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RESEARCH ARTICLE

Estimation of genetic diversity in nutmeg (*Myristica fragrans* Houtt.) selections using RAPD markers

■ **ANU G. KRISHNAN, RESHMA JOHN, ANU CYRIAC AND G.V. SIBLE**

SUMMARY

Nutmeg (*Myristica fragrans* Houtt.) is an evergreen tree and popular spice. In the present study, Random Amplified Polymorphic DNA (RAPD) analysis was performed to assess the genetic diversity among the 19 superior accessions of nutmeg collected from different geographic locations and maintained in the germplasm collections of Regional Agricultural Research Station, Kumarakom, Kerala. This included one released variety IISR Viswasree. Out of 28 RAPD primers tested, 20 were amplified. Out of the 109 loci amplified, 82 were polymorphic with an average polymorphism rate of 72.74 per cent. The number of bands for each primer ranged from 2 to 8. The markers which produced maximum number of polymorphic bands were BB-18 and PO-5. PIC value of the markers ranging from 0.09 (OPA 11) to 0.48 (W-15) with an average of 0.31. The marker index (MI) varied between 0.09 and 2.08 with an average of 1.25. Jaccard's similarity co-efficient of the genotypes ranged between 0.34 – 0.93. Dendrogram constructed based on UPGMA analysis grouped the 19 selected genotypes into two major clusters. The knowledge on genetic diversity of nutmeg can be used for further breeding programmes for getting higher nut and mace yield.

Key Words : Nutmeg, Genetic diversity, RAPD, Dendrogram

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Myristica fragrans Houtt., commonly known as nutmeg, belonging to the family Myristicaceae is an aromatic evergreen tree and widely cultivated tree spice. The family Myristicaceae comprises of 18 genera and 300 species. *M. fragrans* is economically and medicinally popular spice, which is valued for its nut, mace and essential oils. In India, it is cultivated throughout Kerala, Tamilnadu, Karnataka, Goa, Andaman and Nicobar Islands, and Assam. Nutmeg possesses various medicinal properties like hepatoprotective, anti-oxidant, anti-cancerous, anti-diabetic, anti-depressant, hypocholesterolemic, anti- inflammatory, antibacterial and antifungal activity (Asgarpanah and Kazemivash, 2012). It is also used by the traditional healers for treating

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diarrhoea, mouth sores and insomnia (Somani and Singhai, 2008).

Estimation of genetic diversity between plant population is essential for protection, conservation as well as useful application of germplasm. Besides, it is also helpful in the identification of genetic content of important breeding traits associated to breeding purposes (Cruz *et al*., 2007). Molecular markers are considered as powerful tool for analysing the genetic variation as well as elucidating the genetic relationships within and among species. DNA markers like RAPD are successfully used in genetic diversity analysis, genetic mapping studies, molecular phylogenetics, genetic fidelity and marker assisted selection in numerous plant species (Salem *et al.*, 2007 and Kumar *et al*., 2009). RAPD markers are used for characterisation and sex determination in nutmeg (Ganeshiah *et al*., 2000) and for detecting molecular variations between clonal and seedling progenies of nutmeg (Sheeja *et al.*, 2006). They are successfully used in analysing the genetic diversity in several spice crops like *Garcinia gummi-gutta* (Krishnan *et al.*, 2016), large cardamom (Chaudhary *et al*., 2016), *Cinnamomum zeylanicum* (Sandigawad and Patil, 2011), cardamom (Radhakrishnan and Mohanan, 2005) etc.

To develop strategies for the improvement of *Myristica* sp., to conserve the unique germplasm and to detect duplicate samples in the genotype collection it is important to analyse the genetic diversity between members of the species and wild relatives using molecular methods. Limited studies have been conducted on genetic diversity analysis in *Myristica* species using molecular markers (Sheeja *et al.*, 2006, 2008 and 2013). The objective of the present study was to demonstrate the genetic diversity between various superior accessions of *M. fragrans* maintained in Regional Agricultural Research Station, Kumarakom, using RAPD markers.

MATERIAL AND METHODS

Plant material, sample collection and DNA extraction :

The 19 superior accessions of nutmeg available in the germplasm of Regional Agricultural Research Station, Kumarakom, Kerala were selected for the study (Table A). This included the released variety IISR Viswasree and other genotypes collected from different localities of Kottayam district, Kerala.

