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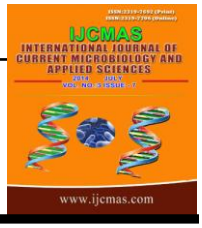


**Original Research Article**

# Genetic Diversity and Relationships of Piper Species Using Molecular Marker

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**A B S T R A C T**

RAPD analysis revealed a high degree of genetic diversity among the species examined in the study, which can contribute to the crop involvement. RAPD analysis can also be used for detecting gene flow between species. Furthermore, this technique is less restricting than other molecular technique RFLP (Restricted Fragment Length Polymorphism), as no hybridization and no use of radioisotopes is required, and it is therefore more convenient for use in research centers in developing countries. Thus our results demonstrate that 4C DNA content and RAPD markers provide an effective tool for detection and evaluation of genetic variation existing among 6 promising species of pipers.The result presented in the present study demonstrated the utility of using RAPD markers to characterize genetic diversity among 6 promising species of piper. Differential polymorphism was noted 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.

**K e y w o r d s**

Genetic Diversity, Piper Species, Molecular Marker

## Introduction

Piper is an important member of *Piperaceae*, have a wide distribution in tropical and subtropical regions. The evergreen forest of the Western Ghats in South India is considered as the center of diversity of *Piper nigrum* and its close relatives. The genus includes important species like *Piper nigrum*; the black pepper, *Piper longum* and *Piper betel*, which are commonly used in the indigenous system of medicine. Among these, black pepper is one among the most widely used spices in the world. It is taxonomically a very difficult genus because of the greater range of variability and minute nature of flowers (Ravindran *et al.*,1992).

However various attempts to classify the Piper species based on morphological, cytological studies by Sharma and Bhattacharya (1959) and chemical constituent data carried out by Sebastian and Sujatha (1996).But differentiation of species through morphological features is inefficient and inaccurate. The use of biochemical and genetic markers for identification of varieties offer a viable alternative method of Williams (1990). According to Lee *et al.*, (1995) DNA based molecular markers are the most useful to detect the levels of genetic diversity in plant genome.

Until recently, several promising species released formally are characterized on the basis of morphological data, Piper content and yield potential. Worldwide demand on Piper necessitate work on conservation of Piper germplasm and their further genetic improvement. Therefore, precise characterization of promising species and determination of genetic variation among those are felt necessary. Until recently, several promising species released formally are characterized on the basis of morphological data, Piper content and yield potential. These characters differ under varying environmental condition thereby posing problem identification of species. Unlike morphological markers, cytological (chromosome numbers, nuclear DNA content) and molecular markers (RAPD,AFLP,ISSR etc.) are not prone to environmental influences and characterize the plants portraying the extent of genetic diversity among taxa (Bennett 1987,Bennett and Smith 1991,Waugh and Powell 1992,Chalmers *et al.*,1994,Das *et al.*,1998,Rodriguez *et al.*,1999,Das *et al.*,2001).Of the different markers, RAPD has been widely used in the last decade in species identification programme (Schnell *et al.*,1995) 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 (Williams *et al.*,1990,Frankel *et al.*,1995). RAPDs reveal similar patterns of genetic diversity when compared with other marker types and can be performed more rapidly than most other methods (Morell *et al.*,1995) and can provide vital information for the development of genetic sampling, conservation and improvement strategies (Waugh and Powell 1992,Chalmers *et al.*,1994).No report has been published so far either on the genetic characterization or on the extent of genetic variations existing among promising species of Piper using 4C DNA content and RAPD analysis.

The present study deals with the insitu DNA estimation and RAPD analysis of 6 promising species of Piper to identify and evaluate extent of genetic variation existing among these.

## Materials and Methods

The present investigation was carried out in the Department of Lifescience Biotechnology Centre, DNA Fingerprinting Laboratory, Govt of Karnataka, Hulimavu, Banerghata Road, Bangalore.Karnataka.India, during the year January-2013 to December-20014.The materials used and methods followed in this study are presented here. The present study selected leaf samples of six piper species plants that were collected from the conservatory of Biotechnology Centre, Hulimavu, Department of Horticulture, Bangalore, Karnataka, India. Which may represent the wide variation prevalent in the genome. The recently matured leaves were collected and used for DNA extraction.

