Identification of Species in *Tripterygium* (Celastraceae) Based on DNA Barcoding

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Species of genus *Tripterygium* (Celastraceae) have attracted much attention owing to their excellent effect on treating autoimmune and inflammatory diseases. However, due to high market demand causing overexploitation, natural populations of genus *Tripterygium* have rapidly declined. *Tripterygium* medicinal materials are mainly collected from the wild, making the quality of medicinal materials unstable. Additionally, identification of herbal materials from *Tripterygium* species and their adulterants is difficult based on morphological characters. Therefore, an accurate, convenient, and stability method is urgently needed. In this work, we developed a DNA barcoding technique to distinguish *T. wilfordii* Hook. f., *T. hypoglaucum* (Lév.) Hutch., and *T. regelii* Sprague et Takeda and their adulterants based on four uniform and standard DNA regions (internal transcribed spacer 2 (ITS2), *matK*, *rbcL*, and *psbA–trnH*). DNA was extracted from 26 locations of fresh leaves. Phylogenetic tree was constructed with Neighbor–Joining (NJ) method, while barcoding gap was analyzed to assess identification efficiency. Compared with the other DNA barcodes applied individually or in combination, ITS2+psbA–trnH was demonstrated as the optimal barcode. *T. hypoglaucum* and *T. wilfordii* can be considered as conspecific, while *T. regelii* was recognized as a separate species. Furthermore, identification of commercial *Tripterygium* samples was conducted using BLAST against GenBank and Species Identification System for Traditional Chinese Medicine. Our results indicated that DNA barcoding is a convenient, effective, and stability method to identify and distinguish *Tripterygium* and its adulterants, and could be applied as the quality control for *Tripterygium* medicinal preparations and monitoring of the medicinal herb trade in markets.

Key words *Tripterygium*; DNA barcoding; internal transcribed spacer 2 (ITS2); *psbA–trnH*

Species of the genus *Tripterygium* (Celastraceae) have a long history of use in traditional Chinese medicine (TCM) for the treatment of autoimmune and inflammatory diseases, including rheumatoid arthritis,1–3 systemic lupus erythematosus,4–6 ankylosing spondylitis,2 psoriasis,5,7 and idiopathic refractory nephrotic syndrome.8 In the Flora of China (Chinese), 9 three species are recognized in the family Celastraceae, such as *Celastrus angulatus* (known as Kupiteng in Chinese), are commonly found as adulterants of *Tripterygium* because of their similar morphological characteristics. In fact, over 50% of the market samples of *T. wilfordii* were found to be contaminated by *C. angulatus*.6 This problem might directly lead to problems with medical safety and effectiveness of the medicine. Accordingly, an accurate, commonly, convenient, and stability method for the identification of *Tripterygium* medicinal plants is urgently needed to guarantee the purity of *Tripterygium* in clinical applications.

Moreover, identification of herbal materials from *Tripterygium* species and their adulterants is difficult based on morphological characters. Si et al. reported that *T. hypoglaucum* and *T. wilfordii* are not distinct owing to the continuity of variation and distribution,13 Liu et al.14 and Law groups15 indicated that *T. hypoglaucum* was not distinct from *T. wilfordii*, while *T. regelii* should be recognized as a separate species. DNA barcoding, a new biotechnology for an accurate, commonly, accurate, and convenient taxonomic identification of plants and animals, has been recently developed and applied in species recognition, exploration, and conservation

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It has been shown that based on several specific DNA regions, medicinal herbs can be identified against other closely related species with high accuracy and reproducibility in a short amount of time. Recently, several candidate DNA regions, such as the internal transcribed spacer 2 (ITS2), megakaryocyte-associated tyrosine kinase (matK), a large subunit of the ribulose-1,4-bisphosphate carboxylase gene (rbcL), and the intergenic spacer photosystem II protein D1-trnA-His (psbA–trnH), or a combination of them have been popularly used as suitable DNA barcodes in plants. 

La-haye et al. recommended matK as a universal DNA barcode for flowering plants. The Plant Working Group of the Consortium for the Barcode of Life (CBOL) officially proposed that rbcL and matK were able to serve as core barcodes, and psbA–trnH or ITS regions could serve as supplemental loci for differentiating plant species. The China Plant BOL Group recommended the addition of nuclear ITS to the matK+rbcL combination as a seed plant barcode that could achieve maximum identification rates, even in closely related species.

In this study, four commonly used DNA barcodes (matK, rbcL, psbA–trnH, ITS2) were employed individually or in combination to identify the species of Tripterygium with the aim of examining the effectiveness of these regions and selecting the best DNA barcode or barcode combination for the authentication of the Tripterygium genus. Moreover, these candidate DNA barcodes were used to distinguish commercial materials in order to evaluate the prospective application of the DNA barcoding method in monitoring crude drugs in the market.

