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Genetic Analysis of the Bletillae Rhizoma and Its Common Adulterants Using Internal Transcribed Spacer 2 Molecular Barcoding

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Authors' contributions

This work was carried out in collaboration between all authors. Author YL designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SL and BJ managed the analyses of the study. Author JW managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was designed to discriminate Bletillae Rhizoma from its common adulterants: *Iris tectorum*, *Polygonatum odoratum* and *Polygonatum cyrtonema* using molecular barcoding.

Place and Duration of Study: Department of Traditional Chinese Medicine, College of Pharmaceutical science, Soochow University, China, between February, 2015 and December, 2015.

Methods: Total genomic DNA was isolated from the leaves of *Bletilla striata* and similar species. The internal transcribed spacer 2 (ITS2) of the ribosomal DNA was sequenced after PCR amplification. A neighbor-joining (NJ) phylogenetic tree was constructed from the ITS2 sequences, using the software MEGA 6.0.

Results: The lengths of the ITS2 sequences of *Bletilla striata*, *Iris tectorum* and *Polygonatum odoratum* were 259 bp, 268 bp and 227 bp, respectively. The secondary structure of the ITS2 from

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Bletilla striata was obviously different in terms of the number, size and angle. The samples were clustered into three classes, and the NJ tree and systematic classification of the plants were consistent.
Conclusion: ITS2 could be used to identify the Bletillae Rhizoma and adulterants rapidly, and promote standardized planting.

Keywords: Genetic analysis; internal transcribed spacer; ITS2; DNA molecular barcoding; Bletillae Rhizoma.

1. INTRODUCTION

The Bletillae Rhizoma is the dry tubers of *Bletilla striata*, which have been used widely as a traditional Chinese medicine. The Bletillae Rhizoma promotes hemostasis with astringents, and detumescence and promotion of granulation. Recently, the price of the Bletilla Rhizome has increased as a result of a decrease in wild resources, which has led to the addition of an increasing number of adulterants from similar species [1]. The rhizomes of *Iris tectorum*, *Polygonatum odoratum* and *Polygonatum cyrtonema* are the most common adulterants. Although traditional methods of identification are professional, they are time-consuming and complex [1]. Thus, there is a need to find a simple and rapid method to distinguish the Bletillae Rhizoma from its similar species adulterants. In general, the divergence of thousands copies of DNA barcoding ITS1 and ITS2 sequences present in the plant nucleus mainly correlated with the direction and rates of morphological divergence of taxa [2].

DNA barcoding has become a popular tool to identify herbal materials [3-5]. This method will be useful to provide better quality control of traditional medicines.

Comparisons of candidate plant DNA barcoding, such as psbA-trnH, matK, rbcL and internal transcribed spacer (ITS) 2, showed that ITS2 is useful to identify the source of a sequence [6]. The effectiveness of ITS2 barcoding to identify medicinal plants using samples from a wide range of taxa was confirmed [7]. Wu et al. [8] supported the use of nuclear ribosomal DNA (nrDNA) ITS and ycf1 as potential DNA barcodes to identify the medicinal plants in *Bletilla* genus and its adulterants. In recent years, the ITS1 and ITS2 sub-regions have been applied as metabarcoding markers [9]. Thus, in this study, total genomic DNA was isolated from *Bletilla striata*, *Polygonatum odoratum* and *Iris tectorum*. The ITS2 of the rDNA was amplified by PCR and sequenced. A neighbor-joining (NJ) tree was constructed using the software MEGA 6.0. The ITS2 sequence could be used for the rapid identification of the Bletillae Rhizoma and its similar adulterants.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples of *Bletilla striata* and its similar adulterants were obtained in the field from Jiangsu, Zhejiang and Guizhou provinces, China. Professor Chunyu Liu at Soochow University identified the species of these samples. All samples are kept at the Chinese medicine Specimen Museum of the college of pharmaceutical science of Soochow University. Fresh leaves were collected and dried using silica gel until DNA extraction. Table 1 shows the samples used in this study.

