the primer OPA 30 shows higher degree of similarity between female parent (hybrid P5PC), their progenies and grandparent Panniyur 5 compared to grandparent *P. colubrinum*.

(ii) OPP 08

The agarose gel profile for the amplification pattern observed in open pollinated progenies, their female parent (Culture P5PC) and grandparents Panniyur 5 and *P. colubrinum* with the primer OPP 08 is shown in Plate 20b. A total of eight clear distinct amplicons were observed and the amplicons ranged in size from 300bp to 1400bp.

Four out of eight loci amplified monomorphic bands, three monomorphic bands present in the open pollinated progenies and their female parent hybrid P5PC as well as grandparents Panniyur 5 and *P. colubrinum*. One amplicon of size 700bp was found polymorphic to *P. colubrinum* whereas monomorphic in female parent (hybrid P5PC) and progenies as well as in grandparent Panniyur 5. Four loci were polymorphic, present in the grandparent *P. colubrinum*. The amplification pattern with the primer OPP 08 shows higher degree of similarity between female parent (hybrid P5PC), their progenies and grandparent Panniyur 5 compared to grandparent *P. colubrinum*.
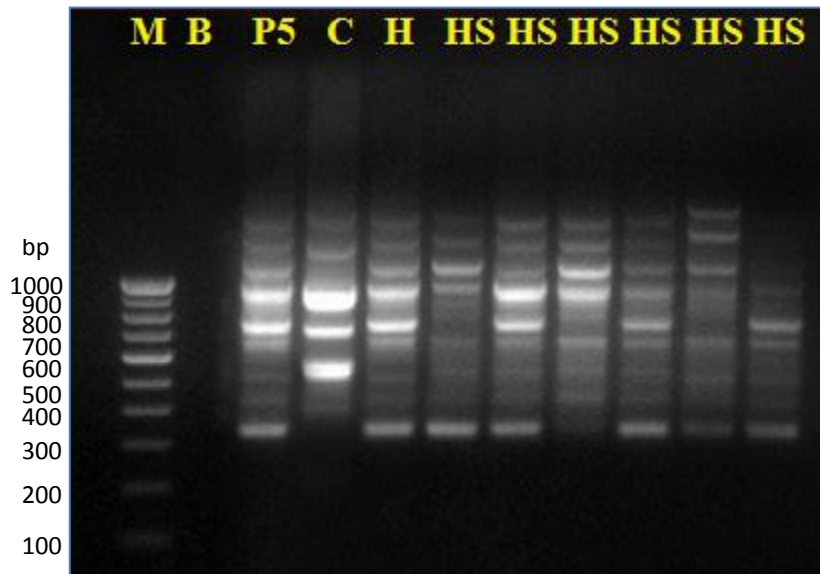
M: 100bp ladder/Marker, B: Control, P5: Panniyur 5,
C: *Piper colubrinum*, H: Hybrid P5PC, HS: progeny of P5PC

a. Amplification with primer OPA 30

M: 100bp ladder/Marker, B: Control, P5: Panniyur 5,
C: *Piper colubrinum*, H: Hybrid P5PC, HS: progeny of P5PC

b. Amplification with primer OPP 08

**Plate 20: Amplification pattern of open pollinated progeny of P5PC and
their parents with RAPD primers OPA 30 and OPA 08**

4.6.3 Amplification with selected SSR primers

The SSR primer sets PN B5, PN E3, PN D10, PnCA9 and PnGT2 were selected for amplifying DNA of open pollinated progenies and grandparents Panniyur 5 and *P. colubrinum*.

(i) PN B5

The agarose gel profile for the amplification pattern observed in open pollinated progenies as well as their grandparents Panniyur 5 and *P. colubrinum* with primer PN B5 is shown in Plate 21a. A total of nine clear distinct loci were observed and the amplicons ranged in size from 250bp to 750bp.

Seven out of nine loci amplified monomorphic bands, present in the open pollinated progenies of hybrid (Culture P5PC) and female grandparent Panniyur 5. Two polymorphic loci were generated in the male grandparent *P. colubrinum*. The amplification pattern with the primer PN B5 shows the all the open pollinated progenies of hybrid P5PC were found to be homozygous for their female grandparent (Panniyur 5) allele and absence of male grandparent (*P. colubrinum*) specific allele.

(ii) PN E3

The agarose gel profile for the amplification pattern observed in open pollinated progenies as well as their grandparents Panniyur 5 and *P. colubrinum* with primer PN E3 is shown in Plate 21b. A total of 13 clear distinct loci were observed and the amplicons ranged in size from 50bp to 300bp.

Eleven out of thirteen loci amplified monomorphic bands of size 250bp and 300bp, present in four open pollinated progenies of hybrid (Culture P5PC) and female grandparent Panniyur 5. Whereas, two progenies amplified only monomorphic band of size 250bp and absence of 300bp sized band. Two polymorphic loci of size 40bp and 50bp were generated in the male grandparent *P. colubrinum*. The amplification pattern with the primer PN E3 shows the all the open pollinated progenies of hybrid P5PC were found to be homozygous for their

female grandparent (Panniyur 5) allele and absence of male grandparent (*P. colubrinum*) specific allele.

(iii) PN D10

The agarose gel profile for the amplification pattern observed in open pollinated progenies as well as their grandparents Panniyur 5 and *P. colubrinum* with primer PN D10 is shown in Plate 21c. A total of 16 clear distinct loci were observed and the amplicons ranged in size from 200bp to 650bp.

Fourteen out of sixteen loci amplified monomorphic bands, present in the open pollinated progenies of hybrid (Culture P5PC) and female grandparent Panniyur 5. Two polymorphic loci were generated in the male grandparent *P. colubrinum*. The amplification pattern with the primer PN D10 shows the all the open pollinated progenies of hybrid P5PC were found to be homozygous for their female grandparent (Panniyur 5) allele and absence of male grandparent (*P. colubrinum*) specific allele.

(iv) PnCA9

The agarose gel profile for the amplification pattern observed in open pollinated progenies as well as their grandparents Panniyur 5 and *P. colubrinum* with primer PnCA9 is shown in Plate 22a. A total of 16 clear distinct loci were observed and the amplicons ranged in size from 100bp to 450bp.

Fourteen out of sixteen loci amplified monomorphic bands, present in the open pollinated progenies of hybrid (Culture P5PC) and female grandparent Panniyur 5. Two polymorphic loci were generated in the male grandparent *P. colubrinum*. The amplification pattern with primer PnCA9 shows the all the open pollinated progenies of hybrid P5PC were found to be homozygous for their female grandparent (Panniyur 5) allele and absence of male grandparent (*P. colubrinum*) specific allele.
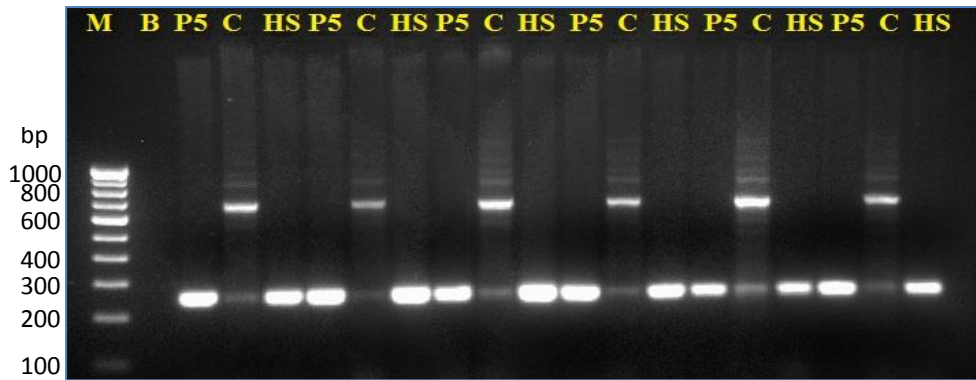
M: 100bp ladder/Marker, B: Control, P5: Panniyur 5, C: *Piper colubrinum*,
H: Hybrid P5PC, HS: progeny of P5PC

a. Amplification with primer PN B5
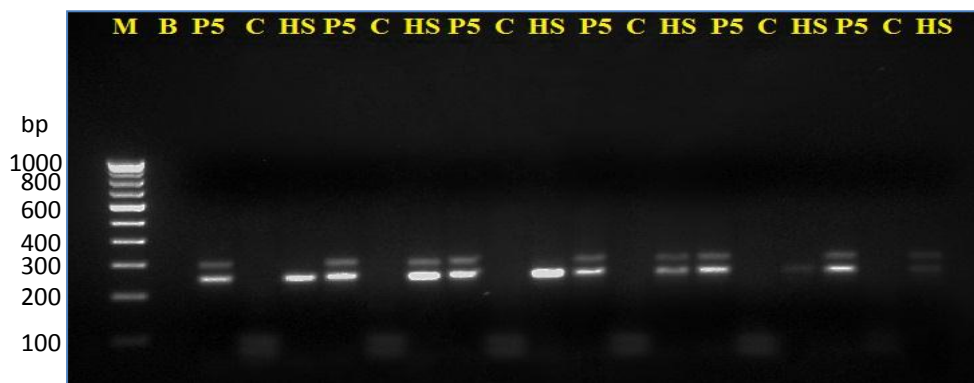
M: 100bp ladder/Marker, B: Control, P5: Panniyur 5, C: *Piper colubrinum*,
H: Hybrid P5PC, HS: progeny of P5PC

b. Amplification with primer PN E3
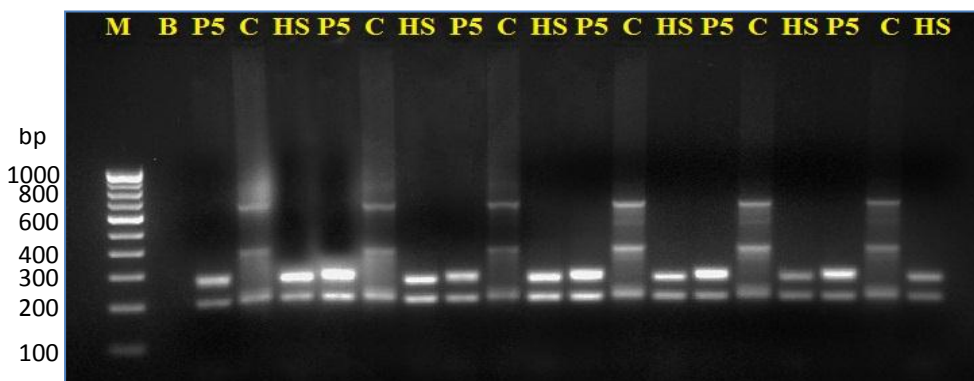
M: 100bp ladder/Marker, B: Control, P5: Panniyur 5, C: *Piper colubrinum*,
H: Hybrid P5PC, HS: progeny of P5PC

c. Amplification with primer PN D10

**Plate 21: Amplification pattern of open pollinated progeny of P5PC and
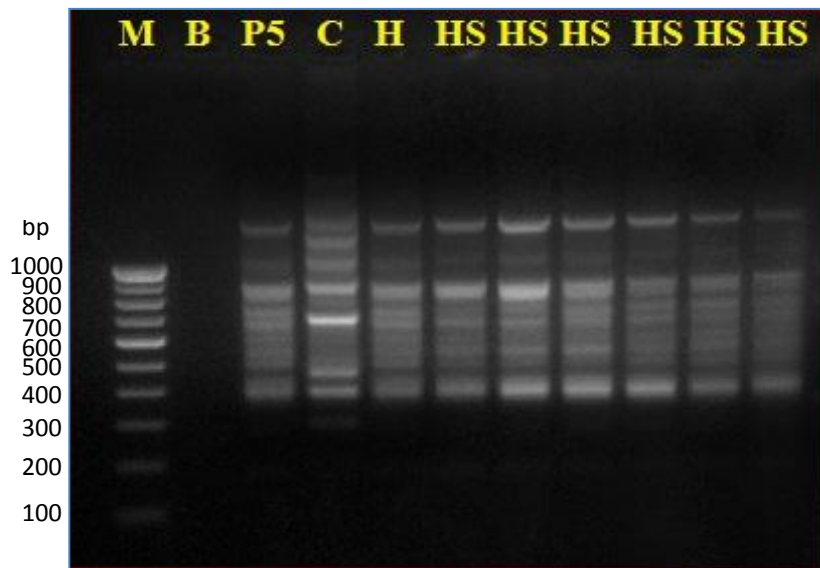their parents with SSR primers PN B5, PN E3 and PN D10**

(v) PnGT2

The agarose gel profile for the amplification pattern observed in open pollinated progenies as well as their grandparents Panniyur 5 and *P. colubrinum* with primer PnGT2 is shown in Plate 22b. A total of 16 clear distinct loci were observed and the amplicons ranged in size from 100bp to 450bp.

