Journal of Plantation Crops, 2006, 34(3) : 558-561

DNA polymorphism in clonal and seedling progenies of an elite nutmeg (*Myristica fragrans* Houtt.) by RAPD

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Abstract

Molecular profiling was done involving clonal and seedling progenies of a dwarf, high yielding, and high quality selection of nutmeg (*Myristica fragrans* Houtt.) very popular for its suitability to humid tropics. The RAPD profiles were analysed for detecting molecular variations and uniqueness in progenies. A total of 405 data points were scored with 20 polymorphic loci. The JSI values ranged from 0.72 to 1.00 showed the close relatedness among the progenies and mother. The clones were found to show definite variations from the mother. Clones No. 12, 15 and seedling No. 11 showed 100% similarity with the mother. The similarity index within clones (96-100%) was considerably more than that between clones and seedlings (83-100%) and within seedlings (76-90%). Twelve unique bands were detected for identification of individuals. The male seedling could be characterized by presence of specific bands of 1300 and 1000 bp amplifiable by OPC 16 and OPA 05 and absence of a band of 1400 and 1200 amplifiable by OPA 01 and 710 bp by OPA 14, respectively.

Key words : *Myristica fragrans*, RAPD, fingerprinting, gender identification

Abbreviations : TE- Tris EDTA; CTAB- Cetyl trimethyl ammonium bromide; PVP- Poly Vinyl Pyrrolidone

Introduction

Nutmeg belonging to the genera Myristica (family Myristicaceae) is the most primitive genus of the family. Different species of *Myristica* are distributed from India to South East Asia to North Australia. Nutmeg cultivation has gained global importance of late mainly due to the hurricanes in Grenada that ravaged its nutmeg based economy.

Two major issues in nutmeg cultivation are identification of sex in juvenile stage and production of uniform quality planting material with very little variation from elite mother. Nutmeg is generally propagated through seedlings and grafting is practiced to combat these problems. Several attempts made for identification of sex of nutmeg seedlings based on morphological and chemical characters (Krishnamoorthy *et al.*, 1996) reported only limited success. Differences between clones and closely related species are not always absolute using morphological characters (Oraguzie, 1998) and distinguishing between clonal and seedling progenies are impossible due to very limited morphological variations in this crop (Krishnamoorthy *et al.*, 1997). In this context identification of variations in clonal and seedling progenies is important, which can help in adoption of suitable propagation method, efficient sampling and utilization of germplasm resources. Early determination of sex with the help of linked markers shall facilitate better management of nurseries and improve production.

As in the present study, studies involving closely related individuals the concept of homology between comigrating bands holds good and RAPD analysis gives more accurate estimates than other molecular markers (Bardakci, 2001). Earlier, RAPD markers have been exploited for characterization and sex determination in nutmeg (Ganeshiah *et al.*, 2000). This paper addresses two major issues of immediate significance in nutmeg.

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Materials and Methods

Plant materials under study are all progenies of an elite high yielding accession of nutmeg, A9/4 released as "IISR- Viswashree" in 2002 by the Indian Institute of Spices Research, Calicut, India. Five clones and six seedling progenies of Viswashree were chosen for the study. It has a vigorous growth habit with a compact canopy and heavy bearing nature. It yields on an average 480 kg of dry mace and 3122 kg dry seed per hectare at the eighth year of planting with low incidence of fruit rot.

Genomic DNA was extracted from young leaves according to the modified CTAB protocol outlined by Murray and Thomson (1980). Extraction buffer contained 2 % PVP and 0.3 % mercaptoethanol. DNA extraction and purification was done as per procedures standardized in the lab. The random amplification was performed by method of Williams *et al.* (1990). A total reaction volume of 25 μl of solution at pH 8.3 containing 10X ready prepared PCR buffer (with 2 mM $MgCl_2$), 100 mM Tris-Cl, 500 mM KCl, 2.5μl dNTP stock (300 μM premixed), 2 μl (5 picomoles) of a single 10-base primer (OPERON Technologies, Inc.) 0.5 U Taq DNA polymerase and 0.5 μl (20 ng) genomic DNA template was used. Out of 45 primers of OPERON Technologies, 10 used for PCR were OPA01, OPA03, OPA05, OPA06, OPA14, OPA17, OPA20, OPB15, OPC06, OPC16, OPE04, OPE06, OPE08, OPE09 and OPE19. The amplification was performed with MJ Research model PTC-100 DNA thermocycler as per the following programme: Initial denaturation at 94°C for 3 min, 34 cycles of 1 min at 94°C (denaturing), 1 min at 45°C (annealing), 1 min at 72°C (extension) and 15 min at 72°C (final extension).

