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Molecular Identification of Chinese Materia Medica and Its Adulterants Using ITS2 and *psbA-trnH* Barcodes: A Case Study on Rhizoma Menispermi

Pei Yang[1*], Xiwen Li[1,2*], Hong Zhou[1], Hao Hu[3], Hui Zhang[4], Wei Sun[2], Yitao Wang[3], Hui Yao[1#]

[1]Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
[2]Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China
[3]State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau, China
[4]Development Center of Traditional Chinese Medicine and Bioengineering, Changchun University of Chinese Medicine, Changchun, China
Email: [#]scauyaoh@sina.com

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Abstract

Rhizoma Menispermi, derived from the rhizoma of *Menispermum dauricum* DC., is one of the most popular Chinese medicines. However Rhizoma Menispermi is often illegally mixed with other species in the herbal market, including *Aristolochia mollissimae* Hance, which is toxic to the kidneys and potentially carcinogenic. The use of DNA barcoding to authenticate herbs has improved the management and safety of traditional medicines. In this paper, 49 samples belonging to five species, including 34 samples of *M. dauricum*, from different locations and herb markets in China were collected and identified using DNA barcoding. The sequences of all 34 samples of Rhizoma Menispermi are highly consistent, with only one site variation in internal transcribed spacer 2 (ITS2) of nuclear ribosomal DNA and no variations in the *psbA-trnH* region. The intra-specific genetic distance is much smaller than inter-specific one. Phylogenetic analysis shows that both sequences allow the successful identification of all species. Nearest distance and BLAST1 methods for the ITS2 and *psbA-trnH* regions indicate 100% identification efficiency. Our research shows that DNA barcoding can effectively distinguish Rhizoma Menispermi from its adulterants from both commercial and original samples, which provides a new and reliable way to monitor com-

[*]These two authors contributed equally to this work.
[#]Corresponding author.

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mercial herbs and to manage the modern medicine market.

Keywords

Rhizoma Menispermi, *Menispermum dauricum* DC., ITS2, *psbA-trnH*, Identification, Adulterants

1. Introduction

Rhizoma Menispermi (Beidougen) is a commonly used traditional Chinese herbal medicine recorded in Chinese Pharmacopoeia as an analgesic and antipyretic drug. Rhizoma Menispermi is derived from the rhizoma of the plant *Menispermum dauricum* DC. (Menispermaceae), which is widely distributed in North China. Recent studies have shown that the alkaloids from *M. dauricum* have various bioactivities, which include anti-arrhythmic and anti-tumor effects [1]. Zhao *et al*. stated that the phenolic alkaloids in the rhizome of *M. dauricum* could protect against brain ischemia injury [2]. In addition, the water-soluble polysaccharides extracted from Rhizoma Menispermi significantly inhibit cell proliferation and DNA synthesis in human ovarian carcinoma SKOV3 cells [3]. Rhizoma Menispermi has become a focus of research because of its newly discovered medicinal properties.

Medication safety is currently a research hot spot, which has become necessary with the increase in market demand for medicinal plants. The rhizome of *Aristolochia mollissimae* Hance (Aristolochiaceae), an adulterant of Rhizoma Menispermi, shows profound nephrotoxicity and carcinogenicity [4]. Radix et Rhizoma Sophorae Tonkinensis (Shandougen), the root and the rhizome of *Sophora tonkinensis* Gagnep., is the most common adulterant in the herb market. Several studies have explored traditional methods to distinguish these two medicinal species [5]. In China, *M. dauricum* is the only species in the genus *Menispermum*; however, medicinal plants in the same family, such as *Stephania tetrandra* S. Moore and *Cocculus orbiculatus* (L.) DC, are commonly interchanged because of their similar morphological characteristics. An accurate identification of these medicinal plants is essential to ensuring the purity and safety of medicines for consumers.

