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DNA Barcoding for Discriminating the Economically Important *Cinnamomum verum* from Its Adulterants

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Traded forms of spice and spice powders are often subjected to admixing with inferior substances by design or default, affecting public health and national prestige. *Cinnamomum verum* (true cinnamon), a high-value spice, is often adulterated with its inferior species such as *C. cassia* and *C. malabatrum*. The presence and detection of the spurious species in traded barks (whole or powder) of true cinnamon is posing problems. DNA markers are now used to detect such adulteration. Here we report the application of a DNA barcoding method to detect these adulterants in traded market samples of true cinnamon using the barcoding loci *rbcL, matK* and *psbA-trnH*. The PCR success rate, sequencing efficiency, inter and intra specific divergence, and occurrence of single nucleotide polymorphisms (SNPs) were utilized to assess the potential of each barcode loci to authenticate *C. verum* from its related adulterants. The amplification and sequencing success was 100% for *rbcL* and *psbA-trnH* while *matK* failed to amplify in the market samples. The locus of *rbcL* showed higher interspecific divergence while *psbA-trnH* exhibited lower interspecific divergence. SNPs specific to *C. cassia* were detected in *rbcL* locus in seven out of the ten market samples studied thereby confirming the presence of *C. cassia* adulteration in commercial samples of true cinnamon. Out of the three loci, *rbcL* locus proved to be efficient in tracing out adulterants in traded cinnamon. The SNP sites in this locus can be exploited in designing *C. cassia* specific primers, enabling kit development for easy detection of adulterants at the band level itself thereby bypassing the cost of sequencing.

Key Words: adulteration; cinnamon; *rbcL*; *psbA- trnH*; SNP; market samples

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INTRODUCTION

Cinnamomum verum Presl (syn: *C.zeylanicum* Blume), the cinnamon of commerce, is an important tree spice belonging to family Lauraceae and is indigenous to Sri Lanka and India. Dried stem bark of the tree is mainly used as a spice and medicine (Abeysinghe et al., 2009; Jayaprakasha and Rao, 2011). The spice is credited with antipyretic, analgesic, antioxidant, anti-inflammatory and stimulating activities (Vijayan and Thampuran, 2004). However, correct taxonomical identity of the specimen plays a significant role in the biological efficacy of the commodity. Incorrect identity not only leads to reduced or ineffective biological activity but also to toxicological problems (Kool et al., 2012).

C. verum barks are frequently adulterated with a rougher, thicker, cheaper, and less aromatic bark of the morphologically similar *C. cassia* (syn. *C. aromaticum*) having a bitter and burning flavor. *C. cassia* is reported to contain 1% coumarin, a naturally occurring flavoring substance known to cause kidney and liver damage in rodents (Lungarini et al., 2008). Some isolated incidents of hepatotoxicity to humans by coumarin intake were also reported by the World Health Organization (WHO, 1995). According to the European Commission, the tolerable level of coumarin in food stuffs is 2 $mgkg^{-1}$. Since *C. cassia* contains high amount of coumarin, heavy consumption of this spice may exceed the tolerable intake (Blahova and Svabodova, 2012). Dried bark of *C. malabatrum,* common in many tropical countries and also rarely seen in homestead gardens in India and Sri Lanka, is also passed off as true cinnamon; however, its toxicity to humans is not yet reported. Identification of true cinnamon from the adulterants based on the physical appearance is difficult and the situation becomes more complicated once it is processed as a value-added product.

Many new analytical approaches such as NMR spectroscopy, mass spectroscopy, protein based methods (immunological screening), chromatographic techniques (HPLC, TLC), and DNA-based approaches are used to validate food authenticity (Fugel et al., 2005; Lukyx and Ruth, 2008; Galimberti et al., 2013). However, DNA markers are the most advanced traceability tools to track the raw materials used in the food industry (Dhanya and Sasikumar, 2010; Galimberti et al., 2013). RAPD, RFLP, ISSR, SSR, and SCAR markers have been used in detection of adulterants in medicinal plants (Hussain and Bedi, 2012; Li et al., 2007), traded spices such as turmeric (Sasikumar et al., 2004), black pepper (Dhanya et al., 2009), oregano (Marieschi et al., 2009), and chili (Dhanya et al., 2011).

