MORPHOMOLECULAR CHARACTERISATION OF THE VARIANTS OF *PIPER NIGRUM* L. VARIETY PANNIYUR 1

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DECLARATION

I hereby declare that this thesis entitled **Morphomolecular characterisation of the variants of *Piper nigrum* L variety Panniyur 1** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree diploma associateship fellowship or other similar title of any other university or society

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CERTIFICATE

Certified that this thesis **Morphomolecular characterisation of the variants of *Piper nigrum* L variety Panniyur 1** is a record of research work done independently by Ms Smitha Bhasi (2005 11 135) under my guidance and supervision and that it has not previously formed the basis for the award of any degree fellowship or associateship to her

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DEDICATED TO

MY BELOVED PARENTS

AND

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LIST OF ABBREVIATIONS

µl	Microlitre
µM	Micromolar
AFLP	Amplified fragment length polymorphic DNA
bp	base pa r
CTAB	Cetyl trimethyl ammon um b om de
CV	Coefficient of V ariation
DNA	deoxy ribonucleic acid
dATP	deoxy Adenosine Tri Phosphate
dCTP	deoxy Cytosine Tri Phosphate
dGTP	deoxy Guanosine Tri Phosphate
dTTP	deoxy Thiam dine Tri Phosphate
dNTPs	deoxy Nucleotide Tri Phosphates
EDTA	Ethylene Diamino Tetra Acet c acid disod um salt
HCl	Hydrochloric ac d
IISR	Indian Institute of Sp ce Resea ch
ISSR	Inter Simple Sequence Repeats
M	Molar
ml	milli litre
mM	Millimolar

NaCl	Sodium Chloride
NaOH	Sodium hydroxide
OD	Optical Density
ng	Nanogram
PCR	Polymerase Chain Reaction
pH	Per Hydrogen
PVP	Poly Vinyl Pyrrolidone
RAPD	Random Ampl fied Polymorphic DNA
RARS	Regional Agricultural Research Station
RFLP	Restriction frag nent length polymorphism
SCAR	Sequence Characterized Amplified Region
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Microsattelite sites
TAE	Tris Acetic acid EDTA
TE	Tris EDTA
Tris HCl	Tris (hydroxyl methyl) amino methane Hydrochloride
VNTR	Variable Number of Tandem Repeats

Introduction

1 INTRODUCTION

Black pepper *Piper nigrum* L. often referred to as the King of spices is the most traded spice crop. India is a major producer, consumer and exporter of black pepper in the world and Indian black pepper is well known for its quality and fetches premium price. India with more than 40 per cent share of the world area under pepper contributes to about 23 per cent of the total pepper production in the world.

Among the thousand reported species in the genus *Piper* about one hundred and ten have are originated in the humid tropical evergreen forest of the Western Ghats of India. Majority of the present day Indian cultivars are land races representing direct introduction from the wild. Advanced cultivars have been derived mostly by clonal selection from landraces, though a few are of hybrid origin. As India is the primary centre of diversity of *P. nigrum* the indigenous genetic resources are reservoirs of useful genes for plant improvement programme. The development of improved cultivars through hybridization has made a major contribution to increased productivity and quality of plants in different crop plants.

The first ever hybrid of black pepper, Panniyur 1 (Uthirankotta x Cheriyakaniyakaadan) is the most popular pepper variety grown in India and has recorded the highest potential yield of 8800 kg dry pepper/ha. It is the most widely cultivated improved varieties of black pepper in Kerala since its release in 1971. It is an early bearing variety and performs well under open conditions and is suitable to almost all pepper growing regions.

Black pepper is propagated vegetatively through rooted cuttings. The propagation can be either through the traditional three nodal cuttings or through the split bamboo method. Generally it has been assumed that there exists no

variation among vegetatively propagated crops (Eckert and Barret 1993) and the off springs are true to type However contradictory to this assumption there are reports on significant variation among clonal propagation in many crops (Mekuria et al 1999 Gabrielsen 1998 Novak et al 2000 Diggle et al 1998 Esselman et al 1999) Similar reports are there among the different black pepper cultivars also (Ibrahim et al 1985 Prasannakumari 2001 Chandy et al 1984 Kanakaswamy et al 1985 Mathew and Mathew 2001) and the variation noticed was mainly in terms of yield

Intra clonal variability was reported in black pepper in the local variety Karimunda (Ratnambal et al 1985) and Pradeepkumar et al (1999) reported intra clonal variability in yield in the hybrid Panniyur 1 at the RARS Ambalavayal Pradeepkumar et al (2003) in another study reported the variability in yield contributing factors and quality parameters in a population of Panniyur 1 plants through cluster analysis The standard deviation was the greatest for berries per spike (SD 17 710) and yield (SD 12 901) the lowest variability was observed for piperine content (SD 0 238) Among the quality characteristics oleoresin content exhibited more variability than piperine content The plants were grouped into five clusters based on their mean performance

Such reports deserve serious concern and in depth analysis as pepper is a leading commercial crop of India, important in the domestic as well as international market The present study was taken up in this context utilising the progeny of the forty variant plants reported by Pradeepkumar et al (2003) from RARS Ambalavayal The objective was to assess the extent of variability with respect to morphological traits including yield parameters as well as the molecular analysis of genetic variability

Review of literature

2 REVIEW OF LITERATURE

Piper is the most diverse genera among basal lineages of angiosperms (Gentry et al 1990) Being the largest genus in the family Piperaceae it consists of more than one thousand species occurring through out the tropical and subtropical regions (Parthasarathy et al 2006) According to Purseglove et al (1981) out of this one hundred and ten are of Indian origin

2 1 BLACK PEPPER A MAJOR SPICE OF KERALA

The genus *Piper* includes *Piper nigrum* (black pepper) *Piper longum Piper colubrinum Piper betle Piper chaba Piper brachystachyum* etc which are commonly used in our indigenous system of medicine (Nazeem et al 2003)

Black pepper often referred to as the King of spices is the most important spice in the world (Pradeepkumar et al 2001) The popular names King of Spices and Black Gold designated to black pepper reveal the importance the crop has attained world over It is believed to have originated in the sub mountainous tracts of the Western Ghats (Rahiman et al 1987 Joseph and Skaria 2001)

India is a major producer consumer and exporter of black pepper in the world (George et al 2005) It has the largest area of 2 11 lakh ha under this crop (Radhakrishnan et al 2004) and earns more than US $70 million foreign exchange per year (Arunkumar et al 2006) Maximum exports were observed during the periods 1999 2000 (42824 metric tonnes) 1996 97 (47893 metric tonnes) and 1993 94 (48743 metric tonnes) (Vasanthakumar 2006)

Apart from India, black pepper is widely cultivated throughout Vietnam Indonesia Malaysia Thailand Tropical Africa Brazil Sri Lanka and China

(Joseph and Skaria 2001) Kerala contributes to more than 90 per cent of area as well as production of black pepper in our country (Vasanthakumar 2006)

2 1 1 Plant description

The black pepper plant is a stout glabrous climbing shrub with small cordate leaves when young gradually getting larger later sending out flowering branches with large leaves and fruits Leaves are simple alternate cordate varying in breadth broadly ovate 5 9 nerved This is a dioecious plant with minute flowers in spike which vary in length Fruit is globose one seeded drupe bright red when ripe Seed is globose (Joseph and Skaria 2001)

2 1 2 Uses of black pepper

Black pepper is used in human dietaries medicine and preservative and in perfumery (Srinivasan 2007)

Black pepper is used in cuisine worldwide at all stages of the cooking process and as a table condiment It is used universally in sauces poultry gravies processed meats snack foods etc Black pepper is added to fruitcakes and gingerbread and is also used as a light seasoning on fresh fruit (Vasanthakumar 2006)

Being a native of Western Ghats pepper forms an important ingredient of several indigenous medicines of India (Vasanthakumar 2006) as well as African and Chinese systems In Ayurvedic system of medicine black pepper is known in different names such as Maricam (killer of poison) Krishna (corrosive) Ooshana (giving burning sensation) and Vellajam (antihelmintic) The whole plant is being used as medicine in various ayurvedic preparations (Nybe and Sujatha 2001) Black pepper can be used as a stimulant carminative digestive stomachic nervine deobstruent resolvent cholagogue diuretic emmenagogue and antiperiodic Pepper is much employed as an aromatic stimulant against

cholera as an antiperiodic for malarial fever and alternative to arthritis disease (Joseph and Skaria 2001)

2 1 3 Value added products

Black pepper contains chiefly resin (chavicin) volatile oil and an alkaloid piperine The presence of resin makes black pepper a stimulant while volatile oil imparts to the odour and aromatic taste and the alkaloid piperine gives the febrifuge property The accumulation pattern of various chemical constituents of commercial importance in black pepper berries of two cultivars showed that oleoresin piperine essential oil and starch show a manifold increase one to two months before complete maturity of the berries Certain wild types of black pepper and local land races are reported to contain more piperine and oleoresin (Mathai et al 1981)

2 1 3 1 Black pepper oleoresin

Black pepper oleoresin is a mirror image of the flavour pungency and aroma components and is obtained by extraction of pepper powder using organic solvents viz ethyl acetate ethylene dichloride hexane ethanol acetone etc When freshly made pepper oleoresin is a dark green, viscous heavy liquid with a strong aroma One kilogram of oleoresin when dispersed on an inert base can replace fifteen to twenty kilogram of spice for flavouring purpose (Vasanthakumar 2006)

2 1 3 2 Piperine

Piperine is produced from oleoresin through centrifugation and its content ranges from four to six per cent in dry pepper and thirty five to fifty per cent in oleoresin (Vasanthakumar 2006) According to Srinivasan (2007) dietary piperine enhances the digestive capacity and significantly reduces the

gastrointestinal food transit time The most far reaching attribute of piperine has been its inhibitory influence on enzymatic drug biotransforming reactions in the liver It enhances the bioavailability of a number of therapeutic drugs It is non genotoxic and possesses anti mutagenic and anti tumor influences

2 1 3 3 Pepper oil

Pepper oil is obtained through steam or water distillation of berries It is used in perfumery and in flavouring (Vasanthakumar ?006) The analysis of volatile components and the odour characteristics of Japanese pepper using gas chromatography showed that geraniol citronellal linalool and methyl cinnamate were perceived to be important to the basic flavour (Jiang and Kubota 2004)

2 1 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 (George et al 2005) As India is the primary centre of diversity of *P nigrum* the indigenous genetic resources are reservoirs of useful genes for plant improvement programmes (Pradeepkumar et al 2001)

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) One of the major research aims of different institutions is developing high yielding good quality varieties of black pepper with tolerance to diseases and pest (Saji and Sasikumar 2006) More emphasis is now given in improvement programmes for quality parameters like piperine

oleoresin and oil rather than bulk pepper as the export of value added products is now gaining importance (Kumar et al 2003)

Twelve improved varieties have been released and a few more are in the pipeline at the AICRPS centre at Panniyur as well as at Indian Institute of Spice Research (IISR) Kozhikode Panniyur1 2 3 4 5 6 & 7 from Pepper Research Station (PRS) Panniyur yielding between 1 27 2 57 tonnes ha and Pancham Pournami Sreekara and Subhakara with 2 3 2 8 tonnes ha IISR Malabar Excel IISR Girimunda and IISR Sakthi with 1 05 2 1 tonnes ha from IISR and PLD 2 with 2 4 tonnes/ha from Central Plantation Crop Research Institute (CPCRI) Palode are promising varieties both in research and in farmer s field with respect to yield and other spike quality parameters Of this Panniyur 1 3 and IISR Malabar Excel and Girimunda are hybrids and others are clonal selection from landraces (Ravindran et al 2000)

So far sixteen improved varieties are developed or recommended for release (Saji and Sasikumar 2006)

2 1 4 1 Panniyur 1

The first ever hybrid of black pepper Panniyur 1 (Uthirankotta x Cheriyakamyakaadan) is the most popular pepper variety grown in India (Pradeepkumar et al 2001) and t has recorded the highest potential y eld of 8800 kg dry pepper ha^{1} (NRCS 1991) Panniyur 1 has been holding the status of one of the widely cultivated improved varieties of black pepper in Kerala s nce its release in 1971(Pradeepkumar et al 2003) It is the most popular variety among the seventy distinct cultivars of black pepper (Mathai et al 1981 George et al 2005)

Panniyur 1 is an early bearing variety and performs well under open situations and is suitable to all pepper growing regions but is not suited to heavily shaded areas (Ravindran and Johny 2000 Saj and Sasikumar 2006) The spikes are long with large berries compact and spiral The average dry yield is 1242 kg/ha and the driage is 35 3 per cent and the potential yield is 8800 kg/ha The yield per vine is 2 2 kg green pepper The maximum length of a spike is 17 cm The maximum number of berries on a spike is 125 Thousand berry weight and volume are 155 g and 145 cc respectively The oleoresin content is 11 8 per cent piperine is 5 3 per cent and essential oil is 3 5 per cent (Edison et al 1991)

2 2 VARIATION IN VEGETATIVELY PROPAGATED CROPS

2 2 1 Vegetative propagation of crops

Most perennial flowering plants combine sexual reproduction with some form of asexual reproduction through vegetative propagation for example by rhizomes bulbils cuttings layering tillering or rooting of surface runners (Cook 1983 Eckert et al 1999) In angiosperms vegetative propagation is extremely wide spread and common (Albert et al 2003) It is considered that vegetative propagation can ensure true to type offsprings and hence can ensure the genetic fidelity

2 2 2 Vegetative propagation in black pepper

Black pepper is usually propagated through pre rooted cuttings Pepper develops different types of aerial shoots

a) Primary stem or climbing stem

b) Runner shoots which originate from the base of the vines

c) Fruit bearing lateral branches

d) Hanging shoots

Different planting materials will produce different types of plants In India generally cuttings from the runner shoots are used or raising rooted planting materials Runner shoots of high yielding and healthy vines in the garden is selected and 2 3 node cuttings are planted either in nursery beds or polythene bags filled with fertile potting mixture This traditional method does not provide enough planting materials to meet the requirements So alternative methods of rapid multiplication techniques were devised for large scale production of rooted pepper cuttings (Package of practices 2007) These include

a) Rapid multiplication of rooted pepper cuttings using split bamboo method

b) Pit method (single node from runner shoots are used for root ng)

c) Rooted lateral or fruiting branches are propagated as bush pepper

2 2 3 Variations among vegetatively propagating or clonal plants

Generally it has been assumed that there exists no variation among vegetatively propagated crops (Eckert 2002) and the off springs are true to type It was assumed that genetic diversity was lower for clonal plants than for non clonal plants (Harper 1977) However a growing body of data indicated that populations of clonal plants could maintain considerable amounts of genetic diversity (Ellstrand and Roose 1987 Hamrick and Godt 1989 Eckert and Barrett 1993 Widen et al 1994) Reports show that asexual species are as genetically diverse as sexual ones (Hamrick and Godt 1989)