Fourteen out of sixteen loci amplified monomorphic bands, present in the open pollinated progenies of hybrid (Culture P5PC) and female grandparent Panniyur 5. Two polymorphic loci were generated in the male grandparent *P. colubrinum*. The amplification pattern with the primer PnGT2 shows the all the open pollinated progenies of hybrid P5PC were found to be homozygous for their female grandparent (Panniyur 5) allele and absence of male grandparent (*P. colubrinum*) specific allele.

4.7 Data analysis

4.7.1 RAPD data analysis

Reproducible, well resolved fragments were scored using Quantity one software (Biorad) and each scorable band was scored as presence (1) or absence (0). The total number of markers observed among the interspecific hybrids and their respective parents based on RAPD analysis with ten decamer primers were 87. The number of scorable markers produced per primer ranged from 6 to 12 and size of the products ranged from 250bp to 1400bp. The total number of polymorphic markers and the percentage of polymorphism were 60 and 68.87 respectively.

Data analysis was done using two different softwares: NTSYS pc version 2.02i and DARwin (Version 5.0.158).

a) NTSYS pc version 2.02i

A genetic similarity matrix as well as UPGMA dendrogram was constructed using the Jaccard's coefficients (Table 20 and Fig. 7a). The pairwise coefficient
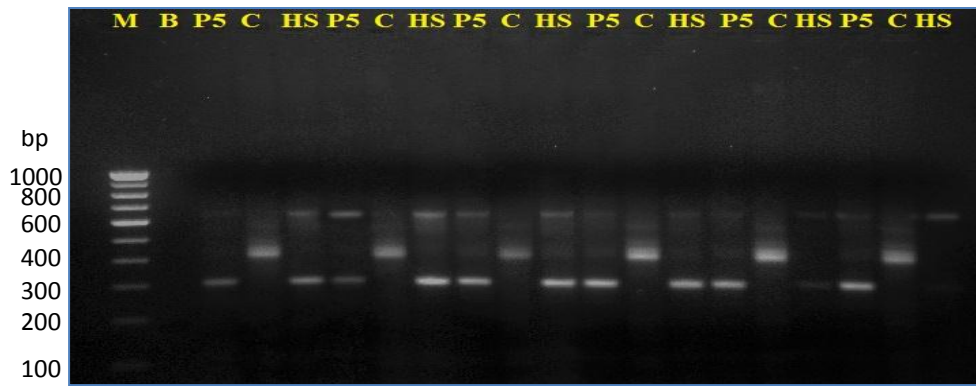
M: 100bp ladder/Marker, B: Control, P5: Panniyur 5, C: *Piper colubrinum*,
H: Hybrid P5PC, HS: progeny of P5PC

a. Amplification with primer PnCA9
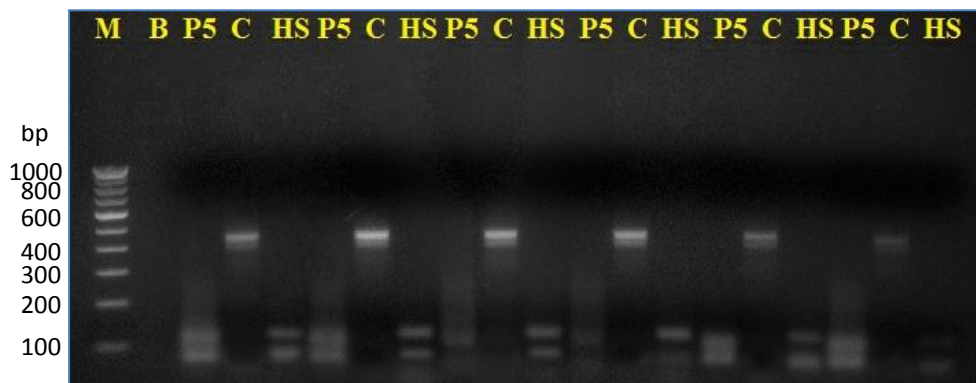
M: 100bp ladder/Marker, B: Control, P5: Panniyur 5, C: *Piper colubrinum*,
H: Hybrid P5PC, HS: progeny of P5PC

b. Amplification with primer PnGT2

**Plate 22: Amplification pattern of open pollinated progeny of P5PC and
their parents with SSR primers PnCA9 and PnGT2**

values varied between 0.2820 and 1.0000. It was observed that all the six putative interspecific hybrids were closely related to their respective female parents. The value of relatedness of hybrids with their parents ranged between 0.2820-1.0000. The highest value (1.0000) for the similarity coefficients was obtained for the hybrid KMPC and UKPC with their female parents Karimunda and Uthirankotta respectively. The value of genetic similarity coefficient i.e. 0.9807, 0.9800, 0.9615 and 0.9795 observed for the hybrids P5PC, P3PC, P2PC and P1PC with their female parents respectively. While, least similarity coefficient values were observed in all the interspecific hybrids with their male parent *P. colubrinum*, and ranged between 0.2820-0.3600.

b) DARwin (Version 5.0.158)

A genetic dissimilarity matrix as well as UPGMA dendrogram was constructed using the Dice coefficients (Table 21 and Fig. 7b). The pairwise coefficient values varied between 0.00 and 0.56. It was observed that all the six putative interspecific hybrids were highly dissimilar to their male parent *P. colubrinum*. The value of dissimilarity of hybrids with their parents ranged between 0.0-0.56. The highest value (0.56) for the dissimilarity coefficients was obtained for the hybrid P3PC with their male parent *P. colubrinum*. The value of genetic dissimilarity coefficient i.e. 0.5294, 0.5145, 0.5151,0.4747 and 0.4705 observed for the hybrids P5PC, P2PC, P1PC, KMPC and UKPC with their male parent (*P. colubrinum*) respectively. While, least dissimilarity coefficient values were observed in all the interspecific hybrids with their respective female parents, and ranged between 0.00-1.9607.

4.7.2 SSR data analysis

Reproducible, well resolved fragments were scored using Quantity one software (Biorad) and each scorable band was scored as presence (1) or absence (0). The eleven polymorphic SSR primer sets amplified a total of 33 alleles with sizes ranges from 50 to 1200bp and average of 3.0 alleles per marker.

Table 20: Genetic similarity matrix of parents and hybrids of interspecific crosses in black pepper analysed by Jaccard's coefficient method of the NTSYS program using RAPD data

	P5	P5PC	P3	P3PC	P2	P2PC	P1	P1PC	KM	KMPC	UK	UKPC	PC
P5	1.0000000												
P5PC	0.9807692	1.0000000											
P3	0.9615385	0.9423077	1.0000000										
P3PC	0.9423077	0.9230769	0.9800000	1.0000000									
P2	0.9245283	0.9423077	0.9230769	0.9038462	1.0000000								
P2PC	0.9622642	0.9433962	0.9615385	0.9423077	0.9615385	1.0000000							
P1	0.8703704	0.8518519	0.9038462	0.8846154	0.8679245	0.9056604	1.0000000						
P1PC	0.8518519	0.8333333	0.8846154	0.8653846	0.8490566	0.8867925	0.9795918	1.0000000					
KM	0.7543860	0.7678571	0.7500000	0.7321429	0.7818182	0.7543860	0.7636364	0.7454545	1.0000000				
KMPC	0.7543860	0.7678571	0.7500000	0.7321429	0.7818182	0.7543860	0.7636364	0.7454545	1.0000000	1.0000000			
UK	0.6349206	0.6190476	0.6557377	0.6393443	0.6557377	0.6612903	0.6393443	0.6500000	0.6779661	0.6779661	1.0000000		
UKPC	0.6349206	0.6190476	0.6557377	0.6393443	0.6557377	0.6612903	0.6393443	0.6500000	0.6779661	0.6779661	1.0000000	1.0000000	
PC	0.3037975	0.3076923	0.2948718	0.2820513	0.3116883	0.3205128	0.3157895	0.3200000	0.3561644	0.3561644	0.3600000	0.3600000	1.0000000

P5- Panniyur 5; P3- Panniyur 3; P2- Panniyur 2; P1- Panniyur 1; KM- Karimunda; UK- Uthirankotta; PC- *Piper colubrinum*

Table 21: Genetic dissimilarity matrix of parents and hybrids of interspecific crosses in black pepper analysed by Dice coefficient method of the DARwin program using RAPD data

	P5	P5PC	P3	P3PC	P2	P2PC	P1	P1PC	KM	KMPC	UK	UKPC	PC
P5	0												
P5XPC	1.96	0											
P3	1.96	2.97	0										
P3XPC	2.97	0.04	1.01	0									
P2	3.92	2.97	0.04	5.05	0								
P2XPC	1.92	0.02	1.96	2.97	1.96	0							
P1	6.93	5.05	6.12	7.07	4.95	0.06	0						
P1XPC	0.08	9.09	6.12	7.21	8.16	0.06	1.03	0					
KM	0.14	0.13	0.14	0.15	0.12	0.14	0.13	0.14	0				
KMXPC	0.14	0.13	0.14	0.15	0.12	0.14	0.13	0.14	0	0			
UK	0.22	0.23	0.20	0.22	0.20	0.20	0.22	0.21	0.19	0.19	0		
UKXPC	0.22	0.23	0.20	0.22	0.20	0.20	0.22	0.21	0.19	0.19	0	0	
PC	0.53	0.52	0.54	0.56	0.52	0.51	0.52	0.51	0.47	0.47	0.47	0.47	0

P5- Panniyur 5; P3- Panniyur 3; P2- Panniyur 2; P1- Panniyur 1; KM- Karimunda; UK- Uthirankotta; PC- *Piper colubrinum*
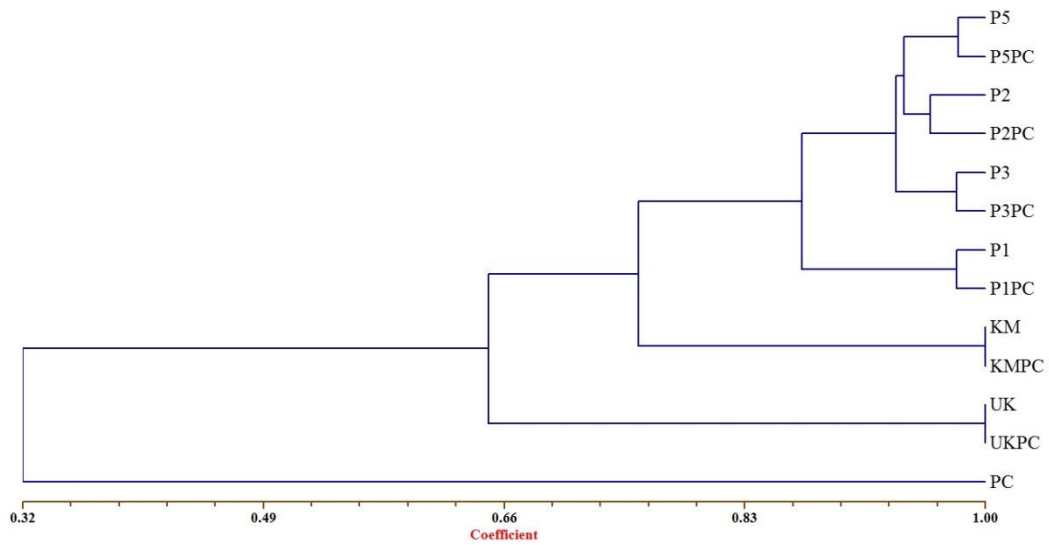
a. Dendrogram (UPGMA) pattern of RAPD analysis using NTSYS
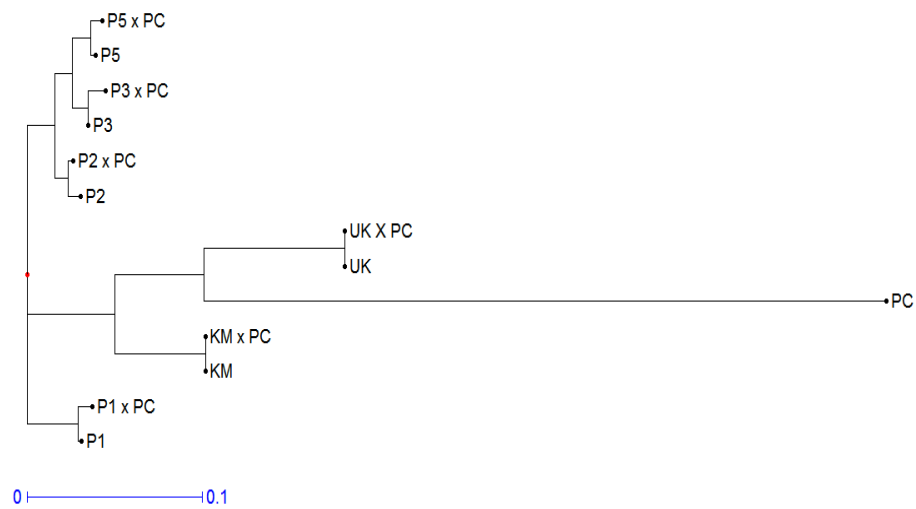
b. Dendrogram (UPGMA) pattern of RAPD analysis using DARwin

Fig. 7: Denrogram derived results of RAPD using NTSYS and DARwin for parents and hybrids of interspecific crosses in black pepper

(P5-Panniyur 5; P3-Panniyur 3; P2-Panniyur 2; P1-Panniyur 1; KM-Karimunda; UK-Uthirankotta; PC-*Piper colubrinum*)

Data analysis was done using two different softwares: NTSYS pc version 2.02i and DARwin (Version 5.0.158).

a) NTSYS pc version 2.02i

A genetic similarity matrix as well as UPGMA dendrogram was constructed using the Jaccard's coefficients (Table 22 and Fig. 8a). The pairwise coefficient values varied between 0.1250 and 1.0000. It was observed that all the six putative interspecific hybrids were exactly similar to their respective female parents. The value of relatedness of hybrids with their parents ranged between 0.1250-1.0000. The highest value (1.0000) for the similarity coefficients was obtained for the all six putative interspecific with their respective female parents. While, least similarity coefficient values were observed in all the interspecific hybrids with their male parent *P. colubrinum*, and ranged between 0.1250-0.1290.

b) DARwin (Version 5.0.158)

A genetic dissimilarity matrix as well as UPGMA dendrogram was constructed using the Dice coefficients (Table 23 and Fig. 8b). The pairwise coefficient values varied between 0.00 and 0.76. It was observed that all the six putative interspecific hybrids were exactly similar to their respective female parent and highly dissimilar to their male parent *P. colubrinum* with value of dissimilarity ranged between 0.75-0.76.

