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ORIGINAL ARTICLE

DNA Barcoding Identification of Kadsurae Caulis and Spatholobi Caulis Based on Internal Transcribed Spacer 2 Region and Secondary Structure Prediction

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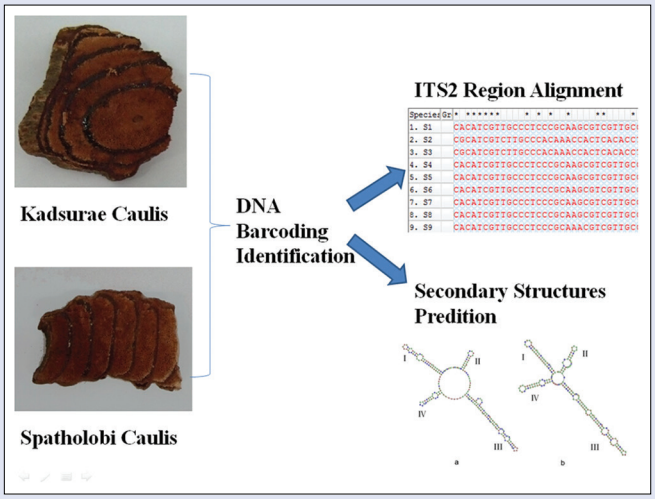
ABSTRACT

Background: Kadsurae Caulis and Spatholobi Caulis have very similar Chinese names. Their commodities were hard to distinguish because their stems were very alike after dried and processed. These two herbal drugs were often mixed in clinical use. **Objective:** Authenticity assurance is crucial for quality control of herbal drugs. Therefore, it is essential to establish a method for identifying the two herbs. **Materials and Methods:** In this paper, we used the DNA barcoding technology, based on the internal transcribed spacer 2 (ITS2) regions, to differentiate Kadsurae Caulis and Spatholobi Caulis. **Results:** The ITS2 of these two herbs were very different. They were successfully differentiated using the DNA barcoding technique. **Conclusions:** DNA barcoding was a promising and reliable tool for the identification of medicinal plants. It can be a powerful complementary method for traditional authentication.

Key words: DNA barcoding, internal transcribed spacer 2, Kadsurae Caulis, Spatholobi Caulis

SUMMARY

- The internal transcribed spacer 2 (ITS2) regions between Kadsurae Caulis and Spatholobi Caulis varied considerably, totally 139 variable sites
- Sample 1 was not Kadsurae Caulis as it labeled, but it should be Spatholobi Caulis in fact based on ITS2 region
- The secondary structure can also separate Kadsurae Caulis and Spatholobi Caulis effectively
- DNA barcoding provided an accurate and strong prove to identify these two herbs.



Abbreviations used: CTAB: hexadecyltrimethylammonium bromide, DNA: deoxyribonucleic acid, ITS2:internal transcribed spacer 2, PCR: polymerase chain reaction

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INTRODUCTION

There is a great variety of herbal drug in clinical use. Some of them are difficult to identify by morphological method for the similar appearance. Moreover, for historical reasons, there has been a phenomenon that some different herbs share the same name, or one herb has different names. This may lead to confusion in clinical application. In recent years, some disastrous events about the herbal drug were caused by the misuse of toxic substitute, for example, Stephaniae Tetrandrae Radix (Fen fang ji) replaced by Aristolochiae Fangchi Radix (Guang fang ji), resulting serious nephropathy.[1]

Kadsurae Caulis (Dianjixueteng in Chinese), the dry stems and rattans of *Kadsurae interior* A.C. Smith, is a member of *Magnoliaceae*'s family.[2] It is the main drug in the formula of the Fufang Dianjixueteng Gao.[2] Its similar substitute, Spatholobi Caulis (Jixueteng in Chinese), the dry stem of *Spatholobus suberectus* Dunn, is belonged to the family of *Fabaceae*.[2] These two herbal commodities are hard to differentiate because they have very similar appearance [Figure 1] and Chinese names, resulting confusion. Consequently, it is important to develop an efficient and accurate method to differentiate these two herbal drugs.