The traditional identification of herbal medicines is mainly based on the morphological characteristics of the source plants, microscopic observations, and the physical form or chemical compositions. These parameters are subjective and can be affected by the external environment, leading to inaccurate classification of herbal authenticity. DNA barcoding has emerged as a cost-effective standard for species identification and has brought a renaissance to the study of taxonomy [6]. DNA barcoding has been proven highly effective in identifying medicinal herbs with high accuracy and reproducibility. The internal transcribed spacer 2 (ITS2) of nuclear ribosomal DNA has been validated as a novel DNA barcode to identify medicinal plant species [7] and is now recommended as the universal DNA barcode for plants [8]. In addition, the China Plant Barcode of Life Group [9] has confirmed that ITS/ITS2 should be incorporated into the core barcode set for seed plants. The chloroplast intergenic spacer, *psbA-trnH*, has been suggested as a candidate barcode sequence after a large-scale study found high variability and efficiency of detection across a broad range of flowering plants [10]. This region is one of the most variable non-coding regions of the plastid genome in angiosperms [11]. In this study, the ITS2 and *psbA-trnH* regions were used to distinguish Rhizoma Menispermi from its adulterants. The extraction of DNA from different tissues of herb species may be hampered by different chemical compositions and growing environments. Leaves and other fresh tissues are generally used in published literatures, whereas roots, which have high polysaccharides and polyphenols content, are rarely used. In this study, *M. dauricum* rhizomas were used to verify the use of DNA barcoding in the identification of *M. dauricum* and its adulterants. Commercial materials were included to evaluate the prospective application of the DNA barcoding method in monitoring crude drugs in the market.

2. Material and Method

2.1. Taxon Sampling

Forty-nine samples of various herbal species were collected from different locations and markets in China (**Table 1**). A total of 4 original plant samples and 30 rhizoma samples of *M. dauricum* were collected from main

Table 1. Detailed information of experimental samples in this study.

Taxon	Sampling part	Voucher No.	Location	GenBank Accession NO.	
				ITS2	*psbA-trnH*
Menispermum dauricum	Rhizoma	YC0146MT01	Chengde, Hebei	KC902480	KC902468
M. dauricum	Rhizoma	YC0146MT02	Chengde, Hebei	KC902481	/
M. dauricum	Leaf	YC0146MT03	Songfengshan, Heilongjiang	KC902482	KC902460
M. dauricum	Rhizoma	YC0146MT04	Songfengshan, Heilongjiang	KC902483	KC902467
M. dauricum	Rhizoma	YC0146MT05	Songfengshan, Heilongjiang	KC902484	KC902466
M. dauricum	Leaf	YC0146MT06	Chifeng, Inner Mongolia	KC902485	KC902465
M. dauricum	Rhizoma	YC0146MT07	Xiaoxinganling, Heilongjiang	KC902486	KC902464
M. dauricum	Rhizoma	YC0146MT08	Fushun, Liaoning	KC902487	KC902463
M. dauricum	Rhizoma	YC0146MT09	Wangqing, Jilin	KC902488	KC902459
M. dauricum	Rhizoma	YC0146MT10	Wangqing, Jilin	KC902489	/
M. dauricum	Rhizoma	YC0146MT11	Fengcheng, Liaoning	KC902490	KC902462
M. dauricum	Rhizoma	YC0146MT12	Fengcheng, Liaoning	KC902491	KC902461
M. dauricum	Leaf	YC0146MT13	Yichun, Heilongjiang	KC902492	KC902458
M. dauricum	Rhizoma	YC0146MT14	Fushun, Liaoning	KC902493	KC902476
M. dauricum	Rhizoma	YC0146MT15	Xiaoxinganling, Heilongjiang	KC902494	KC902475
M. dauricum	Rhizoma	YC0146MT16	Chifeng, Inner Mongolia	KC902495	KC902474
M. dauricum	Rhizoma	YC0146MT18	Qingdao, Shandong	KC902496	KC902473
M. dauricum	Rhizoma	YC0146MT19	Qingdao, Shandong	KC902497	KC902472
M. dauricum	Rhizoma	YC0146MT20	Shangluo, Shaanxi	KC902498	KC902471
M. dauricum	Rhizoma	YC0146MT21	Shangluo, Shaanxi	KC902499	KC902470
M. dauricum	Rhizoma	YC0146MT22	Ziyang, Sichuan	KC902500	KC902469
M. dauricum	Rhizoma	YC0146MT23	Anguo Medicine Market, Hebei	KC902501	KC902479
M. dauricum	Rhizoma	YC0146MT25	Bozhou Medicine Market, Anhui	KC902502	KC902477
M. dauricum	Rhizoma	YC0146MT26	Bozhou Medicine Market, Anhui	KC902503	/
M. dauricum	Rhizoma	YC0146MT28	Drug Store I, Beijing	KC902504	/
M. dauricum	Rhizoma	YC0146MT29	Drug Store I, Beijing	KC902505	/
M. dauricum	Rhizoma	YC0146MT31	Drug Store II, Beijing	KC902506	KC902457
M. dauricum	Rhizoma	YC0146MT32	Drug Store II, Beijing	KC902507	KC902456
M. dauricum	Rhizoma	YC0146MT33	Drug Store III, Beijing	KC902508	KC902455
M. dauricum	Rhizoma	YC0146MT34	Drug Store III, Beijing	KC902509	/
M. dauricum	Rhizoma	YC0146MT35	Drug Store IV, Beijing	KC902510	KC902454
M. dauricum	Rhizoma	YC0146MT36	Drug Store IV, Beijing	KC902511	KC902453
M. dauricum	Rhizoma	YC0146MT37	Drug Store V, Beijing	KC902512	/
M. dauricum	Leaf	PS0345MT01	IMPLAD, Beijing	GQ434390	/