Currently, DNA barcoding is gaining importance for authentication of agri-food commodities. DNA barcoding is a robust technique that uses a short sequence of DNA having invariable nucleotide sequence in all members of the same species but with sufficient divergence to discriminate between the species (Herbert et al., 2003; Shneer et al., 2009). The chloroplast coding regions *rbcL*,

matK, and the noncoding spacer *psbA-trnH* are the ideal loci proposed for the barcoding of plants (Kress and Erickson, 2007; Chase et al., 2007; CBOL plant working group, 2009). This technique has been used in detecting plant based adulterants in medicinal plants, commercial tea packets, olive oils, etc. (Yuan et al., 2011; Stoeckle et al., 2011; Kumar et al., 2011; Vijayan and Tsou, 2010; Srirama et al., 2010). In spices, adulteration detection using DNA barcoding loci viz., *psbA-trnH, matK, rbcL, ITS* and *rpoC1* singly or in combination are reported in Lamiaceae (Guo et al., 2011; Mattia et al., 2010), star anise (Meizi et al., 2012), saffron (Gismondi et al., 2013), and black pepper (Parvathy et al., 2014).

In this study we attempted to detect the presence of *C. cassia* and *C. malabatrum* in traded samples of cinnamon using the barcoding loci *rbcL, matK* and *psbA-trnH*.

MATERIALS AND METHODS

Sample Collection and DNA Isolation

The leaf samples of five accessions each of *C. verum* (37151, 370125 370177, 370179, 370167), *C. cassia* (370417, 370412, 370429, 370401, 370408), and *C. malabatrum* (Collection 1, Collection 2, Collection 3, Collection 4, Collection 5) were obtained from the Experimental Farm of Indian Institute of Spices Research, Peruvanamuzhi, Kozhikode. Ten commercial samples of cinnamon bark were procured from the local market at Kozhikode, Kerala to check the authenticity of the traded product.

Total genomic DNA was isolated in replicates from the leaf samples using Qiagen DNAeasy plant mini kit and from traded bark samples using the protocol of Swetha et al. (2014). The quality of the DNA was estimated by checking the absorbances at the ratio of 260 nm/280 nm.

PCR Amplification and Sequencing

The isolated genomic DNA was amplified using universal primers for *rbcL, matK,* and *psbA-trnH* (Table 1) obtained from IDT Technologies. The amplification was done in a reaction volume of 10 μL containing 1 mM *Taq* buffer with 1.5 mM $MgCl_2$, 1 mM dNTP, 1 pmoleμL^{-1} of forward and reverse primer, 0.4 U *Taq* DNA polymerase (Banglore, Genei, India) and 10-20 ng of genomic DNA in a Vapoprotectant Eppendorf thermocycler. The optimum temperature profiles for the loci are given in Table 2.

The amplicons were resolved in a 1% agarose gel containing 0.5 μgml^{-1} ethidium bromide and documented using Syngene gel doc system. The bands were purified and custom sequenced at Scigenom Pvt Labs Ltd.

Table 1: Primers used for PCR amplification.

Primer name	Sequence	Reference
rbcL a-F	5′ ATG TCA CCA CAA ACA GAG ACT AAA GC3′	Kress and Erickson, 2007
rbcL a-R	5′ GTA AAA TCA AGT CCA CCG CG 3′	
psbA-F	5′ GTT ATG CAT GAA CGT AAT GCT C 3′	Yang et al., 2011
trnH-2	5′ CGC GCA TGG TGG ATT CAC AAT CC 3′	
matK3F	5′ CGT ACA GTA CTT TTG TGT TTA CGA G 3′	Vijayan and Tsou, 2010
matK1R	5′ ACC CAG TCC ATC TGG AAA TCT TGG TTC 3′	

Table 2: PCR conditions for different barcoding loci.

	Loci		
Reaction condition	***rbcL***	***psbA-trnH***	***matK***
Initial denaturation	95°C -4 min	92°C -1 min	94°C -1 min
Denaturation	94°C -30 sec	94°C -1 min	94°C -30 sec
Annealing	55°C -1 min	52°C -1 min	52°C -20 sec
Extension	72°C -1 min	64°C -1 min	72°C -50 sec
Final extension	72°C -10 min	64°C -8 min	72°C -5 min
Total cycles	35	35	35

Sequence Analysis

The contigs were assembled from the forward and reverse sequence reads using DNA Baser (version 3.4) software. Blast analysis was done against the nucleotide database to confirm the sequence originality (Atschul et al., 1997). The generated sequences were aligned using the online tool ClustalW (Larkin et al., 2007) and edited manually by Bioedit (Hall, 1999). Further data analysis was done using Mega 5 (Tamura et al., 2011).

