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Authentication of *Myristica fragrans* Houtt. using DNA barcoding

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ABSTRACT

Myristica fragrans mace, an economically important traded spice is being adulterated with mace of *M. malabarica*, a closely related species. Identification of the genuine mace from its adulterant is difficult owing to the loss of diagnostic morphological characters on drying and storage. Four DNA barcoding loci viz., *rbcL*, *matK*, *psbA-trnH* and Internal Transcribed Spacer (ITS) are compared to analyse *Myristica malabarica* adulteration in traded *Myristica fragrans* mace samples. The potential of *psbA-trnH* as the best barcode over other loci in authentication of *M. fragrans* mace was established by its amplification and sequencing success, high interspecific variation and presence of polymorphic sites. Sixty polymorphic sites and 9 indel regions in *psbA-trnH* locus specific to *M. malabarica* are found in three out of the five market samples studied, thereby confirming the adulteration of traded *M. fragrans* mace with *M. malabarica*.

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1. Introduction

The nutmeg tree, *Myristica fragrans* Houtt, is indigenous to Moluccas in Indonesia but has been successfully grown in other Asian countries such as India, Malaysia, Papua New Guinea, Sri Lanka, and in the Caribbeans, namely Grenada. A range of commercial products derived from the nutmeg tree of which the spices – nutmeg (kernel) and mace or aril covering the seed - are the most commonly known and widely traded; other products are their essential oils, extracted oleoresins and nutmeg butter. Other economically relevant *Myristica* species include *M. argentea* Warb. which produces 'Papuan' nutmegs from Irian Jaya and Papua New Guinea, and *M. malabarica* Lam. which produces 'Bombay mace' from India; both are used as adulterants of *M. fragrans* products.

Myristica genus is represented by 12 species in India, the important ones are *M. fragrans* Houtt., *M. malabarica* Lam., *M. beddomei* King etc. of which *M. fragrans* is the only cultivated species. Introduced to India in the 18th century by the British, it is now cultivated in parts of Kerala, Tamil Nadu and Karnataka (Sasikumar, George, Saji, Rema, & Krishnamoorthy, 2013) in about 20,120 ha in the country as per the estimate in the year 2015 (http://dasd.gov.in/images/kerala/pdf_files/SPICES__AREA_PRODUCTION_AND_PRODUCITIVTY_IN_INDIA.pdf). Apart from its use as a spice in food industry mainly in seasoning of meat products and in sauces, soups and baked goods, nutmeg is prized for its aromatic, therapeutic and aphrodisiac properties since time immemorial (Agbogidi & Azagbaekwe, 2010). Mace and nutmeg are also used in the perfumery and pharmaceutical industries. Though both seed and mace have similar taste qualities; mace is more popular because of its light orange colour in light coloured foods.

World trade in nutmeg (mace and kernel) along with cardamom was 44,887 MT worth US$ 374,296 during 2015 (http://www.trademap.org/tradestat/Country_SelProduct_TS.aspx). The economical value coupled with its high cost has led to the fraudulent adulteration of *M. fragrans* mace with its closely related species *M. malabarica* by unscrupulous dealers.

Mace of *M. malabarica*, a common wild relative of the nutmeg, also known as Bombay mace, is yellow in colour, lack aroma and is of inferior quality mainly exploited as a natural dye source. Morphological differentiation may be possible between the genuine and adulterant commodity in fresh or whole form but discrimination becomes difficult on sample drying, ageing and powdering as they lose their characteristic morphological and diagnostic features.

Physical and chemical methods are available for detecting adulterations, mainly the synthetic ones, in food commodities. Physical methods include analysis of macroscopic and microscopic structural evaluation and other parameters like solubility and bulk density (Dhanya & Sasikumar, 2010). Chemical methods involve chromatographic techniques like High Performance Liquid

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Table 1
Details of reference samples.

Species	No. of sample	Location of collection.
M. fragrans	3	Kozhikode, Kerala,India.
M. fragrans	1	Nagercoil, Tamil Nadu, India.
M. fragrans	1	Andaman and Nicobar Islands, India.
M. malabarica	1	Community Agro Biodiversity Center, M S Swaminathan Research Foundation, Wyanadu, Kerala,India.
M. malabarica	1	Nagercoil, Tamil Nadu, India.
M. malabarica	1	Ernakulam, Kerala,India.
M. malabarica	1	Kerala Forest Research Institute, Thrissur,Kerala, India.
M. malabarica	1	Kozhikode, Kerala, India.

