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MOLECULAR CHARACTERIZATION OF *Piper* SPECIES USING RAPD TECHNIQUE

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Horticulture

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Kerala Agricultural University

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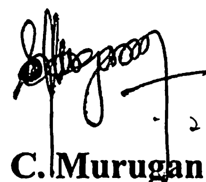
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2002

DECLARATION

I hereby declare that this thesis entitled **"Molecular characterization of *Piper* species using RAPD technique"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.



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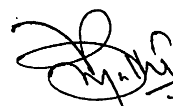

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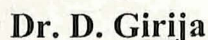

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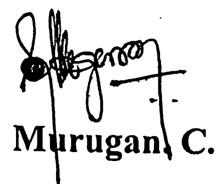

Murugan C.

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Introduction

INTRODUCTION

Black pepper, the king of spices is the dried fruits obtained from the perennial climbing vine, *Piper nigrum* which is indigenous to the tropical forests of Western Ghats of South India. It is one of the important and earliest known spices produced and exported from India. India produces 58,290 t of black pepper from an area of 1.92 lakh ha. It is the highest foreign exchange earner among Indian spices, the value realized being Rs. 21192.50 lakhs through export of 24000 tonnes of black pepper during 2001-02. Kerala contributes about 96 per cent of the total production of India. Pepper is one of the most ancient crops cultivated in India and has probably originated in the South Western Ghats of India, where it is met with in the wild state in the rain forests extending from North Kanara to Kanyakumari. It is also cultivated in Indonesia, Malaysia, Srilanka, Brazil, Thailand and other tropical countries.

The genus *Piper* contains several economically important species like *P. nigrum, P. longum, P. chaba, P. betle* etc. For many years the general problems of differentiation and evolution of the genus had been neglected, although some papers with newly described taxa and regional revisions were published.

Efforts were taken in the recent past to characterise the genus *Piper* using cyto morphological and biochemical (isozymes) markers. However these are dependent on developmental stages and are also influenced by environment. So they cannot depict an exact relationship among the species in the genus. Members of family *Piperaceae* differ greatly in morphology, chromosome number and

ecological adaptation, thereby leading to the description of large number of species. Information on the degree and distribution of genetic variation is essential for developing more efficient ways of evaluating, utilizing and conserving bio-diversity. Conventional methods like morphological and biochemical characterization have limitations. Hence it is essential to use modern molecular marker technology for this purpose.

In recent years, DNA based genetic markers have been developed for the detection of polymorphism, which open up new vistas in genome analysis and in elucidating the evolutionary history of plants. The most reliable method is RFLP analysis but this procedure is time consuming, labour intensive and expensive. Welsh and Mc Clelland (1990) and Williams *et al.* (1990) reported a novel technique called Random Amplified Polymorphic DNA (RAPD) based on the amplification of DNA sequences by polymerase chain reaction (PCR) with random primers. Recently, RAPD technique is extensively applied in plant genetics and clonal identification, pedigree analysis, genetic mapping, QTL mapping, phylogenetic studies, germplasm analysis etc.

Studies on phylogeny and evolution are essential pre-requisites for any crop improvement programme. The present study was conducted during 1999-2001 in the department of Plantation Crops and Spices with the following objectives:

i) To study the genetic polymorphism in *Piper* species at molecular level.

ii) To elucidate species relationship using RAPD analysis.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Phylogenetic and systematic studies in *Piper*

The earliest record of the description of *Piper* of Indian sub-continent was by Rheede (1678) in his *Hortus Indicus Malabaricus*, wherein he described five types of wild pepper including black pepper and long pepper. Linnaeaus (1753) included 17 species from India in his *Species Plantarum*. Roxburgh (1832) described seven species of *Piper* from Indian peninsula. Miquel (1843) included seven wild species from India in his monograph on *Piper*. Wight (1853) in his *Icones Plantarum Indiae Orientalis*, illustrated 16 species, 15 of which were from Indian Peninsula.

Hooker (1886) described 45 species in genus *Piper* of which 29 were assigned to Indian peninsula. Gamble (1925) in his '*Flora of the Presidency of Madras*' described 13 species viz., *P. argyrophyllum*, *P. attenuatum*, *P. galeatum, P. trichostachyon, P. longum, P. hapnium, P. brachystachyum, P. schmidtii, P. hookeri, P. hymenophyllum, P. barberi, P. nigrum* and *P. wightii* from South India. *P. bababudani, P. silentvalleyensis* and *P. pseudonigrum* were not included in Hooker's flora. They were later described respectively by Rahiman (1981), Ravindran *et al.* (1987) and Velayudhan and Amalraj (1992).

Hooker (1886) had included *P. argyrophyllum* and *P. attenuatum* under the section Eupiper along with *P. nigrum*. *P. galeatum* was included in section Muldera along with *P. trichostachyon*. *P. longum, P. chaba, P. betle* and *P. hapnium* were members of section Chavica.

2.2 Morphology

Plants belonging to the genus *Piper* exhibit considerable variation in their morphological characters. Rahiman (1981) observed the morphological variations among them as follows. All the species were climbers except *P. longum*, which was a creeper. *P. nigrum* and *P. bababudani* showed twining habit to a greater extent. Stem girth showed marginal increase from 0.05 cm in *P. longum* followed by *P. hapnium, P. attenuatum, P. argyrophyllum, P. nigrum, P. bababudani* reaching upto 7 cm in *P. trichostachyon* and *P. galeatum.* Samuel *et al.* (1983) reported marked difference in foliar characteristics such as leaf shape, size and venation among the members of the genus *Piper.* The leaf shape varied from broadly ovate in *P. longum* and *P. betle* to lanceolate or ovate lanceolate in *P. argyrophyllum.* As per some of the earlier works, majority of the species had 5-9 principle nerves which were multiple palmately or rarely nerved. Stipules none to two, connate or adnate to the petiole (Hooker, 1886; Hains, 1924; Gamble, 1925; Saldanha and Nicholson, 1976).

Most of the *Piper* species were unisexual. Inflorescences were cylindrical and erect in *P. longum* and *P. hapnium* while filiform and drooping in *P. nigrum, P. bababudani, P. argyrophyllum, P. attenuatum* and *P. galeatum.* Among the species, *P. argyrophyllum* and *P. attenuatum* showed many similarities in morphological characters which made them difficult to distinguish in the case of male plants. But they could be distinguished in female plants based on spike and berry characters (Rahiman, 1981).

P. bababudani was reported by Rahiman in 1981, which according to him was distinguishable from *P. nigrum* by white flowering spikes, irregularly arranged bract, almost sessile stamens and bolder berries which never turn deep red. In other morphological characters they were very similar. *P. hapnium* and *P. longum* resembled in several morphological characters but for the climbing habit, amplexicaul auricles and absence of multiple nerved lamina in *P. hapnium* (Rahiman, 1981). *P. pseudonigrum* is a newly reported species (Velayudhan and Amalraj, 1992) and is related to *P. nigrum* and *P. trichostachyon* in many respects.

Ravindran (1990) suggested a taxonomic key for the *Piper* species occurring in Western Ghats. He subdivided the genus into two sections Pippali and Maricha based on the orientation of spikes, erect or pendent.

Anand (1997) observed that *P. argyrophyllum* and *P. attenuatum* were morphologically similar except for the hairyiness on petiole and under surface of the leaves. *P. bababudani* and *P. nigrum* were very similar in vegetative morphology. The two species differed in bract characters and size and colour of fruits. Except *P. longum* and *P. colubrinum* all the species were climbers. *Piper longum* was a creeper and *P. colubrinum* was a shrub with stilt roots. Among the species only *P. betle* had purple stem colour. *P. chaba* showed distinct dimorphism between orthotrops and runners in leaf shape and venation whereas this distinction was not shown by rest of the species.

Based on the morphological comparisons, a key for species identification was suggested as follows.

A Plants erect shrubs with stilt roots, stem dark green with white patches, leaves elliptic ovate, venation eucamptodromous, inflorescence pendulous, bracts peltate. *P. colubrinum*

A.A. Plants creepers, stipules adnate up to one third the length of petiole, venation acrodromous, inflorescence erect, bracts peltate, fruits cylindrical. *P. longum*

A.A.A. Plants climbers with medium to high amount of adventitious roots with clinging ability.

B. Leaves cordate, stipules adnate up to half the length of petiole, venation campylodromous. *P. betle*

B.B. Leaves lanceolate, exstipulate, venation eucamptodromous, inflorescence erect, bracts peltate, fruits conical, distinct dimorphism between orthotrops and runners. *P. chaba*

B.B.B. Leaves cordate to elliptic ovate, venation acrodromous, inflorescence pendulous, bracts sessile and adnate to rachis, berries conical, turn globose when mature, turning to dark green to black on ripening.

C. Petioles and under surface of leaves hairy *P. argyrophyllum*

C.C. Petiole and both surfaces of leaves glabrous *P. attenuatum*

B.B.B.B. Leaves cordate to ovate, venation campylodromous, inflorescence pendulous.

C. Bracts sessile and adnate to rachis, berries globose, turning to red on ripening. *P. nigrum*

C.C. Spikes fleshy, bracts hooded at the tip, arranged in clear spirals, bigger berries, turning to orange on ripening. *P. bababudani*

2.3 Biometrics

Based on D^2 analysis carried out in both male and female plants of eight species, Rahiman and Bhagavan (1985) found that *P. attenuatum* and *P. argyrophyllum* formed one cluster. *P. trichostachyon* was clustering with *P. galeatum*. *P. nigrum* and *P. longum* retained their identity and formed separate clusters indicating that these species were distinct from all other species.

Ravindran *et al.* (1992) carried out centroid cluster analysis in 11 species of *Piper* based on observation of 30 characters. They had observed that *P. argyrophyllum* and *P. attenuatum* were closely related and formed a single cluster. *P. galeatum* and *P. trichostachyon* were part of second cluster. *P. nigrum* clustered along with *P. wightii* while *P. longum* stood alone. This study led to six clusters of *Piper* genus. They are

Cluster A - *P. attenuatum, P. argyrophyllum*

Cluster B - *P. schmidtii, P. galeatum, P. trichostachyon*

Cluster C - *P. nigrum, P. wightii*

Cluster D - *P. hymenophyllum*

Cluster E - *P. silentvallyensis, P. mullesua*

Cluster F - *P. longum*

2.4 Anatomy

2.4.1 Stem

According to Pal (1981) there was variation in layers of tunica in shoot apices of different species. Anatomical variation in *Piper* species was reported in stem endodermis development also (Bond, 1931).

Van Teighem (1908) grouped the plants in the genus *Piper* into three categories, viz. the plants having one central canal accompanied by peripheral canals, others having only central canal, whilst in the third group they were absent. Murthy (1959) noted the presence of mucilage canal in *P. betle* and their absence in *P. longum*. Pal (1981) reported presence of mucilage canals in *P. betle, P. nigrum* and *P. longum*.

2.4.2 Leaf

Datta and Dasgupta (1977a) reported varying number of layers of cells in hypodermis in *P. nigrum, P. betle* and *P. longum*. According to him, adaxial hypodermis was unilayered in *P. longum* and clearly 2-3 layered in varieties of *P. betle*. Abaxial hypodermis was unilayered in *P. longum*, 1-2 layered in all the varieties of *P. betle* and 2-3 layered in *P. nigrum*.

Leaf epidermal studies by Samuel *et al.* (1984) showed that epidermal cells of *P. betle, P. longum* and *P. argyrophyllum* were much larger than that of *P. nigrum* and *P. attenuatum*. Albeit, morphological features of *P. nigrum* and *P. attenuatum* were quite distinct, there was strong resemblances in their leaf epidermal characteristics.

2.4.3 Root

Datta and Dasgupta (1977b) based on root anatomical studies, proposed the sequential advancement among *Piper* species as *P. longum, P. nigrum* and *P. betle.*

2.5 Cytogenetics

2.5.1 Chromosome number

The cytological investigations by various workers showed wide variations in chromosome number of the same species in the genus *Piper* (Table 1).

2.5.2 Karyomorphology

According to Sharma and Bhattacharya (1959), out of the four species of the genus *Piper* studied, *P. nigrum* possessed one pair of chromosome characteristic to the species. They had also reported that chromosome sizes ranged from 0.6-1.7 μ in *P. longum* with 2n = 48 and 0.6-2.2 μ for another type with n = 24.

In *P. betle,* chromosome size ranging between 0.7-2.5 μ was reported for 2n = 78 varieties (Mathew, 1958) and 0.72-1.2 μ for 2n = 64 varieties (Dasgupta and Datta, 1976). The Karyotypes of *Piper* species showed a gross uniformity in size with 0.56-2.41 μ in *P. betle,* 0.56-2.05 μ in *P. nigrum,* 0.56-1.48 μ in *P. chaba*, 0.74-1.9 μ in *P. attenuatum* and 0.74-1.85 μ in *P. longum. P. attenuatum* showed three Karyotypes while *P. nigrum* and *P. longum* showed only two Karyotypes. *P. betle* mostly showed two Karyotypes but one variety, Jhol

Table 1. Somatic chromosome number reported in *Piper* spp.

Species	Somatic chromosome number (2n)	Reference
1	2	3
P. argyrophyllum	36, 39	Samuel and Bavappa (1981)
	52	Rahiman and Nair (1986)
	36	Anand *et al.* (2000)
	52	Ravindran and Babu (1994)
P. attenuatum	26, 39	Samuel (1986)
	36	Bai and Subramanian (1985)
	52	Jose (1981)
	52	Jose and Sharma (1983, 1984)
	52	Rahiman and Nair (1986)
	52, 104	Ravindran and Babu (1994)
	52	Anand *et al.* (2000)
P. bababudani	52	Rahiman (1981)
	32	Anand (2000)
P. barberi	52	Babu *et al.* (1992)
	52	Mathew and Mathew (1992)
	64	Sharma and Bhatacharya (1959) Dasgupta and Datta (1976)
	78	Mathew (1958)
P. betle	32	Janakiammal (1945) Johanson (1930) Anand *et al.* (2000)
P. boehmeriafolium Wall.	52	Jose and Sharma (1984)
P. brachystachyum	132	Bai and Subramanian (1985)
	132	Ravindran and Babu (1994)
P. chaba Hunter	24	Janakiammal (1945), Anand *et al.* (2000)
	104	Jose and Sharma (1984)
P. chuvya	52	Samuel and Bavappa (1981)
P. colubrinum Lamk.	26	Ravindran and Babu (1994), Anand *et al.* (2000)
P. cubeba Linn.	24	Janakiammal (1945)
	24	Dasgupta and Datta (1976)
	24	Jose and Sharma (1983, 1984)
P. futokazura Sub. *et* Zuce.	24	Yoshida (1960)
P. galeatum ...	40	Bai and Subramanian (1985)
	52	Rahiman and Nair (1986)
	52	Ravindran and Babu (1994)

Contd.

Table 1. Continued

1	2	3
P. geniculatum Sw.	28	Maugini (1951)
P. gibbilimbum	130	Lebot *et al.* (1991)
P. grissico-argenta Yunck.	22	Smith (1966)
P. hapnium	52	Rahiman (1981)
P. hookeri Miq.	60 104	Bai and Subramanian (1985) Rahiman and Nair (1986)
P. hymenophyllum	104	Ravindran and Babu (1994)
P. longum	24 44 48 52 52 52 53 96 32	Tjio (1948) Sampathkumar and Navaneethan (1981) Dasgupta and Datta (1976) Mathew (1958) Jose and Sharma (1983, 1984) Rahiman and Nair (1986) Samuel and Morawetz (1989) Sharma and Bhattacharya (1959) Anand *et al.* (2000)
P. magnificum Trel.	24 26	Dasgupta and Datta (1976) Smith (1966)
P. medium Jacq.	28	Maugini (1953)
P. methysticum	130	Lebot *et al.* (1991)
P. mullesua Ham.	52 104	Samuel (1986) Rahiman and Nair (1986)
P. obliqum	52	Samuel (1986)
P. ornatum N.E.Br.	52 80	Samuel (1986) Sharma and Bhattacharya (1959)
P. peepuloides Roxb.	156	Jose and Sharma (1984)
P. posteltanum	26	Ono (1975)
P. pseudonigrum	32	Anand *et al.* (2000)
P. schmidtii Hook. f.	96	Bai and Subramanian (1985)
P. subpeltatum	24	Johansen (1930)
P. sugandhi	52	Ravindran and Babu (1994)
P. sylvestre Lamk.	26, 39	Samuel and Bavappa (1981)
P. thawaitseii	39, 65	Samuel and Bavappa (1981)
P. trichostachyon	52 52	Rahiman and Nair (1986) Ravindran and Babu (1994)
P. trineuron	26	Samuel and Bavappa (1981)
P. umbellatum	28	Gadella (1972)
P. ungiculatum Ruiz. and Pav.	26 28	Bedi *et al.* (1981) Maugini (1951)

Contd.

Table 1. Continued

1	2	3
P. nigrum	128	Janakiammal (1945)
	104	Mathew (1958),
		Samuel and Bhavappa (1981), Jose and Sharma (1983 and 1984)
	52	Anand *et al.* (2000), Rahiman and Nair (1986), Nair *et al.* (1993)
		Sharma and Bhatacharya (1959)
	48	Dasgupta and Datta (1976)
	36, 60	
P. wichmanii	130	Lebot *et al.* (1991)
P. wightii Miq.	52	Ravindran and Babu (1994)
P. zeylanicum	39	Samuel and Bavappa (1981)
Piper spp. (undetermined)	104	Mathew (1958)

Bangla showed the additional Karyotype which was present in *P. attenuatum* (Jose, 1981; Jose and Sharma, 1985).

2.5.3 Species affinities and evolutionary pattern based on cytogenetics

Sharma and Bhattacharya (1959) had reported that polyploidy might have played a distinct role in the origin of *P. longum, P. nigrum* and *P. betle.* Chromosome numbers were multiples of 12 and 16 in *P. longum* (2n = 24 and 96) whereas in *P. nigrum* it was multiplies of 16, i.e., 48 and 128 respectively. Jose and Sharma (1985) reported chromosome numbers ranging from 24 to 195 in the genus *Piper* and suggested that polyploidy had been an important factor in the evolution of *Piper.* According to them, n = 12 might represent the basic set from which n = 13 might have been derived and 13 became deep seated in the genus *Piper*, possibly due to selective advantage.

Rahiman and Nair (1986) classified *Piper* species as North Indian types with n = 12 and South Indian with n = 13. The North Indian types included the species from transgangetic provinces and South Indian types were from South Indian and Ceylon centres of distribution described by Hooker (1886). They have suggested that the species from these two centres had different evolutionary pathway starting from the basic number of 6 and 7. They also supported the earlier hypothesis of Mathew (1958) that the commonly observed haploid number of 13 among the South Indian types might have arisen from the hybridization of the types with n = 6 and 7.

In the light of an earlier report by Samuel and Bavappa (1981) on the occurrence of different species with 2n = 26, Rahiman and Nair (1986) suggested that 26 chromosome types could be diploids and the commonly observed 2n = 52 types could be considered as tetraploids. They have also suggested that the evolution of genus *Piper* is complicated by different primary basic numbers, amphidiploidy and vegetative propagation.

Samuel and Morawetz (1989) have suggested that long term cultivation is responsible for intraspecific numerical variation in *P. nigrum, P. betle* etc. Since polyploidy, amphidiploidy or diploidy had been reported in these species. Based on the studies from both Old World and New World species and Okada's (1986) result, Samuel and Morawetz (1989) disproved correlation between geographical distribution and ploidy indicated by Mathew (1958) based on different basic numbers.

Mathew (1958) had reported that in spite of gross homogeneity in chromosome morphology between different species, each species or variety was characterised by distinct Karyotype of its own. The presence of a pair of chromosome with supernumerary constrictions which was present only in *P. nigrum* also proved considerable structural alteration in the evolution of the species.

According to Jose and Sharma (1985) the genus *Piper* as a whole represented a homogenous assemblage having general uniformity in Karyotype and with difference in number of nucleolar chromosome from one to four pairs. They

were of the opinion that gene mutation and imperceptible chromosome changes if any, had affected the evolution, both at inter-strain and inter-specific levels. Occassional polyploidy and structural alterations involving principal chromosomes with secondary constriction had taken place. Samuel (1986) had opined that in the genus *Piper*, chromosomes of tetraploid species were much smaller than those of diploid species.

Samuel *et al.* (1986) reported significant interspecific DNA variation between species. They had also noticed that several New World diploid species had higher nuclear DNA than several New or Old World species. DNA content per basic genome was on the whole, lower in cultivated species. In *P. nigrum*, DNA content of wild accessions of *P. nigrum* was approximately double that of cultivated tetraploid variety of same species.

2.5.4 Chemotaxonomy

The flavonoid analysis carried out by Rahiman and Subbaiah (1984) had revealed that *P. argyrophyllum* and *P. attenuatum* were biochemically related as they possessed a percentage similarity of 82 and paired affinity of 33.33 per cent. *P. trichostachyon* showed a close similarity biochemically to *P. galeatum* with paired similarity of 89 per cent and paired affinity of 42.2 per cent. *P. nigrum* and *P. longum* were reported to be very distinct among eight species studied.

