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Identification of medicinal plant *Schisandra chinensis* using a potential DNA barcode ITS2

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Abstract

To test whether the internal transcribed spacer 2 (ITS2) region is an effective marker for using in authenticating of the *Schisandra chinensis* at the species and population levels, separately. And the results showed that the wild populations had higher percentage of individuals that had substitution of C→A at site 86-bp than the cultivated populations. At sites 10-bp, 37-bp, 42-bp and 235-bp, these bases of the *Schisandra sphenanthera* samples differed from that of *S. chinensis*. Two species showed higher levels of inter-specific divergence than intra-specific divergence within ITS2 sequences. However, 24 populations did not demonstrate much difference as inter-specific and intra-specific divergences were concerned. Both *S. chinensis* and *S. sphenanthera* showed monophyly at species level, yet the samples of different populations shown polyphyly at population level. ITS2 performed well when using BLAST1 method. ITS2 obtained 100% identification success rates at the species level for *S. chinensis*, with no ambiguous identification at the genus level for ITS2 alone. The ITS2 region could be used to identify *S. chinensis* and *S. sphenanthera* in the "Chinese Pharmacopoeia". And it could also correctly distinguish 100% of species and 100% of genera from the 193 sequences of *S. chinensis*. Hence, the ITS2 is a powerful and efficient tool for species identification of *S. chinensis*.

Keywords: DNA barcode, ITS2, *Schisandra chinensis*, species, populations

Introduction

Schisandra chinensis (Turcz.) Baill. grows mainly in the northeast China and its mature fruits are famous traditional Chinese medicine recorded in "Chinese Pharmacopoeia". As a traditional medicinal herb, *S. chinensis* has been used as an astringent curing dry cough, asthma, night sweats, nocturnal seminal emissions and chronic diarrhea [1,2]. Modern medical research had proved that *S. chinensis* contains multiple active components used to protect liver [3–5], prevent senility [6–9], restrain oxidation [10,11], improve human body immunity ability [12], regulate the central nervous system and so on [13,14].

Commonly *S. chinensis* and its adulterants are frequently found in the market together. *Schisandra sphenanthera* Rehd. et Wils. is important traditional Chinese medicine recorded in "Chinese Pharmacopoeia", too, but its active components of the kinds and levels are clearly different from *S. chinensis* [15]. Conventional viewpoints are that the medicinal value of *S. chinensis* is better than *S. sphenanthera*. But the two medicines are often mistaken for each other [16]. The identification of the two species of *Schisandra* is difficult when based solely on morphological characteristics. Additionally, some limitations in traditional taxonomy prevent this technique from meeting the complicated demands of species recognition [17]. As such, a method for the simple and accurate authentication of *Schisandra* is indispensible.

As yet some works had been done to differentiation or identification of *Schisandra* species using RAPD, ISSR, *rbcL* and ITS [18–20]. Recently, being part of ITS, ITS2 was relatively easy to be amplified using one pair of universal primers [21,22]. In addition, ITS2 had been found to provide taxonomic signature in systematic evolution [23,24]. The ITS2 region was also a promising potential molecular marker to be used for rapid taxonomic classification [21,22]. To our best knowledge, applying ITS2 region to identify plant materials from *Schisandra* with such a large sample size and geographic range had not been reported. And these studies were not found whether the ITS2 region could be used as a genomic marker to identify different *Schisandra* populations from different ecological environment and geographical distribution. In the current study, we utilized ITS2 as a DNA barcode to distinguish medicinal plants within the *Schisandra* genus and populations in order to ensure their safe, effective application in traditional using.

