TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 151-155
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doi:10.3906/biy-1308-5

Isolation and amplification of genomic DNA from barks of *Cinnamomum* spp.

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Received: 02.08.2013 • **Accepted:** 08.10.2013 • **Published Online:** 02.01.2014 • **Printed:** 24.01.2014

Abstract: *Cinnamomum verum* Presl (syn. *C. zeylanicum* Blume), the cinnamon of commerce, is an important aromatic tree spice having wide applications in perfumery, flavoring, beverages, and medicine. Adulteration of cinnamon with the cheaper and inferior barks of *C. aromaticum* and *C. malabatrum* is a problem. Morphological distinction of the barks is difficult; in the case of powdered barks, the situation is even worse. DNA-based molecular tools are preferred under these circumstances. Isolation of high quality DNA is a prerequisite for molecular studies, but barks contain polysaccharides, polyphenols, and secondary metabolites that hamper DNA isolation. Since attempts at isolating DNA using existing protocols and commercial DNA isolation kits (Qiagen) have failed, a reliable and efficient protocol for the isolation and amplification of genomic DNA from the dried barks of 3 species of *Cinnamomum* (true cinnamon plus 2 spurious species), very recalcitrant materials, was perfected by trial and error. The yield of genomic DNA ranged from 5 to 8.1 μg g^{-1} of dried bark and the absorbance values at 260 nm and 280 nm gave a ratio higher than 1.8, indicating the good quality of DNA. The isolated DNA was PCR-amplified using 3 RAPD primers, 1 barcoding locus (*rbcL*) primer, and restriction digested (*EcoR* V and *Hind* III). Complete restriction digestion and PCR amplification of the isolated DNA confirmed the good quality of the results and supported the efficacy of this protocol to yield DNA that can be utilized in further molecular analysis.

Key words: Adulteration, bark, *Cinnamomum* sp., DNA, RAPD, *rbcL*

1. Introduction

Spices are widely used as medicine and food flavorants. Although there are about 109 species listed as spices in the International Standards Organization (ISO) list, their uses are specific as each of these commodities have different uses in flavoring foods or in medicine. *Cinnamomum aromaticum*, *C. zeylanincum* syn. *C. verum*, *C. burmanii*, *C. loureirii*, and *C. tamala* are included in the ISO list. While barks of the first 4 species are used as food flavorant, in beverages and in medicine, it is the leaves of *C. tamala* that are economically important for flavoring food items.

The genus *Cinnamomum* belongs to the family Lauraceae and consists of about 250 species of plants that are aromatic and flavoring (Leela, 2008). Their antiinflammatory, antidiabetic, and antioxidant activities have popularized the use of *Cinnamomum* in folk medicine (Lee et al., 2010). *C. verum*, a native of Sri Lanka, is known as true cinnamon. It is cultivated in Sri Lanka and India, and the bark of this species forms the most important traded *Cinnamomum*. The dried bark of *C. verum* is used as a spice in flavoring foods like biscuits, cakes, sweets, and pickles (Abeysinghe et al., 2009). However, of late, adulteration of this commodity with the hard, thick, and less aromatic bark of *C. aromaticum* (*C. cassia*, cassia cinnamon or Chinese cinnamon) has been reported, as this species bears close resemblance with the bark of true cinnamon, making fraudulent practices easy (Thomas and Duethi, 2001). This commodity has a bitter and burning flavor. A high amount of coumarin is also present in *C. aromaticum,* which is known to cause liver and kidney damage in rats, mice, and probably in humans (Lungarini et al., 2008). The dried bark of *C. malabatrum,* another *Cinnamomum* species, common in many tropical countries as wild growth and, on rare occasion, in homestead gardens in India and Sri Lanka, is also passed off as true cinnamon. Identification of true cinnamon from adulterant species based on physical traits is very difficult, and the situation is all the more difficult once the commodity loses its physical form (e.g., powder). Incidentally, powdered bark is more frequently used as food flavorant and in medicine.