Asexual modes of reproduction in most artic plants have led to the common assumption that they contain low levels of genetic diversity (Bierzychudek 1985 Peck et al 1998) But investigation of some artic autogamous species e g *Draba* spp (Brochmann et al 1998) *Saxifraga oppositifolia* L (Abbott et al 1995 Gabrielsen et al 1997) and *Silene acaulis* (L) (Abbott et al 1995 Philipp 1997) have revealed high levels of genetic

variation According to Jefferies and Gottlieb (1983) in the artic seashore g ass *Puccinellia phryganodes* (Trin) high levels of clonal variation we e observed within and among population According to Novak et al (2000) clonal diversity was found in populations of the apomixtic vine *Bryonia alba* a herbaceous plant According to Verburg et al (2000) though low clonal diversity was found in young population of *Circaea lutetiana* it was high in the established population Studies of the artic alpine *Carex bigelowii* and the alpine *Carex curvula* demonstrated high levels of clonal variability (Jonsson et al 1996 Steinger et al 1996)

2 2 4 Molecular characterization for assessment of genetic variability in vegetatively propagated crops

One method for assessing variation in vegetatively p opagated crops is through its morphological characterization Since those morphological traits are much governed by complex genetic interactions morphological characterization alone will not be much reliable in assessing genetic diversity (Karp et al 1998) and here the real role- play by molecular markers come

Identification of different clones in populations of clonal plants has been greatly facilitated by the use of molecular markers Since they are genotypic markers (Brettmg and Widrlechner 1995) they are used to study the differences among strains at molecular level They are useful for diversity assessment in a number of plant species (Waugh and Powel 1992) and are direct manifestations of genetic content (Weising et al 1995) They serve as reliable indices of genetic variation

Currently molecular markers are being used not only for the assessment of genetic variability and characterization of germplasm but also for the identification and fingerprinting of genotypes estimation of genetic distances between populations inbred and breeding material detection of monogenic and

qualitative trait loci marker assisted selection identification of e|uence of useful candidate genes etc (Koruzon 2001) The genetic markers can be very well used for clonal identification linkage mapping population diversity taxonomy evolutionary studies determining the genetic fidelity during micro propagation germplasm conservation and so on (Bretting and Widrlechner 1995)

Molecular markers are widely classified into biochemical markers and DNA based markers Biochemical markers have been used since long for the characterization of variation in plants De Michele et al (1991) reported that some isozyme variants are not selectively neutral An allozyme study of the arctic alpine pseudo viviparous grass *Poa alpine* L revealed large within population variation (Nordal and Iversen 1993) In art c and alpine populations of *Polygonum viviparum* L intermediate to high levels of genetic diversities were detected based on isozyme electrophoresis and bulbil colour (Bauret 1996) The allozyme analysis of three alpine populations of the herbaceous perennial *Polygonum viviparum* a plant with no observed sexual reproduction revealed genetic diversity (Diggle et al 1998) Genetic diversity and structure of fifteen populations of *Phragmites australis* were investigated usmg starch gel electrophoresis and the analysis based on seventeen enzyme loci coding for eight enzyme systems showed that there exist a high level of genetic variability (Gou et al 2003) According to Sasikumar (1999) isozyme technology was effectively used in the identification of two interspecific hybrids of *Piper*

Isozymes are unstable markers during plant development and standardization of sampling procedures is sometimes difficult and is considered to be inappropriate as universal markers (Cooke 1984) Because of its plasticity ubiquity and stability DNA is the ideal molecule for analysis of variation (Anollees et al 1991) Therefore the isozymes have been replaced by DNA based molecular markers (Anolles and Trigiano 1997)

The various types of molecular markers utilized to evaluate DNA polymorphism are generally classified as hybridization based markers and Polymerase Chain Reaction (PCR) based markers (Joshi et al 1999) The hybridization based DNA marker techniques utilize labeled nucleic acid molecules as hybridization probes (Anolles et al 1991) Probe molecules range from synthetic oligonucleotides to cloned DNA Some of the important hybridization based DNA techniques are Restriction Fragment Length Polymorphism (RFLP) Hyper Variable Sequences and Variable Number of Tandem Repeats (VNTRs)

PCR based DNA marker techniques utilizes an *in vitro* enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Anolles and Trigiano 1997 Michelli and Bova 1996) Among the PCR based marker techniques the important ones are Amplified Fragment Length Polymorphism (AFLP) Microsatellites Sequence Characterized Amplified Region (SCAR) and Random Amplified Polymorphic DNA (RAPD) As the PCR based DNA markers evolve rapidly enough to be variable within a population they are much suited for detecting genotypic diversity (Esselman et al 1999)

Analysis of variation in clonal populations using molecular markers such as allozymes (Widen et al 1994) and polymerase chain reaction (PCR) based markers like RAPD (Esselman et al 1999 Persson and Gustavsson 2001 Hangelbroek et al 2002 Albert et al 2003) Inter Simple Sequence Repeat (ISSR) (Esselman et al 1999 Li and Ge 2001) and AFLP (Albert et al 2003 Escaravage et al 1998 Suyama et al 2000) is reported in a number of crop species

Differences in molecular patterns have been demonstrated previously within and among different cultivars of the olive tree *Olea europea* which is usually propagated asexually (Mekuria et al 1999) Chen et al (2006) reported

that molecular analysis of three natural populations of *Caldesia grandis* a highly clonal marshy herb revealed a high level of genetic variation at the species level

Assessment of asexual genetic variability in *Agave fourcroydes* using AFLP shows difference at the population level while this pattern is conserved in the samples from the same plant (Infante et al 2003)

According to George et al (2005) ISSR primers were successfully tested for assessing the genetic diversity of spice germplasm including different species of cardamom *Vanilla and Piper* and were also useful in identifying the selected cultivars of black pepper and also hybrids of black pepper

2 2 4 1 Random amplified polymorphic DNA (RAPD)

Polymerase chain reaction in conjunction with random primers is used for fingerprinting genomes (Welch and Mc Clelland 1990) for population biology studies (Astley 1992) identification of genome specific markers and other uses (Williams et al 1990 Erlich et al 1991) The major advantage of this approach lies in the fact that it allows exploration of large genomic portions

Analysis of RAPD offers several advantages The most important advantage is that RAPD is not a labour intensive procedure It is not necessary to construct or maintain a genomic library RAPD requires smaller quantities of genomic DNA than RFLP analysis Also it is less costly compared to RFLP Generation of RAPD is quicker than RFLP and can be used to detect even single gene mutations (Williams et al 1990)

2 2 4 1 1 RAPD in detection of genetic variability

Several authors have applied the RAPD technique to investigate genetic variability and found the technique very efficient and reliable (Brown et al

1993 Munthali et al 1996) RAPD can be used to detect genetic variation at the intra as well as interspecific level (Aboelwafa et al 1995)

RAPD markers were found to be very useful in assessing the genetic variability in vegetatively propagated crops Morphological variations noticed on these crops may or may not result in variations on molecular analysis and several reports are available on the same

According to Palacios and Gonzales (1997) no genetic variability was observed in the rare and endangered *Limonium cavanillesii* using RAPD markers and this was the lowest level of genetic variation detected in plants using RAPD markers RAPD analysis of *Allium ampeloprasum* var *babingtonii* revealed no polymorphism suggesting that all sampled individuals are part of a single clone (Treu et al 2001)

Vega et al (2001) reported one of the lowest levels of polymorphism 0 8% detected for a plant species by RAPD analysis was for *Agave tequilana* var *azul* plants Seven population of *Alternanthera philoxeroides* a clonally propagated aquatic plant on molecular analysis using RAPD and ISSR markers showed that its genetic diversity is extremely low (Wang et al 2005) According to Li et al (2006) RAPD and ISSR markers used to analyze genetic structure of six populations of invasive plant *Eichhornia crassipes* indicate that the genetic diversity is extremely low

Hamrick and Godt (1989) reported that asexual species are as genetically diverse as sexual ones According to Gabrielsen and Brochmann (1998) high levels of diversity are detected in the artic clonal plant *Saxifraga cernua* using RAPD markers

In the clonal grass *Calamagrostis porteri spp insperata* diversity was detected using allozymes RAPD and ISSR markers (Esselman et al 1999)

RAPD analysis of fifteen African planta n landraces revealed no polymorphis n among the landraces but variation within landrace resulted (Newbury et al 2000)

Studies on genetic diversity of nine varieties of *Morus* spp showed that the overall extent of polymorphism was very high and the RAPD data were useful in distinguishing between the nine varieties of *Morus* spp (Bhattacharya and Ranade 2001)

Genetic diversity amongst landraces of a dioecious vegetatively propagated plant betel vine using RAPD was proved by Ranade et al (2004) Significant genetic variation was reported in a perennial clonal aquatic weed *Leersia hexandra* using ISSR technique (Song et al 2006)

According to Prakash et al (2007) the degree of genetic diversity observed between seven species of *Rhus* L a woody genus belonging to the family Anacardiaceae with RAPD markers suggests that this approach could be used for studying the phylogeny of the genus

RAPD markers have been used to characterize germplasm in several important crop species including *Carica papaya* L (Stiles et al 1993) rice (Fukoka et al 1992) apple (Koller et al 1993) and pigeon pea (Ratnaparkhe et al 1995)

According to Hu and Quiros (1992) four RAPD markers could successfully discriminated fourteen broccoli (*Brassica oleraceae italica*) and twelve cauliflower (B *oleraceae botrytis*) cultivars RAPD markers have been used to characterize the three main cultivated sub populations viz Criollo Forestro and Trinitario of cocoa clones (Wilde et al 1992)

Mulcahy et al (1993) characterized twenty five accessions of apple representing eight cultivars (Golden Delicious Delicious Gala Jonathan Jonagold Florina Fior di Cassia and Imperate Dallago) th RAPD which could give a distinctive fingerprint for each of the cultivars

RAPD markers have been used successfully to detect genetic variation among lowland and upland rice cultivars and the genetic characterization and classification of Japonica cultivars into temperate and tropical groups (Yu and Nguyen 1994) Nine primers were used to specify nine genotypes of *Musa* representing AA AAA AAB and BB genotypes through RAPD technique (Howell et al 1994)

RAPD markers were used for fingerprinting genotypes within and between *Annona* species (Renning et al 1995) The use of RAPD analysis for *Mangifera* germplasm classification and clonal identification is reported by Schnell et al (1995) Graham and Mc Nicol (1995) generated RAPD markers from different *Rubus* species in order to access the degree of similarity between species Iqbal et al (1995) used RAPD markers to establish polymorphisms among local sugarcane varieties and polymorphisms were detected

Lashermes et al (1996) have successfully employed RAPD markers to analyze genetic diversity among cultivated and sub spontaneous accessions of *Coffea arabica* Machado et al (1996) reported that RAPD used for assessing the polymorphism and genetic variability between thirty nine mediterranean *Mandarin* genotypes revealed a low level of genetic variation between accessions whereas their hybrids with other *Citrus* species showed greater genetic dissimilarity

Analysis of genetic variability in forty eight coconut types belonging to East African Tall types by RAPDs microsatellite primed PCR and ISTR analysis detected large number of DNA polymorphism and allowed the identification of

single genotypes by individual specific fingerprints (Duran et al 1997) Varghese et al (1997) evaluated the applicability of RAPD markers in 24 clones cultivated rubber tree *Hevea* and the statistical analysis indicated the absence of a distinct geographical grouping because of the breeding history of *Hevea*

According to Verma et al (1999) RAPD analysis allows the identification and discrimination of the individual genotypes of Basmati rice including the identification of duplicates in genetic resource collections A relatively large genetic diversity was observed within the germplasm collection Interspecific and intraspecific/varietal variations were observed in the RAPD analysis of forty two accessions of *Vitis* representing thirteen species (Wang et al 1999) According to Lanham and Brennen (1999) RAPD markers were used to fingerprint and to examine genetic diversity among twelve genotypes of gooseberry Six hazelnut (*Corylus avellana*) cultivars were identified using RAPD markers (Galderisi et al 1999) RAPD analysis was done to determine intra specific variability in *Andrographis paniculata* (Padmesh et al 1999) In the RAPD technique used for cultivar identification of eleven aubergine cultivars out of twelve primers nine revealed polymorphism in cultiva s (Kochieva et al 1999)

RAPD analysis was carried out by Egashira et al (2000) to investigate genetic diversity of Peruvianum–Complex (PC) species of highly polymorphic wild tomato relatives and the genetic relationship between the PC and the Esculentum Complex (EC) species including the cultivated species RAPD technique was used to detect the genetic variation at the level of DNA among aromatic and non aromatic cultivars by Baishya et al (2000) RAPD and SSR markers were used to characterize genetic relationship among forty six accessions in two *Cucumis melo* L subsp (*Cantaloupensis lodorus*) and subsp *agrestis* (Conomon and Flexuosus) groups (Jack et al 2000) Evaluation of the genetic diversity among twenty seven superior tea accessions (*Ca nell a sine sis* Var *sinensis*) from Korea Japan and Taiwan by Kaundun et al (2000) s ng RAPD

PCR markers showed that three primers were sufficient to distinguish all the twenty seven accessions RAPD analysis was used to assess the genetic diversity of clones of a subset collection of wild apple (Vernam and Gebhardt 2000)

According to Choudhary et al (2001) RAPD profiling was successfully employed to distinguish forty eight aromatic rice genotypes and among fifty eight screened primers 96 5% detected polymorphism among the genotypes

RAPD technique used to analyze the genetic diversity of wild and cultivated clone populations of *Ensete ventricosum* (Enset) demonstrated that cultivated clones clustered distinctly from wild samples which suggest that the present day cultivated enset clones have been introduced to domestication from a limited number of wild progenitors (Birmeta et al 2004)