Samuel *et al.* (1984) reported chemotaxonomic markers by phenolic separation in different species of *Piper*. Species specific spots were found in *P. nigrum* and *P. betle*.

Ravindran *et al.* (1992) noted that *P. nigrum* was the only species having the alkaloid piperine and the whole set of terpenoids that contribute typical black pepper flavour.

A comparative study of flavanoid profile by Ravindran (1990) and Ravindran and Babu (1994) indicated *P. galeatum* and *P. trichostachyon* with 87 per cent affinity, *P. attenuatum* and *P. argyrophyllum* with 79 per cent affinity, *P. argyrophyllum* and *P. hymenophyllum* with 78 per cent affinity, *P. galeatum* and *P. sugandhi* with 82 per cent affinity, *P. longum* and *P. mullesua* with 69 per cent affinity *P. longum* and *P. silentvalleyensis* with 35 per cent affinity and *P. mullesua* and *P. silentvalleyensis* with 57 per cent affinity.

2.6 Isoenzyme analysis

Kochhar *et al.* (1984) noted characteristic taxonomic markers for six varieties in betel vine by peroxidase pattern study. They had also observed that different types of same cultivar possessed the same banding pattern in most of the cases.

Isozyme studies (Sebastian *et al.*, 1996) showed three groups of closely related species. The first group included *P. nigrum, P. pseudonigrum, P. bababudani* and *P. galeatum.* The second group consisted of *P. chaba*, *P. hapnium* and *P. colubrinum. P. argyrophyllum* and *P. attenuatum* formed the third group. *P. longum* and *P. betle* were distinct from all others.

Sebastian and Sujatha (1996) found that the isozyme pattern of peroxidase and esterase are different in different plant parts and vary according to the developmental stage.

2.7 Molecular markers in diversity analysis

With the advent of molecular markers, a new generation of markers has been introduced over the last two decades which has revolutionised the biological world. Molecular markers include biochemical constituents (secondary metabolites) and macro molecules such as proteins, DNA etc. are utilised in various fields like taxonomy, physiology, embryology, genetic engineering etc. Ever since their development they were constantly being modified to enhance their utility and being automated. The discovery of PCR was a land mark in this effort. This enabled the development of marker based gene tags, map based cloning, phylogenetic analysis and marker assisted selection etc.

Sreenath *et al.* (1999) reported the procedure for generating silver stained AFLP marker in coffee including 5 selections of *C. arabica, C. canephora and Psilanthus bengalensis.* The results confirmed the maximum genetic distance of *Psilanthus bengalensis* than other two species. Of the 16 primer combination tested, 8 have good amplification with polymorphic nature, indicating the feasibility of AFLP technique for the genome analysis of coffee.

Augustyniak *et al.* (1999) noted the usefulness of organellar markers in determination of genetic diversity in *Brassica napus* and *Lupinus* species. The 2D-PAGE pattern of protein isolated from mitochondria and chloroplast showed the presence of species specific polypeptides. He also reported a high level of specific mitochondrial DNA polymorphism in Lupinus causing t RNA like sequences.

Davis *et al.* (1998) conducted a study to reveal genetic relationship among cultivated Avocado through RFLP analysis. The results showed the high level of polymorphism among 36 cultivars and all cultivars were assigned a unique genotype based on 14 genetic loci and grouped into 3 major clusters corresponding to the 3 major racial groupings in Avacado.

De Vincente *et al.* (1998) reported the level of polymorphism by RFLP in 33 almond genomic and cDNA probes in a set of 53 European and North American cultivars. Eighteen of these probes were reported to be polymorphic with 48 bands. They have clustered the genome in to five different group and also revealed that the group of Spanish cultivars had a lower level polymorphism than the others.

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

2.7.1 RAPD markers and molecular characterisation

RAPD markers have been widely used to reveal the genetic relationship among the different species of a particular genus and intra specific relationship among them. Numerous genera were studied extensively using this technique.

Balakrishna (1995) reported enormous inter and intra populational variation and intra site variation among the 7 populations of *Avicennia marina*

collected from all over India. There were some similarities between the genotypes collected from Bombay and Ennore. The material has been grouped into three different groups based on his study.

Padmesh *et al.* (1999) reported five major groups of *Andrographis paniculata* based on the RAPD analysis of intra specific variability from the plants collected from all over India and south east Asia. He found a close resemblence of a Thailand accession (AP 48) with Indian accession (AP 38). An accession from Assam (AP 29) was found distinctly divergent from the rest of the Indian genotypes.

Duran *et al.* (1997) characterised 48 east African tall coconut genotypes from all over Africa using RAPD techniques. He had reported numerous sub-groups among the genotypes from the same locality and each group with same genotypes from diverse geographical region.

Faccioli *et al.* (2000) reported six clusters among 39 *Osteospermum* clones and cultivated varieties of different origin using UPGMA cluster analysis. He also reported a high level of polymorphism than reported literature using RAPD in *Osteospermum* and found that the cultivated genotypes of *Osteospermum* were often inter specific hybrids.

Koller *et al.* (1993) reported 14 RAPD markers, that can be used to clearly distinguish among the 11 apple cultivars. He also reported enormous inter and intra genotypic variation and high inconsistency in reproducibility in banding pattern.

Rival *et al.* (1998) carried out a study to investigate the genetic fidelity of somatic embryogenesis derived regenerants of oil palm (*Elaeis guineensis*). Of the 387 arbitrary primers used, 259 were successfully used to amplify oil palm genomic DNA with consistent reproducibility but only 73 primers enabled the identification of polymorphism between clones. No inter clonal variability and no difference between mother palms and regenerants could be identified using the total number of markers scored (8900). He also suggested that RAPD technique is not suitable for the detection of the mantled variant phenotype.

Rapaport *et al.* (1998) have reported the use of RAPD markers for the marker linked sex determination loci in the dioecious plant *Salix viminalis* L. with 4 x 4 factorial mating design. Out of 1080 RAPD bands examined, only a single, 560 kb band was reported to be linked to a sex determination locus.

Krutovskii *et al.* (1998) generated RAPD genome maps of Douglas-Fir *Pseudotsuga menziesii* (Mirb) Franco. The results showed that the highly significant clustering in both *P. mramenziesii* and *P. mramenziesii* var. *glauca*. Both trees were linked maps of similar length of 2600 cm and 3000 cm and the total map size ranged from 2800 cm to 3500 cm.

Major *et al.* (1998) characterized 36 selected *Robinia pseudoacacia* L., *Robinia ambigna* Poir and decanisneana hybrids (*R. pseudoacacia x Riviscos venr*) using RAPD markers. The results were compared with the isozymes. It was found that both RAPD and isozymes showed heterogeneity sufficient for clonal identification. Polymorphic bands of 135 numbers were obtained and the analysis of the bands resulted in UPGMA dendrogram which separated the accessions

studied into 35 composite banding pattern. Principle co-ordinate analysis and multidimentional scaling produced compatible ordinations on the basis of RAPD data, and the RAPD and isozymes based ordinations of the samples were found different.

Gidoni *et al.* (1994) carried out a study to test the feasibility of developing cultivar specific RAPD markers in strawberries *Fragaria ananassa* Dutch. The results showed that the variation between closely related varieties was high and that the cultivar used could be distinguished from each other by using four different primers.

Schnell *et al.* (1995) differentiated 25 accessions of mango (*Mangifera indica* L.) and a maternal half sib family using RAPD marker. Out of 80 primers screened 33 did not amplify, 19 were monomorphic, and 28 gave reproducible, polymorphic bands. The results showed that the number of bands generated was primer and genotype dependent and no primer gave unique banding pattern for any of 25 accessions. The maternal half sib family (MHS) clustered together in principle coordinate analysis while the randomly selected accessions were scattered with no apparent pattern.

Wilde *et al.* (1992) have successfully employed RAPD markers to characterise cocoa (*Theobroma cacao*) clones representing three main cultivated sub-populations, criollo, forestero and trinitario. The DNA amplification pattern were found unique to the individual cocoa clones studied. The results showed amplification product of different sizes and wide distinction between the wild

cocoa species and the cocoa cultivars and the lowest degree of similarity between the cultivars. The band map, a graphical method of recording the RAPD data also confirmed the above results.

Million and Chinnappa (2000) studied the genetic diversity in the *Stellaria longipes* complex using RAPD analysis. A total of 47 individual genotypes were used with 11 random primers. The results revealed a close relationship of *S. longipes* to *S. porsildii* than to *S. longifolia* and *S. borealis* in that order based on molecular data. The results support that the diploid species of *S. longifolia* and *S. porsildii* are the progenitors of polyploid *S. longipes*.

Sharma *et al*. (2000) used RAPD, protein, and morphological markers for the variability analysis in *Podophyllum hexandrum* Royle, an endangered medicinal herb of north-west Himalaya. Morphologically, the selected 30 accessions divided into 4 varient groups having 1, 2, 3 and 4 leaves respectively. SDS-PAGE of root protein produced 23 protein markers of which 16 were polymorphic, based on this the accessions were regrouped into 2 groups (A & B) with 3 sub groups (B_1, B_2 and B_3). RAPD analysis revealed high inter and intra population genetic diversity. Out of a total of 76 DNA markers all were polymorphic with co-efficient of similarity index of 0.25 to 0.75 range and the accessions were divided into 6 groups. This study revealed the inability of protein markers to delineate the accessions in to region specific groups but can cluster the total population into different groups and suggested that the RAPD is more suitable for the elucidation of genetic diversity in *P. hexandrum* than protein analysis.

Saikia *et al*. (2000) reported the application of RAPD marker for the assessment of diversity among 24 accessions of *Taxus walliciana* from north

eastern India using 15 random primer for AP-PCR. Out of the 106 distinct bands produced only 48 were polymorphic. Three primers did not produce any polymorphic band at all. The study pointed out the low level of polymorphism and close clustering with small molecular distances which indicated a narrow genetic base among the trees growing in this region.

Chakrabarti *et al.* (1999) studied the genetic similarity analysis among 18 Indian potato cultivars by using RAPD markers. Out of the total 74 bands produced by 12 random primers, 57 were polymorphic. The results of pair-wise genetic similarity analysis on the basis of presence or absence of bands revealed wide range of variability among cultivars, indicating a wide genetic base of Indian potato cultivars. Kufri Swarna viz., (*Solanum vernei*) showed maximum genetic divergence, Kufri Badshah and Kufri Alankar also formed separate clusters. Rest of the cultivars formed two closely related clusters.

Rana *et al.* (1999) studied RAPD based assessment of diversity in 11 indica rice genotypes including 3 basmati indica, 8 non-basmati indica and one japonica genotypes with 13 selected primers. Of the 146 bands generated, 91 were polymorphic with 0.24 to 3.7 kb size. Cluster analysis revealed a high degree of polymorphic value (0.232) between the basmati genotypes and non-basmati indica genotypes. He also reported high degree of polymorphism between indica and japonica genotypes.

Parani *et al.* (2000) carried out a study to reveal ribosomal DNA variation and phylogenetic relationship among *Cajanus cajan* L. and its wild

relatives. The inter specific RFLP's were used to construct a dendrogram which revealed a close relationship between the cultivated species *C. cajan* and its wild species *C. scarabaeoides*. The results did not support the view that *C. cajan* could have evolved through hybridisation between *C. scarabaeoides* and *C. lineatus*. A close relationship between the Australian species *C. scarabaeoides* and *C. reticulatus* to the Indian species *C. cajan* and *C. platycarpus* respectively was also reported. The species *C. goensis* and *C. lineatus* were distantly related to cultivated and wild species.

Sonia *et al.* (2001) reported that the regenerated plants from same calli were having more or less same genetic make up with the mother plants in a study of genetic analysis of somaclonal variation among callus derived plants of tomato.

Jain *et al.* (2000) carried out a study to reveal reproductive behavior and genetic variability in geographically isolated populations of *Rhododendron arboreum* s.sp. *arboreum* and *Rhododendron arboreum* s.sp. *nilagiricum* from Himalayas and tropical montane forests of Nilgiri hills respectively. The results revealed higher genetic variability among individuals of temperate Rhododendron populations than among those of tropical origin. Based on the results of the dendrogram, genome was separated in to two distinct clusters.

2.7.2 Molecular marker studies in *Piper*

Banerjee *et al.* (1999) carried out a study for the molecular basis of genotypic differentiation between 6 male and 25 female plants of *P. longum* using RAPD techniques using 40 decamer random oligonucleotide primers. They have reported two male-sex associated RAPD markers for the first time in *P. longum*.

Philip *et al.* (2000) conducted a study to reveal genotypic and morphogenetic differences among three female varieties of *P. longum*, one variety each from Assam and Calicut and one released variety, Viswam. RAPD analysis revealed that Calicut variety and Viswam were closer (95% similarity) than Assam variety.

Haneesh Babu (2000) conducted a study to assess the genetic stability in tissue culture derived black pepper plants using RAPD analysis with three selected primers. He observed monomorphic banding pattern for the tissue culture regenerants with their respective source plants. He also confirmed the uniformity and genetic stability for the tissue culture regenerants.

Pradeepkumar *et al.* (2001) reported cultivar specific banding pattern in *Piper nigrum*. Total number of markers per primer ranged from 4 to 21 and the range of polymorphic markers per primer ranged from 3 to 21 with a mean of 15.3 polymorphic bands per primer.

Materials and Methods

3. MATERIALS AND METHODS

The present study was carried out in the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Vellanikkara during 1999-2001. Experiments were carried out to standardise the DNA isolation protocol and RAPD analysis in different species of black pepper to elucidate the species relationship in the genus *Piper*.

3.1 Materials used

3.1.1 *Piper* species and accessions

Rooted cuttings of 13 different *Piper* species/accessions (Plate 1) maintained in polybags in the green houses of Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara were used in the study DNA isolation protocols were standardised, using *P. nigrum* var. Panniyur 1.

Table 2. List of species used in the study

Sl. No.	Species	Accession No.	Origin
1	*P. nigrum* Linn.	Panniyur 1 (P 1)	KAU
2	*P. nigrum* Wild	P 49	Western Ghats
3	*P. attenuatum* Ham.	TCR 321 (NBPGR)	Western Ghats
4	*P. chaba* Hunter	P 86	N.E. India
5	*P. longum* Linn. Male	PLD	Dehradun
6	*P. longum* Linn. Bisexual	P 25	Western Ghats
7	*P. betle* Linn.	Kasuri (P 87)	Western Ghats
8	*P. arboreum* Aubl.	P 91	S. America
9	*P. colubrinum*	P 85	S. America
10	*Piper* species	P 90	S. America
11	*P. magnificum*	TCR 266 (NBPGR)	S. America
12	*P. bababudani* Rahiman	TCR 364 (NBPGR)	Western Ghats
13	*P. pseudonigrum* Velayudhan & Amalraj	TCR 315 (NBPGR)	Western Ghats

Piper arboreum

South American Piper

Piper chaba

***Piper longum* (P-25)**

Piper magnificum

***Piper nigrum* (Panniyur 1)**

Plate 1. Some of the *Piper* species studied

OPP 14	CCAGCCGAAC
OPP 15	GGAAGCCAAC
OPP 16	CCAAGCTGCC
OPP 17	TGACCCGCCT
OPP 18	GGCTTGGCCT
OPP 19	GGGAAGGACA
OPP 20	GACCCTAGTC
OPAH 1	TCCGCAACCA
OPAH 2	CACTTCCGCT
OPAH 3	GGTTACTGCC
OPAH 4	CTCCCCAGAC
OPAH 5	TTGCAGGCAG
OPAH 6	GTAAGCCCCT
OPAH 7	CCCTACGGAG
OPAH 8	TTCCCGTGCC
OPAH 9	AGAACCGAGG
OPAH 10	CCTACGTCAG
OPAH 11	TCCGCTGAGA
OPAH 12	TCCAACGGCT
OPAH 13	TGTGGCCGAA
OPAH 14	TGTGGCCGAA
OPAH 15	CTACAGCGAG
OPAH 16	CAAGGTGGGT
OPAH 17	CAGTGGGGAG
OPAH 18	GGGCTAGTCA
OPAH 19	GGCAGTTCTC
OPAH 20	GGAAGGTGAG

3.2 Methods

Standardisation of DNA isolation protocol

Three different methods of DNA isolation were tried with possible modifications in order to standardise the procedure.

3.2.1 Method 1

The procedure was originally standardised for Alfalfa plant by Edwards *et al.* (1991).

Piper arboreum

South American Piper

Piper chaba

Piper longum **(P-25)**

Piper magnificum

Piper nigrum **(Panniyur 1)**

Plate 1. Some of the *Piper* species studied

3.1.2 Equipment and chemicals

The equipment available at the Centre for Plant Biotechnology and Molecular Biology, Department of Plantation Crops and Spices and the Biochemistry laboratory, College of Horticulture, were utilized for the study. The list of equipment and chemicals used in the study is given in Appendix I and II.

3.1.3 List of primers screened

Fifty decamer primers from Operon Technology, USA were screened and twenty primers each of OPAH, OPP and OPF series were selected for further analysis.

Table 3. List of primers screened for RAPD analysis

Primer code	Sequence
OPF 1	ACGGATCCTG
OPF 2	GAGGATCCCT
OPF 3	CCTGATCACC
OPF 4	GGTGATCAGG
OPF 5	CCGAATTCCC
OPF 6	GGGAATTCGG
OPF 7	CCGATATCCC
OPF 8	GGGATATCGG
OPF 9	CCAAGCTTCC
OPF 10	GGAAGCTTGG
OPP 1	GTAGCACTCC
OPP 2	TCGGCACGCA
OPP 3	CTGATACGCC
OPP 4	GTGTCTCAGG
OPP 5	CCCCGGTAAC
OPP 6	GTGGGCTGAC
OPP 7	GTCCATGCCA
OPP 8	ACATCGCCCA
OPP 9	GTGGTCCGCA
OPP 10	TCCCGCCTAC
OPP 11	AACGCGTCGG
OPP 12	AAGGGCGAGT
OPP 13	GGAGTGCCTC

OPP 14	CCAGCCGAAC
OPP 15	GGAAGCCAAC
OPP 16	CCAAGCTGCC
OPP 17	TGACCCGCCT
OPP 18	GGCTTGGCCT
OPP 19	GGGAAGGACA
OPP 20	GACCCTAGTC
OPAH 1	TCCGCAACCA
OPAH 2	CACTTCCGCT
OPAH 3	GGTTACTGCC
OPAH 4	CTCCCCAGAC
OPAH 5	TTGCAGGCAG
OPAH 6	GTAAGCCCCT
OPAH 7	CCCTACGGAG
OPAH 8	TTCCCGTGCC
OPAH 9	AGAACCGAGG
OPAH 10	CCTACGTCAG
OPAH 11	TCCGCTGAGA
OPAH 12	TCCAACGGCT
OPAH 13	TGTGGCCGAA
OPAH 14	TGTGGCCGAA
OPAH 15	CTACAGCGAG
OPAH 16	CAAGGTGGGT
OPAH 17	CAGTGGGGAG
OPAH 18	GGGCTAGTCA
OPAH 19	GGCAGTTCTC
OPAH 20	GGAAGGTGAG

3.2 Methods

Standardisation of DNA isolation protocol

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3.2.1 Method 1

The procedure was originally standardised for Alfalfa plant by Edwards *et al.* (1991).

Chemicals and reagents required

i) TE Buffer (pH 8.0)

Tris HCl

0.3 mM EDTA

ii) 70% Ethanol

iii) Isopropanol

iv) Extraction Buffer (pH 8.0)

200 mM Tris HCl

250 mM NaCl

25 mM EDTA

1% SDS (Sodium dodecyl sulphate)

v) 100 mM ß-mercapto ethanol

Original procedure

Tender leaf was collected in an ice box. 40 mg leaf sample was mascerated in an eppendorff tube at room temperature using plastic pestles until liquid came out of the tissues. To this, 40 µl of extraction buffer was added, the sample vortexed for 10 seconds and the mixture centrifuged at 14,000 rpm for 4 minutes. 300 ml of supernatant was transferred to a fresh eppendorff tube, equal volume of isopropanol was added and the sample kept at -20°C for 30 minutes. After incubation the mixture was centrifuged at 14,000 rpm for 3 minutes at 4°C. The supernatant was then discarded, the DNA pellet washed with 70% ethanol and the pellet was air dried and dissolved in 100 µl of 1/3 X TE Buffer.

Modification 1

Chloroform-iso-amyl alcohol treatment was given inorder to precipitate the proteins.

Modification 2

Added 24 mg PVP while grinding the leaf sample.

Modification 3

Increased the leaf sample (400 mg, 0.5 g, 1 g) with proportionate increase in extraction buffer.

Modification 4

The leaf sample was ground after half an hour freezing.

3.2.2 Method 2

The method was reported by Mondal *et al.* (2000) in tea. This was tried with slight modifications in *Piper* sp.

