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Full Length Research Paper

Authentication of *Illicium verum* using a DNA barcode *psbA-trnH*

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The objective of this research was to test an appropriate DNA barcode from four candidate barcoding loci *(mat*K*, rbc*L*, psbA-trnH* and ITS2) for discrimination between *Illicium verum* and its congeneric toxic adulterants in trade. Twenty fresh samples from 8 species of the genera *Illicium* were collected from four areas of China, and two samples of medicinal material of *I. verum* were purchased respectively, from the market and pharmacy. In this study, PCR amplification success rate, sequencing efficiency, the intra- and inter-specific divergences, and DNA barcoding gap were employed to assess the ability of each barcode for the authentication of *I. verum* and its adultrants. The results showed that *psbA-trnH* exhibited the highest inter-specific divergence among the four DNA barcoding sequences and the highest species identification efficiency at 100% both by BLAST1 and the nearest distance method. In addition, the *psbA-trnH* query sequences of the two dried fruits were compared with the reference sequence by BLAST1, they were identified as *I. verum*. Our findings supported the notion that the *psbA-trnH* region could distinguish *I. verum* from other adulterating species.

Key words: *Illicium verum*, *psbA-trnH*, identification, DNA barcoding, Illiciaceae, medicinal material.

INTRODUCTION

The Chinese star anise *Illicium verum* (Illiciaceae) is commonly used as a spice for culinary purposes, cosmetics and herbal medicine in our daily life all around the world. In China, it is also embodied in the Chinese Pharmacopoeia with a functional treatment of vomiting, abdominal distension and abdominal pain (Chinese Pharmacopoeia Commission, 2010). *I. verum* itself has been safely used in medicinal and edible respects, but most of its congeneric adulterants, which are often misused as *I. verum* can lead to food poisoning by its toxicity. In China, the fruit of *Illicium lanceolatum* contained sikimin is toxic and could induce dizziness, nausea and convulsion (Upton, 2006; Chen and Chen, 2001). In some European countries, *I. verum* is a kind of tea infusion for treating infant colic, often contaminated with *Illicium anisatum* has been repeatedly reported clinical toxicity and advised consumers not to drink teas of its star-shaped fruit by Food and Drug Administration (FDA) (Ize-Ludlow et al., 2004). These intoxications were seriously threatened to both adults and infants' health. It is therefore essential to discriminate *I. verum* correctly with its congeneric adulterant. *I. anisatum*, which contained anisatin and sofrole and caused neurologic and gastrointestinal toxicities (Garzo et al., 2002; Biessels et al., 2002). Safrole with toxicity to human body has been detected in many other congeneric adulterants like *I. lanceolatum*, *Illicium henryi*, *Illicium micranthum*

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and *Illicium simonsii,* whereas not in *I. verum* (Lederer et al., 2006). To avoid drug misuse and ensure food safety, it is indispensable to authenticate *I. verum* and its related species correctly. Microscopy, multi-steps infrared macro-fingerprint and molecular biology methods have been reported to identify *I. verum* and some certain adulterant in previous studies (Joshi et al., 2005; Zhou et al., 2008; Techen et al., 2009). However, these methods are professional, time-consuming and lacking of the universal ability to identify large sample.

DNA barcoding is a new technology that uses a standardized genomic DNA sequence as a barcode to identify species rapidly and efficiently, which has become a hotspot of molecular identification research. The CO1 which is used as a DNA barcode has been proven to be a powerful tool for the discrimination of closed related species in most of the animals (Hebert et al., 2003). In 2009, the Plant Working Group of the Consortium for the Barcode of Life (CBOL) recommended the two locus *rbcL+matK* as core barcodes to identify plants (CBOL Plant Working Group, 2009). The *psbA-trnH* intergenic spacer and ITS/ITS2 were also suggested as barcodes for plant identification in the Third International Barcode Conference in Mexico City (Kress et al., 2005; Chen et al., 2010). In this study, four candidate loci (*matK, rbcL, psbA-trnH* and ITS2) were used to discriminate *I. verum* and its congeneric adulterants.