4.7.3 Combined analysis of RAPD and SSR data

The RAPD and SSR data were combined, the NTSYS pc version 2.02i and DARwin (Version 5.0.158) were used for UPGMA analysis.

a) NTSYS pc version 2.02i

A genetic similarity matrix as well as UPGMA dendrogram was constructed using the Jaccard's coefficients (Table 24 and Fig. 9a). The pairwise coefficient values varied between 0.2820 and 1.0000. It was observed that all the six putative interspecific hybrids were closely related to their respective female parents. The

Table 22: Genetic similarity matrix of parents and hybrids of interspecific crosses in black pepper analysed by Jaccard's coefficient method of the NTSYS program using SSR data

	P5	P5PC	P3	P3PC	P2	P2PC	P1	P1PC	KM	KMPC	UK	UKPC	PC
P5	1.0000000												
P5PC	1.0000000	1.0000000											
P3	0.9375000	0.9375000	1.0000000										
P3PC	0.9375000	0.9375000	1.0000000	1.0000000									
P2	0.9375000	0.9375000	1.0000000	1.0000000	1.0000000								
P2PC	0.9375000	0.9375000	1.0000000	1.0000000	1.0000000	1.0000000							
P1	0.9375000	0.9375000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000						
P1PC	0.9375000	0.9375000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000					
KM	0.7647059	0.7647059	0.8235294	0.8235294	0.8235294	0.8235294	0.8235294	0.8235294	1.0000000				
KMPC	0.7647059	0.7647059	0.8235294	0.8235294	0.8235294	0.8235294	0.8235294	0.8235294	1.0000000	1.0000000			
UK	0.9375000	0.9375000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000	0.8235294	0.8235294	1.0000000		
UKPC	0.9375000	0.9375000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000	0.8235294	0.8235294	1.0000000	1.0000000	
PC	0.1290323	0.1290323	0.1250000	0.1250000	0.1250000	0.1250000	0.1250000	0.1250000	0.1290323	0.1290323	0.1250000	0.1250000	1.0000000

P5- Panniyur 5; P3- Panniyur 3; P2- Panniyur 2; P1- Panniyur 1; KM- Karimunda; UK- Uthirankotta; PC- *Piper colubrinum*

Table 23: Genetic dissimilarity matrix of parents and hybrids of interspecific crosses in black pepper analysed by Dice coefficient method of the DARwin program using SSR data

	P5	P5PC	P3	P3PC	P2	P2PC	P1	P1PC	KM	KMPC	UK	UKPC	PC
P5	0												
P5PC	0	0											
P3	0.04	0.04	0										
P3PC	0.04	0.04	0	0									
P2	0.04	0.04	0	0	0								
P2PC	0.04	0.04	0	0	0	0							
P1	0.04	0.04	0	0	0	0	0						
P1PC	0.04	0.04	0	0	0	0	0	0					
KM	0.16	0.16	0.12	0.12	0.12	0.12	0.12	0.12	0				
KMPC	0.16	0.16	0.12	0.12	0.12	0.12	0.12	0.12	0	0			
UK	0.04	0.04	0	0	0	0	0	0	0.12	0.12	0		
UKPC	0.04	0.04	0	0	0	0	0	0	0.12	0.12	0	0	
PC	0.75	0.75	0.76	0.76	0.76	0.76	0.76	0.76	0.75	0.75	0.76	0.76	0

P5- Panniyur 5; P3- Panniyur 3; P2- Panniyur 2; P1- Panniyur 1; KM- Karimunda; UK- Uthirankotta; PC- *Piper colubrinum*
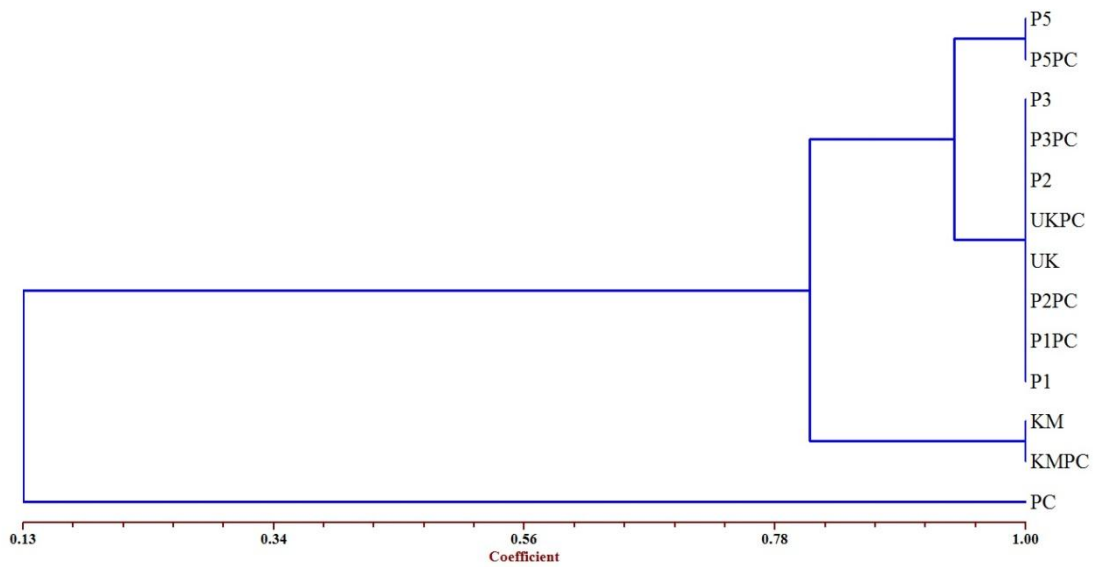
a. Dendrogram (UPGMA) pattern of SSR analysis using NTSYS
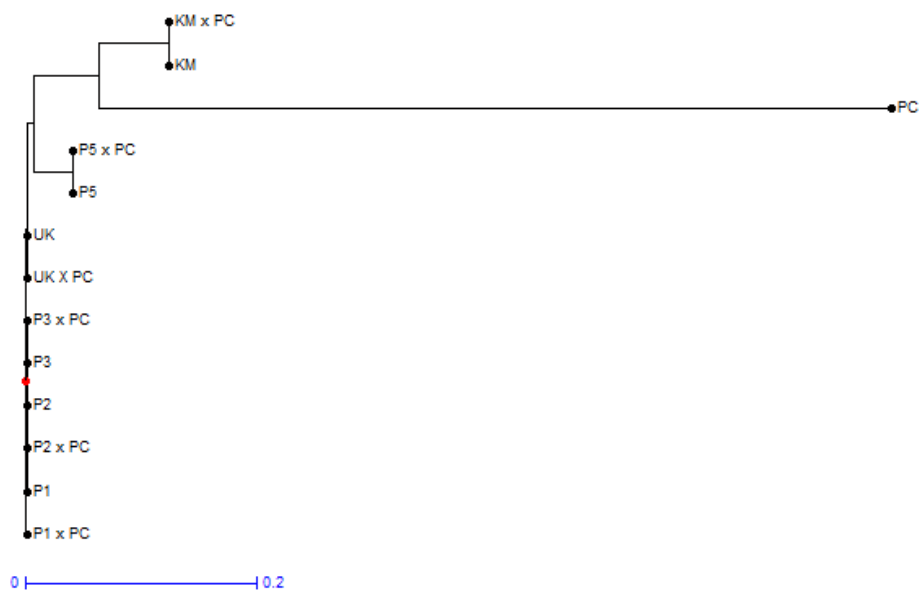
b. Dendrogram (UPGMA) pattern of SSR analysis using DARwin

Fig. 8: Denrogram derived results of SSR using NTSYS and DARwin for parents and hybrids of interspecific crosses in black pepper

(P5-Panniyur 5; P3-Panniyur 3; P2-Panniyur 2; P1-Panniyur 1; KM-Karimunda; UK-Uthirankotta; PC-*Piper colubrinum*)

value of relatedness of hybrids with their parents ranged between 0.2820-1.0000. The highest value (1.0000) for the similarity coefficients was obtained for the hybrid KMPC and UKPC with their female parents Karimunda and Uthirankotta respectively. The value of genetic similarity coefficient i.e. 0.9807, 0.9800, 0.9615 and 0.9795 observed for the hybrids P5PC, P3PC, P2PC and P1PC with their female parents respectively. While, least similarity coefficient values were observed in all the interspecific hybrids with their male parent *P. colubrinum*, and ranged between 0.2820-0.3600.

Based on the proximity matrix obtained from Jaccard's coefficients, Sequential Agglomerative Hierarchical Non-overlapping (SAHN) clustering was done using Unweighted Pair Group Method with Arithmetic averages (UPGMA) method. The dendrogram grouped putative interspecific hybrid and parents in to two main clusters, major Cluster I and a minor Cluster II at 32 per cent similarity. Cluster I consisted of four groups- Group 1 with P5PC, P2PC, P3PC and their respective female parents, diverged at 90 per cent similarity with group 2, that were more closely related. Group 2 with P1PC and their respective female parent Panniyur 1, diverged at 76 per cent similarity with group 3. Group 3 with KMPC and their respective female parent Karimunda diverged at 68 percent similarity with group 4. Group 4 with UKPC and their respective female parent Uthirankotta diverged at 36 per cent similarity with Cluster II. Cluster II consist of male parent *P. colubrinum*, as the most divergent genotype.

b) DARwin (Version 5.0.158)

A genetic dissimilarity matrix as well as UPGMA dendrogram was constructed using the Dice coefficients (Table 25 and Fig. 9b). The pairwise coefficient values varied between 0.01 and 0.60. It was observed that all the six putative interspecific hybrids were highly dissimilar to their male parent *P. colubrinum*. The value of dissimilarity of hybrids with their parents ranged between 0.01-0.60. The highest value (0.6) for the dissimilarity coefficients was obtained for the hybrid P3PC with their male parent *P. colubrinum*. The value of

Table 24: Genetic similarity matrix of parents and hybrids of interspecific crosses in black pepper analysed by Jaccard's coefficient method of the NTSYS program using combined data of RAPD and SSR

	P5	P5PC	P3	P3PC	P2	P2PC	P1	P1PC	KM	KMPC	UK	UKPC	PC
P5	1.0000000												
P5PC	0.9807692	1.0000000											
P3	0.9615385	0.9423077	1.0000000										
P3PC	0.9423077	0.9230769	0.9800000	1.0000000									
P2	0.9245283	0.9423077	0.9230769	0.9038462	1.0000000								
P2PC	0.9622642	0.9433962	0.9615385	0.9423077	0.9615385	1.0000000							
P1	0.8703704	0.8518519	0.9038462	0.8846154	0.8679245	0.9056604	1.0000000						
P1PC	0.8518519	0.8333333	0.8846154	0.8653846	0.8490566	0.8867925	0.9795918	1.0000000					
KM	0.7543860	0.7678571	0.7500000	0.7321429	0.7818182	0.7543860	0.7636364	0.7454545	1.0000000				
KMPC	0.7543860	0.7678571	0.7500000	0.7321429	0.7818182	0.7543860	0.7636364	0.7454545	1.0000000	1.0000000			
UK	0.6349206	0.6190476	0.6557377	0.6393443	0.6557377	0.6612903	0.6393443	0.6500000	0.6779661	0.6779661	1.0000000		
UKPC	0.6349206	0.6190476	0.6557377	0.6393443	0.6557377	0.6612903	0.6393443	0.6500000	0.6779661	0.6779661	1.0000000	1.0000000	
PC	0.3037975	0.3076923	0.2948718	0.2820513	0.3116883	0.3205128	0.3157895	0.3200000	0.3561644	0.3561644	0.3600000	0.3600000	1.0000000