Reagents required

i) PVP (Poly vinyl pyrolidene)

ii) Extraction Buffer (pH 8.0)

2% CTAB

1.4 M NaCl

200 mM EDTA

100 mM Tris HCl

iii) 20 µL ß mercapto ethanol

iv) (24:1) chloroform isoamyl alcohol

v) 3 M sodium acetate

vi) absolute alcohol

vii) 70% alcohol

viii) TE Buffer (pH 8.0)

10 mM Tris HCl

1 mM EDTA

Original protocol

500 mg leaf sample was ground in an autoclaved pre-chilled, mortar and pestle with 300 mg PVP and 4.0 ml of hot (65°C) extraction buffer was added. This mixture was then poured into a centrifuge tube and incubated at 65°C for 45 minutes with gentle shaking occassionally. An equal volume of chloroform isoamyl alcohol was added and thoroughly mixed, centrifuged at 10,000 rpm for 10 minutes and supernatant was transferred with a wide bore pipette to a glass tube. Added sodium acetate (3 M 1/10 vol) and double volume of chilled (-20°C) absolute alcohol. The floating mass of DNA was hooked by a bent tipped glass pasture pipette. Then the DNA pellet was washed with 70% ethanol and absolute alcohol. The pellete was then allowed to air dry and resuspended in 150 μl 1x TE buffer.

Modification 1

PVP was not added while grinding.

Modification 2

DNA was not hooked out by bent tipped glass pasture pipette and direct centrifugation after adding sodium acetate (3 M).

3.2.3 Method 3

The method suggested by Rogers and Bendich (1994) and slightly modified for *Piper nigrum* by Haneeshbabu (2000) was tried in this study also.

Reagents required

i) 2 X CTAB Buffer

2% CTAB (W/V)

100 mM Tris (pH 8)

20 mM EDTA

1.4 M NaCl

1% PVP

ii) 10% CTAB solution

10% CTAB (W/V)

0.7 M NaCl

iii) TE Buffer

10 mM Tris (pH 8.0)

1 mM EDTA (pH 8.0)

iv) Chloroform iso amyl alcohol (24:1 V/V)

v) Isopropanol

vi) Ethanol 70% and 100%

Procedure

One gram leaf sample was ground in a pre-chilled mortar using a pestle, in presence of liquid N2 and transferred to a sterile 50 ml centrifuge tube

containing 5 ml hot 2x CTAB extraction buffer. The ground leaf and extraction buffer mixture was then incubated at 65°C for 15-20 minutes. Equal volume of chloroform isoamyl alcohol was added to the mixture, mixed gently by inversion and centrifuged at 10,000 rpm for 10 minutes at 4°C. The upper aqueous phase was pippetted out and transferred to another tube. $1/10^{th}$ volume of 10% CTAB was added to the tube and mixed gently by inversion. Equal volume of chloroform isoamyl alcohol mixture was added to the mixture, mixed gently to form an emulsion and centrifuged at 10,000 rpm for 10 minutes at 4°C. The aqueous phase was again collected carefully and transferred to another tube. Two third volume of chilled isopropanol was added and mixed gently until the DNA precipitated. The contents were centrifuged at 10,000 rpm for 5 minutes at 4°C to pellet the DNA. The isopropanol was poured off and the pelleted DNA was washed first with 70% alcohol and then with absolute alcohol. The DNA pellet was then air dried and dissolved in 250 µl TE buffer.

3.2.4 Purification of DNA

RNAse treatment

Preparation of RNAse

The Ribonuclease A from sigma USA was used to prepare RNAse. One per cent solution was prepared by dissolving RNAse A in TE buffer at 100°C for 15 minutes. Solution was allowed to cool to room temperature, dispensed into aliquot and stored at -20°C.

Incubation of DNA with RNAse

10 µl RNAse was added to 100 µl DNA sample and kept it for over night digestion at 37°C. Digested sample was mixed with equal volume of chloroform isoamyl alcohol mixture (24:1), by gentle inversion. Mixture was centrifuged at 10,000 rpm for 5 minutes. The aqueous layer was transferred into a fresh tube and two volumes of isopropanol was added (for precipitating the DNA). Then added 10 µl potassium acetate. Mixture was centrifuged at 10,000 rpm for 3 minutes. Then the DNA pellet was washed with 70% ethanol after discarding the supernatant. The DNA pellet was vacuum dried and redissolved in 100 µl TE buffer.

3.2.5 Estimation / Quantification of DNA

The quality and quantity of DNA was estimated by electrophoresis and by spectrophotometry.

Electrophoresis

Reagents

i) TAE Buffer (50x)

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

0.04 M Tris acetate

0.01 M EDTA

ii) Gel loading buffer/tracking dye

0.25% Bromophenol blue

40% sucrose in water

iii) Agarose

iv) Ethidium bromide 1 mg in 1 ml (stock)

Procedure

For preparing 1x TAE Buffer 1 ml was taken from the stock solution (50 x) and the volume was made up to 50 ml with sterile water. Twenty five ml TAE Buffer was taken in a beaker, 250 mg agarose was added and boiled in a microwave oven for one minute. After cooling down to room temperature 1 µl of ethidium bromide was added to this and mixed well. The comb was placed at appropriate position in the gel casting tray. The melted agarose was poured in to the tray and kept for half an hour at room temperature for solidification. The comb was removed after half an hour. The gel was kept in the electrophoresis unit and the tank was filled with 1x TAE buffer. The well side was directed towards cathode. 10 µl of DNA sample (15 µl in case RAPD products), was pipetted out on to parafilm and mixed well with 5 µl of tracking dye. The samples were then loaded carefully in to the well using micro pipette. Electrophoresis was carried out at a constant voltage of 70 V for one to one and half hours till the loading dye had covered two third length of the gel.

Gel documentation

The gel was taken from the electrophoresis unit and viewed under UV light in a UV transilluminator. The image was monitored, and saved in a gel documentation system (Alpha Imager 2000, Alpha Innotech, USA).

Spectrophotometer determination

The DNA samples were diluted 10 times using sterile water and the optical density was determined by reading the absorbance at two specific wave

lengths viz. 260 nm and 280 nm. The 260/280 ratio was then calculated to check the purity. Pure DNA gives a ratio 1.8. The DNA in the sample was quantified as per the equation.

OD of one at 260 nm = 50 μg/ml DNA

3.2.6 Random amplified polymorphic DNA (RAPD analysis)

In RAPD analysis, a single decamer primers were used to amplify the random sequences in total genomic DNA.

The primer length and size of target genome will decide the number of amplified products in RAPD analysis. The resulting products were resolved with electrophoresis technique elaborated earlier, and visualised by ultraviolet illumination of ethidium bromide stained gels.

PCR amplification process involve repeated thermal cycles. The programme standardized by Haneeshbabu (2000) includes,

a) DNA denaturation at 94°C for 1:00 minute

b) Primer annealing to the template DNA at 37.5°C for 1:00 minute

c) Primer extension at 72°C catalysed by Taq DNA polymerase enzyme for 2:00 minutes

d) 44 times repeated running of step a to c

The reaction mixture consisted of the following

i)	Template DNA	- 25 ng/tube
ii)	dNTP's	- 1.5 μl/tube
iii)	Primer	- 2.0 μl/tube

iv) 10x assay buffer with 15 mM $MgCl_2$ - 2.5 µl/tube

v) Taq DNA polymerase - 1.0 µl/tube

vi) Sterile milli Q water - to make up to the required volume

The reaction mixture was prepared as a master mix for the required number of reactions. The aliquot of the master mix (dNTP's - 1.5 µl/tube, Primer - 2.0 µl/tube, 10x assay buffer with 15 mM $MgCl_2$ - 2.5 µl/tube and Taq DNA polymerase - 1.0 µl/tube) was dispensed to 0.5 ml PCR tubes to which template DNA 25 ng/tube and sterile water were added. The control samples were also run without template DNA. The reaction mixtures were centrifuged in a micro centrifuge for mixing the components. The contents were overlaid with 25 µl mineral oil and the PCR tubes were loaded in a thermal cycler (PTC 200 MJ Research, USA).

3.2.7 Primer screening for RAPD

The decamer primers obtained from Operon technologies USA were used for the study. Twenty primers each under OPP and OPAH series and ten primers under OPF series were tried, with the thermal cycle setting mentioned earlier. Genomic DNA from Panniyur 1 was used for standardisation work. The details of the primers tested are given in Table 3. Those primers which gave maximum number of reproducible bands were selected and further used for screening the species of *Piper*.

3.2.8 Statistical methods employed in the study

The data obtained were analysed statistically using the following methods:

(i) Similarity coefficient

Pairwise similarity between genotypes (primer wise and pooled) were calculated using Jaccard's similarity coefficient

$GS_{ij} = a/a+b+c$

Where GS_{ij} is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands shared by i and j, b is the number of bands present in i and absent in j and c is the number of bands present in j and absent in i.

(ii) Construction of dendrogram

The DNA finger print data were used to construct dendrogram by employing unweighted pair group Method of arithmetic averages (UPGMA) using NTSYS programme (Rohlf, 1998) using SAHN coefficient.

Results

4. RESULTS

The results of the research work conducted on "standardization of protocol for genomic DNA isolation" in black pepper, screening of primers for RAPD analysis and molecular characterisation of *Piper* species using RAPD technique are given in this chapter.

4.1 Genomic DNA isolation in black pepper

Three different procedures along with modifications mentioned in chapter 3 were tried for the genomic DNA extraction in black pepper. Results of the experiments are presented in Table 4.

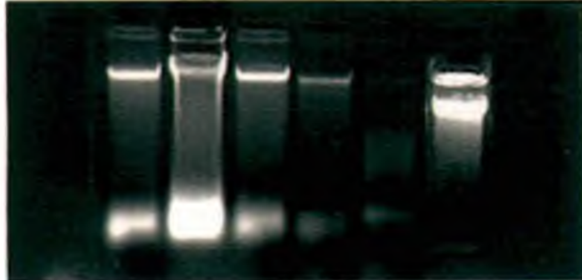
The quality of the DNA isolated was assessed by agarose gel electrophoresis. The results showed that, the original protocol of Edwards *et al.* (1991), with chloroform isoamyl alcohol treatment (IA) gave good quality DNA in sufficient quantity (Plate 2). In the method reported by Mondal *et al.* (2000), the results showed that whenever quantity of sample was more, slight degradation of DNA was observed, DNA quality was also poor with all the modifications. Even though sufficient quantity of good quality DNA was recovered by the DNA isolation protocol standardised by Haneeshbabu (2000), method of Edwards *et al.* (1991) was very simple and did not require expensive chemicals like CTAB, liquid nitrogen etc.

Table 4 shows that the ratio of absorbance at 260/280 nm was between 1.78 and 1.918 in almost all cases. The quantity of DNA extracted by the Edwards method increased with the increase in quantity of leaf samples. It was 4.0 μg/ml for 40 mg leaf samples to 16.4 μg/ml for 1 g leaf sample.

Table 4. The quantity and quality of genomic DNA isolated using different methods

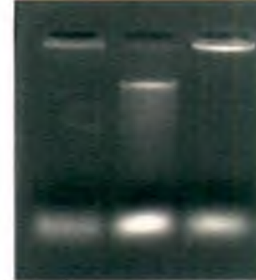
Sl. No.	Method of DNA isolation	Sample quantity	Absorbance		260/280 nm ratio	Quantity µg/ml	Quality	Nature of bands	Total time required for DNA isolation	Remarks
			260 nm	280 nm						
1	Protocol I	40 mg	0.080	0.043	1.86	4.00	Good	Clear narrow	2 hrs	No liquid N_2 No chloroform isoamyl alcohol Treatment
2	Protocol I A	40 mg	0.091	0.051	1.78	4.55	Very good	Clear narrow	,,	No liquid N_2
3	Protocol I B	40 mg	0.083	0.048	1.72	4.15	Poor	Clear narrow	2 hrs	,,
4	Protocol I C	(i) 400 mg (ii) 0.5 g (iii) 1.0	 0.204 0.234 0.323	 0.107 0.125 0.048	 1.87 1.87 1.92	 10.20 11.70 16.40	 Poor Poor Poor	 Partially smeared Partially smeared Totally smeared	 2 ½ hrs ,, ,,	 No liquid N_2 ,, ,,
5	Protocol I D	40 mg	0.061	0.036	1.69	3.05	Poor	,,	,,	,,
6	Protocol II	1.0 g	0.318	0.200	1.58	15.90	Poor	Totally smeared	4 ½ hrs	,,
7	Protocol II A	500 mg	0.218	0.126	1.73	10.90	Poor	,,	,,	,,
8	Protocol II B	500 mg	0.218	0.122	1.78	10.90	Poor	,,	,,	,,
9	Protocol III	1.0 g	0.443	0.232	1.90	22.15	Fair	Partially smeared	4 ½ to 4 hrs	Required liquid N_2

Method by Edwards *et al.*, 1991
(With and without modification)



M1 - Modified with Chloroform Isoamyl Alcohol Treatment
M2 - Modified with PVP grinding
M3a - Increased quantity of leaf sample (0.40 g)
M3b - Increased quantity of leaf sample (0.50 g)
M3c - Increased quantity of leaf sample (1.00 g)
M4 - Modified with half an hour freezing of leaf sample

Method by Mondal *et al.*, 2000
(With and without modification)



2 - Original Protocol
M1 - PVP was not added while grinding
M2 - Modified with direct centrifugation

Method by Rogers and Bendich, 1994
(Without modification)

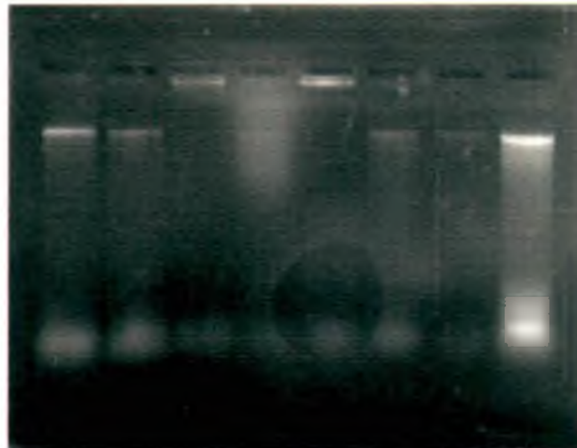


Plate 2. DNA isolation methods

In Mondal's (2000) method, DNA recovered was 10.9 μg/ml for 500 mg leaf sample (II A) and 15.9 μg/ml for 1 g leaf sample (II). In Rogers and Bendich's (1994) method it was 22.15 μg/ml (III).

Protocol of Edwards *et al.* (1991) modified with chloroform isoamyl alcohol treatment (1A) was found superior and sufficient recovery of DNA was obtained. The quality was also good as indicated by the ratio (1.78). Repeated trials confirmed this result. The method was simple and less expensive and hence was selected for further detailed study of *Piper* species.

4.2 Random primer screening

Fifty random primers from Operon Technology, USA were screened with the method mentioned in earlier chapter.

4.2.1 OPP series

The results of 20 OPP series decamer primers are presented in (Table 5). Out of the 20 primers screened, 10 gave good amplification. The number of bands ranged between 1 and 12. Those that gave good amplification were OPP 2, OPP 3, OPP 4, OPP 5, OPP 6, OPP 7, OPP 9, OPP 12, OPP 13, OPP 20. The number of bands produced by these primers were different for each species. A few other primers gave an amplification of average quality and some other with poor quality amplification or no amplification at all. Repeated tests gave same results and the above mentioned primers were selected from OPP primer series for further analysis.

4.2.2 OPF series

Out of 10 primers screened, OPF 1 and OPF 5 gave good amplification with the number of bands ranging between 1 to 8 and 3 to 13 respectively. Others

Table 5. Screening of primers for DNA amplification in *P. nigrum* (OPP series)

Sl.No.	Primers	Number of bands	Quality of the bands	Remarks
1	OPP 1	None (0)	No amplification	Rejected
2	OPP 2	Six (6)	Clear, distinct	Selected
3	OPP 3	Seven (7)	Clear, distinct	Selected
4	OPP 4	Four (4)	One clear, 3 dull but distinct	Selected
5	OPP 5	Ten (10)	Clear, distinct	Selected
6	OPP 6	Eight (8)	Clear, distinct	Selected
7	OPP 7	Nine (9)	Clear, distinct	Selected
8	OPP 8	None (0)	No amplification	Rejected
9	OPP 9	Six (6)	Clear, distinct	Selected
10	OPP 10	None (0)	No amplification	Rejected
11	OPP 11	Two (2)	Clear, distinct	Rejected
12	OPP 12	Twelve (12)	Clear, distinct	Selected
13	OPP 13	Seven (7)	Clear, distinct	Selected
14	OPP 14	One (1)	Clear, distinct	Rejected
15	OPP 15	Three (3)	Clear, distinct	Rejected
16	OPP 16	None (0)	No amplification	Rejected
17	OPP 17	None (0)	No amplification	Rejected
18	OPP 18	Four (4)	Slightly smeared	Rejected
19	OPP 19	One (1)	Clear, distinct	Rejected
20	OPP 20	Five (5)	Clear, distinct	Selected

gave poor amplification of average quality. Number of bands varied from 0 to 12. Subsequent trials confirmed the results. Hence OPF 1 and OPF 5 were selected for further study (Table 6).

4.2.3 OPAH series

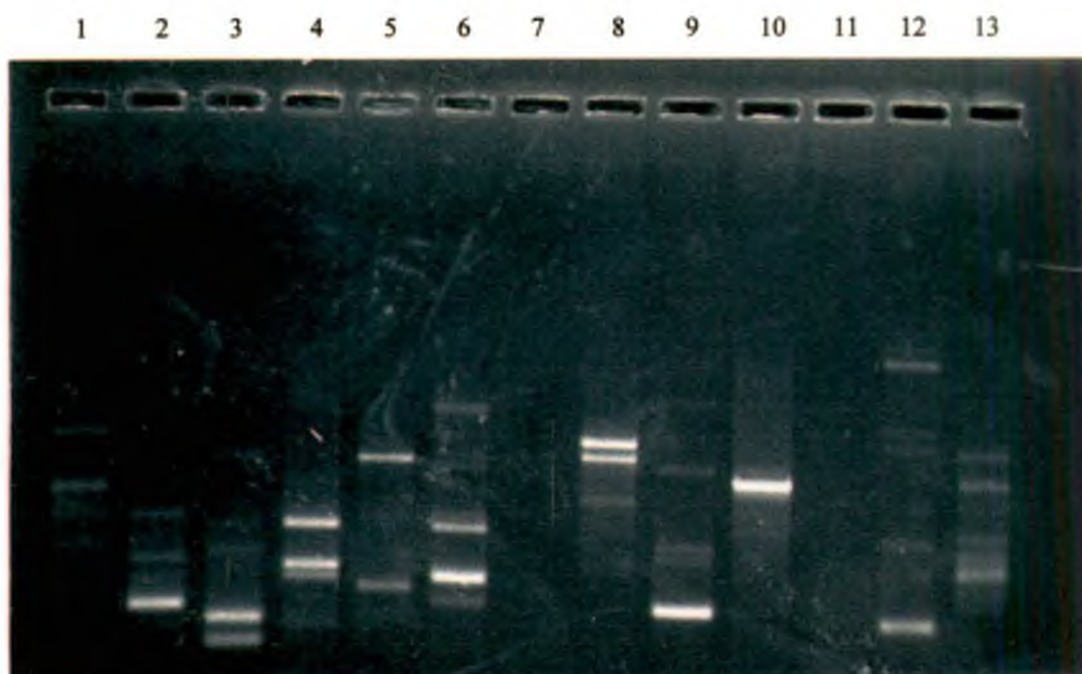
Table 7 and Plate 3 shows the results of screening of 20 primers of OPAH series. Out of these, eight primers gave good amplification with the number of bands ranging between 2 and 9. Number of bands among the primers tested varied from zero to 9. A few primers gave average quality amplification and for some others the quality of amplification was poor. OPAH 6, OPAH 9, OPAH 12, OPAH 13, OPAH 14, OPAH 15, OPAH 18 and OPAH 20 which gave good amplification were selected for further analysis.

The primers for further analysis were selected based on the number of bands, quality of amplification and stability of expression. Those primers which gave more distinct banding pattern with good quality amplification and reproducibility were selected for further analysis.

4.3 Molecular characterization of *Piper* species using selected primers

Primer OPF 1

The RAPD profile of all the species using OPF 1 primer is presented in Plate 4. The number of bands ranged from 1 to 7, *P. attenuatum* with 1 band to *P. chaba* with 7 bands. All the species showed distinct banding pattern. Table 8 shows that, the similarity between the species ranged from 0.00 to 1.00 per cent. Species pairs *P. nigrum* Wild and *P. longum* PLD, *P. nigrum* Wild and *P. longum* P25 and *P. logum* PLD and *P. longum* P25 showed the maximum similarity of 1.00.