RESULTS

High-quality genomic DNA was isolated from *C. verum, C. cassia, C. malabatrum* and traded market samples. The absorbance values at 260 nm/280 nm gave a ratio of 1.8 indicating good quality DNA. The PCR success rate was 100% for all the analyzed loci except *matK,* which did not show any amplification in the market samples and was hence excluded from the study. Amplification of *rbcL* and *psbA-trnH* yielded 600bp and 500bp sized fragments, respectively (Figs. 1 and 2). BLAST analysis confirmed that the sequences showed similarity to the respective loci of genus *Cinnamomum* (Table 3). The generated sequences for *rbcL* and *psbA-trnH* were deposited in GenBank (KF978091-KF978095, KF979087-KF978100, KF878109-KF878113, KF744226-KF744230).

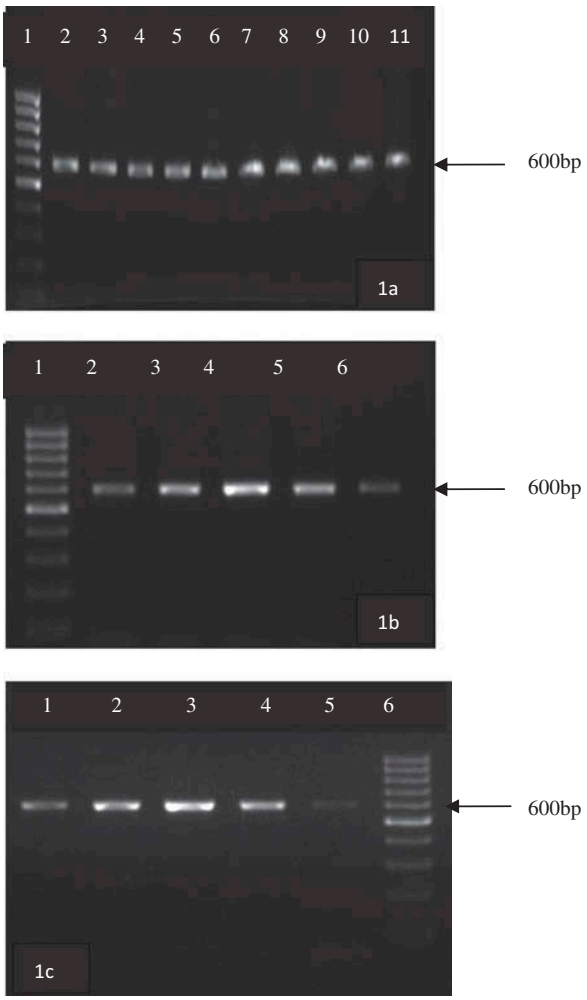

Figure 1. (a) Amplification of *rbcL* locus in *C. verum* and *C. cassia*. (Lane 1- 100 bp ladder (Fermentas), lane 2- *C. verum* 37151, lane 3- *C. verum* 370125, lane 4- *C. verum* 370177, lane 5- *C. verum* 370179, lane 6- *C. verum* 370167, lane 7- *C. cassia* 370417, lane 8- *C. cassia* 370412, lane 9- *C. cassia* 370429, lane 10- *C. cassia* 370401, lane 11- *C. cassia* 370408. (b) Amplification of *rbcL* locus in *C.malabatrum.* Lane 1- 100 bp ladder (Fermentas), lane 2- *C. malabatrum* collection 1, lane 3- *C. malabatrum* collection 2, lane 4- *C. malabatrum* collection 3, lane 5- *C. malabatrum* collection 4, lane 6- *C. malabatrum* collection 5. (c) Amplification of *rbcL* locus in market samples of cinnamon. Lane 1- market sample 1, lane 2- market sample 2, lane 3- market sample 3, lane 4- market sample 4, lane 5 -market sample 5, lane 6- 100 bp ladder (Fermentas).