The basic DNA extraction protocol Dellaporta *et al.,* (1983), was slightly modified following Porebski *et al.,,* (1997). 1g of fresh leaf sample of each plant was grinded using liquid nitrogen and then transferred to a centrifuge tube containing 20 ml of extraction buffer (3% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 1% PVP and 1% B-mercaptoethanol) preheated to 60º C and maintained at this temperature for 1 hour with intermittent shaking. The centrifuge tube was brought to room temperature and 6 ml of chloroform and isoamyl alcohol (24:1) were added. The contents mixed well by inverting the tube gently 25-30 times, then spun at 7,000 rpm for 15 min. The supernatant was transferred

to centrifuge tube and this clean-up step was repeated until a clear supernatant was obtained. Supernatant was kept overnight at 4º C to precipitate DNA by adding half a volume of 5 M NaCl and one volume of isopropanol. The DNA was pelleted by centrifuging at 10,000 rpm for 20 min and the pellet was washed with 70% ethanol. The dried DNA pellet was resuspended in 300 µl of TE (Tris EDTA) buffer. Contaminating RNA was removed by digestion with 10 µg of Rnase for 60 min at 37º C. Proteins were removed by digestion with 25µg of Proteinase-K. The DNA was further purified by extracting twice with an equal volume of phenol followed by an equal volume of phenol: Chloroform (1:1) and finally with an equal volume of chloroform. The DNA was precipitated by the addition of one volume of isopropanol and spun at 5,000 rpm for 5 min; the final pellet was dissolved in 300 µl TE. The DNA concentration was determined using UV-Visible spectrophotometer at 260 nm and 280nm and the quality verified by electrophoresis on a 0.8% agarose gel and diluted to a uniform concentration of 5 ng/µl for RAPD analysis.

For Feulgen cytophotometric estimation of 4C DNA, ten fixed root tips from each species were hydrolyzed in 1N HCL for 12 minute at 60 ºC, washed in distilled water and stained in Schiff's reagent for two hours at 14ºC; each root tip squash was prepared in 75% acetic acid. Ten scorings were made from each slide and 4C DNA was estimated from metaphase chromosomes using NIKON Opitiphot microscope with microspectophotometer following the method of Sharma and Sharma (1980) with monochromatic light at 550 nm. In situ DNA values were obtained on the basis of optical densities which were converted to picograms (pg) by using Van't Hof's (1965) 4C nuclear DNA value (67.1pg) for *Allium cepa* as standard species n-53 of *Allium cepa* was used in our experiment. ANOVA were performed among the nuclear DNA values to find out the differences at the species level.

**DNA Amplification**

**Results and Discussion**

The yield and quality of DNA extracted by following the procedure described earlier was 10-25 ng per µl for every gram of leaf sample. The DNA obtained was amplifiable and of high quality. Spectrophotometer reading of 1.7-1.8 (260nm/280nm) confirmed the quality of DNA.DNA isolated from 500 mg leaf tissue using 20 ml extraction buffer yielded good quality, high molecular weight DNA (above 50 kb). The quality of the DNA was also confirmed by gel electrophoresis.

In this study, 100 Operon random ten-base long, single stranded primers (OPA 1-6 and OPD with 12 primers in each group) were screened using the Piper species, which on an average gave nine bands. The selected 12 primers were used for the screening of species, in which all the bands obtained were either monomorphic / polymorphic and were considered for the precise calculation of genetic diversity.

The basic protocol described elsewhere for PCR was optimized for high quality amplification and intense repeatable banding patterns. However, a reduction in the amplification of fainter bands was noticed with large changes in template DNA concentrations, while too much DNA produced a smear effect, which emphasized the importance of quantification of the DNA for clear amplification.

A representative of the PCR amplification

product of 6 species is shown in fig.1 which yielded sufficient polymorphisms to distinguish between species by Ward's method of analysis with Squared Euclidean distance gave dendrogram. Based on the number of bands all the species were grouped in three clusters (Fig. 2). The dendrogram revealed a maximum similarity between *Piper nigrum* and *Piper longum* species. Every single species/hybrid could be identified using these selected twelve primers.

