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Isolation and amplification of genomic DNA from nutmeg mace

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A reliable and efficient protocol for isolation and amplification of genomic DNA from dried mace of *Myristica fragrans*, was developed. The yield of genomic DNA was 231.4 µg g^{-1} and 306.8 µg g^{-1}, respectively for the samples procured from the farm and market. The absorbance ratio at A260/A280 was greater than 1.8 indicating the good quality of DNA. Complete restriction digestion and PCR amplification of genomic DNA further confirmed the quality of isolated DNA.

Keywords: Nutmeg, *Myristica fragrans*, recalcitrant, restriction digestion, RAPD, *rbcL*, ITS

Introduction

Myristica fragrans Houtt. (Family Myristicaceae) is an aromatic tree spice distributed from India and South East Asia to North Australia and the Pacific Islands, producing two important spices namely nutmeg – the kernel of the seed and mace, the aril of the seed. They are widely used as flavorants in baked goods, confectionaries, puddings, meats, sausages and beverages[1]. Both nutmeg and mace are stimulants, carminative, astringent and aphrodisiac and are used in pharmaceutical preparations for dysentery, stomach ache, vomiting, malaria, rheumatism and early stages of leprosy. The economic importance coupled with its high cost has created a situation favourable for adulteration of the commodity. *M. fragrans* mace is found to be frequently adulterated with mace of *M. argentea* and *M. malabarica*[2]. *M. argentea* has a very pungent aroma while the latter lacks fragrance. Even though morphological identification of these nutmeg maces is possible while fresh and whole to some extent, it is difficult to differentiate the mace when it is dried or powdered. In such a scenario molecular markers come handy in discriminating the adulterated, inferior quality samples from the true specimen. However, for an ideal DNA marker system, an efficient, high quality DNA isolation protocol is a prerequisite. Being a recalcitrant tissue, nutmeg mace poses problems such as co-isolation of highly viscous polysaccharides, polyphenolics, secondary metabolites and degradation by endonucleases[3-5]. Though there are different protocols for isolating DNA from various tissues of nutmeg such as those of leaf[6], traded nutmeg seed[7] etc, there is no protocol for isolating genomic DNA from mace of nutmeg. Any new method to isolate DNA from a recalcitrant tissue can be arrived at only after trial and error studies. Here we describe a method for isolation of genomic DNA from nutmeg mace. This protocol was perfected after trying few of the existing protocols and suitably modifying the protocol developed for dried berries of black pepper[8].

Dried nutmeg mace collected from the Experimental Farm of ICAR – Indian Institute of Spices Research (IISR), Peruvannamuzhi, Kozhikode, Kerala (farm sample) as well as from the local market (market sample) were used for extracting DNA, separately. Initially we tried to isolate the DNA using the already reported protocols of nutmeg leaf[6], traded nutmeg seed[7], recalcitrant dried berries of black pepper[8], okra[9], and *Hibiscus*[10]. As none of these protocols were successful, we modified the protocol of DNA isolation from dried berries of black pepper[8] and used. Stepwise procedures with modifications made are as given below: (modifications done are given in italics).

1 The nutmeg mace samples were powdered and soaked in distilled water overnight. The water was poured off and the mace sample was sun dried. Two gram of the sample was homogenised using 15 ml of preheated extraction buffer (100 mM Tris (pH 8), 20 mM EDTA (pH 8),

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2 M sodium chloride, 2% SDS, 2% PVP, 1% β-mercaptoethanol) in a pre-chilled mortar and pestle and transferred to oakridge tubes.

2 The tubes were incubated at 65ºC for 2 hours with intermittent shaking.

3 Plunged the tubes in ice and brought to room temperature.

4 Added one third volume of 6 M potassium acetate solution and incubated in ice for 1 hour.

5 Equal volume of chloroform:isoamylalcohol (24:1) was added and centrifuged the tubes at 10000 g for 15 minutes at 4ºC.

6 Chloroform : isoamylalcohol extraction was repeated once more. Aqueous phase was transferred to fresh tubes, an equal volume of 30% polyethylene glycol 8000 added and tubes were incubated in ice for 1 hour.

