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Molecular characterization of black pepper (*Piper nigrum*) using RAPD and SSR markers

REMMIA RAGHAVAN[1], S. ELUMALAI[1], K. NIRMAL BABU[2], SHAILAJA HITTALMANI[3]

[1]P.G. Research Department of Plant Biology & Biotechnology,
Presidency College Chennai - 600 065 (India).
[2]Dept of Crop Improvement & Biotechnology, Indian Institute of Spices Research,
Calicut, Kerala - 673 012 (India).
[3]Marker Assisted Selection Laboratory, Department of Plant Breeding and Genetics,
UAS,GKVK, Bangalore - 560 065 (India).

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ABSTRACT

A set of 20 *Piper nigrum* (Black Pepper) accessions were screened to identify the extent of genetic diversity present at the Molecular level using RAPD and SSR markers.. Dendogram constructed based on molecular polymorphism unveiled considerable amount of diversity among the varieties. Among this the SSR markers were found to be useful in discrimination and identification of the genotypes as it gave more genotypic specific bands.

Key words: *Piper nigrum*, SSR, RAPD, genetic diversity.

INTRODUCTION

Black Pepper (*Piper nigrum* L) is one of the oldest spice know to the world *P. nigrum* belongs to the family piperacea. The tropical ever green forests of Western Ghats are considered as the center of origin of Black Pepper[1]. The major center of diversity of the genus piper is central and Northern South America, where 60% of the species are distributed. More than 1000 spps are included in the genus of which 115 are of Indian origin. Black pepper, the most popular spice is cultivated in about 21 countries including India, Indonesia, Brazil, Malaysia, Srilanka, Vietnam, Thailand, China, Mexico, Guatemala. In India, Black pepper is grown predominantly in the states of Kerala, Karnataka and Tamil Nadu and to a certain extent in Maharashtra, AndraPradesh, Andaman and Nicobar Islands and North Eastern states viz, Assam, Meghalaya, Manipur and Arunachal Pradesh.

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 of molecular is the use of molecular markers as a tool to detect the extent and structure of Genetic variation[3,4] Providing insight into the diversity of crop- varieties and potential contributions represented by their wild relatives.

Microsatellite markers have become the DNA markers of choice for a wide range of application in genetic mapping and genome analysis genotype identification of Variety protection[5] seed purity evaluation and germ plasm conservation, diversity studies[6], paternity determination and pedigree analysis[7] give and quantitative trait locus analysis[8] and Markers assisted breeding[9]. In measuring genetic diversity assigning lines to

heterotic groups and genetic fingerprinting , Microsatellite provides Power of discrimination equal to greater than that RFLP in an more cost effective manner and case of study the objective of this study were to assess the extent of genetic diversity and relationship among 20 *Piper nigrum* genotypes.

MATERIAL AND METHODS

Plant material and DNA extraction

Five Land Races and 13 advanced cultivars and 2 wild accessions of P*iper nigrum* collected from IISR Experimental farm, Peruvannamuzhi were used in the present study (Table 1) DNA was extracted according to the CTAB method with Minor modifications[2]. Leaf tissue of 2g was ground to fine powder in liquid N_2, followed by incubation in 15 ml of preheated extraction buffer (4% W/V CTAB, 1.4 M Nacl, 100 mm Tris-HCL PH 800, 20mm EDTA, 1.4m Nacl, 100mm Tris-HCL, PH 8-0, 20 mm EDTA, 2% PVP and 0.1 % V/V and Mercaptoethanol) for 2hr at 55°C. The homogenate was extracted once with chloroform: Iso- amyl alcohol (24:1) and centrifuged at 15000 rpm at 25°C. Nucleic acids were precipitated in 0.6 volume of isopropanol and collected by centrifugation (15,000rpm, 15 min, 25°C). The precipitate was dissolved in TE- Buffer (100Mm Tris HCL PH : 8.0, 1 mm EDTA). DNA was treated with bovine pancreatic RNase and extracted with phenol: chloroform (1:1) and chloroform: iso-amyl alcohol (24:1) in succession. DNA was quantified in a Fluorometer and dissolved to appropriate dilution in TE Buffer.

SSR and RAPD Primers

15 RAPD primers and 9 SSR primers were used in the study are mentioned in the Table 2(A,B) as follows. The Primers were obtained from Sigma –Aldrich,USA.PCR reactions were carried out in a Eppendorf Thermocycler.

PCR amplification for RAPD

The PCR was performed in a 20µL reaction mixture comprising 50 ng of template DNA, 2µL 10X assay Buffer, 25 Mm $Mgcl_2$ (1µL), 100 mm each DNTP's (dATP, dGTP, DCTP and dTTP), .85 U Taq DNA polymerase 10 pmolar primer, PCR reaction were carried out on a perkin Elmer 9600 DNA thermal cycler, After a predenaturation step of 4min at 94°C, amplification reaction were cycled 40 times at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min followed by 5 min at 72°C, was followed by completion of the primer extension on an Eppendorf thermal cycler.