### MATERIALS AND METHODS

#### Plant Materials

A total of 35 specimens (from 26 locations of fresh leaves, shown in Supplementary Fig. S1) belonging to three species of *Tripterygium* were used to evaluate the four candidate DNA barcodes. However, among 35 specimens, 25 individuals were successfully amplified and sequenced using four DNA barcodes, as shown in Table 1. All of the *Tripterygium* specimens were collected from June 2014 to October 2014 in 9 Provinces of China, including Sichuan, Guizhou, Chongqing, Hunan, Jiangxi, and Guangxi provinces. The fresh leaves and roots of *T. wilfordii*, *T. hypoglaucum*, and *T. regelii* are shown in Fig. 1. All samples were identified by Prof. Xianyou Qu from the Chongqing Academy.

### Table 1. *Tripterygium* Taxa Samples, Collection, Voucher Numbers, and Accession Numbers Specimen Information

| Species          | Voucher no. | NCBI accession no. | Locality of collection | Date of collection |
|------------------|-------------|---------------------|------------------------|-------------------|
|                  |             | ITS2                | matK                   |                   |
| *T. hypoglaucum* | KMS1-5      | K644425             | K644450                | Puwei, Sichuan    | 21-Jun-2014     |
|                  | KMS2-1      | K644426             | K644451                | Hengshan, Sichuan | 24-Jun-2014     |
|                  | KMS5-1      | K644427             | K644452                | Manning, Sichuan  | 26-Jun-2014     |
|                  | KMS6-1      | K644428             | K644453                | Leishan, Guizhou  | 20-Jul-2014     |
|                  | KMS6-2      | K644429             | K644454                |                   |                 |
|                  | KMS7-1      | K644430             | K644455                | Jianhe, Guizhou   | 22-Jul-2014     |
|                  | KMS9-1      | K644431             | K644456                | Wulong, Chongqing | 25-Jul-2014     |
|                  | KMS11-1     | K644432             | K644458                | Jiangjin, Chongqing| 27-Sep-2014    |
|                  | KMS17-1     | K644433             | K644458                | Lichuan, Hubei    | 3-Sep-2014      |
|                  | KMS18-1     | K644434             | K644459                | Hengshan, Hunan   | 31-Aug-2014     |
|                  | KMS20-1     | K644435             | K644460                | Wugang, Hunan     | 2-Sep-2014      |
|                  | KMS22-2     | K644436             | K644461                | Dali, Yunnan      | 30-Sep-2014     |
|                  | KMS23-1     | K644437             | K644462                | Nanjian, Yunnan   | 1-Oct-2014      |
|                  | KMS23-2     | K644438             | K644463                |                   |                 |
|                  | KMS24-2     | K644439             | K644464                | Baoshan, Yunnan   | 3-Oct-2014      |
| *T. wilfordii*   | LGT12-1     | K644440             | K644465                | Longquan, Zhejiang| 26-Aug-2014     |
|                  | LGT14-2     | K644441             | K644466                | Wuyuan, Jiangxi   | 28-Aug-2014     |
|                  | LGT15-1     | K644442             | K644467                | Xiushui, Jiangxi  | 29-Aug-2014     |
|                  | LGT16-3     | K644443             | K644468                | Tongcheng, Hubei  | 30-Aug-2014     |
|                  | LGT16-6     | K644444             | K644469                |                   |                 |
| *T. regelii*     | DBLGT25-3   | K644445             | K644470                | Tonghua, Jilin    | 21-Sep-2014     |
|                  | DBLGT26-1   | K644446             | K644471                | Jiangyu, Jilin    | 22-Sep-2014     |
|                  | DBLGT27-1   | K644447             | K644472                | Linjiang, Jilin   | 23-Sep-2014     |
|                  | DBLGT28-1   | K644448             | K644473                | Changbaishan, Jilin| 25-Sep-2014    |
|                  | DBLGT28-10  | K644449             | K644474                |                   |                 |
| *C. orbiculatus* | —           | KJ716428             | KF022392               | GenBank            | —               |

—: Not acquired in this study.
of Chinese Materia Medica. All corresponding voucher specimens were deposited in the Herbarium of the Institute of Medicinal Plant Development at the Chinese Academy of Medical Sciences in Beijing, China. Fresh leaves were dried on silica gel. The 14 commercial samples that were purchased from the markets had already been dried. There is no specific permission required for these locations/activities, and the field studies did not involve endangered or protected species.

