Phylogeny and Identification Analysis of *Materia Medica* from *Angelica* L. （*Umbelliferae*） and its Adulterants based on DNA Barcoding

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Abstract: *Angelica* L., an herbaceous perennial genus of Umbelliferae, is one of the most commonly used herb drugs which is mainly used for enriching blood, antiviral and anti-inflammatory. However, the *materia medica* of *Angelica* were often misused and substituted by its adulterants. In this study, DNA barcoding was performed to analyze the systematics between *Angelica* and its adulterants. Three chloroplast barcodes (*psb*A-*trn*H, *rbc*L and *mat*K) and two nuclear barcodes (ITS and ITS2) were employed and evaluated in terms of identification efficiency, intra-specific variation, inter-specific divergence, barcoding gap and phylogenetic tree (neighbor joining (NJ) tree and maximum likelihood (ML) tree). The results showed that DNA barcoding could discriminate the species and *materia medica* from *Angelica* and its adulterants. Chloroplast barcodes were performed better than nuclear sequences in identification efficiency, intra- and inter-specific variation and barcoding gap. The *psb*A-*trn*H sequence was more suitable for identifying the species and *materia medica* within *Angelica* and its adulterants. This study proposes a paradigm for phylogeny analysis and identification of *materia medica* and its adulterants, which is helpful for the safety in clinical application and resource utilization of herb drugs.

Keywords: *Angelica*; *materia medica*; DNA barcoding; systematics; identification

INTRODUCTION

The genus *Angelica* L. is mainly distributed in North Temperate Zone and New Zealand, which consists of 80 species throughout the world. The coumarins, volatile oil, terpenoid and sterols are the main chemical compounds in the *Angelica* L (Yao, 2003). The genus *Angelica* L. possess the pharmacological activity of enriching blood, improving immune function, abirritation, antibacterial, antiviral and anti-inflammatory(Li, 20096). There are about 45 species distributed in China, which is extremely polymorphic in leaf morphology, subterranean structures and fruit anatomy (Vasil'eva and Pimenov, 1991). Among them, 17 species within *Angelica* L. are used as herb drugs in common and endemic usage. While three species, *A. sinensis*, *A. pubescens* and *A. dahurica*, are recorded in Chinese Pharmacopoeia (Commission, 2015). With the increasing demand for medicinal material, the *materia medica* of the species within *Angelica* L., and adulterants from other genera, such as *Ligusticum jeholense*, *Conioselinum vaginatum*, *Notopterygium franchetii*, *Peucedanum decursiva*, *Actaea asiatica*, *Levisticum officinale* and *Seselopsis tianschanica*, are flooded into the market for cost concern. The researches for identifying *A. sinensis* were mainly focused on evaluating the quality grade in different producing areas, or analyzing the phylogeny between the species within the genus of *Angelica* L. (Zhao, 2006; Wang et al., 2014; Yuan et al., 2015). The previous studies on identification of *A. sinensis* and its adulterants used chemical analysis, which were complicated and time wasted (Wang, 2012). The molecular marker technology, such as inter-simple sequence repeat (ISSR), 5S-rRNA spacer domains and rDNA internal transcribed spacer (ITS), was employed to identify in previous research(Zhao et al., 2003; Zhang, 2011; Xin, 2013; Zhang, 2014). However, the species for the adulterants were incomplete, and the analysis method were limited.

DNA barcoding is an accurate and rapid method of identifing species useing a short standardized DNA sequence. It was first proposed in 2003 by Hebert, and had been employed in molecular systematics and species identification in animals and plants widely(Hebert et al., 2003; Hollingsworth et al., 2011; Xin, 2013). The nuclear ITS sequence was proposed as the core barcode for seed plants, and the ITS2 was recommended as the novel barcode for identifying medicinal plants (China Plant et al.,

2011). The *psb*A-*trn*H sequence is the most variable plastid barcode in angiosperms and is easily amplified in most land plants, especially in medicinal plant (Kress et al., 2005; Feng et al., 2014; Zheng et al., 2014). The *rbc*L and *mat*K sequences were recommended as the suitable barcode combination for land plants (Group, 2009). The five commonly used barcodes were widely used in identifying species of plants. In this study, we employed the five common DNA barcodes to identify the *materia medica* from the genus *Angelica* L. and its adulterants. The identification efficiency, inter-specific divergence and intra-specific variation and barcoding gap were analyzed and calculated for evaluating the DNA barcodes by bioinformatics analysis. The NJ tree and ML tree were built to present phylogenetic relationship between *materia medica* from *Angelica* L. and its adulterants. This work is aimed to propose a scientific and easy method for discriminating the *materia medica* within the genus of *Angelica* L. and its adulterants, which is important for the safe application of the medicinal material within the genus *Angelica* L.