PCR amplification for SSR

The PCR was performed in a 20µL reaction mixture comprising 20 ng of template DNA, 2µL 10X assay Buffer, 25 Mm $Mgcl_2$ (1µL), 100 mm each DNTP's (dATP, dGTP, DCTP and dTTP), .85 U Taq DNA polymerase 10 pmolar primer, PCR reaction were carried out on a perkin Elmer 9600 DNA thermal cycle.Temperature DNA was initially denatured at 94°C for 5 min ,followed by 35 cycles of 94°C for 1 min denaturation ,primer annealing temperature between 57°C to 68°C FOR 1 min and 2 min primer extension at 72°C.Final 5 min incubation at 72°C was followed for completionof primer extension on an Eppendorf thermal cycler.

Gel scoring and data analysis

Amplified DNA samples were analyzed by electrophoresis on 1.4% and 3%agarose gels in 1xTBE Buffer. Clear and well resolved bands were scored for presence (1 or absence (O). (Fig. 1) Statistical analysis was carried out using STATISTICA Package. The program used was treeing with raw input data. The main parameter, which guided the joining process, is unweighed pair group method with Arithmetic mean (UPGMA) and Euclidean distance was computed. The relationship among 20 Black Pepper accessions was portrayed graphically in the form of a Dendogram.

RESULTS AND DISCUSSION

Molecular profiles were developed with 15 RAPD Primers and 9 SSR primers Good polymorphism was observed between the genotypes for most of the primers studied. A total of 130 markers were obtained with 15 RAPD Primers .Out of which 71 markers were polymorphic and 51 were monomorphic. RAPD primer OPE-20 produced maximum numberof markers[13]. Out of which 10 were polymorphic and 2 were monomorphic. Among the 9 SSR primers studied[5], were Polymorphic(PNF1, PND10, PNA5, PNG11 and PNH8), rest of the primers produced

Table 1: Piper accessoins used in the present investigation

S. No.	Name	Place of collection
1.	PANNIYUR- 1	KANNUR
2.	PANNIYUR- 2	KANNUR
3.	PANNIYUR 3	KANNUR
4.	PANNIYOR 4	KANNUR
5.	PANNIYUR 5	KANNUR
6.	SREEKARA	CALICUT
7.	SHUBAKARA	CALICUT
8.	POURNAMI	CALICUT
9.	PANCHAMI	CALICUT
10.	MALABAR EXCEL	CALICUT
11.	THEVAM	CALICUT
12.	GIRIMUNDA	CALICUT
13.	SHAKTHI	CALICUT
14.	KARIMUNDA	IDUKKI
15.	KALLUVALLY	WAYANAD
16.	KUTHIRAVALY	CALICUT
17.	BALAN KOTTA	CALICUT
18.	PERUM KODI	IDUKKI
19.	Piper nigrum (WILD5422	PALAGHAT
20.	Piper nigrum (WILD)5569	KANNUR

Table:5 Operon primers which showed polymorphism for developing RAPD profiles

S. No.	Oligo Name	GC %	Sequence (5'-3')
1	OPA08	60%	GTGACGTAGG
2	OPA02	70%	TGCCGAGCTG
3	OPA05	60%	AGGGGTGTTG
4	OPB20	60%	GGACCCTTAC
5	OPB14	70%	TCCGCTCTGG
6	OPE12	70%	TTATCGCCCC
7	OPE05	60%	TCAGGGAGGT
8	OPE06	60%	AAGACCCCTC
9	OPE18	70%	GGACTGCAGA
10	OPE20	60%	AACGGTGACC
11	OPF09	60%	CCAAGCTTCC
12	OPF10	60%	GGAAGCTTGG
13	OPM04	70%	GGGGGTTGTC
14	OPU17	70%	ACCTGGGGAG
15	OPW11	60%	CTGATGCGTC

Monomorphic(PNB5,PNE3,PNH4,PNB9,) bands. The PCR products were run on 4% Polyacrylamide gel electrophoretic system to check the extent of polymorphism. These polymorphic loci amplified approximately of size between 120-210bp. The

Table 6: List of SSR Primers used in the Study

Locus	Primer sequence (5'-3')
PN A5	F 5' CTTCCAGACCAATAATCAACTT 3'
	R 5' ATCCCAAAATACACAACAATTC 3'
PN B5	F 5' GTTTTGAATGGGTCGGTGAT 3'
	R 5' ATTGTTCTGATTTCTTCGTTATTG 3'
PN B9	F 5' AGTATTGGTTGTTTCTCTC 3'
	R 5' ATGTAAAATCGATAGTCCTCA 3'
PN E3	F 5' TTTGTGTCCTCTCCCTCTCC 3'
	R 5' AAGACTAAATAGGCAAGGCAAA 3'
PN F 1	F 5' ACTTCAGTGCTATTTTTATCTTCC 3'
	R 5' CCAACGCCCACTCTCAT 3'
PN G11	F 5' TTACTAGTGTCCACCCCCACT 3'
	R5' TCGATGGAAAGTCACCCTCT 3'
PN H4	F 5' CTTTTCCCACAATTCAGTCTCG 3'
	R 5' ACCCATGCGTGTATCTTCTCAG 3'
PN D10	F 5' GTGTTACCTTTGGGGCATTCA 3'
	R 5' TGTGTCAGGGCATCAAACC 3'
PN H 8a	F5' TGTGTCTTTTATATTTTTGATG 3'
	R5' TATTAGTAGTTCTCCCTTTTGA3'