Molecular markers are useful in discriminating adulterants from the genuine products in cases where there is high physical resemblance between the entities, or in the instances of change of the physical form. The first step in any DNA-based discrimination technique is the isolation and amplification of DNA.

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DNA isolation protocols for problem species need to be specially developed (Cingilli and Akçin, 2005). Genomic DNA isolation from wood is difficult due to the presence of proteins, polysaccharides, and phenolic compounds of the lignin pathway that act as strong inhibitors of DNA extraction (Finkeldey et al., 2010). The presence of polysaccharides and polyphenols will contaminate the DNA preparation and make it unsuitable for further analysis (Cheng et al., 1997; Karaca et al., 2005). A desired protocol for such specimens can be arrived at only through trial and error. Here we describe a method to isolate genomic DNA from the dried barks of 3 species of *Cinnamomum*.

2. Materials and methods

2.1 DNA isolation

Dried barks of *C. verum*, *C. aromaticum,* and *C. malabatrum* were collected from the Experimental Farm at Peruvannamuzhi, Kozhikode, Kerala. The dried barks were turned into a fine powder using a Cyclotech 1083 sample mill.

The stepwise protocol developed for the isolation of genomic DNA is described below. Though the basic protocol adopted was that of Asif and Cannon (2005), many modifications were required to get good quality amplifiable DNA.

1. One gram of the powdered sample was homogenized using 10 mL of preheated hexadecyl trimethylammonium bromide (CTAB) extraction buffer (100 mM Tris base (Himedia), 20 mM ethylenediaminetetraacetic acid (EDTA) (Himedia), 3 M sodium chloride (Sigma), 5% CTAB (Sigma), 1% polyvinylpyrrolidone (PVP) (soluble) (Sigma), and 0.3% β mercaptoethanol (Himedia).
2. The tubes were incubated at 65 °C in a shaking water bath with constant stirring at 200 × *g* for 2 h.
3. The tubes were cooled to room temperature and an equal volume of chloroform (Merck):isoamylalcohol (Merck) (24:1, v/v) was added.
4. The tubes were centrifuged at 1118 × *g* for 15 min at 4 °C.
5. The aqueous phase was transferred to another tube and one third volume of 3 M sodium acetate (pH 5.2) (Sigma) and two thirds volume of chloroform: isoamylalcohol (24:1, v/v) were added.
6. The tubes were centrifuged at 1118 × *g* for 15 min at 4 °C and the aqueous phase was taken to another tube.
7. Ice cold isopropanol (Merck) was added in equal volumes and kept for incubation at –40 °C overnight.
8. The tubes were centrifuged at 4472 × *g* for 20 min.
9. The pellet was washed with 70% ethanol.
10. The pellet was dried and dissolved in sterile nuclease-free water or TE buffer.

The concentration of DNA was determined using the absorbance values at 260 nm using a Biophotometer (Eppendorf, Germany). The quality of the DNA was estimated by checking the absorbance ratios at 230/260 nm and 260/280 nm. The purity of the DNA was also confirmed by 0.8% agarose gel electrophoresis. The bands were observed and documented using a Syngene gel doc system.

2.2 Restriction digestion

The quality of the DNA was further checked by performing single restriction analysis. Single restriction digestion was done using 6 units of *EcoR* V and *Hind* III (Genei, Bangalore), separately. Reactions were carried out at 37 °C for 4 h and the digested products were resolved on 1% agarose gel.

2.3 RAPD analysis

The DNA quality was confirmed by random amplified polymorphic DNA technique using decamer primers OPA10 (5'-GTGATCGCAG-3'), OPAF05 (5'-CCCGATCAGA-3'), and OPJ18 (5'-TGGTCGCAGA-3') synthesized by IDT technologies. PCR was performed following the protocol of Syamkumar (2008) in 25 µL reaction volume in a thermal cycler (Eppendorf, Germany). Amplified products were loaded in a 1.8% agarose gel containing 0.5 µg mL^{-1} of ethidium bromide and documented by a gel documentation system (Syngene gel doc system).