The traditional authentication by macroscopic and microscopic method requires an experienced specialist for examination. Another method is to test the chemical components or markers. However, the chemical profiles may be affected by the growing environment, harvest season,

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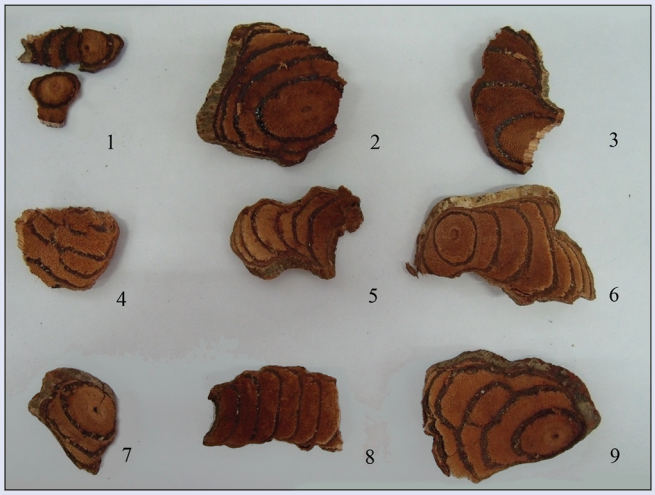

Figure 1: The appearance of the nine samples

storage conditions, or other external factors. In contrast, authentication at the DNA level provides more reliability because DNA is a stable macromolecule that is not affected by external factors and is found in all tissues.[3]

DNA barcoding is a novel technique for identifying biological specimens using short DNA sequences from either nuclear or organelle genomes.[3] An ideal DNA barcode should be short enough to be amplified from archival specimens using universal primers. In addition, most of the medicinal materials available in the market are dry and have been stored for long periods, this may lead to DNA degradation in these materials.[4] Thus, it is very difficult to amplify long DNA regions from some of these materials. The internal transcribed spacer 2 (ITS2) is part of the eukaryotic nuclear rDNA cistron and lies between the 5.8S and the 28S rRNA.[5] The ITS2 region is fast-evolving to allow classification at the species level, containing highly conserved priming sites and is highly reliable for DNA amplification and sequencing. ITS2 is considered to have evolved in concert, which leads to a homogenization of all the copies of this gene throughout the genome and in most organisms ITS2 was treated as a single locus. Thus, the ITS2 region might be a suitable marker for taxonomic classification.[4] In recent years, there have been some reports on identifying medicinal plants using ITS2. Gao *et al*. studied the ITS2 regions of 114 samples in *Fabaceae* and showed that ITS2 region is an effective marker for use in authenticating of the family *Fabaceae*.[6] Besides, the ITS2 secondary structure can be predicted on the homology basis. It was based on the Needleman–Wunsch algorithm leading to global optimal alignments between a sequence with a known structure and a novel sequence.[5] Zhang *et al*. reported that the herb "Mu tong" were successfully identified using short fragments of 250 bp ITS2 sequences, together with their secondary structure. Their analysis strengthens the potential of ITS2 as a promising DNA barcode because it incorporates valuable secondary structure information that will help improve discrimination between species.[7] Therefore, we selected ITS2 region to establish the DNA barcoding method together with the secondary structure in this study.

MATERIALS AND METHODS

Apparatus

An Electronic balance (KERN ABT 220-5DM, 0.1 mg, Germany), a thermostatic water bath (Shanghai Yarong Biochemical Instrument Factory, China) and an eppendorf centrifuge 5417R (Eppendorf AG, Hamburg, Germany) were used for sample preparation and DNA extraction. Polymerase chain reaction (PCR) amplification was performed on the K960 thermal cycler (Hangzhou Jingle Scientific Instrument Co., Ltd., China).

Table 1: Samples collection

Samples	Labeled	Collection place
S1	Kadsurae Caulis	Yunnan, China
S2	Kadsurae Caulis	Yunnan, China
S3	Kadsurae Caulis	Yunnan, China
S4	Spatholobi Caulis	Guangdong, China
S5	Spatholobi Caulis	Guangdong, China
S6	Spatholobi Caulis	Guangdong, China
S7	Spatholobi Caulis	Guangdong, China
S8	Spatholobi Caulis	Guangdong, China
S9	Spatholobi Caulis	Guangdong, China

Chemicals and reagents

A total of nine commodity samples retailed as Dian-Jixueteng and Jixueteng were purchased from different pharmacies in Yunnan and Guangdong provinces [Table 1]. Cetyltrimethylammonium bromide (CTAB), NaCl, ethylenediaminetetraacetic acid (EDTA), chloroform, isopropanol, isoamyl, and mercaptoethanol were analytical grade and manufactured by Tianjin Zhiyuan Chemical Reagent Factory (Tianjin, China). Polyvinylpyrrolidone (PVP), Tris-HCl buffer (pH 8.0), TE buffer, TAE buffer, agarose, *Taq* PCR Master Mix (×2, blue dye), and SanPrep Column DNA Gel Extraction Kit were purchased from Sangon Biotech (Shanghai, China). Goldview (MYM Biological Technology Co., Ltd., USA) was used for agarose gel electrophoresis.