MATERIAL AND METHODS

Research material

A total of 22 samples belonging to 8 species were collected from different locations in China (Table 1S). The 20 fresh samples were identified by Prof. Jingyun Cui, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences and the voucher samples were deposited in the herbarium of the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences. The two crude fruits of *I. verum* were purchased from the supermarket and pharmacy.

DNA extraction, amplification and sequencing

DNA extraction and PCR amplification were performed according to the previous studies (CBOL Plant Working Group, 2009; Kress et al., 2005; Chen et al., 2010; Chiou et al., 2007; Sungkaew et al., 2009).

The extraction method of dried fruits was improved according to the following: Grinding the seeds with liquid nitrogen and taking it in the 56°C water bath overnight. The ITS2 PCR reaction condition was also improved, which consisted of an initial denaturation for 5 min at 94°C, 30 cycles of 1 min denaturation at 94°C, 2 min annealing at 55°C and 1 min extension at 72°C and a final extension at 72°C for 7 min. The sequences of the four DNA regions were assembled by CodonCode Aligner V3.7.1 (CodonCode Co., USA). The ITS2 region was obtained by trimming 5.8S and 26S region based on Hidden Markov Model. The sequences were aligned by Clustal W, and the intra- vs inter-specific genetic distances were computed by MEGA4.0 based on the K2P model (Tamura et al., 2007).

The intra-specific variation was reflected by the average intra-specific distance and coalescent depth (Chen et al., 2010; Meyer and Paulay, 2005). The inter-specific divergence was evaluated by the average inter-specific distance and minimum inter-specific distance (Chen et al., 2010; Meyer and Paulay, 2005; Meier et al., 2008). The distribution of intra- vs. inter-specific variability was represented by the DNA barcoding gap (Meyer and Paulay, 2005; Lahave et al., 2008). Wilcoxon signed-rank tests and two-sample tests were used as previously described (Lahave et al., 2008; Kress and Erickson, 2007). BLAST1 and the nearest distance method were used for identification (Chen et al., 2010; Ross et al., 2008).

RESULTS AND DISCUSSION

The PCR amplification efficiency of *rbcL* was 100%, and those of the others were all 95% (Table 2S). The sequencing efficiency of *psbA-trnH* and *matK* were both 100% that of *rbcL* was 95%, and the ITS2 was the lowest (84.2%). The GC contents of *psbA-trnH*, *matK*, *rbcL* and ITS2 were 35.3, 35.6, 44.1 and 59.6%, respectively. The inter-specific divergence of *psbA-trnH* showed the highest, followed by ITS2, while *matK* provided the lowest. For the intra-specific variation, ITS2 exhibited the highest, followed by *psbA-trnH*, while *rbcL* and *matK* showed both lower (Table 3S). Wilcoxon signed rank texts showed that *psbA-trnH* exhibited the highest inter-specific divergence and had all significant differences in variations with *matK*, *rbcL* and ITS2 (Table 4S). The distributions of intra- and inter-specific variation of four barcodes were showed by a scale of 0.002 distance units. Although, the “barcoding gap” of the four candidate sequences were all not obvious, *psb*A-*trn*H performed better (Figure 1). Wilcoxon two-sample tests demonstrated that the inter-specific divergences for the four barcodes were significantly higher than that of intra-specific variations, *psbA-trnH* exhibited the most significant difference among the four barcodes (Table 4S).

Finally, we used BLAST1 and the nearest distance method to test the ability of different regions for authentication of *I. verum* and its adulterants. The results revealed that the *psbA-trnH* locus exhibited the highest identification success rate of 100% at the species level (Table 2S). The success rate of identification of *rbcL* and ITS2 were 50 and 52.6%, respectively, both using BLAST and the nearest distance. The success rate for *matK* using the nearest distance and BLAST1 methods were 10.53 and 36.84%, respectively. The discrepancy was caused by the different calculation model of the two methods. The nearest distance was represented by an assessment based entirely on sequence similarity so that the actual indel variation of *matK* between *Illicium dunnianum* var. *latifolium* and *I. simonsii* was not reflected. The BLAST1 method which integrated across top-ranking hits as the species identification performed better (Ross et al., 2008)

Furthermore, we analyzed the *psbA-trnH* genetic variation of *I. verum* and its adulterants. There was the

Table 1S. Plant materials used in this study.