2.4 Amplification of chloroplast coding *rbcL* region

The chloroplast gene *rbcL* was amplified using universal primers *rbcLa F* 5'-ATGTCACCACAAACAGAGACT-AAAGC-3' and *rbcLa R* 5'-GTAAAATCAAGT CCACCCRCG-3' (Kress and Erickson, 2007) obtained from IDT technologies. 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 of genomic DNA in a thermalcycler (Eppendorf, Germany). The reaction conditions were a predenaturation step at 94 °C for 4 min, 35 repeated cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. A final extension was given at 72 °C for 10 min. The amplified products were resolved on a 1% agarose gel and documented (Syngene Geldoc system).

3. Results

Attempts at isolating DNA from *Cinnamomum* barks using a Qiagen kit (Figure 1) and the protocols of Asif and Cannon (2005) (Figure 2), Syamkumar et al. (2003), Syamkumar et al. (2005), Echevarria-Machado et al.

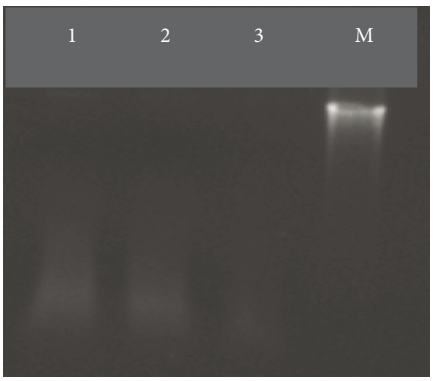

Figure 1. DNA isolated using Qiagen kit. Lane 1: *C. verum;* lane 2: *C. aromaticum;* lane 3: *C. malabatrum;* M: human genomic DNA (from blood cells) marker (Genei, Bangalore).

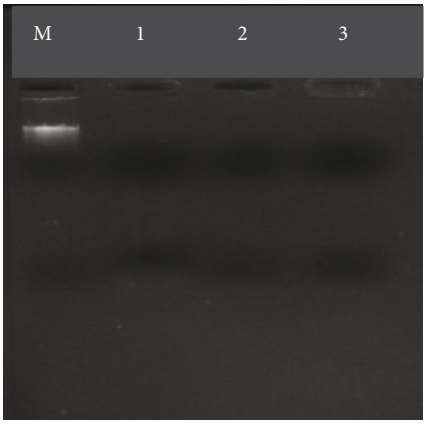

Figure 2. DNA isolated by Asif and Cannon's (2005) protocol. M: Human Genomic DNA marker (blood cells) (Genei, Bangalore); lane 1: *C. verum;* lane 2: *C. aromaticum;* lane 3: *C. malabatrum.*

(2005), Dhanya et al. (2007), and Novaes et al. (2009) were not successful. Hence, we developed a new protocol by modifying the protocol of Asif and Cannon (2005) by increasing the concentration of CTAB to 5%, sodium chloride to 3 M and addition of sodium acetate during chloroform-isoamylalcohol extraction.

The solid to liquid ratio in the present case was 1 g:10 mL extraction buffer. The addition of sodium acetate (pH 5.2) along with chloroform:isoamylalcohol extraction helped in better removal of polysaccharides and proteins. Overnight incubation at –40 °C increased the yield of DNA. The yield of DNA was in the range of 5–8.1 μg g^{-1} of dried bark. The absorbance ratios at 260/280 nm and 230/260 nm were higher than 1.8 and 2, respectively, indicating the good quality of the DNA. The quality of the DNA was also checked on 0.8% agarose gel electrophoresis (Figure 3). Complete digestion of DNA by both the enzymes (*EcoR* V and *Hind* III) confirmed the purity of DNA (Figure 4). RAPD analysis performed as per the method of Williams et al. (1990) modified by Syamkumar (2008), produced a distinct banding pattern (Figures 5a–5c). Amplification of genomic DNA using the universal primers for the *rbcL* locus coding for the large subunit of the RUBISCO enzyme produced a band of 600 bp (Figure 6). This band may be further sequenced to detect SNPs or indels specific to the species. Amplifications of the DNA further confirmed that the DNA is of high quality, free from polysaccharides and other contaminants.