MATERIALS AND METHODS

Plant and materials

The materia medica from *Angelica* L. and its adulterants were collected from Gansu, Yunnan, Hunan, Liaoning, Beijing and Xinjiang provinces of China (Table 3). All corresponding voucher specimens were deposited in the Herbarium of the Institute of Medicinal Plant Development at the Chinese Academy of Medical Sciences in Beijing, China. The identification of the collected samples was performed by Prof. Lin Yulin (Institute of Medical Plant Development) on the basis of morphological characteristics. The sequences of psbA-trnH were submitted to GenBank database, and the obtained accession numbers were KJ999545-KJ999588.

DNA extraction, PCR amplification and sequencing

The samples were naturally dried and 30mg of dried root material was used for the DNA extraction. Samples were rubbed to powder for two minutes at a frequency of 30r/s in a FastPrep bead mill (Retsch MM400, Germany). The total genomic DNA was isolated from the powder of root according to the manufacturer's instructions (Plant Genomic DNA Kit, Tiangen Biotech Co., China). Based on the introduction, we made the following modifications to the protocol: chloroform was diluted with isoamyl alcohol (24:1 in the same volume) and buffer solution GP2 with isopropanol (same volume). The powder, 700 μL of 65 ℃ GP1 and 1 μL β-mercaptoethanol were mixed for 10-20 s before being incubated for 1 h at 65 ℃. Then, 700 μL of the chloroform:isoamyl alcohol mixture was added and the solution was centrifuged for 5 minutes at 12000 rpm (~13400×g). Supernatant was removed and placed into a new tube before adding 700 μL isopropanol and blending for 15-20 minutes. The mixture was centrifuged in CB3 spin columns for 40 s at 12000 rpm. The filtrate was discarded and 500 μL GD (adding quantitative anhydrous ethanol before use) was added before centrifuging at 12000 rpm for 40 s. The filtrate was discarded and 700 μL PW (adding quantitative anhydrous ethanol before use) was used to wash the membrane before centrifuging for 40s at 12000rpm. This step was repeated with 500 μL PW, followed by a final centrifuge for 2 minutes at 12000 rpm (~13400×g) to remove residual wash buffer. The spin column was dried at room temperature for 3-5 minutes and then centrifuged for 2 minutes at 12000 rpm to obtain the total DNA.

PCR reaction conditions and universal DNA barcode primers used for the ITS, ITS2, and psbA-trnH barcodes were presented in Table S1 15, 18, 20). PCR amplification was performed on 25 μL reaction mixtures containing 2 μL DNA template (20-100 ng), 8.5 μL ddH2O, 12.5 μL 2×Taq PCR Master Mix (Beijing TransGen Biotech Co., China), and 1/1 μL forward/reverse (F/R) primers (2.5 μM). The reaction mixtures were amplified in 9700 GeneAmp PCR system (Applied Biosystems, USA). The Amplification products were visualized by electrophoresis on 1% agarose gels. Purified PCR products were sequenced in both directions using the ABI 3730XL sequencer (Applied Biosystems, USA).

Sequences assembly, alignment, and data analysis

Sequencing peak diagrams were obtained, proofread and then assembled using a CodonCode Aligner 5.0.1 (CodonCode Co., USA). In order to better analysis the phylogeny and systematics of the materia medica from Angelica L. and its adulterants, we downloaded the sequences of all the species within Angelica L. and its adulterants in terms of five commonly used DNA barcodes, two nuclear barcodes (ITS and ITS2) and three chloroplast barcodes (psbA-trnH, rbcL and matK) (Table S2). Furthermore, the ITS2 sequences were cut from the obtained ITS sequences using HMMer annotation method based on Hidden Markov model (Keller et al., 2009). All the sequences were aligned using ClustalW by MEGA 6.0 software (Tamura et al., 2013). The GC content and genetic distance were calculated by MEGA after sequence alignment. The NJ tree and ML tree were built and analyzed the phylogeny and systematics of the materia medica within Angelica L. and its adulterants. To evaluate the identification success rate of five DNA barcodes, the identification efficiency, barcoding gap and inter- and intra-species variation were employed by bioinformatics analysis (Meyer and Paulay, 2005; Ross et al., 2008).

