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Optimization of DNA isolation and PCR parameters in *Myristica* sp. and related genera for RAPD and ISSR analysis.

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Abstract

An efficient protocol for isolation of DNA from wild and related genera of Myristica rich in polysaccharides and polyphenols was developed. The protocol utilizes CTAB (3%), 1.5% PVP and 0.3% ß-mercaptoethanol for isolation and RNase and phenol chloroform extraction for purification. The yield of DNA ranged from 25-175 µg/g of fresh leaf tissue, with Knema andamanica giving the highest yield. The present method yielded 10 times higher than the old methods. Characteristic patterns were generated on digestion of DNA by EcorI and Hind III restriction enzymes. PCR parameters were optimized using random primers (OPERON Technology, USA). DNA concentration at 20 ng/reaction, annealing temperature of 45°C, 0.3 mM dNTP in presence of 0.5 U of Taq DNA polymerase, and 2.0 mM $MgCl_2$ and MJ Research Gene Thermocycler was best. Successful amplification by ISSR and RAPD primers indicated that DNA is of good quality and free of polysaccharides and polyphenols.

Key words: DNA isolation, optimization, PCR, polymorphism, restriction digestion

Abbreviations: CTAB, cetyl trimethylammonium bromide; ISSR, inter simple sequence repeats; PCR, polymerase chain reaction; PVP, polyvinylpyrrolidone; RAPD, random amplified polymorphic DNA.

Intruduction

Nutmeg (*Myristica fragrans* Houtt.) belongs to the family Myristicaceae is an important spice, valued mainly for its nut, mace and essential oils, used as condiment and in medicine.

The National Repository of Nutmeg Germplasm at Indian Institute of Spices Research, Calicut has 482 accessions of *Myristica* including 405 accessions of *M. fragrans* and nine wild species. Accessions with high sabinene coupled with low myristicin and elemicin are preferred in confectionary. In order to develop strategies for the improvement of *Myristica* and to preserve the unique germplasm, it is necessary to have an understanding of the genetic diversity among members of the species and wild relatives preferably using molecular means. A major constraint in the application of molecular techniques to *Myristica* is isolation of good quality DNA with high yield. Since the biochemical composition of plant tissues vary in different species, DNA isolation protocols need to be optimized for each species (Weising *et al.*, 1995). DNA isolation from Myristica is cumbersome

mainly due to the high content of polyphenols and polysaccharides that is found to co precipitate with the DNA besides the degradation of DNA due to endonucleases that hampers DNA restriction, amplification and cloning.

Several protocols have been tried for isolation of DNA from *Myristica* (Murray and Thompson, 1980; Doyle and Doyle, 1990; Chen and Ronald, 1999; Khanuja *et al*., 1999) but are found unsuitable due to brown colour and viscous nature of the pelleted DNA, besides the degrading and denaturing of DNA. Some of the wild species like M. prainii, *M. amygdalina*, *M. andamanica* and *Knema andamanica* are found to be nearly recalcitrant to DNA isolation due to very high amount of polyphenolics. In this background we have developed a simple, rapid and cost effective method for DNA isolation from leaves of *Myristica* and related wild genera. This protocol is a modification of the principal protocols in vogue for DNA isolation from various plant species. Present study also attempts to optimize RAPD parameters for *Myristica* DNA. Quality of DNA being the most crucial requirement for successful PCR, we have evaluated the efficiency and reliability of the present method of isolation by restriction digestion and amplification by ISSR and random primers.

Materials and methods

Plant Material

The plant material used for optimization of DNA isolation and PCR protocol was that of a released variety of Myristica fragrans viz., IISR Viswashree and nine wild species of Myristica viz., *M. malabarica, M. fatua, M. beddomeii, M. prainii, M. andamanica, M. amygdalina, Knema andamanica, Gymnocranthera canerica*, and an unidentified species of *Myristica* maintained in the germplasm repository of IISR at Peruvannamuzhi, Calicut.