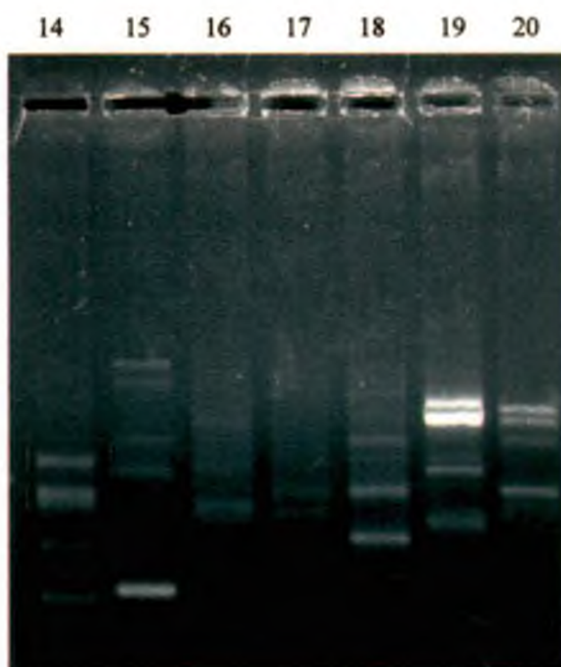


Plate 3. Primer screening for OPAH series (OPAH 1 to 20)

Plate 4. RAPD profile of *Piper* species for the primer OPF1

Table 6. Screening of primers for DNA amplification in *Piper nigrum* (OPF series)

Sl.No.	Primers	Number of bands	Quality of the bands	Remarks
1	OPF 1	6	Clear, distinct	Selected
2	OPF 2	3	Clear	Rejected
3	OPF 3	0	No amplification	Rejected
4	OPF 4	4	Fully smeared	Rejected
5	OPF 5	13	Clear, distinct	Selected
6	OPF 6	5	Smeared	Rejected
7	OPF 7	0	No amplification	Rejected
8	OPF 8	7	Fully smeared	Rejected
9	OPF 9	1	Primer to primer amplification	Rejected
10	OPF 10	1	,,	Rejected

Table 7. Screening of primers for DNA amplification in *P. nigrum* (OPAH series)

Sl.No.	Primers	Number of bands	Quality of the bands	Remarks
1	OPAH 1	Three (3)	Smeared	Rejected
2	OPAH 2	Three (3)	One is clear, two is smeared	Rejected
3	OPAH 3	Two (2)	Clear but less no. of bands	Rejected
4	OPAH 4	Three (3)	Cleared but less no. of bands	Rejected
5	OPAH 5	Three (3)	Cleared but less no. of bands	Rejected
6	OPAH 6	Seven (7)	Clear, distinct	Selected
7	OPAH 7	None (0)	No amplification	Rejected
8	OPAH 8	Three (3)	Clear but less no. of bands	Rejected
9	OPAH 9	Six (6)	Clear, distinct	Selected
10	OPAH 10	One (1)	Clear, prominent	Rejected
11	OPAH 11	None (0)	No amplification	Rejected
12	OPAH 12	Six (6)	Clear, distinct	Selected
13	OPAH 13	Seven (7)	Clear, distinct	Selected
14	OPAH 14	Six (6)	Clear, distinct	Selected
15	OPAH 15	Seven (7)	Clear, distinct	Selected
16	OPAH 16	Three (3)	Totally smeared	Rejected
17	OPAH 17	Five (5)	Totally smeared	Rejected
18	OPAH 18	Five (5)	Clear, distinct	Selected
19	OPAH 19	Five (5)	Partly smeared	Rejected
20	OPAH 20	Four (4)	Clear, distinct	Selected

P. nigrum was genetically wide apart from *P. arboreum*, *P. attenuatum* and *P. magnificum* with 0.00 per cent similarity. The species pair *P. chaba* and *P. betle* showed a similarity of 0.714 whereas the species *P. attenuatum*, *P. magnificum* and *P. nigrum* showed least similarity with all the species studied. The species *P. nigrum* Wild has shown highest similarity with all the species except *P. attenuatum* and *P. magnificum.*

OPF 5

RAPD banding pattern of all the species were distinct for OPF 5 (Plate 5). The number of bands ranged from 3 to 11. Similarity index of banding pattern were calculated (Table 9). The highest similarity of (1.00) was observed between the species pair *P. arboreum* and *P. chaba.* The next higher similarity of 0.800 was observed in the species pair *P. nigrum* P1 and *P. longum* PLD. The lowest similarity of 0.166 was observed between *P. longum* P25 and *P. attenuatum.* The species *P. nigrum* P1, *P. nigrum* Wild, *P. longum* PLD, *P. colubrinum*, *P. chaba*, *P. betle* and *P. arboreum* have shown high similarity among themselves. Whereas *P. attenuatum* showed least similarity with other species in the range of 0.166 to 0.500.

OPP 2

All the species showed specific banding pattern (Plate 6). The number of bands were varying from one to eight. Table 10 shows the similarity index ranging between 0.00 to 0.800. The highest similarity was observed between *P. nigrum* P1 and *P. nigrum* Wild (0.800). The species pairs *P. nigrum* Wild - *P. attenuatum*

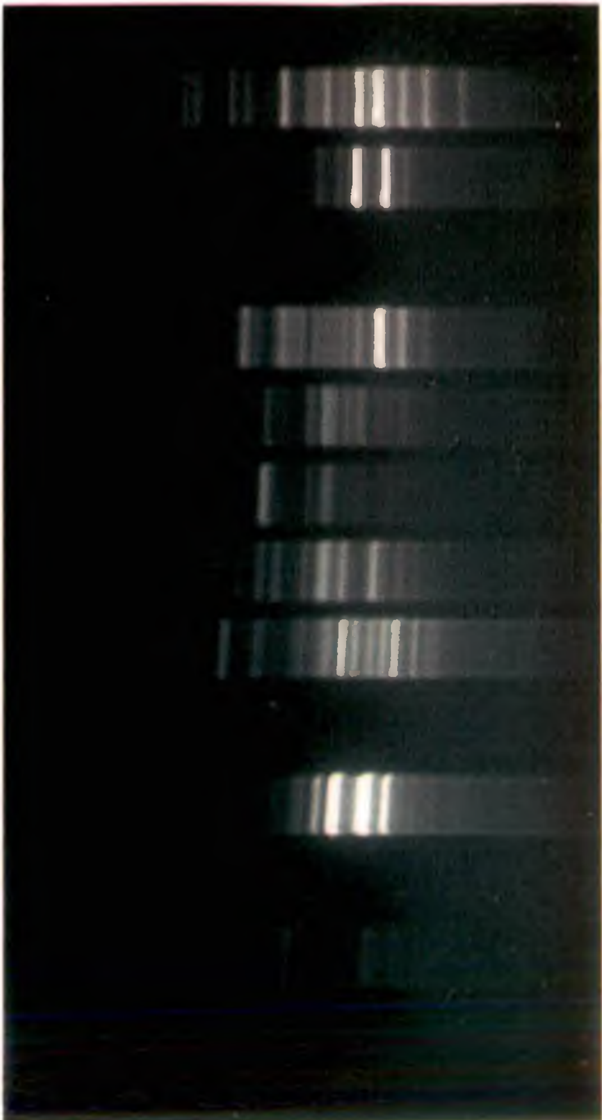


Plate 5. RAPD profile of *Piper* species for the primer OPF5

Plate 6. RAPD profile of *Piper* species for the primer OPP2

Table 10 Genetic similarity index of *Piper* spp. for the primer OPP2

```
" SIMQUAL: input=A:\opp2, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 9L 9  0
Pl PnW PlD P25 Pcol Pch Pb Parb Patt
  1.0000000
  0.8000000  1.0000000
  0.1250000  0.1428571  1.0000000
  0.0000000  0.0000000  0.2500000  1.0000000
  0.2000000  0.2500000  0.2500000  0.0000000  1.0000000
  0.3333333  0.1666667  0.4000000  0.0000000  0.3333333  1.0000000
  0.3333333  0.4000000  0.4000000  0.0000000  0.3333333  0.2000000  1.0000000
  0.3333333  0.1666667  0.1666667  0.0000000  0.0000000  0.2000000  0.2000000  1.0000000
  0.5000000  0.6000000  0.3333333  0.2500000  0.2500000  0.1666667  0.1666667  0.1666667  1.0000000
```

Table 11 Genetic similarity index of *Piper* spp. for the primer OPP3

```
" SIMQUAL: input=A:\Opp3, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 11L 11  0
Pl PnW PlD P25 Pcol Pch Pb Parb Patt SA Pbababud
  1.0000000
  0.4000000  1.0000000
  0.6000000  0.0000000  1.0000000
  0.6000000  0.0000000  1.0000000  1.0000000
  0.8000000  0.2000000  0.7500000  0.7500000  1.0000000
  0.5000000  0.0000000  0.7500000  0.7500000  0.6000000  1.0000000
  0.2000000  0.0000000  0.3333333  0.3333333  0.2500000  0.2500000  1.0000000
  0.1666667  0.0000000  0.2500000  0.2500000  0.2000000  0.5000000  0.0000000  1.0000000
  0.1666667  0.0000000  0.2500000  0.2500000  0.2000000  0.5000000  0.0000000  1.0000000  1.0000000
  0.4000000  0.0000000  0.6666667  0.6666667  0.5000000  0.5000000  0.5000000  0.3333333  0.3333333  1.0000000
  0.1666667  0.0000000  0.2500000  0.2500000  0.2000000  0.5000000  0.5000000  0.3333333  0.3333333  0.3333333  1.0000000
```

showed the next highest similarity of 0.600 whereas the species *P. longum* P25 showed least similarity with all other species in the similarity range of 0.00 to 0.250. All the species studied showed least similarity among themselves with respect to this primer. *P. arboreum* showed complete distinctness with all other species with similarity of 0.00 to 0.33.

OPP 3

The RAPD profile for OPP 3 (Plate 7) showed three prominent monomorphic bands in all the species except South American species. The remaining banding pattern was distinct among the species. The total number of bands were varied from 1 to 6. The results showed that the similarity index among *Piper* species was ranging between 0.000 to 0.800 per cent. The species pair *P. nigrum* P1 and *P. colubrinum* showed the maximum similarity of 0.800. The next highest similarity of 0.750 was observed between the species pairs of *P. longum* PLD and *P. colubrinum*, *P. longum* PLD and *P. chaba*, *P. longum* P25 and *P. colubrinum* and *P. longum* P25 and *P. chaba*. The species *P. nigrum* Wild showed complete distinctness with all the species studied, in the similarity range of 0.00 to 0.20, whereas *P. bababudani*, *P. betle*, *P. arboreum* and *P. attenuatum* showed least similarity among themselves and all the species studied (Table 11).

OPP 4

The results showed (Plate 8) distinct banding pattern among species with the number of bands ranging from 2 to 10. Similarity index of banding pattern was calculated (Table 12). The similarity index was ranging from 0.00 to 1.00. The

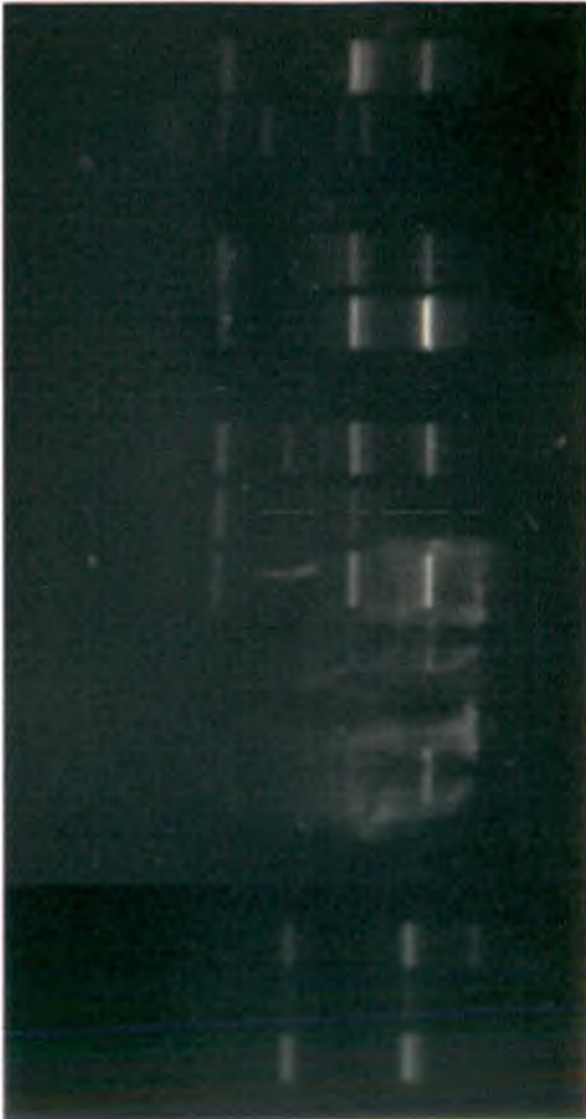
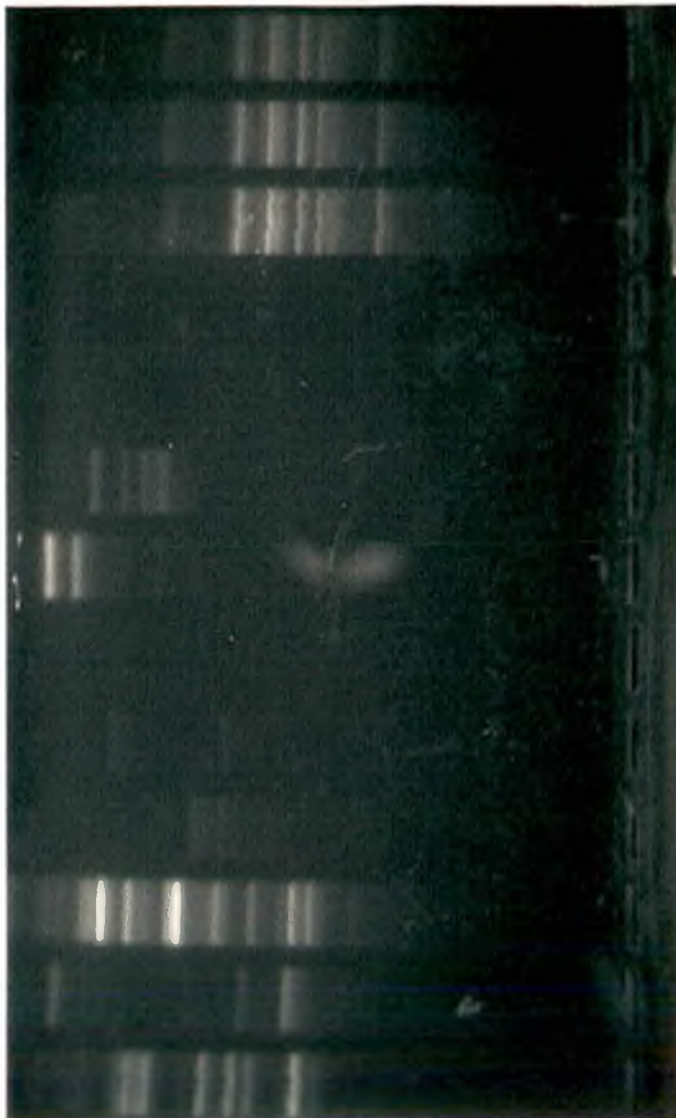


Plate 7. RAPD profile of *Piper* species for the primer OPP3

Plate 8. RAPD profile of *Piper* species for the Primer OPP4



P. nigrum (P1)

P. nigrum Wild (P49)

P. longum (PLD)

P. longum (P25)

P. colubrinum (P85)

P. chaba (P86)

P. betle (P87)

P. arboreum (P91)

P. attenuatum (TCR321)

Table 12 Genetic similarity index of *Piper* spp. for the primer OPP4

```
" SIMQUAL: input=A:\Opp4, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 9L 9  0
Pl PnW PlD P25 Pcol Pch Pb Parb Patt
  1.0000000
  0.4285714  1.0000000
  0.4285714  1.0000000  1.0000000
  0.4285714  1.0000000  1.0000000  1.0000000
  0.0000000  0.0000000  0.0000000  0.0000000  1.0000000
  0.0000000  0.0000000  0.0000000  0.0000000  1.0000000  1.0000000
  0.1000000  0.3636364  0.3636364  0.3636364  0.2500000  0.2500000  1.0000000
  0.2000000  0.2500000  0.2500000  0.2500000  0.2500000  0.2500000  0.3750000 1.0000000
  0.1428571  0.3333333  0.3333333  0.3333333  0.1666667  0.1666667  0.6250000 0.6000000  1.0000000
```

Table 13 Genetic similarity index of *Piper* spp. for the primer OPP5

```
" SIMQUAL: input=A:\Opp5, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 11L 11  0
Pl PnW PlD P25 Pcol Pch Pb Parb Patt SA Ppseu
  1.0000000
  0.8000000  1.0000000
  0.7500000  0.6000000  1.0000000
  0.4000000  0.6000000  0.2000000  1.0000000
  0.4000000  0.6000000  0.2000000  1.0000000  1.0000000
  0.4000000  0.6000000  0.2000000  0.5000000  0.5000000  1.0000000
  0.4000000  0.3333333  0.2000000  0.2000000  0.2000000  0.5000000  1.0000000
  0.8000000  0.6666667  0.6000000  0.3333333  0.3333333  0.3333333  0.6000000 1.0000000
  0.2222222  0.3333333  0.2500000  0.2500000  0.2500000  0.2500000  0.1111111 0.2000000  1.0000000
  0.6000000  0.8000000  0.4000000  0.7500000  0.7500000  0.7500000  0.4000000 0.5000000  0.3750000  1.0000000
  0.5000000  0.4000000  0.2500000  0.2500000  0.2500000  0.6666667  0.6666667 0.4000000  0.1250000  0.5000000  1.0000000
```

111720

species pairs *P. nigrum* Wild and *P. longum* PLD, *P. nigurm* Wild and *P. longum* P25, *P. longum* PLD and *P. longum* P25 and *P. chaba* and *P. colubrinum* have shown the similarity of 1.00. The species *P. colubrinum* showed complete distinctness with all the species studied except *P. chaba*. The species *P. arboreum, P. longum* P25 and *P. attenuatum* have shown least similarity with other species studied.

OPP 5

The RAPD profile (Plate 9) of all the species showed distinct, banding pattern, specific to them. The number of bands ranged from 3 to 10. The similarity index ranged from 0.11 to 1.00 (Table 13). The species pair *P. colubrinum* and *P. longum* P25 showed the maximum similarity of 1.00. The species pairs *P. nigrum* P1 and *P. nigrum* Wild, *P. nigrum* P1 and *P. arboreum* and *P. nigrum* Wild and South American species had shown the next highest similarity of 0.800. The species pairs *P. nigrum* P1 and *P. longum* PLD, *P. longum* P25 and South American spp., *P. colubrinum* and South American spp. showed a similarity of 0.750, whereas the species *P. pseudonigrum* and *P. attenuatum* has shown the least similarity range of 0.11 to 0.66 with all the other species studied.