According to Wolf et al (1995) RAPD marker technique has potential applications in the identification registration and protection of black pepper accessions Efforts were done in *Piper longum* to find out the genetic difference among the varieties using RAPD analysis by Philip et al (2000) Molecular characterization of black pepper cultivars using RAPD markers was successfully done by Pradeepkumar et al (2003)

2 2 4 1 2 RAPD and linkage maps

RAPD assay has been used by several groups as an efficient tool for identification of markers linked to agronomically important traits which are introgressed during the development of near isogenic lines Traits of interests studied include jointless pedicel (Wing et al 1994) disease resistance (Martin et al 1991) and spotted wilt virus resistance (Chaque et al 1996) in tomato anthracnose resistance in mango (Subramanian et al 1996) scab resistance in apple (Hong et al 1997 Tartarini 1996) leaf minor resistance (Moriera et al

1999) and lettuce infectious yellows virus resistance in *C ci s elo* (Mc Creight 2000)

The three RAPD markers viz OPH 11 OPJ 06 and OPL 15 were found to have significant association with the trait seed oil content in Indian mustard (Sharma et al 1999)

Genetic linkage maps have been created in banana (Faure et al 1993) sweet cherry (Stockinger et al 1996) *Citrus* (Christofani et al 1999) rose (Debener and Mattiesch 1999) and oil palm (Moretzsohn et al 2000) using RAPD

In an effort to map the loci affecting the cooking quality traits in basmati rice a doubled haploid population from the basmati indica (Hasan Serai) into non basmati japonica (Xiang Nuo 4) hybrid generated earlier was genotyped using 121 RAPD markers and a linkage map was constructed Single factor analysis of variants revealed significant association with some of the markers and cooking quality traits (IARI 1999)

According to Moury et al (2000) four RAPD markers were successfully used to determine the hypersensitive resistance to tomato spotted wilt virus (TSWV) in pepper

2 2 4 1 3 RAPD and taxonomic studies

RAPD markers have been widely used for taxonomic and related studies Demeke et al (1992) investigated the potential use of RAPD for taxonomic studies in *Brassica Sinapis* and *Raphanus* taxa Results showed that *Raphanus sativus* and *Sinapis alba* were distinct from the *Brassica* taxa

Dunemann et al (1994) investigated the use of RAPD markers for taxonomic studies in *Mallus* Eighteen accessions of w ld species and twenty seven apple cultivars were tested with twenty nine pre selected primers The analysis of the bands using unweighted pair group arithmetic average showed the relationship among the cultivars which was in agreement with the known lineage A dendrogram generated for wild species gave relationships that were in accordance with the known phylogenetic information

The technical simplicity of the RAPD technique has facilitated its use in the analysis of phylogenetic relationships in several plant genera e g roses (Debener et al 1996) blueberry (Levi and Rowland 1997) barley (Noli et al 1997) *Cymbidium* (Obara okeyo and Kako 1998) etc

The genetic closeness of various species of *Vanda* was determined using RAPD markers Strip leaved *Vanda* sp (including *Vanda sanderiana*) and *Ascocentrum miniatum* were more closely related to each other than to the terete leaved *Vanda* sp studied RAPD analysis supported the suggestion that terete leaved *Vanda* trees and *V hookeriana* be classified in the separate genus *Papilionanthe* and that *V sanderiana* should remain in the genus *Vanda* (Lim et al 1999)

According to Nazeem et al (2003) RAPD can be successfully used to evaluate the genetic diversity among the nine important *Piper* species

According to Renuka et al (2005) RAPD markers were found to be useful in analyzing the diversity among seven *Piper* species

2 2 4 1 4 RAPD and soma clones

RAPD analysis was used to detect genetic variation in micropropagated Cavendish bananas (Damasco et al 1996) Four different types of somaclonal

a s ere de f ed and cl aracterized n ba a a p l nts ge e ated by ne ste c lture vl ich allo s tl e el m nat o of off tvp es before pl t g (Walther et al 1997) RAPD vas appl ed to onitor the gc et c f del t of micropropagated meado v fescue viz *Fest ca p ate s s* (Valles et al 1993) Norway spruce (Heinze and Sche nidt 1995) and strawberries (Kumar et al 1995) Accord ng to Lu et al (1996) RAPD were useful for establishing a genetic bas s for so naclonal variation in rice Somaclonal variants were reported in *T t c aestivi* (Bro vn et al 1993) populus (Rani et al 1995) beet (Munthal et al 1996) peach (Hashmi et al 1997) tomato (Hong et al 1999) grapes (Verd sson et al 1999) and pigeon pea (Prasannalatha et al 1999) using RAPDs Plants regenerated by somatic embryogenesis from long term callus cultures derived from five garl c cult vars subjected to RAPD analysis revealed variation (Al Zahi n et al 1999)

In the genus *P pe* RAPD technique has been successfully ut l zed n identify ng somaclonal variants of *P pe lo g* (Parani et al 1997) Analysis of the genetic f del ty of micro propagated plants of black pepper using both morpholog cal and molecular characterizations reported that m cropropagated plants are morphologically similar and RAPD and ISSR profiling also d d not sho v detectable variations (Prabhu and Kumaran 2005)

2 2 4 1 5 RAPD and hybrids

Wang et al (1994) reported RAPD fingerprint ng as a convenient tool for the identification protection and parentage determ nation of rice hybrids Their study ncluded rice plants selected n Northern China (each compris ng the male sterile the restorer the hybrid F1 and the ma ntainer l nes of rice cultivar) and the results obtained vere useful for ident f cat on of each single plant l ne

Truksa an l Procl azka (1996) reported d fferent band ng pattern base l l e DNA polyn era e t sed for test g three l nes of c cumber use l for t e

pr duc of l ybrid see ls Lo le el of p olymoip h s n as obtn ne l l cl
i d cated tl at RAPD vas not s itable for ve fy ng l e h brid ty of seeds

RAPD markers have been successfully used to test the patern tv of Japanese pair hybrid (Banno et al 2000) RAPD markers are no v v dely used for the dentificnation of artificial and natural hybrids n d fferent crops Sheng et al (2000) reported that RAPD techn ques have been used for the ident ficat on of hybrids and the r parent determination as vell

According to George et al (?005) one of the proble ns faced by pepper breeders was the difficulty in identifying true hybrids from the crossed progenies before planting RAPD can be successfully i sed in selecting true hybrids based on shared bands in male parent and offspr ng at the early stage of the plant

According to Jooju et al (2005) RAPD analysis can help identification of true hvbrids of black pepper The primer used in the study vas found useful n ident fication of hybrids in the cross HP780 x *P ig* (Wild)

According to K shore et al (2005) genet c analys s of black pepper hybrids and their parents based on RAPD revealed a moderate degree of divers ty among the cult vars examined

2 2 4 1 6 RAPD for identification of somatic hvbrids

RAPDs ha e been used to cl aracterize nolecularly both nterspecific and intraspecific somatic hybrids

Ba rd et al (1992) proposed RAPD for the identification of inter and tra spec f c so at c l ybrids along tl sexual hybrids at a early stage n potato

Four RAPD primers were found successful for the identification of hybrids between *Solanum torvum* and *Solanum incanum* and the somatic hybrids showed a combination of the parental banding pattern whereas regenerants from one of the parents had a similar banding pattern as that of the parent (Xu et al 1993)

2 2 4 1 7 RAPD in sex determination

Early identification of sex in dioecious plants like papaya (Somri 1998) and nutmeg (Shibu et al 2000) was possible with the help of RAPD markers

Male sex associated RAPD markers were identified for the first time in *Piper longum* (Banerjee et al 1999) The markers could successfully differentiate genotypically between the male and female parents

In the molecular characterization of black pepper and related twenty species using RAPD polymorphism both male and female line showed genetic variation and formed different clusters (Sinoj 2005) According to Kripa (2005) RAPD polymorphism using female plants of twenty two species of *Piper* showed polymorphism

2 2 5 Variation in genus *Piper*

Ravindran et al (1992) reported that the genus *Piper* is known to have wide distribution in tropical and pan tropical region Around three thousand binomials have been reported in this genus all over the world

According to Ravindran et al (1992) it is taxonomically a very difficult genus because of greater range of variability among the species and minute nature of flowers In this regard Howard (1973) states that the family Piperaceae is one of the worst messes in plant taxonomy The reasons of this variability are

considered t be du e to the natu re of breedin. yste ı s Eff cient pollen dispe sal neci anis n is absent in *Pipe* establishing an effecti e solat on barrier between population un ts and indiv iduals This barrier prevents the free gene flow and thus the population will remain discrete (Ravindran et al 1990)

Various attempts to classify the *Piper* species based on morphological cytological (Sharma and Bhattacharya 1959) and chemical constituent data (Rahiman 1984 Sebastian et al 1996 Sebastian et al 2000) have been carried out But all these classification failed to create a concrete grouping of *Piper* species

The genus *Piper* thus warrant the application of the more relevant genotypic marker assisted classification systems for the genome analysis Nazeem et al (2003) utilized dominant markers such as RAPD and AFLP for the evaluation of relatedness among nine *Piper* species including *Piper nigru n P lo igum P colubr inum P chaba P ar bo eum* etc and the results showed high variability among the species

2 2 5 1 Morphological variations in *Piper nigrum*

Majority of the present day Indian cultivars of black pepper are land races representing direct introduction from the wild (Ibrahim et al 1984) Considerable variations exist among the landraces with respect to an array of plant morphological characters giving them the status of distinct plant types each with its own characteristic features (Mathew et al 2006) In black pepper cultivars greater amount of variation exist for yield when compared to components of yield such as spike and berry characters (Ibrahim et al 1985 Ravindran and Babu 1994 Prasannakumari 2001) There are also several reports on intraspecific variabil ty n *Pipe ig u r* (Chandy et al 1984 Kan ikaswamy et al 1985 Mathew and Mathe v 2001) Howe ver these group ngs w ere not based on D^2 analys s Multivariate analysis n f fty cult rs

of bla k pepper n Kerala s ng Malnalob s D nalys s slo e l tl n ca be gro pe nto el e cl sters (Matl e et al 2006) Matl e e al (2007) h s also done tl e assessment and co ser ation of intra pec f c anabil ty n *P pe* g occurr ng in the Western Ghats of Indian Peninsula

2 2 5 2 Molecular variation in *Piper nigrum*

Molecular characterization of th rteen landraces and nine advanced cult vars of *Pipe g u n* L using RAPD markers showed varat on among the samples and cultivar specific bands (Pradeepkumar et al 2001)

RAPD analysis in eight cultivated types and four related spec es of black pepper by Hareesh (2005) showed that all the cultivated types tested genet cally d ffer from each other to a large extent The RAPD prof les also indicated that cult vars Sreekara and Subhkara d ffer from each other thou gh t s d ff cult to d st nguish between them morpholog call v

Molecular and morpholog cal characterization of seven black pepper lines by Sreedevi (2005) showed that all exhibited co nmon features w th respect to morphology and RAPD band ng pattern Assessment of genetic fidelity of black pepper regenerated from somatic embryos using morphological characters and RAPD showed 100 per cent uniformity between regenerated plants and with the orig nal parent (Das et al 2005)

Identification and examinat on of the genetic similarity in cultivated *P g* anet es Iruman yan Kar munda Panniyur l A mp r yan and *P atte at* based on RAPD markers reve led a moderate degree of divers ty (K shore 2005) RAPD markers proved ts ut l ty for a alysing the genetic relat onsh p of selected *P pe* spec es of South Ind a by Raji (2005)

Accord ng to Hidayath (2005) RAPD a id ISSR markers were tou n l to be useful i tl e assessment of genetic d vers ty among twelv e released var et es of black pepper According to Renuka et al (2005) a single ISSR primer could genetically differentiate seven species of black pepper under study and ISSR has proved to be of better option in genetic d versity studies Standardization of ISSR profiling was done in *Piper nigrum* by Kumaran (2005) and used the technique for studying the genetic fidelity of micropropagated plants

According to Reddy (2005) ISSR marker is found to be useful in discriminating cultivated varieties of black pepper Microsatellites used to genetically differentiate different cultivars of black pepper revealed high genetic variability among cultivars with up to one base pair resolution between piper species and within cultivars of the same species (Joy and Sonia 2006)

According to Nazeem et al (2003) AFLP can be successfully utilized for the evaluation of genetic relatedness among nine *Piper* species

2 2 5 3 Variation in Panniyur 1

Ratnambal et al (1985) reported that there exists intraclonal variability in black pepper variety Karimunda

Pradeepkumar et al (1999) reported that some of the true to type vegetatively propagated vines of Panniyur 1 in the RARS Ambalavayal found to exhibit variation in yield potential under the identical environment of soil and other physical factors In the analysis of yield spanning over twenty years plants with high mean yield and low standard deviation have been identified a nong the vines of Panniyur 1

Accord ng to another study by Pradeepkumar et al (2003) for the analysis of va ab l ty in y eld contr buting factors and qu l tv parameters n a r nd mly

selected popu lat on of Pann yur l nvolv ng forty t vo vines throughout h erarchical Euclidian cluster analys s (Spark 1973) considerable var at on of characters under study and the highest standard dev ations was observed for berries/spike (SD 17 710) and yield (SD12 901) while lowest for piperine content (SD 0 238) The correlation analysis of the data has revealed that all the yield contributing factors have positive influence on the final yield of the crop The clones can be clustered ideally to five clusters based on their mean performance It points towards the existence of phenotypic variability in Panniyur 1

According to Ibrahim et al (1985) in black pepper greater amount of variation exist for yield when compared to components of yield such as spike and berry characters but the heritability is the lowest and high correlation of yield with spike length and berries spike 1 was observed (0 31 and 0 44 respectively)

According to Shujan et al (2005) biochemical and physiological parameters influencing productivity in black pepper studies using biochemical constituents and isozyme profiling showed that reducing sugar starch total carbohydrate and protein content present in leaves and stem of juvenile pepper vines may influence productivity

Studies on variation in yield and growth performance of cuttings derived from top middle and bottom nodal explants of the five high yielding varieties viz Panchami Pournami Panniyur 1 Panniyur 3 and Panniyur 5 revealed intraclonal variability in black pepper (Manoj 2005)