Analysis of diversity based on PCR fragments amounted to saturating the genome. The analysis of 6 species of Piper suggested that diversity is moderate to high and has shown even differences also.

**Table.1** C Nuclear DNA Content in Six Piper Species. (*P. Nigrum, P.Mullesua, P. Longum, P. Betel, P. Retrofactum* and *P.chaba* )

| ***Species of Piper*** | **4C nuclear DNA content (pg)** | **Number of polymorphic bands(OPA 1-6)** | **Number of polymorphic bands (OPD 1-6)** | **% of polymorphic bands** |
|---|---|---|---|---|
| *P. nigrum* | 8.74±0.04 | 09 | 09 | 18.36 |
| P.mullesua | 8.58± 0.06 | 09 | 11 | 20.40 |
| *P. longum* | 7.31± 0.05 | 07 | O6 | 13.26 |
| *P. betel,* | 6.81 ±0.05 | 13 | 11 | 24.48 |
| *P. retrofactum* | 7.57 ±0.05 | 06 | 00 | 06.12 |
| *P.chaba* | 4.20 ±0.06 | 09 | 08 | 17.34 |

**Table.2** Synthetic Oligonucleotide OPA 1-6 and OPD 1-6 used as Primers for Amplification of Piper DNA

| **Primer No.** | **Sequence** | **Total no. of fragments amplified** |
|---|---|---|
| OPA-01 | CAGGCCCTTC | 09 |
| OPA-02 | TGCCGAGCTG | 09 |
| OPA-03 | AGTCAGCCAC | 07 |
| OPA-04 | AATCGGGCTG | 13 |
| OPA-05 | AGGGGTCTTG | 06 |
| OPA-06 | GGTCCCTGAC | 09 |
| OPD-01 | ACCGCGAAGG | 09 |
| OPD-02 | GGACCCAACC | 11 |
| OPD-03 | GTCGCCGTCA | O6 |
| OPD-04 | TCTGGTGAGG | 11 |
| OPD-05 | TGAGCGGACA | 00 |
| OPD-06 | ACCTGAACGG | 08 |

**Fig.1** Gel Profile of Piper species (*P. Nigrum P.mullesua, P. Longum, P. Betel, P. Retrofactum* And *P.chaba* Amplified with Selected Primers like OPA 1-6 and OPD 1-6

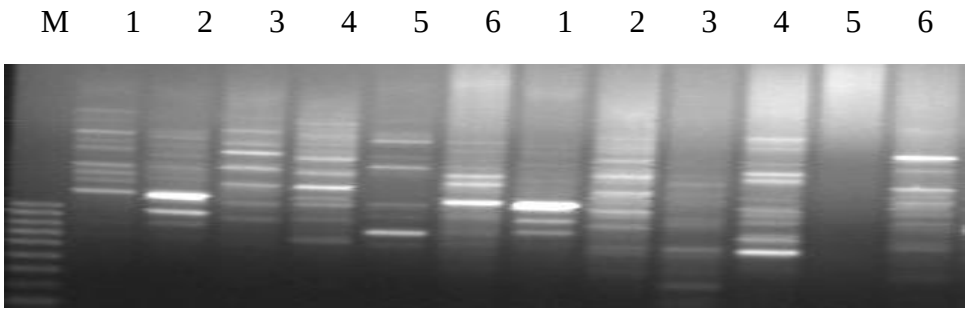
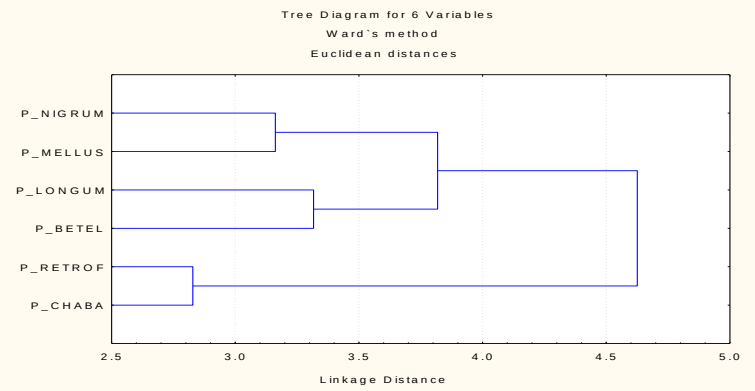