The amplification products were separated on 1.8 % agarose (Biogene Reagents Inc.) on 1X TAE buffer (40 mM Tris acetate, pH 8.2, 1mM EDTA) and stained with ethidium bromide (BDH, Cat No. 214-984-6) to a final concentration of 0.5 mg/ml. The electrophoresis was carried at 30 volts for 12 h. The banding patterns were recorded using a gel documentation system (BioRad) and image profiles and molecular weight of each band were determined by Alpha Imager. Electrophoretic patterns were reconstructed according to the presence or absence of clear, visible and reproducible bands (Williams *et al.*, 1990). The most intense monomorphic band from each accession with each primer was used as reference to calibrate the different lanes for amount of DNA present. When there was no monomorphic band, the band with highest frequency in each sample was used for calibration. Within each lane, bands were scored present if their intensity was at least 10% that of monomorphic reference band within that lane. Markers were scored as present (1) and absent (0) for each accession. Band sharing data were used to calculate genetic similarities and UPGMA algorithm was employed to determine the genetic relationships. All analyses were performed using NTSYS-pc software (version 1.7).

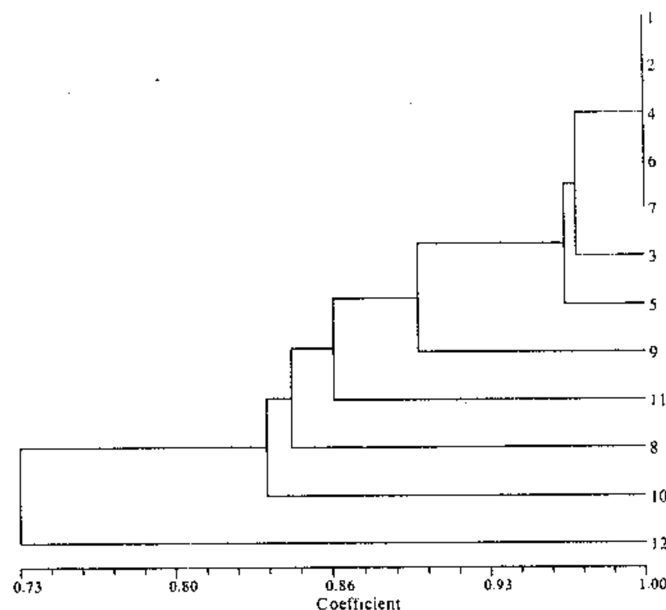

Fig. 2. Dendrogram showing the genetic relatedness of the progenies of elite nutmeg, IISR Viswashree. 1-5: clonal progenies; 6: Viswashree mother; 7-12: seedling progenies;

Results and Discussion

Number of amplified products per primer ranged from 80 (OPC 06) to 18 (OPA 05). All together 405 bands were produced. The bands ranged from 2500 bp (OPC 06) to 225 bp (OPA 14). All the individuals under study gave scorable bands (Fig.1). Number of polymorphic loci observed was 20 with a total of 160 making the mean number of polymorphic bands/primer as 16. Primers OPA 01, OPA 05 and OPA 14 gave the maximum number of polymorphic loci of three each. All the primers showed monomorphic loci, the maximum given by OPA 01 (4). Extensive amount of variation for the total and average number of bands formed by each primer within each individual was observed. All the primers taken together, the maximum number of bands was found in male seedling (37) and minimum in seedling No. 14 and clone No. 14 (31 each).

UPGMA was done assuming that amplification products differing in electrophoretic mobilities are non-allelic and products with same mobilities are allelic. Reproducible results could be obtained in this study, using specific combination of primer and DNA template combination. Extreme care was taken not to alter any experimental parameter. Genetic similarity was

calculated assuming that comigrating amplified bands are allelic. Homology of RAPD products has been previously demonstrated in different species of *Glycine* and *Allium*. RAPD analysis was shown to identify accepted relationships between genomes of *Musa* and *Brassica*, hence the concept that comigrating bands are allelic seems to be realistic. The JSI values in the present study ranged from 0.72 to 1.00 (Fig 2.). It could be inferred that high genetic affinities were present among the individuals due to their common parentage. Dendrogram grouped all the clones and seedlings into one cluster, with the male seedling separated from the rest. Clones No. 12, 15 and seedling No. 11 showed 100% similarity with the mother. Two of the clones viz. No. 13 and No. 16 showed 96 and 93% similarity. It is interesting to note that clones showed definite variations from the mother and clone No. 13 was very close to seedling No. 16. Clonal selections are expected to be extremely difficult to differentiate using anonymous markers. All the seedlings clustered separately from the mother and the clonal progenies; however, one of the seedlings (No. 11) showed very close relationship with clones, distinguishable only by the absence of a band of 101 bp amplifiable by OPC 06.