According to Shahanas et al (2005) the possibility of intraclonal variability in Panniyur 1 due to the position of the cuttings (top middle bottom) using RAPD technique revealed no intraclonal variability at the genetic level

Materials and methods

3 MATERIALS AND METHODS

The study entitled Morphomolecular characterization of variants of *P pe i g m* L variety Panniyur 1 was conducted at the Department of Plant Biotechnolo_y College of Agriculture Vellayani Thiruvananthapuram and in the Block V of Pann yur 1 at the Regional Agricultural Research Station (RARS) Ambalavayal during the year 2006 2007 Details regarding the experimental materials used and methodology adopted for various experiments are presented in th s chapter

Stem cuttings of the forty plants that showed variation among a population of Panniyur 1 in a 4 ha plot at the RARS vere planted in another plot in July 2000 This thesis is based on the morphological and molecular characterization of the above forty variant plants of Panniyur 1 These variant plants were denoted as V1 to V40 serially Among the forty plants ten plants vere severely affected bv foot rot and hence replanted

3 1 Morphological analysis

The morphological observations for the studv vere taken by consulting the descriptor of black pepper (IPGRI 1995) The major morphological characters for which observations were taken include

1 Internodal length (cm)

2 Length of lamina (cm)

3	Breadth of lamina (cm)
4	Shape of leaf lamina
5	Spike yield per plant (kg)
6	Number of spike per plant
7	Length of spike (cm)
8	Number of berries per spike
9	1000 berry weight (g)
10	Drying percentage (%)

The observations were recorded as given below

1) **Internodal length** Average of five randomly selected internodal lengths

2) **Length of lamina** Average of five randomly selected mature leaves measured from the base of the midrib to the tip

3) **Breadth of lamina** Average of five randomly selected mature leaves measured at the maximum width

4) **Shape of lamina** The shape of leaf lamina was recorded from the leaves of the lateral branches

5) **Spike yield per plant** Data from the records at the RARS Ambalavayal

6) **Number of spikes per plant** Data from the records at the RARS Ambalavayal

7) **Length of spike** Average of five randomly selected spikes

8) **Number of berries per spike** A ıge of f e ra ıdo ı ly selected sp kes

9) **Thousand berry weight** Average of ell de eloped t venty f e berries taken from f e rındomly selected sp kes and converted for thousand berr es

10) **Drying per cent** Data from the eco ds at the RARS Ambalavayal

3 1 1 Statistical analysis

All tl e recorded observat ons except the sp ke yield and number of sp kes per plant ere subjected for stat stical analysis s nce replant ng caused considerable aria ıon among the plants n those elin ı nated characters Tl e sim larity coeff c ent as constructed us ng Heiraıcl al Euclidean cluster analys s and a de drogra n as constructed to analyze the d stance bet veen the clones (Spark 197ɔ)

3 2 Molecular analysis

3 2 1 DNA ısolatıon

For ısolatıon of genomic DNA tender leaf tissues ere used The leaves collected vere stored at 80 ^{0}C (Sanyo Ultra Low) C TAB nethod vas follo ved for ısolatıo ı (Doyle and Doyle 1987) F ve gran leaf tissue per sample as chopped coarselv and vashed tl oroughly ın d st lled vater T vo per cent (0 l gram per sample) Poly Vinyl Pyrrolidoı e (PVP) vas added and the sample v as ground vell ın frozen l qu d n troge ı us ng mortar and pestle The f ne po vder was then transferred to lɔ ml extract on buffer (?% v/v C TAB 1 4 M NaCl 100 mM T ıs HCl (pH 8) ?0 mM EDTA a ıd 0 l°o v β mercaptoe h ınol) and subjected to an cubat on te peratu e of ɔɔ C for 2 l ours The supe natant as transferred to ı l ɔ l tube

ı d eacl ube ɔs treated th ɔ00 µ l pl enol cl lo oto m soɔ ı yl alcol ol (2ɔ ?4 1) ɔnd ce ıt fuged at 7000 ɔpm 4 C foı 7 n ıı utes Equal olun e of chlorofo ɔ soamyl ɑlcohol (24 1) vas added to he supernɔtant ɑ d ceı tr fuged ɑt 7000 ɪp n 4^{0}C foı 7 mınutes and further precipıtated usmg 100% (ɔ tımes olume) ch lled ethanol The prec pıtate vas centrıfuged at 10000 ɪpm 4 ^{0}C ɑ ıd ɔashed t v ce v th 70% etl anol and d ssolv ed ın 80 µl TE buffer (10 mM Tr s HCl pH 8 1 mM EDTA) and thıs was stored at 4 ^{0}C

All the n aterıals used ın the preparation and storage of reagents ıncludıng reagent bottles conıcal flasks centrıfuge tubes spatula glass rods funnels and t ps of m crop pettes were autoclaved before use

3 2 2 Quantıfıcatıon of DNA

DNA quantıfıcatıon vas carr ed out vıth the help of UV spectrophotoı ıeter (Spectron c Genesıs 5) The spectropl oto neter vas calıbrated at 260 nm a ɔd 280 m wavelength usıng TE buffer The opt cal dens ty (O D) of the DNA sa ɔple d ssolved ın TE buffer was recorded at both 260 nm and 280 nı ɔ S nce the O D of 1 0 at 260 nn represent ɔ0 µgml of DNA the quant ty of DNA ın the sample vas est mated by employıng the followıng formula

Amount of DNA (µgml) A260 x ɔ0 x Dılutıon fɔctor (vhere A260 ıs absorbance at ?60 nm)

The qual ty of DNA could be judged from the ratıo of the O D values recorded at 260 nm and 280 nm A rɑt o of 1 8 ındıcates good qualıty DNA The DNA sɔmples ere ɔlso analyzed for the r qual ty throuɡh electrophoresıs us ng 0 8 ^{0}o aga ose gel

3.2.3 RAPD analysis

The DNA samples were first screened with thirty five arbitrarily designed decamer primers supplied by Operon Inc., CA, USA. Out of them, fifteen primers which produced the highest number of bands were selected for amplifying DNA from all the forty samples.

The components of the amplification reaction were optimized and a typical 25 µl PCR mixture comprised 20 ng genomic DNA; 2.5 µl 10X assay buffer; 2 mM $MgCl_2$; 200 mM each of dATP, dGTP, dCTP and dTTP; 0.75U Taq DNA polymerase and 1 mM primer. PCR reactions were carried out in a Programmed Thermal Cycler (PTC 100, M. J. Research. Inc). After a pre-denaturation step of 4 minutes at 94 °C, amplification reactions were cycled 40 times at 94 °C for 1 minute, 35 °C for 1 minute and 72 °C for 2 minutes followed by 5 minutes at 72 °C. Amplification products were separated by electrophoresis on 1.2% agarose gel in 1X TAE buffer (0.04 M Tris Acetate, 0.001 M EDTA).

3.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit (Genie, Bangalore). Various conditions required for carrying out the gel electrophoresis were standardised. The required amount of agarose was weighed out (0.8 per cent for visualizing the genomic DNA and 1.2 per cent for visualizing the amplified products) and added to 1X TAE buffer. Uniform dissolution of agarose was achieved by boiling in microwave oven. After cooling to about 50 ^{0}C, ethidium bromide was added to a final concentration of 0.5µgml^{-1}. The

mixture was poured immediately to preset template with appropriate comb. After solidification, the comb and sealing tapes were removed and the gel was mounted in an electrophoresis tank filled with 1X TAE running buffer. The gel was completely covered on the surface by the buffer. The DNA sample was mixed with required volume of gel loading buffer (0.25% bromophenol blue, 30% glycerol, 70% sterile water). Each well was loaded with 15µl of sample. One of the well was loaded with 1.0µl of DNA molecular marker along with gel loading buffer. Electrophoresis was performed at 55 volts (running voltage @ 5 V/cm; where cm = distance between cathode and anode) until the loading dye reached three fourth of the length of the gel. The gel was visualized and documented using Gel documentation unit (Bio-Rad).

The PCR product was scored for the presence (+) or absence (-) of bands. The number of monomorphic bands, number of polymorphic bands was recorded. Thus banding pattern of all the fifteen primers for the forty samples were scored as 1 and 0 in the excel sheet and subjected for further statistical analysis.

3.2.5 Data analysis

A genetic similarity matrix was constructed using Jaccard's similarity coefficient values and this matrix was subjected to an unweighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. All these computations were carried out using NTSYS-pc version 2.02 (Rohlf, 1998) software and the dendrogram constructed was used to asses the association and distance between the variants under study.

Results

4. RESULT

Investigation on the 'Morphomolecular characterisation of the variants of *Piper nigrum* L. variety Panniyur-1' was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani and in the Block V of Panniyur-1 at the RARS, Ambalavayal during the year 2006-2007. The results of the investigations are presented in this chapter.

4.1 Morphological analysis

Observations on different morphological traits like internodal length (cm), length of lamina (cm), breadth of lamina (cm), shape of lamina, number of spike per plant, spike yield per plant (kg), length of spike (cm), number of berries per spike, thousand berry weight (g) and drying percentage (%) were taken from the forty variant plants of Panniyur-1 maintained at the RARS (Plate1). The observations (Table 1) were subjected to Euclidian cluster analysis.

1) **Internodal length**: The internodal length in the forty plants (Table 2) ranged from 4.9 to 8.9 cm and the mean value was 6.96 cm. The lowest value (4.9 cm) was recorded by V39 and the highest (8.9 cm) by V3. Out of the forty plants under study twenty seven plants recorded the value ranging from 6-7 cm.

2) **Length of lamina**: The value varied from 13-19 cm with a mean length of 16.02 cm. The lowest value was recorded by V26 and V31 (Plate2 A) and the highest by V38 and V40 (Plate2 B). Among the forty plants, twenty recorded a value ranging from 16-17 cm.

Table 1. Morphological traits of forty plants of black pepper variety Panniyur-1.

Plant No.	IL (cm)	LL (cm)	BL (cm)	NS	SY (kg)	SL (cm)	B/S	BW (g)	D (%)
V1	6.6	16.2	12.3	85.0	1.2	12.5	62	150	30.0
V2	7.2	16.5	11.2	50.0	3.2	10.5	60	150	27.1
V3	8.9	13.8	8.7	40.0	0.6	14.0	83	150	28.8
V4	5.5	14.5	10.5	56.0	1.2	12.5	44	150	29.4
V5	8.6	13.2	12.0	10.0	0.9	14.5	96	150	21.6
V6	7.1	16.0	12.2	102.0	2.4	15.5	99	150	25.0
V7	8.4	18.0	12.5	87.0	1.1	16.5	67	150	25.5
V8	6.7	16.5	13.5	63.0	2.8	17.5	82	150	22.4
V9	7.2	15.8	11.8	77.0	1.3	19.0	105	150	27.6
V10	8.1	16.5	12.5	70.0	1.8	14.5	61	150	23.9
V11	6.7	17.0	11.5	20.0	1.8	15.0	81	150	26.0
V12	5.3	16.4	12.7	40.0	0.6	17.5	72	150	24.0
V13	7.5	16.0	11.0	23.0	1.8	19.0	89	150	27.3
V14	6.0	16.0	11.0	52.0	1.9	16.5	54	150	26.1
V15	7.5	15.5	11.5	42.0	0.6	16.0	50	150	26.7
V16	8.2	17.5	13.0	41.0	0.9	14.5	71	200	23.9
V17	7.2	15.5	10.5	28.0	1.8	16.0	76	150	30.0
V18	8.5	15.0	10.5	122.0	2.4	17.5	77	150	33.2
V19	6.6	17.5	11.5	46.0	1.7	16.0	79	150	33.3
V20	6.5	18.0	13.0	75.0	1.1	13.0	64	200	39.5
V21	7.2	17.0	12.0	37.0	1.3	15.5	76	150	37.5
V22	6.7	16.0	12.5	42.0	1.5	17.5	95	150	30.0
V23	7.8	17.0	13.5	10.0	0.9	17.5	56	150	28.9
V24	7.2	15.0	11.0	63.0	2.2	17.0	71	150	28.0
V25	7.7	17.0	13.0	127.0	3.0	20.0	100	150	27.0
V26	6.5	13.0	9.5	56.0	2.0	16.0	91	150	26.0
V27	8.0	13.5	10.0	30.0	1.2	13.5	57	200	32.1
V28	6.5	18.0	13.0	63.0	2.9	16.5	102	200	36.9
V29	6.7	15.5	10.9	67.0	1.1	19.5	92	150	29.2
V30	7.1	16.0	12.0	35.0	1.3	15.0	78	150	26.5
V31	6.5	13.0	9.0	10.0	1.0	17.0	75	150	26.3
V32	7.4	14.5	11.5	158.0	3.7	17.0	99	200	31.6
V33	5.4	16.5	11.7	23.0	2.3	18.0	78	200	30.6
V34	7.1	15.5	9.7	NA	NA	16.5	99	150	36.4
V35	6.4	17.0	12.7	10.0	1.0	18.5	97	200	37.8
V36	5.4	14.5	12.0	40.0	1.0	12.5	68	200	35.0
V37	5.8	16.0	12.5	160.0	3.9	16.5	73	200	33.0
V38	7.9	19.0	13.5	90.0	2.0	20.0	109	200	31.1
V39	4.9	16.5	12.5	10.0	0.5	17.5	71	150	35.6
V40	6.0	19.0	12.5	32.0	2.2	15.4	78	200	34.2