P5- Panniyur 5; P3- Panniyur 3; P2- Panniyur 2; P1- Panniyur 1; KM- Karimunda; UK- Uthirankotta; PC- *Piper colubrinum*

Table 25: Genetic dissimilarity matrix of parents and hybrids of interspecific crosses in black pepper analysed by Dice coefficient method of the DARwin program using combined data of RAPD and SSR

	P5	P5PC	P3	P3PC	P2	P2PC	P1	P1PC	KM	KMPC	UK	UKPC	P C
P5	0												
P5PC	0.012												
P3	2.36223	0.1746											
P3PC	3.1746	0.04	0.008										
P2	3.9370	3.1746	3.1746	0.04									
P2PC	2.3255	0.0312	0.0156	2.3622	0.0156								
P1	6.3492	0.072	0.04	4.8387	0.056	3.9370							
P1PC	0.072	8.0645	4.8387	5.6910	6.4516	4.7619	0.0810						
KM	0.1451	0.1382	0.1382	0.1475	0.1219	0.136	0.1311	0.1404					
KMPC	0.1451	0.1382	0.1382	0.1475	0.1219	0.136	0.1311	0.1404	0				
UK	0.1875	0.1968	0.1653	0.1746	0.1653	0.1627	0.1746	0.168	0.1774	0.1774			
UKPC	0.1875	0.1968	0.1653	0.1746	0.1653	0.1627	0.1746	0.168	0.1774	0.1774	0		
PC	0.5748	0.5714	0.5873	0.6	0.5714	0.5625	0.568	0.5645	0.5284	0.5284	0.5275	0.5275	0

P5- Panniyur 5; P3- Panniyur 3; P2- Panniyur 2; P1- Panniyur 1; KM- Karimunda; UK- Uthirankotta; PC- *Piper colubrinum*
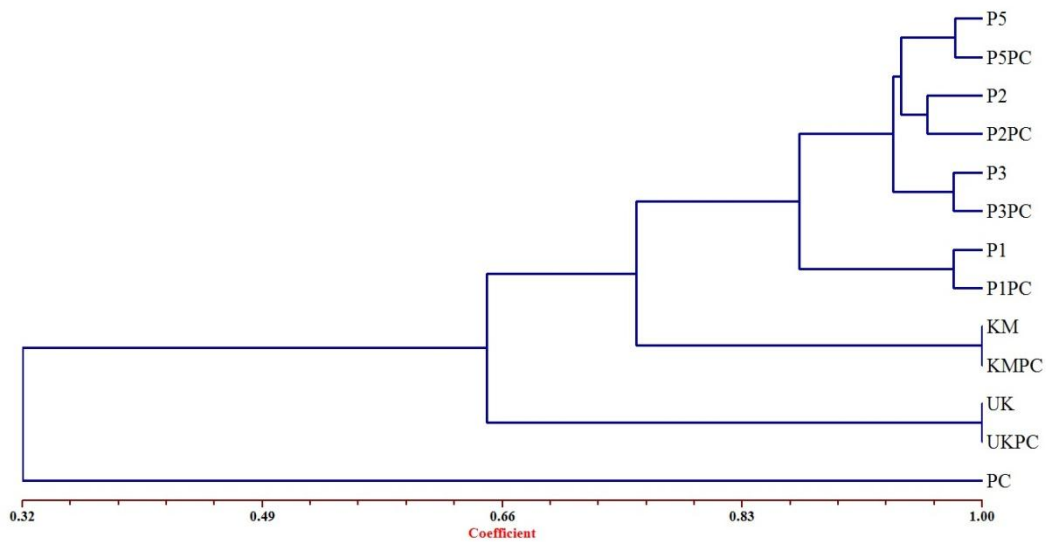
a. Dendrogram (UPGMA) pattern of combined RAPD and SSR analysis using NTSYS
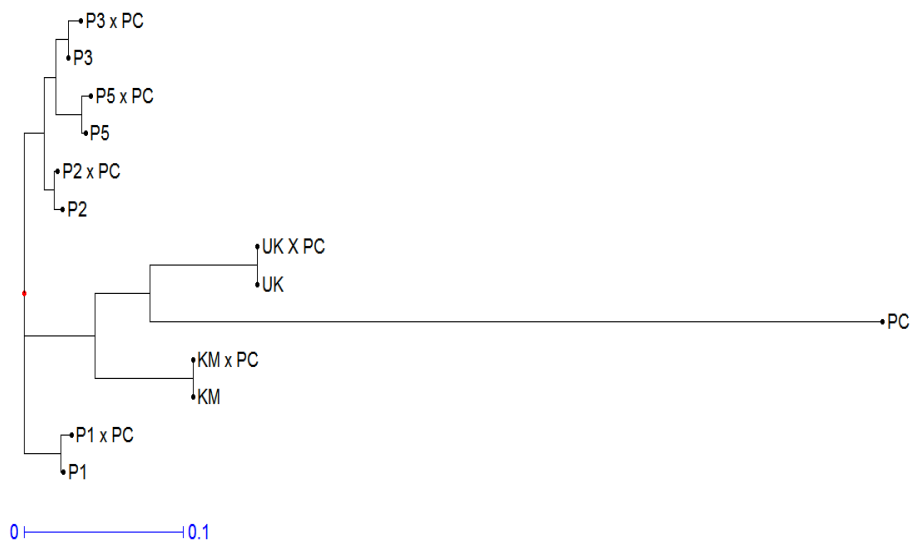
b. Dendrogram (UPGMA) pattern of combined RAPD and SSR analysis using DARwin

Fig. 9: Combined denrogram derived results of RAPD+SSR using NTSYS and DARwin for parents and hybrids of interspecific crosses in black pepper

(P5-Panniyur 5; P3-Panniyur 3; P2-Panniyur 2; P1-Panniyur 1; KM-Karimunda; UK-Uthirankotta; PC- *Piper colubrinum*)

genetic dissimilarity coefficient i.e. 0.5714, 0.5625, 0.5645, 0.5284 and 0.5275 observed for the hybrids P5PC, P2PC, P1PC, KMPC and UKPC with their male parent (*P. colubrinum*) respectively. While, least dissimilarity coefficient values were observed in all the interspecific hybrids with their respective female parents, and ranged between 0.00-0.0810.

Discussion

5. DISCUSSION

The genus *Piper* includes the most valuable economically important spice crop, black pepper (*Piper nigrum* L.) the 'King of Spices'. Apart from Asian countries such as Malaysia, India, Indonesia, Thailand, Vietnam, China and SriLanka, it is also cultivated in Brazil and Madagascar. The Western Ghats of Indian peninsula is the primary centre of origin of the black pepper (Ravindran *et al.*, 1997). By habit, it is a perennial woody climber. Kerala, the southernmost state of India occupies a considerable portion of the Western Ghats and is a rich source of wild relatives of this spice crop. The hot and humid climate of the sub-mountainous tracts of Western Ghats is ideal for its cultivation and hence Kerala has the highest area and production of black pepper in India.

Although, India is one of the leading producers of black pepper in the world, the productivity of the crop is low due to various factors among which infestation by *Phytophthora capsici* has been identified as a major factor. The pest damage accounts for up to 40-50 per cent losses in yield in endemic areas (Sarma *et al.*, 1991). At present, chemical control measures using insecticides are practised to manage the pest. However, the increasing demand by importing countries for "clean spices" free from pesticide residues, have necessitated the need for breeding resistant varieties.

Crosses between species of same or different genera are commonly known as wide crosses. Such crosses have contributed to plant improvement (Dewey, 1977; Whelan and Conner, 1989; Jauhar, 1993; Jiang *et al.*, 1994). In recent years, crosses between crop plants and their wild relatives have attracted significant attention as sources of desirable characteristics for genetic improvement of crops. However, such wide crosses are not always successful and a detailed study on molecular biology, cytology, embryology, pollen germination, *etc.* is essential to determine the real cause of crossability failure so that the existing barriers could be overcome (Prabhakaran *et al.*, 1995).

The utilization of wild relatives in hybridization programme in black pepper is limited because of cross incompatibility and hybrid sterility caused by the difference in chromosomal status between black pepper species. The numbers of attempts made are many, the results obtained are inconsistent and the success obtained is very low (Sasikumar *et al*., 1999).

In this direction, Vanaja *et al*. (2008) crossed the wild species *P. colubrinum* which is resistant to "*Phytophthora* foot rot" with cultivated *P. nigrum* species and have reported the characters of one of the hybrids *viz.*, P5PC. Seven crosses were made using different cultivars / high yielding varieties of *P. nigrum* as female parents and *P. colubrinum* as male parent and the hybrids are maintained at Pepper Research Station (PRS), Panniyur. However, morphologically all the hybrids resemble the female parents except for the setting of the berries. Hence the present study was undertaken with six of the above mentioned interspecific hybrids maintained at PRS, Panniyur *viz.*, P5PC (Panniyur 5 x *P. colubrinum*), P3PC (Panniyur 3 x *P. colubrinum*), P2PC (Panniyur 2 x *P. colubrinum*), P1PC (Panniyur 1 x *P. colubrinum*), KMPC (Karimunda x *P. colubrinum*), UKPC (Uthirankotta x *P. colubrinum*) and respective parents with a view to characterise the putative hybrids and to identify true inter-specific hybrids by comparing with the parents using molecular markers.

Many of the reports suggested that identification of true hybrids based on morphology alone is not sufficient in perennial crops like black pepper. The reasons are, morphological markers are influenced by environmental factors and frequently lacks the resolving power to identify hybrids at the juvenile stage (George *et al*., 2005). Therefore, in the present investigation, the combinations of morphological marker, together with molecular markers RAPD and SSR were used for precise and efficient testing of hybridity.

5.1 Morphology of interspecific hybrids and their parents

Several vegetative, reproductive and qualitative characters were identified as varietal characters. Among the vegetative characters, plant growth habit, leaf

area, production of orthotrope and plagiotrope shoots as well as number and direction of growth of lateral branches were found suitable to distinguish the hybrids and parental cultivars.

5.1.1 Vegetative traits (Quantitative)

The mean length and width of lamina and consequently leaf area were found to differ significantly among six parental genotypes indicating that these leaf characters have varietal significance as reported earlier (Kanakamany, 1982; Ibrahim *et al*., 1985a; Sujatha and Namboothiri, 1995). The present investigation also provided an additional proof for this fact. All the interspecific hybrids resembled the respective female parents for the various leaf characters studied. As leaf matures, leaf area increased in *P. nigrum* genotypes and the hybrids while L/W ratio decreased indicating a greater increase in the width of leaf. But in the male parent *P. colubrinum*, leaf area as well as L/W ratio showed increase indicating a greater increase for leaf length upon maturity.

Angle of insertion of laterals on the main shoot is an important varietal character. Chandy and Pillai (1979) and Pillai *et al*. (1985) also pointed out that in black pepper, the angle of insertion of laterals on main shoot vary from variety to variety, with some varieties having drooping branches and others somewhat horizontal or semi erect or almost erect branches. The present study also supported the fact. This morphological character was reported to have a direct bearing on photosynthetic efficiency of the plant (Sujatha and Namboothiri, 1995) since the intensity of sunlight falling on the leaves varies accordingly. Erect branches were observed in the male parent *P. colubrinum*. Semi erect branches were observed in the hybrid KMPC their female parent Karimunda. In other hybrids and respective female parents, the angle was greater, giving a horizontal arrangement of laterals. In all the cases none of the hybrids resembled the male parent with respect to this character also. For the stem characters recorded, *viz*. internodal length and thickness as well as nodal thickness in both ortho and plagiotropes, the hybrids resembled the female parents. All the orthotrope

characters recorded higher values for *P. colubrinum* than the female parents and hybrids.

5.1.2 Reproductive traits (Quantitative)

Reproductive traits related to spike and berries in the interspecific hybrids were found to differ from both parents. In all the six crosses except Karimunda x *P. colubrinum,* hybrids showed lower values than the respective female parents for the various spike characters such as number of spikes per branch, spike length, spike peduncle length, fresh and dry spike weight. In KMPC, the average number of spikes per lateral branch was slightly higher than the female parent Karimunda.

Eight berry characters viz., number of berries per spike, weight and volume of thousand fresh and dried berries, number and percentage of well developed and under developed berries per spike and fruit setting percentage were compared between the putative hybrids and the respective female parents. The percentage of developed and under developed berries and correspondingly the fruit setting per cent in spikes were significantly low in all the six hybrids compared to the female parents. These characters could be recorded only for the female parents and the hybrids as there was no berry setting in *P. colubrinum*. In other crops also, low fruit setting percentage in interspecific crosses have been reported. In sesame, Kulkarni (2006) reported that capsule setting was observed in all the attempted crosses but setting percentage was very low and there were differences in viability and germination percentage of crossed seeds. Similarly, Ramanathan (1950), Shi (1993), Qu *et al*. (1994) and Ramesh *et al*. (2003) obtained shriveled seeds from the crosses involving wild species *S. occidentale* and *S. radiatum* (female parent) and *S. indicum* (male parent).