DNA Isolation

Fresh leaves were collected and stored in iceboxes until reaching the laboratory after which they were dipped in liquid nitrogen and stored at -80°C until extraction of DNA. For optimization experiments the composition of DNA extraction buffer used was as described by Doyle and Doyle (1990) with CTAB at 1, 2, 3, 4% and SDS at 0.5, 1, 1.5, 2% concentrations. The pH of the buffer was adjusted to either 8 or 9. The DNA isolation was done following the CTAB protocol (Murray and Thompson 1980) with modifications, described below:

Protocol

1. Grind the plant material in liquid nitrogen (2.5 g fresh tissue).
2. Preheat extraction buffer with 0.3% -mercaptoethanol and add PVP (1.5%) powder directly to the tube.
3. Transfer the ground material to 30 ml polypropylene tube; incubate at 65 C for 45 minutes in a shaking water bath.
4. Plunge the tubes in ice immediately and bring to room temperature
5. Add an equal volume of chloroform: isoamyl alcohol (24:1) and mix by inverting the tubes several times, keep for 10 minutes at room temperature.
6. Centrifuge at 12,000 g for 10 min. at 25°C
7. Carefully transfer the aqueous phase, add 2/3rd V ice cold isopropanol.
8. Incubate the tubes at -20°C for 2 hours or keep overnight
9. Centrifuge the tubes at 12,000 g for 10 min at 4°C
10. Discard the supernatant; add 70% ethanol, wash the pellet, dry and dissolve in water.
11. Add RNase (100 g/mL) to the DNA solution and incubate for one hour at 37°C.
12. Add equal volume of buffer saturated phenol: chloroform: isoamyl alcohol (25:24:1) mix properly and centrifuged at 12,000 g for 10 min at 4°C.
13. Extract the supernatant with an equal amount of chloroform:isoamyl alcohol (24:1).
14. Add sodium acetate (3 M; 1/10 V) and ice-

cold isopropanol and keep at -20°C for 30 min.

15. Centrifuge at 12000 g for 10 min at room temperature.
16. Precipitate DNA and wash with 70% ethanol, air dry, suspended in 100 µl of water and reprecipitate using 100% ethanol
17. Centrifuge at 12,000 g for 10 minutes and wash in 70% ethanol, air dry pellet and store.

The purity of the DNA sample was determined by measuring absorbance at A260 nm in a UV spectrophotometer and A260/A280 ratio was evaluated. Molecular weight and concentration of the DNA were estimated using agarose gel electrophoresis on 0.8% agarose and visualized by ethidium bromide staining against Human Genomic DNA (Bangalore Genei). The quality of DNA was ascertained through restriction digestion using Ecor1 and Hind III (Bangalore Genei, India) both by single and double digestion as per the manufacturer's protocol.

Optimisation of PCR Parameters

RAPD was performed as per Williams et al., (1990). The amplification profiles involved an initial denaturation at 93°C for 3 min, 40 cycles of denaturation at 93°C for 1 min, annealing at 40°C for 1 min, extension at 72°C for 1 minute and a final extension at 72°C for 10 min. Amplification products were resolved on a 2% agarose gel stained with ethidium bromide and electrophoresed at 80 V for 2.5 hours. The gels were photographed and visualized in a Gel documentation system (Alpha Imager, USA) and raw gel images were recorded through molecular Analyst Software/PC version 99.04).

Factors like DNA concentrations (10, 20, 30, 40 and 50 ng of DNA in 25 µl reaction volume), Mg+2 (1-10 mM), Taq DNA Polymerase (0.5, 1.0, 1.5 U in combination with 0.1, 0.2, 0.3, 0.5, 1.0, 1.25 and 1.5 mM dNTP) and three brands of Taq DNA polymerase (Bangalore Genei; and Biogene, USA viz., Sure Taq and Doctor Taq), annealing temperature (37°C, 40°C, 45°C, 50°C), different thermocyclers (MJ Research, PTC 200 and Gene Amp PCR systems 9700) and primers singly and in combination were tried for optimization of the amplification condition. All the PCR optimization experiments were repeated twice using primers OPA 01 and OPC 06 (OPERON technologies, USA). In case of the experiments involving primer pairs, OPA 01, OPA 06 and OPA 03, OPA 05 were used in pairs. About 45 random primers were screened and 13 that gave good amplification were selected for analysis of genetic relationship among the accessions. ISSR PCR was done according to Johnson *et al.* (2006).