A favorable barcode should possess higher interspecific distance than the intraspecific distance. The average intra and interspecifc distance for *rbcL* and *psbA-trnH* loci of *C. verum* and their adulterants are given in Table 4. The average intra-specific distance within *C. verum, C. cassia,* and *C. malabatrum* was zero while the average interspecific distance between *C. verum* and its

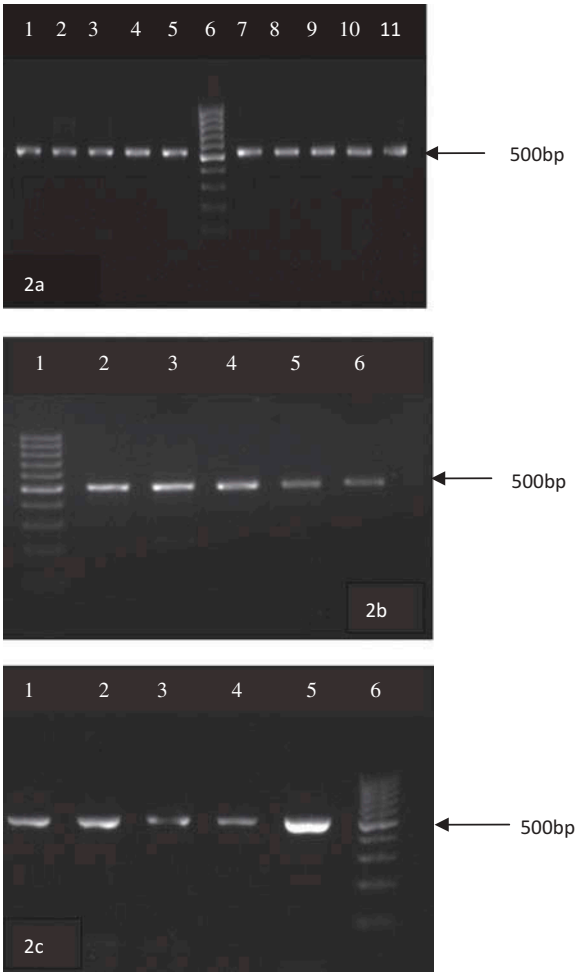

Figure 2. (a) Amplification of *psbA-trnH* locus in *C. verum* and *C. cassia*. (Lane 1- *C.verum* 37151, lane 2- *C.verum* 370125, lane 3- *C. verum* 370177, lane 4- *C. verum* 370179, lane 5- *C.verum* 370167, lane 6- 100 bp ladder (Fermentas), lane 7- *C. cassia* 370417, lane 8- *C. cassia* 370412, lane 9- *C. cassia* 370429, lane 10- *C. cassia* 370401, lane 11- *C. cassia* 370408. (b) Amplification of *psbA-trnH* locus in *C. malabatrum*. (Lane 1- 100bp ladder (Fermentas), lane 2- *C. malabatrum* collection 1, lane 3- *C. malabatrum* collection 2, lane 4- *C. malabatrum* collection 3, lane 5- *C. malabatrum* collection 4, lane 6- *C. malabatrum* collection 5. (c) Amplification of *psbA-trnH* locus in market samples of cinnamon. Lane 1- market sample 1, lane 2- market sample 2, lane 3- market sample 3, lane 4- market sample 4, lane 5 -market sample 5, lane 6- 100 bp ladder (Fermentas).

adulterant species was 0.198 and 0.007, respectively for *C. cassia* and *C. malabatrum* for *rbcL* locus. The *psbA-trnH* locus gave an intra specific distance of 0.269 for *C. verum* and interspecific distance of 0.352 and 0.194 for *C. cassia* and *C. malabatrum*, respectively. It shows that the interspecifc distance of *rbcL* is greater than its intraspecific distance while for *psbA-trnH* the intraspecific distance is greater than the interspecific distance thus excluding the potential of *psbA-trnH* as an ideal barcode.

Table 3: Blast analysis of *rbcL* and *psbA-trnH* of *Cinnamomum* spp.

Species	TOP HIT plant from GenBank (accession no)	Query Coverage %	% Identity	Alignment Length(bp)	Mismatch	Gap	E_Value
rbcL locus							
C. verum	*C. cappara -coronde* (JQ843682)	100	100	570	0	0	0
C. cassia.	*C. camphora* (GU135257)	99	99	561	1	0	0
C. mala-batrum	*C. cappara -coronde* (JQ843682)	100	100	510	0	0	0
psbA-trnH locus							
C. verum	*Cinnamomum* sp.SGS-2011 (JN9884671)	97	99	419	2	0	0
C. cassia	*C. bejolghota* (GQ298266)	99	99	433	4	1	0
C. mala-batrum	*C. aromaticum* (HM019388)	50	96	398	5	2	4e-48

Table 4: The average intraspecific and interspecific distances of *C. verum* and their adulterants.