7 Centrifuged the tubes at 12,000 g for 20 minutes at 4ºC. Washed the pellet using 70% ethanol. Dried and dissolved the pellet in sterile distilled water or TE buffer.

The concentration of DNA was determined using the absorbance values at 280 nm using a Biophotometer (Eppendorf, Germany). The purity of the DNA was determined using the absorbance ratios at 260 nm and 280 nm. The quality of the DNA was also checked by running the samples on a 0.8% agarose gel containing 0.5 µg ml^{-1} ethidium bromide at 100 V for 1 hour. Gel was documented using Syngene Gel Documentation System. The quality of the DNA was further checked by performing single restriction analysis using 6 units of *Eco*R V, *Hae* III and *Hind* III (Genei, Bangalore), separately. Digestion reactions were carried out at 37°C for 4 hours and the digested products were resolved on a 1% agarose gel.

The DNA quality was confirmed by random amplified polymorphic DNA technique using decamer primers OPA10 (5'- GTGATCGCAG - 3'), OPA07 (5'-GAAACGGGTG-3'), OPD11 (5'-AGCGCCATTG-3') and OPJ18 (5'-TGGTCGCAGA-3') synthesized by IDT technologies (USA). PCR was performed in 25 µl reaction mixture containing 1 mM *Taq* buffer, 2 mM $MgCl_2$, 0.3 mM dNTP, 2 mM primer, 0.2 U *Taq* DNA polymerase, 20 ng of genomic DNA in a thermal cycler (Eppendorf, Germany). The reaction conditions were a pre-denaturation step at 93°C for 3 minutes, 40 repeated cycles of denaturation at 93°C for 1 minute, annealing at 45°C for 1 minute, extension at 72°C for 1 minute. A final extension was given at 72°C for 10 minutes. Amplified products were loaded in a 1.8% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide and documented by a Syngene Gel Documentation System. DNA quality was further confirmed by amplification of two barcoding loci viz *rbcL* and ITS using universal primers synthesised from IDT technologies (USA) (Table 1). The reaction was performed in a 10 µl reaction volume consisting of 1 mM *Taq* buffer containing 1.5 mM $MgCl_2$ (Genei,, Bangalore), 1 mM forward and reverse primer, 1 U *Taq* polymerase (Genei, Bangalore) and 10-20 ng genomic DNA in a thermal cycler (Eppendorf, Germany). The temperature profile for the two loci is given in Table 2. The amplicons were resolved on a 1% agarose gel and documented using Syngene gel documentation system.

DNA isolation is the preliminary step in the molecular characterisation of any individual[11]. Extraction of DNA from recalcitrant tissue like mace is difficult due to the high amount of polysaccharides, polyphenols, secondary metabolites and pigments contained in it. Polysaccharides and polyphenols interfere with enzyme such as restriction enzymes in Southern blotting and *Taq* polymerase in polymerase chain reaction[12]. Continuous trial and error methods are required to isolate DNA from such recalcitrant tissues[13].

Though attempted to isolate the mace DNA using protocols of nutmeg leaves, traded nutmeg seeds, okra, *Hibiscus*, recalcitrant black pepper berries, they were not successful (Fig. 1a-e). The modification

Table 1 — List of primers used

Primer Name	Primer sequence
rbcL a-f	5' ATG TCACCA CAA ACA GAG ACTAAAGC 3'
rbcL a-r	5' GTA AAA TCA AGT CCA CCG CG 3'
ITS-2	5'-GCTGCGTTCTTCATCGATGC-3'
ITS-3	5'-GCATCGATGAAGAACGCAGC-3'