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Material and methods

Plant materials

In our study, 217 samples – 193 samples of *S. chinensis* and 24 samples of *S. sphenanthera*, which belonged to 24 populations from two species, were collected from 17 counties of nine provinces in China between June and August 2012 (Tab. 1). All subjects were identified by Professor Bing Wang, Liaoning University of Traditional Chinese Medicine, China. The voucher samples were deposited in the herbarium of Liaoning University of Traditional Chinese Medicine.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from silica gel-dried leaves according to the protocol associated with the Plant Genomic DNA Kit (Tiangen Biotech Co., China). Polymerase chain reaction (PCR) amplification of the ITS2 region was carried out in the Peltier Thermal Cycler PTC0200 (BioRad Lab Inc., USA) using approximately 30 ng of genomic DNA as a template in a 25-µl reaction mixture [1× PCR buffer without $MgCl_2$, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer (synthesized by Sangon Co., China)], and 1.0 U of Taq DNA Polymerase (Biocolor BioScience & Technology Co., China). ITS2 primer and PCR amplification for the ITS2 region were conducted as described previously [25]. The PCR products were run on a 1.2% agarose gel in 0.5× TBE buffer and purified with the TIANGel Midi Purification Kit (Tiangen Biotech Co., China). The purified PCR products were sequenced on an ABI 3730XL sequencer (Applied Biosystems Inc.) using the amplification primers.

Sequence alignment and data analysis

Contig assembly and the generation of consensus sequences were performed using CodonCode Aligner ver. 3.0 (CodonCode Co., USA). The ITS2 sequences from GenBank were subjected to hidden Markov model (HMM) model analysis of HMMer annotation to remove the conserved 5.8S and 26S (or equivalent) rRNA sequences [26–28]. And we also used HMMer based annotation on well-curated fungal sequences to search for downloaded ITS2 sequences to remove the possible contaminated sequences of fungi. The sequences were then aligned using Clustal W and the genetic distances were computed using MEGA 5.0 according to the Kimura 2-parameter (K2P) model [29,30]. The average intra-specific distance, coalescent depth and Theta were calculated to evaluate the intra-specific variation using the K2P model [22,31]. The average inter-specific distance, the minimum inter-specific distance and Theta prime were used to represent inter-specific divergences [22,31,32]. The distributions of intra- vs. inter-specific variability were compared using DNA barcoding gaps [22,31,33]. Wilcoxon two-sample tests were performed as described previously [22,33,34]. Two methods of species identification, including NJ tree and BLAST1-based methods, were performed as described previously [35]. In the BLAST1 method, correct identification means that the best basic local alignment search tool (BLAST) hits of the query sequence is from the expected species; ambiguous identification means that the best BLAST hits for a query sequence is found to be those of several species including the expected species; incorrect identification means that the best BLAST hits of the query sequence is not from the expected species.

Tab. 1 Plant samples of *Schisandra* used in the present study.

Species	Population codes	Collected place	Statement	Number	Accession number
Schisandra chinensis (Turcz.) Baill.	BG	Donggang, Liaoning, China	Cultivated	BG1-8	AB558158.1
	BJ	Yanqing, Beijing, China	Wild	BJ1-2	AB558158.1
	CH	Wangqing, Jilin, China	Cultivated	CH1-10	AB558158.1
	FX	Fengcheng, Liaoning, China	Cultivated	FX1-4	AB558158.1
	KD	Kuandian, Liaoning, China	Wild	KD1-10	AB558158.1
	HG	Donggang, Liaoning, China	Cultivated	HG1-5	AB558158.1
	LH	Fusong, Jilin, China	Wild	LH1-10	AB558158.1
	LJ	Linjiang, Jilin, China	Wild	LJ1-10	AB558158.1
	MJ	Mudanjiang, Heilongjiang, China	Wild	MJ1-10	AB558158.1
	NM	Jiagedaqi, Neimenggu, China	Wild	NM1	AB558158.1
	QC	Cixiangguan Qianshan,	Wild	QC1-10	AB558158.1
	QS	Nangou Liaoning, China	Wild	QS1-10	AB558158.1
	QX	Xianrentai	Wild	QX1-10	AB558158.1
	S	Tieli, Heilongjiang, China	Wild	S1-10	AB558158.1
	SZ	Tieli, Heilongjiang, China	Cultivated	SZ1-10	AB558158.1
	TP	Chicheng, Hebei, China	Wild	TP1-10	AB558158.1
	WQ	Wangqing, Jilin, China	Wild	WQ1-10	AB558158.1
	YS	Panshi, Jilin, China	Wild	YS1-10	AB558158.1
	YT	Yantai, Shandong, China	Wild	YT1-15	AB558158.1
	ZE	Fengcheng, Liaoning, China	Cultivated	ZE1-8	AB558158.1
	ZH	Zhuanghe, Liaoning, China	Wild	ZH1-10	AB558158.1
	ZZ	Fengcheng, Liaoning, China	Cultivated	ZZ1-10	AB558158.1
Schisandra sphenanthera Rehd. et Wils.	LB	Lingbao, Henan, China	Wild	LB2-15	AF263437.1
	PL	Pinglu, Shanxi, China	Wild	PL1-10	AF263437.1

