Identification of plant materials containing ephedrine alkaloids based on DNA barcoding and TaqMan real-time PCR assay

Yaqin Zheng¹ · Han Gao¹ · Ming Song¹ · Yunhan Lin¹ · Jiajia Fan¹ · Yaqin Zhang¹ · Xia Liu¹

Received: 17 September 2019 / Revised: 14 June 2021 / Accepted: 28 August 2021 / Published online: 13 October 2021
© The Author(s) 2021

Abstract

An intentional or inadvertent mixing of plant materials containing ephedrine alkaloids, especially *Ephedra*, is illegal. In order to better detect plant materials containing ephedrine alkaloids in export and smuggling, DNA barcoding combined with a TaqMan real-time PCR-based assay were used in this study. We collected 201 samples from 18 species belonging to four genera distributed in three families to amplify two barcoding markers, internal transcribed spacer 2 (ITS2) and *psbA-trnH*. 175 ITS2 sequences and 136 *psbA-trnH* sequences were obtained. Alignments and the neighbor-joining tree indicated that ITS2 showed a better discrimination of species than *psbA-trnH*. In addition, based on the sequence comparison of the ITS2 region from 18 species, three sets of primer/probes were designed using the real-time PCR assay platform. A sensitivity test showed the above primer/probe sets were specific and sensitive (as little as 0.0050 ng/μL target DNA) to identify species containing ephedrine alkaloids. Hence, the combination of novel DNA barcoding and the TaqMan real-time PCR-based technique are promising tools for the identification of plant materials containing ephedrine alkaloids. It is beneficial to standardize market circulation and detection of plant materials containing ephedrine alkaloids.

Keywords Ephedrine alkaloids · *Ephedra L.* · DNA barcoding · ITS2 · *PsbA-trnH* · TaqMan real-time PCR

Introduction

Beginning in 1995, the China Food and Drug Administration (CFDA) enacted several laws to regulate the use of ephedrine alkaloids. One of the most publically known restrictions is the policy for cold medications. More notably, the US Food and Drug Administration (FDA) Import Alert 54-13 regarding the dietary supplements and bulk dietary ingredients containing ephedrine alkaloids, which went into effect on December 14, 2012. Consequently, plant materials

containing ephedrine alkaloids have triggered a wide concern in public health care safety around the world.

In China, plant species containing ephedrine alkaloids are generally found in the genus *Ephedra*, known as *ma huang* in Mandarin Chinese. *Ephedra* is the only genus in *Ephedraceae*, and it includes approximately 50 species around the world (Price 1996). There are ca. 14 *Ephedra* species in China, distributed mainly along shores, rocky beaches, and in sandy soils (Committee 1999). Apart from *Ephedra* (Caveney et al. 2001), some species also are known to contain ephedrine alkaloids, including *Pinellia* (Araceae), *Typhonium* (Araceae), and *Sida* (Malvaceae) (Caveney et al. 2001; Khatoon et al. 2005). Some species that do not contain ephedrine alkaloid and are easy to mix with ephedra herbs include *Equisetum hyemale* L., *Equisetum ramosissimum* Desf. et al.

Based upon a literature review, we identified 18 species containing ephedrine alkaloids distributed in China. Among them, *Ephedra sinica* Stapf, *Ephedra intermedia* Schrenk et C.A. Mey., *Ephedra equisetina* Bge., and *Pinellia ternata* (Thunb.) Breit. are described in the Chinese Pharmacopoeia, serving as a remedy for asthma, respiratory tract diseases, and vomiting in traditional Chinese and Indian medical

Communicated by J. Van Huylbroeck.

Yaqin Zheng and Han Gao have contributed equally to this work.

✉ Yaqin Zhang
zhangyaqin09@163.com

✉ Xia Liu
lrx1125@126.com

Yaqin Zheng
15927143211@163.com

¹ School of Chemistry, Chemical Engineering and Life Sciences, Wuhan University of Technology, Luoshi Road, Hongshan District, Wuhan 430070, China

systems (Committee 2015). *Sida cordifolia* Linn. is rarely used in China, but it is known as *Bala* in Ayurvedic medicine and widely used to treat illnesses like pulmonary tuberculosis, rheumatism, hematuria, urinary and heart diseases in India (Khatoon et al. 2005).

In addition to disease prevention, medicinal plants containing ephedrine alkaloids are widely used in health care products for fat loss, which are effective and popular, but often ephedrine alkaloids are added to these pills without proper labeling. Use in such a way may increase the risk of heart attack, stroke, and even death if misused (Haller and Benowitz 2002; Samenuk et al. 2002). Furthermore, medicinal plants containing ephedrine alkaloids have served as an important source of amphetamine in clandestine laboratories (Lee et al. 2006) and the lawless smuggling of medicinal plants containing ephedrine alkaloids continues to be a problem. Hence, it is urgent to develop a valid and sensitive method to effectively identify and detect plant materials containing ephedrine alkaloids to standardize the market circulation and ensure clinical safety.