Results and discussion

DNA yield from very young leaves of Myristica was poor with large amounts of RNA. DNA isolated from older leaves showed low recovery and contamination with protein. Third leaf from the shoot tip was found to give good quality of DNA in comparison to young shoot tips and older leaves with A260/A280 ratio as 1.74. Choice of appropriate tissue is very important for DNA extraction and earlier reports also suggested better quality and yield of DNA from rapidly expanding leaves, one to two nodes from shoot tip (Mauro et al., 1992; Lodhi et al., 1994). The DNA obtained was of high molecular weight (~23 Kb), showed no shearing and gave clear bands. DNA yield from different treatments ranged from 2.4 to 150 ng/g of leaf tissue for CTAB and 4-30 ng/g in case of SDS (Figure 1a). Among the different concentrations of CTAB used (1%, 2%, 3%, 4%) at pH 8 and pH 9, it was found that CTAB of all concentration at pH 9 gave good DNA yield without RNA contamination (Figure 1b). The DNA yield obtained from CTAB at 2%, 3% and 4% concentrations was 40, 150 and 125 ng, respectively with A260/A280 ranging from 1.74-1.86. DNA yield in presence of SDS was lower than that of CTAB at both the pH tried. Addition of PVP in powder form was found to enhance efficiency of the protocol. Though PVP at 2% levels improved the colour of DNA, it was found

to interfere with PCR amplification. Similarly plunging the tubes in ice immediately after incubation at 65°C before chloroform: isoamyl alcohol treatment also improved the quality and yield of DNA. Since we have used sodium acetate and isopropanol for purification, the excess of sodium salt were removed by resuspending the pellet in water and precipitating in 100% ethanol.

The yield of DNA obtained in the present study is comparable with earlier reports in plants having high phenol content like sweet potato, oil palm and other dicotyledonous plants (Couch and Fritz, 1990; Varadarajan et al., 1991; Jack *et al*., 1995) and several times higher than that reported in Myristica fragrans (Ganeshiah *et al.*, 2000). The yield and quality of DNA obtained from wild and related genera of Myristica is listed in the Table 1; Figure 1c.

The purity of DNA was further confirmed upon restriction digestion using enzymes Ecor I and Hind III singly and in combination and a characteristic smear was obtained on agarose (Figure 2).

Table 1. Yield and purity of DNA from Myristica species and related genera

No.	Species	Yield of DNA µg/g fresh leaf	OD value A_{260}/A_{280}
1	*M. fragrans*	150	1.75
2	*M. beddomeii*	120	1.70
3	*M. malabarica*	150	1.70
4	*M. prainii*	25	1.61
5	*M. fatua*	115	1.67
6	*M. andamanica*	100	1.69
7	*K. andamanica*	175	1.75
8	*M. amygdalina*	125	1.72
9	Unidentified sp.	160	1.67
10	*Gymnocranthera canerica*	100	1.67

DNA isolated by this method could be stored at -20 oC for more than one year and could be amplified by PCR. Our method is novel in the case that it does not use ultra centrifugation and CsCl but employs a few simple modifications to the principal protocols in vogue for isolation of DNA from recalcitrant species like wild Myristica. Successful double and single digestions with Ecor 1 and Hind III indicates that the present method is suitable for isolating DNA for sensitive experiments like AFLP, RFLP, Southern blotting etc.

