



Genetic Diversity Analysis Of Selected Varieties Of *Piper Nigrum* Using RAPD

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Abstract: The *Piper nigrum* (*Piper Longum*) is a major plantation crop in tropical regions. Germplasm characterization using RAPD will be useful in accessing genetic diversity among natural population. Random Amplified Polymorphic DNA (RAPD) technique is a protocol sensitive which limit cross comparison of information generated by this method with others. Effectiveness of the present RAPD analysis in revealing the genetic diversity is evident from the amplification products. Present molecular marker study was done using five *Piper nigrum* varieties (P1, P2, P3, P4 & P5) collected from Regional Pepper Research Station, Panniyoor of kannur district, Kerala, India. Five random primers RP1, RP2, RP3, RP4, RP5, RP6, RP7, RP8, RP9 & RP10 were selected from 20 initially screened were used in molecular marker studies amplified 198 different reproducible bands. The number of bands per primer product ranged from 300 bp-1.5 kb. All the primers reveal polymorphism between clones. Out of 198 scored, 193 bands were found to be polymorphic. Maximum number of polymorphic bands (26) was obtained with the primer RP4, RP6 and RP10. The average number of polymorphic bands per primer is 19.3. Based on the bands obtained we can say that the level of genetic diversity among the varieties is high. The present study reveals the high genetic diversity among the pepper populations from Panniyoor clones. This information would be useful to study more genetic diversity analysis on Pepper species from different areas of the state and outside. The current molecular marker studies can be used to develop an effective molecular marker system for marker assisted selection and breeding for an effective crop improvement programme.

INTRODUCTION

Black pepper often referred as “king of spices” is incredibly popular spice since ancient times. Black pepper is a flowering vine in the family of Piperaceae, in the genus of piper and known scientifically as **Piper nigrum**. Black pepper is native to south Indian state Kerala and is extensively cultivated there and elsewhere in tropical regions. Morphological characters have been used extensively to study diversity of different form in the past. In recent years, attempts to study biodiversity at molecular level have gained importance. Among DNA based approaches for crop-improvement, the first step is the use of molecular markers as a tool to detect the extent and structure of Genetic variation providing insight into the diversity of crop- varieties and potential contributions represented by their wild relatives.

Random Amplified Polymorphic DNA (RAPD) is a PCR-based technology. The method is based on enzymatic amplification of target or random DNA segments with arbitrary primers. This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermal cyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals. However, due to the stochastic nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification. RAPDs are DNA fragments amplified by the PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1–10 genomic sites simultaneously. Amplified products (usually within the 0.5–5 kb size range) are separated on agarose gels in the presence of ethidium bromide and view under ultraviolet light and presence and absence of band will be observed. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites. Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands that are complementary to the primer. Of the different markers, RAPD has been widely used in the last decade in species identification programme and in assessing genetic variations among different taxa at DNA level because of its cost effectiveness and simple operation without requiring prior knowledge of species DNA sequences. RAPDs reveal similar patterns of genetic diversity when compared with other marker types and can be performed more rapidly than most other methods and can provide vital information for the development of genetic sampling, conservation and improvement strategies. M. Shivashankar et al., (2014) got the result in 6 species of piper showing variation in percentage of polymorphic bands from 24.48% to 06.12% using 12 primers of OPA 1-6 and OPD 1-6 while studying Differential Polymorphism. Lisong Hu et al., (2015) conducted studies in De Novo Assembly and Characterization of Fruit Transcriptome in Black Pepper (*Piper nigrum*). Jameel M Al-Khayri et al., (2022) conducted a genetic and morphological diversity assessment of five kalanchoe genotypes by SCoT, ISSR and RAPD-PCR markers carried out a comparative study and genetic diversity in Salvia (Lamiaceae) using RAPD Molecular Markers. Yamen AS Hamdan et

al., (2024) conducted a genetic diversity assessment of Palestinian Okra Landraces (*Abelmoschus esculentus* L.) Through RAPD Marker. Tutik Nurhidayati *et al.*, (2025) studied genetic diversity of Indonesian rice varieties for salinity tolerance using RAPD markers.