Plant morphology and chemical analysis methods have long been used to identify plant materials containing ephedrine alkaloids (Sun et al. 2004; Kim et al. 2005; Li et al. 2008; Chatterjee et al. 2013). Nevertheless, if the diagnostic morphological features are not visible or overlooked, it is difficult to identify. For example, when the specimen is processed, it may no longer harbor its classical morphological features. Furthermore, chemical variability of the plant materials may obstruct the verification of the botanical traceability, as chemical composition varies with geographic condition and post-harvest processing (Figueredo et al. 2017).

Sequence-based technology has proven to be a powerful tool to authenticate species. DNA barcoding is diagnostic technology that uses a short, standard DNA fragments to distinguish species. It stands out for its advantages in generality and practicality without being affected by the morphological identification experience (Gu et al. 2013). In plants, the Consortium for the Barcode of Life (CBOL) recommends the combination of *matK* and *rcbL* as the core barcode. This combination has a high degree of species differentiation between genera and a low rate of species identification within genera (Hollingsworth et al. 2009). Recently, the internal transcribed spacer 2 (ITS2) has been shown to be useful in exploring systematic relatedness under genera and species (Goudsmit et al. 2003; Schultz and Wolf 2009). ITS2 sequence had an obvious advantage in the success rate of amplification and sequencing and the comparative similarity (Chen et al. 2010). And it was proposed to be a core barcode and *psbA-trnH* to be a complementary barcode for identifying medicinal plant species (Li et al. 2015). Many previous studies have validated that the combination of ITS2 and *psbA-trnH* possesses much more recoverability, universal

sequencing applicability, and species discrimination ability (Xiang et al. 2013; Han et al. 2016; Mishra et al. 2016; Carrotero and Hoyos 2018; Ha et al. 2018).

Real-time PCR is a technique that can quantify the nucleic acid copies based on different fluorescences. TaqMan real-time PCR is characterized by its high specificity and sensitivity, and has been widely applied in molecular fields such as clinical microbiology, gene expression, tumor immunology, detection of minimal residual lesions, and polymorphism (Ohst et al. 2015; Xu et al. 2016; Kading et al. 2017). Alasaad developed a TaqMan real-time PCR-based assay to identify *Fasciola* spp (Alasaad et al. 2011). Wu distinguished toxic *Aristolochia manshuriensis* from *Akebia quinata* to prevent renal damage using TaqMan real-time PCR-based assay (Wu et al. 2015).

In the present study, ITS2 and *psbA-trnH* were amplified from 201 samples, spanning four genera and three families. The *Ephedra* group included 128 samples from 11 species. The *Araceae* group contained 53 samples from four species. The *Sida* group included 20 samples from three species. TaqMan real-time PCR assays were conducted to detect medical plant materials containing ephedrine alkaloids. The aims of this study were to: (1) study the diversity of two barcode regions in several species and genera and (2) develop an assay to discriminate the different groups (*Ephedra* vs *Pinellia/Typhonium* vs *Sida*).

Materials and methods

Materials

Two-hundred and one sample belonging to *Ephedra*, *Pinellia*, *Sida*, and *Typhonium* were collected from Xinjiang, Inner Mongolia, Sichuan, Gansu, Beijing, Guangxi, Hainan, Yunnan, and Hubei Provinces (Table s1). All the samples were morphologically identified by Wei Sun at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. The stem and root samples collected from the field were dried on silica gel. The majority of *Ephedra* collected in Xinjiang were dried naturally. All corresponding voucher samples were further deposited in the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China. Forty-five ITS2 sequences and 17 *psbA-trnH* sequences were downloaded from GenBank for comparative and complementary analyses (Table s2).

DNA extraction

Total genomic DNA was extracted using Plant Genomic DNA Kits (Tiangen Biotech Co., China) following the manufacturer's protocol with the following adjustments: repeated

chloroform:isoamylol (24:1, v/v) treatment and a final DNA precipitation using ice cold isopropanol to precipitate DNA (Moyo et al. 2008).

DNA amplification and sequencing using primers of ITS2 and *psbA-trnH*

The ITS2 region was amplified from genomic DNA using two pairs of primers, S2F/3R or P3/E4 (Table S3) (Chen et al. 2010; Li et al. 2011). The *psbA-trnH* sequences were amplified using the universal primers: the *psbAF* and *trnHR* and another pair of primer fwd PA and rev TH (Table S3) (Chen et al. 2010; Li et al. 2011). The PCR reaction mixture consisted of 2 μ L (~35 ng) DNA, 12.5 μ L of 2 $\times$ Taq PCR Master Mix (Aidlab Biotechnologies Co., China), 1.0 μ L of 2.5 μ mol·L⁻¹ forward and reverse primers, respectively (synthesized by Sangon Biotech (Shanghai) Co., China), and distilled deionized water in a final volume of 25 μ L. General PCR conditions were conducted as previously reported (Kress et al. 2005). The PCR products were examined with 1.0% agarose gel electrophoresis and were sequenced bidirectionally using a 3730XL sequencer (Applied Biosystems, USA).