Young leaf samples were collected from the genotypes during early morning hours. DNA was extracted using the method described by Sheeja *et al*. (2008). PCR amplification was carried out in an Agilent Sure Cycler 8800 (Agilent Technologies, USA) using 28 decamer arbitrary primers (Operon technologies, USA). PCR was performed in a final reaction volume of 25 µL contained deionised water 16.5 µL, *Taq* buffer (10x) 2.5 µL, $MgCl_2$ (25mM) 1 µL, RAPD primer (10 pmol/ µL) 2 µL, dNTP mix (2.5mM) 1 µL, *Taq* DNA polymerase (2 U/µL) 1 µL, Template DNA (25 ng/µL) 1 µLThe PCR programme was designed for 40 cycles with an initial denaturation for 3 min at 94^0C followed by denaturation at 94^0C for 30 sec, annealing at 37^0C for 1 min, extension for 1 min at 72^0C and final extension 72^0C for 15 min. Amplified products were visualized on 2 per cent agarose gel and documented using a BioRAD Gel Documentation System (Bio-Rad Laboratories, Hercules, CA).

Table A : Selected nutmeg accessions and their site of collection for genetic diversity analysis using RAPD markers

Sr. No.	Genotypes/Accession	Place of collection
1.	ACC1	IISR Viswasree (a released variety)
2.	ACC2	Kadapoor
3.	ACC3	Vaikom
4.	ACC4	Vaikom
5.	ACC5	Piravom
6.	ACC6	Piravom
7.	ACC17	Piravom
8	ACC7	Chengalam
9.	ACC8	Chengalam
10.	ACC9	Arunootimangalam
11.	ACC21	Arunootimangalam
12.	ACC22	Arunootimangalam
13.	ACC12	Poovarani
14.	ACC19	Poovarani
15.	ACC13	Elikulam
16.	ACC14	Chengalam
17.	ACC20	Pinakanad
18.	ACC15	Kanjirappally
19.	ACC11	Kanjirapally

Data analysis :

For each RAPD primer the amplified products were scored as present (1) or absent (0). Faint and unclear bands were not considered. The binary data obtained were used to estimate the polymorphism by dividing the number of polymorphic bands with total number of bands. The polymorphism information content was calculated as PIC= 2fi (1-fi), where fi is frequency of amplified

allele (band present) and (1-fi) is frequency of null allele (band absent) for the allele *i* (Roldan-Ruize *et al*., 2000). MI was determined as product of PIC and number of polymorphic bands per assay unit. Marker index (MI) = PIC × No. of polymorphic bands (Powell *et al.*, 1996). The genetic relationship was analysed using the software programme numerical taxonomy and multivariate analysis system (NTSYS-pc, Version 2.02e, Exeter Software, NY, USA) (Rohlf, 1998). The SIMQUAL program was used to generate Jaccard's co-efficient of similarity (Jaccard, 1908). The dendrogram was constructed based on unweighted pair group with arithmetic averages (UPGMA) using Shan module of NTSYS-pc (Sneath and Sokal, 1973). Principal co-ordinate analysis (PCoA) was performed using EIGEN procedure in NTSYS.

RESULTS AND DISCUSSION

In the current study, a total of 28 RAPD primers were tested for the 19 selected accessions of nutmeg, out of which 20 primers generated clear consistent amplification profiles. The number of loci amplified for each marker ranged from two (OPAB-01 and W-15) to eight (GO-3 and OPM-18) and the amplicon size varied between 300-1250 bp. Among the 109 amplified bands, 75.2 per cent were polymorphic with a mean of five polymorphic fragments per primer (Table 1). The primers which produced maximum number of polymorphic bands were BB-18 and PO-5. The percentage of polymorphism ranged from 33 per cent (OPA-11) to 100 per cent (OPJ-18, BB-18, PO-5 and OPC-09) with an average value of 72.74 per cent. Sheeja *et al*. (2013) observed a high level of polymorphism (98.1%) among different species of *Myristica* and related genera using 30 RAPD primers. However, the percentage of polymorphism obtained in this study was higher when compared to previous RAPD analysis in *Garcinia gummi-gutta* (68.76 %) and sugarcane (44.9 %) (Krishnan *et al.*, 2016 and Kawar *et al.*, 2009). It is reported that the ability to resolve genetic variation is related to the number of polymorphisms detected by the marker system (Sivaprakash *et al.*, 2004).

