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Isolation and PCR Amplification of Genomic DNA From Traded Seeds Of Nutmeg (*M. Fragrans*)

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Abstract

An efficient protocol for the isolation of high molecular weight DNA from powder of traded nutmeg seeds (*Myristica fragrans*) is described which will aid in protecting the intellectual property rights of those who trade nutmeg in the world market. The method involves a modified 3 % CTAB (hexadecyltrimethylammonium bromide) procedure with 2M NaCl, 0.3 per cent β -mercaptoethanol and 1.5% polyvinylpyrrolidone. The yield of the DNA obtained from the samples varied from 4 to 6 ug/g tissue. The isolated DNA was subjected to Polymerase Chain Reaction (PCR) amplification using a random decamer primer 5'GGACCCTTAC3' and showed distinct and reproducible band patterns. The protocol has trade implications as it will help in the PCR characterization of traded nutmeg seeds and powders from different regions and thus help in tracing the geographical origin of the commodity.

Keywords: Nutmeg, *Myristica fragrans*, RAPD, PCR, DNA, spice, powder, recalcitrant

1. Introduction

Nutmeg (*Myristica fragrans*), belonging to a family Myristicaceae, is an important spice. World production of nutmeg is about 12000 tons per year and the annual world demand is 9000 tons. The East Indian and the West Indian nutmeg dominates International trade in nutmeg, with a negligible quantity of wild 'Bombay' nutmeg (*Myristica malabarica*) imported by USA. Other producing countries include India, Malaysia, Papua New Guinea, Sri Lanka and a few Caribbean Islands. USA is the biggest individual market for whole nutmegs. Nutmeg, mace, their oleoresins and essential oils are used in the food, beverage, and pharmaceutical industries. Although whole nutmeg is available, powdered nutmeg is more popular (Krishnamoorthy and Rema, 2001).

A geographical indication (GI) is a sign used on goods which possess a specific geographical origin and have qualities or a reputation that are due to that place of origin. GIs are finding increasing use worldwide which reflects that economic stakes involved in the commercial use of geographical names are high. GIs have a link to a territory and the biodiversity components are usually the resources that sustain them (Larson, 2007). Development of DNA based molecular markers will help to protect the traded commodities through GI by curtailing unfair trade practices. The first and foremost step in molecular characterization of the biological commodity is isolation of good quality DNA from the commodity of concern.

Conventional methods used for authenticity testing pose certain limitations especially when the products are biological in nature. Structural evaluation for example, requires expertise in analyzing the macroscopic and microscopic features of plant parts, especially those that are ground to very fine powders or degraded due to poor storage or processing. Many closely related materials are not easily distinguished at chemical level. Most of the chemical standards are too expensive and rare or no marker compound has been identified for that particular food (Dhanya and Sasikumar, 2010).

Since DNA is present in the majority of the cells of an organism, identical information is obtained from any appropriate sample from the same source, regardless of the tissue of origin. Additionally, through the

acquisition of sequence data, DNA can potentially provide more information than even protein, due to the degeneracy of the genetic code and the presence of many non-coding regions. (Lockley and Bardsley, 2000).

A protocol to isolate and amplify DNA from traded powder of nutmeg is needed for two reasons *viz.* tracing the geographical origin of the traded nutmeg powders using DNA fingerprinting techniques, to determine quality or authenticity of marketed nutmeg powders using molecular profiling techniques. In addition, preserving the leaf material for a long time is difficult and the leaf itself is not available during the off-seasons. In this paper, we describe the optimized PCR amplifiable genomic DNA isolation protocol from seed powder of traded nutmeg (*M. fragrans*).

2. Materials and methods

2.1 DNA isolation

Export grade seeds of *M. fragrans* were used in the study. Three locally available market samples of nutmeg were also taken for DNA isolation. The seeds were ground to a fine powder and used for DNA extraction as per the protocol given below:

1. The Extraction buffer (3%CTAB, 150mM Tris, 30mM EDTA, 2M NaCl) was preheated at 60°C and 0.3% β-mercaptoethanol and 1.5% PVP was added freshly to the buffer.
2. 1 gm of seed powder was taken and 5 ml of the extraction buffer was added; the samples were mixed.
3. The tubes were incubated at 65 °C for 90 min with intermittent gentle inversions.
4. The samples were plunged in ice immediately to bring to room temperature. An equal amount of buffer saturated phenol: chloroform: isoamylalcohol (25:24:1) was added.
5. The tubes were mixed, and incubated for 15 min with one or two intermittent inversions.
6. The tubes were centrifuged at 12000 rpm for 15 min at 25 °C.
7. The aqueous phase was carefully transferred to fresh tubes, and equal amount of chloroform: isoamylalcohol (24:1) was added.
8. The tubes were mixed, and incubated for 20 min with two or three intermittent inversions.
9. The tubes were centrifuged at 12000 rpm for 15 min at 25 °C.
10. The aqueous phase was carefully transferred to fresh tubes, 2/3rd volume of ice cold isopropanol was added.
11. The tubes were incubated overnight at -20 °C.
12. The tubes were centrifuged at 12000 rpm for 15 min at 25 °C.
13. The supernatant was discarded, and the pellet was dissolved in nuclease free water.
14. The aqueous solution was transferred to 2 ml eppendorf tubes, and RNase 10 ug/ml was added to the solution and the samples were incubated for 1 hour at 37 °C.
15. An equal volume of buffer saturated phenol: chloroform: isoamylalcohol (25:24:1) was added and was mixed, the tubes were incubated for 10 min at room temperature.
16. An equal volume of chloroform: isoamylalcohol was added and was mixed by inverting tubes several times; the tubes were incubated for 10 min at room temperature.
17. The aqueous phase was transferred in a fresh tube, 2/3rd volume of ice cold isopropanol was added and the tubes were incubated for 2 hours at -20 °C.
18. The tubes were centrifuged at 12000 rpm for 20 min at 25 °C.
19. The supernatant was discarded, 70 % ethanol was added for washing.
20. The DNA pellet was air-dried and dissolved in nuclease free water.

1.1 Optimisation of PCR parameters and RAPD analysis

PCR amplification of the isolated DNA was done using random decamer primer obtained from BioServe Biotechnologies, Ltd., India. The RAPD reaction performed in a 25 ul reaction volume with the amounts of 50 ng genomic DNA, 0.4 mM dNTPs, 10 picomols primer, 2.5 mM MgCl₂ and 0.5U Kapa 2G robust Taq DNA polymerase (Saf Labs Pvt. Ltd. Mumbai, India) gave best results. BioRad MyCycler™ Thermocycler (96 wells, serial no: 580BR 10084) was used. The thermal profile used was initial denaturation at 96°C for 3

min, 35 cycles of denaturation at 96 °C for 1 min, annealing at 45 °C for 1 min, extension at 72 °C for 1 min, and a final extension of 72 °C for 1 min. The amplified products were separated on a 2 % agarose gel, running at 60 volts for 4 hours visualized by staining the gel in 0.5 ug/ml ethidium bromide, and documented with gel documentation system (3UV™ Benchtop Transilluminator,UVP).