Distance	Locus	
Intraspecific distance	*rbcL*	*psbA-trnH*
C. verum	0	0.269
Interspecific distance		
C. verum & *C. cassia*	0.198	0.352
C. verum & *C. malabatrum*	0.007	0.194

Sequence analysis of *rbcL* locus showed the presence of three single nucleotide polymorphisms specific to *C. cassia* at positions 54, 55 and 304 (Table 5). In seven out of the ten market samples studied (Market sample 1, Market sample 2, Market sample 6, Market sample 7, Market sample 8, Market sample 9, and Market sample 10) these specific SNPs were found. The SNPs were further verified by checking in more accessions (5) of *C. cassia* thus confirming the adulteration of these market samples with *C. cassia.* The alignment of *psbA-trnH* showed a SNP at position 89 common to both *C. cassia* and *C. malabatrum* which was also present in seven of the market samples. Though the SNP was able to discriminate between *C. verum* and the adulterants we could not attribute it to any of the two adulterant species thereby limiting the scope of this locus.

Table 5: SNP table of *rbcL* locus.

	Position of SNP and Nucleotide substituted		
Species	**54**	**55**	**304**
C. verum	A	G	T
C. cassia	G	A	C
C. malabatrum	A	G	T
Market sample1	G	A	C
Market sample 2	G	A	C
Market sample 6	G	A	C
Market sample 7	G	A	C
Market sample 8	G	A	C
Market sample 9	G	A	C
Market sample 10	G	A	C

DISCUSSION

Traded forms of spices and spice powders are often subjected to admixing or substitution with inferior substances (Franco, 2011). This may be done by design or default, and in either case the consequence will be significant.

As per the FAO statistics 2011, the annual global production of true cinnamon is 198874 tonnes which is far below the global demand. The situation is thus an incentive for fraudulent practices in the trade of true cinnamon. Barks of *C. cassia*, another species of *Cinnamomum*, grown widely in China, Indonesia and Southeast Asia and *C. malabatrum* come handy in this regard for adulterating the true cinnamon and trading the commodity in the guise of *C. verum*. This aspect is reported widely in the print media in India.

Among the three barcoding loci studied, *rbcL* was found ideal for detecting *C. cassia* adulteration in traded true cinnamon based on the parameters such as amplification and sequencing success and higher interspecific divergence than intraspecific divergence. Amplification and sequencing success are essential criteria for an ideal barcode. Similarly a higher interspecific divergence is another important prerequisite (Kress and Erickson, 2007; Hollingsworth et al., 2009; Vijayan and Tsou, 2010; Meizi et al., 2012). In the present study, *matK* did not give consistent amplification. Difficulty in amplification of this locus has been previously reported (Ford et al., 2009; Pettengill and Neel, 2010; Mattia et al., 2010; Wang et al., 2012). *psbA-trnH* was also excluded from the study as it did not yield any informative SNPs to discriminate between the two adulterant species besides having high intraspecific divergence than interspecific divergence, which is not a characteristic of an ideal barcode locus. With the same locus, Mattia et al. (2010) reported a higher intraspecific divergence over interspecific divergence in *Origanum* samples.

Authentication of traded true cinnamon was possible using the barcoding locus *rbcL*. The presence of single nucleotide polymorphisms specific to *C. cassia* in seven of the market samples out of the ten studied has confirmed the significance and extent of *C. cassia* adulteration in commercial samples of true cinnamon. The *rbcL* locus proved efficient in detecting the adulteration using Single Nucleotide Polymorphism (SNP). These SNP sites in the *rbcL* locus can be exploited to design *C. cassia* specific primers enabling kit development for easy detection of adulterants at the band level thereby bypassing the cost of sequencing. The efficiency of *rbcL* locus as an ideal barcode has been already reported by Kress et al. (2007) and CBOL plant working group (2009). *rbcL* in combnination with *matK* was used in the molecular authentication of the ethnomedicinal plant *Sabia parviflora* (Sui et al., 2011). Nair et al. (2013) have also suggested the utility of this locus in the molecular distinction of two *Morinda* (*M. reticulata* and *M. umbellate)* species on the basis of SNPs.

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