2.2 DNA Extraction, PCR Amplification and Sequencing

Samples of approximately 100 mg were ground into a powder using liquid nitrogen and 2% polyvinylpyrrolidone (PVP) with a mortar and pestle. Total genomic DNA was subsequently extracted using a Plant Genomic DNA Isolation Reagent (Sangon Biotech, Shanghai Co., Ltd. China), following the recommended protocol. The extracted DNA concentration of all samples was determined using a Q3000 type micro ultraviolet spectrophotometer (Bio-Rad Company, Hercules, CA, USA), and optical density values at 260 and 280 nm of each DNA sample were determined. One pair of universal primers, forward (5′-GCGATACTTGGTGTGAAT-3') and reverse (5′-GACGCTTCTCCAGACTACAAT-3'), were designed to PCR amplify the ITS2 region of *Bletilla striata* and its adulterants. PCR was performed in 25μl reaction mixtures, containing 12.5 μl of 2 × Taq Master Mix (Sangon Biotech), 1 μl of each PCR primer (10 μM), and 1 μl of DNA extract, and the total volume was adjusted to 25 μl with sterile deionized water. PCR

Table 1. Source of samples

Species	Origins	Accession number
Bletilla striata (Thunb.exA.Murray) Rchb.f.	Guiyang,Guizhou province; Bozhou, Anhui province; Yuxi, Yunnan province; Dabieshan, Sichuan province	
B. striata	Guiyang,Guizhou province	KX663341
B. striata	GenBank	KJ405410
Iris germanica	Dushu Lake of Suzhou city,Jiangsu province	KX663343
I. tectorum	Guizhou province	
I. tectorum	GenBank	KP006792
Polygonatum odoratum(Mill)Druce	Hangzhou Botanical Garden,Zhejiang province ; Guiyang,Guizhou province	KX663342
P. cyrtonema Hua	Guiyang,Guizhou province	

amplification was conducted according to the following procedure: 94°C for 3 min; 30 cycles of 94°C for 50 s, 53.9°C for 50 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis through a 1% agarose gel in 0.5 × TAE buffer with 0.5 μg/ml Gel Red, at 100 V constant voltage for 0.5 h. To estimate the size of the separated fragments, a DNA ladder was loaded in the first lane of each gel. The PCR products were sequenced bidirectionally using an ABI 3730XL sequencer (Sangon Biotech, Shanghai, China).

2.3 Phylogenetic Analysis

The 5.8S and 28S sections of the obtained sequences and those from GenBank were removed with the assistance of a Hidden Markov model (HMM) [10], and the remaining intermediate sequence is the ITS2 sequence. All ITS2 sequences were included in the phylogenetic analysis, which was performed using software MEGA 6.0 [11]. A neighbor-joining (NJ) tree [12] was constructed with the following parameters: The bootstrap method was conducted with 1000 replicates, the substitution model was Kimura-2-parameter (K2P) [13], and gaps were treated as missing data (complete deletion). Intraspecies and interspecies sequence divergence was also calculated using the K2P model, and gaps were treated as missing data. The secondary structures of the ITS2 sequences were predicted at the ITS2 database website-(http://its2.bioapps.biozentrum.uni-wuerzburg.de/) [14].

3. RESULTS

3.1 DNA Quality

Table 2 shows the qualities of the extracted DNAs. DNA was extracted from leaves. The results indicated that the leaf DNAs were of sufficient quality for use in subsequent experiments. Fig. 1 shows that ITS2 PCR amplification of all sample are good and can be used for sequencing.