3. Results and discussion

The problem with the recalcitrant tissues is the difficulty in obtaining contamination free, high-quality genomic DNA, without which further analyses or manipulations are not feasible. The purer the DNA, the more reliable the result becomes because precise, reliable and reproducible results are often not obtained using contaminated nucleic acid (Varma *et al.*, 2007). The methods for DNA extraction need to be adjusted to each plant species and even for each tissue due to the plethora of primary and secondary metabolites in plants (Dhanya and Sasikumar, 2010). Thus the preferred method of DNA extraction for a given tissue can only be determined through trial and error methods (Remya *et al.*, 2003; Syamkumar *et al.*, 2004). Four different DNA isolation methods were tried to isolate high-quality DNA suitable for PCR amplification from traded nutmeg seed powders (Doyle and Doyle, 1987; Syamkumar *et al.*, 2003; Syamkumar *et al.*, 2005; Sheeja *et al.*, 2008). Most of them failed to yield DNA or yielded DNA that was completely sheared, dark brown in colour and was not amplifiable (Fig. 1a). The method proposed by Sheeja *et al.*, 2008 needed to be optimized to give better yield and quality of genomic DNA from nutmeg seeds. The optimum solid-liquid ratio was found to be 1 gm powder per 5 ml extraction buffer. Increase in amount of tissue above 1 gm led to the formation of clumps as well as adherence of tissue to the tube wall, resulting into incomplete or no extraction. The incubation time duration is determined by the choice of tissue as generally leaf cells are easier to crack than seed cells, and it is easier for DNA to release from leaf cells than from seeds. Thus increasing the incubation time to 90 min from 60 min for nutmeg seeds resulted in higher yield and quality of genomic DNA. RNase concentration of 10 ug/ml was adequate to purge the RNA. The nutmeg seeds being the storage tissues contain a high amount of polysaccharides, polyphenols and secondary metabolites. Polyphenols and polysaccharides bind to nucleic acids during DNA isolation and interfere with subsequent reactions (Prittila *et al.*, 2001). It is reported that increase in CTAB and NaCl concentrations increase the yield of cellular DNA. CTAB is generally used as a detergent to separate out polysaccharides. Similarly NaCl concentration greater than 1.5 M removes the polysaccharides (Syamkumar *et al.*, 2005). In addition, increase in the concentration of PVP, β -mercaptoethanol are also useful for increasing the yield and quality of DNA (Prittila *et al.*, 2001). Use of potassium acetate removes most of secondary metabolites and polysaccharides from the DNA resulting in better yield of high-molecular weight DNA (Dhanya *et al.*, 2007). But this didn't work for nutmeg powder. Also the use of PEG instead of isopropanol for precipitation did not yield DNA. The spectrophotometric measurements at 260 and 280 nm of the DNA obtained from the modified protocol gave an absorbance ratio (A260/A280) of 1.6-1.7 indicating pure DNA. The yield ranged from 4–6 ug/g of dried tissue. The quality of DNA was also checked by agarose gel (2%) electrophoresis. A sharp conspicuous band of high molecular weight DNA was observed (Fig. 1b). The DNA isolated by this modified protocol was consistently amplified with random decamer primers used for PCR amplification.

The absorbance ratio of the isolated DNA at 260/280 (1.6–1.7) and consistent amplification pattern in PCR using RAPD markers revealed that the genomic DNA isolated using the modified protocol from the traded nutmeg powder is of good quality (Fig. 3a). The protocol may also be applied to other dry plant tissues rich in polysaccharides and polyphenolic compounds.

4. Acknowledgements

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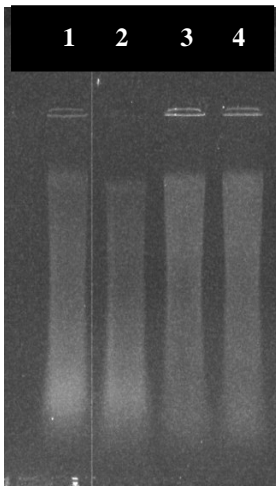


Fig. 1a. DNA isolated from traded nutmeg seed and marketed powders using Doyle and Doyle protocol. Lane 1: powdered *Myristica fragrans* traded seed, Lanes 2, 3, 4: Market samples of traded *M. fragrans* seed powder

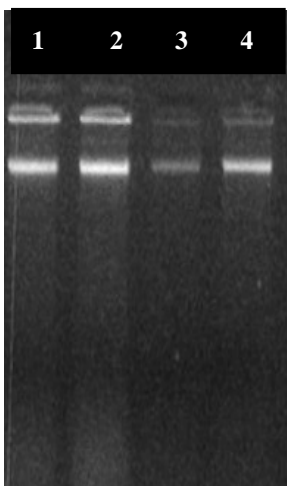


Fig. 1b. DNA isolated from traded nutmeg seed and marketed powders using modified CTAB method. Lane 1: powdered *Myristica fragrans* traded seed, Lanes 2, 3, 4: Market samples of traded *M. fragrans* seed powder

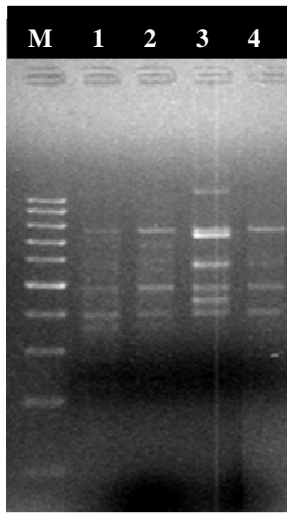


Fig. 3a: RAPD profile of DNA isolated from powdered seed of *M. fragrans* and traded market samples with: primer (5'GGACCCTTAC3') M: Marker (1 kb DNA ladder); Lane 1: *M. fragrans* seed powder; Lane 2, 3, 4: Market samples of traded *M. fragrans* seed powder

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