Table 2 — PCR reaction conditions for the barcoding loci

Loci	PCR reaction condition					
	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Total number of cycles
rbcL	95ºC- 4 min	94ºC - 30 sec	52.5ºC - 1 min	72ºC - 1 min	72ºC - 10 min	35
ITS	94ºC - 5 min	94ºC - 30 sec	56ºC - 45 sec	72ºC - 45 sec	72ºC - 10 min	40

in the black pepper protocol done such as overnight soaking of mace in water, replacing CTAB with 2% SDS, incorporating 2% PVP and 1% β-mercaptoethanol in extraction buffer and increasing the concentration of potassium acetate to 6 M, yielded good quality DNA. In the present study, overnight soaking of the mace samples softened the tissue and removed the skin pigments and phenolic substances contained in it. Water soaking is reported to be helpful in DNA isolation from recalcitrant materials[14-16]. Though CTAB is widely used in as an extraction buffer for DNA isolation and the same was used by nutmeg leaf and traded nutmeg seed DNA isolation as well, we found SDS a better option for cell lysis and releasing DNA. CTAB extraction buffer could not guarantee the elimination of some polysaccharides and lipids[17]. Increasing sodium chloride concentration to 2 M from 1.5 M helped to remove the polysaccharides efficiently from the DNA preparation[18]. Incorporation of PVP and β-mercaptoethanol helped in the efficient removal of polyphenols and secondary metabolites. PVP binds to phenolics forming complex hydrogen bonds and co-precipitates with cell debris upon lysis during chloroform-isoamylalcohol extraction[13,19]. Potassium acetate contributed to the efficient removal of secondary metabolites and polysaccharides thereby increasing the quality of DNA[7-8]. The superiority of polyethylene glycol (PEG) over isopropanol / ethanol precipitation in yielding DNA preparation free from contaminants is already reported[8]. The ability of PEG to remove coloured pigments from the DNA preparation has also been stressed[20]. The yield of DNA obtained from the farm sample and market sample was 231.4 µg g^{-1} and 306.8 µg g^{-1}, respectively. The absorbance ratio (A260/A280) of 1.83 and 1.79 indicated the purity of the DNA of the farm sample and market sample, respectively. The quality of DNA was also checked on 0.8% agarose gel electrophoresis (Fig. 1f). Complete digestion of DNA obtained by single restriction analysis using *Eco*R V, *Hae* III and *Hind* III also confirmed the DNA purity (Fig. 2). Amplification of DNA by RAPD primers produced distinct scorable banding pattern (Fig. 3a & 3b) while *rbcL* locus produced an amplicon of 600 bp in both samples (Fig. 4). The nuclear region ITS gave a band size of 500 bp for *M. fragrans* and market sample (Fig. 5). Amplification of the isolated DNA using RAPD primers, the chloroplast coding *rbcL* locus and the nuclear ITS region provides further evidence for the applicability of this DNA in molecular analysis like genetic diversity studies and DNA barcoding.

This protocol is simple, short, efficient and cost effective and can be employed to develop molecular marker based identification techniques to discriminate