Sample pretreatment

About 150 mg of each sample was grinded and added into a 2 ml tube, respectively. And then, the samples were washed by 0.1 mol/L Tris-HCl buffer (pH 8.0) twice. The supernatant was discarded.

Total DNA extraction

Genomic DNA of all the materials was extracted using a modified CTAB method. The 3 × CTAB extracting buffer contained 3% (w/v) CTAB, 1.4 mol/L NaCl, 0.1 mol/L Tris-HCl buffer, 0.5 mol/L EDTA, 1% PVP (w/v), and 0.2% (v/v) mercaptoethanol. The prewashed sample was extracted with 1 ml CTAB buffer under 65°C water bath for 1 h. The sample was shaken gently every 15 min. After the sample was cool to room temperature, 1 ml chloroform-isoamyl (24:1) was added, shaken tenderly and centrifuged (12,000 rpm/min for 10 min) to collect the supernatant. This procedure was repeated again. And then, 0.6 ml cool isopropanol was added into the collected supernatant and stored under −20°C for 2 h. Afterward, sediment was collected after centrifuged (12,000 rpm/min for 10 min). The sediment was washed by 75% cool ethanol twice. The obtained sediment was dried and dissolved by 200 µl TE buffer and stored under −20°C.

Polymerase chain reaction amplification and DNA sequencing

DNA barcodes were amplified by PCR using universal primers (S2F: 5'-ATG CGA TAC TTG GTG TGA AT-3' and S3R: 5'-GAC GCT TCT CCA GAC TAC AAT-3'). Each 25 µl reaction mixture contained 12.5 µl *Taq* PCR Master Mix, 1 µl Genomic DNA (about 20–50 ng), 1 µl of each 10 µM primer, 1 µl $MgCl_2$ (25 mmol/L), and ddH_2O 8.5 µl.

The PCR conditions for amplification were 1 cycle 94°C for 5 min; 40 cycles of 94°C 30 s, 56°C 30 s, and 72°C 45 s; and 1 cycle 72°C for 10 min, and hold 4°C. To detect successfully amplified products and the possible contamination of negative controls, PCR products were examined on 2% agarose gels stained with Goldview and visualized under ultraviolet light. Amplified products were purified following the manufacturer's protocol for SanPrep Column DNA Gel Extraction. And then, they were sent to Sangon Biotech Co., Ltd., China for DNA sequencing.

Sequence alignment and analysis

The sequences were analyzed by MEGA 5.0. The secondary structures were predicted according to the database and website (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) established by Schultz *et al.*[5,8,9]

RESULTS

Authenticity assurance is crucial for quality control of natural products. It is essential to develop different approaches to authenticate the natural products as each approach has advantages that complementary to one another.[10]

The results of DNA barcoding showed a good differentiation. The ITS2 regions of all samples were successfully amplified from total DNA and sequenced. The ITS2 regions between Kadsurae Caulis and Spatholobi Caulis varied considerably, totally 139 variable sites. The results showed that the ITS2 regions of Samples 2 and Sample 3 were 227 bp in length; Sample 1 and Samples 4–9 were 207 bp in length [Table 2]. It was also worth noting that the ITS2 region of Sample 1 was the same as Samples 4–9. This indicated that Sample 1 was not Kadsurae Caulis as it labeled, but it should be Spatholobi Caulis in fact. The neighbor-joining tree was built by MEGA 5.0 [Figure 2]. Sample 1 and Samples 4–9 were clustered into one group, whereas Samples 2–3 were clustered into another group. The genetic distance was calculated by MEGA 5.0, based on Kimura-2-parameter model. The interspecies distance (d_{inter}) between Kadsurae Caulis and Spatholobi Caulis was 1.722. The intraspecies distance (d_{intra}) of Spatholobi Caulis was 0.007.