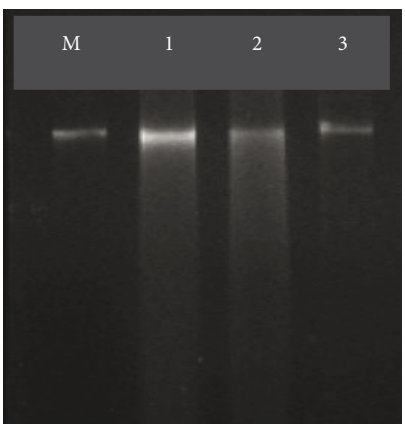

Figure 3. DNA isolated by the modified protocol. M: Human Genomic DNA marker (blood cells) (Genei, Bangalore); lane 1: *C. verum;* lane 2: *C. aromaticum;* lane 3: *C. malabatrum.*

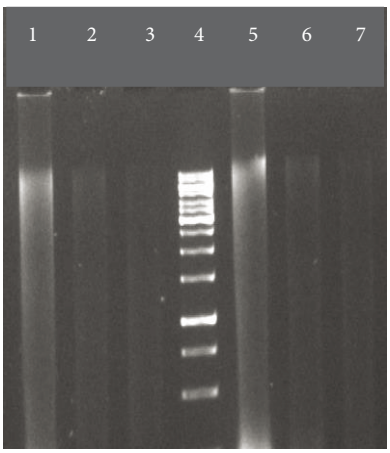

Figure 4. Restriction digestion of genomic DNA isolated from the bark of cinnamon with *Hind* III and *EcoR* V, respectively. Lane 1: *C. verum:* lane 2: *C. aromaticum;* lane 3: *C. malabatrum;* lane 4: 1 kb ladder (Fermentas); lane 5: *C. verum;* lane 6: *C. aromaticum;* lane 7: *C. malabatrum.*

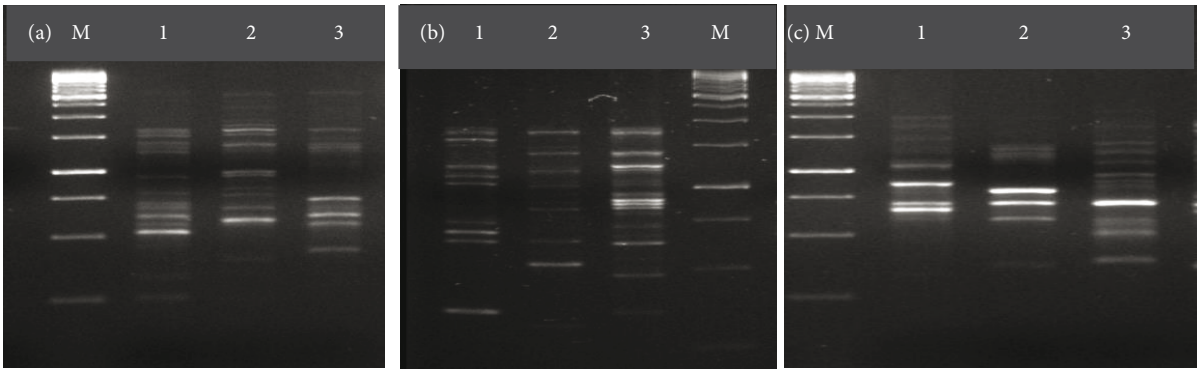

Figure 5. RAPD profile of DNA isolated from barks of *C. verum*, *C. aromaticum,* and *C. malabatrum* with: (a) primer OPAF-05, (b) primer OPJ18, and (c) primer OPA-10. Lane 1: *C. verum;* lane 2: *C. aromaticum;* lane 3: *C. malabatrum;* M: marker (1 kb ladder) (Fermentas).

