Journal of Medicinal Plants Studies
Year: **2014**, Volume: **2**, Issue: **2**
First page: (**69**) Last page: (**75**)
ISSN: **2320-3862**
Online Available at **www.plantsjournal.com**

Received: 01-01-2014
Accepted: 29-02-2014

Journal of Medicinal Plants Studies

Diversity studies for characterization of spike branching black pepper (*Piper nigrum* L.) type collected from Idukki district of Kerala

Vimarsha HS [1*], Rajmohan K [2], Soni KB [2], Swapna Alex [2]

1. Vimarsha H. S. Ph. D. Scholar, College of Agriculture, GKVK, Bangalore-650065.
   *[Email: vimarsh135@gmail.com, Tel: +91-9743833808]
2. Professor, Department of Plant Biotechnology, COA, Vellayani, Thiruvananthapuram, Kerala - 695 522.

Black pepper bears single pendulous spike. The yield potential of any of these improved varieties of India, bearing pendulous spikes is yet meet the half level productivity challenge posed by leading countries. Malaysia and Brazil nations have highest records of productivity. Thought India is the center of origin for black pepper species with huge diversity, we still lack the potential varieties that yield above average produce compared to global productivity levels. Finding the new diverse germplasm for exploiting and transferring the traits that associates with yield and yield related characters are of much need in present context. Spike branching is a yield associated trait. Recently, a spike branching variant is observed in Idukki district of Kerala. Present investigation is aimed at delineating parentage or the decendence of this unknown cultivar. RAPD analysis is done to find the phylogeny of this variant. The local cultivar, vellamundi depicted near relativeness (74%) as compared to wide cluster formed by famous local cultivar karimunda that diverged from this variant.

Keyword: Black pepper, Spike branching, RAPD, Phylogeny.

1. Introduction

India holds the top rank in production and export of spices. Black pepper, referred to as 'king of spices' accounts for major spice export and income to farmers. India is the center of origin for black pepper and exhibit huge diversity for this species. Diversity account of more than 6000 spices have been recorded in the International Plant Name Index (www.ipni.org) database. India is one of major contributor for species diversity, however, productivity potential is unsatisfactory.

Productivity of Indian black pepper is very less, compared to that in other countries [1]. Majority of the black pepper fields are cultivated with land races or with popular high yielding hybrid varieties like Panniyur-1 [2]. India has varieties which can yield more than 3000 kg ha-1. However, the average productivity is very less (320 kg/ha) compared to Malaysia (2000 kg/ha), Brazil (1571 kg/ha) and Indonesia (800 to2000 kg/ha) [1]. Irrespective of the prevailing agro climatic conditions, indiscriminate use of cultivars may be one of the several reasons for low productivity [3]. The hybridization programs by many research stations have yielded improved varieties with higher productivity and quality [2]. However, the status of productivity has lot of potential to be upgraded.

India Accounts for huge diversity for black pepper. According to Parthasarathy *et al.* [4]. over the thousands reported species of Piper, 111 are of Indian origin which are distributed throughout the tropical and subtropical regions. Central and South America has the maximum diversity (~700) for Piper species [5], which accounts for 60 per cent of species diversity in Piper. More than 300 species were recorded from Southern Asia, out of which Piper nigrum L. is believed to have

originated in the Western Ghats region of India [6, 7], still there are many unidentified species present in core areas of tropical forests of India. Present study is on one such uncharacterized variant recently found in hilly regions of Western Ghats of Kerala.

Spike branching in black pepper is very rare, though, considerable variation in spike length, floral composition, fruit number and size are reported [5]. In cultivar Aimpiriyan, formation of rudimentary spikes on main spikes are also reported. The concept of spikelet matches to the proliferating spikes, where spikelet literally means "little spike" referring to its similarity to an indeterminate branching inflorescence, developing within the larger inflorescence [8]. Mutation in the floral meristem control genes of black pepper could also result in branching of inflorescence. Scientists from Indian Institute of Spices Research have reported a new spike variant with hundred percent of its spikes, proliferating. The vegetative progenies observed to be true-to-type [9].

RAPD technique, to investigate genetic variability was found to be efficient and reliable [10, 11]. According to Nazeem *et al*. [12] RAPD technique can be successfully used to evaluate the genetic diversity among the Piper species. Renuka [13] also found RAPD markers useful in analyzing the diversity among Piper species. Studies on variation in yield and growth performance of cuttings derived from top, middle and bottom nodal explants of five high yielding varieties viz. Panchami, Pournami, Panniyur-1, Panniyur-3 and Panniyur-5 revealed intraclonal variability [14].