RESULTS

Sequence information and identification efficiency

The five commonly used DNA barcodes were evaluated for identifying the *materia medica* from *Angelica* L. and its adulterants (Table 1). A total of 724 sequences were obtained from the experiment and GenBank database. The sequence numbers of ITS and ITS2 were more than other three barcodes. Meanwhile, 200 sequences of ITS2 were cut from the ITS sequences obtained from GenBank database. The GC content of ITS and ITS2 were higher than other three chloroplast barcodes (Table 2). The results of genetic distance showed that the chloroplast barcode *psb*A-*trn*H possessed higher genetic distance between *Angelica* and its adulterants, followed by ITS2 and ITS sequence. The identification efficiency calculated based on BLAST1 and K-2-P Nearest distance method showed that chloroplast barcodes were higher than nuclear barcodes. Though the identification efficiency is different between two methods, the order and trend of five barcodes were consistent. The chloroplast *rbc*L sequence was performed better than other four DNA barcodes, followed by *psb*A-*trn*H and *mat*K sequences.

Table 1 Taxon Sampling information of Angelica and its adulterants

Experiment Number	Species	Sampling spot	Collection time

DG1-DG20	*Angelica sinensis*	Gansu	China	2012.5
QG1-QG16	*Angelica sinensis*	Yunnan	China	2012.6
D1-D7	*Angelica biserrata*	Hunan	China	2012.6
LGB1-LGB8	*Ligusticum jeholense*	Liaoning	China	2011.7
XJ1-XJ3	*Conioselinum vaginatum*	Xinjiang	China	2011.7
QH1-QH7	*Notopterygium franchetii*	Gansu	China	2012.10
ZH1-ZH2	*Peucedanum decursiva*	Hunan	China	2012.6
SM	*Actaea asiatica*	Beijing	China	2012.7
TDG1-TDG5	*Levisticum officinale*	Beijing	China	2012.7
XG1-XG8	*Seselopsis tianschanica*	Yunnan	China	2012.6

Table 2 The information of identification efficiency for five barcodes

Markers		**ITS2**	**ITS**	**matK**	**rbcL**	**psbA-trnH**
Number of sequences		312	268	31	23	90
Average GC content/%		55.90	55.22	35.53	43.97	28.60
Genetic distance	Min	0.0000	0.0000	0.0000	0.0000	0.0000
	Max	2.1221	0.6028	0.2628	0.0923	2.0112
	Average	0.2327	0.1113	0.0201	0.0160	0.3474
Identification efficiency/%	BLAST 1/%	16.03	26.12	77.42	47.83	36.67
	Nearest Distance/%	62.82	74.63	80.65	82.61	81.11

Intra-specific variation and inter-specific divergence analysis

Six parameters were employed to analyze the intra-specific variation and inter-specific divergence for evaluating the utility of five DNA barcodes (Table 3). The ideal barcodes should possess smaller intra-specific variation and bigger inter-specific divergence, so that DNA barcoding could distinguish species between the same genus and closely related species, while performed stably between the samples of the same species. The theta (average intra-specific distance), coalescent depth (the maximum intra-specific distance) and all intra-specific distance were shown the intra-specific variation, and the theta prime (average inter-specific distance), minimum inter-specific distance and all inter-specific distance were shown the inter-specific divergence. The results indicated that the *rbc*L sequence possessed the smaller intra-specific variation, followed by *mat*K and ITS sequences. The *psb*A-*trn*H sequence possessed the bigger inter-specific divergence, followed by ITS2 and ITS sequences. The results demonstrated that the chloroplast barcodes had smaller intra-specific variation, while the nuclear barcodes possessed bigger inter-specific divergence.