Sequence analysis

A Codon Code Aligner V 3.7.1 (CodonCode Co., USA) was used to proofread and assemble the sequencing peak diagrams. The ITS2 region was obtained based on the Hidden Markov model (HMM) to remove the 5.8S and 28S rRNA sections at both ends of the sequence on the ITS2 database (<http://its2.bioapps.biozentrum.uni-wuerzburg.de/>) (Keller et al. 2009). The *psbA-trnH* region was obtained after trimming the *psbA* gene and *trnH* gene by referencing the submitted sequence noted clearly in GenBank. All of the ITS2 and *psbA-trnH* sequences were deposited in GenBank, omitting accessions with identical sequence information. The GenBank IDs are as follows: all *psbA-trnH* sequences range from KX779046–KX779070, the ITS2 sequences for *Ephedra* range from KX779071–KX779088, and the ITS2 sequences for *Sida* are KX779089–KZ779111. The ITS2 sequences for *Pinellia* and *Typhonium* were KM236606, KM236618, KM236622, KM236659.

To evaluate the variability and the discriminating ability, MEGA 6.0 and Clustal W were applied. And the neighbor-joining (NJ) tree analysis was used for specie identification.

Designed primers and probes

To develop the TaqMan Real-time PCR, we firstly designed three groups of primers and probes. We divided the studied plant species into three groups according to their homology. In addition to the 11 studied species, another four species distributed in China without acquisition of samples, *E. saxatilis*, *E. gerardiana*, *E. likiangensis*, and *E. minuta*, were downloaded from GenBank to design the primers and probe for the *Ephedra* group. Primers and probes were designed by Applied Biosystems 7500 real-time quantitative PCR software—primer Express 3.0 using default parameters. Three groups of probes were purified using HPLC, all of the 5' ends of the oligonucleotides were attached to FAM (6-carboxy-fluorescein) reporter dye, and the 3' ends were labeled with a TAMARA (6-carboxy-tetramethyl-rhodamine) quencher dye. Both the primers and probes were synthesized by Invitrogen (Life Technologies, China; Table 1).

To guarantee the accuracy of TaqMan Real-time PCR, the specificity of the three pairs of primers was tested as follows. The genomic DNA was firstly examined by the amplification of ITS2 region using universal primers mentioned above. Once the quality of DNA was examined, the DNA was used as template to conduct regular PCR using the designed specific primers. In *Ephedra* group, all the 21 haplotypes from 141 *Ephedra* samples and one representative sample from *Pinellia* and *Sida* were amplified using the specific primers of *Ephedra* group. The amplification products were inspected with gel electrophoresis. The same assays were conducted in the *Pinellia* and *Typhonium* group, *Sida* group.

TaqMan real-time PCR assay

Amplification reactions contained 0.5 μ L of 10 mol·L⁻¹ primer, 0.5 μ L of 10 mol·L⁻¹ of TaqMan probe, 10 μ L of 2 $\times$ Goldstar TaqMan Mixture with ROX (TaKaRa Biomedical Technology (Beijing) Co. China), 1 μ L of DNA solution (replaced by water for negative controls), and nuclease free water in a final volume of 20 μ L. Cycling conditions for the

Table 1 The primer and probe of three sets

Set	Left primer	Right primer	Taqman probe
<i>Ephedra</i>	5'-GCGGTCGGTTGAAATRCAR-3'	5'-AATAAMTCCCGCCTTYGRTG-3'	5'-TCCGACGGTGGGAGGTTGC-3'
<i>Sida</i>	5'-GGTTGAAGTGTGGGCGGAA-3'	5'-GAATTGACGAGCACACACGA-3'	5'-TGGCTTCCCGTGCCTCACC-3'
<i>Pinellia</i> and <i>Typhonium</i>	5'-YGVGGGATGCGGAGAWTGG-3'	5'-TTMYCTCSGAYGRHYGGGTT-3'	5'-CCGYGCACAAGGRYRGGMCC-3'

According to the homology, the experimental species were divided into three groups. Three groups primers and probes were designed

PCR consisted of a 10 min start up denaturation step at 94 °C, followed by 40 cycles of amplification for 15 s at 94 °C and 1 min at 60 °C.

Assessment of sensitivity of the TaqMan assay

To evaluate the specificity, all the 42 haplotypes covered 201 ephedrine alkaloids containing plant samples were used as template to develop the TaqMan Real-time PCR. The sensitivity of our assay was assessed using a tenfold dilution series (between 80 ng/μL and 8 ng/μL) and a twofold dilution series (between 8 ng/μL and 0.0050 ng/μL) of *E. sinica* gDNA. The detection limit was based on the final dilution at which the fluorescent signal of the TaqMan probes can still be exponentially amplified.