MATERIALS AND METHODS

SAMPLE COLLECTION

The present study selected leaf samples of 5 *Piper nigrum* varieties (Panniyoor 1, 2, 3, 4 and 5) that were collected from Pepper Research Station, Panniyoor, Thaliparamba, Kerala, India, which may represent the wide variation prevalent in the genome. The recently matured leaves were collected and used for DNA extraction.

DNA ISOLATION

Total cellular genomic DNA was isolated and purified from young leaves by the CTAB extraction procedure (Doyle and Doyle 1990). Young leaves were ground to very fine powder in liquid nitrogen in a mortar and pestle. CTAB buffer was added and centrifuged at 10,000 g for 10 min. Following extraction with an equal volume of Phenol: chloroform: isoamylalcohol (25:24:1), the aqueous phase was transferred to new centrifuge tube where RNA was eliminated by treatment with RNase. The aqueous phase was re-extracted twice with chloroform and DNA was precipitated by the addition of 0.6 volumes of ice-cold isopropanol (100%).

AGAROSE GEL ELECTROPHORESIS

The quality of the isolated DNA was checked by agarose gel electrophoresis. Two µl of loading buffer was added to 8 µl of DNA and the samples were loaded to 0.8% agarose gel prepared in 0.5X TBE buffer. Electrophoresis was carried out at 70 Volts for nearly 2 hours until the bromophenol dye front has migrated to the bottom of the gel. Staining was carried out with 0.5 µg/ml of ethidium bromide. The gel was visualized using gel documentation system and was photographed under UV light.

PCR AMPLIFICATION

8 µl of template DNA, 4µl of RAPD primer, 25µl PCR master mix and 13µl nuclease free water were added into microfuge tube. The solution was mixed gently and amplification were carried out using the following reaction condition (**Table 1**)

Temperature	94°C	94°C	35°C	72°C	94°C	38°C	72°C	72°C
Time	5 min	45 s	1 min	1.5 min	45 s	1 min	1 min	10 min
Steps	Initial denaturation	Denaturation	Annealing	Extension	Denaturation	Annealing	Extension	Final extension
Cycles		8 cycles			35 cycles			

Table 1. PCR conditions for the RAPD profile of *Cocos nucifera*

RESULTS AND DISCUSSION

Characterisation and quantification of genetic diversity have been a major goal in evolutionary biology and plant breeding. Study of genetic polymorphism provide scientific basis for utilization of germplasm resources efficiently in crop improvements. The *Piper nigrum* (*Piper Longum*) is a major plantation crop in tropical regions. Currently, Vietnam is the world's largest producer and exporter of pepper, producing 34% of the world's *Piper nigrum* crop.

Molecular markers are powerful techniques that reflect variation at the DNA sequence level. The molecular markers are present in unlimited numbers compared to phenotypic markers; hence they detect diversity at very high resolution. The molecular markers are helpful in crops for screening of germplasm for new sources of variation, identification of genotype marking of single gene controlling commercially important trait, morphing quantitative trait loss, study of genetic intergression, population genetics and anatomic studies. Molecular markers in contrast to phenotypic markers are independent of environment and they reflect variations at the DNA sequence level. Germplasm knowledge is quite important in breeding programme, since it is a source of variation that can be used for selection.



Figure. 4 Genomic DNA isolated from different varieties of *Piper nigrum*. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

Characterization and evaluation of black pepper, based on morphological and agronomical traits is time consuming, labour intensive and subject to environmental effects. Therefore, sensitive and reliable techniques for assessing genetic diversity and screening agronomic traits are necessary to establish effective collecting and gene banking strategies. Random amplified Polymorphic DNA is useful for the Characterization of DNA from plants and other organisms. This technique involves short oligo nucleotide primers that are arbitrarily selected to amplify a set of DNA fragments randomly distributed throughout the genome. The genetic resources of *Piper nigrum* in Asiatic region was characterized using fruit morphological and molecular characters throughout the region.