Reproductive trait related to spike and berries were found to vary between the *P. nigrum* genotypes used as female parents indicating the importance of these characters for varietal identification. The typical long spike with bold, round berries of Panniyur 1 was reported earlier by several workers (Pillai *et al*., 1987; Babu and Ravindran, 1992 and Ibrahim *et al.*, 1986a). The varietal differences

with respect to these traits were also reported in Panniyur 2, Panniyur 4, Karimunda and Subhakara (Sujatha, 2001; Pillai *et al*., 1989; Babu and Ravindran, 1992). Karimunda recorded the shortest spikes (Prasannakumari *et al*., 2001). The present study also supported these observations.

5.1.3 Qualitative characters

Based upon the descriptor of black pepper and *Piper* species (IPGRI-Biodiversity International, 1995) each of the variety was characterised for the qualitative traits also. It was found that many characters were more or less identical even between varieties but there were some which exhibited variation.

Characters such as anthocyanin pigmentation on various parts (leaf sheath, tender leaves, tender stem, petiole and stipules), leaf shape, leaf base, stoutness of stem, clinging ability of adventitious roots, berry shape, berry colour on maturity and time taken for maturity *etc*., were found to vary between varieties. In the present study, the hybrids resembled the female parent *P. nigrum* for the characters such as plant growth habit, leaf shape, arrangement, venation, texture, spike orientation, and spike shape. In general appearance, the hybrids are more inclined to the cultivated species *P. nigrum.* The wild species *P. colubrinum* showed elliptic, glabrous membranous, slightly wavy and eucamptodromous leaves, while evenly arranged glabrous coriaceous and campylodromous leaves were observed in all the hybrids and their female parents.

The leaf shape was identified as a varietal character. Large cordate leaves were characteristic of the variety Panniyur 1 and its hybrid P1PC also showed the same character whereas in typical Karimunda, small, elliptic leaves helped in identification of the variety (Kanakamany, 1982; Sujatha, 1991) and its hybrid KMPC also showed similar type of leaves.

Anthocyanin pigmentation of stipule was reported in many varieties of black pepper and was found to have a dominant recessive intra allelic interaction wherein; the allele favouring pigmentation was dominant (Ibrahim *et al*., 1986b). Hybrids P1PC, P5PC, P3PC as well as female parents Panniyur 1 and Panniyur 3

and the male parent *P. colubrinum* were found devoid of such pigmentation while hybrids P2PC, KMPC, UKPC as well as female parents Panniyur 5, Panniyur 2, Karimunda and Uthirankotta were pigmented.

The plant growth habits differed significantly between *P. colubrinum* compared to the hybrids and their female parents. The climbing (on support) growth habit observed in all the six hybrids and their respective female parents, while trailing (on the ground) growth habit was observed in the male parent *Piper colubrinum*.

The stilt roots were observed in male parent *P. colubrinum*, while absent in all the six hybrids and their respective female parents. Also the climbing roots were absent in the male parent *P. colubrinum*, while many climbing roots were observed in the hybrids and their respective parents.

The spike colour, orientation and shape differ significantly in male parent *P. colubrinum* compared to hybrids and their female parents. Yellow, erect and cylindrical shaped spikes were observed in *P. colubrinum*, while green, prostrate and filiform spikes were observed in all the six hybrids and their respective female parents. The vigorous nature of vine with strong stout stem, bold and spherical berries were characteristic of Panniyur 1, Panniyur 3 and Panniyur 5 whereas Panniyur 2 was with ovate leaves, moderately sized berries. These observations are in agreement with earlier reports (Pillai *et al*., 1985; Ibrahim *et al*., 1985b, Sujatha, 1991, Babu and Ravindran, 1992. Also moderately sized berries and small elliptic leaf shapes were characteristic of Karimunda (Kanakamany, 1982; Sujatha, 1991). The present study also supported the fact.

5.2 Molecular characterization

The cultivated black pepper appears to be an ideal candidate for characterisation using molecular markers; variability available in the black pepper is very high. The assessment of intra and inter-varietal variation using the present system of characterisation based on morphological descriptor alone is not sufficient. The reasons are, it is time consuming as both vegetative and

reproductive characters have to be recorded, is influenced by environment and also, the existing morphological keys are not sufficient to identify true hybrids. Molecular markers in conjugation with morphological markers will be the ideal method to characterize any line or variety.

5.2.1 DNA isolation

Pale green tender leaves (1st or 2nd leaf from tip) were used for DNA extraction from hybrids and their corresponding parents of six interspecific crosses in black pepper. The protocol suggested by Rogers and Bendich (1994) modified by using 4x CTAB extraction buffer yielded good quality DNA. The electrophoresed DNA showed distinct bands without shearing. The major problem encountered in the isolation and purification of high molecular weight DNA from plants species is the degradation of DNA due to endonuclease, polyphenols and other secondary metabolites that directly and indirectly interfere with subsequent enzymatic reactions as reported by Weishing *et al*. (1995) and Matasyoh *et al*. (2008). On grinding, the leaves of black pepper turned brown immediately due to phenolic oxidation. The quinones produced were known to be powerful oxidising agents, which damage DNA and proteins (Weising *et al*., 1995). The addition of antioxidant like β-mercaptoethanol and sodium metabisulfite in the extraction buffer or during grinding was found effective. Similar results were seen also in coffee, which is a crop with high amount of phenols (Ram and Sreenath, 1999).

5.2.1.1 Purification and Quantification of DNA

The quality of the DNA was tested by subjecting it to agarose gel electrophoresis as well as spectophotometric method. DNA was visualized on 0.8 per cent agarose gel under UV light by ethidium bromide staining. A DNA sample was reported as high quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasingf and Peffley, 1998). The DNA extracted showed high amount of RNA as a smear below it. To remove RNA, RNase A was used. Use of RNase A was reported by several workers (Raval *et al*., 1998; Wettasingf and Peffley, 1998; Gallego and Martinez, 1996). In the

present investigation, the RNase treated DNA sample on electrophoresis showed a high molecular weight DNA, which formed a single band just below the well. This indicted that the DNA under test was of good quality.

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

5.3 Molecular marker analysis

In most of the crop improvement programmes through introgression breeding, wide crosses especially using wild species are taken up. Similarly in black pepper, *Phytophthora* foot rot is serious disease causing heavy yield loss in every year. With the objective of transferring *Phytophthora* resistance, from *Piper colubrinum* to *Piper nigrum*, these two were crossed (Vanaja *et al*., 2008). Ambiguous morphological characters necessitated to go for molecular markers to establish the hybridity.

Molecular marker technology provides novel tools for DNA fingerprinting as well as assessing variation within parental lines and testing the genetic constitution of hybrids. Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based marker. Most of the molecular marker are developed by PCR (Polymerase Chain Reaction) technology and amplifies unique regions on the genomic DNA based on the primers designed for DNA amplification. In the present study, two such PCR based marker systems, RAPD and SSR were utilized for molecular characterization and hybridity testing of interspecific crosses in black pepper.

5.3.1 RAPD analysis

The RAPD technique was developed by Williams *et al.* (1990) and the technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary decamer primers. In RAPD markers, polymorphism results from the changes in the sequence of the primer binding site. Usually, RAPD markers are dominant in nature (Waugh and Powell, 1992) because polymorphisms are detected as the presence or absence of bands. RAPD patterns are very sensitive to slight changes in amplification conditions giving problems of reproducibility and necessity for extensive standardization to obtain reproducible results (Penner *et al.*, 1993). Technical problems associated with application of RAPD technique in the field of genetic variation research have been reported by many workers (Lynch and Milligan, 1994; Rajput *et al.*, 2006). Use of high quality DNA is shown to be a key factor in obtaining reproducible RAPDs bands (Penner *et al.*, 1993). In the present study, the use of high quality DNA helped in getting reproducible bands using the standardised conditions for the thermal cycler.

However the advantages of RAPD include simplicity, rapidity, requirement for only a small quantity of DNA, and ability to generate numerous polymorphism (Cheng *et al.*, 2007). Therefore, it has been used for molecular characterisation in several crop plants such as *Oryza sativa* L. (Hashemi *et al.*, 2009), *Ficus carica* L. (Khadari *et al.*, 1995), *Capsicum annuum* (Hulya, 2003) and *Mangifera indica* L. (Adato *et al.*, 1995). Similar kinds of work have been reported by various workers in *Piper* using RAPD markers (Pradeepkumar *et al.*, 2003; Nazeem *et al.*, 2005; Keshavachandran *et al.*, 2005; Budiguppe *et al.*, 2007; Sen *et al.*, 2010). Recently, RAPD markers have been widely used by various workers for identification of hybrids and testing genetic purity of hybrid in various crops. They have been used in Black pepper (George *et al.*, 2005) Sugarcane (Zhang *et al.*, 2008), Cotton (Mehetre *et al.*, 2004; Seyal *et al.*, 2008; Dongare *et al.*, 2010), Papaya (Magdalita *et al.*, 1997), Sorghum (Akhare *et al.*, 2008), Barly (Hoffman *et al.*, 2003), Maize (Asif *et al.*, 2006), Cabbage (Liu *et al.*, 2007), Chilli (Mongkolporn and

Dokmaihom, 2004), Chrysanthemum (Chung *et al*., 2000) and Rose (Kaul *et al*., 2009).

5.3.1.1 DNA amplification conditions for RAPD

The amplification conditions standardized in the present study were suited to Mastercycler personal thermalcycler from Eppendorf (USA). Also, the annealing temperature was identified as the most critical with respect to number of amplified fragments and reproducibility of result. Cipriani *et al.* (1996) and Erlich *et al*. (1991) suggested 37ºC as the best, and hence only time was varied for this step.

The amplification pattern produced by the different combinations of the ingredients of the reaction mixture indicated that the most important factor affecting the specificity and yield of amplification were concetration of $MgCl_2$ in the buffer as well as concentration and type of DNA polymerase enzyme. In the present investigation, the enzyme used was *Taq* DNA polymerase supplied by Genei, Bangalore. The molarities of primers as well as dNTPs were also found to affect the intensity and number of amplifications. All reactions were performed in 20 µl final volume.

5.3.1.2. Primer Screening for RAPD

From the 30 random deacamer primers, 10 primers selected on the basis of screening test considering the number, intensity and consistency of bands, were found useful for screening the black pepper vines. These ten primers produced polymorphic bands between *P. nigrum* and *P. colubrinum* and showed some male parent specific bands which could be used to check the hybrids in different crosses. The RAPD markers generated using the selected primers were visualised by electrophoresis, in a 1.3 per cent agarose gel stained with ethidium bromide.

5.3.1.3. Characterisation using RAPD

The amplification pattern with selected 10 primers showed that all the six putative interspecific hybrids resembled their respective female parents which are

P. nigrum genotypes. Each hybrid and the respective parents were compared based on the total bands produced by the 10 primers. In all the six cases, the total number of bands produced ranged from 73-79 out of which 24-27 were monomorphic between the parents and respective hybrids and were not useful in detecting the hybridity. The remaining bands which are polymorphic between the two parents in each cross were compared with the banding pattern in hybrids. The results reveal that in hybrids KMPC and UKPC banding pattern is exactly similar to the respective female parent Karimunda (22 amplicons) and Uthirankotta (24 amplicons). The bands specific to *P. colubrinum* but absent in Karimunda and Uthirankotta were absent in the respective hybrids also. For the other four hybrids also all the amplicons in the female parents except one or two were present in the respective hybrids. But the bands present in *P. colubrinum* but absent in the respective female parents were absent in the hybrids P1PC, P2PC, P3PC and P5PC also. These results indicate that with respect to the ten random primers selected in the present study, the putative hybrids are genetically similar to the female parents. However, in hybrids P1PC, P3PC and P5PC one band each was absent compared to female parents. But absence of band in hybrids and male parent cannot be considered as a similarity between the two. In P2PC one additional band was detected which was absent in Panniyur 2. But it was found to be a *P. nigrum* specific band as it was present in all other *P. nigrum* genotypes and hybrids but absent in *P. colubrinum*.

The number of fragments generated per primer varied from 6 to 12 using 10 Operon primers and size of the products ranged from 250bp to 1400bp. Similar number of fragments were reported in same genus (Pradeepkumar *et al*., 2003; Wadt *et al*., 2004; Sen *et al*., 2010) and in other plants such as *Capsicum* (Rodriguez *et al*., 1999) and Indian tomato (Archak *et al*., 2002).