Table 2. DNA quality and concentration of Bletillae Rhizoma and similar species

Samples	A260/280	Concentration (ng/μl)
Bletillae Rhizoma	1.45(Guizhou)	165.6
	1.58(Yuxi)	183.3
	1.6(Bozhou)	111.8
	2.02(Dabieshan)	152.6
*B. striata (*Guizhou*)*	1.92	119.6
Iris germanica (Suzhou)	1.75	988.5
*I. tectorum (*Guizhou*)*	1.83	481.6
*Polygonatum odoratum (*Guizhou*)*	1.81	363.9
*P. cyrtonema (*Guizhou*)*	1.86	235.5

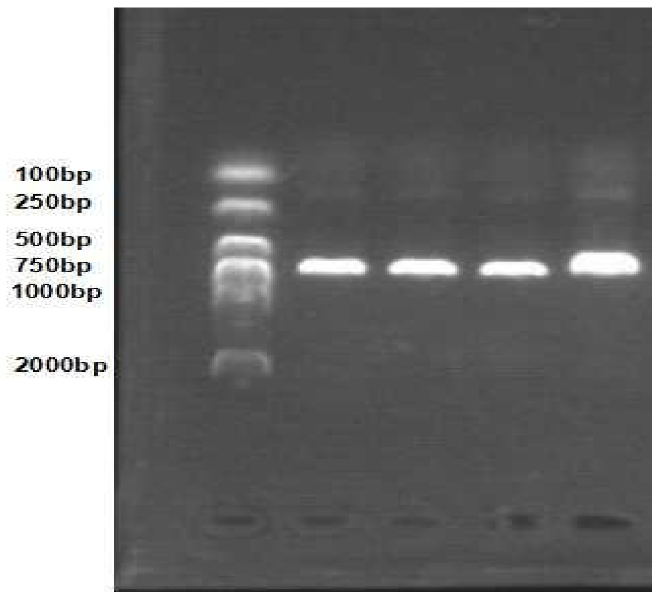

Fig. 1. ITS2 PCR amplification of Bletillae Rhizoma and its common adulterants. 1. *Bletilla striata* (Guizhou) 2. *Iris tectorum* (Guizhou) 3. *I. tectorum* (Suzhou) 4. *Polygonatum odoratum*

3.2 ITS2 Sequence Analysis and Intra-and Inter-species Variations

The lengths of the ITS2 sequences from four samples (*Bletilla striata* and *Iris tectorum* from Guizhou, *Iris tectorum* from Suzhou, and *Polygonatum odoratum*) were 259 bp, 268 bp, 268 bp and 227 bp after deletion of the 5.8S and 28S rDNAs and alignment using the MUSCLE method, respectively. The average GC contents of the ITS2 regions of these four samples were 62.2%, 67.5%, 67.2% and 61.8%, respectively. *Bletilla striata* from Guizhou was compared with the sequence from GenBank and showed three interspecies variable sites (Table 3). For *Iris tectorum*, there were seven interspecies variable sites among the samples from Guizhou, Suzhou and GenBank (Table 4).

Nucleotide analysis of the ITS2 region could provide more information about inter and intraspecies divergences. The average intraspecific genetic distance was calculated by the Kimura-2-parameter model [13]. The interspecific diversities ranged from 0 to 0.01 between *Bletilla striata* from Guizhou and *Bletilla striata* from GenBank; from 0 to 0.03 between *Iris tectorum* from Guizhou, Suzhou and GenBank; and the intraspecific distance ranged from 0.74 to 0.82 between *Bletilla striata* from Guizhou and the other species examined (Table 5).

Table 3. Sequence alignment of *Bletilla striata* from Guizhou and GenBank

Samples	Variable sites		
	22	**139**	**171**
Bletilla striata (Guizhou)	G	A	G
*B. striata (*GenBank*)*	A	G	A

Table 4. Sequence alignment of *Iris tectorum* from Guizhou, Suzhou and GenBank

Samples	Variable sites						
	42	**63**	**76**	**85**	**102**	**177**	**207**
Iris tectorum (Guizhou)	A	A	G	G	G	C	A
I. tectorum (Suzhou)	G	G	T	C	C	G	A
I. tectorum (GenBank)	G	G	T	C	C	G	G

Table 5. Kimura-2-parameter(K2P) genetic distance of Bletillae Rhizoma and similar species