A number of unique bands could be identified, which are useful in designating the individuals under study

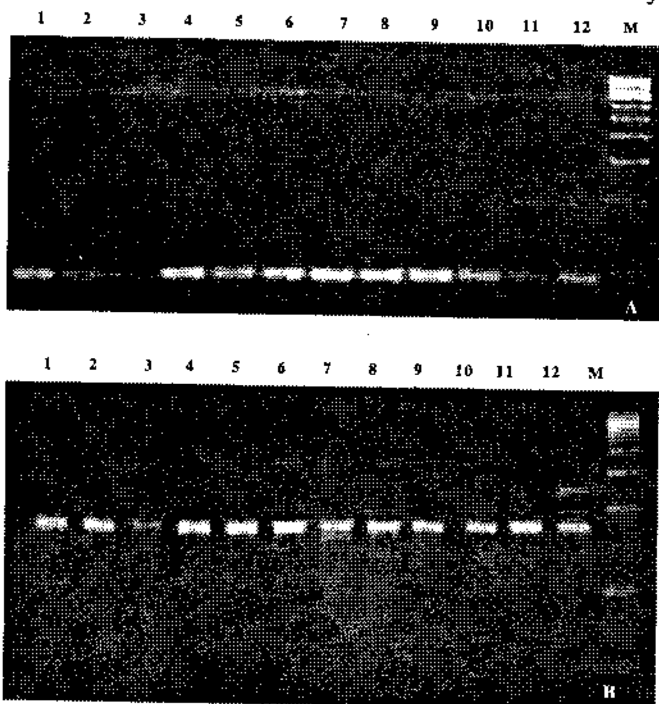

Fig. 1. RAPD profiles of progenies of elite nutmeg, IISR Viswashree. A : OPC 16; B : OPA 05 primers. M-500 bp ladder, Lane 1-5 : clones, Lane 6 : Viswashree, Lane 7-11 : seedlings; Lane 12: male seedling, arrows indicate male specific band in seedling No. 12

(Table 1.). Though clonal progenies could not be distinguished from each other, all the seedling progenies could be designated with unique bands, for specific identification. No single primer could distinguish all the progenies (Table 1). The similarity index within clones (96-100%) was considerably more than that between clones and seedlings (83-100%) and within seedlings (76-90%). As expected, clones closely resembled parent (96-100%), while seedlings showed variations (86-100%). The variability induced due to sexual crossing in nutmeg is hereby confirmed. However, lack of adequate genetic variability for many important attributes has been reported in nutmeg progenies (Krishnamoorthy *et al.*, 1997). The study thus confirms absence of obvious relationship between the extent of morphological variations and that of changes at the DNA level already reported in rice (Mezencev *et al.*, 1997). It is also indicated that to conserve the unique qualities of the genetically superior nutmeg, clonal propagation is better. Nutmeg being dioecious in nature variations is expected as in case of other dioecious plants like *Cannabis sativa*, where high degree of polymorphism existed and plant-to-plant variation could be observed (Forapani *et al.*, 1999). Earlier workers suggested use of similar molecular techniques for identification in case of a confusion or misidentification of genotypes where not much of a morphological variation whatsoever exists (Forapani *et al.*, 1999). A high potential of RAPD markers in detecting molecular polymorphism between individuals of an accession (Virk *et al.*, 1995), phenotypically pure stocks (Olufowote *et al.*, 1997) and intra varietal variations among donor plants (Mezencev *et al.*, 1997) has been demonstrated.