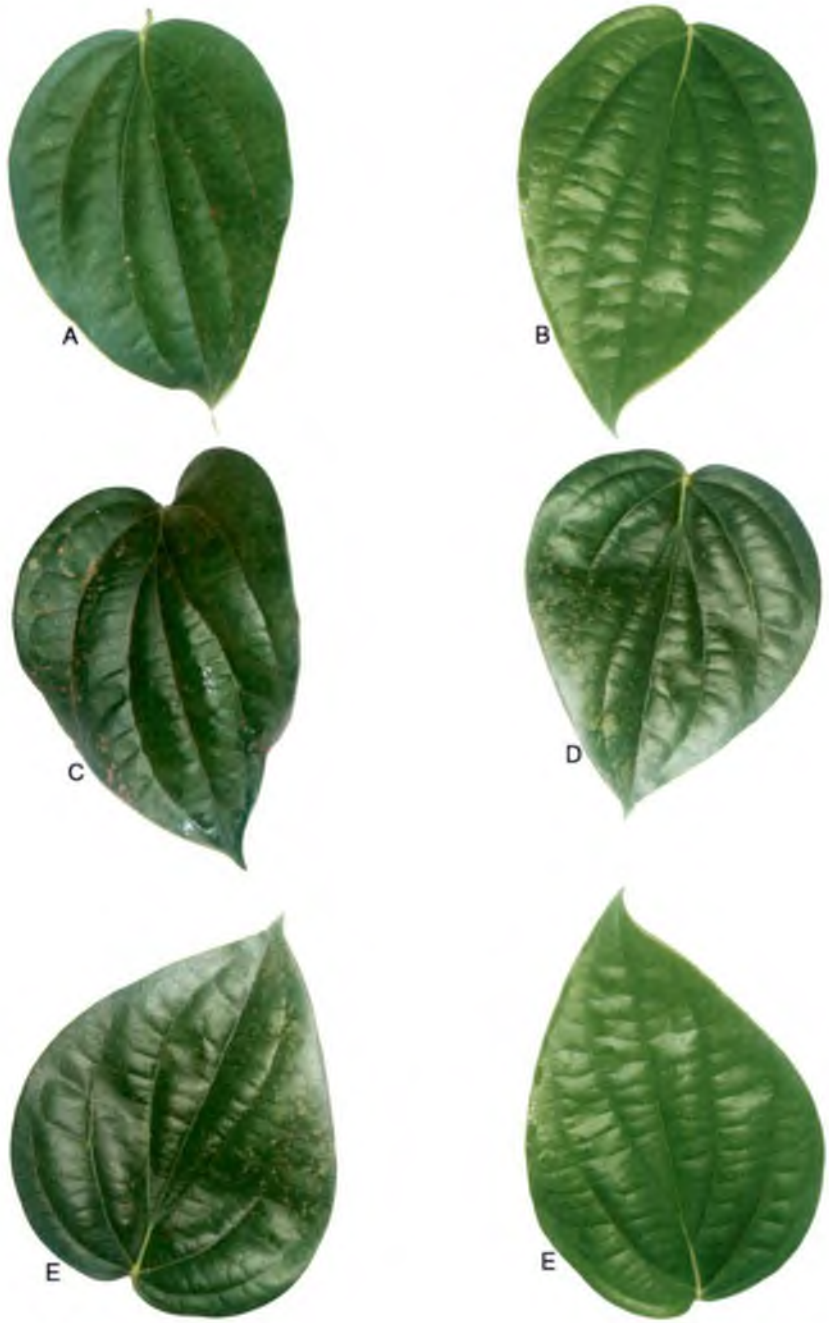
Abbreviations : **IL** - Internodal length (cm). **LL** - Length of leaf (cm). **BL** - Breadth of lamina (cm), **NS** - Number of spike per plant, **SY**- Spike yield per plant (kg), **SL** - Length of spike (cm). **BS** - Number of berries per spike, **BW** - 1000 berry weight (g), **D %** - Drying percentage (%), **NA** - Not available.

Table 2. Range and Frequency of character states of quantitative characters in the forty plants of black pepper variety Panniyur-1.

Sl . No.	Character	Character state	Range	Frequency
1	Internodal length (cm)	Low Medium High	4-5 6-7 8-9	6 27 7
2	Leaf length (cm)	Low Medium High	13-15 16-17 18-19	15 20 5
3	Leaf Breadth (cm)	Low High	8-10 11-14	9 31
4	Number of spike per plant	Low Medium High	10-50 60-100 110-160	24 10 5
5	Spike yield per plant (kg)	Low Medium High	0.5-1.85 1.75-2.85 2.85-3.85	25 10 4
6	Length of spike (cm)	Low Medium High	10.5-14.5 15.5-19.5 20.5-24.5	12 27 1
7	Number of berries per spike	Low Medium Medium-High High	44-64 65-85 86-106 106-109	9 18 12 1
8	1000 berry weight (g)	Low Medium High	150-170 171-191 192-212	30 0 10
9	Drying percentage (%)	Low Low-Medium Medium Medium- High High	21-24 25-28 29-32 33-36 37-40	5 15 11 6 3

Plate 1- Variants of Panniyur-1 maintained at the RARS,Ambalavayal-Field view

Plate 2 - Leaf Characteristics of the Variants of Panniyur -1



A - Leaf with minimum length

B - Leaf with maximum length

C - Leaf with minimum breadth

D - Leaf with maximum breadth

E - Leaf showing cordate shape

Plate 3 - Spike characteristics of the variants of Panniyur -1

A - Spike with minimum length
B - Spike with maximum length
C - Spike with minimum number of berries
D - Spike with maximum number of berries
E - Spike with spiral and compactly arranged berries
F - Spike with less spiral and less compactly arranged berries

ɔ) **Breadth of lamina** The value varied from 8 7 13 ɔ cm with a mean breadth of 11 7 cn The lo vest value vas recorded by V3 (Plate2 C) and the h ghest by V?3 and V38 (Plate2 D) Among the forty plants th rty one plants recorded a value ranging from 11 14 cm

4) **Shape of lamina** The shape of the leaves of all forty plants was cordate (Plate 2 E)

5) **Number of spike per plant** The value varied from 10 160 Nos with a mean value of 54 8 The lowest number of spikes were observed in V23 V31 V35 and V39 (10 Nos) and the maximum in V37 (160 Nos)

6) **Spike yield per plant** The value varied from 0 ɔ 3 85 kg with a mean yield of 1 63 kg The lowest spike yield was recorded by V39 (0 5 kg) and the maximu n by V37 (3 9 kg) Majority of the plants (25 Nos) recorded a value rang ng from 0 5 1 85 kg

7) **Length of spike** The value varied from 10 5 20 0 cm with a mean spike length of 16 12 cm The lowest value was recorded in V2 (Plate3 A) and the highest in V25 and V38 (Plate3 B) Majority of the plants (27 Nos) recorded a value ranging from 15 ɔ 19 5 cm

8) **Number of berries per spike** It ranged bet veen 44 109 with the mean value of 78 43 The lowest value was recorded in V4 (Plate3 C) and the highest in Vɔ8 (Plate 3 D) Eighteen plants showed a value ranging from 65 85 The berries were arranged n a compact and spiral manner in most of the plants (Plate3 E) Ho vever n some of the plants the spiral nature and compactness was comparatively less (Plate ɔ F)

9) **Thousand berry weight** It sho ed a ange of 150 200 g and the nean alue vas 16ɔ 75 Th rty plants reco ded a valu rangn g f om 150 170 g

10) **Drying per cent** It sho ved a range of 21 39 ɔ per cent and the mean value vas 29 62 The h ghest value vas recorded by V20 and the lowest by Vɔ F fteen plants sho ved value ranging from 2ɔ 28 %

4 1 1 Statistical analysis

All the observat ons except the spike yield per plant and number of spikes per plant vere subjected for Euclidean cluster analysis As some of the plants vere eplanted the traits spike yield per plant and number of sp kes per plant were excluded for stat st cal a alysis Among the morphological characters studied the max mum variation vas ioticed n the number of berries per sp ke (44 109) with a Coeff c ent ot varat on (CV) of 20 9ɔ follo ved by drying percentage (21 39 5%) v th a CV 1ɔ 38 The lo vest CV (9 36) vas recorded for length of lamina Tl e Standard Dev atio ı (SD) vas maximum for thousand berry veight (22 61) follo ved by number of berries per sp ke (SD 16 432) (Table ɔ)

Table 3 Mean Standard deviation and Coeff c ent of variation of the morpholog cal tra ts n fortv plants of Pann yur 1 variety of black pepper

Character	Mean	St ndard Deviation (SD)	Coefficient of Variation
Internodal length (cm)	6 96	0 973	1ɔ 98
Length of leaf lam na (cm)	16 02	1 499	9 36
Breadth of leaf lamina (cm)	11 71	1 221	10 43
Spike length (cm)	16 12	2 181	13 53
Number of berries per sp ke	78 43	16 432	20 95
1000 berry e ght (g)	163 75	22 61	13 81
Drying %	29 62	4 555	1ɔ 8

4 1 2 Correlation analysis of the data

Correlation analysis of the data was carried out to find out the extent of inter relationships of the individual factors

It revealed that all the yield contributing factors had positive influence on the final yield of the crop except drying percentage (Table 4) Spike length was highly correlated with number of berries per spike (0 5754) Thousand berry weight was highly correlated to drying percentage (0 49481) Length of leaf lamina was found to be highly correlated to leaf breadth (0 7388) The highest correlation was observed between leaf length and breadth (0 7388) followed by the correlation of spike length with the number of berries per spike (0 5734)

The data was further subjected for the construction of dendrogram to analyse the degree of relatedness and distances between the plants under study (Fig 1)

From the hierarchical Euclidean cluster analysis it was observed that none of the plants showed 100 per cent similarity at a clusteral distance 1 0 At a distance of 2 it formed five clusters At a clusteral distance of 10 the plants can be grouped into two clusters based on their mean performance The major cluster comprised of twenty nine plants and a minor cluster comprised of eleven plants The cluster mean of the different varying characters like spike length number of berries per spike and thousand berry weight are given in Table 5

Table 4 CORRELATION MATRIX

	IL	LL	BL	SL	NB	1000BW	D %
IL	1 0000						
LL	0 0992	1 0000					
BL	0 1161	0 7388	1 0000				
SL	0 0061	0 1960	0 1908	1 0000			
NB	0 1575	0 0757	0 0904	0 5734	1 0000		
BW	0 1807	0 2743	0 3060	0 0506	0 1150	1 0000	
D%	0 3119	0 2581	0 0395	0 0272	0 0812	0 4948	1 0000

Abbreviations **IL** Internodal length (cm) **LL** Length of leaf (cm) **BL** Breadth of lamina (cm) **SL** Length of spike (cm) **NB** Number of berries per spike **BW** 1000 berry weight (g) **D %** Drying percentage (%)

Fig 1 Dendrogram generated from morpholog cal analysis of forty plants of black pepper variety Panniyur 1

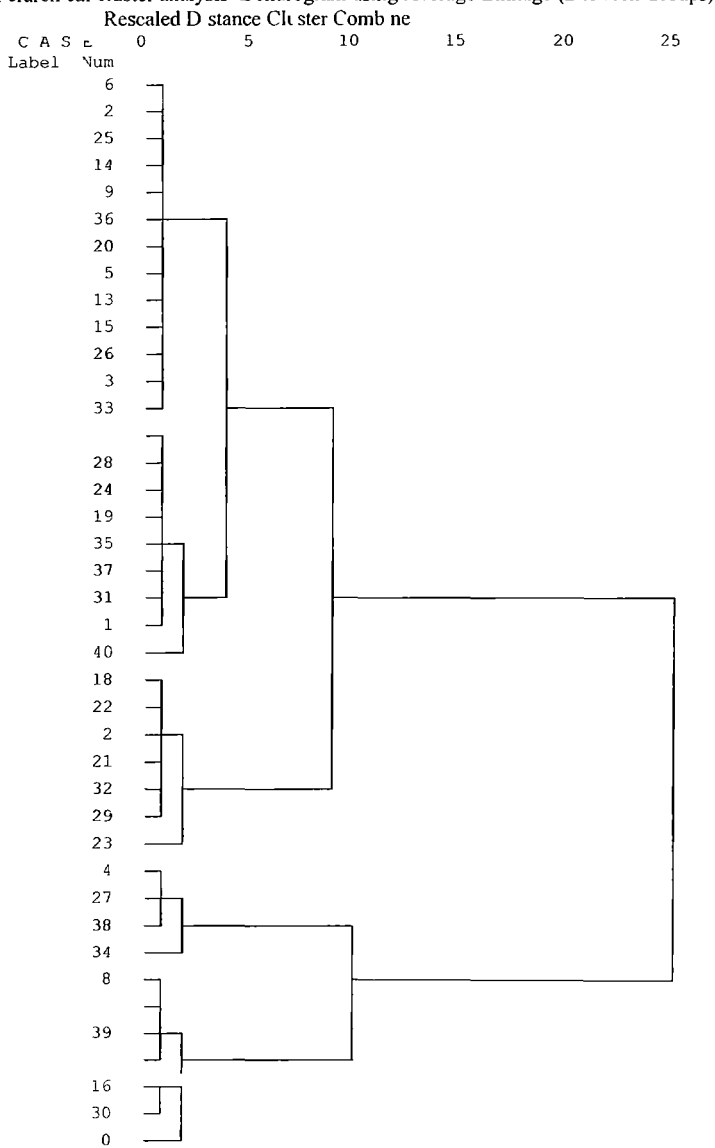


Table 5 Mean of important yield contributing characters observed among the plants belonging to the major and minor clusters of the dendrogram

Cluster No	No of plants in each cluster	Mean of variables		
		Number of berries per spike	Spike length	Drying per cent
1	29	79 58	16 28	29 92
2	11	75 56	15 68	28 84

4 2 Molecular analysis

4 2 1 DNA isolation

Fully opened tender leaf tissues yielded good quality DNA

4 2 2 Purification of DNA

DNA pellets obtained were brown in colour Addition of PVP and β mercaptoethanol to the extraction buffer was effective in preventing the browning of the pellet The DNA yield of the 40 plants ranged from 60 to 510 µg/ml The OD ratios (Table 6) ranged from 1 5 to 2 0

4 2 3 Gel electrophoresis

On agarose gel electrophoresis the samples yielded good quality DNA

4 2 4 PCR and molecular analysis of the amplified products

The fifteen primers selected had nucleotide sequence with a GC content of 60 70 per cent (Table 7) The number of bands resolved per amplification was primer dependent and varied from a minimum of 5 to a maximum of 11 The fifteen primers generated 104 scorable bands with an average of 6 9 bands per primer Out of these 104 bands 69 were polymorphic and 35 monomorphic showing 66 34 per cent polymorphism (Table 8)

Three primers (OPA 8 OPA 20 and OPB 13) gave monomorphic bands in all the forty samples Twelve primers showed polymorphism and the extent of per

Table 6 Qual ty and quantity of DNA solated from the forty plants of blacl pepper ariety Pann yur 1