The secondary structure can also separate Kadsurae Caulis and Spatholobi Caulis effectively. From Figure 3, the ITS2 secondary structures of Kadsurae Caulis and Spatholobi Caulis were significant different. Helix IV was relative conservative that both Kadsurae Caulis and Spatholobi Caulis had two loops. The main differences were Helix I, II, and III. Kadsurae Caulis had three loops on the stem of Helix I and one loop on Helix II, whereas Spatholobi Caulis had just one loop on Helix I but two loops on Helix II. For Helix III, although both Kadsurae Caulis and Spatholobi Caulis had four loops, the size and location were different. Moreover, Helix III of Kadsurae Caulis was shorter in length. Moreover, the angles among Helix I, II, III, and IV were not the same.

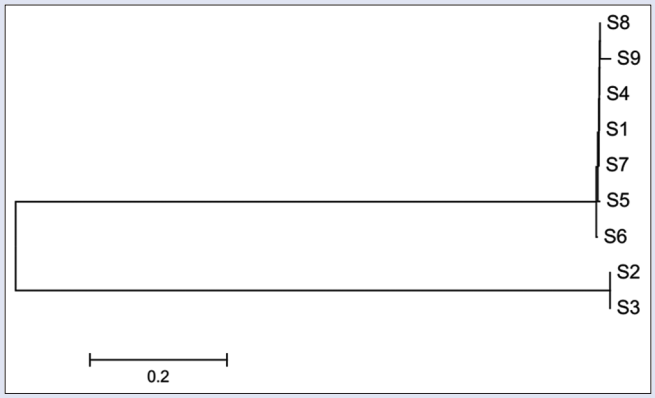

Figure 2: The neighbor-joining tree of the nine samples

Table 2: Properties of the internal transcribed spacer 2 region of the nine samples

Samples	Length (bp)	GC content (%)	Variable sites
			1111111222222223333334455555566666677
			2901246891234789026789581237890246780 34
S1	207	70.53	ATGCCTCGCAGCGGTTGGGGCGTCCCCAGCGCTTCGGTG
S2, S3	227	58.15	GCTTGCAAACCACACACTAAGTCTGGGGAAATGCTCCAC
S4, S7, S8	207	70.53	ATGCCTCGCAGCGGTTGGGGCGTCCCCAGCGCTTCGGTG
S5, S6	207	70.01	ATGCCTCGCAGCGGTTGGGGCGTCCCCAGCGCTTCGGTG
S9	207	69.08	ATGCCTCGCAACGGTTGGGGCGTCCCCAGCGCTTCGGTG
			1111111111111111111111111111111111111111111111111111111
			7778888888999999999000000011111111222222233333333344444444555555555
			5690123679012456789012356923456789015678912345789023456780124 56
			GCAAAATAGTCGTGTCGGGCGAGCAACAATGGTGGAGCGACCTCGAACCGTCGATCGGCGGGC
			CTTCTTGGTGGCGAAAAACGAGTTCGACGGATGTCGCATCGTGGTTTAAAGACCCTCTGTTTG
			GCAAAATAGTCGTGTCGGGCGAGCAACAATGGTGGAGCGACCTGTAACCGTCGATCGGCGGGC
			GCAAAACAGTCGTGTCGGGCGAGCAACAATGGTGGAGCGACCTGTAACCGTCGATCGGCGGGC
			GCAAAATAGTCGTGTCGGGCGAGCAACAATGGTGGAGCGACCTGTAACCGTCGATCGGCGGAC
			1111111111111111111111111111111111111222222
			5666666677777777888888999999999000000
			7014568901456789023689012456789023457
			CGCGTGGGACTCCGACCCTGGTCCCGGACGCTTCGGG
			TCGAATCTCGATCCTAGGATCAGGACCTTAGGAGCAA
			CGCGTGGGACTCCGACCCTGGTCCCGGACGCTTCGGG
			CGCGTGGGACTCCGACCCTGGTCCCGGACGCTTCGGG
			CGCGTGGGACTCTGACCCTGGTCCCGGACGCTTCGGG

Numbers above the variable sites are their positions in the multiple sequence alignment