Chromatography (HPLC), spectroscopic methods like Nuclear Magnetic Resonance Spectroscopy (NMR) and electrophoretic methods like capillary electrophoresis that differentiates the samples based on their variation in chemical profile (Galimberti et al., 2013). But molecular methods are dominant over other approaches due to their accuracy, effectiveness and non-dependence on age, environmental factors, storage and processing conditions, especially for the biological adulterants (Balachandran, Mohanasundaram, & Ramalingam, 2015; Heubl, 2013). Authentication of commodities by DNA barcoding, a recently evolved molecular technique is gaining wide acceptance now over other DNA based methods due to its universality (Galimberti et al., 2013) and reliability (Heubl, 2013).

DNA barcoding employs short nucleotide stretches called barcodes that possess nucleotide variation to discriminate and identify species (Hebert, Cywinska, Ball, & de Waard, 2003). The Consortium of Barcode for Life (CBOL) has proposed *rbcL* coding for large subunit of ribulose 1,5 bisphosphate and *matK* coding for maturase as core barcodes, and *psbA-trnH* spacer and nuclear ITS region as supplementary barcodes in plants. DNA barcoding has been used to detect adulteration in different commodities like medicinal plants (Ganie, Upadhyay, Das, & Sharma, 2015; Newmaster, Grguic, Shanmughanadhan, & Ramalingam, 2013; Srirama et al., 2010), tea (Stoeckle et al., 2011), olive oil (Kumar, Kahlon & Chaudhary, 2011), spices like saffron (Gismondi, Fanali, Labarga, Cailoa, & Canini, 2013), black pepper (Parvathy et al., 2014), turmeric (Parvathy, Swetha, Sheeja, & Sasikumar, 2015), cinnamon (Swetha, Parvathy, Sheeja, & Sasikumar, 2014), star anise (Meizil et al., 2012) and members of family Lamiaceae (de Mattia et al., 2011).

Here we attempted to test the potential of DNA barcoding as an authentication tool for *M. fragrans* mace using the loci *rbcL*, *matK*, *psbA-trnH* and ITS.

2. Materials and methods

2.1. Sample collection

Fresh leaves were collected from five samples of *M. fragrans* and *M. malabarica* from different locations (Table 1) to create a reference database to authenticate the traded nutmeg mace. The reference samples were identified by Dr. Rema J and Dr. Saji K.V., Principal Scientists, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India. Five samples of traded nutmeg mace were collected from different shops in Kozhikode, Kerala, India.

2.2. Genomic DNA isolation

Genomic DNA was isolated from 100 mg of the reference leaf samples using Qiagen DNA easy kit (Qiagen, Germany) and from the traded mace samples using the protocol developed in our lab. 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 grams of the sample was homogenised using 15 ml of preheated extraction buffer (100 mM Tris (Sigma, USA) (pH-8), 20 mM EDTA (Sigma, USA) (pH-8), 2 M sodium chloride (Sigma, USA), 2% SDS (Himedia, Mumbai), 2% PVP (Sigma, USA), 1% β-mercaptoethanol (Himedia, Mumbai) in a prechilled mortar and pestle and transferred to oakridge tubes. The tubes were incubated at 65 °C for 2 h with intermittent shaking. The tubes were brought to room temperature by plunging in ice. One third volume of 6 M potassium acetate (Himedia, Mumbai) solution was added and the tubes were incubated in ice for 1 h. An equal volume of chloroform (Merck, Germany):isoamylalcohol (Merck, Germany) (24:1) was added to the tubes and centrifuged at 10,000 g for 15 min at 4 °C. Chloroform:isoamylalcohol extraction was repeated once more, aqueous phase transferred to fresh tubes and an equal volume of 30% polyethylene glycol 8000 (Sigma, USA) was added and tubes were incubated in ice for 1 h. The tubes were centrifuged at 12,000 g for 20 min at 4 °C. The pellet was washed using 70% ethanol, dried and dissolved in sterile nuclease free water.

2.3. PCR amplification and sequencing

Amplification of genomic DNA was carried out using universal primers of the barcoding loci *matK*, *rbcL*, *psbA-trnH* and Internal Transcribed Spacer (ITS) synthesized by Integrated DNA Technologies (IDT), (USA) (Table 2). The reactions were carried out in 50 µl volume containing 20–50 ng genomic DNA, 1X *Taq* assay buffer with 1.5 mM $MgCl_2$ (Takara, Japan), 1mM dNTP mix (Takara, Japan),

Table 2
Primers used for PCR amplification.