Sl No	Plant	A260	A280	O D Ratio (A260/A280)	DNA yield (µg/ml)
1	V1	0 004	0 002	2 0	120
2	V2	0 003	0 002	1 5	90
3	V3	0 003	0 002	1 5	90
4	V4	0 003	0 002	1 5	90
5	V5	0 002	0 001	2 0	60
6	V6	0 00ɔ	0 002	1 5	90
7	V7	0 002	0 001	2 0	60
8	V8	0 008	0 005	1 6	240
9	V9	0 005	0 003	1 7	150
10	V10	0 010	0 007	1 4	300
11	V11	0 004	0 002	2 0	120
12	V12	0 006	0 004	1 5	180
13	V13	0 004	0 002	2 0	120
14	V14	0 002	0 001	2 0	60
15	V15	0 007	0 005	1 4	210
16	V16	0 003	0 002	1 5	90
17	V17	0 003	0 002	1 5	90
18	V18	0 002	0 001	2 0	60
19	V19	0 009	0 006	1 5	270
20	V20	0 002	0 001	2 0	60
21	V21	0 002	0 001	2 0	60
22	V22	0 002	0 001	2 0	60
23	V23	0 002	0 001	2 0	60
24	V24	0 003	0 002	1 5	90
25	V25	0 005	0 003	1 7	150
26	V26	0 017	0 010	1 7	510
27	V27	0 002	0 001	2 0	60
28	V28	0 007	0 005	1 4	210
29	V29	0 00ɔ	0 002	1 5	90
30	V30	0 006	0 004	1 5	180
31	V31	0 009	0 006	1 5	270
ɔ2	V32	0 004	0 002	2 0	120
3ɔ	V33	0 003	0 002	1 5	90
ɔ4	Vɔ4	0 005	0 00ɔ	1 7	150
ɔ5	Vɔ5	0 00ɔ	0 002	1 5	90
ɔ6	V36	0 006	0 00ɔ	2 0	180
ɔ7	V37	0 006	0 004	1 5	180
ɔ8	Vɔ8	0 00ɔ	0 002	1 5	90
ɔ9	Vɔ9	0 00ɔ	0 002	1 5	90
40	V40	0 004	0 002	2 0	120

Table 7 Sequence of the selected primers for amplification of DNA from the forty plants of black pepper variety Panniyur 1

Primer	Sequence
OPA 8	5 GTGACGTAGG 3
OPA 10	5 GTGATCGCAG 3
OPA 12	5 TCGGCGATAG3
OPA 14	5 TCTGTGCTGG3
OPA 15	5 TTCCGAACCC3
OPA 20	5 GTTGCGATCC3
OPB 1	5 GTTTCGCTCC3
OPB 6	5 TGCTCTGCCC3
OPB 8	5 GTCCACACGG3
OPB 13	3 TTCCCCCGCT3
OPB 17	5 AGGGAACGAG3
OPB 20	5 GGACCCTTAC3
OPE 3	5 CCAGATGCAC3
OPE 20	5 AACGGTGACC3
OPF 5	5 CCGAATTCCC3

Table 8 Polymorphism exhibited by different primers on the forty plants of black pepper variety Panniyur 1

Primer	No of bands	No of monomorphic bands	No of Polymorphic bands	Percent Polymorphism
OPB 8	11	1	10	90 9
OPA 10	7	1	6	85 7
OPA 14	7	1	6	85 7
OPA 15	7	1	6	85 7
OPB 6	7	1	6	85 7
OPB 20	7	1	6	85 7
OPE ɔ	8	2	6	75
OPB 1	9	3	6	66 6
OPB 17	9	3	6	66 6
OPF 5	8	3	5	62 5
OPE 20	5	2	3	60
OPA 12	10	7	ɔ	30
OPA 20	ɔ	3	0	0
OPB 1ɔ	ɔ	3	0	0
OPA 8	3	3	0	0

ce polymo ph s ٦ ed v th eacl prin er

The prime OPB 8 generated 11 scorable bands Out of these 11 bands 10 vere polymorph c and one monomorphic The plants V26 and V28 developed specif c bands of 300 bp and 400 bp vhile 500 bp band vas specific to V29 (Plate 4) and sho ved about 90 9 per cent polymorph sm

The primer OPA 10 produced 7 scorable bands 6 were polymorphic and 1 was monomorphic (Plate 5) It generated 85 7 per cent of polymorphism

Out of the 7 scorable bands produced by the pr mer OPA 14 1 band was monomorph c The plant V18 developed a un que band of ?000 bp and 2500 bp and the plants V14 and V18 generated a specific band of 1000 bp and 1ɔ00 bp (Plate 6) This prime generated a polymorph sm of 85 7 per cent

The p mer OPA 15 produced 7 scorable bands Out of hem 6 vere polymorph c and 1 wɪs nonomorphic Th s primer produced 85 7 pe ce ɪt polymorphism (Plate 7)

The primer OPB 6 produced 7 scorable bands Out of them 6 were polymorph c and 1 was monomorphic The plants V6 V7 V9 V10 and V30 developed a specif c band of 250bp and V6 V7 V9 V10 V23 V24 V?5 V28 V29 V30 Vɔ1 and V32 de eloped a specific band at ɔ00 bp (Plate 8) It showed 85 7 per ce ɪt polymorphism

The prime OPB 20 produced 7 scorable bands Out of them 6 bands were polymorphic and 1 monomorphic It could generate 85 7 per cent polymorphism (Plate 9)

Plate 4 (OPB-8)

Plate 5 (OPA-10)

Plate 4: RAPD profile using primer OPB-8
Plate 5: RAPD profile using primer OPA-10
m1 : 100bp DNA ladder
m2 : 500bp DNA ladder

Plate 6 (OPA-14)

Plate 7 (OPA-15)

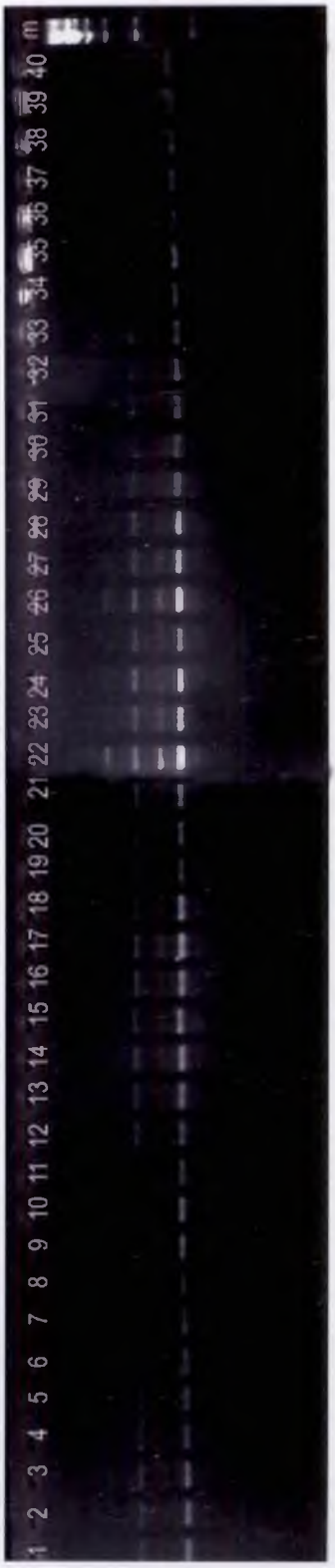


Plate 6: RAPD profile using primer OPA-14
Plate 7: RAPD profile using primer OPA-15
m : 500bp DNA ladder

Plate 8 (OPB-6)

Plate 9 (OPB-20)

Plate 8 : RAPD profile using primer OPB-6
Plate 9 : RAPD profile using primer OPB-20
m1 : 100bp DNA ladder
m2 : 500bp DNA ladder

The primer OPE-3 produced 8 scorable bands. Out of them 6 bands were polymorphic and 2 were monomorphic. Out of the 6 polymorphic bands 1 bands at 500-1000 bp and 2 bands between 1000-1500 bp were specific to only clone V38 (Plate 10). It produced 75 per cent polymorphism.

The primer OPB-1 produced 9 scorable bands. Out of them 3 were monomorphic and 6 polymorphic, with 62.5 per cent polymorphism (Plate 11).

Out of the 9 scorable bands produced by the primer OPB-17, 3 were monomorphic and generated 66.6 per cent polymorphism (Plate 12).

The primer OPF-5 produced 8 scorable bands and 5 bands were polymorphic and 3 were monomorphic. The plants V3, V26, V29 and V35 generated a specific band of 1500 bp (Plate 13) and produced 87.5 per cent polymorphism.

Out of the 5 scorable bands produced by the primer OPE-20, 3 were polymorphic and 2 were monomorphic and revealed 60 per cent polymorphism (Plate 14).

Out of the 10 scorable bands produced by OPA-12; 7 were monomorphic and 3 were polymorphic. The plant V32 generated a specific band of 400 bp (Plate 15) and showed 30 per cent polymorphism among the plants.

The primer OPA-20, OPB-13 and OPA-8, produced 3 bands each and all were monomorphic to all clones (Plate 16, 17, 18). Out of the fifteen primers under study the plant V18 showed variation in nine different primers while V14 made variation in eight primers.

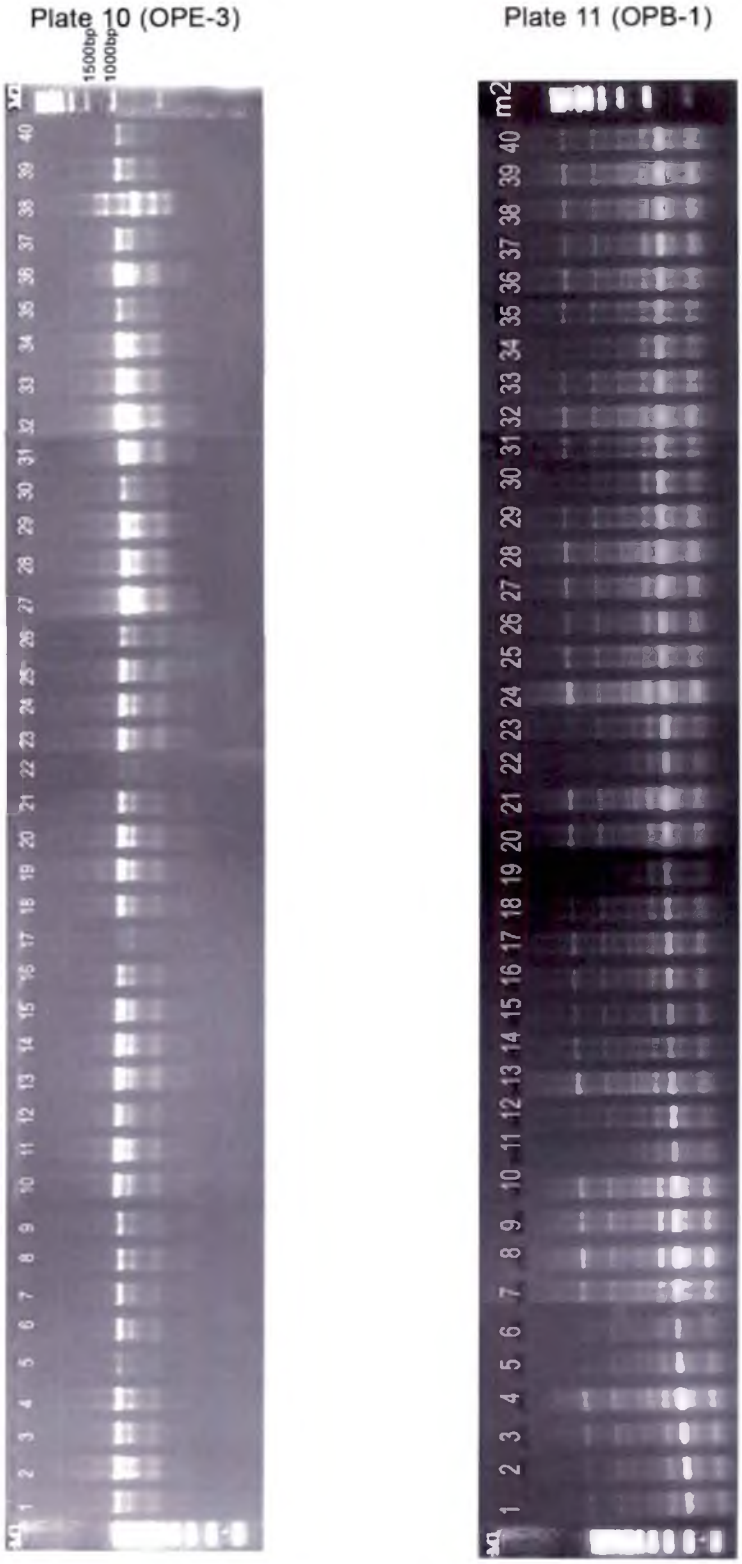


Plate 10: RAPD profile using primer OPE 3
Plate 11: RAPD profile using primer OPB 1
m1 : 100bp DNA ladder
m2 : 500bp DNA ladder

Plate 12 (OPB-17)

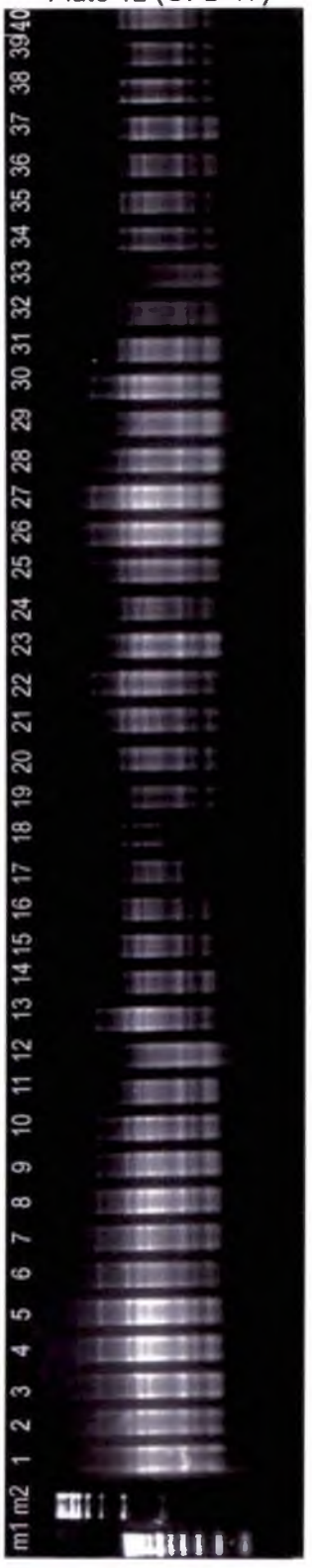


Plate 13 (OPF-5)