Results

Analysis of the sites mutation of ITS2 sequences

Base mutation at site 86-bp was found in large quantities of samples in *S. chinensis* populations. And the wild populations had higher percentage of individuals that had substitution of C→A at site 86-bp than the cultivated populations, Qianshan in Liaoning province 63%, WQ 60% and TP 70%. Also, base mutation at sites 58-bp and 227-bp were found in samples of LB13, PL1 and PL7 in *S. sphenanthera* populations, but the *S. chinensis* populations were not found (Tab. 1, Tab. 2). At sites 10-bp, 37-bp, 42-bp and 235-bp, these bases of all the *S. sphenanthera* samples differed from the samples of *S. chinensis*. So the samples of *S. chinensis* and *S. sphenanthera* could be distinguished accurately based on these base sites (Tab. 3).

Tab. 2 The sites mutation in the samples of *S. chinensis* and *S. sphenanthera*.

Site	58-bp	86-bp	183-bp	227-bp	229-bp
BG	C	C	G	C	G
CH1,3		A			
FX2,3		A			
HG1,4		A			
LH2,5		A			
LJ7,8		A			
QC1,4,5,6,7,8,9		A			
QS2,3,5,9		A			
QX1,2,4,5,6,8,9,10		A			
SZ6,7,8,10		A			
TP1,2,3,4,7,8,10		A			
WQ4,5,6,7,8,9,		A			
YS2,5,7,9		A			
ZE3		A			
ZH6,9		A			
ZZ6		A			
KD2			T		
ZZ2			A		T
LB13	T				
PL1,7	T			T	

The ITS2 sequences of the samples of BG population stands for consensus ITS2 sequences of *S. chinensis* and *S. sphenanthera*.

Tab. 3 The different sites between the samples of *S. chinensis* and *S. sphenanthera*.

Sites	10-bp	37-bp	42-bp	235-bp
BG	A	T	G	T
LB	T	C	T	A
PL	T	C	T	A

The ITS2 sequences of the samples in BG population stands for consensus ITS2 sequences of *S. chinensis*.

Measurement of DNA divergence for ITS2

The lengths of the ITS2 sequences used for the analyses were 231 bp. We used six metrics to characterize inter- vs. intra-specific variations in ITS2 sequences [22,31,32]. As shown in Tab. 4, species showed significant levels of inter-specific divergence within ITS2 sequences. Relatively lower levels of intra-specific divergence were found with calculations for the three metrics. Therefore, the ITS2 region of the *Schisandra* species, with lower levels of genetic divergence within species than between species, again showed that might be used as a genomic marker for the identification of the two species.

To evaluate the reliability that the ITS2 region could be used as a genomic marker for the identification of the different populations in *Schisandra*, we also characterized the intra- and inter-specific variations in ITS2 sequences. At population level, we used the same six metrics to characterize inter- vs. intra-specific variations. As shown in Tab. 4, the populations showed levels of inter-specific divergence within ITS2 sequences were fairly near the levels of intra-specific divergence that were found with calculations for the three metrics except Coalescent depth. Wilcoxon two-sample tests also showed no significant difference between the mean of the inter-specific divergences and the intra-specific variations in populations (Tab. 5). Based on the results of this experiment, the ITS2 region of the *Schisandra* did not possess apparent intra- and inter-specific variation gaps of these populations with the two species. The ITS2, with the same genetic divergence within populations and among populations, should not be used as a genomic marker for identifying the populations of *S. chinensis* and *S. sphenanthera*.

Tab. 4 Analyses of inter-specific divergence between congeneric species, populations and intra-specific variations in ITS2 sequences in *Schisandra*.