The variety Aimpiriyan occasionally show rudimentary spike branching trait. Recently, cultivar "Kathirinmelkkathir" has been reported to have profuse spike branching character [15]. However, the details regarding growth pattern and quality are not available. Present black pepper variant, an elite germplasm is yet to be classified and the parentage information is to be elucidated. However, for the better part, it bears yields as twice as compared to well know varieties like Paniyur-1 and other famous local cultivars. Considering the impact this variant could create, Department of Biotechnology, College of Agriculture, Kerala Agriculture University, Trivandrum has taken up initial approaches to multiply and maintain this variant by tissue culture and field planting for various studies.

Present study is aimed at finding the parentage or the relatives of this variant as the parentage information is not available. All the varieties, local cultivars and wild type are collected from various parts of same farm to decode the relatives by RAPD analysis. Future studies are aimed at qualitative studies also such as piperine, oleoresin and oil content analysis.

2. Materials and Methods

2.1 Sample Collection

Plant samples of black pepper showing spike branching (Pepper Tekkan–as named by the farmer) were collected from an innovative farmer, Thomas T.T of Kanjiar village, near Kakkatikada, Kattappana taluk, Idukki district. Plant samples of seven varieties and cultivars from the same farm viz., Karimunda, Kumbakal Kodi, Arayan Mundi, Vellamundi, Naraya Kodi, Panniyur 2 and Panniyur 4 were also collected. Semi mature leaves were collected and transported to laboratory in ice boxes.

2.2 DNA Extraction

Modified C-TAB method developed by Kalisz Lab [16] was used for genomic DNA isolation of all eight black pepper samples. For five milliliter of extraction buffer (2% w/v C-TAB; 3 M NaCl; 100 mM Tris-HCl of pH 8, 0.5M EDTA) 200 mg (4% w/v) of Poly Vinyl Pyrrolidone (PVP) was added prior to DNA extraction. The PVP was dissolved completely by warming the buffer, using a water bath. Thereafter, 25 µl (0.5% v/v) of β-mercaptoethanol was added to the extraction buffer, mixed well and kept warm in water bath.

Next, the deep frozen leaves were washed and dried using sterile blotting papers. One gram of leaf sample was chopped and transferred to a dry,

sterile mortar. Chopped material was ground well to a fine powder using liquid nitrogen. Warm extraction buffer was added immediately and the mixture was homogenized. The extract was then subjected to incubation at 55 to 60 degree celsius for sixty minutes with intermittent shaking. The tubes were then brought back to room temperature. The mixture was centrifuged at 10,000 rpm for 8 minutes at 4 °C and the supernatant was collected. For each 500 µl of supernatant, 250 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and slowly mixed by gentle inversions for 2-3 minutes and centrifuged at 7000 rpm for 7 minutes. Then, the supernatant was extracted twice with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 7000 rpm.

The DNA from the supernatant was then precipitated with chilled 100 per cent ethanol and stored overnight at -200 ^{0}C. The precipitate was then centrifuged at 14,000 rpm and washed twice with 70 per cent ethanol and the pellet was air dried for 15 minutes. Thereafter, the pellet was slowly dissolved in 60 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and stored at -200 ^{0}C (Rotek deep freezer). Spectrophotometric analysis of the extracted DNA samples was made for determining quality and quantity of DNA (Table 1).

Table 1: DNA quantification and quality check for PCR studies.

Sl. No.	Plant	A260	A280	O. D. Ratio (A260/ A280)	DNA Yield (µg/g)
1	Karimunda	0.012	0.007	1.71	360
2	Kumbukal Kodi	0.007	0.004	1.75	210
3	Arayan Mundi	0.015	0.008	1.87	450
4	Spike branching type	0.014	0.008	1.75	420
5	Vellamundi	0.006	0.004	1.5	180
6	Naraya Kodi	0.021	0.012	1.75	630
7	Panniyur 2	0.017	0.009	1.88	510
8	Panniyur 4	0.02	0.011	1.81	600

Table 2: RAPD primers used for phylogeny and diversity analysis

Sl. No.	Primer	Sequence	GC content (%)
1	OPA 8	5’ GTGACGTAGG 3’	60
2	OPA10	5’ GTGATCGCAG 3’	60
3	OPB 8	5’ GTCCACACGG 3’	70
4	OPB10	5’ CTGCTGGGAC 3’	70
5	OPF 3	5’ CCTGATCACC 3’	60
6	OPP1	5’ GTAGCACTCC 3’	60
7	OPS12	5’ CTGGGTGAGT 3’	60
8	OPU13	5’ GGCTGGTTCC 3’	70

2.3 RAPD-PCR

Many RAPD studies have been reported at the molecular level with the genus Piper. RAPD analysis is one of the necessary techniques in order to begin an extensive molecular taxonomic study on spike branching black pepper. Furthermore, no reports on molecular comparisons of spike branching pepper with landraces, local cultivars and improved varieties of black pepper are available.