RAPD markers provide an effective tool for detection and evaluation of genetic variation existing among promising species of piper. It demonstrated the utility of using RAPD markers to characterize genetic diversity among promising species of piper. Differential polymorphism was noted in 3 species of piper showing variation in percentage of polymorphic bands. Of the different markers, RAPD has been widely used in the last decade in species identification programme and in assessing genetic variations among different taxa at DNA level because of its cost effectiveness and simple operation without requiring prior knowledge of species DNA sequences.

Genomic DNA from *Piper nigrum* was isolated in good quality and quantity (**Fig. 4**). 10 random primers RP1, RP2, RP3, RP4, RP5, RP6, RP7, RP8, RP9 and RP10 were used in molecular marker studies amplified 198 different reproducible bands (**Fig 5 to Fig 14**). Nine primers revealed 100% polymorphism between clones.

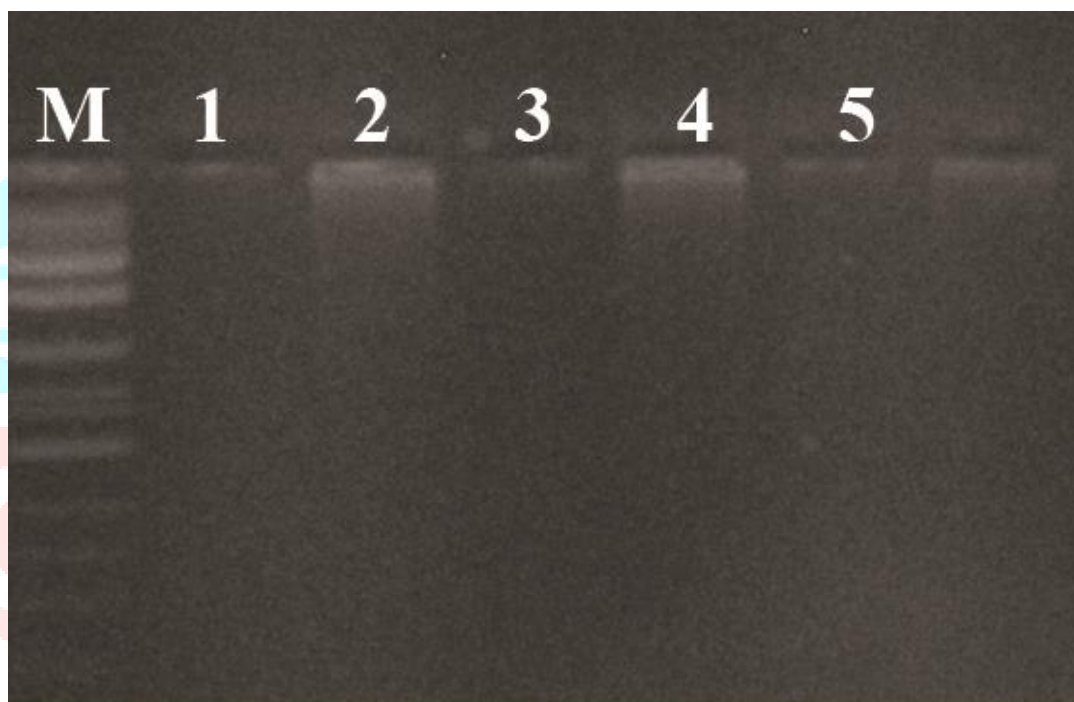


Figure. 5 RAPD profile of *Piper nigrum* using random primer 1. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