Results

Efficiency of the PCR amplification

In the *Ephedra* group, the success rates of PCR amplification of ITS2 and *psbA-trnH* sequences were 88.4 and 92.2%, respectively. Considering *Sida*, the PCR amplification success rates for the two regions were both 45%. However, in the *Pinellia* and *Typhonium* group, the success rate of the PCR amplification for ITS2 was 100% using the primer pair

P3/E4, and 62.5% using the pair S2F/3R. The primer pair PA/TH for the *psbA-trnH* region had 12.5 and 75% success amplification rates, respectively, for *P. ternata* and *P. pedatisecta*. Considering the low amplification rate for *psbA-trnH* in *Pinellia* and *Typhonium*, another primer pair, fwd/rev, was utilized. Nevertheless, the amplification rate success for *psbA-trnH* in *P. ternata* was 36.1, and only 30.8% of the PCR products were sequenced. No successful amplification was obtained for *P. cordata* and *T. flagelliforme* using the PA/TH and fwd/re primer pairs.

Alignment of ITS2 sequences and *psbA-trnH* sequences

The GC content was calculated and variable sites were analyzed, the characteristics of the ITS2 sequences and *psbA-trnH* sequences of the ephedrine alkaloids containing plants were summarized in Table 2 and Table 3, respectively.

The length of the obtained ITS2 region from the 11 species of *Ephedra* in this study was 251 bp with 21 haplotypes (Figure S1). The length of the ITS2 region for *P. ternata*, *P. cordata*, *P. pedatisecta*, and *T. flagelliforme* was 251, 250, 252, and 239 bp, respectively. For these four species, the aligned length of ITS2 was 255 bp, with 16 existing haplotypes and 71 variable sites in total. The length of ITS2 among *Sida* species was 230 bp. Only two haplotypes and two nucleotide variation sites were detected among *Sida* spp.

Table 2 The characteristics of ITS2 sequences of the ephedrine alkaloids containing plants

Species Name	Sequence number	Sequence length (bp)	GC content (%)	Haplotype number	No. of variable sites	No. of Pi sites	No. of singletons
<i>Ephedra sinica</i>	23	251	65.4	1	0	0	0
<i>Ephedra intermedia</i>	32	251	65.4	3	2	0	2
<i>Ephedra equisetina</i>	26	251	65.1	5	5	3	2
<i>Ephedra monosperma</i>	9	251	65.5	3	5	5	0
<i>Ephedra przewalskii</i>	15	251	65.2	2	1	0	0
<i>Ephedra intermedia</i> var. <i>tibetica</i>	4	251	65.4	1	0	0	0
<i>Ephedra distachya</i>	10	251	65.4	2	2	1	1
<i>Ephedra glauca</i>	6	251	65.4	1	0	0	0
<i>Ephedra regeliana</i>	4	251	65.3	1	0	0	0
<i>Ephedra lomatolepis</i>	10	251	65.4	1	0	0	0
<i>Ephedra fedtschenkoae</i>	2	251	65.0	1	0	0	0
<i>Pinellia ternata</i>	37	251	73.8	11	7	3	4
<i>Typhonium flagelliforme</i>	2	239	69.0	1	0	0	0
<i>Pinellia cordata</i>	3	250	72.0	2	3	1	1
<i>Pinellia pedatisecta</i>	14	252	73.8	2	3	0	3
<i>Sida rhombifolia</i>	3	230	55.9	2	2	2	0
<i>Sida alnifolia</i>	3	230	56.1	1	0	0	0
<i>Sida cordifolia</i>	4	230	55.7	2	2	0	2

In the table, ITS2 sequence number, G + C content, sequence length, haplotype number, variable site number and other information of *Ephedra* group, Araceae group and *Sida* group were shown

Table 3 The characteristics of *psbA-trnH* sequences of the ephedrine alkaloids containing plants

Specie Name	Sample number	Sequence length(bp)	GC content (%)	No. of haplo-type	No. of variable sites	No. of Pi sites	No. of single-tons	No. of deletions
<i>Ephedra sinica</i>	22	461	38.0	2	1	0	1	N
<i>Ephedra intermedia</i>	31	461	38.1	2	0	0	0	2/31
<i>Ephedra equisetina</i>	33	461,473	38.1	2	0	0	0	3/33
<i>Ephedra monosperma</i>	10	473	38.2	2	1	0	1	N
<i>Ephedra przewalskii</i>	12	461	38.0	1	0	0	0	N
<i>Ephedra intermedia</i> var. <i>tibetica</i>	4	461	38.0	1	0	0	0	N
<i>Ephedra distachya</i>	11	461	38.0	1	0	0	0	N
<i>Ephedra glauca</i>	4	461	38.0	1	0	0	0	N
<i>Ephedra regeliana</i>	5	461	38.0	2	0	0	0	2/5
<i>Ephedra lomatolepis</i>	3	461	38.0	1	0	0	0	N
<i>Ephedra fedtschenkoae</i>	3	473	38.1	2	0	0	0	2/3
<i>Pinellia ternata</i>	3	629	20.4	2	1	0	1	N
<i>Pinellia pedatisecta</i>	7	490	14.6	3	6	0	6	N
<i>Sida rhombifolia</i>	4	432–454	20.6	4	17	10	6	3/4
<i>Sida alnifolia</i>	2	341	20.7	2	3	0	3	N
<i>Sida cordifolia</i>	3	375–381	20.3	2	1	0	1	N