Samples name	Voucher number	Sampling location	Genbank access number			
			psbA-trnH	***rbcL***	***matK***	**ITS2**
Illicium verum	LMZ001MT01	Kunming, Yunnan	JQ003528	JQ003507	JQ003488	JQ003472
I. verum	LMZ001MT02	Nanning, Guangxi	JQ003529	JQ003508	JQ003489	JQ003473
I. verum	LMZ001MT03	Guangzhou, Guangdong	JQ003536	JQ003516	JQ003496	JQ003480
I. verum	LMZ001MT04	Guangzhou, Guangdong	JQ003540	JQ003520	JQ003500	——
I. verum	LMZ001MT05	Guangzhou, Guangdong	JQ003543	JQ003522	JQ003503	JQ003484
I. verum	LMZ001MT06	Guangzhou, Guangdong	JQ003542	JQ003521	JQ003502	JQ003483
I. micranthum	LMZ002MT01	Xishuangbanna, Yunnan	——	JQ003509	——	——
I. micranthum	LMZ002MT02	Xishuangbanna, Yunnan	JQ003530	JQ003510	JQ003490	JQ003474
I. micranthum	LMZ002MT03	Xishuangbanna, Yunnan	JQ003531	JQ003511	JQ003491	JQ003475
I. micranthum	LMZ002MT04	Xishuangbanna, Yunnan	JQ003532	JQ003512	JQ003492	JQ003476
I. lanceolatum	LMZ003MT01	Nanning, Guangxi	JQ003534	JQ003514	JQ003494	JQ003478
I. lanceolatum	LMZ003MT02	Nanning, Guangxi	JQ003535	JQ003515	JQ003495	JQ003479
I. simonsii	LMZ004MT01	Xishuangbanna, Yunnan	JQ003538	JQ003518	JQ003498	JQ003482
I. simonsii	LMZ004MT02	Xishuangbanna, Yunnan	JQ003539	JQ003519	JQ003499	——
I. modestum	LMZ006MT01	Xishuangbanna, Yunnan	JQ003546	JQ003525	JQ003506	JQ003487
I. jiadifengpi	LMZ007MT01	Guangzhou, Guangdong	JQ003541	——	JQ003501	——
I. jiadifengpi	LMZ007MT02	Guangzhou,Guangdong	JQ003533	JQ003513	JQ003493	JQ003477
I. henryi	LMZ009MT01	Guangzhou, Guangdong	JQ003537	JQ003517	JQ003497	JQ003481
I. dunnianum var. *latifolium*	LMZ010MT01	Guangzhou, Guangdong	JQ003544	JQ003523	JQ003504	JQ003485
I. dunnianum var. *latifolium*	LMZ010MT02	Guangzhou, Guangdong	JQ003545	JQ003524	JQ003505	JQ003486
I. verum	LMZ001MT07	——	JQ003526	——	——	——
I. verum	LMZ001MT08	——	JQ003527	——	——	——

average of 0.0013 genetic variation distance within *I. verum,* the maximum intra-specific variation of it was 0.0021. While the average of inter-specific K2P distance was 0.0141. The inter-specific K2P distance between *I. verum* and its adulterants ranged from 0.0042 (*I. lanceolatum*) to 0.0256 (*I. somonsii*). Two dried fruits were obtained from supermarket and pharmacy in order to verify the identification results. Both of the samples were identified as *I. verum* because of 100% similarity with *I. verum* reference sequence in the *psbA-trnH* database by BLAST1.

In order to ensure food and drug safety, the researchers aimed to select a practical and powerful tool to authenticate closely related species. The *psbA-trnH* candidate barcode is one of the most variation sequences in the chloroplast genome because of its faster evolutionary rate. In addition, it presents a conserved region at both ends that can be easily amplified with universal primers, and another critical advantage of it is short enough for degraded edible plant amplification. There is a potential application in authenticating some dried and processed material which is common in our real life. This is what morphological identification can't achieve because of its own limitation. In recent years, the applicability of the *psbA-trnH* intergenic spacer for species identification across medicinal plants to edible plants has been verified by previous studies (Bruni et al., 2010; Sun et al., 2011; Song et al., 2009; Yao et al., 2009). In this study, *psbA-trnH* had 95% amplification efficiency and 100%

Table 2S. The description of the four studied DNA barcoding sequences.