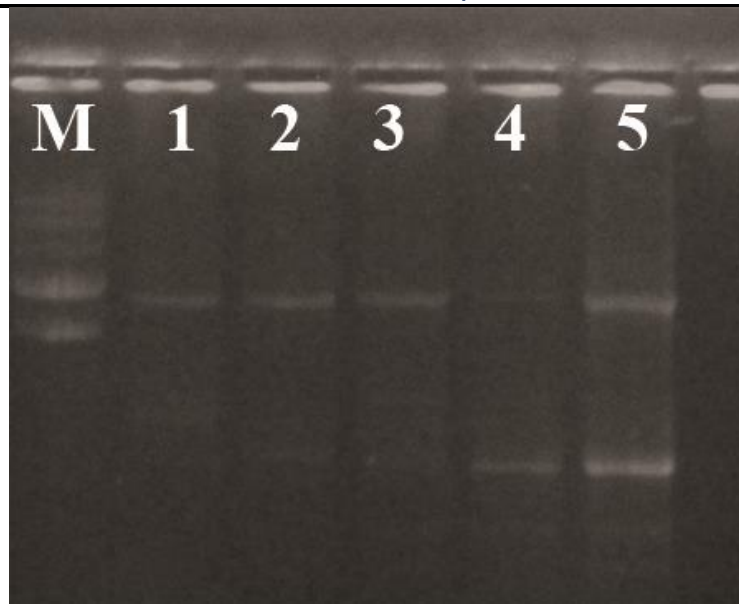


Figure. 6 RAPD profile of *Piper nigrum* using random primer 2. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5



Figure. 7 RAPD profile of *Piper nigrum* using random primer 3. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

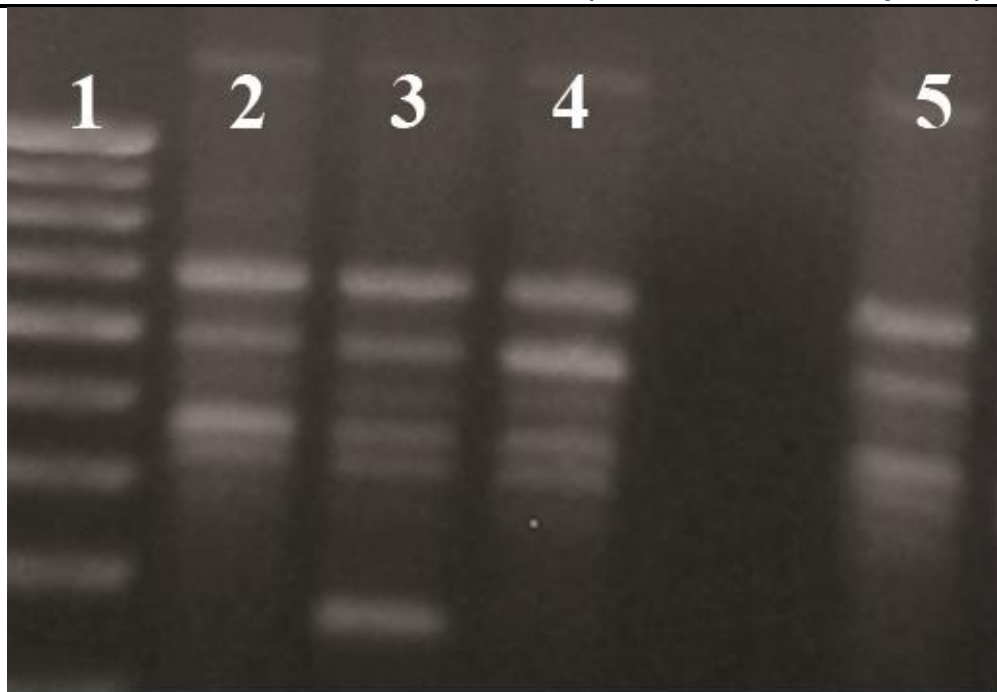


Figure. 8 RAPD profile of *Piper nigrum* using random primer 4. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