Primer name	Sequence (5′-3′)	Reference
rbcL af	5′ ATG TCA CCA CAA ACA GAG ACT AAA GC3′	Kress & Erickson, 2007
rbcL ar	5′ GTA AAA TCA AGT CCA CCG CG 3′	
matK 3F	5′ CGT ACA GTA CTT TTG TGT TTA CGA G 3′	Vijayan & Tsou, 2010
matK 1R	5′ ACC CAG TCC ATC TGG AAA TCT TGG TTC 3′	
psbA f	5′GTT ATG CAT GAA CGT AAT GCTC 3′	Yang, Zhang, Liu, Zhang, & Ji, 2011
trnH R	3′CGT AAC AAG GTT TCC GTA GGT GAA 5′	
ITS 4	5′ TCC TCC GCT TAT TGA TAT GC 3′	Abeysinghe, Wijhesinghe, Tachida, & Yoshda, 2009.
ITS 5A	3′ CCT TAT CAT TTA GAG GAA GGA G 5′	

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Table 3
PCR conditions for the barcoding loci.

Reaction condition	Locus		
	rbcL	*psbA-trnH*	*ITS*
Initial denaturation	95 °C–4 min	94 °C–3 min	94 °C–5 min
Denaturation	94 °C–30 s	94 °C–1 min	94 °C–1 min
Annealing	52.5 °C–1 min	54.5 °C–1 min	56 °C–1 min
Extension	72 °C–1 min	72 °C–1 min	72 °C–1 min
Final Extension	72 °C–10 min	72 °C–10 min	72 °C–10 min
Number of cycles	35	35	40

Table 4
Average intraspecific and interspecific analysis of *rbcL* and *psbA-trnH*.

Distance	*rbcL*	*psbA-trnH*
All intraspecific distance	0.003 ± 0.002	0.057 ± 0.011
Coalescent depth	0.011 ± 0.004	0.166 ± 0.024
All interspecific distance	0.004 ± 0.002	0.771 ± 0.098
Minimum interspecific distance	0	0.748 ± 0.092

1 pmol μL^{-1} of forward and reverse primers and 1.5 U *Taq* DNA polymerase (Takara, Japan) in the Agilent Sure cycler 8800 thermocycler. Temperature profiles were optimized by putting gradient PCR for *rbcL*, *psbA-trnH* and ITS loci (Table 3). *matK* was amplified following Stoeckele et al. (2010).

The amplified products were purified using QIA quick PCR purification kit (Qiagen, Germany) and bidirectionally sequenced at Scigenom Labs, Cochin, Kerala, India.

2.4. Data analysis

Contigs were assembled from the forward and reverse sequences using Cap 3 software (Huang & Madan, 1999) and they were queried against the nucleotide database of Genbank employing the BLAST algorithm (Atschul et al., 1997) to confirm the species identity. Sequences obtained for coding regions *rbcL* and *matK* were translated using online tool Expasy (Gasteiger et al., 2003) and the identity of the protein sequences were confirmed by Protein Blast. The nucleotide sequences generated were deposited in the Genbank database of NCBI.

Alignment of the obtained sequences were done using MUSCLE algorithm (Edgar, 2004). Sequences were then trimmed using Bioedit (Hall, 1999) and further analysed in Mega 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The genetic distances were calculated using nucleotide model K2P from pairwise global alignment in which gaps and missing data were removed by the pairwise deletion option and the intraspecifc and interspecific divergence were assessed. Wilcoxon two sample test was performed to test the intraspecific and interspecific distance of barcode. A neighbor joining tree was constructed using the Kimura 2 parameter (K2P) with a bootstrap support of 1000 replications to determine the confidence estimate of the tree. During the analysis all the positions containing gaps and missing data were eliminated.

3. Results

3.1. DNA isolation, amplification and sequencing success

High quality genomic DNA was isolated from all samples. Amplification was 100% successful in the tested barcoding loci except for *matK* locus (66.67%). Amplicons of 600bp, 900bp, 750bp and 450bp were obtained for *rbcL, matK*, ITS and *psbA-trnH* loci, respectively (Supplementary file 1).