Table 3 Analysis of inter-specific divergence and intra-specific variation for five barcodes

Marker	ITS2	ITS	*mat*K	*rbc*L	*psb*A-*trn*H
Theta	0.0613±0.1252	0.0486±0.1206	0.0026±0.0024	0.0002±0.0005	0.0521±0.0536
coalescent depth	0.1793±0.4055	0.0900±0.1802	0.0032±0.0032	0.0004±0.0007	0.1666±0.4278
All intraspecific distance	0.0787±0.3141	0.0133±0.0523	0.0023±0.0027	0.0003±0.0006	0.1388±0.0000
Theta prime	0.0817±0.0386	0.0620±0.0216	0.0062±0.0034	0.0024±0.0016	0.0731±0.0370
minimum interspecific distance	0.0139±0.0167	0.0138±0.0124	0.0029±0.0023	0.0015±0.0011	0.0124±0.0122
all inter-specific distance	0.1252±0.1540	0.0800±0.0487	0.0079±0.0055	0.0032±0.0017	0.1278±0.2110

Barcoding gap

Barcoding gap presents the divergence of inter- and intra-species, which indicates the distribution frequency of genetic distance. The ideal DNA barcode possesses separate, non-overlapping distribution between specimens. In this study (Figure 1), the overlap between intra- and inter-specific genetic distances of five DNA barcodes were all unconspicuous. The distribution of *mat*k and *rbc*L sequence were concentrated on 0-0.03, which indicated that the intra- and inter-specific variation were smaller than other three barcodes. The genetic distance in ITS, ITS2 and *psb*A-*trn*H sequences were evenly distributed. Compared with ITS and ITS2 sequences, *psb*A-*trn*H sequence performed smaller genetic distance distribution of intra-species, and bigger genetic distance distribution of inter-species relatively. The results of barcoding gap indicated that the *psb*A-*trn*H sequence was suitable for distinguishing the *materia medica* within *Angelica* L. and its adulterants.

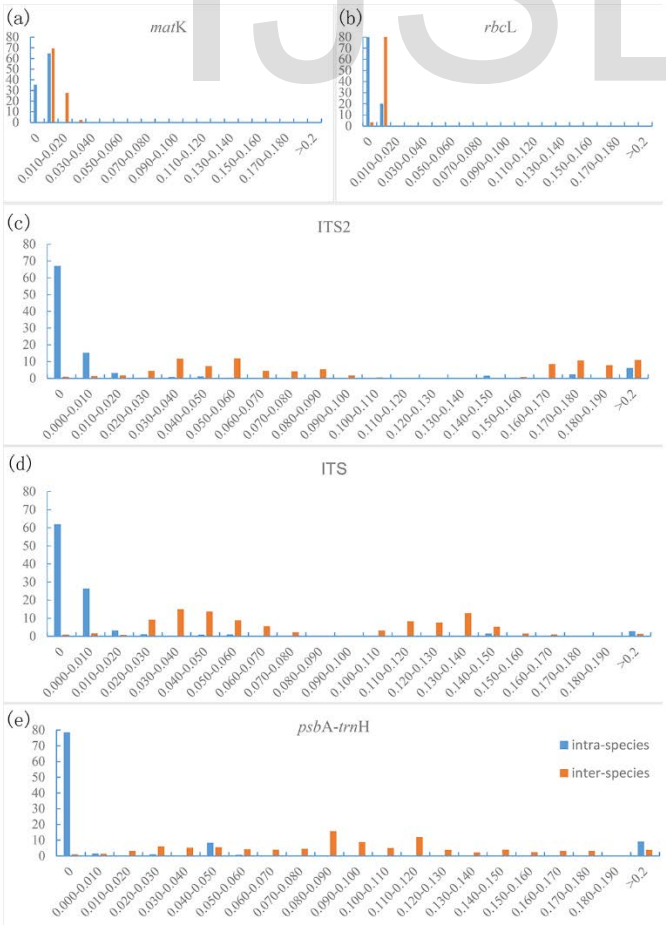

Figure 1. a Barcoding gap of *mat*K sequence for *Angelica* and its adulterants; **b** Barcoding gap of *rbc*L sequence for *Angelica* and its adulterants; **c** Barcoding gap of ITS2 sequence for *Angelica* and its adulterants; **d** Barcoding gap of ITS sequence for *Angelica* and its adulterants; **e** Barcoding gap of *psb*A-*trn*H sequence for *Angelica* and its adulterants.