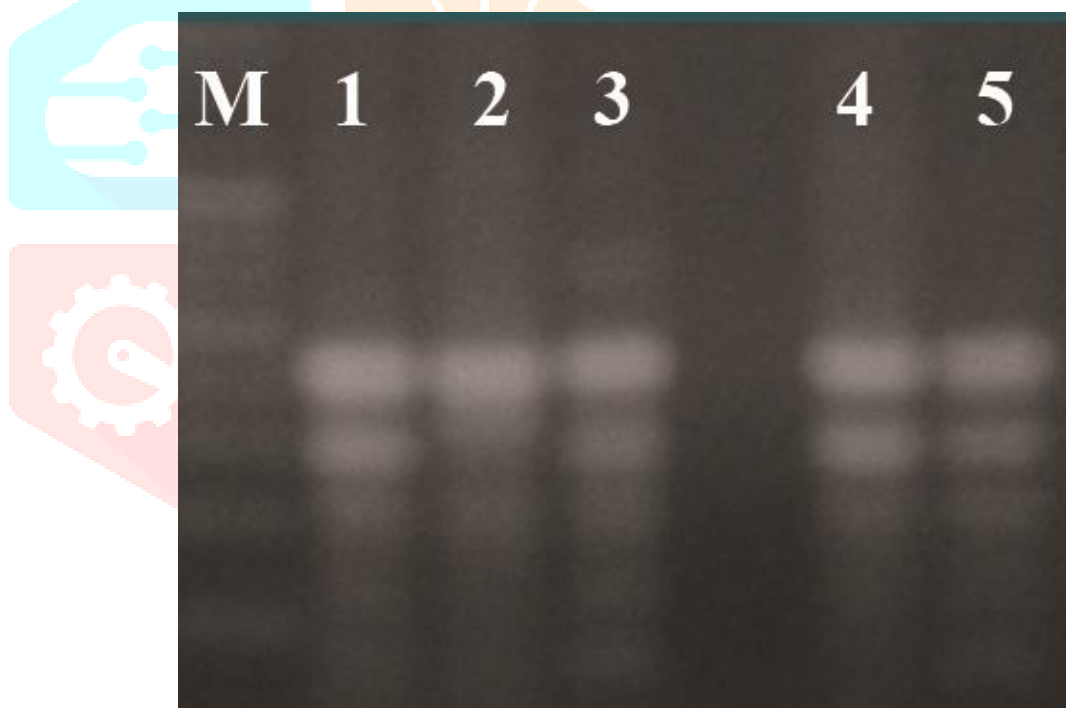


Figure. 9 RAPD profile of *Piper nigrum* using random primer 5. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

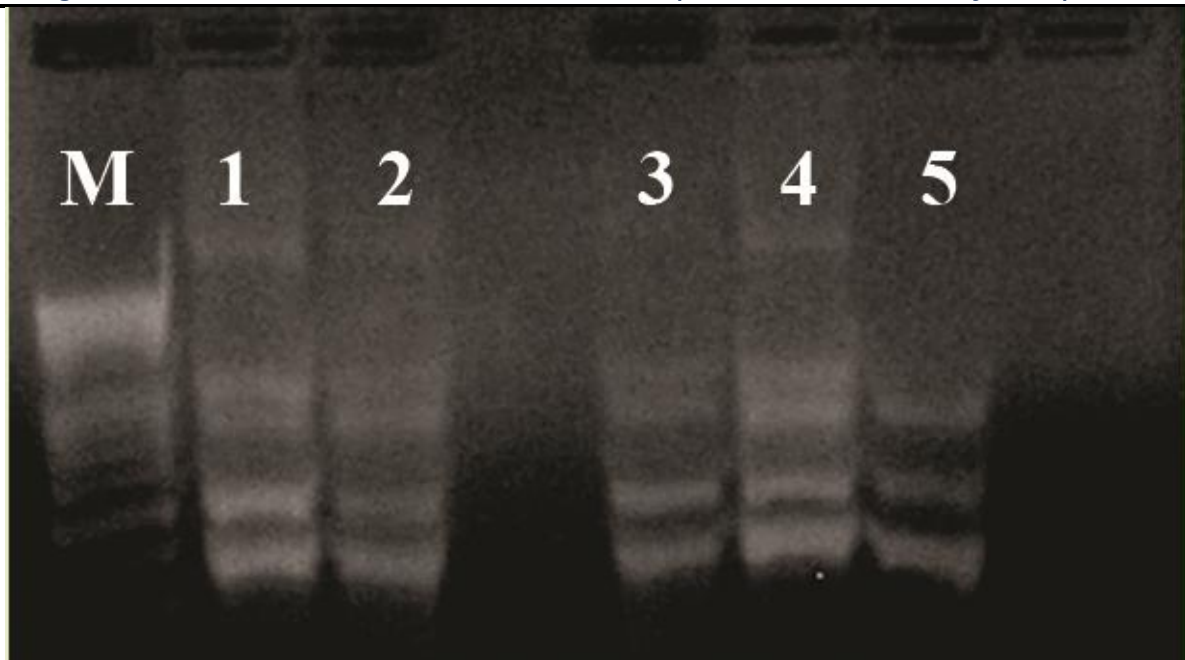


Figure. 10 RAPD profile of *Piper nigrum* using random primer 6. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