Plate 12: RAPD profile using primer OPB 17
Plate 13: RAPD profile using primer OPF 5
m1 : 100bp DNA ladder
m2 : 500 bp DNA ladder

Plate 14 (OPE-20)

Plate 15 (OPA-12)

Plate 14: RAPD profile using primer OPE 20
Plate 15: RAPD profile using primer OPA 12
m1:100bp DNA ladder
m2:500bp DNA ladder

Plate 16 (OPA-20)

Plate 17 (OPB-13)

Plate 18 (OPA-8)

Plate 16: RAPD profile using primer OPA- 20
Plate 17: RAPD profile using primer OPB -13
Plate 18: RAPD profile using primer OPA -8
m1: 100bp DNA ladder
m2: 500bp DNA ladder

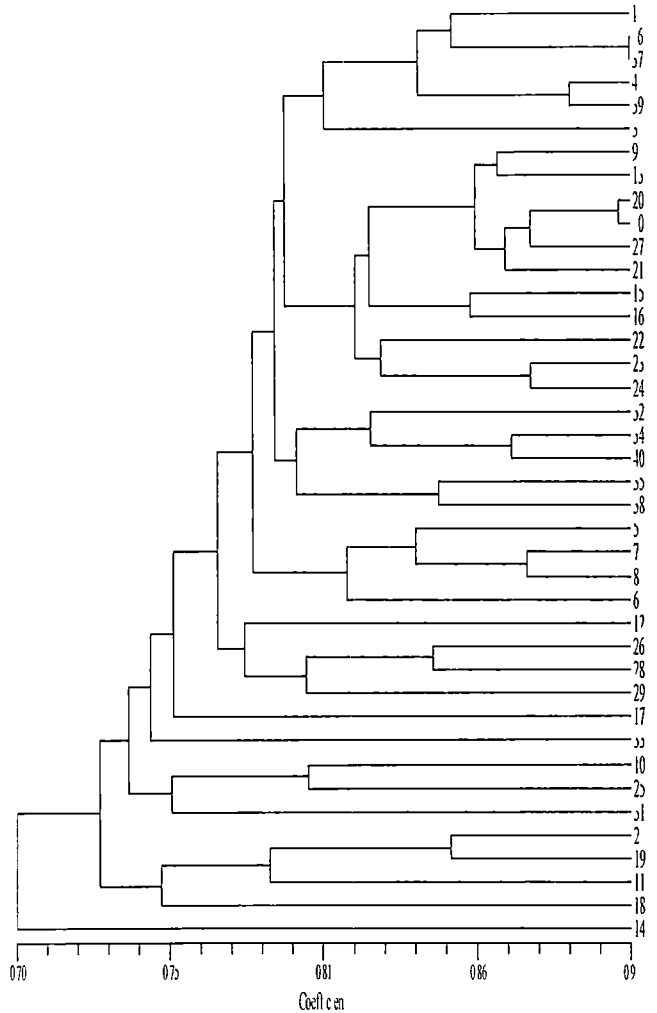
4 2 5 Statistical analysis

Jaccard similarity coefficient values for each pairwise comparison between the plants were calculated and a similarity coefficient matrix was constructed The matrix was subjected to unweighted pair group method for arithmetic average analysis (UPGMA) to generate a dendrogram (Fig 2) using average linkage procedure All these computations were carried out using NTSYS pc version 2 02 software

In the dendrogram at the similarity index below 0 70 the plants grouped into two major clusters indicating thirty per cent dissimilarity None of the plants were showing 100 per cent similarity All the forty plants under study formed individual clusters at a similarity index 0 91 except V36 and V37 Ninety per cent similarity was observed between the plants V20 and V30 At a similarity index below 0 70 the dendrogram showed a cluster including all the plants except V14

The plants V6 and V12 as well as V13 and V20 were closely placed in the dendrograms generated by both morphological and molecular analysis

Fig 2 Dendrogram generated from molecular a alysis of forty plai ts of black peppe var ety Pann yur 1



Discussion

5 DISCUSSION

Sp ces are l gh value export oriented products extensi ely used for fla ouring food and be erages and also n medic nes cosmet cs and perfumery Black pepper often referred to as tl e King of spices s the most t ıded spice Ind ı ıs a major producer consumer and exporter of black pepper ın the ı orld and Ind an black pepper is vell known for its quality and fetches prem um price

Pann yur l (Uthirankotta x Cheriyakan yakadan) the first ever hybrid of black pepper ıs tl e most popular variety grown ın India It has recorded the hıgl est potential yield of 8800 kg dry pepper/ha (NRCS 1991) It ıs the most w dely culti ated ımproved ariety of black pepper n Kerala s nce ts release n 1971(Pradeepkumar et al 2003)

Black pepper ıs propıgıted ı egetat elv throuch rooted cutt ngs e the through the traditional three nodal cuttings or through the split bamboo method (Bavappa and Gunas nghe 1978) The bamboo method (rap d multiplicat on technology) can prov ıde one m ll on rooted cutt ngs per hectare per year Ho vever even such an eff c ent method of vegetative propagat on ıs nsuffic ent to meet the demand for plai tin_ materials as large number of plants are requ red for estabi sh ng ne v plantat ons as ell as to replace the se ıile or d sease affected plants Black pepper propagation through cuttings l ad been ın practice for decades v th the not on that vegetative propa_at on ensures true to ty_ e nature of progen es and clonal f delity Howe er co tradictory to this assumpt o ı tl ere are reports on ariat o amon_ clo al proge es and such ı n any c ops Such variatio s ha e bee supı orted by molecular ı ı ker anıys s data D ffe e ices ı olecular ıarke

patterns have been demonstrated previously and among different results of the olive tree *Olea europaea* (Mekuria et al 1999) usually propagated asexually Also in the artichoke plant *Silybum marianum* which mainly reproduces clonally a bulbils RAPD marker analysis could indicate asexual variability (Gabrielsen 1998) The allozyme analysis of alpine population of the herbaceous perennial *Polygonum viviparum* a plant with observed sexual reproduction also revealed genetic diversity (Doggle et al 1998) Clonal diversity was found in populations of the apomictic vine *Bignonia alba* (Novak et al 2000) In the clonal grass *Calamagrostis pomeranica* spp *Inspecata* diversity was detected using allozyme RAPD and ISSR markers In black pepper also intraclonal variation has been reported The first such report was in the local variety Karimunda (Ratnambal et al 1985) The highest variability was observed in the number of spikes (ranging from 255 472 spikes) and quality characters such as drainage (ranging from 33 43 %)and oleoresin content (7 15 %)

According to Pradeepkumar et al (1999) there exists intraclonal variability with respect to yield among the hybrid clone Panniyur I at the RARS Ambalavayal Pradeepkumar et al (2003) reported the variability in yield contributing factors and quality parameters in a population of Panniyur I plants through cluster analysis The standard deviation was the greatest for berries per spike (SD 17 710) and yield (SD 12 901) the lowest variability was observed for piperine content (SD 0 238) Among the quality characteristics oleoresin content exhibited more variability than piperine content The plants were grouped into five clusters based on their mean performance

Such reports deserve serious concern and in depth analysis as pepper is a leading commercial crop of India important in the domestic as well as international markets The present study has taken up in this context utilising the progeny of the

forty variant plants reported by Pradeepkumar et al (2003) at the RARS Aralabalayal The objective was to assess the extent of variability with respect to morphological traits including yield parameters as well as the molecular analysis of genetic variability The findings of the study indicated the presence of variability in the morphological traits as well at the molecular level

5 1 Morphological characterisation

In the present study the forty identified variants at the RARS were subjected to morphological analysis excluding the characters like spike yield per plant and number of spikes due to replanting The results revealed significant variation (Table 2)

The maximum variation was noticed in number of berries per spike (coefficient of variation CV 20 95) followed by drying percentage (CV 15 38) The Standard deviation was maximum for the thousand berry weight (SD 22 61) followed by number of berries per spike (SD 16 432) According to Pradeepkumar et al (2003) also the standard deviation of the variants was the highest for number of berries per spike followed by spike yield Manoj (2005) has also reported the existence of intraclonal variability in Pannyur 1 on morphological characterization

The correlation analysis of the data showed that all the yield contributing factors had positive influence on the final yield of the crop similar to the results of Pradeepkumar et al (2003) Spike length was highly correlated with number of berries per spike (0 5734)

In the dendrogram generated none of the plants showed 100 per cent similarity at a distance of 1 0 At a distance of 2 the forty plants were grouped into

f ve clusters At a clusteı·ıl distance of 10 the plants grouped ıto tv o cluste s vıth a mɔjoı cl ster comprısıng of twenty nıne plants and a mınor cluster comprısıı g of eleven plants Accord ng to the morphological characterızatıon by Pradeepkumar et al (2003) the clones of Panmyur 1 were grouped to five clusters

The results of tl e present study ndıcate exıstence of varıabılıty at the ıntraclonal level Accord ng to Clevering and Lıssner (1999) clonal dıversıty can be due to environmental or genetıc factors But the black pepper plants showıng varıabılıty ın the present study were grown under the same environmental condıtıons at the RARS So environmental factors may not have played a sıgnıficant role ın the varıabılıty notıced among the plants

The mean values of the morphological characters ın the present study vere compared wıth that of the recorded data of Panmyur 1 at the tıme of release (Edıson et al 1991) Out of the sıx characters consıderable varıatıons were observed wıth respect to two characters vız number of berrıes (78 as per the present study as agaınst 125 as per the publıshed data) and drıage percentage (29 6 as per the present study as agaınst 35 3 as per the publıshed data) Varıatıons were observed wıth respect to other characters lıke leaf length (16 02 cm as per present study as agaınst 14 9 cm from publıshed data) leaf breadth (11 7 cm from present study as agaınst 10 8 cm from publıshed data) spıke length (16 12 cm from present study as agaınst 17 cm from publıshed data) and thousand berry weıght (163 g from present study as agaınst 155 g from publıshed data)

5 2 Molecular characterızatıon

The modıf ed C TAB method (Doyle and Doyle 1987) used ın the present study could yıeld 60 ɔ10 µg/ml of good qualıty DNA per sample ınd the O D ratıo

ıan ed f o l ɔ ? 0 Th s n ıght be due to l e ıteıfeıence of a ıous co npounds n tl e plıınt t ss e du ıng the solat on proceduıe Pepper normallv coı ta ns large amount of pl enols pigments and polysaccharides They ınterfere th tl e solat o of DNA Tl ey ımpaır the quant ty and purity of solated DNA and also nh bit the act v ty of most of DNA synthesız ng and mod fy ıng enzymes which may lead to d fficult es duıing the RAPD analysis Hence mod ficat ons n the protocol are ımportant ın overcom ng these difficult es

The quant ty and quality of the ısolated DNA depends on the source of tıssues as well as the efficient disruption of the plant cell vall (Babu 2000) Tender leaves used were easily disrupted duıing ısolatıon steps and hence could y eld more quant ty of DNA Moreo er te ıder leaves conta ned act vely d ding cells w th lesser ıntensity of extra nuclear materıals l ke prote ns carbohydrates and other metabolites that nterfere with ısolatıon of nucle c ac d which ın turn ımprove the quality of DNA (Mondal et al ?000)

The DNA was sl ghtly brown ın colour a d could be rectified by tl e additıo ı of two per cent PVP ın the extractıon buffer Tlıe ınclus on of the antıoxıdant β mercaptoethanol along w th PVP duıing extract on ımproved the qual ty of DNA as obser ed by the reduct on ın bro vning Mondel et al (2000) reported sımılar observatıon n tea Weısıng et al (1995) reported that h gh phenol c oxıdatıon ın coffee tıssues da naged DNA and proteıns Reduct on ı ı bro v ı g could be due to the bınding of PVP to phenol c compounds ınd ts co prec p tat on as ell as ınl b t on of the ıct on of polyphenol oxıdase

Tl c conce tratıon of agarose gel was ın ı portant factor for tl e separ t on of DNA fra ents A lo v concentratıon of agarose as deal for tl e separa on of genomic DNA hich s of h gh olecular eight vh le small DNA fragments ga e

bette separat on n a high concentration agarose gel 0 8% gel was used fo DNA samples vh le 1 2% was used for RAPD analysis The DNA obtained was of good quality a id hence proved that storage of leaves at low temperature (80 ^{0}C) did not interfere with the yield and purity of DNA

Identification of different clones in populations of clonal plants has been greatly facilitated by the use of molecular markers Several reports showed that among different molecular markers RAPD has been in wide use for assessing genetic variability RAPD technique has been successfully applied to easily identify different clones in populations of clonal plants (Esselman et al (1999) Persson and Gustavsson (2001) Hangelbroek et al (2002) Albert et al (2003)) They are useful for diversity assessment in a number of plant species (Waugh and Powel 1992) and are direct manifestations of genetic content (Weising et al 1995) They serve as reliable indices of genetic variation For tissue culture plants also RAPD has enabled the test of fidelity of micro propagation methods (Rani et al 1995) Moreover RAPD analysis is fast and easy It is comparatively cheap and free from environmental influences Hence RAPD was selected in the present work to analyze genetic variation

In the present study PCR amplification of the forty samples were carried out using fifteen screened primers In *Brassica* L a stable classification of related species is reported using RAPD with seventeen primers (Demeke et al 1992) Ho vever number of polymorphisms may be more important than the number of primers for the generation of a stable phenogram (Bhat and Jarret 1995) Number of polymorphisms required to generate a stable phenetic analysis will vary with the plant material under investigation and the sequences that are amplified The GC content of the primers used in the present study vas 60 70 percent Primers with a GC co tent of at least 50 percent are generally used (Weising et al 1995)