NJ and ML tree

The NJ and ML tree are mainly employed to analyze the phylogeny and systematics of distinctly related species, which can present the relationship between species visually. In our study (Figure 2, 3 and S1), the NJ and ML tree of five DNA barcodes within *Angelica* L. and its adulterants were performed and compared. The results showed that the species were divided into separate clade, which demonstrated that the NJ and ML tree could distinguish the species of *Angelica* L. and its adulterants.

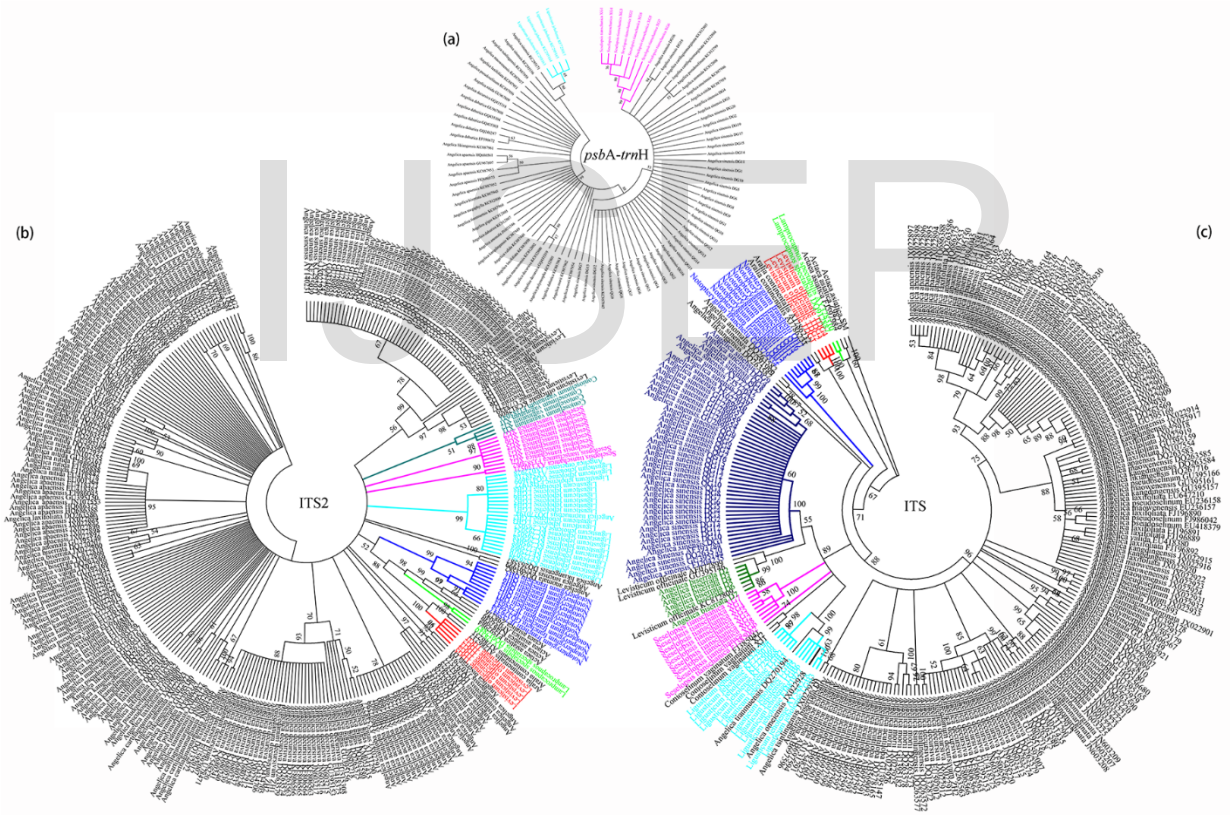

Figure 2. a The NJ tree of *psb*A-*trn*H sequence built for *Angelica* and its adulterants; **b** The NJ tree of ITS2 sequence built for *Angelica* and its adulterants; **c** The NJ tree of ITS sequence built for *Angelica* and its adulterants.