Regarding optimization of PCR parameters, a DNA concentration of 20 ng in 25µl reaction volume was found to give distinct scorable bands. Amplification was visible only up to 40 ng, beyond which the amplification pattern was not distinct. Upadhyay et al. (2004) also reports better amplification in coconut at 20 ng. Regarding $MgCl_2$, no amplification was observed at 1.5 mM concentration. At a concentration of 2 mM $MgCl_2$, the DNA amplification was clear with maximum polymorphism, above which, a reduction in number and intensity of bands were observed. Use of relatively high concentration of $MgCl_2$ of 4.5 mM concentration is also reported in same species (Upadhyay *et al.*, 2001). In coconut also, $MgCl_2$ of 3 and 4 mM gave optimum intensity of bands (Manimekalai *et al.*, 2004).

With respect to annealing temperatures, maximum polymorphism and clear pattern was obtained at 45°C, below which the pattern was faint. Increasing annealing temperature above 45°C lead to diffusion of bands. Annealing temperature of 37°C is commonly used for RAPD. But in the case of Myristica it was observed that 45°C gave best results. Innis & Gelfand (1990) observed that annealing temperature in the range of 55°C to 72°C yielded the best results. Among the three brands of Taq DNA polymerase used, Sure Taq gave the maximum scorable and distinct polymorphic bands. Taq polymerases of different brands were used for amplification. The observation denotes that amplification profiles can vary with the Taq DNA polymerase used. Earlier workers, (Sobral and Honeycut, 1993) have also reported that different polymerases gave different profiles. It is observed that for