Source: Menezes., *et al.,*

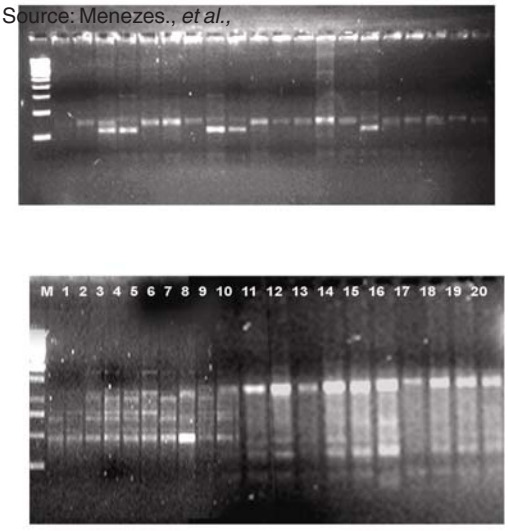

Fig. 1: Profiling of 20 *Piper nigrum* genotypes with RAPD primer OPA02 and SSR primer pnb5

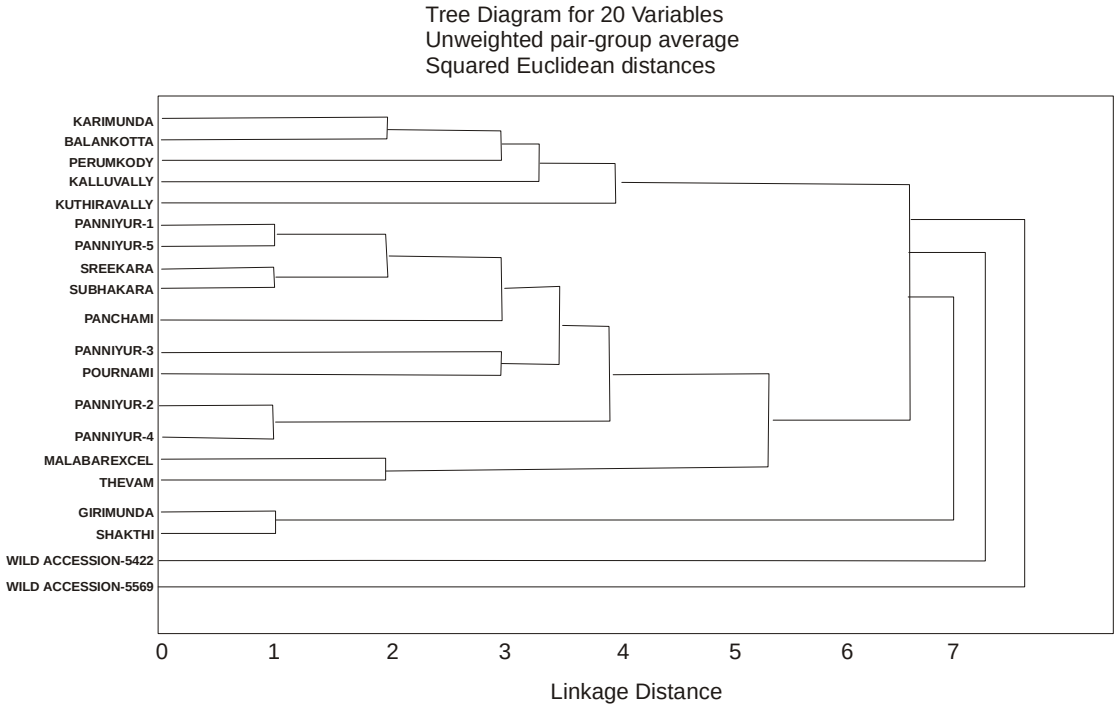

Fig. 7: Combined dendogram of *Piper nigrum* genotyps constructed from RAPD and SSR markers

maximum number of alleles amplified were at an average of 2 bands for each polymorphic primer pairs.

CONCLUSION

Grouping of individuals based on RAPD and SSR indicate a similarity in code region of the genome and hence common morphological traits. The molecular markers identified using RAPD and SSR techniques in the present study could be used in the identification of pepper genotypes quickly. Among this the SSR markers were found to the useful in discrimination and identification of the genotypes and it gave more genotypic specific bands. The genetic diversity obtained in this study might be useful in future strategies for evaluation of desired genotypes. Such molecular data would be useful for detecting DNA patterns unique for a given accession or a set of accessions . This will through some light on the pepper crop improvement and boost our domestic needs as well as the earnings of foreign exchange

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