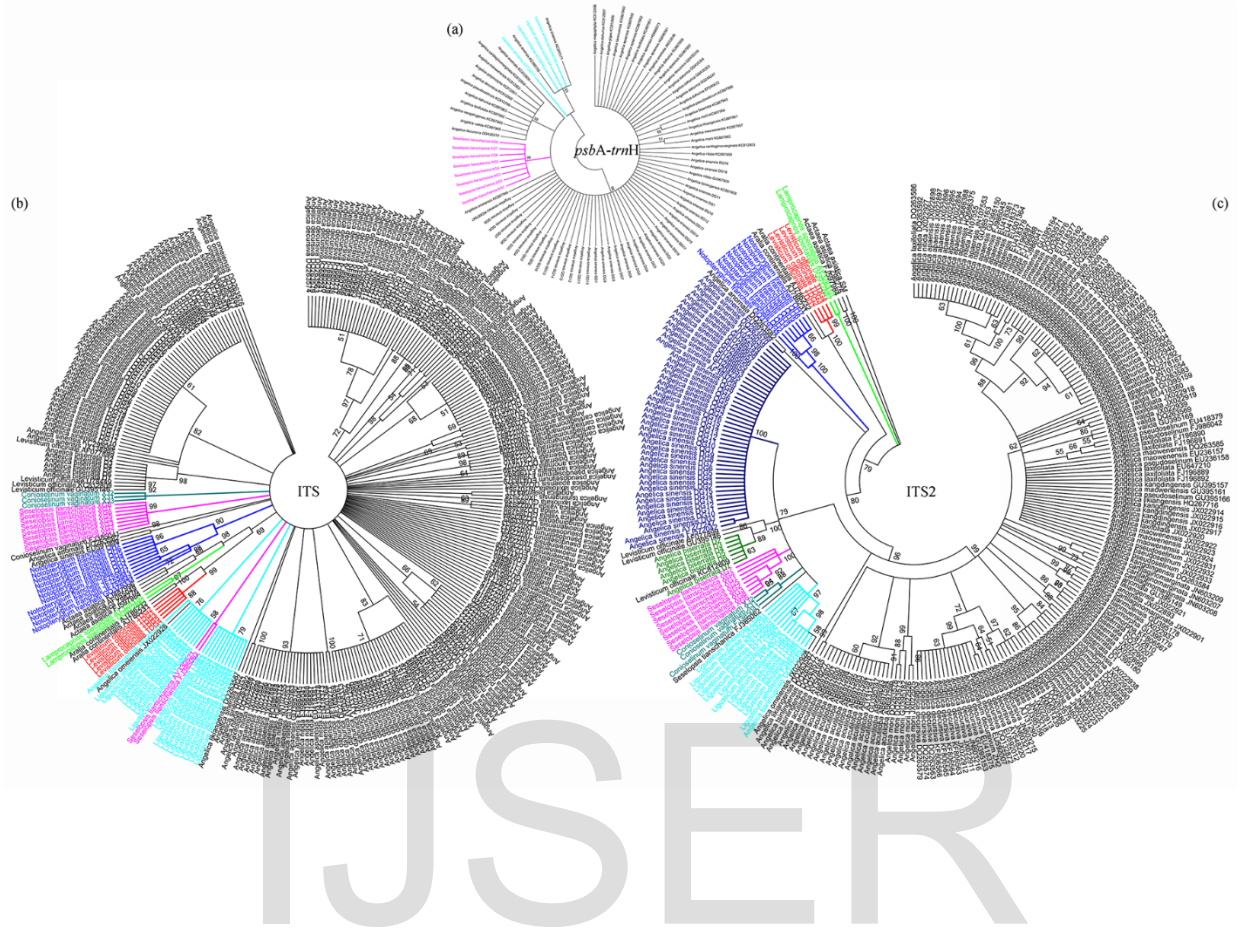

Figure 3. a The ML tree of *psb*A-*trn*H sequence built for *Angelica* and its adulterants; **b** The ML tree of ITS2 sequence built for *Angelica* and its adulterants; **c** The ML tree of ITS sequence built for *Angelica* and its adulterants.