Marker	*psbA-trnH*	ITS2	*matK*	*rbcL*
GC content/%	35.3	59.6	35.6	44.1
Efficiency of PCR amplification/%	95.0	95.0	95.0	100
Success rate of sequencing/%	100	84.2	100	95.0
Indentification efficiency/% nearest distance	100	50	10.53	52.63
Blast 1	100	50	36.84	52.63

Table 3S. Analysis of inter-specific divergence and intra-specific variation.

Marker	*psbA-trnH*	ITS2	*matK*	*rbcL*
All inter-specific distance	0.0153±0.0089	0.0111±0.0063	0.0008±0.0007	0.0017±0.0012
All intra-specific distance	0.0015±0.0014	0.0017±0.0029	0.0001±0.0004	0.0000±0.0000
Minimum inter-specific distance	0.0060±0.0065	0.0044±0.0044	0.0004±0.0006	0.0008±0.0011
Coalescent depth	0.0018±0.0017	0.0033±0.0042	0.0002±0.0005	0.0000±0.0000

Table 4S. Wilcoxon singed rank test for intra-specific variation.

W+	W-	Correlation between the variation of intra-specific n, p	Result
psbA-trnH	*rbcL*	W+=78. W-=0. n=21. p≤0.001	*psbA-trnH*>*rbcL*
psbA-trnH	*matK*	W+=91. W-=0. n=22. p≤0.001	*psbA-trnH*>*matK*
psbA-trnH	ITS2	W+=15. W-=40. n=15. p≤0.202	*psbA-trnH*=ITS2
rbcL	*matK*	W+=0. W-=3. n=21. p≤0.18	*rbcl*=*matK*
rbcL	ITS2	W+=0. W-=36. n=15. p≤0.012	*rbcl*<ITS2
matK	ITS2	W+=3. W-=52. n=15. p≤0.012	*matK*<ITS2

species authentication. Based on variable sites of the *psbA-trnH* alignment, *I. verum* can be distinguished from its toxic adulterants obviously by naturally occurring indel variation and single nucleotide polymorphisms. Additionally, the maximum variation within *I. verum* is smaller than the minimum variation between *I. verum* and other adulterants. Although, DNA extraction from dried fruit is difficult because of degradation, DNA was successfully extracted by improving the extraction kit. And the two dried fruits are both accurately identified as *I. verum*.

The results demonstrates that the *psbA-trnH* can be used as a barcode with great practical value to identify dried fruit of *I. verum* and its closely toxic adulterants owning to its excellent discrimination power. Techen et al. (2009) identified *I. anisatum* from *I. verum* using ITS fragment length polymorphisms with endonucleases (Techen et al., 2009). Compared with this method, DNA barcoding which is applied to degraded material doesn't need to design specific primers, thus it exhibits advantages of superior repeatability and convenience. In conclusion, our study proved that the *psbA-trnH* would play an important role in identification of the spice Chinese star anise from its adulterants. Not only does it establish a database about the *psbA-trnH* sequences of *I. verum* and its adulterants, it also successfully discriminates the purchasing dried fruits as *I. verum*. Furthermore, this study may provide a valuable method for the raw and processed edible plant material identification.

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Supporting information

Plant materials used in this study as supporting information; The description of the four studied DNA barcoding sequences; Analysis of inter-specific divergence and intra-specific variation; Results of Wilcoxon signed-rank tests of intra- vs inter-specific divergence among the loci and two sample tests showing the distribution of intra-vs inter-specific divergences.