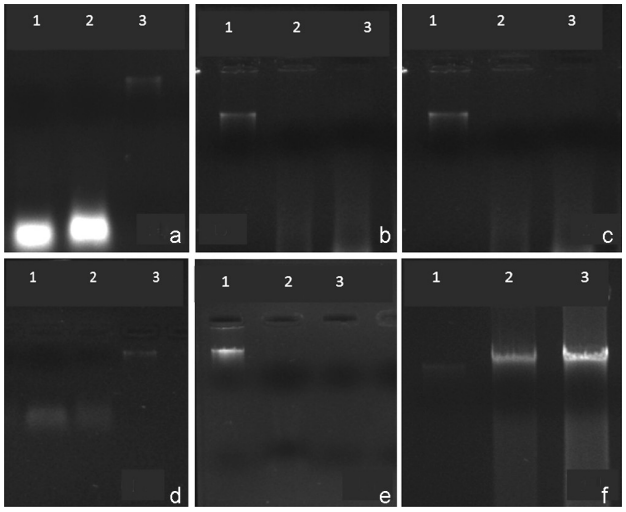

Fig. 1 — (a) Isolation of nutmeg mace DNA using protocol of Sheeja *et al* (2008); Lane 1- Farm sample, lane 2- Market sample, lane 3- Human genomic DNA (blood cells) (Genei, Bangalore), (b) Isolation of nutmeg mace DNA using protocol of Lele *et al* (2011); Lane 1- Farm sample, lane 2- Market sample, lane 3- Human genomic DNA (blood cells) (Genei, Bangalore), (c) Isolation of nutmeg mace DNA using protocol of Singh and Kumar (2012); Lane 1- Human genomic DNA (blood cells) (Genei, Bangalore), lane 2- Farm sample, lane 3- Market sample, (d) Isolation of nutmeg mace DNA using protocol of Reddy (2009); Lane 1- Human genomic DNA (blood cells) (Genei, Bangalore), lane 2- Farm sample, lane 3- Market sample, (e) Isolation of nutmeg mace DNA using protocol of Dhanya *et al* (2007); Lane 1- Farm sample, lane 2- Market sample, lane 3- Human genomic DNA (blood cells) (Genei, Bangalore), and (f) Isolation of mace DNA using modified protocol of Dhanya *et al* (2007); Lane 1- Human genomic DNA (blood cells) (Genei, Bangalore), lane 2- Farm sample, lane 3- Market sample.

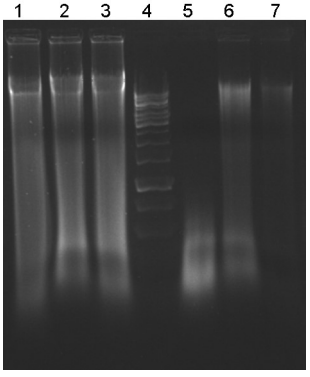

Fig. 2 — Restriction digestion of nutmeg mace genomic DNA with *Hae* III / *Eco*RV / *Hind* III ,respectively. Lane 1 to 3- Market sample, lane 4- 1 Kb ladder (Fermentas), lane 5 to 7- Farm sample.

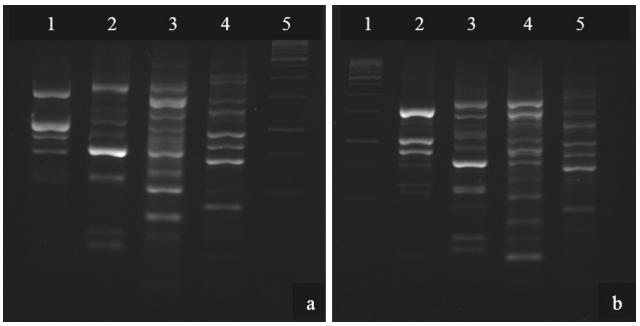

Fig. 3 — (a) RAPD profile of mace DNA isolated from the farm sample. Lane 1- Primer OPA 07, lane 2- Primer OPA 10, lane 3- Primer OPD 11, lane 4- Primer OPJ 18, lane 5- 1 Kb ladder (Fermentas) and (b) RAPD profile of mace DNA isolated from the market sample. Lane 1- 1 Kb ladder (Fermentas), lane 2- Primer OPA 07, lane 3- Primer OPA 10, lane 4- Primer OPD 11, lane 5- Primer OPJ 18.

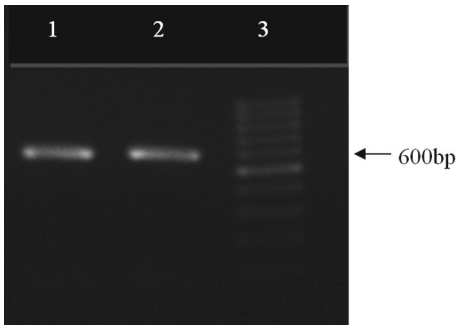

Fig. 4 — Amplification of *rbcL* locus. Lane 1- Farm sample, lane 2- market sample, lane 3- 100 bp ladder (Fermentas).

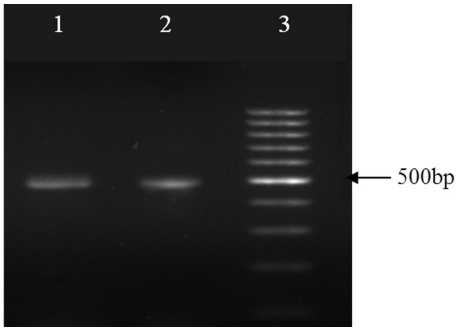

Fig. 5 — Amplification of ITS locus. Lane 1- Farm sample, lane 2- market sample, lane 3- 100 bp ladder (Fermentas).

traded true nutmeg mace from the adulterant species and also in molecular taxonomic studies of the genus.

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