

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

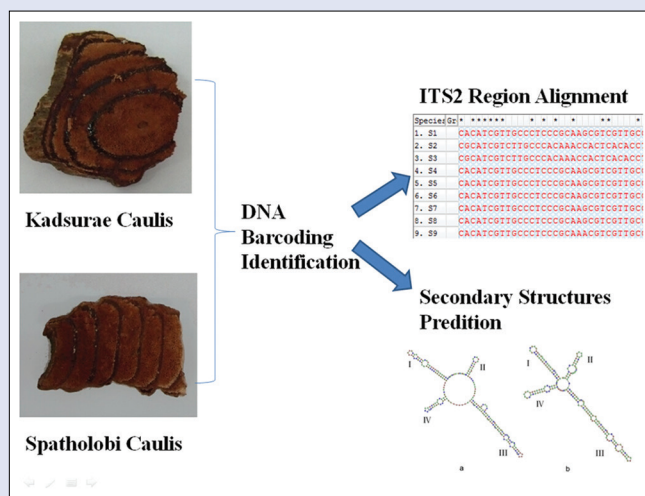
Background: *Kadsurae Caulis* and *Spatholobi Caulis* have very similar Chinese names. Their commodities were hard to distinguish because their stems were very alike after dried and processed. These two herbal drugs were often mixed in clinical use. **Objective:** Authenticity assurance is crucial for 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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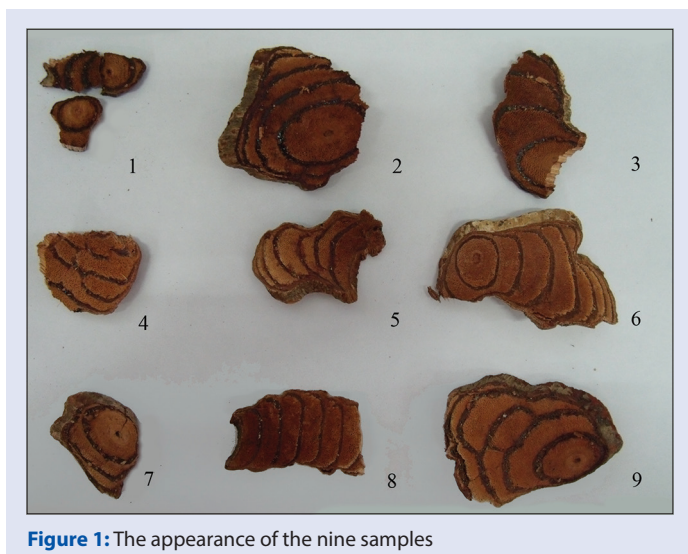


Figure 1: The appearance of the nine samples

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

Table 1: Samples collection

Samples	Labeled	Collection place
S1	Kadsuræ Caulis	Yunnan, China
S2	Kadsuræ Caulis	Yunnan, China
S3	Kadsuræ 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

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).

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 MgCl₂ (25 mmol/L), and ddH₂O 8.5 µl.

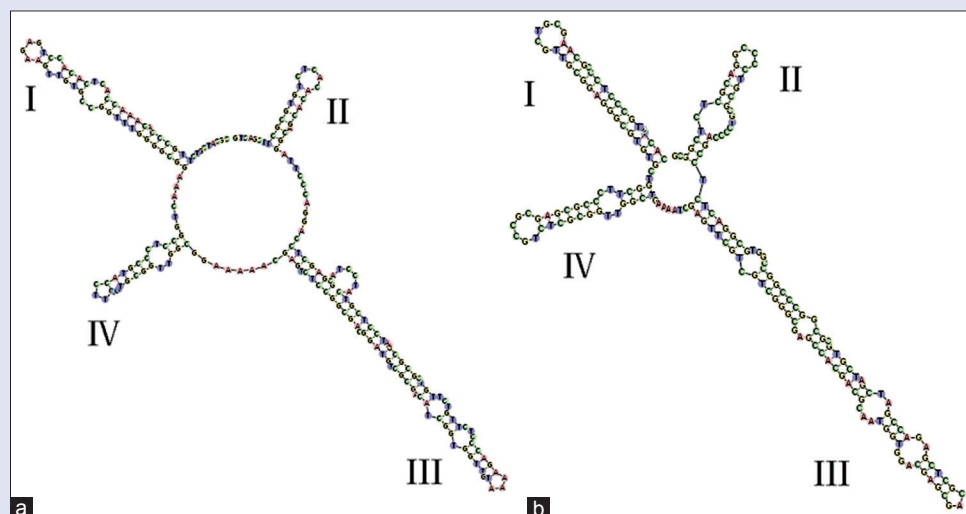


Figure 3: The secondary structures of internal transcribed spacer 2 region (a) *Kadsuræ Caulis*; (b) *Spatholobi Caulis*

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 *Kadsuræ 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 *Kadsuræ Caulis* and *Spatholobi Caulis* can cluster into two groups, respectively. Therefore, the ITS2 region was an appropriate DNA barcode for identifying *Kadsuræ 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 *Kadsuræ Caulis* from *Spatholobi Caulis*.

CONCLUSIONS

In this paper, *Kadsuræ Caulis* and *Spatholobi Caulis* were successfully differentiated based on the ITS2 region and secondary structure prediction. Sample 1 labeled as *Kadsuræ 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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