OPP 6

RAPD profile of *Piper* species for the primer OPP 6 showed that the species *P. nigrum* P1, *P. nigrum* Wild, *P. betle*, *P. attenuatum* and South American spp. were distinct from other species and among themselves (Plate 10 and Table 14). The similarity index ranged from 0.00 to 1.00. The highest similarity of 1.00 has

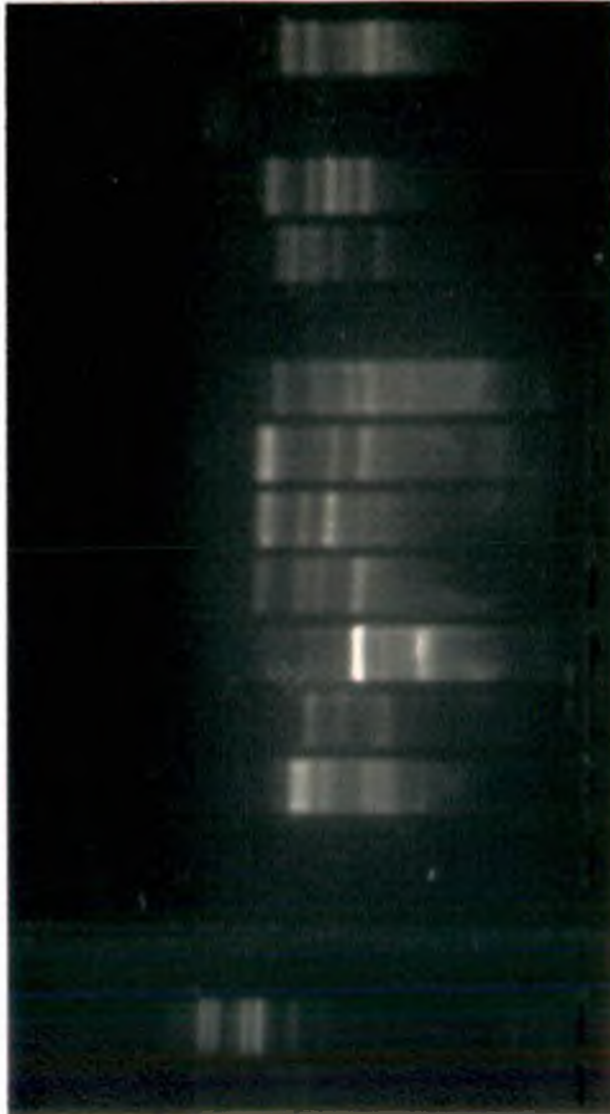


Plate 9. RAPD profile of *Piper* species for the primer OPP5

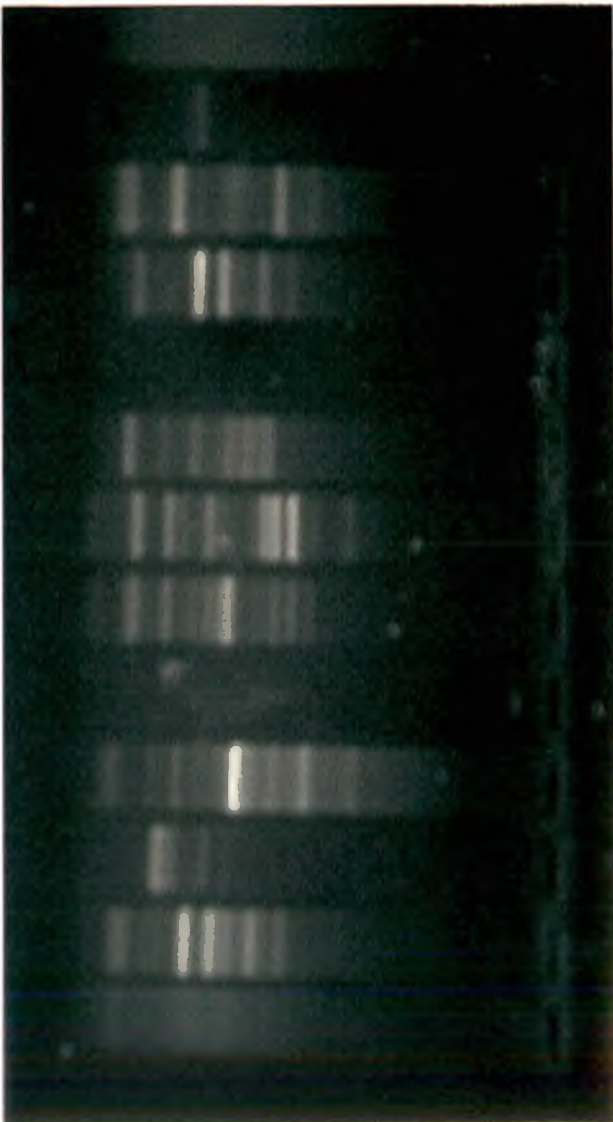


Plate 10. RAPD profile of *Piper* species for the primer OPP6

observed between *P. attenuatum* and South American spp. The next highest similarity of 0.833 was observed between *P. colubrinum* and *P. arboreum*. The species pair *P. nigrum* Wild and *P. longum* PLD showed a similarity of 0.750. The South American species *P. arboreum* and *P. betle* had shown least similarity with all other species and among themselves with respect to this primer.

OPP 7

The RAPD profile of OPP 7 is presented in Plate 11. The total number of bands ranged from 1 to 10. All the species showed distinct banding pattern with similarity index ranging from 0.000 to 0.889 (Table 15). The species pair *P. betle* and *P. chaba* showed the similarity of 0.889. The next highest similarity of 0.750 was recorded in the species pairs of *P. nigrum* P1 and *P. betle* and *P. longum* PLD and *P. longum* P25, whereas the species *P. nigrum* Wild has shown the least similarity range of 0.000 to 0.250 with all the species studied. The species *P. arboreum* and *P. attenuatum* also recorded very low similarity with all other species studied.

OPP 9

Table 16 shows a similarity index range of 0.000 to 1.000 among species for OPP 9. The total number of bands ranged from 3 to 7. All the species showed distinct banding pattern (Plate 12). The highest similarity of 1.00 was observed between the species pairs of *P. colubrinum* and *P. chaba*, *P. colubrinum* and *P. arboreum* and *P. chaba* and *P. arboreum*. The next highest similarity of 0.857 was observed between the species pairs of *P. longum* P25 and *P. colubrinum*,

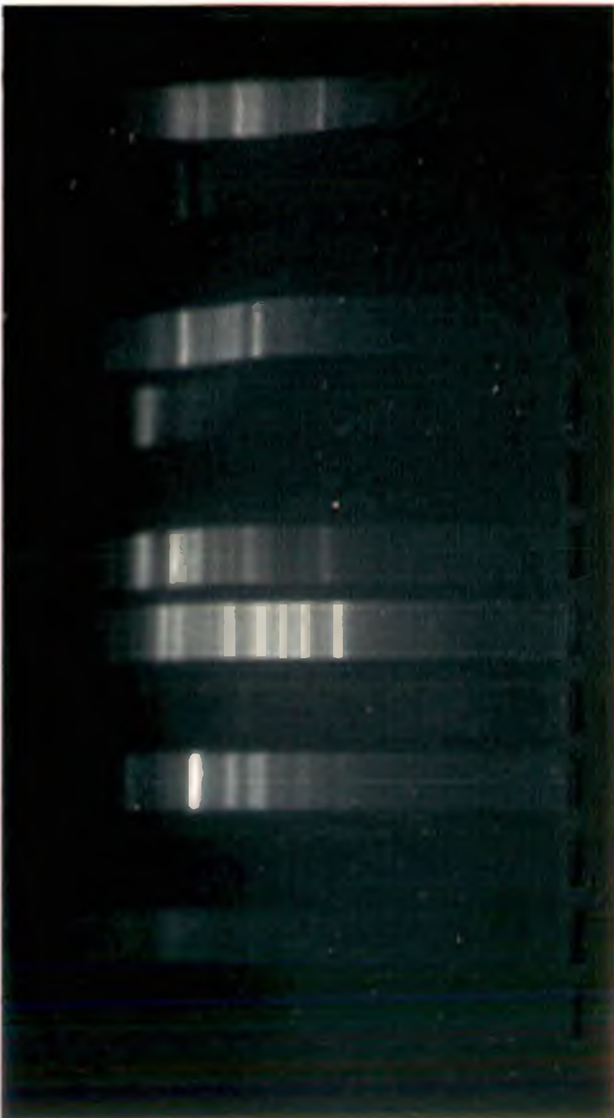


Plate 11. RAPD profile of *Piper* species for the primer OPP7

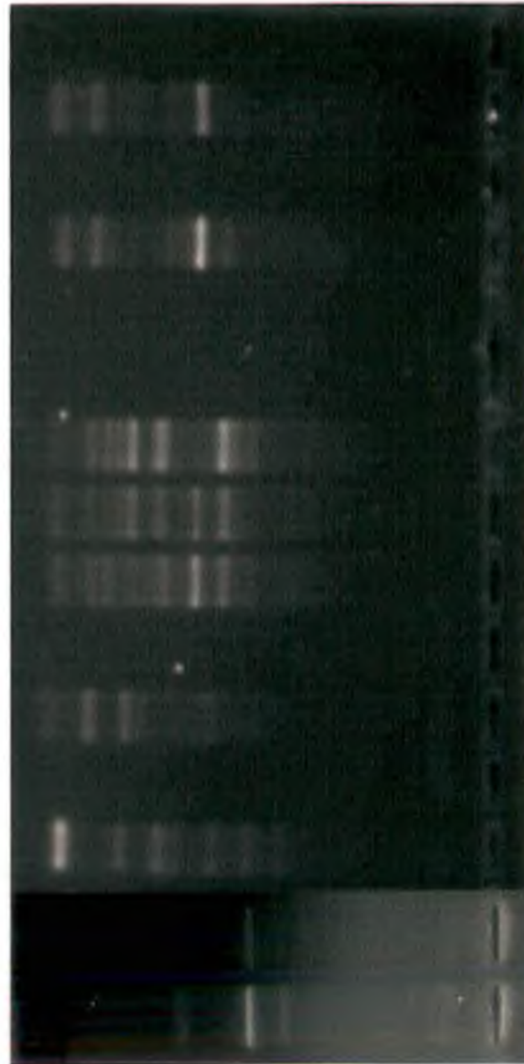


Plate 12. RAPD profile of *Piper* species for the primer OPP9

Table 16 Genetic similarity index of *Piper* spp. for the primer OPP9

" SIMQUAL: input=A:\opp9, coeff=J
" by Rows, += 1.00000, -= 0.00000
3 9L 9 0

Pl	PnW	PlD	P25	Pcol	Pch	Pb	Parb	Patt
1.0000000								
0.3333333	1.0000000							
0.2857143	0.7500000	1.0000000						
0.5714286	0.2857143	0.4285714	1.0000000					
0.7142857	0.4285714	0.5714286	0.8571429	1.0000000				
0.7142857	0.4285714	0.5714286	0.8571429	1.0000000	1.0000000			
0.4285714	0.3333333	0.5000000	0.8333333	0.7142857	0.7142857	1.0000000		
0.7142857	0.4285714	0.5714286	0.8571429	1.0000000	1.0000000	0.7142857	1.0000000	
0.3333333	0.0000000	0.1666667	0.5000000	0.4285714	0.4285714	0.6000000	0.4285714	1.0000000

Table 17 Genetic similarity index of *Piper* spp. for the primer OPP12

" SIMQUAL: input=A:\opp12, coeff=J
" by Rows, += 1.00000, -= 0.00000
3 9L 9 0

Pl	PnW	PlD	P25	Pcol	Pch	Pb	Parb	Patt
1.0000000								
0.2222222	1.0000000							
0.1428571	0.2500000	1.0000000						
0.2500000	0.7500000	0.3333333	1.0000000					
0.6000000	0.3000000	0.1111111	0.2000000	1.0000000				
0.3636364	0.3333333	0.0000000	0.2222222	0.5454545	1.0000000			
0.4545455	0.4444444	0.1111111	0.3333333	0.6363636	0.7000000	1.0000000		
0.3750000	0.1428571	0.0000000	0.1666667	0.3000000	0.5000000	0.4444444	1.0000000	
0.4545455	0.4444444	0.1111111	0.3333333	0.6363636	0.8888889	0.8000000	0.4444444	1.0000000

P. longum P25 and *P. chaba* and *P. longum* P25 and *P. arboreum*. The next highest similarity of 0.833 was observed between *P. longum* P25 and *P. betle*. The species pair *P. longum* PLD and *P. nigrum* Wild had shown the similarity of 0.750. The species *P. nigrum* P1, *P. longum* P25, *P. colubrinum* and *P. arboreum* had showed more similarity with all the species studied.

OPP 12

The genetic similarity among the species for OPP 12 is presented in the Table 17. The genetic similarity index ranged from 0.000 to 0.889. The total number of bands ranged from 3 to 11 (Plate 13). The species pair *P. chaba* and *P. attenuatum* had shown the maximum similarity of 0.889. The next highest similarity of 0.800 was observed between *P. betle* and *P. attenuatum*, whereas the species *P. nigrum* Wild showed 0.750 similarity with *P. longum* P25. The species *P. longum* PLD, *P. longum* P25 and *P. arboreum* has shown very less similarity with all other species studied with respect to this primer. The species *P. longum* PLD showed complete distinctness with low similarity range of 0.000 to 0.333 with all the species studied.

OPP 13

Table 18 shows that the similarity index ranged between 0.000 to 1.000. The total number of bands ranged from 2 to 11 (Plate 14). The highest similarity of 1.00 was observed in the species pairs of *P. nigrum* Wild and *P. longum* PLD, *P. nigrum* Wild and *P. longum* P25 and *P. longum* PLD and *P. longum* P25. The species pairs *P. betle* and *P. arboreum* and *P. nigrum* P1 and *P. chaba* have shown

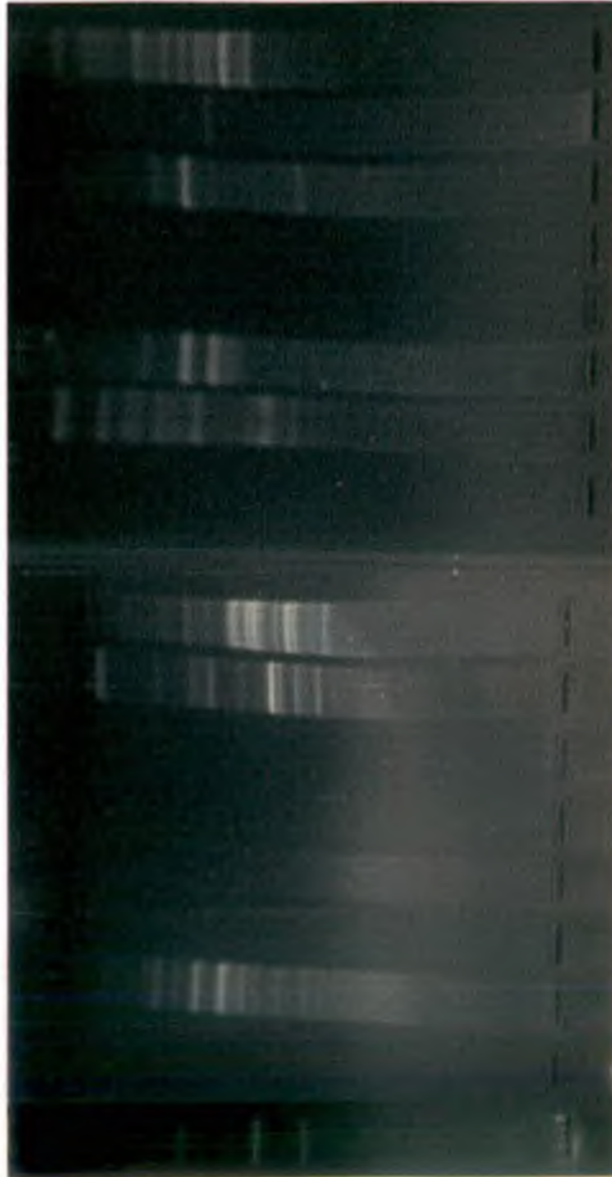


Plate 13. RAPD profile of *Piper* species for the primer OPP12

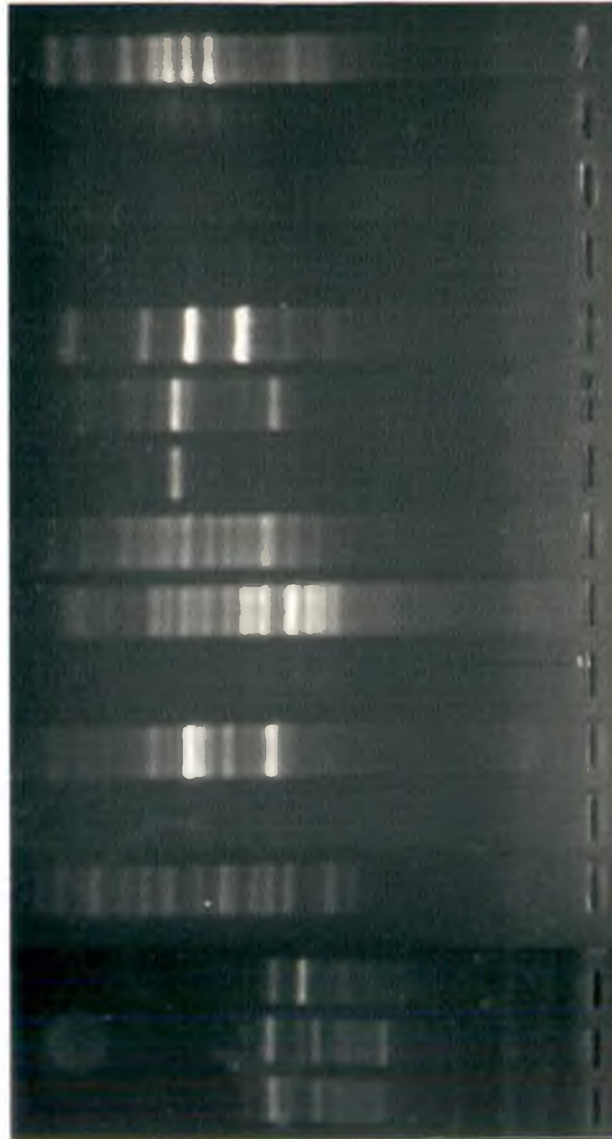


Plate 14. RAPD profile of *Piper* species for the primer OPP13

Table 18 Genetic similarity index of *Piper* spp. for the primer OPP13

```
" SIMQUAL: input=A:\opp13, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 11L 11  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt Ppseud Pbaba
  1.0000000
  0.3636364  1.0000000
  0.3636364  1.0000000  1.0000000
  0.3636364  1.0000000  1.0000000  1.0000000
  0.3000000  0.1111111  0.1111111  0.1111111  1.0000000
  0.8000000  0.5000000  0.5000000  0.5000000  0.4444444  1.0000000
  0.6000000  0.3000000  0.3000000  0.3000000  0.5714286  0.77777781.0000000
  0.5000000  0.3333333  0.3333333  0.3333333  0.6666667  0.6666667  0.8571429 1.0000000
  0.3000000  0.2500000  0.2500000  0.2500000  0.3333333  0.3000000  0.2222222 0.2500000  1.0000000
  0.2000000  0.2857143  0.2857143  0.2857143  0.4000000  0.3333333  0.4285714 0.5000000  0.1666667  1.0000000
  0.1111111  0.0000000  0.0000000  0.0000000  0.2500000  0.1111111  0.1428571 0.1666667  0.2500000  0.3333333  1.0000000
```

Table 19 Genetic similarity index of *Piper* spp. for the primer OPP20

```
" SIMQUAL: input=A:\opp20, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 9L 9  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt
  1.0000000
  0.1666667  1.0000000
  0.0000000  0.0000000  1.0000000
  0.7142857  0.1666667  0.0000000  1.0000000
  0.5555556  0.1250000  0.0000000  0.7500000  1.0000000
  0.1111111  0.0000000  0.0000000  0.1111111  0.2000000  1.0000000
  0.3000000  0.1428571  0.0000000  0.4444444  0.6666667  0.3750000  1.0000000
  0.2500000  0.0000000  0.0000000  0.2500000  0.3333333  0.1428571  0.3750000 1.0000000
  0.5555556  0.1250000  0.0000000  0.5555556  0.7777778  0.2000000  0.6666667 0.5000000  1.0000000
```

the similarity of 0.857 and 0.800 respectively whereas the species *P. pseudonigrum* and *P. bababudani* has shown very less similarity with all other species studied (similarity range 0.000 to 0.500). The species *P. attenuatum* has also shown least similarity with all other species studied (similarity range 0.166 to 0.333).

OPP 20

The Table 19 shows that the genetic similarity index among the species ranged from 0.000 to 0.777 for OPP 20. The number of bands ranged from 1 to 11 (Plate 15). The species pairs *P. colubrinum* and *P. attenuatum*, *P. colubrinum* and *P. longum* (P25) and *P. nigrum* (P1) and *P. longum* (P25) showed the similarity of 0.777, 0.750 and 0.714 respectively, whereas the species *P. longum* PLD showed complete distinctness with all other species studied with 0.000 per cent similarity. The species *P. nigrum* Wild, *P. chaba* and *P. arboreum* showed least similarity with all other species studied with respect to this primer.

OPAH 6

The genetic similarity was calculated for all the species for OPAH 6 (Table 20). The similarity index ranged from 0.000 to 1.000. The total number of bands ranged between 1 to 7 (Plate 16). The species *P. longum* PLD has shown a similarity of 1.000 with *P. longum* P25. The species pairs *P. nigrum* P1 and *P. longum* PLD, *P. nigrum* P1 and *P. longum* P25 and *P. attenuatum* and *P. betle* showed the similarity of 0.750 among themselves, whereas the South American spp. showed least similarity ranging between 0.000 to 0.333 with all the other species studied. The species *P. colubrinum*, *P. chaba* and *P. attenuatum* showed very less similarity with respect to this primer.