In the table, the *psbA-trnH* sequence number, G + C content, sequence length, haplotype number, variable site number, insertion or deletion and other information of the *Ephedra* group, Araceae group and *Sida* group were shown. “N” means no insertion or deletion in the sequence

For *psbA-trnH* sequences, the GC content among *Ephedra* species was approximately 38.0%. The sequence length in eight *Ephedra* spp. was 461 bp, and the sequence length was 473 bp in *E. fedtschenkoae* and *E. monosperma*. However, the length *psbA-trnH* in *E. equisetina* was 461 bp and 473 bp. From the multiple sequence alignments of *psbA-trnH* of *Ephedra*, we found that the differences consisted of insertions, deletions, and single nucleotide changes. Therein, only *E. sinica* showed a C/T transition (1/19) and *E. monosperma* showed A/C transversion (4/10). Notably, the single nucleotide was a “C” at site 300 in *E. monosperma* in the four *psbA-trnH* sequences obtained from individuals collected in Sichuan, while sequences from individuals collected in Xinjiang and Gansu read as “A”. Compared with the corresponding *psbA-trnH* sequence downloaded from GenBank, deletions or insertion of 12 bases were found in *E. intermedia*, *E. regeliana* and *E. fedtschenkoae*, which was the base “TTGGATTTCCTG”. In all the 33 *psbA-trnH* sequences of *E. equisetina*, 3 experimental sequences existed 12 bases deletion compared with other experimental sequences and those downloaded by GenBank.

Phylogenetic analysis

In this study, a phylogenetic tree based on Kimura 2 parameter (K2P) model was constructed for both ITS2 and *psbA-trnH* sequences. The NJ-tree based on ITS2 sequences

demonstrated that the three groups clustered into three clades (Fig. 1). In the *Ephedra* clade, the haplotypes of *E. intermedia*, *E. przewalskii*, *E. distachya* dispersed to different group. The remaining nine *Ephedra* species clustered into one subclade. In the *Pinellia* and *Typhonium* clade, four species grouped into four subclades, illustrating monophyly for these genera. The three *Sida* species included in this study grouped into one clade.

The NJ tree based upon *psbA-trnH* sequences was constructed without *P. cordata* and *T. flagelliforme*, as no sequences were available on GenBank or obtained in our study (Fig. 2). The *psbA-trnH* NJ tree showed that *Ephedra*, *Sida*, *Pinellia*, grouped into three clades. Seventeen haplotypes of 11 *Ephedra* species grouped into one clade, with no subclades, and *P. ternata* and *P. pedatisecta* grouped into two branches. Four species of *Sida* clustered together.

The specificity of the designed primers

The ITS2 regions of all the 42 haplotypes of ephedrine alkaloids containing plant materials, including *Ephedra*, *Pinellia*, *Typhonium* and *Sida*, were successfully amplified firstly (Figure S2). We demonstrated that the genomic DNA was successfully extracted and could be used as templates to evaluate the specificity of primers. All 21 haplotype samples from *Ephedra* generated PCR products of 166 bp,

Bootstrap was repeated for 1000 times, and the results showed that the self-developing support rate was $\geq 50\%$

Fig. 2 The NJ tree of plant materials containing ephedrine alkaloids constructed with *psbA-trnH* sequences

Fig. 3 The specific assay of primers of *Ephedra* group. M: DL2000; 1: *Ephedra sinica*; 2–4: three haplotypes of *Ephedra intermedia*; 5–9: five haplotypes of *Ephedra equisetina*; 10–12: three haplotypes of *Ephedra intermedia*; 13–14: two haplotypes of *Ephedra*

przewalskii; 15: *Ephedra intermedia* var. *tibetica*; 16–17: two haplotypes of *Ephedra distachya*; 18: *Ephedra glauca*; 19: *Ephedra regeliana*; 20: *Ephedra lomatolepis*; 21: *Ephedra fedtschenkoae*; 22: one haplotype of *Pinellia ternata*; 23: one haplotype of *Sida rhombifolia*

observed with the primers and probe from the *Pinellia* and *Typhonium* group or the *Sida* group. Four species of *Pinellia* and *Typhonium* showed normal fluorescence amplification only when the primers and probe from the *Pinellia* and *Typhonium* group was used. The same is true for the *Sida*

group. Results from the Real-time PCR showed that all haplotypes generated normal fluorescence amplification curves with CT < 35.