Measurement	Species	Populations
All inter-specific distance	0.0236 ±0.0381	0.0070 ±0.0157
Theta prime	0.0236 ±0.0381	0.0061 ±0.0013
The minimum inter-specific distance	0.0236 ±0.0381	0.0037 ±0.0111
All intra-specific distance	0.0078 ±0.0550	0.0070 ±0.0520
Theta	0.0064 ±0.0019	0.0067 ±0.0228
Coalescent depth	0.0217 ±0.1184	0.0294 ±0.1147

Assessment of the intra- vs. inter-specific differences of ITS2 sequences

To perform a preliminary examination of inter- and intra-specific variation, we investigated the distribution of genetic distance in classes of 0.006 distance units. Only a slight overlap in inter/intra-specific variation of the ITS2 was found in our study (Fig. 1a). The inter-specific distance equaled to zero for 0% of the samples. Also, most of the *Schisandra* species in some studies were found to have a unique sequence in the ITS2 [36,37]. This will provide a useful way to authenticate different ITS2 species. To perform a preliminary examination of inter- and intra-specific variation among the populations of the *Schisandra*, then we studied the distribution of genetic distance too. And notable overlap in inter/intra-specific variation of ITS2 was found (Fig. 1b). The inter- and intra-specific distance less than 0.006 reached for 97.45% and 95.25% of the samples with these populations.

Tab. 5 Wilcoxon two-sample tests for distribution of intra- vs. inter-specific divergences.

Data sources	No. of inter-specific distances	No. of intra-specific distances	Wilcoxon W	*P* value
species	1	2	#	#
populations	232	24	2498.0	0.185

"#"stands for species were not analyzed, because the No. of inter- vs. intra-specific distances were few.

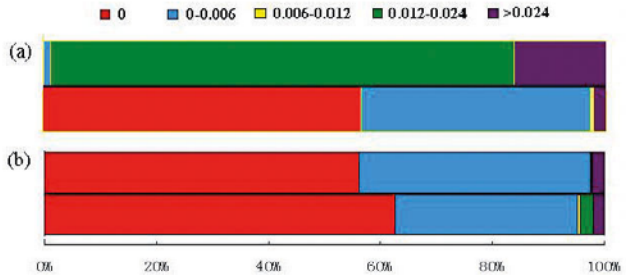

Fig. 1 Relative distribution of inter-specific divergences between congeneric species/population and intra-specific variations for ITS2 sequences. The colored bars in each box represent inter-specific (above) and intra-specific (below) genetic distances. **a** Species. **b** Population.

NJ tree and BLAST1-based methods were used to identify the samples

Two dendrograms were constructed on account of neighbor joining (NJ; Fig. 2). The same species were classed together obviously. The species of *S. chinensis* and *S. sphenanthera* all showed monophyly, and the two species could be differentiated obviously (Fig. 2a). The samples of different populations showed polyphyly at population level. And the samples of different populations were classed together, *S. sphenanthera* populations, for example (Fig. 2b). So the samples of different populations and geographical origins could not be differentiated by NJ tree-based method.

ITS2 performed well when using BLAST1 method. ITS2 obtained 100% identification success rate at the species level for *S. chinensis*, with no ambiguous identification at the genus level for ITS2 alone. *S. sphenanthera*, the success rate of ITS2 was 0% at the species level, but reached up to 100% at the genus level.

Discussion

A rapid and accurate method to authenticate species from the family Schisandraceae is very important to ensure the safe, effective usage of drugs made from the two medicinal herbs. To our knowledge, this was the first time that the ITS2 region was used to identify plant materials from *Schisandra* with such a large sample size and geographic range. In the study, ITS2 was found to be a sufficiently variable DNA region between *S. chinensis* and *S. sphenanthera* species as determination by genetic divergences, and ITS2 also demonstrated a high capability of successful discrimination. ITS2 can be a powerful marker for taxonomy studies, identifying species and solving taxonomic problems.