**Fig.2** Tree Diagram/Dendrogram Depicting the Genetic Relationship of 6 Piper Species




## Diversification in DNA Amount

The 4C DNA values were reported for the first time in 6 species of piper significant differences of 4C DNA were recorded between species 8 having minimum 4.2Picogram to maximum 8.74 Pico gram nuclear DNA content such intraspecific variations are in close agreement with the reports of other workers (Furuta *et al.*,1975, Price *et al.*, 1980, Das *et al.*, 1998). The consistency in the DNA amount at the species level in the repeated experiment revealed the stable 4C DNA content in each species of piper. The DNA amount differed significantly and the difference in the DNA content depend on the repetitive DNA amount (Flavetl *et al.*,1977). The variability in the DNA content in different species might be attributed to the loss or addition of many repeats in the genomes through alteration in the micro-and macro-environment during evolution in the selection of new species (Price *et al.*, 1980, Das etal.1998). The variability of DNA amount could be attributed to loss or addition of highly repetitive DNA sequence rather than AT or GC rich sequences in a genome (Martel *et al.*,1997). Which is reached a certain level and got stabilized during micro-evolution and gradual selection. (Table 1).

Clustering of speciesThe squared Euclidean distances was calculated using the data on 6 piper species with 12 Primers OPA 01- 06, OPD 01-06). Highest distance was observed

between *Piper nigrum* and *Piper chaba*. The lowest distance was recorded between *Piper retrof* and *Piper chaba* .

The distance within species was least (2.84 units) and highest (4.6 units) and intermediate distance between (3.82 units). However this was in line with the results obtained in Wards genetic diversity calculations.

The dendrogram (Fig.2) showed three distinct clusters namely 1. *Piper nigrum* and *Piper mellus*. Cluster 2 included *Piper longum* and *Piper betel* and cluster 3 showed *Piper retrof* and *Piper chaba*.From the pattern of clustering, it was pertinent that RAPD technique was efficient in segregating species into different clusters. More significantly, the clustering had been largely successful in retaining the relationship between species as proposed by Schilling and Heiser (1981). In the light of current study at species level, it can be seen in the clustering pattern that the series were clearly distinguished in piper species. The association of the species observed in the present day was similar to the pattern observed by Sivloap and Solodenko (1998) and Khalid *et al.,* (1999).

**Differential Polymorphism of DNA**

The result presented in the present study demonstrated the utility of using RAPD markers to characterize genetic diversity among 6 promising species of piper. Differential polymorphism was noted 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 (Table 1). The observed high proportion of polymorphic loci reveals profound variation among the piper species. Significant genetic variations by RAPD have also been reported in other species at Species level (Colombo *et al.,* 1998, Das *et al.,* 1998, Huang *et al.,* 2003).Wide genetic distances determined by Euclidean Wards genetic distance reveals relatively high genetic variation among 6 species. The considerable polymorphism detected in the present day also illustrated genetic diversity among piper species of the same origin as reported among coffee species (Sera *et al.,* 2003). The observed differences among 6 piper species could be ascribed to the fluctuating micro and macro climatic conditions of habitat. Ward's analysis of RAPD data also reveals that all piper species belonging to the state of Karnataka are genetically closer. The greater sensitivity of RAPD obtained in the result to species diversity may be derived from rapid evolution of non-coding, repetitive D N A sequence detected by R A P D s. This hypothesis has been corroborated from (Plomion *et al.,* 1995).

On comparison of six species in, it can be observed that though they differ largely in their, phenotypic expression, but the genetic difference as observed from DNA fingerprints was found to be low. The genetic variation as detected by RAPD analysis opens up the avenue for the proper identification and selection of the species that could be used for varietal identification and planning for future breeding programme.

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