The results of a series TaqMan qPCR assays on diluted genomic DNA of *E. sinica* showed that fluorescence

Fig. 4 The specific assay of primers of *Pinellia* and *Typhonium* group. M: DL2000; 24–34: eleven haplotypes of *Pinellia ternate*; 35: *Typhonium flagelliforme*; 36–37: two haplotypes of *Pinellia cordata*;

38–39: two haplotypes of *Pinellia pedatisecta*; 40: *Ephedra sinica*; 41: one haplotype of *Sida rhombifolia*

Fig. 5 The specific assay of primers of *Sida* group. M: DL2000; 42–43: two haplotypes of *Sida rhombifolia*; 44: *Sida alnifolia*; 45–46: two haplotypes of *Sida cordifolia*; 47: *Ephedra sinica*; 48: one haplotype of *Pinellia ternate*

amplification curves were exponential at 0.0050 ng/μL and the fluorescence signal was no longer normal at 0.0025 ng/μL. This indicated that the minimum amount of genomic DNA of *E. sinica* detected with the TaqMan qPCR was about 0.0050 ng/μL (Figure S3).

Discussion

The comparison of the ITS2 and *psbA-trnH* barcodes used for identification of ephedrine alkaloid containing species

An ideal barcode should be easily amplified and sequenced (Taberlet et al. 2007). In the present study, 175 ITS2 sequences and 136 *psbA-trnH* sequences from 201 specimens of 18 species were obtained, indicating that the utilized ITS2 primers are more universally applicable and

can be utilized to trace botanical origin from ephedrine alkaloid containing plant species. This can be attributed to several reasons: (i) the length of ITS2 is much smaller than *psbA-trnH*, varying between 230 and 252 bp, which makes it easier to amplify and sequence; (ii) The efficiency of PCR amplification and sequencing of ITS2 is higher than *psbA-trnH*; and (iii) High-quality sequencing peak diagrams of *psbA-trnH* of *Sida* were difficult to obtain because of the existence of poly A or T sequence.

The *psbA-trnH* region is more applicable to distinguish *Ephedra*, as the entire region is highly conserved, and *E. monosperma*, *E. equisetina*, and *E. fedtschenkoae* individuals had unique insertions, which could be used for identification purposes. The stable single change at site 300 in *E. monosperma* could provide geographical information because the *psbA-trnH* sequences from *E. intermedia*, *E. regeliana*, and *E. fedtschenkoae* did not match the *psbA-trnH* sequences obtained from GenBank. However, the 12 base insertion or deletion could be due to geographical variation (Lahaye et al. 2008) or pseudogene amplification. For *E. sinica*, *E. glauca*, *E. lomatolepis*, *E. distachya*, *E. intermedia* var. *tibetica*, and *E. przewalskii*, both ITS2 and *psbA-trnH* sequences proved inappropriate to differentiate among species. A more informative barcode is needed to distinguish these species.

In this study, we did not experiment with easily mixed species that did not contain ephedrine. Because compared to ephedrine-containing species, their genetic distance and sequence are quite different. Our results indicated that two universal primer pairs, PA/TH and fwd/rev, showed no successful amplification and sequencing in *P. cordata* and *T. flagelliforme*, which suggested that more applicable primers need to be designed.

DNA barcoding and TaqMan probe technology were used to trace the ephedrine alkaloid containing plant materials

A previous study documented that specific primers based on the difference of *psbA-trnH* in *Ephedra* can identify *Ephedra* DNA in dietary supplements using PCR (Tehen et al. 2006). However, in our work, *psbA-trnH* cannot be amplified and successfully sequenced in several species. In addition, some species also had no referenced *psbA-trnH* sequences in GenBank. ITS2 stood out for its universality and accessibility. Therefore, we selected ITS2 to design primers and probes. The TaqMan assay was specific and sensitive enough to detect as little as 0.0050 ng/μL of DNA in our samples. In addition, under normal PCR conditions, results showed no smeared or invisible bands when visualized using electrophoretic analysis at ~0.0050 ng/μL of DNA.

The significance of using DNA barcoding and TaqMan probe technology

Although the supervision and administration of medical plant materials containing ephedrine alkaloids are strict, cases of smuggling are still prevalent. The identification of medical plant materials containing ephedrine alkaloids is of great importance not only for ensuring safety but for controlling reasonable market circulation. The present work developed a rapid, specific, and highly sensitive method to detect plant materials containing ephedrine alkaloids. We tested 18 species in this study, covering the most common medical species containing ephedrine alkaloids in China, and more than 90% of the ephedrine alkaloid containing plant species listed on the FDA Poisonous Plant Database and European Food Safety Authority. Because of our large sample size, we had a high success rate in identifying existing ephedrine-containing species. If new ephedrine-contained species appear, the false positive or false negative results might occur due to the lack of database information. In case of false negative result, we need to further sequence the new species and determine whether these primers and probes are suitable for them by comparing the sequence information with the current ephedrine-containing species. For the false positive result, we could further detect the content of ephedrine by chemical method to ensure the accuracy of result.