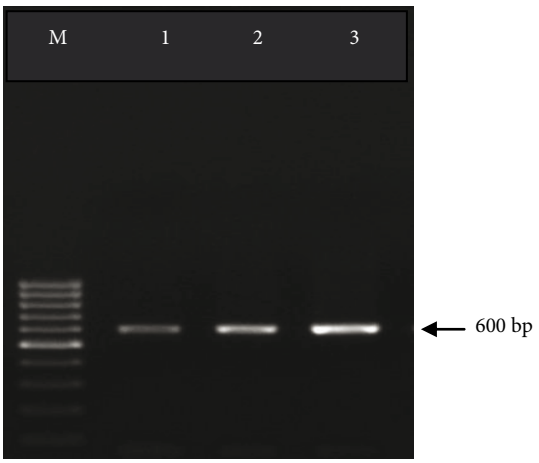

Figure 6. Amplification of the *rbcL* locus. M: Marker (100 bp ladder) (Fermentas); lane 1: *C. verum;* lane 2: *C. aromaticum;* lane 3: *C. malabatrum.*

4. Discussion

Bark/wood is rich in sap, polysaccharides, proteins, lignins, tannins, secondary metabolites, and pigments (de Filipis and Magel, 1998). The presence of polysaccharides in DNA will make it viscous and gum-like, causing difficulty in loading (Sablok et al., 2009). Polysaccharide contamination also inhibits *Taq* polymerase activity (Karaca et al., 2005; Singh and Kumar, 2010). The oxidized polyphenols bind to DNA and hinder the downstream processing of DNA, like restriction digestion and PCR amplification (Sahu et al., 2012). A method for isolating DNA from such recalcitrant tissues can be perfected only through trial and error (Dhanya et al., 2007).

The yield of DNA from barks/woody tissue is reported to be low (50 ng μL^{-1}) (Asif and Cannon, 2005). The DNA isolated from the dried wood of *Cunninghamia lanceolata* could not be visualized in agarose gel due to partial degradation of the DNA extract (Rachmayanti et al., 2009; Tang et al., 2011; Jiao et al., 2012). Degradation may be due to the mechanical treatment employed for harvesting the bark or decomposition of wood by microorganisms. Drying of wood/bark will also result in cell death, following which the DNA is fragmented into very small pieces (Finkedeley et al., 2007). Hence successful amplification of DNA fragments by PCR is considered to be more appropriate than the spectrophotometric estimation of quality and quantity of DNA (Finkeldey et al., 2010).

In the present study, a protocol was developed to isolate high quality DNA from barks of 3 *Cinnamomum* spp. by increasing the concentrations of CTAB and sodium chloride. The inclusion of PVP in the extraction buffer is helpful in removal of polyphenols (Karaca et al., 2005), while increasing the concentrations of sodium chloride and CTAB have helped in the efficient removal of polysaccharides. It has been reported that sodium chloride concentrations greater than 0.5 M, along with CTAB, can remove polysaccharides during DNA extraction (Syamkumar et al., 2005, Sahu et al., 2012). The addition of PVP to the extraction buffer can reduce polyphenol contamination, as it covalently binds with these compounds and coprecipitates during chloroform extraction (Ibrahim, 2011). Ethanol precipitation, though reported to be better than isopropanol to increase the yield and quality of DNA (Remya et al., 2004), did not work in the present study.

As of late, DNA barcoding is becoming significant in the phylogenetic study of many plant species (Türktaş et al., 2012). This DNA isolation protocol may be employed to develop molecular marker-based identification techniques useful in molecular taxonomic studies of the genus and also to discriminate true cinnamon from adulterant species. Thus this study will be useful in ensuring food safety/standards and safeguarding public health.

Acknowledgments

We are thankful to the Director of the Indian Institute of Spices Research (IISR) for his support and encouragement in carrying out this work. Financial support provided by the Ministry of Food Processing Industries, Govt. of India, New Delhi, is gratefully acknowledged. The authors also acknowledge Mr PA Mathew, Principal Scientist & Scientist in Charge (Rtd.), IISR Experimental Farm, Peruvannamuzhi, Kozhikode, for the bark samples.

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