Continued

Sophora tonkinensis	Root	YC0104MT01	Drug Store II, Beijing	KC902514	KC902515
So. tonkinensis	Root	YC0104MT02	Drug Store III, Beijing	KJ766117	KJ766122
So. tonkinensis	Root	YC0104MT03	Tianlin County, Guangxi	KJ766116	KJ766123
So. tonkinensis	Root	YC0104MT04	Meilin County, Guangxi	KJ766118	KJ766124
So. tonkinensis	Leaf	PS0228MT01	Nanning, Guangxi	GQ434351	GQ434960
Aristolochia mollissima	Leaf	YC0514MT01	Nanyang, Henan	KJ766113	KJ766119
A. mollissima	Leaf	YC0514MT02	Nanyang, Henan	KJ766114	KJ766120
A. mollissima	Leaf	YC0514MT03	South China Botanical Garden, Guangdong	KJ766115	KJ766121
Stephania tetrandra	Leaf	PS0348MT01	Danfeng, Shaanxi	/	GQ434988
St. tetrandra	Leaf	PS0348MT02	Xuancheng, Anhui	/	GQ434989
St. tetrandra	Root	YC0200MT01	Anguo Medicine Market, Hebei	/	KJ766125
St. tetrandra	Root	YC0200MT02	Anguo Medicine Market, Hebei	/	KJ766126
Cocculus orbiculatus	Leaf	PS0353MT02	Xuancheng, Anhui	GQ434395	GQ434990
C. orbiculatus	Leaf	PS0353MT03	Chengdu, Sichuan	GQ434396	GQ434991
C. orbiculatus	Leaf	PS0353MT04	Putian, Fujian	GQ434397	GQ434992

"/" means the sample failed to obtain the sequence.

producing areas and different drug stores and markets. Additional 15 samples of species, which were often confused as *M. dauricum*, were also obtained. All samples were identified by Professor Yulin Lin of Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences, Peking Union Medical College. The samples were deposited in the herbarium of the institute.

2.2. DNA Extraction, Amplification and Sequencing

The surface of the rhizoma samples was first wiped with 75% ethanol, and the outer epidermis was scraped off. Approximately 40 mg for rhizoma samples and 20 mg for the leaves were used to extract genomic DNA using Bioteke DNA Exaction Kit (Bioteke Co., Beijing). For the rhizome samples, 1% PVP (Polyvinylpyrrolidone) was mixed before they were crushed. Then the crushed samples with lysate were heated to 56˚C by water bath for 8 h - 12 h. The succeeding steps were consistent with those used for the leaf samples. The primers and Polymerase Chain Reaction (PCR) conditions were previously described [7] [12]. All amplified products were bi-directionally sequenced. Data analysis was conducted according to the methods used in previous study [8].

2.3. Sequence Alignment, Genetic Analysis and Species Identification

Intra- and inter-specific genetic distances were computed with MEGA 5.0 [13] on the basis of the Kimura 2-Parameter (K2P) model. The sequence lengths and GC contents are listed in **Table 2**. Phylogenetic trees were constructed according to ITS2 and *psbA-trnH* regions with the use of the neighbor-joining (NJ) method, with 1000 bootstraps, in MEGA 5.0. Finally, BLAST1 and Nearest Distance methods were applied to species identification, as described in previous studies [7] [14].