Conclusion

In this paper, we evaluated the feasibility of the ITS2 and *psbA-trnH* region as candidate barcodes to discriminate the 201 ephedrine alkaloids containing samples. ITS2 proved

advantageous over *psbA-trnH*. Further, based on DNA barcoding, we developed a specific and sensitive TaqMan real-time PCR assay (as little as 0.0050 ng/μL target DNA) to distinguish plant species containing ephedrine alkaloids. Our study demonstrated that DNA barcoding along with TaqMan technology can be used to detect plant materials containing ephedrine alkaloids rapidly and sensitively. This study could be an assistant method to detect plant materials containing ephedrine alkaloids. In particular, it may also have general guiding implications in the examination of dietary supplements and ephedrine alkaloid containing powdered materials.

Author contribution statement YZ and HG analyzed the data and wrote the manuscript; MS, YL and JF collected the materials and performed the experiments; XL and YZ designed and guided experiments. All the authors agreed on the contents of the paper and have no conflicting interests to declare.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11738-021-03304-3>.

Acknowledgements This research was supported by the Major Scientific and Technological Special Project for “Significant New Drugs Creation” (No. 2014ZX09304307).

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Alasaad S, Soriguer RC, Abu-Madi M, El Behairy A, Jowers MJ, Banos PD et al (2011) A TaqMan real-time PCR-based assay for the identification of *Fasciola* spp. *Vet Parasitol* 179(1–3):266–271. <https://doi.org/10.1016/j.vetpar.2011.01.059>
- Carrero D, Hoyos R (2018) Molecular identification and genetic diversity of *Lutzomyia gomezi* (Diptera: Psychodidae) using DNA barcodes in Cordoba. *Colomb Trop Biomed* 35(1):100–110
- Caveney S, Charlet DA, Freitag H, Maier-stolte M, Starratt AN (2001) New observations on the secondary chemistry of world ephedra (ephedraceae). *Am J Bot* 88(7):1199–1208
- Chatterjee A, Kumar S, Chattopadhyay SK (2013) A validated HPLC-PDA method for identification and quantification of two bioactive alkaloids, ephedrine and cryptolepine, in different *Sidaspecies*.