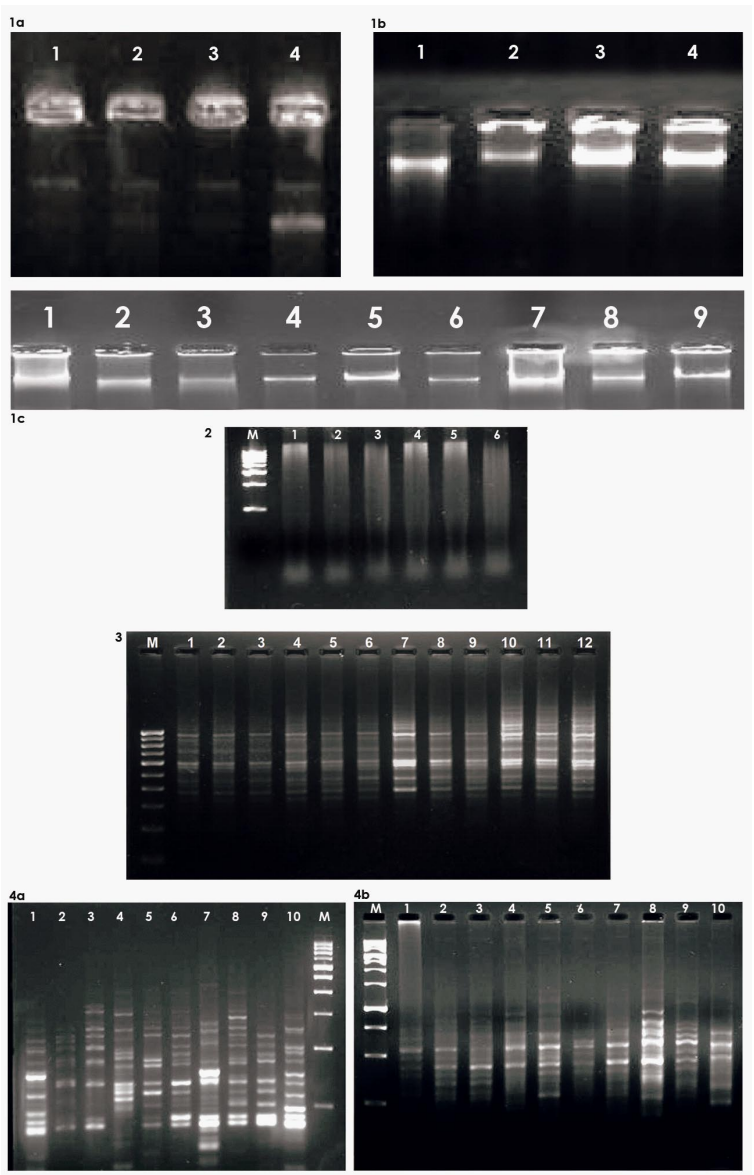

Figure 1: Agarose gel of total genomic DNA isolated from *M. fragrans* using different concentrations of CTAB (a) original protocol, (b) present protocol. Lanes 1-4: DNA obtained using 1,2,3,4 % CTAB extraction buffer c) genomic DNA isolated from different *Myristica* species lane 1- *M. fragrans*, 2- *M. malabarica*, 3- *M. beddomeii*, 4- *M. prainii* 5- *M. fatua*, 6- *M. andamanica*, 7- *K. andamanica*, 8- *M. amygdalina*, 9- Unidentified species, 10- *Gymnocranthera canerica*

Figure 2: Agarose gel electrophoresis of digested genomic DNA isolated as per the new protocol from *M. fragrans* (1-3) and *M. fatua* (4-6). M- 1 Kb ladder; Lanes 1, 4: EcoRI, Lanes 2, 5: Hind III, Lanes 3, 6: double digestion.

Figure 3: PCR amplification of 20 ng DNA at varying concentrations of dNTP and Taq DNA polymerase in *M. fragrans*. Lanes 1-3: dNTP 0.15 mM with 0.5, 1.0. 1.5 U of Taq, Lanes 4-6: dNTP 0.2 mM with 0.5, 1.0. 1.5 U of Taq, Lanes 7-9: dNTP 0.3 mM with 0.5, 1.0. 1.5 U of Taq, Lanes 10-12: dNTP 0.4 mM with 0.5, 1.0. 1.5 U of Taq.

Figure 4: Representative amplification pattern of DNA from ten species of *Myristica* a: RAPD profile obtained with OPB 20 primer. b: ISSR profile obtained using ISSR 11 primer. M- 1 Kb ladder, 1- *M. fragrans*, 2- *M. malabarica*, 3- *M. beddomeii*, 4- *M. prainii*, 5- *M. fatua*, 6- *M. andamanica*, 7- *K. andamanica*, 8- *M. amygdalina*, 9- Unidentified species, 10- *Gymnocranthera canerica*

obtaining consistency in banding profiles and for better comparison of experiments, the same Taq DNA polymerase should be used. Sure Taq (Biogene, USA) though cheap, gave good results in case of *Myristica*.

It is observed that 0.3 mM dNTP in presence of Taq DNA polymerase at 0.5 U gave clear and specific bands (Figure 3). dNTP concentrations below 0.15 mM gave no amplification at all, while those above 0.4 mM showed fused banding pattern. However lower concentrations are known to give higher fidelity and specificity (Williams, 1989).

Between the two different thermal cyclers used viz. Gene Amp PCR systems 9700 and MJ Research PTC 200 slight variation in profiles was discovered. When random primers were used in pairs, some of the bands prominent in single primer amplifications were missing and some new bands were generated. A few fragments were identical to that in case of single primers. Primer pairs when used in combination led to reduced polymorphism. Fragments with annealing sites for 2 different primers at the ends do not form hairpin structures and these primers will not be out competed by internal hairpin formation (Caetano *et al*., 1992) to be occurring in fragments having identical primers at both ends. As suggested by Weising *et al*. (1995) it is advisable to use more single primers than using primer pairs.

The DNA was tested for amplification using random primers and ISSR primers. In case of the wild species, purification using RNase and phenol- chloroform extraction (twice) was inevitable for successful amplification by PCR, while in the accessions of Myristica fragrans it was not essential. The DNA isolated as per the above protocol was successfully amplified using 15 RAPD and seven ISSR primers as represented in Figures 4 a, b.

The procedure described here is simple, efficient, cheap and works well for isolating high quality DNA from elite accessions and wild species of *Myristica*. This is the first report of isolation of genomic DNA and amplification by ISSR and RAPD marker from wild and related genera of *Myristica*.

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