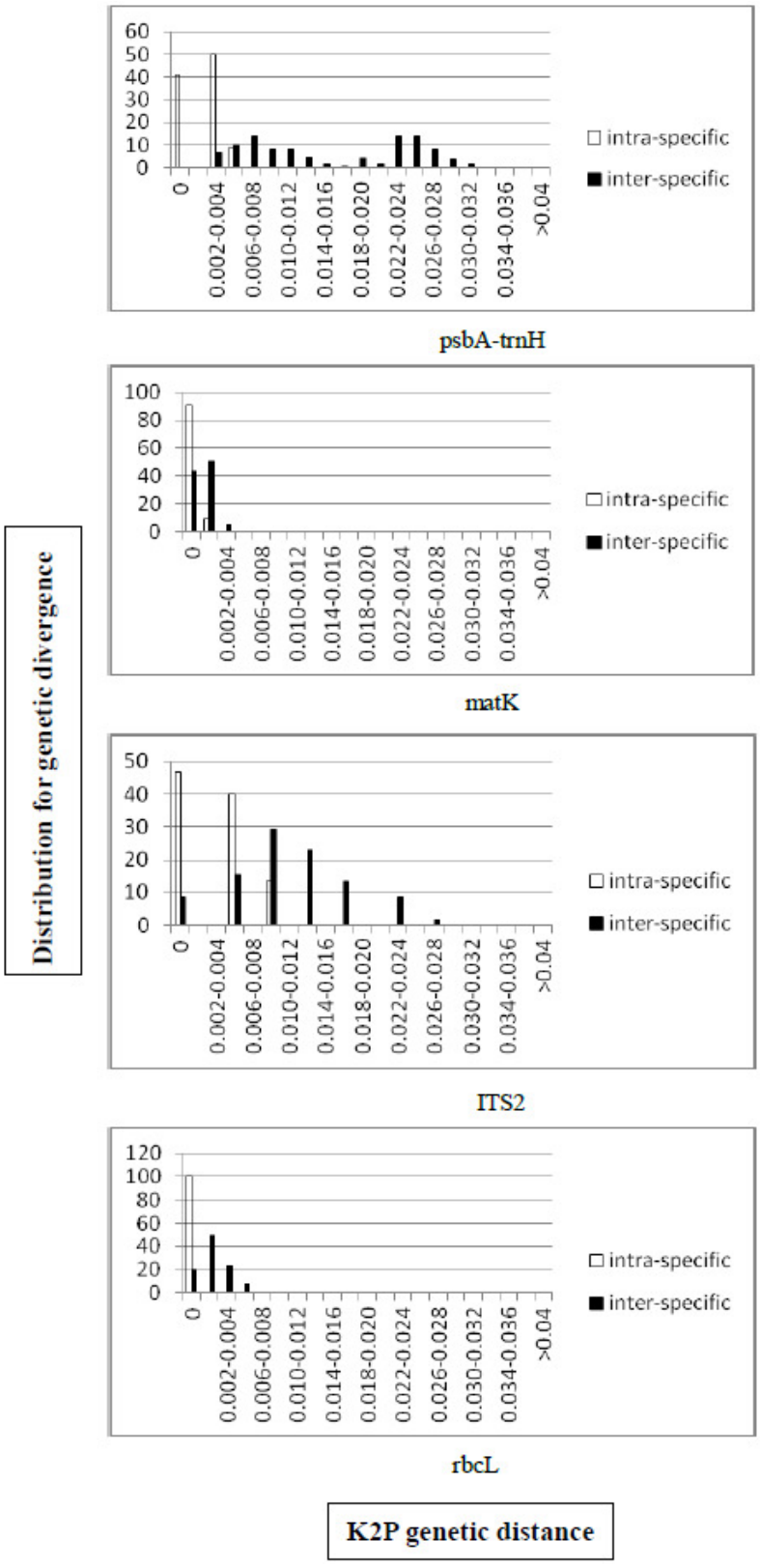

Figure 1. The barcoding gap between interspecific and intraspecific divergences for four candidate barcodes. Histograms showing the relative distribution of pairwise (y-axes), inter-specific (black bar) and intra-specific (white bar), divergences were calculated using K-2-P model, barcoding gaqs were assessed by Wilcoxon two-sample tests ($P<0.05$).

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