The DNA samples of 7 non-spike branching and single spike branching black pepper type were screened with many arbitrarily designed decamer primers supplied by Operon Inc., CA, USA. Out of twelve, eight primers that produced good banding pattern were selected for DNA amplification of collected cultivars/varieties (Table 2).

The components of the reaction mixture were optimized and a typical 20 µl PCR mixture comprised of 30 ng genomic DNA; 2.5 µl 10X assay buffer; 3 µl dNTP mix (4mM each); 1 pM primer. PCR reaction was carried out in a Programmable Thermal Cycler (PTC 100, M J Research, Inc). Pre-denaturation was done at 94 °C for about 4 minutes, and then 40 cycles of amplification were set at 94 °C for 1 minute, 35 °C for 45 seconds, 72 °C for 1minute and 30 seconds. Final extension was set at 72 °C for 5 minutes. The amplified products were separated on 1.4 per cent agarose gel in 1x TAE buffer.

2.4 RAPD Data scoring

The PCR products were scored for the presence (+) or absence (-) of bands on electroporated gels. The numbers of monomorphic and polymorphic bands were recorded. Thus, banding pattern of all eight primers for eight samples were scored as 1 and 0 in Microsoft XL sheet and subjected for statistical analysis.

The genetic similarity matrix was constructed using Jaccard's similarity coefficient values and this matrix was subjected to an unweighted pair-group method for arithmetic average analysis (UPGMA) to generate dendrogram using average linkage procedure. All these computations were carried out using NTSYS-pc version 2.02 [17] software and the dendrogram constructed was used to assess the association and distance between the varieties under study.

3. Results and Discussion

3.1 Spike Characters

Young spikes were greenish yellow in colour and did not show any branching, but almost all matured spikes were profusely branching. Branching spikes had peculiar protruding bracts, compared to normal ones (Fig. 1). Peduncle length was 1.3 to 2.1 cm. Wide variation in spike length was also observed, ranging from 9.4 cm to 18.6 cm

Although main spikes were branching irregularly, they showed complete indeterminate growth status. Seed setting was also irregular and loose on branched spikes and number of berries per spike varied from 60 to 240. More than 4 spikes per lateral branches were observed. Number of spike per plant varied from 87 to 162. Berry shape was round and bold.

Fig 1. Spike branching black pepper type collected from T.T. Thomas of Idukki District of Kerala

3.2 RAPD Analysis

Among the 16 primers tested for amplification, eight primers were selected for the RAPD analysis. The number of bands resolved per amplification was primer dependent and varied from 7 to maximum of 14 (Fig 2). The primers altogether generated 83 scorable bands with an average of 10.37 bands per primer. Out of these 83 bands produced altogether, 13 bands were monomorphic and 70 bands were polymorphic showing 83.57 per cent polymorphism (Table 3).

Table 3: Percent polymorphism depicted by RAPD primers

Primer	No. of bands	Monomorphic bands	Polymorphic bands	% Polymorphism
OPA 8	7	1	6	85.71
OPA 10	10	3	7	70.23
OPB 8	15	1	14	93.33
OPB 10	8	2	6	75.45
OPF 3	7	1	6	85.71
OPP 1	11	1	10	90.92
OPS 12	12	2	10	83.33
OPU 13	13	2	11	84.61

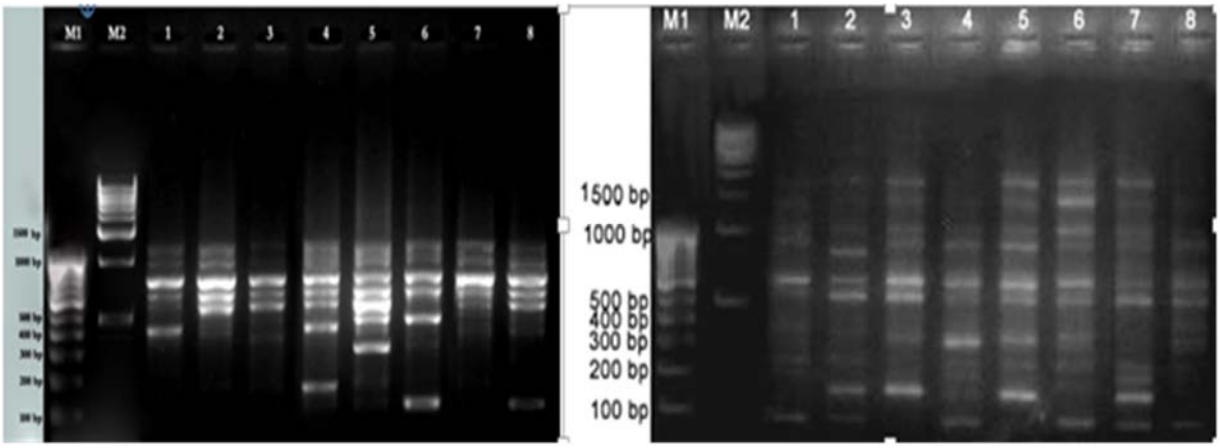

Fig 2: RAPD profile of varieties for RAPD primers OPB 10 and OPB 8.