Sequencing was 100% successful for *rbcL*, *psbA-trnH* and ITS loci but ITS locus generated mixed sequence data in most of the samples thus limiting its potential as a barcode. BLAST analysis of *rbcL, matK* and *psbA-trnH* sequences showed maximum identity to the respective locus of *Myristica* genus. Nucleotide sequences obtained for *rbcL*, *matK* and *psbA-trnH* were submitted to Genbank database of NCBI (KT367808, KT367809, KT380141, KT380142, KT445277, KT445278). *rbcL* and *matK* sequences were translated to protein sequences and they showed 100% identity to ribulose 1,5 bisphosphate and maturase sequences of genus *Myristica*.

The length of sequences obtained were in the range of 803-866bp, 516-585bp and 204-397bp for *matK*, *rbcL* and *psbA-trnH* sequences respectively. *matK* was excluded from further analysis as the locus failed to amplify in the traded mace samples. *rbcL* and *psbA-trnH* sequences of the reference and market samples were aligned and trimmed to a final length of 425bp and 217bp, respectively.

3.2. Analysis of intraspecific and interspecific divergence

An ideal DNA barcode should have a higher interspecific divergence than intraspecific divergence. The intraspecific divergence

Table 5
Polymorphic sites in *psbA-trnH* locus.

Species	2	3	4	6	7	9	11	12	17	19	20	38	41	49	54	56	57	75	77	80
M. fragrans	T	A	C	C	T	T	T	A	T	A	C	T	A	A	C	T	A	T	A	A
M. malabarica	G	C	T	T	A	C	A	C	G	C	T	C	C	G	T	C	G	A	C	T

Table 6
Polymorphic sites in *psbA-trnH* locus.

Species	81	82	83	84	86	89	90	91	108	109	111	112	120	121	124	134	139
M. fragrans	T	T	T	A	A	A	A	A	G	A	C	A	A	C	A	A	C
M. malabarica	A	A	A	C	G	G	T	G	A	C	A	G	C	T	C	T	T

Table 7
Polymorphic sites in *psbA-trnH* locus.

Species	142	147	150	155	157	158	159	160	165	168	169	177	178	180	181	198
M. fragrans	A	G	A	A	A	A	T	C	G	A	G	C	T	G	T	C
M. malabarica	C	T	G	T	T	T	A	A	T	G	T	T	G	T	C	T

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Table 8
Polymorphic sites in *psbA-trnH* locus.

Species	200	203	205	206	207	213	215
M. fragrans	C	C	G	G	G	G	T
M. malabarica	T	T	T	T	T	T	A

Table 11
Indels in *psbA-trnH* locuS.

Species	186	187	188	192	210
M. fragrans	C	A	G	A	–
M. malabarica	–	–	–	–	T

was assessed by two parameters-all intraspecific distance (mean of all intraspecific K2P distances between all samples collected within each species with more than one representative) and maximum intraspecific distance or coalescent depth (maximum intraspecific distance within each species). Two parameters viz., all interspecific distance (mean of all K2P distances between all species in the genus with atleast two species) and minimum interspecific distance (minimum interspecific distance within each genus with at least two species) were calculated to determine the interspecific divergence (Table 4).

Table 4 clearly indicates that the intraspecifc distance and coalescent depth parameters of *rbcL* locus are greater than its interspecific distances. *rbcL* failed as a barcode as the intraspecific distance was greater than interspecific distance, which is contradictory to the ideal barcode criterion.

The interspecific variation of *psbA-trnH* sequences of *M. fragrans* and *M. malabarica* was less than the intrapecific distance parameters (Table 4). The Wilcoxon two sample test also showed that the interspecific distance of *psbA-trnH* was significantly higher than its intraspecific distance ($P < 0.0001$) and the z value obtained was 5.710.Thus this locus meets the requirement of an ideal barcode. The intraspecific variation exhibited by the reference species may be due to the different locations from which they were collected.

3.3. Identification of polymorphic sites and phylogenetic analysis

psbA-trnH exhibited high sequence variation between *M. fragrans* and *M. malabarica* species. Sixty polymorphic sites (Tables 5–8) and 9 indels (positions 25-37, 44-47, 59-61, 101-103, 115-118, 171-174, 186-188, 192 and 210) were identified in the alignment between these two species (Tables 9–11). Out of the five market samples analysed, three showed SNPs and indels similar to *M. malabarica* thus pointing to a possible substitution of *M. fragrans* with *M. malabarica* samples.