DISCUSSION

A. sinensis is commonly used herb drugs, which possesses the pharmacological activity for tonic, antineoplastic and anti-inflammatory and gynecological diseases (Cao et al., 2010). *A. sinensis* is the material of many Chinese patent medicine, such as danggui buxue decoction and Taohongsiwu decoction. With the increasing demand, the adulterants were swarmed into the market. The research on identification for *A. sinensis* and its adulterants were limited. In this study, DNA barcoding was employed to analyze the phylogeny and systematics of the *materia medica* within *Angelica* L. and its adulterants. The results showed that DNA barcoding could distinguish the species of *Angelica* L. and its adulterants. The identification efficiency of chloroplast barcodes was higher than nuclear barcodes calculated by BLAST1 and K-2-P nearest distance method. The intra- and inter-specific variation was analyzed in terms of six parameters. The results showed that chloroplast barcodes had smaller intra-specific variation, while the nuclear barcodes possessed bigger inter-specific divergence, and the *psb*A-*trn*H sequence was performed well than other four DNA barcodes. The analysis of barcoding gap

indicated that the *psb*A-*trn*H sequence possessed smaller genetic distance distribution of intra-species, and bigger genetic distance distribution of inter-species. The NJ and ML tree showed that DNA barcoding could identify the species within *Angelica* L. and its adulterants.

Previous research were mainly focused on the identification of *A. sinensis* from different producing areas and commodity grade using chemical and molecular marker analysis (Zhao, 2006; Wang, 2010; Wang et al., 2014). Relative to the different quality grade, the adulterants were deserved more attention. The genus of *Angelica* is consist of 23 species in China, which 17 species are used for medicinal material. According to the Chinese pharmacopoeia, *A. sinensis*, *A. dahurica* and *A. biserrata* are recorded for medicinal use. Moreover, the adulterants, such as *L. jeholense*, *C. vaginatum*, *P. decursiva* and *L. officinale*, are easily confused in the market and clinical application. Huang et al. employed the DNA barcodes to identify the species and *materia medica* within *Angelica* L., and indicated that the ITS sequence provided the best discriminatory power in identification the species of *Angelica* L (Yuan et al., 2015). The genus of *Angelica* and allied genera from the Hengduan Mountains of China were analyzed by nrDNA ITS sequence, and the results demonstrated that the ITS sequences was contributed to infer the phylogenetic affinities and historical biogeography (Feng et al., 2009). However, the research were not focused on resolving the confusion between the *materia medica* within *Angelica* L. and its common adulterants, which possessed important application significance and value for resource utilization of *A. sinensis*. Therefore, we employed the five DNA barcodes (two nuclear barcodes and three chloroplast barcodes) to analyze the phylogeny and systematics within the *Angelica* L. and its adulterants. The results demonstrated that the chloroplast barcodes were more suitable for identifying the species and *materia medica* of *Angelica* L. and its adulterants. Moreover, the *psb*A-*trn*H sequence was performed better than other four DNA barcodes. In addition, the further systematic research should focus on the comprehensive sampling of *Angelica* and its adulterants throughout the distribution around the world to better understand the phylogenetic relationship between the genus *Angelica* and its closely related species.

CONCLUSION

Herein, we proposed DNA barcoding to identify and analyze the phylogeny and systematics of the species and *materia medica* from *Angelica* L. and its adulterants. The results indicated that the DNA barcoding could distinguish the species between the genus *Angelica* and its adulterants. The chloroplast region *psb*A-*trn*H sequences was performed better than other barcodes, which was suitable for identifying the *materia medica* form *Angelica* L. and its adulterants. The finding provides practical significance for the safety guarantee in clinical and medicinal application of the *materia medica* from *Angelica* L.