3. Results

3.1. PCR, Sequencing Efficiency and Sequence Characteristics

Most genomic DNA samples showed smearing on agarose gel electrophoresis, which indicated DNA degradation. However, most of the samples were successfully amplified except the ITS2 sequence of *S. tetrandra*, suggesting that amplification of both ITS2 and *psbA-trnH* was not affected by the degradation of genomic DNA.

Table 2. Sequence characteristics of ITS2 and *psbA-trnH* of *M. dauricum* and its adulterants.

	ITS2	*psbA-trnH*
Amplification efficiency of *M. dauricum* (%)	100	100
Sequencing efficiency of *M. dauricum* (%)	100	76.5
Length of *M. dauricum* (bp)	203	315
Amplification efficiency of all taxa (%)	91.8	100
Sequencing efficiency of all taxa (%)	91.8	83.7
Length of all taxa (bp)	181 - 277	215 - 656
Aligned length (bp)	331	743
GC content range in *M. dauricum* (%)	52.7	24.4
GC content range (mean) in all taxa (%)	49.3 - 74.4 (62.8)	23.6 - 41.4 (26.6)
Number (and %) of variable sites in all taxa	163 (49.2)	319 (42.9)

The amplification efficiency of *psbA-trnH* was 100%, whereas the sequencing efficiency of *psbA-trnH* was 76.5% (**Table 2**). We failed to obtain the ITS2 sequence for *S. tetrandra* by using the universal ITS2 primer pairs described by Chen *et al*. [6]. However, the *psbA-trnH* region was obtained from all the samples of this species.

The sequence length of ITS2 in *M. dauricum* was 203 bp, with only one variable site observed in 34 sequences. The result of inter-specific sequence alignment showed that the length of ITS2 was 331 bp with 163 bp variable sites (49.2%). The sequence length of *psbA-trnH* in all *M. dauricum* samples was 315 bp, which indicated high consistency. The length of *psbA-trnH* sequences alignment was 743 bp with high variability (42.9%) across taxa. The GC contents of ITS2 and *psbA-trnH* of *M. dauricum* were 52.7% and 24.4%, which averaged 62.8% and 26.6%, respectively across all taxa (**Table 2**). One of the adulterants, *A. mollissima*, had the highest GC content with 74.4% in the ITS2 locus.

3.2. Intra- and Inter-Specific Genetic Distances and Species Identification

The intra- and inter-specific K2P distances of ITS2 and *psbA-trnH* of *M. dauricum* and its adulterants are presented in **Table 3**. All the experimental samples of *M. dauricum* were highly conserved in ITS2 and *psbA-trnH* regions. The maximum intra-specific distance (0.005 in ITS2) was due to a single C-A variation at the 82 bp site. The inter-specific K2P distances of the two sequences were expectedly higher, with an average of 0.457 and 0.428 for ITS2 and *psbA-trnH* respectively. NJ trees indicated that both sequences allowed the successful identification of all species; with each separate species grouped into distinct clades (**Figure 1** and **Figure 2**). All the sequences were examined by the Nearest Distance and BLAST1 methods. The successful identification rates of the two regions were 100% with the two methods.

4. Discussion

Fresh leaves have been traditionally used as the raw material for DNA barcoding. Currently, DNA barcoding is used to identify traditional Chinese medicines derived from other source tissues such as Rhizoma et Radix Notopterygii [15], Radix Gentianae Macrophyllae [16], Herba Ephedrae [17], and Flos Lonicerae Japonicae [18]. Chiou *et al*. [19] described that DNA extracted from various medicinal tissues and processed materials was usually degraded and even contaminated by microorganisms. For root and rhizome medicinal materials, the accumulation of secondary metabolites, such as polysaccharides and polyphenols, can affect the quantity and purity of the extracted DNA and the success of downstream applications, including PCR. The general approaches to improve the quality and quantity of DNA extracted from medicinal parts include extending the water bath time to 8 h - 12 h at 56˚C, increasing the amount of the sample, and simultaneously increasing the use of beta-mercaptoethanol and PVP. In addition, extracting the crushed samples by buffer for two to three times [20] will help to remove the polysaccharides and polyphenols in the medicinal parts of crude drugs. In this study, 36 rhizoma samples were collected, including 16 dried and long-stored rhizoma samples of commercial materials, and successfully