(M1: 100 bp ladder; M2: 500 bp ladder; 1: Karimunda; 2: Kumbakal Kodi; 3: Arayan mundi; 4: Spike branching type; 5: Vellamundi; 6: Naraya Kodi; 7: Paniyur-2 and 8: Panniyur-4)

3.3 Statistical Analysis

Jaccard's similarity coefficient values for each pair wise comparison between the plants were calculated and a similarity coefficient matrix was constructed. The matrix was subjected to un-weighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram (Fig. 3) using average linkage procedure. All these computations were carried out using NTSYS-pc software version 2.02.

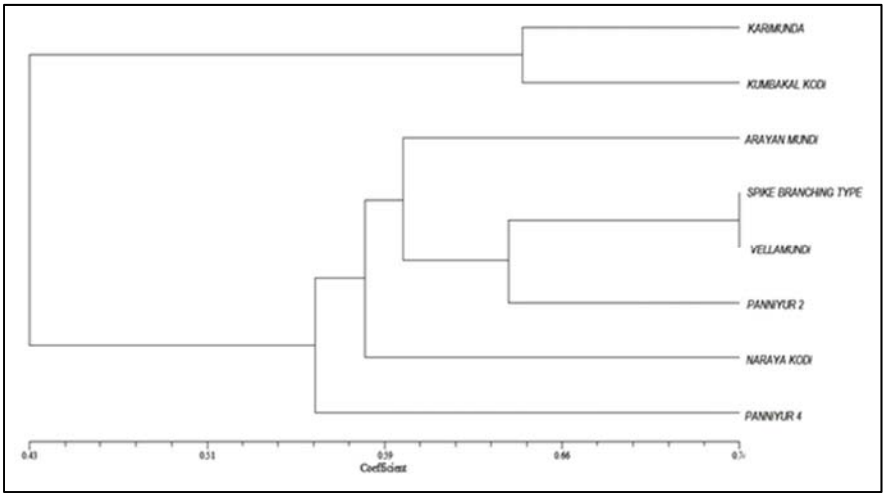

Fig 3: Dendrogram generated from molecular analysis of eight black pepper cultivars and varieties from Idukki district of Kerala**.**

In the dendrogram, divergence for all the varieties under study started at 0.43 coefficient, indicating only 43 percent similarity among cultivars/varieties. Two big clusters formed at the same similarity index. Two of the varieties; Karimunda and Kumbakal kodi formed a separate cluster, indicating the wide divergence over other six varieties. However, both the cultivars showed only 64.6 per cent similarity. Divergence for the biggest cluster started at coefficient of 55.6% indicating the initiation of divergence for six of the varieties of same cluster. Maximum of 74% similarity was obtained for the cultivar Vellamundi and Spike branching black pepper type.

As an initial approach to elucidate the decendence of this new variant of black pepper, RAPD analysis was done. Main idea behind this analysis was to support the hypothesis that this variant might have originated from the cultivars present in same farm. The farmer, T. T. Thomas is an innovative farmer who does extensive grafting experiments in his farm. He has been awarded ICAR germplasm collection award in the year 2007 [18]. All the varieties and cultivars in his farm were subjected to RAPD analysis and it is elusive that this variant has genomic similarity with vellamundi (74%) which is a local cultivar. In our other studies, Karimunda represented gene conservation for *tfl*1, which is believed to be involved in inflorescence variations.

However, in this study the wilt resistant local cultivar (karimunda) formed a separate cluster indicating the genomic diversity, but possibly conserved for some of the alleles that confers inflorescence architecture. It will be an economically important trait to introgress into disease resistant genomic background which may yield wilt resistant varieties. Presently as we speak, many plantations in Western Ghats are suffering plant losses due to devastating wilt disease, for which most prominent high yielding varieties such as Panniyur-1 and Panniyur-2 are highly susceptible. This variant is also found to be resistant to common pest and diseases of black pepper.

4. Acknowledgement

We would like to thank Mr. T. T. Thomas of Kanjiyar Village of Idukki district of Kerala for providing us the plant samples. We also like to thank Kerala Agricultural University for the financial support of the research.

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