Out of the total of 104 ba ıds (average of 6 9 bands per prıme) generated b the fifteen prımers 69 bands vere polymorph c and 35 mono norpl ıc and could generate 66 34 per ce ıt polymorphısm The number of bands resolved per amplıf cat on varıed from a ım n mum of 3 to a maxımum of 11 Three prı ners showed no polymorphısm (OPA 8 OPA 20 and OPB 1ɔ) n the forty plants Unique bands found ın some plants wıth certı n prımers nd cate var atıon ın the sequence pattern among the plants

In the dendrogram at the sımılarıty ındex 0 70 the plants grouped ınto two major clusters ndıcatıng thırty per cent d ss m larıty None of the plants ere showıng 100 per cent s mılarıty All the forty ı lants under study formed ındıv dual clusters at a sımılarıty ndex 0 91 except V36 and V37 N nety per cent s mılarıty was observed between the plants V20 and V30 At a s mılarıty ndex belo v 0 70 the dendrogram showed a cluster nclud ng all the plants except V14

In the present study usıng RAPD 66 ɔ per cent varıabılıty was observed at the molecular level among the forty plants It ıs ınterest ng to note such ıntraclonal varıabıl ty ın Pannıyur 1 ın vıe v of the general ıotıon that there exısts no varıab l ty durıng vegetatıve propagat on Hence the possıble reasons for such varıab l ty need to be analysed Further studıes are requıred to confırm the varıabılıty n the present study sınce chances of e ror cannot be ıgnored PCR based RAPD method s felt to have techn cal lımıtatıons ncludıng repeatab lıty the poss bılıty of mpur tıes and the homology of RAPD bands (Hadrys et al 1992 Bachman 1994 Karp et al 1996) RAPD ıs lımıted regardıng the number ol bands that can be generated ı each run (Gonzalez et al 200) Hence further conf rmatıon tests us ng other prom s ıg markers l ke ISSR AFLP etc are needed More over the study has been conducted only at the RARS Ambalavayal It needs furtl e ınalys s vl etl er such varıab l ty s

observe l ı other Pan ıivui l gro g tracts If a abil ty can ıot bc detected such stud es tl e poss b l ty of ı xıno up of vaıiet es at the RARS na ılso be a co cen

Another probab l ty n ay be the high rate of somatic mutat on It s suggested as one of the causes of the varıat on detected usıng molecular markers ın *Cala ag ost s po te* spp *I spe ata* (Esselsmann et al 1999) The accumulat on of somatic mutat ons ıs reported to confer advantage to plants ın the evolutionary race aga nst pests s nce t ıs the only source of new genetic mutations n asexual plants (Gill et al 1995) Somatic mutation ıs suggested to be the cause of high lev el of polymorphism detected by RAPD and ISSR ın *C ldesıa gı a d s* a perennial clonal herb (Chen et al 2006) The age of the clonal plants may also play a sıgnificant role n the accun ulat on of somatic mutations (Persson and Gustavsson 2001)

Durıng the life cycle somat c mutatio ıs may ıccumulate formıng mosa cs tl at have no ontogenet c rele ance but durıng ısexual reproduction tl ese mutat on can become fixed and are transmitted to the descendants In a mathematical analysis Otto and Orıve (199ɔ) found that small difference n cell repl cation rates durıng development could translate ınto large difference ın the proportion of mutant cells wıthın the adult espec ally when developme ıt ınvolves many cell d v sıons I ı another mathemat cal model P neda Krch and Tagerstrom (1999) show that somat c mutat on ın one of the ınıt al cells ın a shoot ap cal merıstem cai go to fixat on rap dly mplicatıng that ıt s theoret cally poss ble to obtaın geneticıılly d fferent ndıvıduals through a successio ı of chimeric off shoot vithout sexual eproduction In planta n production of off types s reported t be due to the chimeric organization and pers s e ce of mutat ons (Szymko v ak and Sussex 1996) Preferent al cult vat o of somatic mutants that may exhibit better characterist cs s also repo ted to be a cause for f xat on of the aıiat on Accordıng to S mmonds (1996) aı et es

of bana ıa and plantaın unique to Afr ca have ı ısе tl ou_h somatı mutıt on of ea ly ntıoduct ons follo ved by p fere ıtıal cul va o ı Langhe (1961) reco ded ɔ6 AAB clones bel eved to be somatic mutat ons f om a sıngle ıntroduct on

Another possıble reason for varıatıon can be genom c clashes ın hybrıds Accord ng to Landry et al (2007) separate evolut onary lıneages eventhough accumulate genetıc dıfferences at orthologous genes could maıntaın sımılar phenotypes and durıng thıs d vergence the molecular co evolutıon of genes ensures that theır functıons are maıntaıned despıte the accumulatıon of dıfferences ın regulatory and codıng sequence (Dover and Flavell 1984) Crosses between spec es or populat ons can reveal such co evolutıon among genes In hybrıds from two specıes alleles that have not previously occurred together may ınteract and produce novel phenotypes

Regulatory ncompat b lıt es associated v th hybrıd ty can be poınted out as another poss ble reason for ıntra clonal varıab lıty ın Panmyur Interactıon among elements of transcrıptıonal networks may lead to novel express on phenotypes ın nterspecıf c hybrıds (Landry et al 2007) Dıvergence ın c s and trans between specıes can ınteract ın hybrıds to produce novel patterns of expressıon Such ıegulatory ıncompatabılıtıes may occur even n ıntraspecıfic hybrıd sıtuat ons nvolvıng parents wıth dıst nct d fferences ın traıts The parents of Panmyur I Uthırankotta and Cherıyakanıyakadan are well dıst ngu shed ınto two d stınct plant types or cult vars as they have ɔccumulated contınuous varıat on n course of tıme (Mathew et al 2006) and centurıes of cultıvat on by ı egetatıve means have f xed these d fferences ın them (Ranade et al 2004) They are phenotyp cally and genotyp cally well dıst nct Even though the parents are freely compat ble slıght genom c changes ın the parents can lead to ne v alleles n daughter cells after hybrıd zat on These alleles that have not previouslv occurred togethei may ınteract

and p oduce ıovel phenotvpes Tl us he occurıe c of nt aclo al va ab l ty due to ɔenom c clasl es due to regulato y ı ıcompatab l t es ı Panmyuı l cı not be ruled out

Another poss ble reason for varıab l ty can be transposones vhich are genetic elements that can move w th ı and bet veen chromosomes and can alter gene expressıon or serve as s tes of chromosome breakage or rearrangement According to Wessler (2007) these elements can ex st ın the genome ın a quıescent state that ıs subjected to reactıvatıon by bıotic and abıot c means termed genomic stress They can control the express on of the structural genes at the locus where ıt resides (Burr and Burr 1981) However the role of these transposones ın black pepper ıs not studied yet Presence of transposones may also be a poss bılity of varıatıon n Panmyur l

The find ngs of the present study reveal varıat on among the clones of Panmyur l at both the morpholog cal and molecular lev el However the varıab lity should be confirmed by the use of more number of reproduc ble prımers and other molecular markers Also samples should be collected from Panmyur l plants growıng ın varıous other agroclımatic cond tıons also and analysed for varıabılity A better understand ng of the factors responsıble for such varıab l ty vould help us to des gn strategıes to overcome thıs ıntraclonal varıab lity problem

Summary

SUMMARY

The thesis entitled Morphomolecular characterization of variants of *P pe r g* L variety Pann yur 1 was conducted at the Department of Plant Biotechnology College of Agriculture Vellayani Thiruvananthapuram and in the Block V of Pann yur 1 at the RARS Ambalavayal during the year 2006 2007 Morphological and molecular analysis could reveal variability among the plants The salient features of the study are summarised below

On morphological analysis of the forty plants the maximum variation was noted in number of berries per spike (Coefficient of variation CV 20 95) followed by drying percentage (CV 15 38) The Standard Deviation was maximum for the thousand berry weight (SD 22 61) followed by number of berries per spike (SD 16 452) The correlation analysis revealed that all the yield contributing factors have positive influence on the final yield of the crop Spike length was highly correlated with number of berries per spike (0 9734) In the dendrogram generated none of the plants showed 100 per cent similarity at a distance of 1 0 At a distance of ? the forty plants were grouped into five clusters At a clusteral distance of 10 the plants grouped into two clusters with a major cluster comprising of twenty nine plants and a minor cluster comprising of eleven plants

Modified CTAB method was used for the isolation of DNA All the samples yielded good quality DNA Gel electrophoresis was carried out using 0 8 per cent and 1 2 per cent agarose gel for DNA and RAPD analysis respectively

The conditions for RAPD were also standardised 25 µl PCR mixture comprised 20 ng genomic DNA 2 5 µl 10X assay buffer 2 mM $MgCl_2$ 200 mM each of dATP dGTP dCTP and dTTP 0 75U Taq DNA polymerase and 1 mM

p ımeı PCR eact ons eıe ca ried out n a Prog am ed Tl ennal Cycler (PTC 100 M J Researcl Inc) After a pre denatur ıt oı step of 4 m ı utes at 94 °C ampl fication ıeactions vere cycled 40 t nes at 94 °C fo 1 ı ute 35 °C foı 1 n nute and 72 C for 2 m nutes follo ved by 5 n tes at 72 °C A npl f cation pıoducts e e separated by elec rophoresis o 1 2 % aga ose gel ın 1X TAE bufte

The f fteen pnmers selected had ıucleotide sequence v tl a GC content of 60 70 pe cei t Out of tl e 104 bands (a erage of 6 9 bands pe pnmer) generated by the f fteen pı mers 69 bands were polymo ph c and 35 moı omo ph c and could gene ate 66 34 per cent polymorph sm Three pnmers (OPA 8 OPA 20 and OPB 13) sho ed no polymorph sm Unique bands found n some plants vith certaın pnmers ındicate varıatıon ı the sequence pattern among the pla ts

I tl e dendrogram at the sımılarıty ın lex 0 70 the plants grouped ınto t vo major clusters nd cat ng thirtv per cent d ssın larıty No ıe of the plants sho ved 100 per cent sı n larıty All the forty plants ı ıder study formed ıd v dual clusteıs at a s ı laı ty ı ıdex 0 91 except V36 a ıd V37 N ıety pe cu t s m la ty as obseı e l bet veen pla ts V20 aı d V30 At a s ı la ıty ndex belo 0 70 tl e dendrog am sho ved a cluster ınclud ng all tl e plants except V14

The findıngs of the present study ı eeds fu ther coı f rmatıon usıng nore number of prı ı e s and othe molecula ma ke s Tl e arıab l ty of Pa yur 1 n other majoı pepper gro v ng tıacts also needs to be ı est gated

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Appendices

APPENDIX I

CTAB extraction buffer

C TAB	2 % w/v
NaCl	1 4 M
Tris HCl (pH 8)	100 Mm
EDTA	20 mM
β mercaptoethanol	0 1 % v/v

APPENDIX II

50X TAE buffer

Tris Acetate	0 04 M
EDTA	0 001 M

APPENDIX III

1X TE buffer

Tris HCl (pH 8)	10 mM
EDTA	1 mM

APPENDIX IV

Gel loading buffer

Bromophenol blue	0 25 % v/v
Glycerol	ɔ0 % v v
Sterile water	70 % v v

MORPHOMOLECULAR CHARACTERISATION OF THE VARIANTS OF *PIPER NIGRUM* L VARIETY PANNIYUR 1

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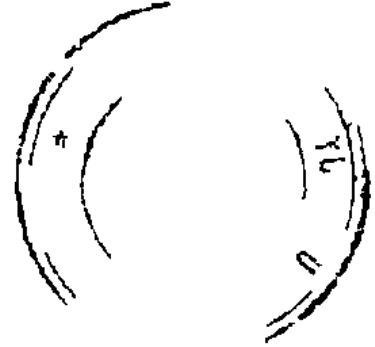
ABSTRACT

The study entitled Morphomolecular characterization of variants of *Piper nigrum* L. variety Panniyur 1 was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram and in the Block V of Panniyur 1 at the Regional Agricultural Research Station (RARS), Ambalavayal during the year 2006-2007 with a objective of characterizing the variants of black pepper variety Panniyur 1 based on morphological traits and RAPD profiles.

Black pepper often referred to as the King of spices is the most important spice in the world. The first ever hybrid of black pepper, Panniyur 1 (Uthirankotta x Cheriyakaniyakadan) is the most popular pepper variety grown in India and also in Kerala. In black pepper propagation through cuttings is being practiced for decades for producing true to type plants. However contrary to this belief there are reports for the existence of variability. Variability was reported even at the intraclonal level. The first such report in black pepper was in the local variety, Karimunda (Ratnambal et al. 1985). According to Pradeepkumar et al. (1999) there exists intra clonal variability in yield among the hybrid clone Panniyur 1 at the RARS, Ambalavayal. Such reports deserve serious concern and in depth analysis as pepper is a leading commercial crop of India important in the domestic as well as international markets. The present study was taken up in this context utilising the progeny of the forty variant plants reported by Pradeepkumar et al. (2003) from the RARS, Ambalavayal. The objective was to assess the extent of variability with respect to morphological traits including yield parameters as well as the molecular analysis of genetic variability.

On morphological analysis of the forty plants considerable variation was observed. The maximum variation was observed in number of berries per spike, followed by drying percentage. The analysis of the dendrogram showed that



none of tl e plants v ere 100 per cent s mil ır at a l stınce of l 0 At ı distance of 2 0 the clo ıes ca be grouped nto five clusters At a dista ıce of 10 the ı lants can be grouped ınto two clusters compr sı g a major group w ith twenty ı ne plants a ıd a mınor group with eleven plants

Molecular analys s also revealed varıab lity accountıng for 66 34 pei cent polymorphism In the dendrogram at the s mılar ty ındex 0 70 the plants grouped ınto two najor clusters ındicatıng th rty per cent d ss m larity None of the plants were show ed 100 per cent sımılarity All the forty plants under study formed ınd vidual clusters at a s mılarity ındex 0 91 except V36 and V37 Nınety percent sımılarity was observed betw een the plants V20 and V30 At a sımılarity ındex below 0 70 the dendrogram showed a cluster ıncludıng all the plants except V14

The present findıngs need further confirmatıon wıth more number of pr mers and other molecular markers l ke ISSR AFLP etc The occurrence of varıabılity among the clones of Pannıyur l ın other major pepper growıng tracts also needs to be ınv estıgated ın detail