PIC value evaluates the efficiency of markers, in addition it is also used to select appropriate marker for phylogenetic and genetic mapping studies (Anderson *et al.*, 1993 and Powell *et al.*, 1996). It reflects the allele diversity and frequency among the varieties. In the

Table 1 : RAPD data obtained with 20 random primers analysed on 19 accessions of *M. fragrans*

Primer	Sequence	Total number of bands	Percentage of polymorphic bands	Percentage of monomorphic bands	PIC	MI
OPAB-01	CCGTCGGTAG	2	50	50	0.45	0.45
OPB-17	AGGGAACGAG	5	80	20	0.32	1.28
W-15	ACACCGGAAC	2	50	50	0.48	0.48
GO-3	GAGCCCTCCA	8	87.5	12.5	0.27	1.87
NO-5	ACTGAACGCC	6	83	16	0.36	1.83
OPM-16	GTAACCAGCC	4	50	50	0.27	0.54
OPJ-18	TGGTCGCAGA	4	100	0	0.37	1.51
AB-16	CCCGGATGGT	6	83	17	0.39	1.95
OPA-1	CAGGCCCTTC	7	71	29	0.26	1.30
OPN-10	ACAACTGGGG	4	75	25	0.36	1.10
OPA-11	CAATCGCCGT	3	33	66	0.09	0.09
BB-18	CAACCGGTCT	7	100	0	0.27	1.89
OPA-2	TGCCGAGCTG	6	66	33	0.23	0.94
PO-5	CCCCGGTAAC	6	100	0	0.30	1.84
OPC-09	CTCACCGTCC	5	100	0	0.27	1.35
OPA-4	AATCGGGCTG	7	71.4	28.5	0.27	1.38
OPA-9	GGGTAAACGCC	7	71.4	28.5	0.28	1.44
OPC-7	GTCCCGACGA	7	28.5	71.4	0.32	0.65
OPM-18	CACCATCCGT	8	75	25	0.34	2.08
OPC-13	AAGCCTCGTC	5	80	20	0.25	1.02
Total		109				
Average		5.45	72.74	27.09	0.31	1.25

present study, PIC value of the markers ranged from 0.09 to 0.48 (Table 1). The lowest and highest PIC value was observed for the primers OPA-11 and W-15, respectively. It coincides with the previous reports in *Myristica* sp. where similar range of PIC values was obtained (Sheeja *et al.*, 2013). The marker index (MI) for the primers used in this study varied from 0.09 to 2.08 with an average of 1.25.

The Jaccard's similarity co-efficient values ranged from 0.34 – 0.93 (Table 2). The lowest similarity index (0.34) was observed between the accessions ACC5 and ACC20, while the accessions ACC22 and ACC19 showed highest similarity index (0.92). Sheeja *et al.* (2013) reported that the genetic distance based on Jaccard's similarity co-efficients ranged from 0.23 to 0.67 between seven *Myristica* sp., two related genera and an unidentified species. Uyoh *et al.* (2014) reported an average similarity index of 0.13-0.88 obtained with 10 RAPD primers for 21 accessions of African nutmeg (*Monodora myristica*).

A dendrogram based on UPGMA cluster analysis grouped the 19 genotypes into two main clusters, I and II with similarity co-efficient ranging from 0.4-0.93 (Fig.1). Cluster I comprised of 17 genotypes. Genotypes within cluster I are further grouped into four sub clusters except four accessions (ACC20, ACC14, ACC3 and ACC7) which individually formed separate OTUs showing less similarity with other genotypes. The first sub cluster Ia comprised ACC1(Viswasree), ACC17, ACC2, ACC8, ACC9, ACC22 and ACC19. Sub cluster Ib included ACC4 and ACC6. ACC12 and ACC13 formed sub cluster Ic. Sub cluster Id consisted of ACC 15 and ACC11. Within sub cluster Ia ACC22 and ACC 19 appeared to be closer to each other with a similarity co-efficient of 0.93. The two genotypes ACC5 and ACC21 were distinguished from all others and grouped into second cluster (II). The dendrogram clearly indicates

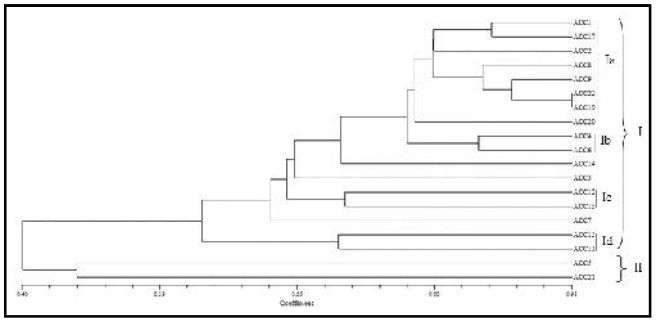

Fig. 1 : Dendrogram obtained from 19 accessions of nutmeg with UPGMA based on Jaccard's co-efficient

Table 2 : Jaccard's co-efficient of similarity between 19 nutmeg selections based on RAPD data analysis