Per cent polymorphism between *P. nigrum* and *P. colubrinum* varied from 50-100 using the 10 decamer and 11 SSR primers. In the present study, it was observed that the percentage of polymorphism at inter-species was relatively high (68.87%) when comparing the result with other studies using the same method

(Rajput *et al*., 2006). Callejas and Ochando (2002) and Wadt *et al*. (2004) reported that higher percentage polymorphism scored with RAPD markers between two species is probably due to preferential amplification of non-coding repetitive regions of the genome that may elude natural selection. Moreover, variation in chromosome number exists among the two species (Ravindran, 2000b). There are chances of ambiguity in the homology of RAPD bands especially in the context of varying chromosome number among *Piper* species. Ambiguity can be due to co-migration of nonhomologous DNA fragments (Adams and Rieseberg, 1998), which can be resolved by using more robust molecular markers like SSR.

Among ten decamer primers used, only six primers (OPA 08, OPA10, OPA 30, OPAH 09, OPC 09 and OPA 28) produced seven unique cultivar specific bands. Maximum numbers (two) of unique bands were produced by primer OPAH 09. The primer OPAH 09 produced a unique band of size 1400bp in female parent Panniyur 1 and its hybrid P1PC as well as a unique band of size 300bp in Uthirankotta and its hybrid UKPC. The primers OPA 8, OPA10 and OPA 28 also produced one unique band each of size 700bp, 400bp and 600bp respectively in Uthirankotta and UKPC. The primers OPC 09 and OPA 30 produced one unique band each of size 1200bp and 800bp respectively in Karimunda and Panniyur 5 as well as their hybrids.

Species-specific RAPD markers are also reported in same genus by Pradeepkumar *et al*. (2003). The species-specific bands could be quantitative trait loci (QTL) which could exhibit some desirable characters such as resistant to some fungal diseases which can be used for marker assisted selection (MAS). Several workers detected and analyzed the QTLs based on RAPD markers (Grandclement and Thomas, 1996; Chague *et al*., 1997; Sharma *et al*., 2002) in different plants. Also there are reports that QTLs are used for MAS (Khan *et al*., 2007). Some promising RAPD markers could be sequenced and so converted to a SCAR marker (Paran and Michelmore, 1993) to study desirable traits as has been done in other crops (Parka *et al*., 2004; Janila and Sharma, 2008). Such specific

RAPD markers can be generated as genetic tags for *Piper* species in future that would be helpful in cultivation and selection programs for the species.

The RAPD profiles in the present study displayed a high degree of inter-varietal and inter-special polymorphism of *Piper* which confirms suitability of RAPD markers for discrimination of the *Piper* species. In brief, the study yielded highly reproducible RAPD fingerprints, which proved as reliable and useful tool for hybridity testing and the analysis of genetic variation in *Piper* plants. This observation and the identification of unique specific markers (private alleles) are significant steps toward realizing the goal of management, cultivation, and conservation of *Piper* resource in Peninsular India.

5.3.2 SSR analysis

Simple Sequence Repeats, also referred as microsatellites observed in eukaryotic genome are unique to individuals with respect to the number of repeats and regions flanking the repeats. They are highly informative molecular markers, among the array of molecular markers available, microsatellite or Simple Sequence Repeats (SSR) marker remains the favourite choice for verification of hybridity and genetic diversity analysis (Joy *et al*., 2011) as they are co-dominant, multiallelic, hypervariable, highly abundant and uniformly dispersed in plant genomes.

Menezes *et al.* (2009) reported nine microsatellite loci in *Piper nigrum.* Recently, Joy *et al*. (2011) also reported seven microsatellite loci and that have been used for analysis of genetic diversity of popular black pepper genotypes in South India. Massive efforts are being conducted for germplasm characterization in various commercial crops, but such a type of study is less initiated in this spice crop due to lack of sequence information. Therefore, the developed polymorphic informative microsatellite markers have added a new and powerful dimension to black pepper breeding and could be utilized to identify genotypes, true hybrids and can ensure significant advancement in research focusing towards fine mapping, linkage analysis and association studies of candidate genes. SSR marker

are also reported and exploited in various other crops for genetic diversity analysis (Kebede *et al*., 2007), evaluation of mapping population (Lee *et al*., 2004), marker assisted breeding (Yu *et al*., 1996) and hybridity testing in many economically important crops such as Cotton (Asif *et al*., 2009), Soybean (Akkaya *et al.*, 1992), Tomato (Benor *et al*., 2008), Maize (Pongsai *et al*., 2009) and interspecific hybrid of sugarcane (Cordeiro *et al*., 2000).

5.3.2.2. DNA amplification conditions for SSR

Before conducting any genome characterization study, optimization of concentration of different reagents and temperature regimes used in polymerase chain reaction (PCR) is necessary. However, annealing temperature and concentration of $MgCl_2$ are important parameters for any marker system, which need optimization. The genomic DNA of parental line was used to optimize the PCR conditions. Amount of template DNA strongly influences the outcome of the reaction. More than 30ng/20µl give the premium amplification (Henegariu *et al*., 1997), also in the present study; 30ng/20µl was found optimum. Optimization of $MgCl_2$ is an important factor for precise amplification. In these experimental studies, 2mM of $MgCl_2$ was found optimum in 20µl final reaction volume. The Mg ions binds tightly to the phosphate sugar backbone of nucleotides and nucleic acids, and variation in the $MgCl_2$ concentration has strong effects on nucleic acid interactions (Ely *et al*., 1998). Variations in $MgCl_2$ concentration below 4mM can improve performance of PCR by affecting specificity (higher concentration lowers the specificity, lower concentration raise specificity) (Blanchard *et al*., 1993). Moreover, concentration of dNTPs in reaction mixture is also strongly correlated to the Mg ions concentration due to the interaction between mononucleotides and the Mg^{2+}. A higher concentration of Mg^{2+} allows amplification with a higher concentration of dNTPs that is not seen at lower Mg^{2+} concentrations (Blanchard *et al*., 1993).

In Polymerase Chain Reaction, 1-3 units of *Taq* Polymerase is normally used in 25µl final volume. Higher *Taq* Polymerase concentration (above

4units/25μl) can generate nonspecific products and may reduce the yield of the desired product (Saiki, 1989). However, in the present study, 3unit/20μl reaction was used to amplify the loci without non-specific products. Annealing temperature is one of the most important parameters that need adjustment in the PCR. The normal range of annealing temperature is 36-75°C. Optimal annealing temperature (T_a) of primers was determined by testing in the range of ±5°C from the theoretical annealing temperature. The annealing temperature 58°C was found optimum to amplify with primer pairs PN B5, PN E3, PN F1, PN G11, PN H4 and PN D10. Similarly, 53.7°C were found optimum for primer pairs PnGT2 and PnGATA10. Also, 53°C were found optimum for primer pairs PnAG30 and PnCA9.

5.3.2.3. Characterisation using SSR

In the present investigation, nineteen primers specific to black pepper and thirty five other plant specific SSR primers used to screen the parents, eleven SSR primers were selected based on Menezes *et al.* (2009) and Joy *et al.* (2011) reports, to identify true hybrids from the six interspecific crosses. In spite of the fact that microsatellite loci are useful genetic markers, their development in species without substantial DNA sequence data required rapid and efficient protocols. Even though black pepper is a major spice consumed throughout the world, very few available public databases are known for *Piper* or its closely related species, which was a real hurdle to initiate the SSR primer isolation (Joy *et al.,* 2011).

Thirty five other plant specific SSR primers used to screening because, the ability to use the same SSR primers, available from public database, from different plant species, known as ''cross-transferability'', is an alternative for species with no available microsatellite markers. But this depends on the extent of conservation of the sequences in the primer binding sites flanking the microsatellite loci and also on the stability of these sequences during evolution (Dos Santos *et al.*, 2007).

The amplification pattern of selected primers were visualised by electrophoresis, in a high resolution 2 per cent agarose gel stained with ethidium bromide, Eight markers showed inter-specific (between *Piper nigrum* and *Piper colubrinum*) and 4 markers showed inter-varietal polymorphism. These polymorphic markers amplified a total of 33 alleles with sizes ranges from 50 to 1200bp and average of 3.0 alleles per marker. Similar number of allele per primer reported in pigeon pea (Burns *et al*., 2001; Saxena *et al*., 2010). The amplicons produced in the present study by the selected primer pairs were similar to the allele size reported in the same cultivars of *P. nigrum* by Mogalayi (2011).

In present investigation, the amplification pattern with 11 selected SSR primers showed all the six putative interspecific hybrids resembled with their female parent. In black pepper, George *et al*. (2005) reported a banding pattern of hybrids exactly similar to the female parent in some populations derived from certain crosses in the study of identification of hybrids using male parent specific RAPD and suggested that, the majority of black pepper cultivars are bisexual type but some are predominantly female. Controlled pollination studies indicated that during the course of hybrid development in black pepper, chances like the formation of progenies without the paternal relationship like apomixis could occur and the developed seeds will give false results. In present study also, a banding pattern exactly similar to the female parent was observed in the populations derived from interspecific crosses, which also may point to the apomictic property of selected lines of black pepper. Therefore, the present study may also be extended to confirm the mode of reproduction by apomixis versus self-pollination, or cross-fertilization.

In genus *Piper*, Sasikumar *et al*. (1999) crossed interspecific cultivar, twenty cultivars of *P. nigrum* as female with *P. barberi* and *P. attenuatum*. In these interspecific crosses, success was obtained only with cv. Aimpiriyan and cv. Karimunda, probably due to the genetic combination of the cultivars/species. Cytologically the species had a same somatic chromosome number of 2n=52. In earlier attempt also, interspecific crosses in *Piper* species with view to transfer

resistance to *Pollu* beetle or *Phytophthora* foot rot resistance were not successful due to incompatibility problem (IISR, 1992). Similar type of problem was also observed in other crop species (Prabhakaran *et al*., 1995).

In sesame, inter-specific crosses for transfer of disease resistance gene from wild species to cultivated species reported by several workers, wild species of sesame S. *malabaricum*, resistance to diseases like *Phyllody* and insect pest *Antigastra* crossed with cultivated species *S. indicum*. Cytologically both the species had a same somatic chromosome number of 2n=26, their F1 hybrids have normal meiosis and are fertile (Kobayashi, 1999; Nayar, 1995). In crosses *Saccharum malabaricum* as female and *Saccharum indicum* as male, the F1 hybrids were male sterile (Thangavelu, 1994 and Prabhakaran *et al*., 1995). This would indicate some nuclear cytoplasmic interactions and differences between two species. Ochatt *et al.* (2004) suggested that, many intergeneric and interspecific crosses have not been successful because of existence of incompatibility between genomes of the two species.

In the present study, high degree of inter-specific (*P. nigrum and P. colubrinum*) polymorphisms was observed. Among eleven primers, eight primers found highly polymorphic to *P. colubrinum*. Four primers (PN A5, PN E3, PN H4, and PN D10) produced unique cultivar specific allele. Primer PN A5 produced allele of size 75bp in Karimunda and hybrid KMPC, similarly, amplification with primers PN E3 and PN D10 produced allele of size 300bp in all the female parent and hybrid except Karimunda and their hybrid KMPC. Also Primer PN H4 produced allele of size 200bp in all the female parents and hybrids except Panniyur 5 and their hybrid P5PC. This result indicates that cultivar Panniyur 5 and Karimunda are genetically highly dissimilar in species *P. nigrum*.

Joy *et al*. (2011) also reported that Karimunda and wild black pepper (*P. colubrinum*) are highly diverse in 35 studied cultivars, and this diverse nature might have been contributed by the mutational events that accumulated during the course of its evolution or by any external factors. This uniqueness of Karimunda

and *P. colubrinum* was in exact correlation with the already reported distinctiveness based on morphological data (Jose and Sharma, 1984; Ravindran, 1991; Ravindran *et al*., 1997) and AFLP fingerprint analysis (Joy *et al.*, 2007). Five of eleven selected SSR loci showed a PIC value greater than 0.5 which enabled to classify these SSR markers as very informative markers. 17 out of 25 SSR markers were classified as highly informative markers as their PIC value was higher than 0.5 in a study conducted for the isolation of microsatellite markers in hop (Stajner *et al*., 2005).

5.4 Molecular charaterization of open pollinated progeny of hybrid P5PC

Fruits produced by the open pollinated partially fertile hybrid (Culture P5PC) were harvested at the fully mature stage and germinated using sand in trays. Seeds started germination after 40 to 45 days with 12 per cent germination rate. These results are consistent with the reports of Kulkarni (2006). The low vigour of some interspecific hybrid seedlings (hybrid weakness or absence of viability) were attributed by Hadley and Openshaw (1980) to incompatibility between the genomes of the two parental species. However when the germinated seedlings were subjected to molecular charaterization along with parents using RAPD and SSR primers selected based on PIC values, most of the hybrid seedling showed banding pattern almost similar to their female parent (P5PC) and grandparent Panniyur 5 with 1 or 2 polymorphic bands. Here also none of the hybrids shared bands specific to the male parent *P. colubrinum*.