A neighbor joining tree constructed based on bootstrap support of 1000 replicates clustered *M. fragrans* and *M. malabarica* into two well separated clades (Fig. 1). Two market samples clustered along with *M. fragrans* proving its authenticity while three of them clustered with *M. malabarica* further confirming the substitution of *M. fragrans* mace with inferior quality *M. malabarica* mace.

4. Discussion

Spice authentication is a matter of primary concern due to globalization in trade. Adulteration of spices may be caused either by default or design with closely related species to achieve economical gain, leading to erosion of the biological property. High value spices are often adulterated with their inferior substitutes (Dhanya & Sasikumar, 2010). *M. fragrans*, used widely as a spice, is often substituted with its counterfeit entity, *M. malabarica*. Even though colour of the fresh mace differ in the two species, red in case of *M. fragrans* and yellow in case of *M. malabarica*, seasoned mace in both the species are of same colour (yellow) and sensory discrimination of the commodities is also difficult. This comes handy for substituting or adulterating the genuine nutmeg mace with that of *M. malabarica*. Even though there have been reports of adulteration of nutmeg mace with that of *M. malabarica* mace, there was no reliable tool to detect the adulteration.

DNA barcoding is a robust technique based on the amplification of short nucleotide regions that are conserved at the species level and has applications in food authentication (Galimberti et al., 2014; Scarano & Rao, 2014), species identification, (Anvarkhah, Khajeh-Hosseini, Mohassel, Panah, & Hashemi, 2013; Jiang et al., 2011), biodiversity studies (Lahaye et al., 2008) etc. Preservation of the barcode regions in the processed foods facilitates its use in quality control, guaranteeing food safety and minimizing food piracy (Barcaccia, Lucchin, & Cassandro, 2016). An ideal barcode should fulfill the following criteria (i) easy to amplify and sequence, (ii) PCR product size not exceeding 1kb (iii) possess high interspecific variation than intraspecific variation (Wong, But, & Shaw, 2013).

In the present study *rbcL*, *psbA-trnH* and ITS loci showed 100% amplification efficiency while traded samples failed to amplify *matK*. Amplification failure of this locus has been reported earlier also in spices like traded cinnamon bark (Swetha et al., 2014); mint, thyme and rosemary (de Mattia et al., 2011).

ITS locus has been reported as an efficient barcode for authentication in medicinal plants (Pang, Shi, Song, Chen & Chen, 2013; Chen et al., 2010) but in the present study the contig sequences could not be assembled from the forward and reverse reads rendering it of no relevance. Also majority of the samples gave mixed sequence data. The messy sequence data is attributable to the presence of the gene in multiple copies due to incomplete concerted evolution and simultaneous sequencing of these multiple variants result in sequence data that cannot be further analysed thereby limiting its application as a potential barcode candidate (Holligsworth, 2011).

Though *rbcL* had high amplification and sequencing success, it

Table 9
Indels in *psbA-trnH* locus.

Species	25	26	27	28	29	30	31	32	33	34	35	36	37	44	45	46	47
M. fragrans	G	G	A	A	A	A	A	A	T	G	C	A	T	–	–	–	–
M. malabarica	–	–	–	–	–	–	–	–	–	–	–	–	–	T	A	T	T

Table 10
Indels in *psbA-trnH* locus.

Species	59	60	61	101	102	103	115	116	117	118	171	172	173	174
M. fragrans	–	–	–	–	–	–	–	–	–	–	A	A	A	C
M. malabarica	G	T	C	C	T	G	G	T	T	T	–	–	–	–