- Biomed Chromatogr 27(12):1720–1725. <https://doi.org/10.1002/bmc.2985>
- Chen S, Yao H, Han J, Liu C, Song J, Shi L et al (2010) Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One* 5(1):e8613
- Committee (1999) Flora of China. Science Press, Beijing
- Chinese Pharmacopoeia Commission (2015) Chinese pharmacopoeia. China Medical Science Press, Beijing
- Figueredo G, Özcan MM, Chalchat JC, Chalard P, Çelik B, Özcan MM (2017) The effect of harvest years on chemical composition of essential oil of Basil (*Ocimum minimum* L.) leaves. *J Essent Oil Bear Plants* 20(6):1–5
- Goudsmit N, Coleman E, Seckinger R, Wolitzky R, Stanford A, Corcoran C et al (2003) A brief smell identification test discriminates between deficit and non-deficit schizophrenia. *Psychiatry Res* 120(2):155–164. [https://doi.org/10.1016/S0165-1781\(03\)00194-X](https://doi.org/10.1016/S0165-1781(03)00194-X)
- Gu W, Song J, Cao Y, Sun Q, Yao H, Wu Q et al (2013) Application of the ITS2 region for barcoding medicinal plants of Selaginellaceae in pteridophyta. *PLoS One* 8(6):e67818. <https://doi.org/10.1371/journal.pone.0067818>
- Ha TT, Huang NT, Hung NP, Guiguen Y (2018) Species identification using DNA barcoding on processed Panga catfish products in Viet Nam revealed important mislabeling. *Turk J Fish Aquat Sci* 18:457–462
- Haller CA, Benowitz NL (2002) Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. *N Engl J Med* 343(25):1833–1838
- Han J, Pang X, Liao B, Yao H, Song J, Chen S (2016) An authenticity survey of herbal medicines from markets in China using DNA barcoding. *Sci Rep* 6:18723. <https://doi.org/10.1038/srep18723>
- Hollingsworth P, Forrest L, Spouge J, Hajibabaei M, Ratnasingham S, Chase M et al (2009) A DNA barcode for land plants. *Proc Natl Acad Sci* 106(31):12794–12797. <https://doi.org/10.1073/pnas.0905845106>
- Kading RC, Wadhwa A, Wilkins K, Gao J, Gigante CM, Zhao H et al (2017) A pan-lyssavirus Taqman real-time RT-PCR assay for the detection of highly variable rabies virus and other lyssaviruses. *PLOS Negl Trop Dis* 11(1):e0005258
- Keller A, Schleicher T, Schultz J, Müller T, Dandekar T, Wolf M (2009) 5.8S–28S rRNA interaction and HMM-based ITS2 annotation. *Gene* 430(1–2):50–57. <https://doi.org/10.1016/j.gene.2008.10.012>
- Khatoun S, Srivastava M, Rawat A, Mehrotra S (2005) HPTLC method for chemical standardization of Sidaspecies and estimation of the alkaloid ephedrine. *J Planar Chromatogr-Mod TLC* 18(105):364–367. <https://doi.org/10.1556/JPC.18.2005.5.5>
- Kim HK, Choi YH, Erkelens C, Lefeber AW, Verpoorte R (2005) Metabolic fingerprinting of ephedra species using 1H-NMR spectroscopy and principal component analysis. *Chem Pharm Bull* 53(1):105–109
- Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. *Proc Natl Acad Sci* 102(23):8369–8374
- Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F, Gigot G et al (2008) DNA barcoding the floras of biodiversity hotspots. *Proc Natl Acad Sci* 105(8):2923–2928
- Lee JS, Han EY, Lee SY, Kim EM, Park YH, Lim MA et al (2006) Analysis of the impurities in the methamphetamine synthesized by three different methods from ephedrine and pseudoephedrine. *Forensic Sci Int* 161(2–3):209–215. <https://doi.org/10.1016/j.forsciint.2006.02.054>
- Li YW, Zhou FQ, Zhang SP, Li ZY, Kong QY (2008) Studies on morphology and histology of *Pinellia pedatisecta*. *Chin Med Mat* 31(2):206–209
- Li D, Liu J, Chen Z, Wang H, GE X, Zhou S et al (2011) Plant DNA barcoding in China. *J Syst Evol* 49(3):165–168. <https://doi.org/10.1111/j.1759-6831.2011.00137.x>
- Li X, Yang Y, Henry RJ, Rossetto M, Wang Y, Chen S (2015) Plant DNA barcoding: from gene to genome. *Biol Rev* 90(1):157–166. <https://doi.org/10.1111/brv.12104>
- Mishra P, Kumar A, Nagireddy A, Mani DN, Shukla AK, Tiwari R et al (2016) DNA barcoding: an efficient tool to overcome authentication challenges in the herbal market. *Plant Biotechnol J* 14(1):8–21. <https://doi.org/10.1111/pbi.12419>
- Moyo M, Amoo SO, Bairu MW, Finnie JF, Van Staden J (2008) Optimising DNA isolation for medicinal plants. *S Afr J Bot* 74(4):771–775. <https://doi.org/10.1016/j.sajb.2008.07.001>
- Ohst T, Kupsch C, Gräser Y (2015) Detection of common dermatophytes in clinical specimens using a simple quantitative real-time TaqMan polymerase chain reaction assay. *Br J Dermatol* 174(3):602–609. <https://doi.org/10.1111/bjd.14198>
- Price RA (1996) Systematics of the gnetales: a review of morphological and molecular evidence. *Int J Plant Sci* 157(6 Suppl):S40–S49
- Samenuk D, Link MS, Homoud MK, Contreras R, Theohardes TC, Wang PJ (2002) Adverse cardiovascular events temporally associated with ma huang, an herbal source of ephedrine. *Mayo Clin Proc* 77(1):12–16. <https://doi.org/10.4065/77.1.12>
- Schultz J, Wolf M (2009) ITS2 sequence-structure analysis in phylogenetics: a how-to manual for molecular systematics. *Mol Phylogenet Evol* 52(2):520–523. <https://doi.org/10.1016/j.ympev.2009.01.008>
- Sun S, Zhou Q, Liu J, Huang H (2004) Study on the identification of standard and false BanXia by two-dimensional infrared correlation spectroscopy. *Spectrosc Spect Anal* 24(4):427–430
- Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A et al (2007) Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Res* 35(3):e14. <https://doi.org/10.1093/nar/gkl938>
- Techen N, Khan IA, Pan Z, Scheffler BE (2006) The use of polymerase chain reaction (PCR) for the identification of ephedra DNA in dietary supplements. *Planta Med* 72(3):241–247
- Wu L, Sun W, Wang B, Zhao H, Li Y, Cai S et al (2015) An integrated system for identifying the hidden assassins in traditional medicines containing aristolochic acids. *Sci Rep* 5(1):11318
- Xiang L, Song J, Xin T, Zhu Y, Shi L, Xu X et al (2013) DNA barcoding the commercial Chinese caterpillar fungus. *FEMS Microbiol Lett* 347(2):156–162. <https://doi.org/10.1111/1574-6968.12233>
- Xu H, Ma Y, Zhang Y, Pan Z, Lu Y, Liu P et al (2016) Identification of cathepsin K in the peritoneal metastasis of ovarian carcinoma using in-silico. *Gene Expression Analysis Journal of Cancer* 7(6):722–729

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.