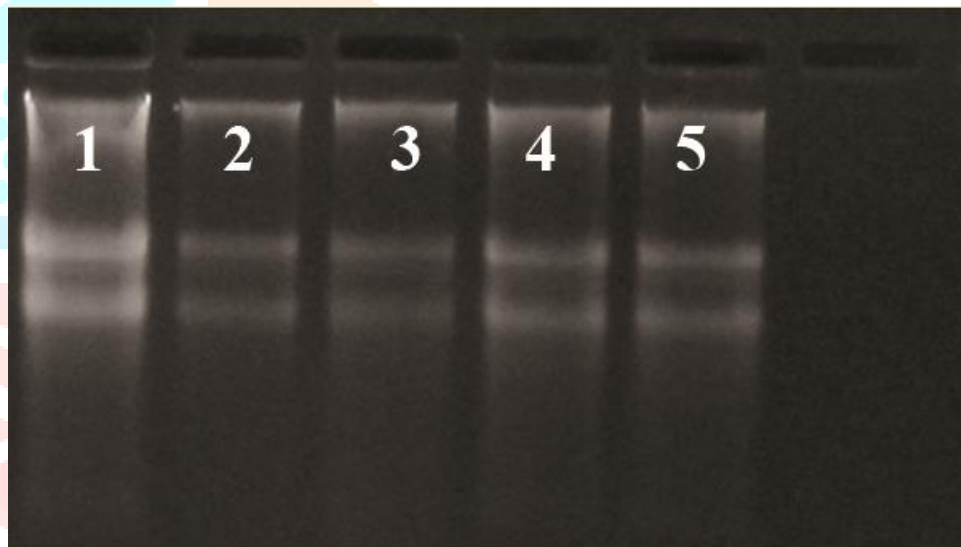


Figure. 11 RAPD profile of *Piper nigrum* using random primer 7. M = Mol wt. marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

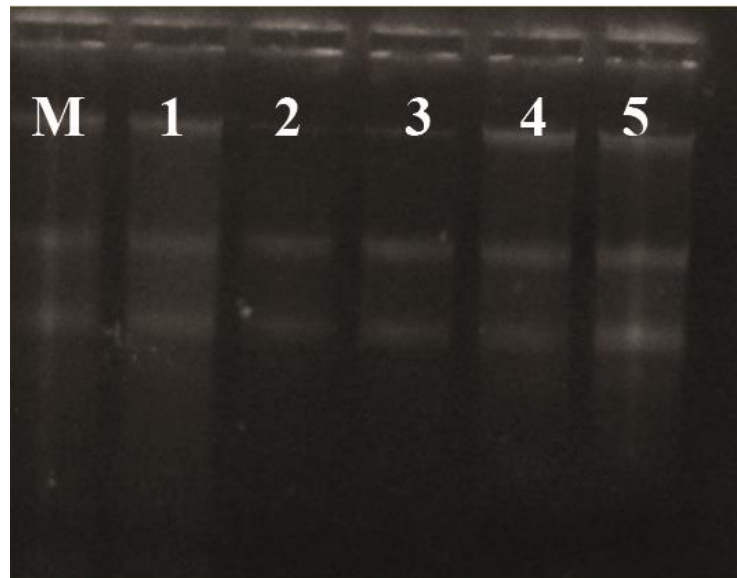


Figure. 12 RAPD profile of *Piper nigrum* using random primer 8. M = Mol wt marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5