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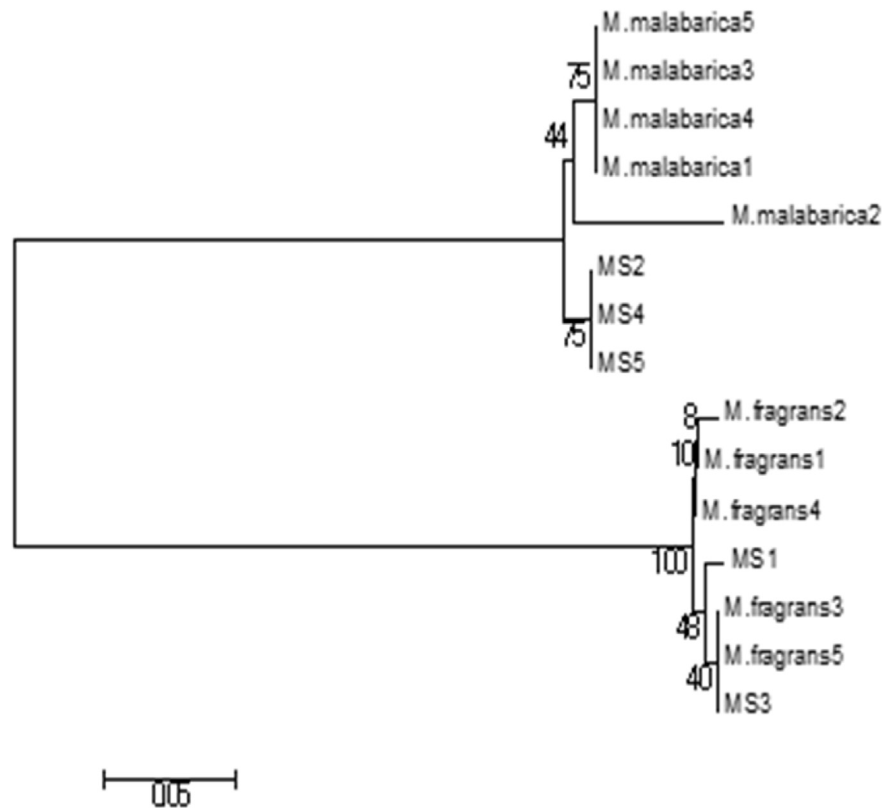

Fig. 1. A consensus NJ tree constructed based on *psbA-trnH* sequences with a boot strap support of 1000 replicates.

was not suitable for authentication as it showed low species resolution and a higher intraspecific variation and absence of polymorphic sites. The low species resolution of *rbcL* locus is widely reported in a number of species like Dalbergia (Bhagwat, Dholakia, Kadoo, Balasundaran, & Gupta, 2015), Roscoea (Zhang, Duan, & Zhou, 2014), Salvia (Wang et al., 2013), Gentiana (Wong et al., 2013), Composonura (Newmaster, Fazekas, Steves, & Janovec, 2007) and family Lauraceae (Liu, Chen, Song, Zhang, & Chen, 2012). Intraspecific variation of *rbcL* was also reported (Little & Jeanson, 2013).

psbA-trnH is one of the widely used barcoding loci in plants. It has been used for discriminating *Piper nigrum* (Parvathy et al., 2014), *Ocimum* species (Christina & Annamalai, 2014), *Scutellaria baicalensis* (Guo,Wang, Su, Zhang & Zhou, 2011), *Lonicera japonica* (Sun et al., 2011), *Phyllanthus* species (Srirama et al., 2010), medicinal plants of Polygonaceae (Song et al., 2009), *Illicium verum* (Meizil et al., 2012), *Crocus sativus* (Gismondi et al., 2013) etc. from its adulterants.

In the present study, *psbA-trnH* was found to be the ideal locus for distinguishing *M. fragrans* from *M. malabarica.* The length of sequences obtained was shorter and was in the range reported by Guo, Wang, Su, Zhang, and Zhou (2011) in *Scutellaria* species. The interspecific variation of the locus was much higher than the intraspecific variation thereby conforming to the criteria of an ideal barcode. Geographical isolation resulting in intraspecific variations in *psbA-trnH* locus was previously reported in *Composonura* genus of Myristicaceae family (Newmaster et al., 2007) and *Crocus sativus* (Gismondi et al., 2013).

Sixty polymorphic sites and 9 indels identified in the *psbA-trnH* sequence alignment points to the high sequence variation of this locus. Though indels in this locus are said to be non informative for species discrimination as their occurrence does not correlate with a species (Zhang, Meng, Wen, & Rao, 2015), the indels observed here were specific either to *M. fragrans* or *M. malabarica.*

The polymorphic sites in *psbA-trnH* locus can be employed to develop species specific primers for *M. malabarica* in order to facilitate its detection bypassing sequencing expense. These primers can also be used by food safety agencies to screen samples to check for its authenticity.

5. Conclusion

DNA barcoding was proved to be a successful tool for authentication of *M. fragrans. psbA-trnH* locus detected *M. malabarica* adulteration in three out of the five market samples analysed thereby demonstrating the extent of adulteration in traded nutmeg mace. The adoption of DNA barcoding as an authentication tool by food safety agencies can safeguard the interests of both consumers and traders.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2016.10.004.

Conflict of interest

The authors declare that they do not have any conflict of interest. We also declare that we do not have any affiliations with or involvement in any organization or entity with financial interest or non financial interest in the subject matter or materials discussed in this manuscript.

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