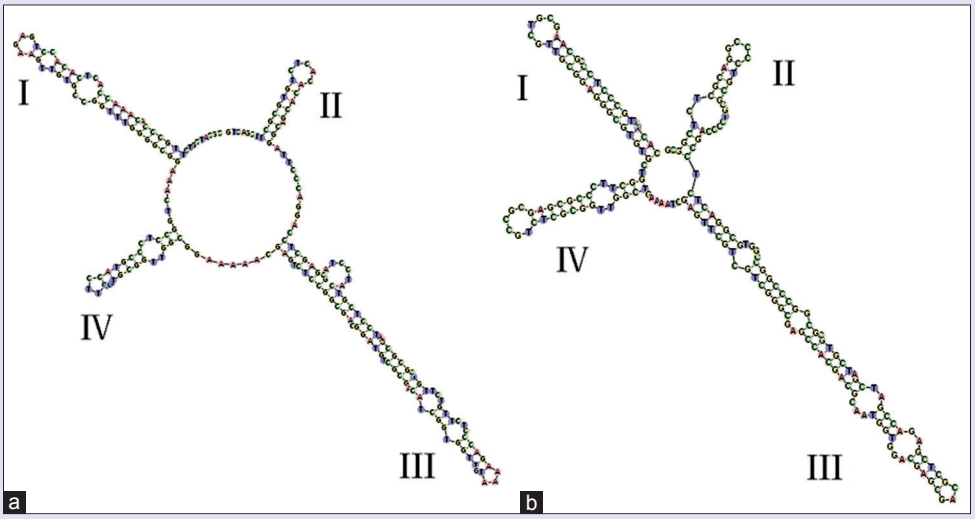

Figure 3: The secondary structures of internal transcribed spacer 2 region (a) Kadsurae Caulis; (b) Spatholobi Caulis

DISCUSSION

It is easy to obtain high-quality DNA from fresh or silica gel dried plant samples because the DNA was not degraded seriously. However, the conditions are more complicated for the commercial medicinal materials because the DNA may degrade severely after stoving and long-time storage. In addition, there are a plenty of various secondary metabolites in the cells such as polysaccharides, polyphenols, and resin. These compounds will coprecipitate with DNA and form insoluble sticky jelly-like substance, seriously affecting the quantity and quality of DNA, as well as PCR amplification. Therefore, it is important to pretreat the samples before the DNA extraction.[11]

To remove part of the polysaccharides and pigments, we washed the samples by 0.1 mol/L Tris-HCl buffer (pH 8.0) twice before the DNA dissolved. In addition, we raised the CTAB concentration to 3% so that the DNA can dissolve out more easily. This can also remove the polysaccharides. We added 1% PVP into the CTAB buffer to chelate the polyphenols. Besides, 0.2% mercaptoethanol was added into the CTAB buffer to prevent polyphenols oxidation.

A desirable DNA barcode should process high interspecific divergences and low intraspecific variations. The Consortium for the Barcode of Life suggested comparing the interspecific and intraspecific distances to estimate the identification effectiveness of the selected barcode. An ideal "barcoding gap" should be interspecific divergences significantly larger than intraspecific divergences. If d_{inter}/d_{intra} is smaller than one, it may not be a suitable DNA barcode.[11] In this study, d_{inter}/d_{intra} was larger than one. The ITS2 sequences of Kadsurae Caulis and Spatholobi Caulis were nearly different that more than 60% of the loci were not the same. Moreover, there were only five variable sites among the Spatholobi Caulis samples, i.e. Sample 1 and Samples 4–9. This indicated that the ITS2 region was relative intraspecific stable. Besides, the neighbor-joining tree also showed that samples of Kadsurae Caulis and Spatholobi Caulis can cluster into two groups, respectively. Therefore, the ITS2 region was an appropriate DNA barcode for identifying Kadsurae Caulis and Spatholobi Caulis.

The secondary structure of the ITS2 region was very intuitional. It can also provide a lot of information to differentiate species, for example, the number of loops on the helix, the length of the helix, and the angles between each helix.

The results showed that it was feasible to use sequence alignment and secondary structure comparison to accurately distinguish Kadsurae Caulis from Spatholobi Caulis.

CONCLUSIONS

In this paper, Kadsurae Caulis and Spatholobi Caulis were successfully differentiated based on the ITS2 region and secondary structure prediction. Sample 1 labeled as Kadsurae Caulis was found out to be Spatholobi Caulis. DNA barcoding provided an accurate and strong proof to identify these two herbs. This technology is a reliable and effective mean for the differentiation of substitutes and adulterants.

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Conflicts of interest

There are no conflicts of interest.

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