With the wide distribution and quite variable leaf shape, *S. sphenanthera* was mistaken for *S. chinensis* more often [16]. And it was hard to identify the two commercial products too. ITS2 sequences of *S. chinensis* were different from that of *S. sphenanthera* samples by 4 bases. Therefore, the molecular results supported the view of the traditional taxonomists and could distinguish the two species accurately. When identifying *S. chinensis* by NJ tree-based method, the 193 samples of *S. chinensis* were clustered as a clade. And all samples of *S. chinensis* were successfully identified using BLAST1-based method. In the study, ITS2 obtained 0% identification success rate at the species level for *S. sphenanthera*, with no ambiguous identification at the genus level for ITS2 alone by BLAST1-based method. But the two species of *S. chinensis* and *S. sphenanthera* could be distinguished by NJ tree and BLAST1-based methods.

When identifying *S. sphenanthera* by BLAST1-based method, the best BLAST hit of the query sequence of the samples with *S. sphenanthera* contain several species – *Schisandra viridis* A. C. Smith, *Schisandra glaucescens* Diels in Bot. and *Schisandra rubriflora* (Franch.) Rehd. et Wils. *S. sphenanthera* is distributed mainly over Shanxi, Henan, Hubei, Hunan, Sichuan, Anhui, Zhejiang, Jiangxi and Guizhou provinces, China. A point worth emphasizing was that these samples of S. sphenanthera were collected by members of task group in Lingbao city Henan province and Pinglu city Shanxi province, China, and the species and sources were accurate, definite. The best BLAST hit of the query sequence of the samples with *S. sphenanthera* were not our expectation for a few reasons. First, we could easily found these species overlaped in geographical distribution [16]. So these species could produce gene flows in the cross areas each other, theoretically. And the divergence, in a way, was reduced; Second, ITS2 cannot solve all the species determination problems. For example, in Caragana, *Caragana tibetica* and *Caragana ordosica* were found to have identical ITS2 sequences [38–42], but they were already reported to be two different species based

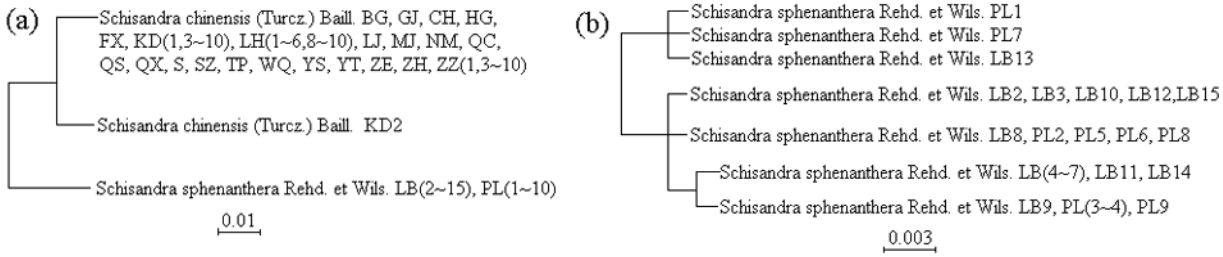

Fig. 2 The NJ tree method on account of ITS2 sequences. **a** The two species of *Schisandra chinensis* and *S. sphenanthera*. **b** The populations with *S. sphenanthera*.

on their ITS sequences [40]. Thus, other DNA marker(s) might be valuable when investigating certaingenus and broad plant taxa such that complete species identification could be achieved in *Schisandra*, *psbA-trnH* [33,43–45], for instance.

The ITS2 region of the *Schisandra* species could be used as a genomic marker for the identification of the two species with *S. chinensis* and *S. sphenanthera*, but could not be used as a genomic marker to identify different populations or origins of the two species. The present study showed the different populations of the two species were found to have identical ITS2 sequences, respectively. It could only make a primary judgment of the species' origin with the wild or cultivated samples based on test results.

Conclusion

In this study, ITS2 was examined for its usefulness in identifying medicinal species of *Schisandra*. Our findings showed that the ITS2 region could be used to identify *S. chinensis* and *S. sphenanthera* in the "Chinese Pharmacopoeia". And could also correctly distinguish 100% of species and 100% of genera from the 193 sequences of *S. chinensis*. Hence, ITS2 is a powerful and efficient tool for species identification of medicinal plants and even for a broad series of *Schisandra* plant taxa.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: research designing: BW; conducting experiments: XL, YZ, HY, LX, JZ, BX; analyzed the data and wrote the paper: XL, RH.

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