	1	2	3	4	5	6
1. *Bletilla striata* (GenBank)						
2. *B. striata* (Guizhou)	0.01					
3. *Iris tectorum*（Suzhou)	0.80	0.78				
4. *I. tectorum*（Guizhou)	0.82	0.80	0.03			
5. *I. tectorum* (GenBank)	0.81	0.80	0.00	0.03		
6. *Polygonatum odoratum* (Guizhou)	0.75	0.74	0.38	0.41	0.38	

In this study, the intra-species minimum genetic distances of samples (0.74) were greater than the interspecies maximum genetic distances (0.01). Therefore, it would be easy to distinguish the samples using the ITS2 region.

3.3 Prediction and Analysis of the Secondary Structure of ITS2

The secondary structure of ITS2 provided the most accurate phylogenetic analysis [15], and ITS2 secondary structure information correlated with the biological species concept [16]. We predicted the secondary structure of the samples using the ITS2 site (http://its2.bioapps.biozentrum.uni-wuerzburg.de/). Generally speaking, the secondary structures of the samples contained a central loop and four helical regions (arms): I, II, III and IV (Fig. 2). There was no significant difference in the number, size and angle of the arm loops in samples of the same species. However, there were significant differences among the different species. The three species *Bletilla striata*, *Iris tectorum* and *Polygonatum odoratum* could be identified directly based on the RNA secondary structures of their ITS2 regions.

3.4 Phylogenetic Analysis

The phylogenetic tree was constructed using MEGA6.0, as shown in Fig. 1, with 1000 bootstrap replicates, for the ITS2 sequences. The four samples were clustered into class I, II or III (Fig. 3). Class I included *Iris tectorum* from Guizhou, GenBank and Suzhou; these three samples and *Polygonatum odoratum* clustered into class II. The class I, class II and *Bletilla striata* then clustered into class III. The phylogenetic tree and systematic classification of the plants were consistent.

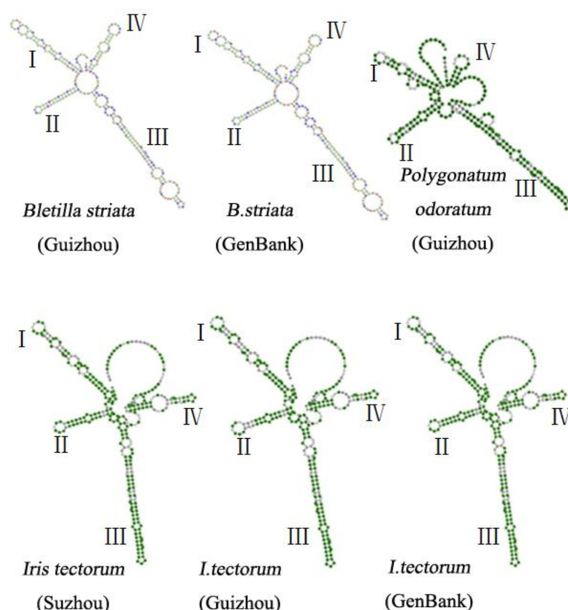

Fig. 2. The secondary structure of ITS2 of Bletillae Rhizoma and its common adulterants

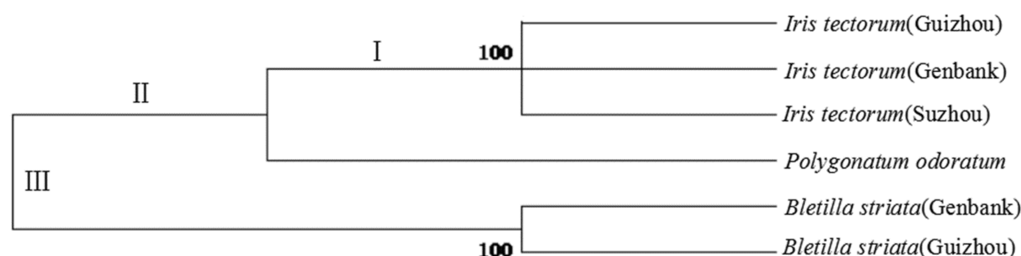

Fig. 3. The NJ tree of Bletillae Rhizoma and its common adulterants with the ITS2 sequences