Table 1. List of unique bands

Band	Primer	Comments
1400 bp	OPA 01	Absent in the male seedling
1200 bp	OPA 01	Absent in the male seedling
800 bp	OPA 03	Absent in clone no. 14
1000 bp	OPA 05	Present in male seedling
710 bp	OPA 14	Absent in male seedling
600 bp	OPA 17	Absent in seedling no. 15
2500 bp	OPC 06	Absent in clone no. 16
1090 bp	OPC 06	Absent in clone no. 16
620 bp	OPC 06	Absent in seedling no. 16
1300 bp	OPC 16	Present in male seedling
179 bp	OPE 08	Present in seedling no. 13
750 bp	OPE 09	Absent in clone no.14

The male seedling showed an average similarity of 72.4% with the clones and 71.6% with seedlings and 73% with the mother. The male seedling could be characterized by presence of specific bands of 1300 and 1000 bp amplifiable by OPC 16 and OPA 05, respectively

and absence of a band of 1400 and 1200 amplifiable by OPA 01 and 710 bp by OPA 14, respectively. It is likely that these markers are linked to sex determining loci, though the sex determination mechanism is not yet characterized in nutmeg. Lemos *et al.* (2002) identified a sex specific marker in papaya and a PSDM was isolated. In *Myristica,* female specific bands were isolated by RAPD (Ganeshiah *et al.*, 2000). SCAR markers were developed for sex determination in alien crops (Yakubov *et al.*, 2005; Urasaki *et al.*, 2002). However, extensive studies involving sex pooled DNA samples from more number of individuals and primers are needed to confirm this. For molecular markers linked to specific genes there is a need for screening large number of RAPD primers (Kafkas *et al.*, 2001), usually more than 500.

References

Bardakci, F. 2001. Random amplified polymorphic DNA (RAPD) markers. *Turk. J. Biol.* **25**: 185-196.

Forapani, S., Carboni, A., Castellani, E., Mandolino G. and Ranalli, P. 1999. RAPD markers for potato germplasm characterization. *J. of Gen. and Breed.* **53**: 43-147.

Ganeshaiah, K.N., Ravishankar, K. V. , Anand Lalitha, Shibu, M.P., Uma Shanker. 2000. Identification of sex specific DNA marker in the dioecious tree, nutmeg. *Plant Genetic Resources Newsletter* **121**: 59-61.

Kafkas, S., Cetiner, S. and Perl-Treves, R. 2001. Development of sex associated RAPD markers in wild *Pistacia* species. *J. of Horticultural Science and Biotechnology* **76**: 242-246.

Krishnamoorthy, B., Sasikumar, B. and Rema, J. 1996. Genetic variability and segregation of sex in nutmeg (*Myristica fragrans* Houtt.). *J. Plantn. Crops* **24**: 468-72.

Krishnamoorthy, B., Sasikumar B., Rema, J., Johnson K., George and Peter, K. V. 1997. Genetic resources of Tree spices and their conservation in India. *Plant Genetic Resources News Letter* **111**: 53-58.

Lemos, E.G.M., Silva, C.L.S.P. and Zaidan, H. A. 2002. Identification of sex in *Carica papaya* L. using RAPD markers. *Euphytica* **127**: 179-184.

Mezencev, N., Ghesquiere, A., Marmey, P., Combes, M. C., Guiderdoni, E., 1997. Assessment of RAPD markers to detect genetic change in protoplast-derived rice plants. *J. Genet. & Breed* **51**: 97-102.

Olufowote, J.O., Xu, Y., Chen, X., Park, W. D., Beachell, H. M., Dilday, R. H., Goto, M., Mc Couch, S. R. 1997. Comparative evaluation of within-cultivar variation of rice (*Oryza sativa* L.) using micro satellite and RFLP markers. *Genome* **40**: 370-378.

Urasaki, N., Tokumoto, M., Tarora, K., Ban, Y., Kayano, T., Tanaka, H., Oku, H., Chinen, I. and Terauchi, R. 2002. A male and hermaphrodite specific RAPD marker for papaya (*Carica papaya* L.). *Theoretical and Applied Genetics* **2**: 281-285.

Virk, P.S., Ford–Llyod, B.V, Jackson, M.T., Newbury, H.J. 1995. Use of RAPD for the study of diversity within the plant germplasm collections. *Heredity* **74**: 170-179.

Williams, J.G.K., Hanafey, M.K., Rafalski, J.A., Tingey, S.V. 1993. Genetic analysis using random amplified polymorphic DNA markers. *Methods in Enzymol.* **218**: 704-740.

Wilkie, S.E., Isaac, P.G. and Slater, R.J. 1993. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. *Theor. Appl. Genet.* **86**: 497-504.

Yakubov, B., Barazani, O. and Golan-Goldhirsh, A. 2005. Combination of SCAR primers and touchdown PCR for sex identification in *Pistachia vera* L. *Scientia Horticulturae* **103**: 473-478.