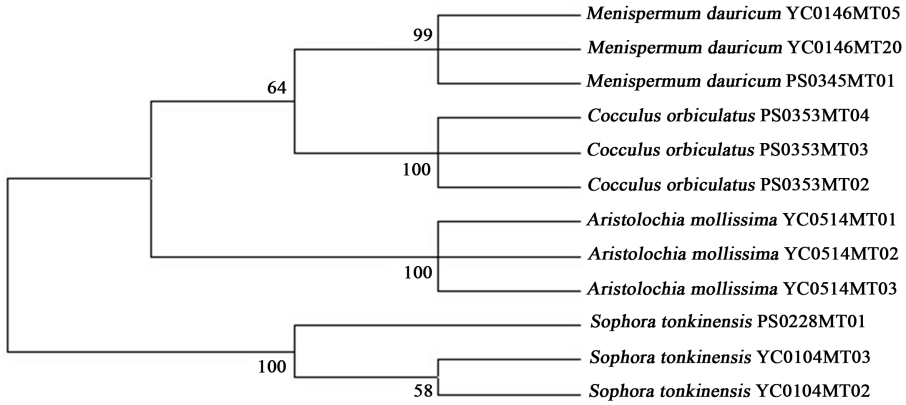

Figure 1. Phylogenetic tree of Rhizoma Menispermi and its adulterants constructed with the representative ITS2 sequences using NJ method. The bootstrap scores (1000 replicates) are shown (≥50%) for each branch.

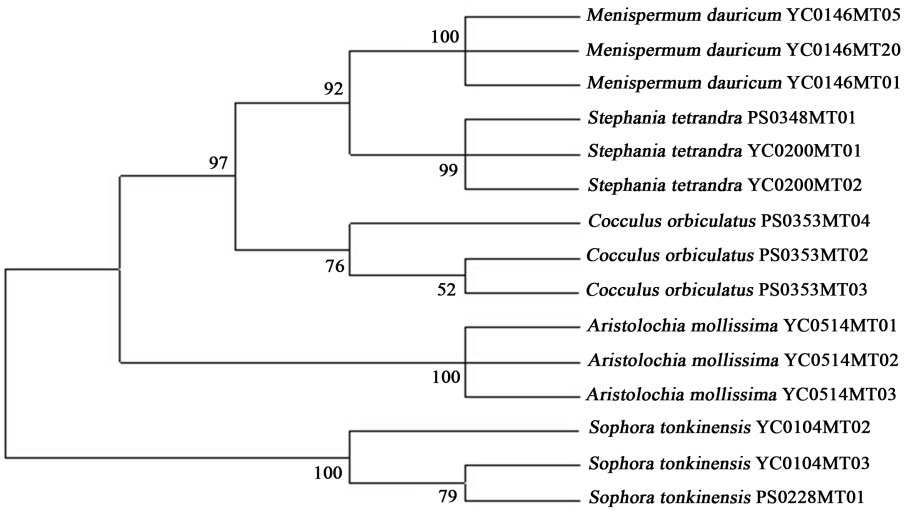

Figure 2. Phylogenetic tree of Rhizoma Menispermi and its adulterants constructed with the representative *psbA-trnH* sequences using NJ method. The bootstrap scores (1000 replicates) are shown (≥50%) for each branch.

Table 3. The K2P genetic distances of ITS2 and *psbA-trnH* sequences of Rhizoma Menispermi and its adulterants.

K2P genetic distances	Range of genetic distances (mean)	
	ITS2	*psbA-trnH*
Intra-specific distances of *M. dauricum*	0 - 0.005 (0.000)	0 (0.000)
Inter-specific distances between Rhizoma Menispermi and its adulterants	0.035 - 0.509 (0.457)	0.033 - 0.624 (0.428)

amplified by PCR. The result showed that the primers used were universal, and the ITS2 and *psbA-trnH* loci were easy to amplify. Wiping with 75% ethanol and scraping the outer epidermis helped prevent the risk of soil fungal contamination to a certain extent. No fungal sequence interference was observed during the experiments. Xu *et al*. [21] verified the operability of ITS2 locus between *S. tonkinensis* and its adulterants, including *M. dauricum*, using sequences downloaded from GenBank and several plant materials. DNA barcoding could identify fresh original plant samples of *S. tonkinensis* and its adulterants. However, no medicinal material samples were included. In this study, we focused on testing the applicability of DNA barcoding to distinguish medicinal materials.