4. DISCUSSION

The largest amount and the highest quality of Bletillae Rhizoma is produced in Guizhou. With the price of Bletillae Rhizoma increasing annually, the number of similar species adulterants is gradually increasing. Therefore, Bletilla striata is a good economic crop, and the artificial breeding study is very slow progress. Molecular identification might represent a good alternative. Zhang et al. [17] isolated 10 novel microsatellite makers for *Bletilla striata* using the biotin–streptavidin capture method, and tested their transferability to the other two congeneric species: *Bletilla ochracea* and *Bletilla formosana*. Their results showed that the 10 microsatellite loci could be used as novel markers for further investigation of the conservation genetics of *Bletilla striata* and its congeneric species [17]. However, in recent years, DNA barcoding identification has emerged as a useful tool for species identification; in particular, the ITS2 region is an efficient marker to authenticate traditional Chinese materials. Zhao et al. [18] studied the identification of the Bletillae Rhizoma and its same genus adulterants using single nucleotide polymorphisms (SNPs) in the ITS2 region. Their studies indicated the SNPs loci of rDNA ITS2 region could identify Bletillae Rhizoma and its adulterants. Our results indicated that ITS2 is effective for distinguishing the Bletillae Rhizoma and its adulterants. However, the ITS2 barcode is not perfect for identifying the Bletilla Rhizome. In previous research, the ITS2 sequence of *Polygonatum cyrtonema* could not be amplified; therefore, other sequences were used instead, such as trnC-petN, psbA-trnH and the matK gene [19-20].

In addition, successful DNA extraction is a prerequisite to identify herbal materials using DNA barcoding [21]. Where a medicinal herb is derived from the leaves or stems, DNA extraction is relatively easy. However, the Bletilla Rhizome is processed by placing it in boiling water or steaming it, before drying to a semi dry state [22]. Thus, the quality of the DNA extracted from tubers of the Bletillae Rhizoma was very poor (Table 2); therefore, we extracted DNA from the leaves of *Bletilla striata* and its adulterants, which provided better quality DNA.

The ITS2 secondary structures of all species comprise a central loop (the main loop) and four helical regions (arms), each of which has a different size and number of arm loop structures [23-24]. There was little difference in the secondary structure of ITS2 of *Bletilla striata* from Guizhou and from GenBank; and the structures of the ITS2 region from *Iris tectorum* from Suzhou, Guizhou and GenBank were the same. However, the secondary structures of the ITS2 regions from *Bletilla striata, Iris tectorum* and *Polygonatum odoratum* were significantly different. Not only in their size, position, number and angle of helix arm loop from different angles, but also the center loop shapes were different. The center loop of ITS 2 secondary structure of *Bletilla striata* has a variable area, that of *Polygonatum odoratum* has four, and that of *Iris tectorum* has three. Thus, secondary structure prediction could be used to identify the Bletillae Rhizoma and its adulterants.

Meanwhile, the phylogenetic tree of *Bletilla striata* and its similar adulterants constructed using the ITS2 sequences could easily distinguish the three species. Moreover, the clustering observed was consistent with the traditional classification. *Polygonatum odoratum* and *Iris tectorum* belong to the order Liliflorae, and *Bletilla striata* belongs to the order microspermae.

At present, the herbal resources of the Bletillae Rhizoma are scarce on the market. If we could standardize the cultivation of *Bletilla striata*, and establish a good agriculture practice area, the problem of similar adulterants would be solved to a certain extent.

5. CONCLUSION

The results obtained from this research strongly suggest that ITS2 sequence is a good molecular barcoding for identification the Bletillae Rhizoma and similar species rapidly, and can also be used in the standardization of *Bletilla striata* cultivation.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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