5.5 Data analysis

The amplification pattern observed in RAPD and SSR analysis was scored and analysed for hybrid identification of six interspecific crosses. The computer package NTSYS pc version 2.02i (Rohlf, 2005) and DARwin (Version 5.0.158) were used for cluster analysis. In the dendrogram, all the six interspecific hybrids showed highest similarity with their respective female parents and value of similarity ranges from 96 (Panniyur 1, Panniyur 2, Panniyur 3 and Panniyur 5) to
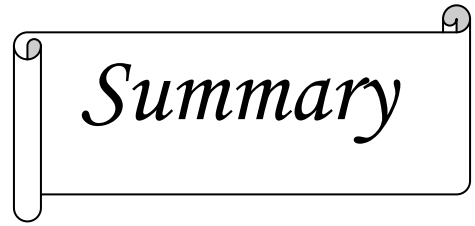
100 per cent (in Karimunda and Uthirankotta). All interspecific hybrids showed greater diversity from the male parent *P. colubrinum* (52-72%).

The markers were efficient in revealing the varietal differences of the various genotypes of *P. nigrum* and grouped each of the putative hybrid along with the respective genotype used as the female parent. Based on the genetic similarity, the diverse nature of Karimunda, Uthirankotta and *P. colubrinum* were identified and this diverse nature might have been contributed by the mutational events that accumulated during the course of its evolution or by any external factors. This uniqueness of Karimunda was in exact correlation with the already reported distinctiveness based on molecular data (Sen *et al*., 2011) and morphological data (Jose and Sharma 1984; Ravindran 1991; Ravindran *et al*., 1997) and AFLP fingerprint analysis (Joy *et al*., 2007). An overall comparison of two *Piper* sp. made us to conclude that *P. colubrinum* was more diverse as reported earlier (Sen *et al*., 2011).

However, the interspecific hybrids developed should be further evaluated for conclusive evidence of hybridity as all the hybrids resemble the respective female parents with respect to morphology and molecular markers included in the present study. At morphological level, only differences between the hybrids and respective female parents are the low berry setting nature of all the hybrids and change in shoot tip colour in the hybrid P5PC. At molecular level, hybrids KMPC and UKPC are 100 per cent similar to their female parents Karimunda and Uthirankotta respectively. The hybrids from Panniyur varieties differed from the respective parents with respect to absence of one band each in P1PC, P3PC and P5PC. Even though the same band was absent in *P. colubrinum*, this cannot be considered as a conclusive proof for similarity to *P. colubrinum* and the hybrids. In P2PC one additional band detected compared to Panniyur 2 was found to be a *P. nigrum* specific band as it was in all other *P. nigrum* genotypes but absent in *P. colubrinum*.

Future line of work includes further investigation of the reason for low berry setting. The possibility of apomictic reproduction has to be verified. Also the number of black pepper specific SSR markers reported is insufficient to cover the large genome of black pepper. Hence more SSR markers as and when they are available as well as efficient markers like AFLP and SNP may be included for genome wide coverage during screening of these hybrids to develop hybrid specific markers.

Summary

6. SUMMARY

The study entitled "Molecular characterization and testing hybridity of interspecific crosses in black pepper (*Piper nigrum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2010-2012. The objective of the study were to characterize the partially fertile interspecific hybrid (Culture P5PC) from the cross *P. nigrum* x *P. colubrinum* tolerant to *Phytophthora* foot rot and to test the hybridity of putative F_1 hybrids using Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) markers. Putative interspecific hybrids developed at Pepper Research Station (PRS), Panniyur by crossing six popular varieties/cultivars of *Piper nigrum* with *Piper colubrinum* were collected from Pepper Research Station (PRS), Panniyur and maintained at CPBMB, COH were used for the study.

The salient findings of the study are stated below:

1. Morphological observations were recorded for 15 vegetative and 12 reproductive characters from the field grown vines of parents and putative hybrids from six interspecific crosses. The data recorded for all the 15 vegetative characters in the six hybrids were similar to the respective female parents and differed from that of the male parent.

2. Among the vegetative characters plant growth habit, leaf area, production of orthotrope (runner) and plagiotrope (lateral) shoots were found efficient distinguish the hybrids and parental genotypes.

3. Various characters of leaf, stem showed significant difference from the male parent, but were on par with the female parents. Spike characters i.e. spike length, peduncle length and berries per spike were on par with the female parents and other spike character, no. of spike per branch, fresh and dry weight of spike in hybrids showed significant difference with female parents and the male parent. Berry characters, weight of thousand fresh
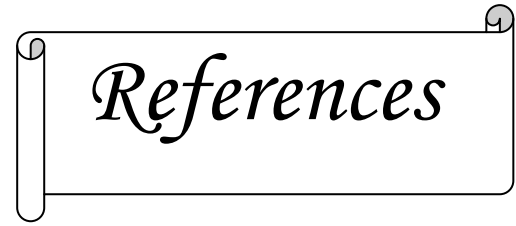
and dry berries as well as berry setting percentage differed significantly between hybrids and female parents.

4. The protocol for genomic DNA isolation was standardised. The protocol suggested by Rogers and Bendich (1994) with slight modification (4% CTAB) was found to be the most appropriate for isolation of DNA from black pepper varieties. The RNA contamination was completely removed through RNase treatment.

5. The quality and quantity of DNA was analyses by NanoDrop^R ND-1000 spectrophometer. The absorbance ratio ranged from 1.80-1.89, which indicated good quality DNA and the recovery was high. This DNA was suitable for RAPD and SSR analysis.

6. Protocol for RAPD and SSR assay in black pepper were standardised with the various quantities of DNA, PCR mixtures and conditions for DNA amplification.

7. 30 RAPD primers and 54 SSR primer pairs were screened for the ability to amplify DNA fragments. Out of these 10 RAPD primers and 11 SSR primer pairs were selected based on the number of the bands and nature of amplicons.

8. A total of 87 RAPD markers were generated using 10 selected decamer primers. Most of the markers were similar in all the six hybrids and their respective female parents and none of the hybrids shared a band that is specific to *P. colubrinum*. RAPD primer OPA 30 as well as OPA 28 and OPAH 09 produced one unique band in variety Panniyur 5 and cultivar Uthirankotta and their corresponding hybrids respectively. Decamer primers OPA 17 and OPC 09 produced one unique band each in cultivar Karimunda, Uthirankotta and their respective hybrids.

9. RAPD primers OPC 08 and OPC 09 amplified one band each in Panniyur 5 which was absent in *P. colubrinum* and the hybrid P5PC1. Primers OPA

28 amplified one band in Panniyur 5 and Panniyur 2 which was absent in *P. colubrinum* and their respective hybrids. Primer OPA 10 showed one polymorphic band present in Panniyur 3 but that was absent in *Piper colubrinum* and their respective hybrid.

10. A total of 33 SSR markers were generated using 11 SSR primer pairs. Out of the 11 SSR primers pairs, 9 primers pairs clearly distinguishing between *P. nigrum and P. colubrinum*. Primer PN A5 generated marker specific to cultivar Karimunda and respective hybrid KMPC.

11. The scored data based on RAPD and SSR banding was used to construct a dendrogram using the NTSYS pc version 2.02i and DARwin (version 5.0.158) showed highest similarity of all the interspecific hybrids with their respective female parents and value of similarity ranges from 96 percent in Panniyur1, Panniyur 2, Panniyur 3 and Panniyur 5 to 100 per cent in Uthirankotta and Karimunda.

12. All interspecific hybrids showed greater diversity from male parent *Piper colubrinum* and ranged from 52 to 72 per cent.

13. The Resolving power (Rp) of the RAPD and SSR primers was calculated and the values ranged between ranged between 5.6 and 13.8 for RAPD primers and 2.0 and 3.69 for SSR primers. The RAPD primer OPA17 and SSR primers PN E3 and PN D10 showed high resolving power.

14. The Polymorphic Information Content (PIC) calculated and ranged between 0.72 and   0.87 for RAPD primers and 0 to 0.57 for SSR primers indicating the suitability of primers to detect polymorphism. RAPD primers OPA 30 and OPP 08 also SSR primers PN E3, PnCA9 and PnGT2 reported highest PIC values.

15. The seeds produced by reported hybrid Culture P5PC were subjected to germination and seedling obtained were characterized using two RAPD and five SSR primers selected based on PIC. Most of the seedling showed

banding pattern similar to Panniyur 5 and hybrid P5PC. RAPD primer OPA 30 showed polymorphic bands which was present in Panniyur 5 and hybrid P5PC but absent in three seedlings.

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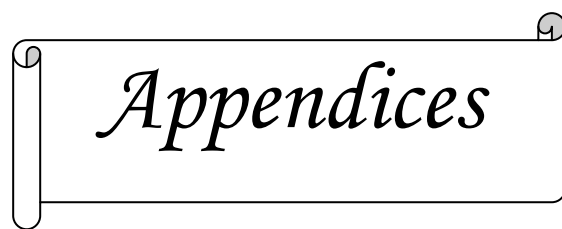
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Appendices

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge	:	Kubota, Japan
Horizontal electrophoresis System	:	Biorad, USA.
Thermal cycler	:	Mastercycler personal, Eppendorf and Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	:	GelDoc-ItTS™ Imaging System UVP(Inc. CA)
Nanodrop® ND-1000 Spectrophotometer	:	Nanodrop®Technologies lnc. USA

ANNEXURE II

Reagent required for DNA isolation

Reagents:

1. 4x CTAB extraction buffer (100 ml)

CTAB	:	4 g

(Cetyl trimethyl ammonium bromide)

Tris HCl	:	1.21 g
EDTA	:	0.745 g
NACl	:	8.18 g
PVP	:	1.0 g

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

2. CTAB (10 per cent, 100 ml)

CTAB	:	10 g
NACl	:	4.09 g

3. Chloroform- isoamylalcohol (24:1)

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

4. Chilled isopropanol

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

5. Ethanol (70 per cent)

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

6. TE buffer (pH8, 100 ml)

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

7. TAE buffer (50 X)

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8)	:	100 ml

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

ANNEXURE III

Composition of Buffers and Dyes used for Gel electrophoresis

1. TAE Buffer 50X

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

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

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

ANNEXURE IV

List of Operon decamer primers used for screening black pepper samples

Sl. No.	Primer	Nucleotide Sequence
1	OPA 6	5'-GGTCCCTGAG-3'
2	OPA 8	5'-GTGACGTAGG-3'
3	OPA 9	5'-GGGTAACGCC-3'
4	OPA 10	5'-GTGATCGCAG-3'
5	OPA 11	5'-CAATCGCCGT-3'
6	OPA 12	5'-TCGGCGATAG-3'
7	OPA 17	5'-GACCGCTTGT-3'
8	OPA 21	5'-CAGGCCCTTC-3'
9	OPA 22	5'-TGCCGAGCTG-3'
10	OPA 24	5'-AATCGGGCTG-3'
11	OPA 26	5'-GGTCCCTGAC-3'
12	OPA 28	5'-GTGACGTAGG-3'
13	OPA 29	5'-GGGTAACGCC-3'
14	OPA 30	5'-AGGTGACCGT-3'
15	OPA 36	5' -AGCCGGGAA-3'
16	OPA 38	5'-AGTGCATTCA-3'
17	OPA 39	5'-CAAACGTCGG-3'
18	OPC 01	5'-TTCGAGCCAG-3'
19	OPC 08	5'-TGGACCGGTG-3'
20	OPC 09	5'-CTCACCGTCC-3'
21	OPC14	5'-TGCGTGCTTG-3'
22	OPF 09	5'-CCAAGCTTCC-3'
23	OPF16	5'-GGAGTACTGG-3'
24	OPP 08	5'-ACATCGCCCA-3'
25	OPU 01	5'-ACGGACGTCA-3'
26	OPU 02	5'-CTGAGGTCTC-3'
27	OPU 03	5'-CTATGCCGAC-3'
28	OPU 13	5'-GGCTGGTTCC-3'
29	OPAH 06	5'-GTAAGCCCCT-3'
30	OPAH 09	5'-AGAACCGAGG-3'