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SUPPLEMENTARY MATERIALS

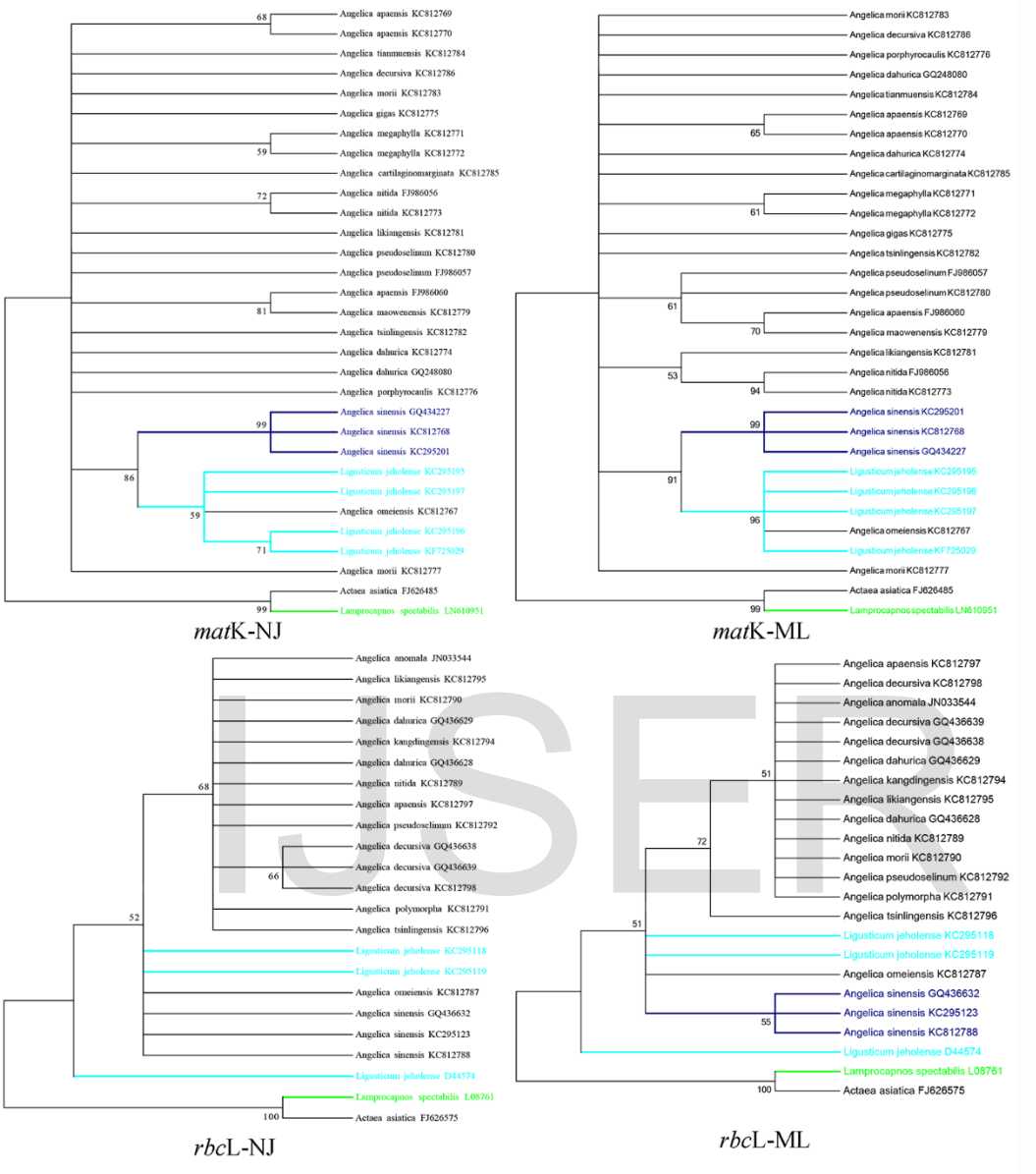

Figure S1. The NJ and ML tree of *mat*K and *rbc*L sequences for Angelica and its adulterants.

Table S1. Primers and PCR reaction conditions for DNA barcodes.

Primer name		Primer sequences(5'-3')	PCR reaction condition
ITS2	2F	ATGCGATACTTGGTGTGAAT	94℃ 5min;
	3R	GACGCTTCTCCAGACTACAAT	94℃ 30s, 56℃ 30s,
			72℃ 45s, 40cycles;
			72℃ 10min;
ITS	4R	TCCTCCGCTTATTGATATGC	94℃ 5min;
	5F	GGAAGTAAAAGTCGTAACAAGG	94℃ 1min, 50℃ 1min,
			72℃ 1.5min+3s/cycle, 30cycles;

			72℃ 7min;
*psb*A	fwdPA	GTTATGCATGAACGTAATGCTC	94℃ 4min;
*trn*H	rev TH	CGCGCATGGTGGATTCACAATCC	94℃ 30s, 55℃ 1min, 72℃ 1min, 35 cycles; 72℃ 10min;