	ACC1	ACC2	ACC3	ACC4	ACC5	ACC6	ACC17	ACC7	ACC8	ACC9	ACC21	ACC22	ACC12	ACC19	ACC13	ACC14	ACC20	ACC15	ACC11
ACC1	1.000																		
ACC2	0.760	1.000																	
ACC3	0.656	0.705	1.000																
ACC4	0.782	0.753	0.652	1.000															
ACC5	0.396	0.373	0.428	0.406	1.000														
ACC6	0.820	0.763	0.637	0.838	0.390	1.000													
ACC17	0.850	0.828	0.701	0.774	0.362	0.785	1.000												
ACC7	0.632	0.681	0.625	0.652	0.355	0.661	0.676	1.000											
ACC8	0.802	0.797	0.630	0.746	0.357	0.780	0.845	0.608	1.000										
ACC9	0.805	0.776	0.613	0.773	0.380	0.783	0.810	0.635	0.840	1.000									
ACC21	0.348	0.388	0.350	0.400	0.452	0.406	0.371	0.379	0.371	0.394	1.000								
ACC22	0.750	0.819	0.671	0.743	0.367	0.777	0.791	0.625	0.861	0.863	0.402	1.000							
ACC12	0.608	0.633	0.661	0.628	0.410	0.661	0.652	0.584	0.630	0.613	0.403	0.647	1.000						
ACC19	0.763	0.783	0.710	0.780	0.402	0.791	0.780	0.638	0.824	0.876	0.439	0.927	0.685	1.000					
ACC13	0.642	0.666	0.621	0.616	0.431	0.647	0.761	0.646	0.757	0.666	0.423	0.680	0.709	0.671	1.000				
ACC14	0.714	0.689	0.647	0.662	0.406	0.694	0.736	0.647	0.684	0.733	0.428	0.726	0.632	0.716	0.666	1.000			
ACC20	0.746	0.789	0.648	0.696	0.342	0.750	0.763	0.626	0.828	0.762	0.336	0.779	0.605	0.769	0.636	0.701	1.000		
ACC15	0.632	0.611	0.560	0.583	0.428	0.637	0.619	0.514	0.608	0.680	0.500	0.625	0.645	0.638	0.630	0.681	0.584	1.000	
ACC11	0.523	0.552	0.500	0.457	0.458	0.530	0.529	0.483	0.507	0.535	0.543	0.521	0.586	0.536	0.573	0.562	0.527	0.703	1.000

that genetic variation existed between the 19 selected accessions of nutmeg. The clustering pattern in the dendrogram does not correlate with geographic locations from where the genotypes were collected, indicating that there is no location specificity among genotypes. Similar results were observed in *Garcinia cambogi*a (Tharachand *et al*., 2015), *Garcinia gummi-gutta* (Krishnan *et al.,* 2016) and *Ricinus communis* (Gajera *et al*., 2010). The PCoA analysis was comparable to the cluster analysis (Fig. 2). The two genotypes ACC 5 and ACC 21 appear to be distinct from other genotypes in the PCoA.

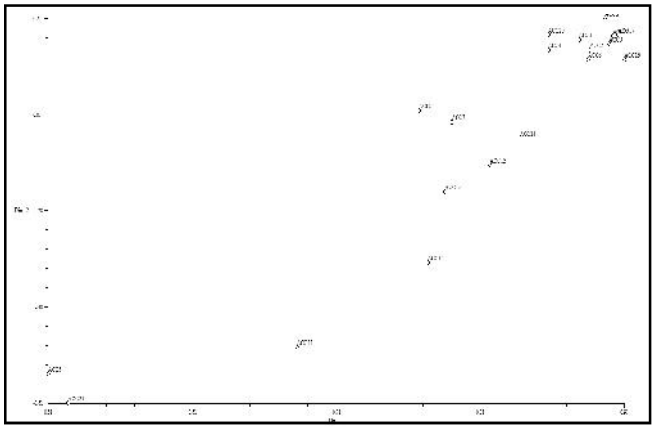

Fig. 2 : Principle co-ordinate analysis (PCoA) plot of genetic diversity of nutmeg accessions with RAPD primers

Conclusion:

Molecular characterization of germplasm is essential for maintaining the identity, purity and for proper conservation of unique germplasm. The study was conducted to analyse the genetic diversity that existed between the 19 superior accessions of nutmeg maintained in farm of RARS, using RAPD markers. The RAPD analysis categorised the 19 accessions into two main clusters. However, further studies using other molecular markers like inter simple sequence repeat (ISSR) markers and simple sequence repeat (SSR) markers have to be carried out for a wide understanding of genetic diversity in nutmeg accessions, which will aid in the detection of duplicate samples and for selecting, establishing and maintaining core collection for further crop improvement programmes.

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