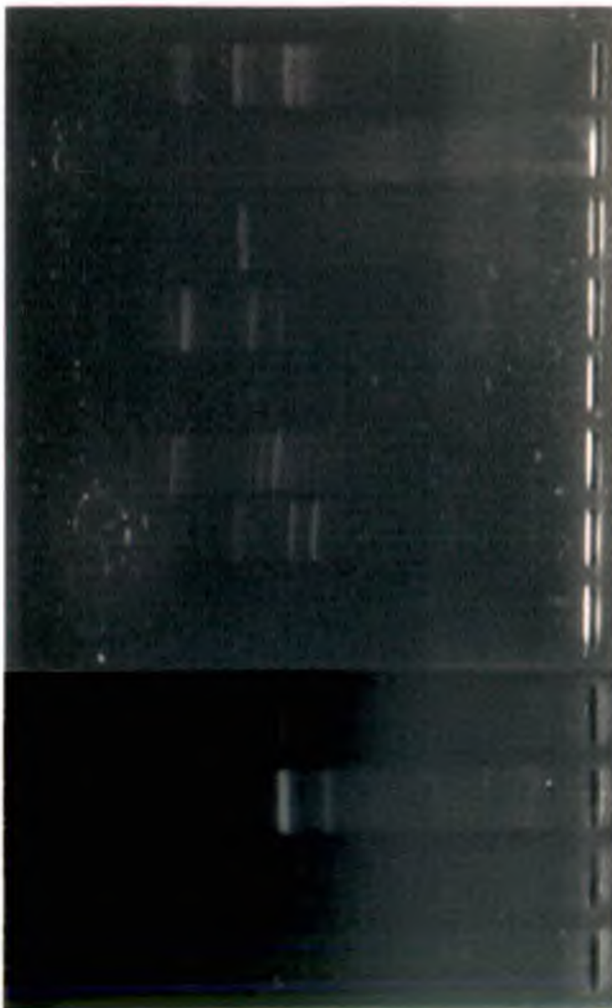
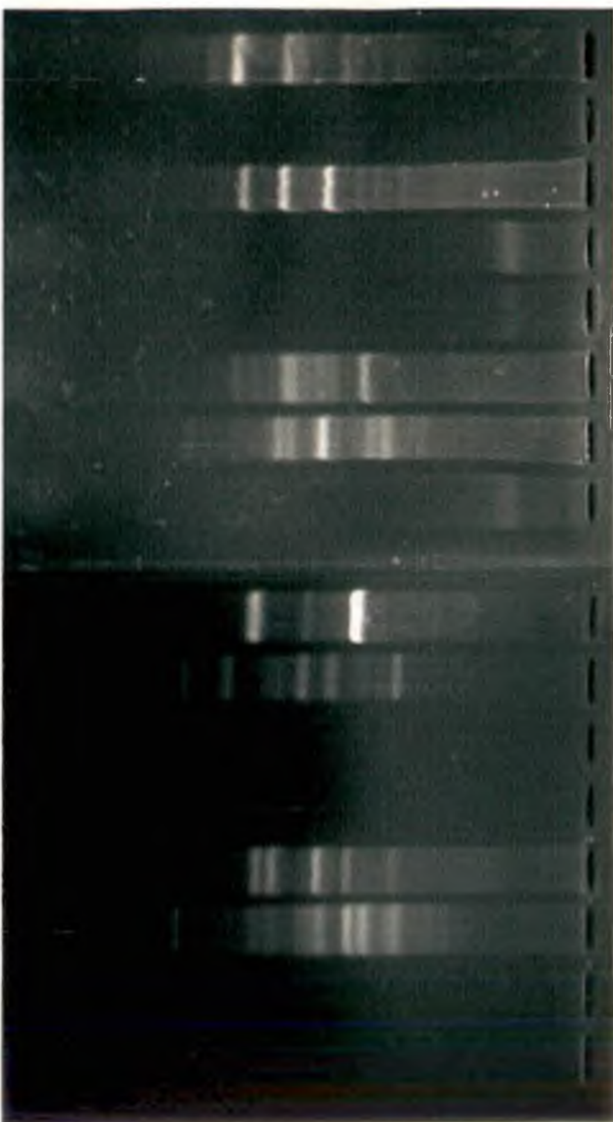


Plate 15. RAPD profile of *Piper* species for the primer OPP20



P. nigrum (P1)

P. nigrum Wild (P49)

P. longum (PLD)

P. longum (P25)

P. colubrinum (P85)

P. chaba (P86)

P. betle (P87)

P. arboreum (P91)

P. attenuatum (TCR321)

Piper Species (P90)

Plate 16. RAPD profile of *Piper* species for the primer OPAH6

Table 20 Genetic similarity index of *Piper* spp. for the primer OPAH6

```
" SIMQUAL: input=A:\opah6, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 10L 10  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt SA
  1.0000000
  0.4000000  1.0000000
  0.7500000  0.3333333  1.0000000
  0.7500000  0.3333333  1.0000000  1.0000000
  0.3333333  0.2857143  0.5000000  0.5000000  1.0000000
  0.0000000  0.2000000  0.2000000  0.2000000  0.1666667  1.0000000
  0.5000000  0.4000000  0.4000000  0.4000000  0.3333333  0.0000000  1.0000000
  0.6000000  0.5000000  0.5000000  0.5000000  0.4285714  0.1666667  0.6000000 1.0000000
  0.4000000  0.3333333  0.6000000  0.6000000  0.5000000  0.2000000  0.7500000 0.5000000  1.0000000
  0.0000000  0.0000000  0.0000000  0.0000000  0.2000000  0.0000000  0.3333333 0.2000000  0.2500000  1.0000000
```

Table 21 Genetic similarity index of *Piper* spp. for the primer OPAH9

```
" SIMQUAL: input=A:\opah9, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 10L 10  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt Pmag
  1.0000000
  0.2000000  1.0000000
  0.0000000  0.0000000  1.0000000
  0.6666667  0.2000000  0.0000000  1.0000000
  0.5714286  0.1666667  0.0000000  0.8333333  1.0000000
  0.5000000  0.0000000  0.0000000  0.2857143  0.4285714  1.0000000
  0.4285714  0.2000000  0.0000000  0.6666667  0.8333333  0.5000000  1.0000000
  0.3333333  0.0000000  0.0000000  0.3333333  0.5000000  0.4000000  0.3333333 1.0000000
  0.2857143  0.0000000  0.0000000  0.5000000  0.6666667  0.3333333  0.5000000 0.7500000  1.0000000
  0.0000000  0.0000000  1.0000000  0.0000000  0.0000000  0.0000000  0.0000000 0.0000000  0.0000000  1.0000000
```

OPAH 9

The similarity among the species for OPAH 9 is presented in the Table 21. The similarity between the species ranged between 0.000 to 1.000. The total number of bands also ranged from 1 to 8 (Plate 17). *P. longum* PLD has shared 1.000 per cent similarity with *P. magnificum*. The species pairs *P. colubrinum* and *P. betle* and *P. longum* (P25) and *P. colubrinum* showed a similarity of 0.833. The species pair *P. arboreum* and *P. attenuatum* has shown a similarity of 0.750, whereas the species *P. magnificum* has shown complete distinctness with all other species studied except *P. longum* P25. *P. nigrum* Wild showed least similarity with all other species studied with respect to this primer.

OPAH 12

The genetic similarity among the species ranged from 0.000 to 0.889 (Table 22). The species *P. attenuatum* showed high similarity of 0.889 with *P. colubrinum*. The species *P. longum* PLD showed a genetic similarity of 0.667 with *P. longum* P25. The species *P. nigrum* P1 showed a genetic similarity of 0.500 with *P. colubrinum*. *P. nigrum* P1, *P. colubrinum* and *P. attenuatum* showed distinct banding pattern with more number of bands. The species *P. arboreum*, *P. chaba* and *P. betle* showed very little similarity among themselves and with other species (Plate 18).

OPAH 13

Table 23 shows that the species *P. arboreum* showed maximum genetic similarity of 0.750 with species *P. nigrum* Wild, *P. longum* PLD and *P. chaba* for

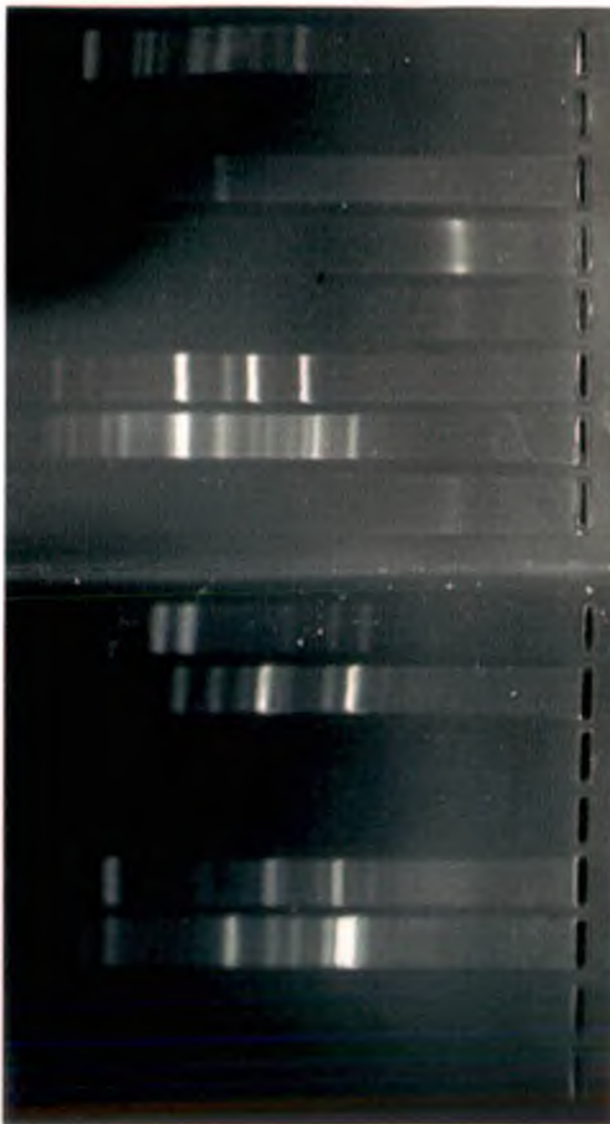


Plate 17. RAPD profile of *Piper* species for the primer OPAH9

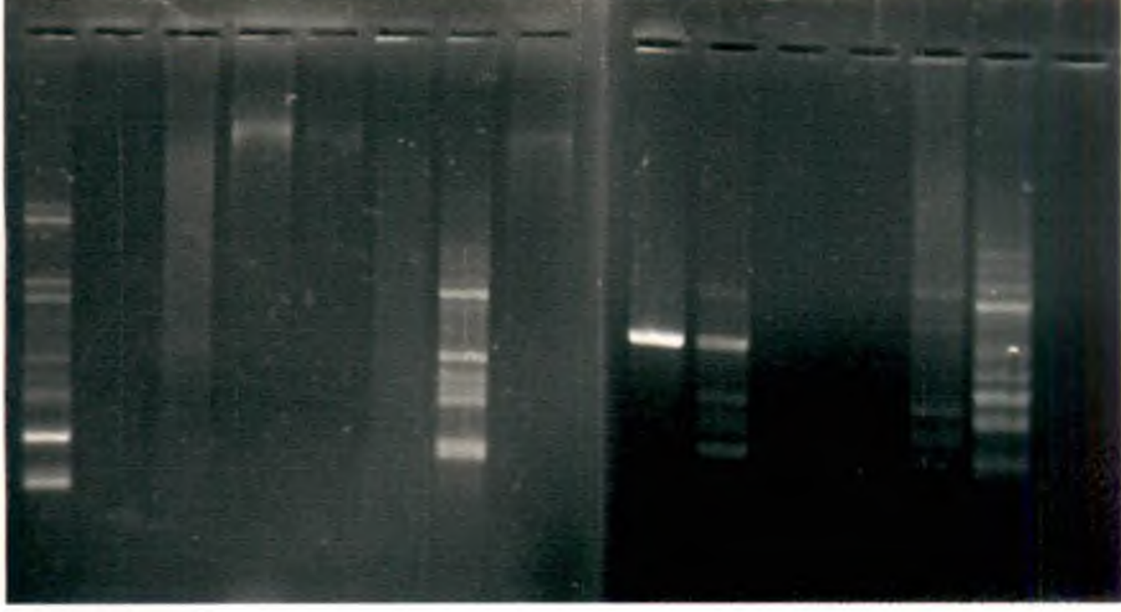


Plate 18. RAPD profile of *Piper* species for the primer OPAH12

Table 22 Genetic similarity index of *Piper* spp. for the primer OPAH12

" SIMQUAL: input=A:\opah12, coeff=J
" by Rows, += 1.00000, -= 0.00000
3 9L 9 0

P1	PnW	PlD	P25	Pcol	Pch	Pb	Parb	Patt
1.0000000								
0.3333333	1.0000000							
0.1000000	0.0000000	1.0000000						
0.2000000	0.2000000	0.6666667	1.0000000					
0.5000000	0.3333333	0.1000000	0.2000000	1.0000000				
0.0000000	0.0000000	0.0000000	0.0000000	0.1111111	1.0000000			
0.3000000	0.1666667	0.2000000	0.4000000	0.4444444	0.2500000	1.0000000		
0.1111111	0.3333333	0.0000000	0.0000000	0.1111111	0.0000000	0.0000000	1.0000000	
0.4166667	0.3750000	0.1111111	0.2222222	0.8888889	0.1250000	0.3333333	0.1250000	1.0000000

Table 23 Genetic similarity index of *Piper* spp. for the primer OPAH13

" SIMQUAL: input=A:\opah13, coeff=J
" by Rows, += 1.00000, -= 0.00000
3 10L 10 0

P1	PnW	PlD	P25	Pcol	Pch	Pb	Parb	Patt	Pmag
1.0000000									
0.3333333	1.0000000								
0.3333333	0.7142857	1.0000000							
0.2000000	0.4285714	0.6666667	1.0000000						
0.1250000	0.4444444	0.6250000	0.3750000	1.0000000					
0.0000000	0.5000000	0.5000000	0.4285714	0.6250000	1.0000000				
0.0000000	0.1428571	0.1428571	0.2000000	0.2857143	0.3333333	1.0000000			
0.2500000	0.7500000	0.7500000	0.5000000	0.6666667	0.7500000	0.2500000	1.0000000		
0.2500000	0.2857143	0.2857143	0.4000000	0.2500000	0.1250000	0.0000000	0.2222222	1.0000000	
0.2500000	0.2857143	0.2857143	0.4000000	0.2500000	0.1250000	0.0000000	0.2222222	1.0000000	1.0000000

OPAH 13. *P. nigrum* P1 showed no similarity with *P. chaba,* and *P. betle. P. nigrum* Wild showed no similarity with *P. betle. P. attenuatum* was totally distinct from *P. betle* with a similarity index of 0.000. The species *P. nigrum, P. betle* and *P. attenuatum* showed less genetic similarity with rest of the species. The species *P. arboreum, P. longum* PLD and *P. colubrinum* showed distinctness with respect to more number of bands and more similarity percentage. The total number of bands varied from 2 to 9 (Plate 19). The species pairs *P. nigrum* Wild and *P. longum* PLD showed a genetic similarity of 0.714. The species pairs *P. longum* PLD and *P. longum* P25, *P. longum* PLD and *P. colubrinum, P. colubrinum* and *P. chaba* and *P. colubrinum* and *P. arboreum* showed the high similarity of 0.667, 0.625, 0.625 and 0.667 respectively.

OPAH 14

The similarity index ranged between 0.000 to 0.800 (Table 24). The species pairs *P. arboreum* and *P. attenuatum* showed the maximum similarity of 0.800. The species pairs *P. nigrum* P1 and *P. nigrum* Wild, *P. longum* PLD and *P. longum* P25 showed the next highest similarity of 0.667. The similarity index of 0.600 was shown in the species pairs of *P. arboreum* and *P. chaba* and *P. arboreum* and *P. betle.* The species *P. chaba* showed zero percentage relationship with *P. nigrum* Wild. *P. longum* PLD, *P. nigrum* Wild, *P. attenuatum, P. bababudani* and *P. pseudonigrum* showed less affinity among themselves and with other species. *P. attenuatum, P. colubrinum* and *P. nigrum* showed more number of bands and they were distinct from each other. The total number of bands observed ranged from 0 to 9 (Plate 20).

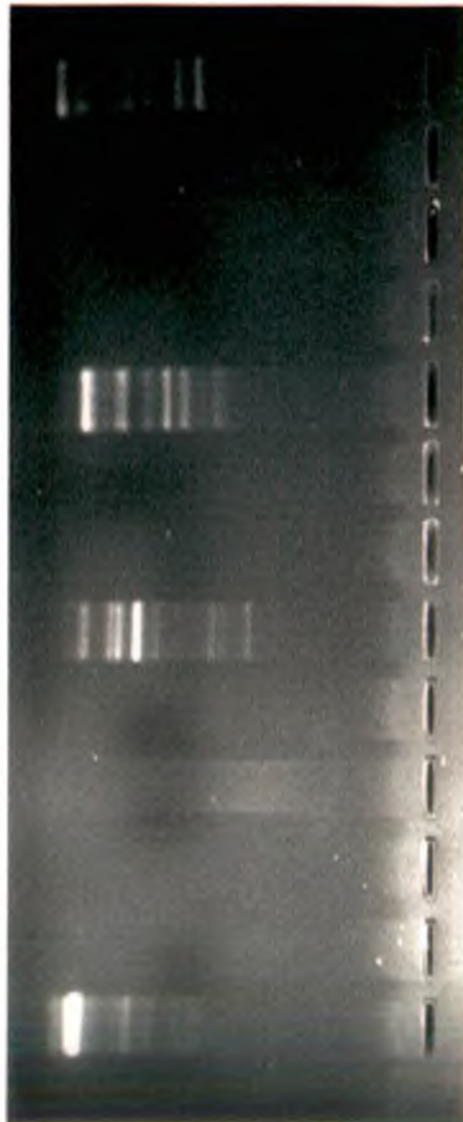


Plate 19. RAPD profile of *Piper* species for the primer OPAH1

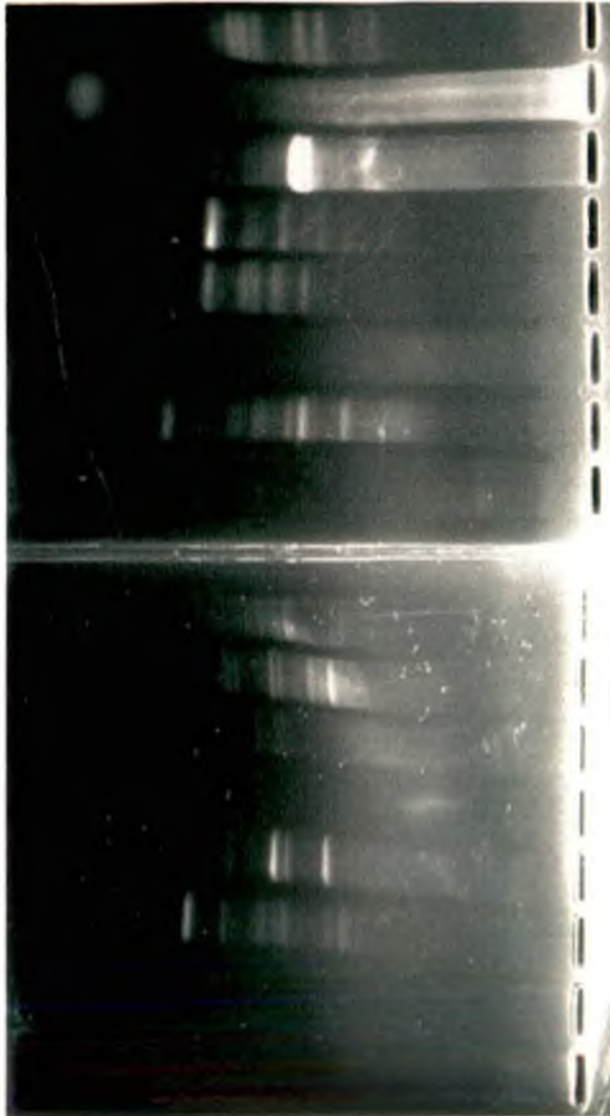


Plate 20. RAPD profile of *Piper* species for the primer OPAH14

Table 24 Genetic similarity index of *Piper* spp. for the primer OPAH14

```
" SIMQUAL: input=A:\opah14, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 11L 11  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt Pbaba Ppseud
  1.0000000
  0.6666667  1.0000000
  0.2500000  0.1428571  1.0000000
  0.3333333  0.2500000  0.6666667  1.0000000
  0.3750000  0.2857143  0.5000000  0.5714286  1.0000000
  0.2500000  0.0000000  0.3333333  0.4285714  0.5000000  1.0000000
  0.4285714  0.3333333  0.1428571  0.2500000  0.2857143  0.3333333  1.0000000
  0.2500000  0.1428571  0.1428571  0.2500000  0.2857143  0.6000000  0.6000000  1.0000000
  0.2222222  0.1250000  0.2857143  0.3750000  0.2500000  0.5000000  0.5000000  0.8000000  1.0000000
  0.1666667  0.0000000  0.0000000  0.0000000  0.0000000  0.2500000  0.2500000  0.2500000  0.2000000  1.0000000
  0.2857143  0.1666667  0.1666667  0.5000000  0.3333333  0.4000000  0.1666667  0.1666667  0.1428571  0.0000000  1.0000000
```

Table 25 Genetic similarity index of *Piper* spp. for the primer OPAH15

```
" SIMQUAL: input=A:\opah15, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 11L 11  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt Ppseud Pbababud
  1.0000000
  0.8888889  1.0000000
  0.3750000  0.3333333  1.0000000
  0.1250000  0.1111111  0.3333333  1.0000000
  0.8888889  1.0000000  0.3333333  0.1111111  1.0000000
  0.1111111  0.2222222  0.0000000  0.0000000  0.2222222  1.0000000
  0.1111111  0.2222222  0.0000000  0.0000000  0.2222222  1.0000000  1.0000000
  0.4444444  0.5555556  0.1428571  0.0000000  0.5555556  0.1666667  0.1666667  1.0000000
  0.7777778  0.8888889  0.3750000  0.1250000  0.8888889  0.2500000  0.2500000  0.6250000  1.0000000
  0.3750000  0.3333333  1.0000000  0.3333333  0.3333333  0.0000000  0.0000000  0.1428571  0.3750000  1.0000000
  0.1250000  0.1111111  0.3333333  0.0000000  0.1111111  0.0000000  0.0000000  0.2000000  0.1250000  0.3333333  1.0000000
```

OPAH 15

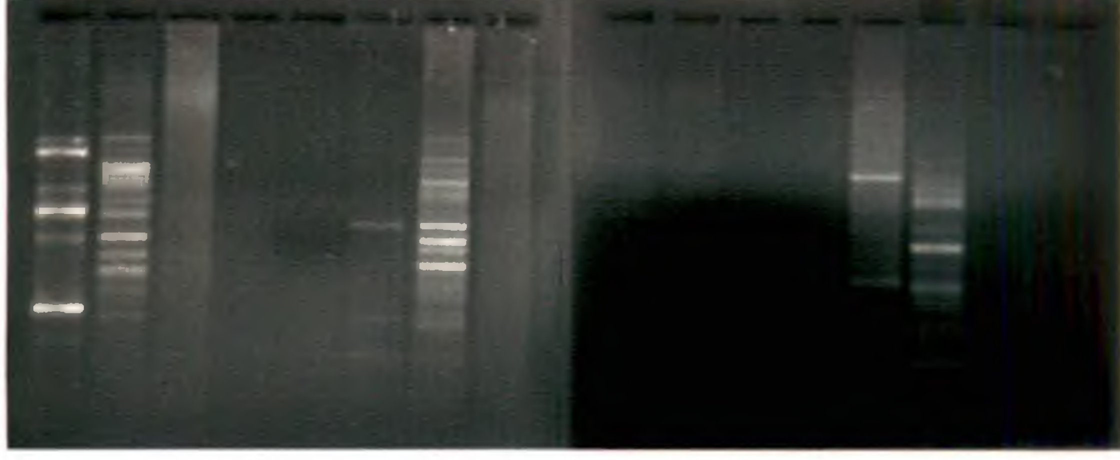
The species pairs *P. nigrum* P1 and *P. nigrum* Wild, *P. nigrum* P1 and *P. colubrinum*, *P. nigrum* Wild and *P. colubrinum*, *P. attenuatum* and *P. nigrum* Wild and *P. attenuatum* and *P. colubrinum* showed highest genetic similarity i.e., 0.889 for OPAH 15 primer (Table 25). The species pairs *P. longum* PLD and *P. chaba*, *P. longum* P25 and *P. chaba*, P. longum PLD and *P. betle*, *P. longum* P25 and *P. betle*, *P. longum* P25 and *P. arboreum* showed zero per cent genetic similarity between themselves. The species *P. pseudonigrum*, *P. bababudani*, *P. chaba* and *P. betle* has shown low similarity with all other species and among themselves whereas *P. attenuatum*, *P. nigrum* P1 and *P. colubrinum* showed maximum number of bands and similarity among themselves (Plate 21).