Figure. 13 RAPD profile of *Piper nigrum* using random primer 9. M = Mol wt marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

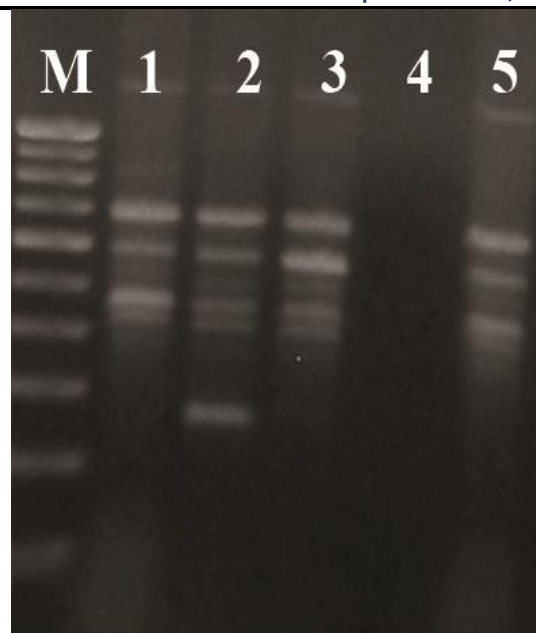


Figure. 14 RAPD profile of *Piper nigrum* using random primer 10. M = Mol wt marker; Lane 1 = P1; Lane 2 = P2; Lane 3 = P3; Lane 4 = P4; Lane 5 = P5

Out of 198 scored, 193 bands were found to be polymorphic. Maximum number of polymorphic bands (26) was obtained with the primer RP4, RP6 and RP10. The average number of polymorphic bands per primer is 19.3. The number of bands per primer product ranged from one to twenty six. Average number bands per primer are 19.8. The size of the amplified product ranged from 300 bp to 1.5 kb (**Table 1**).

SL.NO	PRIMER	NATURE OF BANDS		TOTAL NO. OF BANDS	SIZE RANGE OF BAND
		MONOMORPHIC	POLYMORPHIC		
1	RP1	5	0	5	300bp- 1.5 kb
2	RP2	0	15	15	300bp-1.5 kb
3	RP3	0	23	23	500bp-1.5 kb
4	RP4	0	26	26	500bp-1.5 kb
5	RP5	0	25	25	500bp-1.5 kb
6	RP6	0	26	26	500bp-1.5 kb
7	RP7	0	14	14	500bp-1.5 kb
8	RP8	0	18	18	500bp-1.5 kb
9	RP9	0	20	20	500bp-1.5 kb
10	RP10	0	26	26	500bp-1.5 kb
		5	193	193	500bp-1.5 kb

Table 1: Analysis of banding patterns of RAPD profile of *Piper nigrum* using random primers

Polymorphic DNA uses only one short primer with an arbitrary sequence and bind at many complementary sites in the genomic DNA, amplifying a variety of differentially sized fragments that can be separated by electrophoresis to give specific banding pattern. Being a simple and non-radioactive technique is quite sensitive and used to detect genetic variation in several living things.

The present study suggests that there is high genetic diversity among Panniyoor varieties. This information would be useful to study more genetic diversity analysis on *Piper* species from different areas of the state and outside. This information would be useful to study more genetic diversity analysis on piper species from different areas of Kerala. The current RAPD profile can be used to develop an effective molecular marker system for marker assisted selection and breeding for an effective crop improvement programme. Because of the diverse genetic base of the breeding stock utilized for the hybridization programme the parents of most of the clones used in the study were previously released varieties. This led to the diversification of the genetic base of the evolving new varieties.

CONCLUSION

RAPD is useful for the characterization of DNA from plants and other organisms. The current RAPD profile can be used to develop an effective molecular marker system for marker assisted selection and breeding for an effective crop improvement programme in *Pepper*. The RAPD bands show that Pepper plants are genetically heterogeneous as a high level of variability was observed. This high level of genetic variability may be due to the different geographical locations from which the isolates were obtained or it could indicate that the isolates may have originated from the same species with a wide genetic base or from closely related species

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