New technology contributes to the success of identification methods. In the 2010 edition of *Chinese Pharmacopoeia*, allele-specific diagnostic PCR was recorded as a new method of identifying *Zaocys dhumnades* (Cantor). DNA barcoding technology uses universal primers and the same PCR reaction conditions across samples to construct a reference database. These factors increase the value of such a method in practical applications. The Food and Drug Administration approved DNA barcoding for seafood identification [22]. In the contemporary medicine market, this technology will help stop the spread of fake and adulterant materials with similar appearance to true medicines. Kool *et al*. [23] used root samples sold by herbalists in Marrakech, Morocco, to determine the applicability of DNA barcoding to modern drug markets. The results of their study showed that *rpoC*1, *psbA-trnH*, and ITS had high sequencing success rates, and combining all three loci allowed the identification of the majority of the market samples up to the genus level. DNA barcoding was used to successfully detect substitution and contamination in herbal products in North America [24]. These two previous studies indicated that DNA barcoding can be applied to the identification of commercial herbs. In the current study, we collected 16 commercial medicinal materials, including Rhizoma Menispermi, Radix et Rhizome Sophorae Tonkinensis, and Radix Stephaniae Tetrandrae. DNA is expected to show increasing degradation with an increase in storage time in dried commercial root drugs. We show that all the commercial materials were effectively identified, and the results were consistent with the information indicated on the medicinal drug labels. Sequence conservation in commercial medicinal materials and original samples from a single species indicated that DNA barcoding can consistently identify commercialized medicinal plants.

The *psbA-trnH* region is among the most variable non-coding regions in the chloroplast genome, and this variation means that this intergenic spacer can offer high levels of species discrimination [11]. However, Chase *et al*. [25] stated that a high rate of insertion/deletion may hamper application to barcoding. Agarose gel electrophoresis demonstrated that both ITS2 and *psbA-trnH* had high amplification efficiency. However, the sequencing efficiency of *psbA-trnH* sequences was almost 10% lower than that of the ITS2 sequences, even if bidirectional sequencing was used. This result was likely due to poly-A and poly-T structures in the *psbA-trnH* intergenic spacer. When a continuous repeated A or T structure emerges, the quality of the followed sequence will decrease, so the determination of the complete intergenic spacer is limited. The sequencing efficiency caused a 23.5% decline compared with the amplification efficiency in *M. dauricum*. In all samples used in this study, we also observed continuous repeated nucleotide structures belonging to Menispermaceae and *S. tonkinensis* (Leguminosae).

DNA barcoding technology has contributed to the development of taxonomy [6] and authentication, especially in traditional Chinese herbal medicine. A stable and accurate method is urgently needed for the circulation and standard management of traditional Chinese herbal medicines. This study is the first to use DNA barcoding to authenticate Rhizoma Menispermi (particularly commercial variants) and its adulterants. The stability and accuracy of the two regions (ITS2 and *psbA-trnH*) were examined. The intra-specific genetic distances and sequence alignment indicated that *M. dauricum* from different production areas had few variable sites in ITS2 and *psbA-trnH* sequences. The existence of multiple copies of the ITS2 region in plants may affect the stability of the obtained sequences. However, Song *et al*. [26] showed that despite multiple intra-genomic variants, the use of the major variants alone was sufficient for phylogenetic construction and species determination in most cases. The ITS2 sequences of *M. dauricum* were highly consistent; intra-specific variations were nearly absent. Multi-copies of ITS2 did not affect sequence amplification, sequencing, and analysis in *M. dauricum*. The high conservation of the ITS2 region was also discovered in *Panax ginseng* and *Panax quinquefolius* [27].

5. Conclusion

In conclusion, both ITS2 and *psbA-trnH* can be successfully amplified from commercial and natural samples of the important traditional Chinese medicine, Rhizoma Menispermi and its adulterants. As a result, accurate species identification and validation of product authenticity are facilitated. Compared with the ITS2 regions, the *psbA-trnH* sequence should not be the first choice for the DNA barcoding of Menispermaceae species on the basis of high poly-N structures and reduced sequencing efficiency. However, in consideration of the results for *S. tetrandra*, *psbA-trnH* can still be used as a complementary barcode for ITS2 in species identification.

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