OPAH 18

RAPD banding pattern of all the species were distinct for OPAH 18. The number of bands ranged from 1 to 9 (Plate 22). Similarity of banding pattern was calculated (Table 26). The highest similarity was observed in *P. nigrum* P1 and *P. nigrum* Wild (0.800). The species pairs *P. attenuatum* and *P. betle and P. attenuatum* and *P. arboreum* showed the next highest genetic similarity of 0.750, whereas the South American spp. and *P. chaba* showed complete distinctness with all the species with least genetic similarity.

OPAH 20

The genetic similarity among species ranged from 0.000 to 0.778 for OPAH 20 (Table 27). *P. attenuatum* showed 0.778 per cent genetic similarity with



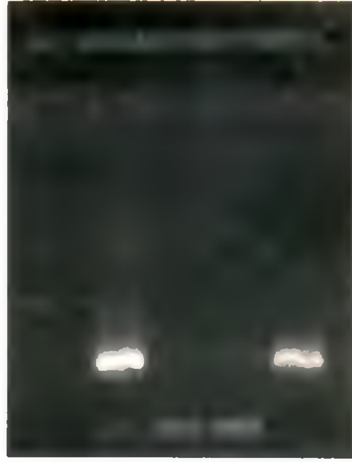


Plate 21. RAPD profile of *Piper* species for the primer OPAH15

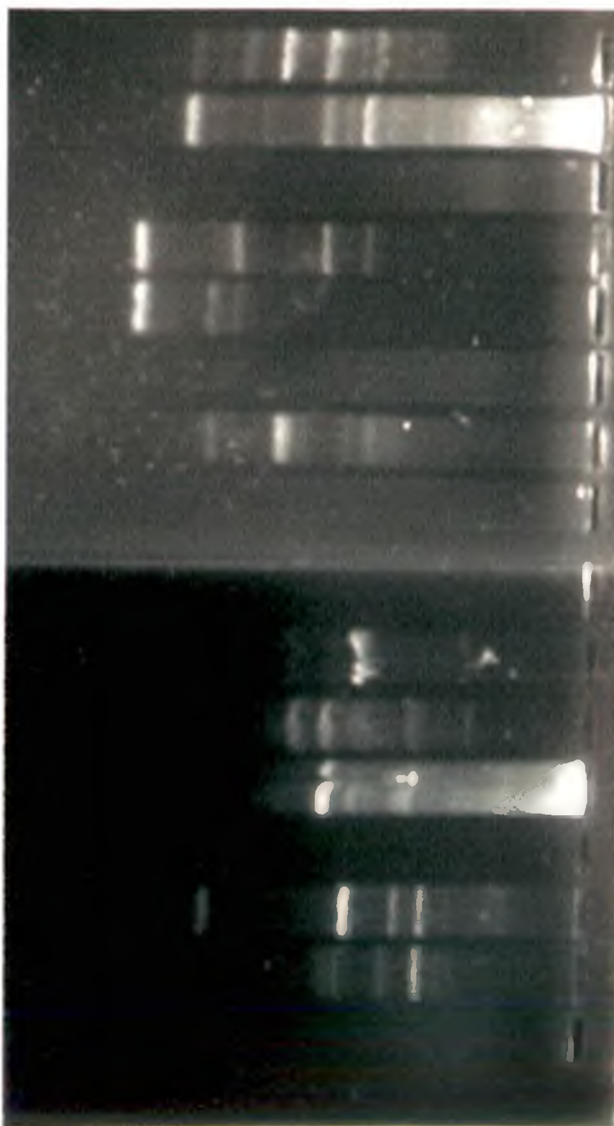


Plate 22. RAPD profile of *Piper* species for the primer OPAH1

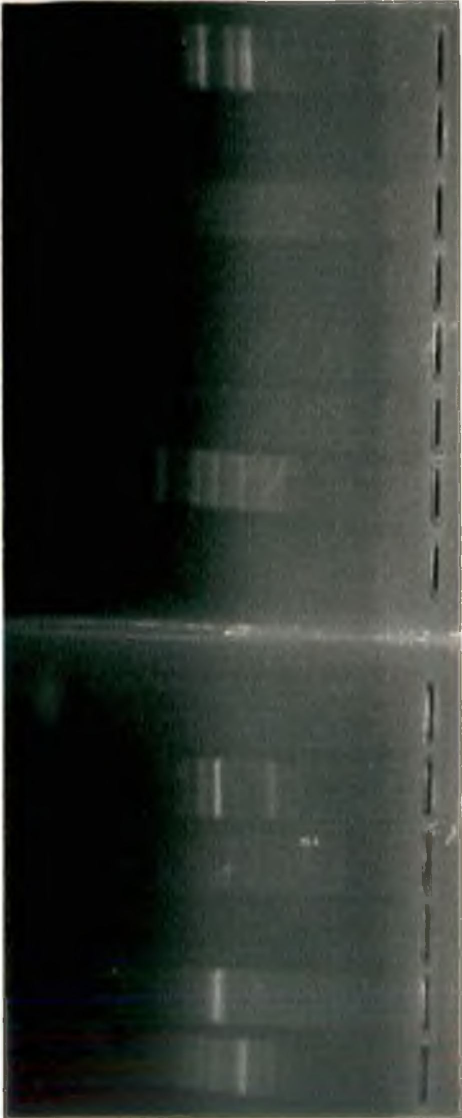


Plate 23. RAPD profile of *Piper* species for the primer OPAH20

Table 26 Genetic similarity index of *Piper* spp. for the primer OPAH18

```
" SIMQUAL: input=A:\opah18, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 10L 10  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt SA
  1.0000000
  0.8000000  1.0000000
  0.4285714  0.5000000  1.0000000
  0.1428571  0.1666667  0.6000000  1.0000000
  0.3333333  0.1666667  0.3333333  0.2000000  1.0000000
  0.0000000  0.0000000  0.0000000  0.0000000  0.0000000  1.0000000
  0.2857143  0.3333333  0.2857143  0.0000000  0.1666667  0.2500000  1.0000000
  0.1250000  0.1428571  0.2857143  0.1666667  0.4000000  0.2500000  0.6000000 1.0000000
  0.1428571  0.1666667  0.1428571  0.0000000  0.2000000  0.3333333  0.7500000 0.7500000  1.0000000
  0.0000000  0.0000000  0.0000000  0.0000000  0.0000000  1.0000000  0.2500000 0.2500000  0.3333333  1.0000000
```

Table 27 Genetic similarity index of *Piper* spp. for the primer OPAH20

```
" SIMQUAL: input=A:\opp20, coeff=J
" by Rows, +=   1.00000, -=   0.00000
3 9L 9  0
P1 PnW P1D P25 Pcol Pch Pb Parb Patt
  1.0000000
  0.1666667  1.0000000
  0.0000000  0.0000000  1.0000000
  0.7142857  0.1666667  0.0000000  1.0000000
  0.5555556  0.1250000  0.0000000  0.7500000  1.0000000
  0.1111111  0.0000000  0.0000000  0.1111111  0.2000000  1.0000000
  0.3000000  0.1428571  0.0000000  0.4444444  0.6666667  0.3750000  1.0000000
  0.2500000  0.0000000  0.0000000  0.2500000  0.3333333  0.1428571  0.3750000 1.0000000
  0.5555556  0.1250000  0.0000000  0.5555556  0.7777778  0.2000000  0.6666667 0.5000000  1.0000000
```

P. colubrinum. High degree of genetic similarity was observed in the species pairs of *P. nigrum* P1 - *P. longum* P25 (0.714) and *P. longum* P25 - *P. colubrinum* (0.750). *P. attenuatum* and *P. betle* showed highest degree of genetic distance with all other species (Plate 23) whereas *P. longum* PLD was totally distinct from all other species with similarity index of zero. The species *P. nigrum* Wild and *P. chaba* showed least similarity with all other species studied.

4.4 Pooled analysis for all the primers

Pooled analysis of similarity index of nine piper species is given in Table 28. Only nine species/accessions (*P. nigrum* P1, *P.* nigrum Wild, *P. longum* PLD, *P. longum* P25, *P. colubrinum*, *P. chaba, P. betle, P. arboreum* and *P. attenuatum*) were used for pooled analysis because only in these species all the 20 primers studied showed amplification.

The genetic similarity was calculated employing Jaccard's coefficient using NTSYS programme. The species pair *P. longum* (PLD) and *P. longum* (P25) showed the maximum similarity of 0.550 followed by the similarity of 0.492 for the species pair of *P. attennuatum* and *P. colubrinum.*

P. nigrum has shared 0.482 per cent similarity with *P. colubrinum.* The species pair *P. chaba* and *P. betle* showed a similarity index of 0.479. *P. nigrum* Wild showed similarity of 0.457 with *P. longum* PLD. The species pair *P. nigrum* and *P. nigrum* Wild has shown the similarity index of 0.423. Whereas the species pair *P. nigrum* Wild and *P. chaba* showed only 0.244 to 0.457 similarity. For *Piper longum* PLD the similarity ranged between 0.251 to 0.550 whereas it was from 0.312 to 0.550 for *P. longum* P25. The South American species *P. colubrinum*

Table 28 Genetic similarity index among Piper spp. for 20 random primers

```
" SIMQUAL: input=A:\pooleddata.NTS, coeff=J
" by Cols, +=   1.00000, -=   0.00000
3 9L 9  0
P.nigrumP1 P.nigrumW P.longumPLD P.longumP25 P.colubrinum P.chaba P.betle
P.arboreum P.attenuatum
  1.0000000
  0.4230769  1.0000000
  0.3529412  0.4571429  1.0000000
  0.3649635  0.3565217  0.5500000  1.0000000
  0.4829932  0.3357143  0.3381295  0.4104478  1.0000000
  0.3266667  0.2444444  0.2945736  0.3281250  0.4460432  1.0000000
  0.3819444  0.2824427  0.2945736  0.3492063  0.4565217  0.4796748  1.0000000
  0.3785714  0.3613445  0.3416667  0.3120000  0.4444444  0.4666667  0.3968254 1.0000000
  0.3581081  0.3178295  0.2518519  0.3129771  0.4926471  0.2957746  0.4153846 0.4015748  1.0000000
```

showed relatively higher similarity with all other species studied. The range was between 0.335 to 0.492. The similarity range for *P. chaba* was from 0.244 to 0.479, whereas it was from 0.282 to 0.479 for *P. betle* with other genotypes. The species *P. arboreum* showed relatively high similarity with all the species studied. It was between 0.312 to 0.466. For *P. attenuatum* the similarity ranged between 0.251 to 0.492.

4.5 Dendrogram of *Piper* species using RAPD data

The RAPD profile of nine species/accessions with 20 primers were analysed using the UPGMA method using NTSYS programme. The genetic distances (D) were used to construct a dendrogram of the nine species (Fig. 1).

The nine species were classified into two clusters with four subclusters (Table 29). The major cluster A at 39 per cent similarity level included two sub clusters namely A1 and A2. The major cluster A was having four OTUs (Operational Taxonomic Units) viz., *P. nigrum* P1, *P. nigrum* Wild, *P. longum* P25, and *P. longum* PLD. The subcluster A1 had two OTUs *P. longum* PLD, and *P. longum* P25 with 55 per cent similarity between them. In the sub cluster A2. *P. nigrum* P1 showed 42 per cent similarity with *P. nigrum* Wild.

The major cluster B at 41 per cent similarity level included two subclusters namely B1 and B2. The major cluster B was having five OTUs viz., *P. colubrinum*, *P. attenuatum*, *P. chaba*, *P. betle* and *P. arboreum*. The subcluster B1 was having two OTUs viz., *P. colubrinum* and *P. attenuatum*. *P. chaba*, *P. betle* and *P. arboreum* formed the subcluster B2 within the cluster B. Around 49 per cent similarity was observed between *P. colubrinum* and *P. attenuatum*. Around 48 per cent similarity was observed between *P. chaba* and *P. betle*.

Fig.1 DENDROGRAM OF NINE PIPER SPECIES FROM RAPD DATA USING UPGMA CLUSTERING

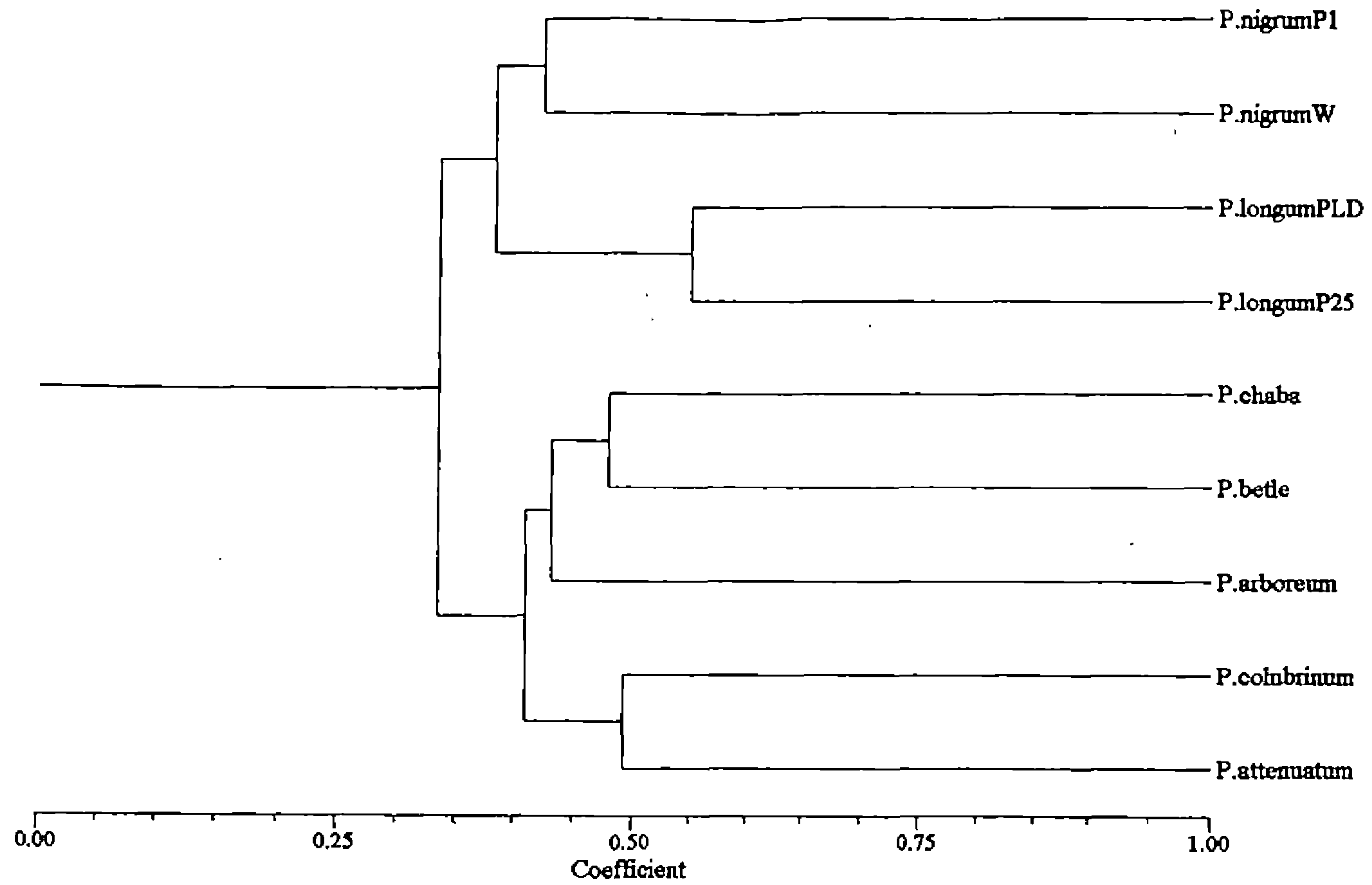


Table 29. Clustering of the *Piper* species based on RAPD data

Sl. No.	Major Cluster	Subcluster	No. of species (OTS)	Name of the species
1	A	A1	2	*P. nigrum* *P. nigrum* Wild
		A2	2	*P. longum* PLD *P. longum* P 25
2	B	B1	2	*P. colubrinum* *P. attenuatum*
		B2	3	*P. chaba* *P. betle* P. *arboreum*

Discussion

5. DISCUSSION

5.1 Standardisation of DNA isolation protocol

Among the three methods tested for total genomic DNA isolation of *Piper,* the protocol by Edwards *et al.* (1991) modified with chloroform isoamyl alcohol treatment was most simple, did not require liquid nitrogen and gave sufficient quantity DNA with good quality. Since the procedure did not require liquid nitrogen, DNA isolation was more economic also. Other modifications tried such as chloroform isoamyl alcohol treatment, grinding with PVP, proportional increase of leaf sample and half an hour freezing of leaf sample did not yield superior quality or more quantity of DNA. The quantity of DNA recovered was proportionate with the amount of leaf sample used for isolation. However, the DNA was found degraded whenever the amount of leaf sample was increased. With increase in leaf sample, the DNA gave slightly smeared to totally smeared bands.

The chloroform isoamyl alcohol treatment was found to give good quality, DNA with less impurity. The usefulness of chloroform isoamyl alcohol treatment to precipitate protein and improve the quality of DNA of black pepper was reported by Haneeshbabu *et al.* (2000). The RNASe treatment was found effective in order to remove RNA from the DNA sample and the quality and purity of the sample improved with the treatment. This is in line with the reports of Bendick (1997), Haneeshbabu *et al.* (2000) and Mondal *et al.* (2000). The amount of DNA recovered in the Edwards *et al.* (1991) method was comparable with Haneeshbabu *et al.* (2000) method. Since one RAPD reaction mixture required 25 to 50 ng of DNA, the DNA recovered in this method was found sufficient for further RAPD analysis.

5.2 Selection of primers for screening

Forty random deccamer primers were used for screening the species. The most important spice crop of the genus, *Piper nigrum*, was used for screening the primers. All the primers did not amplify well in the species. Species to species variation was also noticed in the amplification. Similar trends have been reported in other crop species also (coffee, alfalfa etc.).

From the fifty primers used for screening twenty primers were finally selected for RAPD analysis of *Piper* species. Table 30 shows pair wise similarity among *Piper* species for 20 selected primers..As it is very clear from the table, the species pairs showed high degree of variation in similarity for different primers.