ANNEXURE V

List of SSR primers used for screening black pepper samples

Sl. No.	Name of Primers	Sequence
1	PN A5	**F** 5'-CTTCCAGACCAATAATCAACTT-3' **R** 5'-ATCCCAAAATACACAACAATTC-3'
2	PN B5	**F** 5'-GTTTTGAATGGGTCGGTGAT-3' **R** 5'-ATTGTTCTGATTTCTTCGTTATTG-3'
3	PN B9	**F** 5'-AGTATTGGTTGTTTCTCTC-3' **R** 5'-ATGTAAAATCGATAGTCCTCA-3'
4	PN E3	**F** 5'-TTTGTGTCCTCTCCCTCTCC-3' **R** 5'-AAGACTAAATAGGCAAGGCAAA-3'
5	PN F1	**F** 5'-ACTTCAGTGCTATTTTTATCTTCC-3' **R** 5'-CCAACGCCCACTCTCAT-3'
6	PN G11	**F** 5'-TTACTAGTGTCCACCCCCACT-3' **R** 5'-TCGATGGAAAGTCACCCTCT-3'
7	PN H4	**F** 5'-CTTTTCCCACAATTCAGTCTCG-3' **R** 5'-ACCCATGCGTGTATCTTCTCAG-3'
8	PN H8a	**F** 5'-TGTGTCTTTTATATTTTTGATG-3' **R** 5'-TATTAGTAGTTCTCCCTTTTGA-3'
9	PN D10	**F** 5'-GTGTTACCTTTGGGGCATTCA-3' **R** 5'-TGTGTCAGGGCATCAAACC-3'
10	PC-b8	**F** 5'-AGCAGATACGCATGACCGTA-3' **R** 5'-CTTGCTAGTCTCGATTGCAC-3'
11	PC-b12	**F** 5'-GCTGGTGCTGGTGCCCCTTA-3' **R** 5'-TGCTGCCGCTGCTTTTGACG-3'
12	PC-b13	**F** 5'-AACGCGTTGAACGATGCATC-3' **R** 5'-CATATAGTCGTGCTGAGAGG-3'
13	PnCA25	**F** 5'-GTGTGTTATTTGTCTCTGGGTTTTTCC-3' **R** 5'-CTTTGGGGCACTCTAACCATCGTCTG-3'
14	PnCA88	**F** 5'-CACAT TTTTTCTTACATTGCG-3' **R** 5'-GATTATGGGCTGCCGGATTT-3'
15	PnGT119	**F** 5'-CCCAACTTCAGAATGATTATACAC- 3' **R** 5'-CTGGGCAGTAACAAACATA-3'
16	PnAG30	**F** 5'-ACTAAGGCTAATGTGATAACCTGAGG-3' **R** 5'-ATCCCTGGATGGAAATTTGAAGGCTTGC-3'
17	PnGT2	**F** 5'-CTAGAGAGTAACAGTTATCACTTCACAGC-3' **R** 5'-CTA GCAAATTTGTTCTCTAATTCACATGT-3'
18	PnGATA10	**F** 5'-CTCCCAGTTATACAACATCACAACCTAGCAC-3' **R** 5'-AGAGGCTTGTCTTAGTTGTGCTCGGGA-3'
19	PnCA9	**F** 5'-TCATCAATCACACCTAAAAGAAGGCTATCC-3' **R** 5'-ATGTGGCTATGGGGAACGGTCAGGGGT-3'
20	LE aat001	**F** 5'-GATGGACACCCTTCAATTTA-3' **R** 5'-TCCAAGTATCAGGCACACC-3'

Sl. No.	Name of Primers	Sequence
21	LE aat002	**F** 5'- GCGAAGAAGATGAGTCTAGAGCATAG-3' **R** 5'-CTCTCTCCCATGAGTTCTCCCTTC-3'
22	LE ac001	**F** 5'-TGCCTTCCATCTAACCAATC-3' **R** 5'-CTGTGGCAAATATGTCCCTAAG-3'
23	LE at001	**F** 5'-GCGCGAGCTCTCTCTGATCTCT-3' **R** 5'-TTGTAATTGCATCGGCCACG-3'
24	LE at002	**F** 5'-TACTGCATTTCAGGTACATACTCATC-3' **R** 5'-ATAAACTCGTAGACCATACCCTC-3'
25	LE at003	**F** 5'- GAGAAGTTGGTGCATTCATAAC-3' **R** 5'-AAACAGTAAACCAAACACTTGC-3'
26	LE at004	**F** 5'- GCCAGTTGATCATCATCATGAGTACA-3' **R** 5'-AGAAGCCAATGAAGTGAGTGTTGC-3'
27	LE at005	**F** 5'- TGCAGCCTTTGGGTAAAC-3' **R** 5'-ATAGTTTGAAGAGAGGGAGAAAG-3'
28	LE at006	**F** 5'- CATAATCACAAGCTTCTTTCGCCA-3' **R** 5'-CATATCCGCTCGCTCGTTTCGTTAGTAAT-3'
29	LE at007	**F** 5'-GCCCTAGATCTCACAAGCC-3' **R** 5'-CACAAAGCTGAATGATACGAAG-3'
30	LE at008	**F** 5'-AAGCGCGAGCTCTCTCTGATCTC-3' **R** 5'-CCACGATCTCCGCCATATGC-3'
31	LE at009	**F** GCCCAGGTAAAAGCAATGTGC-3' **R** 5'-AGCAAACCTAGGGACAGATCATA-3'
32	LE at010	**F** 5'- TGGCTCTGCTCAACTCAAGAACTAC-3' **R** 5'-CACGTGAGGTTAGCCAGTGCATC-3'
33	LE at011	**F** 5'- TGGGCTGACTTCGAGTTTG-3' **R** 5'-CGAGAAAGGGCAGAGAATG-3'
34	LE at012	**F** 5'-CGGCAAAGGGACTCGAATTC-3' **R** 5'-GTGGCGGAGTAGAAACCTTAGGA-3'
35	LE at013	**F** 5'- ATCACAAGCTTCTTTCGCCACA-3' **R** 5'-ACCCATATCCGCTCGTTTCG-3'
36	LE at014	**F** 5'-TGTGTTGCGTCATTACCACTAAC-3' **R** 5'-CCCAACCACCAATACTTTCC-3'
37	LE at015	**F** 5'-GGATTGTAGAGGGTGTTGTTGG-3' **R** 5'-TTTGATAATGACTTTGTCGATG-3'
38	LE at016	**F** 5'- CCCAAATGCTATGCAATACA-3' **R** 5'-AGTTCAGGATTGGTTTAAGGG-3'
39	LE at017	**F** 5'- TGAGAACAACGTTTAGAGGAGCTG-3' **R** 5'-GGGGCAGAATCTCGAACTC-3'
40	LE at018	**F** 5'- CGGCGTATTCAAACTCTTGG-3' **R** 5'-GCGGACCTTTGTTTTGGTAA-3'

Sl. No.	Name of Primers	Sequence
41	LE at019	F 5'- TGCCTCTCTTCAAAGATAAAGC-3' R 5'-CGGAAAGTTCTCTCAAAGGAG-3'
42	LE act001	F 5'- AATCATCAACTTTAAACTGTGACAC-3' R 5'-TGCATTGAGATGATGAGTCGTTGG-3'
43	LE aat003	F 5'- CTTCAGGTGGAAATATGAACAC-3' R 5'-AAGCAGGTGATGTTGATGAT-3'
44	LE aat004	F 5'- CAGGATCAGAACAGCGATG-3' R 5'-CCACTGGTATCCATCTTTCAC-3'
45	LE aat005	F 5'- GGTCATGCAGGTTGGATTAC-3' R 5'-AACCTTCCTTCCTATTGGC-3'
46	LE aat006	F 5'- GCCACGTAGTCATGATATACATAG-3' R 5'-GCCTCGGACAATGAATTG-3'
47	LE aat007	F 5'-CAACAGCATAGTGGAGGAGG-3' R 5'-TACATTTCTCTCTCTCCCATGAG-3'
48	LE aat008	F 5'-GAGTCAACAGCATAGTGGAGGAGG-3' R 5'-CGTCGCAATTCTCAGGCATG-3'
49	LE agat001	F 5'- TCCAGATAGTCAGTCAGTCAGACAGC-3' R 5'-TCTCTATCTTTAAGAGTGGGAGAAC-3'
50	LE aga001	F 5'-TTCTTCACTGTTGACAGAGAGAC-3' R 5'-CATTAGTTGAGAGTGATACCGC-3'
51	LE ag001	F 5'- GCTCGAGCACATATAGAAGAGAATCA-3' R 5'-CCATTTCATCATATCTCTCACCTTGC-3'
52	LE ag002	F 5'- AGACGCTTCGACGGGGTTTA-3' R 5'-AGGACAGGTGAATGGGTCAAAGA-3'
53	LE ag003	F 5'-ACCCTAAAACTAACGACATTCAACG-3' R 5'-TTCGTGGACTAATGTATGAAGTGTACC-3'
54	LE aac001	F 5'- AGGAAGAGCGTGAGTCTGAAC-3' R 5'-TCCTGCGCCACTTTAGAG-3'

MOLECULAR CHARACTERIZATION AND TESTING HYBRIDITY OF INTERSPECIFIC CROSSES IN BLACK PEPPER (*Piper nigrum* L.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Although India is one of the leading producer of the economically important spice crop black pepper (*Piper nigrum* L.), the productivity of the crop is low due to various factors among which infestation by *Phytophthora capsici* has been identified as a major factor. Wild relatives of crops are valuable sources of desirable characteristics for genetic improvement of crops. Attempts were made in the past to transfer genes for disease resistance through interspecific hybridization in black pepper also using the related species like *P. attenuatum* (Sasikumar *et al.*, 1999), *P. colubrinum* (Vanaja *et al.*, 2008) *etc*. However, the major problem was cross incompatibility and hybrid sterility caused by the difference in chromosome number between the different species. As it is a perennial crop, a reliable method for identification of hybrids at the early stage of development, preferably at seedling stage itself, is essential. Hence the study entitled "Molecular characterization and testing hybridity of interspecific crosses in black pepper (*Piper nigrum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2010-2012. The objectives of the study were to characterize the partially fertile interspecific hybrid (Culture P5PC-1) from the cross *P. nigrum* x *P. colubrinum* tolerant to *Phytophthora* foot rot and to test the hybridity of putative F_1 hybrids developed at Pepper Research Station (PRS), Panniyur, using Randomly Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) markers.

Six interspecific hybrids produced by crossing two cultivars (Uthirankotta, Karimunda) and four high yielding varieties (Panniyur 1, Panniyur 2, Panniyur 3 and Panniyur 5) of *P. nigrum* as female parents and *P. colubrinum* as male parent were used for the study. Morphological observations were taken from the field grown vines of parents and putative hybrids maintained at PRS, Panniyur. The hybrids resembled the respective female parents for the most of the leaf, stem and spike characters

recorded as per the descriptor for *Piper* sp. except for berry setting percentage which was found significantly low in hybrids compared to respective female parents.

For SSR and RAPD assay, genomic DNA was extracted from the first leaf from tip of the stem of all the plants using the CTAB procedure reported by Rogers and Bendich (1994) with slight modification which yielded good quality DNA for further analysis. Thirty RAPD primers and fifty four SSR primer pairs were screened with DNA of black pepper var. Panniyur 5 for amplification and those which gave reliable distinct banding pattern were selected for further analysis. Genomic DNA of six interspecific hybrids and their parents were amplified with 10 selected decamer primers and 11 SSR primer pairs.

The presence or absence of the markers were scored and entered into a binary data matrix and was used for calculating the similarity coefficient using Dice coefficient (Nei and Li, 1979) using software DARwin (Version 5.0.158) and Jaccard's coefficient (Jaccard, 1908) using software NTSYS pc version 2.02i (Rohlf, 1993). Cluster analysis was done using the UPGMA method and dendrograms were constructed by neighbor joining. The marker data were analyzed separately as well as in combination for the two marker systems.

In the dendrogram with NTSYS pc and DARwin, all the interspecific hybrids showed highest similarity with their respective female parents and values of similarity ranges from 96 per cent (in Panniyur 1, Panniyur 2, Panniyur 3 and Panniyur 5) to 100 per cent (in Uthirankotta, Karimunda). All interspecific hybrids showed greater diversity from the male parent *P. colubrinum* (52-72%). The markers were efficient in revealing the varietal difference of the various genotypes of *P. nigrum* and grouped each of the putative hybrid along with the respective genotype used as the female parent. However, with respect to RAPD assay, one band each present in female parent was absent in the hybrids P5PC and P2PC.

The Polymorphic Information Content (PIC) worked out for the different primers ranged between 0.72 to 0.87 in RAPD and 0.23 to 0.57 in SSR analysis; indicating the capacity of the primers selected to distinguish hybrids. Based on PIC, five SSR and two RAPD primers selected for further charaterization of open pollinated progeny of hybrid culture P5PC-1.

The seeds produced by the partially fertile hybrid (Culture P5PC-1) (Vanaja *et al*., 2008) were subjected to germination test and the seedlings obtained from viable seeds were used for molecular characterization using RAPD and SSR primers selected based on PIC, along with the reported hybrid P5PC-1 and grandparents Panniyur 5 and *P. colubrinum*. Most of seedlings showed SSR banding pattern similar to Panniyur 5 and female parent. In RAPD, primer OPA 30 showed a polymorphic band which was present in Panniyur 5 and the Hybrid P5PC-1 but absent in three seedlings.

The present study using 87 RAPD and 33 SSR markers and the morphological characters revealed significantly high similarity of interspecific hybrids to respective female parents. However the low berry setting percentage as well as the presence of one or two polymorphic bands in two hybrids needs further investigation. Also the number of black pepper specific SSR markers reported is insufficient to cover the large genome of black pepper. Hence more SSR markers as and when they are available as well as efficient markers like AFLP and SNP may be included for genome wide coverage during screening of these hybrids to develop hybrid specific markers.