5.3 Primer to primer variation in similarity among species

For example in the species pair P1 and PnW the similarity ranged between 0 to 0.89. Similarity of the two accession of *P. longum* and *P. nigrum* (P1) ranged between 0 to 0.8. It was surprising to note that the P1 showed very low level of similarity with *P. attenuatum* with only one primer showing a slightly high level of similarity of 0.778 (Table 30, col 4). This is inspite of the fact that both species originated in the western ghats, shared the same chromosome number of 2n=52 and showed fairly good morphological similarity (Anand, 1997). On the other hand Panniyur 1 showed a similarity above 0.7 for three primers studied with *P. colubrinum* and for two primers with *P. arboreum,* both being taxa from South America. PnW showed similarities varying from 0 to 1 with *P. longum* PLD, P25 and *P. colubrinum* for different primers. In the case of *P. longum,* the two

Table 30. Pair-wise similarity index in the genus *Piper* for 20 selected primers

	1	2	3	4	5	6	7	8	9	10	11	12
	P1-PnW	P1-PLD	P1-P25	P1-P. atten	P1-P.betle	P1-P.chaba	P1-P.colu	P1-P.arbo	PnW-PLD	PnW-P25	PnW-P.atten	PnW-P.betle
OPF1	0.250	0.250	0.250	0.000	0.400	0.285	0.000	0.250	1.000	1.000	0.333	0.600
OPF5	0.600	0.800	0.272	0.300	0.600	0.636	0.636	0.636	0.750	0.250	0.500	0.714
OPP2	0.800	0.125	0.000	0.500	0.333	0.333	0.200	0.333	0.142	0.000	0.600	0.400
OPP3	0.400	0.600	0.600	0.167	0.200	0.500	0.800	0.167	0.000	0.000	0.000	0.000
OPP4	0.428	0.428	0.428	0.142	0.100	0.000	0.000	0.200	1.000	1.000	0.333	0.363
OPP5	0.800	0.750	0.400	0.222	0.400	0.400	0.400	0.800	0.600	0.600	0.333	0.333
OPP6	0.167	0.333	0.500	0.200	0.250	0.285	0.667	0.500	0.750	0.333	0.250	0.000
OPP7	0.000	0.428	0.285	0.250	0.750	0.667	0.444	0.285	0.000	0.000	0.250	0.000
OPP9	0.333	0.285	0.571	0.333	0.428	0.714	0.714	0.717	0.750	0.285	0.000	0.333
OPP12	0.222	0.142	0.250	0.454	0.454	0.363	0.600	0.375	0.250	0.750	0.444	0.444
OPP13	0.364	0.364	0.364	0.300	0.600	0.800	0.300	0.500	1.000	1.000	0.250	0.300
OPP20	0.166	0.000	0.714	0.555	0.300	0.111	0.555	0.250	0.000	0.166	0.125	0.142
OPAH6	0.400	0.750	0.750	0.400	0.500	0.000	0.333	0.600	0.333	0.333	0.333	0.400
OPAH9	0.200	0.000	0.666	0.285	0.428	0.500	0.571	0.333	0.000	0.200	0.000	0.200
OPAH12	0.333	0.100	0.200	0.416	0.300	0.000	0.500	0.111	0.000	0.200	0.375	0.166
OPAH13	0.333	0.333	0.200	0.250	0.000	0.000	0.125	0.250	0.714	0.428	0.285	0.142
OPAH14	0.666	0.250	0.333	0.222	0.428	0.250	0.375	0.250	0.142	0.250	0.125	0.333
OPAH15	0.888	0.375	0.125	0.777	0.111	0.111	0.888	0.444	0.333	0.111	0.888	0.222
OPAH18	0.800	0.428	0.142	0.142	0.285	0.000	0.333	0.125	0.500	0.166	0.166	0.333
OPAH20	0.166	0.000	0.714	0.555	0.300	0.111	0.555	0.250	0.000	0.166	0.125	0.142

Contd.

Table 30. Continued

	13	14	15	16	17	18	19	20	21	22	23	24
	PnW-P.chaba	PnW-P.colu	PnW-P.arbo	PLD-P25	PLD-P.atten	PLD-P.betle	PLD-P.chaba	PLD-P.colu	PLD-P.arbo	P25-P.atten	P25-P.betle	P25-P.chaba
OPF1	0.428	0.250	0.500	1.000	0.333	0.600	0.428	0.250	0.500	0.333	0.600	0.428
OPF5	0.750	0.750	0.750	0.333	0.375	0.750	0.600	0.777	0.600	0.166	0.428	0.333
OPP2	0.166	0.250	0.166	0.250	0.333	0.400	0.400	0.250	0.166	0.250	0.000	0.000
OPP3	0.000	0.200	0.000	1.000	0.250	0.333	0.750	0.750	0.250	0.250	0.333	0.750
OPP4	0.000	0.000	0.250	1.000	0.333	0.363	0.000	0.000	0.250	0.333	0.363	0.000
OPP5	0.600	0.600	0.666	0.200	0.250	0.200	0.200	0.200	0.600	0.250	0.200	0.500
OPP6	0.333	0.500	0.600	0.500	0.200	0.250	0.285	0.666	0.500	0.000	0.200	0.666
OPP7	0.000	0.142	0.000	0.750	0.333	0.500	0.444	0.571	0.166	0.400	0.375	0.333
OPP9	0.428	0.428	0.428	0.428	0.166	0.500	0.571	0.571	0.571	0.500	0.833	0.857
OPP12	0.333	0.300	0.142	0.333	0.111	0.111	0.000	0.111	0.000	0.333	0.333	0.222
OPP13	0.500	0.111	0.333	1.000	0.250	0.300	0.500	0.111	0.333	0.200	0.300	0.500
OPP20	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.555	0.444	0.111
OPAH6	0.200	0.285	0.500	1.000	0.600	0.400	0.200	0.500	0.500	0.600	0.400	0.200
OPAH9	0.000	0.166	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.600	0.285
OPAH12	0.000	0.333	0.333	0.666	0.111	0.200	0.000	0.100	0.000	0.222	0.400	0.000
OPAH13	0.500	0.444	0.750	0.666	0.285	0.142	0.500	0.625	0.750	0.400	0.200	0.428
OPAH14	0.000	0.285	0.142	0.666	0.285	0.142	0.333	0.500	0.142	0.375	0.250	0.428
OPAH15	0.222	1.000	0.555	0.333	0.375	0.000	0.000	0.333	0.142	0.125	0.000	0.000
OPAH18	0.000	0.166	0.142	0.600	0.142	0.285	0.000	0.333	0.285	0.000	0.000	0.000
OPAH20	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.555	0.444	0.111

Contd.

Table 30. Continued

	25	26	27	28	29	30	31	32	33	34	35	36
	P25-P.colu	P25-P.arbo	P.betle-P.atten	P.chaba-P.atten	P.colu-P.atten	P.arbo-P.atten	P.chaba-P.betle	P.colu-P.betle	P.arbo-P.betle	P.colu-P.chaba	P.chaba-P.arbo	P.colu-P.arbo
OPF1	0.250	0.500	0.200	0.142	0.500	0.333	0.714	0.400	0.600	0.285	0.428	0.250
OPF5	0.500	0.333	0.500	0.375	0.375	0.375	0.555	0.750	0.555	0.777	1.000	0.777
OPP2	0.000	0.000	0.166	0.166	0.250	0.166	0.200	0.333	0.200	0.333	0.200	0.000
OPP3	0.750	0.250	0.000	0.500	0.200	1.000	0.250	0.250	0.000	0.600	0.500	0.200
OPP4	0.000	0.250	0.500	0.166	0.166	0.600	0.250	0.250	0.375	1.000	0.250	0.250
OPP5	1.000	0.333	0.111	0.250	0.250	0.200	0.500	0.200	0.600	0.500	0.333	0.333
OPP6	0.571	0.428	0.000	0.000	0.333	0.400	0.000	0.166	0.000	0.375	0.428	0.833
OPP7	0.428	0.000	0.333	0.300	0.375	0.166	0.888	0.500	0.222	0.600	0.333	0.250
OPP9	0.857	0.857	0.600	0.428	0.428	0.428	0.714	0.714	0.714	1.000	1.000	1.000
OPP12	0.200	0.166	0.800	0.888	0.636	0.444	0.700	0.636	0.444	0.545	0.500	0.300
OPP13	0.111	0.333	0.222	0.300	0.333	0.250	0.777	0.571	0.857	0.444	0.666	0.666
OPP20	0.750	0.250	0.666	0.200	0.777	0.500	0.375	0.666	0.375	0.200	0.142	0.333
OPAH6	0.500	0.500	0.750	0.200	0.502	0.500	0.000	0.333	0.600	0.166	0.166	0.428
OPAH9	0.833	0.333	0.500	0.333	0.666	0.750	0.500	0.833	0.333	0.428	0.400	0.500
OPAH12	0.200	0.000	0.333	0.125	0.888	0.125	0.250	0.444	0.000	0.111	0.000	0.111
OPAH13	0.375	0.500	0.000	0.125	0.250	0.222	0.333	0.285	0.250	0.625	0.750	0.666
OPAH14	0.571	0.250	0.500	0.500	0.250	0.800	0.333	0.285	0.600	0.500	0.600	0.285
OPAH15	0.111	0.000	0.250	0.250	0.888	0.625	1.000	0.222	0.166	0.222	0.166	0.555
OPAH18	0.200	0.166	0.750	0.333	0.200	0.750	0.250	0.166	0.600	0.000	0.200	0.400
OPAH20	0.750	0.250	0.666	0.200	0.777	0.500	0.375	0.666	0.375	0.200	0.142	0.333

accessions studied shared 100 per cent similarity (coefficient 1.000) for 5 primers. However, for two primers, the similarity was 0 between the two species (Col 6). PLD also showed similarity range in between 0 to 1 with PnW. *P. longum* (P25) showed a similarity as high as 1 with *P. colubrinum,* more than 0.7 similarity for 15 primers. The two taxa were earlier reported to be widely differing in morphology, chromosome number (Anand, 1997 and 2000) and isozyme characters (Sebastian *et al.*, 1996). *P. attenuatum* showed similarity range between 0 to 0.8 with *P. betle,* 0 to 0.89 with *P. chaba* and 0.13 to 1.00 with *P. arboreum. P. betle* showed similarity index ranging between 0 to 1.0 with *P. chaba* and 0 to 0.86 with *P. arboreum. P. chaba* showed the similarity ranging between 0 to 1 with *P. colubrinum* and *P. arboreum.* So also *P. arboreum* showed a similarity ranging between 0 to 1 with *P. colubrinum* for different primers as it is very evident from the table, the species pairs differed widely in genetic similarity from primer to primer. This clearly indicates the necessity of using more number of primers for getting reliable information on species affinity. In the current study 20 primers were used for analysis. Rajkumar *et al.* (2000) had reported that 20 primers were the optimum number for genetic relationship study with RAPD. He had used up to 40 primers for analysis and observed that 20 primers were the optimum number above which there was no significant difference in the pooled data on genetic similarity.

Pooled analysis of similarity using Jaccard's coefficient is presented in Table 28. A phenetic tree was constructed using the same RAPD data using UPGMA clustering (Fig.1). As can be seen from the table and dendrogram, the

similarity among the nine *Piper* species ranged between 0.24 and 0.55. Maximum similarity (0.55) was between two accessions of *Piper longum* viz. PLD, a collection from Dehradun and P25, a collection from Western Ghats. Other accessions which clustered together were *P. colubrinum* and *P. attenuatum* (similarity 0.49) and *P. chaba* and *P. betle* (similarity 0.48). *P. arboreum* showed a similarity of 0.47 with *P. chaba*, 0.44 with *P. colubrinum* and 0.40 with *P. betle*. *P. attenuatum* showed a pooled similarity of 0.41 with *P. betle* and 0.40 with *P. arboreum*. *P. betle* showed similarities as high as 0.48 with *P. chaba* and 0.46 with *P. colubrinum*. *P. chaba* also showed a similarity of 0.45 with *P. colubrinum*.

P. colubrinum showed a high level of similarity of 0.48 with *P. nigrum* (P1) and 0.41 with *P. longum* (P25). On the other hand the similarity of *P. colubrinum* with other accessions of the two species was only 0.34. *P. longum* (PLD) was closely related to *P. nigrum* Wild (0.46) whereas its similarity with *P. nigrum* P1 was only 0.35. The two accessions of *P. nigrum* showed a similarity of 0.423 indicating that the difference between the two were more than 0.57. Even though it is surprising to note such low level of similarity within con specific taxa, the earlier works in *P. nigrum* support the current findings. Kanakamony (1985) grouped 45 accessions of *P. nigrum* into 40 groups due to high level of morphological variation within the species. Sebastian *et al.* (2000) also reported SI as low as 0.40 between accessions of *P. nigrum*. The probable reason for high level of variation with in *P. nigrum* was suggested as high level of outcrossing followed by vegetative propagation whereby variability once created is getting fixed. The result

of the current study supports that the polymorphism within *P. nigrum* is not only at morphological or biochemical but depicted at DNA level as well. In the current study *P. colubrinum* was found to have high level similarity with *P. attenuatum* (0.49). The two taxa had widely diverse geographic origins such as South America and Western Ghats of South India, very different in morphology (Anand, 1997) and quality characters and widely varying chromosome numbers 2n = 52 (*P. nigrum*) and 2n = 26 (*P. colubrinum*) (Anand *et al.*, 2000). Isoenzyme studies also showed similarity (as low as 0.16 to 0.17) between the two taxa (Sebastian *et al.*, 1996). The findings of Rahiman and Subbiah (1984), Rahiman and Bhagavan (1985), Ravindran *et al.* (1992) and Sebastian *et al.* (1996) that the species *Piper nigrum* and *P. longum* were distinct from each other also did not get supporting results in the current study. Such deviations in conventional taxonomic relationship based on DNA data were reported earlier also (Chakrabarti *et al.*, 1999, Padmesh *et al.*, 1999). Williams *et al.* (1990) had reported that RAPD loci are likely to be found within non coding regions or not being closely linked to structural genes. This is probably the reason for differences observed sometimes between conventional markers like morphological and isoenzyme markers on one hand and DNA markers on the other. Phenotypic markers cannot identify the genetic differences of non-coding genes.

Similarity matrix was subjected to Sequential Agglomerative Heirarchial Nested Clustering (SAHN). A phenetic dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) by Sneath and

Sokel (1973) using NTSYS package. As can be seen from Fig.1 four primary clusters were obtained. In cluster 1 *Piper longum* (PLD) grouped with *P. longum* P25. Cluster 2 had *P. colubrinum* and *P. attenuatum*. Cluster 3 grouped *P. chaba* and *P. betle* and cluster 4 had two accessions of *Piper nigrum*.

Earlier studies conducted at this institute had shown that *P. chaba* and *P. betle* differed in morphology (Anand, 1997). The two species were reported to have different 2n number (Anand *et al.*, 2000) and differed in isozyme zymogram with similarity ranging between 0.10 to 0.18 (Sebastian *et al.*, 1996).

To conclude, in the current study an attempt was made to study the genetic variation in thirteen accessions of *Piper* using RAPD techniques. Species pairs showed much variation from primer to primer for the similarity coefficient. Pooled analysis of similarity among the nine accessions of *Piper* which gave amplification for all 20 selected primers showed that the accessions within a taxa clustered together as expected. However, clustering of different species in the genus was not always in the expected level. The probable reason attributed was non-coding regions of the genome and nucleotides beyond structural genes getting amplified and producing band. A more detailed study in the genus with more number of species and more accessions within taxa is warranted.

Summary

6. SUMMARY

The study "Molecular characterization of *Piper* species using RAPD technique" was conducted in the Department of Plantation Crops and Spices and CPBMB, College of Horticulture, Vellanikkara during the period from December 1991 to August 2001. The major objectives were to study the genetic polymorphism in *Piper* species at molecular level and to elucidate species relationship using RAPD analysis. Molecular studies included standardisation of DNA isolation protocol, primer screening and the RAPD analysis using Polymerase Chain Reaction (PCR). The results of the studies are summarised below:

DNA isolation protocol was standardised with three different methods with various possible modifications. Fully expanded immature leaves were used for the DNA isolation. Among the different methods tried, the protocol by Edwards *et al.* (1991) with the modification of chloroform isoamyl alcohol treatment was found good in terms of both quality and quantity of the DNA recovered, simplicity and economy of the technique. In all the three methods, proportionate DNA degradation was found with the increasing amount of leaf sample. Even the liquid nitrogen, polyvinyl pyrolydane (PVP) grinding and half an hour freezing of leaf sample failed to check the DNA degradation.

The RNase treatment, inorder to remove RNA from the DNA sample was found effective in getting good quality DNA without any RNA interference.

Primer screening

Fifty decamer primers (20 each from OPP, OPAH series and 10 from OPF series) from Operon Technology USA were screened with Panniyur 1 (*P. nigrum*) DNA samples. Twenty (2 from OPF series, 12 from OPP series, 8 from OPAH series) primers were selected for further RAPD analysis based on the number of bands produced, stability and reproducibility of the banding pattern.

RAPD analysis

RAPD analysis of 13 species/accessions of *Piper* was done using the twenty selected decamer primers with the standard thermo cycler settings. The experiments with each primer was repeated twice in order to get reproducible and stable results.

Genetic similarity of *Piper* species using RAPD data

The Similarity among *Piper* spp. ranged between 0.000 to 0.550 for twenty primers studied. The species pair *P. nigrum* (P1) - *P. longum* (PLD) and *P. nigrum* (P1) - *P. longum* (P25) showed a similarity of 0.550. The next highest similarity of 0.492 was observed between *P. attenuatum* and *P. colubrinum*. The species *P. nigrum* shared 0.482 per cent similarity with *P. colubrinum*. *P. nigrum* P1 shared similarity of 0.423 with *P. nigrum* Wild. The species pair *P. nigrum* Wild and *P. chaba* shared a similarity of only 0.244. The species pair *P. attenuatum* and *P. longum* (PLD) showed a genetic similarity of 0.251 only in pooled analysis. Results of the similarity studies were subjected to cluster analysis (SAHN). A phenetic dendrogram was constructed using Unweighted Pair Group Method of

Arithmatic Averages (UPGMA). Four clusters were obtained comprising of two accessions of *P. nigrum* in the first (A1), two accessions of *P. longum* in the second (A2), *P. colubrinum* and *P. attennuatum* forming the third (B1) and *P. chaba*, *P. bettle* and *P. arboreum* forming the fourth cluster (B2).

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* Originals not seen

Appendices

APPENDIX-I

List of major equipments used for the study

1	Spectrophotometer	Spectronic ® Genesys-5 Spectronic instruments, Inc. USA
2	Refrigerated high speed centrifuge	Kubota, Japan
3	Water purification system	Millipore, Germany
4	Deep freezer	Sanyo, Japan
5	Electroic balances	Sartorious
6	Laminar flow	Kirloskar, India
7	Cytocentrifuge	Spinwiss
8	Electrophoresis system	Huefer, USA, Biotech Chennai, Genie Bangalore
9	Thermocycler	Peltire PTC 200 M.J.Research, USA
10	Transilluminator	Hero Lab, Germany
11	Gel documentation system	Alpha infotech, USA
12	Ice flaking machine	Ice Matics

APPENDIX-II

List of important chemical used in the study

1	Cetyl trimethyl ammonium bromide (CTAB)	E. Merck, Germany
2	Sodium dodysil sulphate (SDS)	Sigma, USA
3	ß-Mercopto ethanol	E. Merck Germany
4	Ethidium Bromide	Sigma, USA
5	Agarose	Genei, Bangalore
6	Taq DNA polymerase	Genei, Bangalore
7	PCR reagents (10 x buffer NTPS)	Genie, Bangalore
8	Decamer primers	Operon, USA
9	Bromophenol blue	Sigma, USA
10	Liquid N2	Madras Oxyacetylene Company, Coimbatore
11	Mineral oil	Sigma, USA
12	Tris base	Sigma, USA
13	EDTA	Sigma, USA

MOLECULAR CHARACTERIZATION OF *Piper* SPECIES USING RAPD TECHNIQUE

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The study on "Molecular characterization of *Piper* species using RAPD techniques" was conducted in the Department of Plantation Crops and Spices and CPBMB, College of Horticulture, Vellanikkara, Thrissur from December, 1999 to August, 2001. Material included 13 species/accessions of the genus *Piper* including *Piper nigrum* Linn. These were analysed with 20 selected oligomer decamer primers using PCR technique and agarose gel electrophoresis.

The selection of ideal DNA isolation technique was done based on observations of purity of bands, quality of the DNA bands and quantity of DNA recovered. The protocol of Edwards *et al.* (1991) was selected for DNA isolation with additional step of chloroform-isoamyl alcohol treatment.

Fifty decamer primers were screened using DNA of *P. nigrum* and 20 were selected based on the number of bands produced, reproducibility of the bands and distinctness of the RAPD profile.

RAPD profile of the 13 species of *Piper* were compared for genetic similarity index (using Jaccard's coefficient), with all 20 selected primers separately. Pooled similarity of the twenty primers put together was found out for 9 species which gave amplification for all the 20 selected primers. The results of pooled analysis was subjected to cluster analysis (SAHN) and phenetic dendrogram was constructed employing UPGMA.

Four clusters were obtained, comprising of two accessions of *P. nigrum* in the first, two accessions of *P. longum* in the second, *P. colubrinum* and *P. attenuatum* forming the third cluster and *P. chaba*, *P. betle* and *P. arboreum* forming the fourth.