**DNA Extraction, Amplification, and Sequencing** Total genomic DNA was extracted from 20–30 mg of dried leaves or 40–50 mg of dried leaves following the manufacturer’s instructions using the Plant Genomic DNA Kit (Tiangen Biotech Co., Beijing, China) after the material was ground for 2 min at 50 Hz using a DNA extraction grinder (SCIENTZ-48, China). Initial incubation was at 65°C in 700 µL GP1 buffer (Tiangen Biotech Co.) for 1 h for dried leaves or 2 h for roots. A 25 µL PCR amplification was performed in a 9700 GeneAmp PCR system (Applied Biosystems Co., U.S.A.) according to the procedure provided by Chen et al. 21) For ITS2, matK, rbcL, and psbA–trnH DNA barcodes, universal primers and general PCR conditions are presented in Supplementary Table S1. The PCR products were run on a 1.0% agarose gel in 0.5×Trisborate ethylenediamine tetraacetic acid (TBE) buffer and purified with a TIANgel Midi Purification Kit (Tiangen Biotech Co.). An ABI 3730XL sequencer (Applied Biosystems Co.) was used to sequence double-stranded, purified PCR products.

**Sequence Assembly, Alignment, and Analysis** Sequence assembly and consensus sequence generation were performed using CodonCode Aligner V 3.7.1 (CodonCode Co., U.S.A.), and bilateral primers were removed. ITS2 sequences were annotated and delimited based on the Hidden Markov Model (HMM) method. 26) To verify whether these four candidate barcodes were competent for family or genus assignment in authenticating Tripterygium, ITS2, matK, rbcL, and psbA–trnH DNA barcodes, universal primers and general PCR conditions are presented in Supplementary Table S1. The PCR products were run on a 1.0% agarose gel in 0.5×Trisborate ethylenediamine tetraacetic acid (TBE) buffer and purified with a TIANgel Midi Purification Kit (Tiangen Biotech Co.). An ABI 3730XL sequencer (Applied Biosystems Co.) was used to sequence double-stranded, purified PCR products.

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Intraspecific variation and interspecific divergence were analyzed through six parameters with the aim of assessing the utility of the four DNA barcodes. Three parameters (average intraspecific distance, theta and average coalescent depth) were calculated to compare the intrasequence variability of the four markers. Three additional parameters (average interspecific distance, average theta prime and smallest intersequence depth) were calculated to compare the intrasequence variability of the four markers. As shown in Table 3, ITS2 had the highest mean interspecific divergence, followed by matK, rbcL, and psbA-trnH. The highest level of mean intraspecific variation was shown in psbA-trnH, followed by ITS2, while rbcL exhibited the lowest level. Therefore, in terms of intra- and interspecific variation, ITS2 and psbA-trnH are the preferable options to distinguish the species of Tripterygium.

**Barcoding Gap Analysis**

The barcoding gap between intraspecific and interspecific distance was determined by graphing the distribution of variation in K2P distances for ITS2, matK, rbcL, and psbA-trnH. We compared the minimum interspecific divergence with the maximum intraspecific divergence to evaluate whether there were any DNA barcoding gaps. As described by Collins et al. and Chen et al., a dot above the 1:1 slope means that there is a barcoding gap for this species for a specific marker, whereas a dot below the 1:1 slope implies no barcoding gap. The “maximum intraspecific divergence” is the maximum genetic distance of each species. The comparison species were both *T. wilfordii* and *T. regelii* when the interspecific divergence of *T. hypoglaucum* was calculated. When the interspecific divergence of *T. wilfordii* was calculated, the comparison species were *T. hypoglaucum* and *T. regelii*. The minimum interspecific divergences vs. maximum intraspecific divergences are depicted in Fig. 2, and the points corresponding to the three species of *Tripterygium* can be seen above the 1:1 line. As seen from Fig. 2, three of the same plots represent a species and each symbol represents a specific marker region, and most of the
points were distributed in the lower left corner of the scatter plot, implying that the three species using majority of DNA regions had no DNA barcoding gaps. For ITS2 and combined the barcode of ITS2 + psbA–trnH, *T. regelii* had DNA barcoding gaps (Supplementary Table S3). Moreover, the ITS2 region had a higher interspecific variation than the other regions, followed by the *psbA*–*trnH* region, which was in accordance with the above results.

**Species Identification Based on the NJ Tree**

NJ is a general statistical criterion in widespread use for the inference of molecular phylogenies, which visually revealed the relationship between species.\(^3^4,^3^5\) Furthermore, an NJ tree is considered the simplest and most widely used method in barcoding research, and specimens of the same species are always observed to be closely grouped.\(^3^6\) In order to directly evaluate the ability of the four barcodes in species differentiation, *Celastrus orbiculatus* was chosen as outgroup. The NJ trees were constructed for each individual region and their combinations, and the bootstrap scores are shown (≥50%) for each branch (Supplementary Figs. S2 to S14, Fig. 3).

For the *matK* and *rbcL* regions, all of the species could not be distinguished from each other. Thus, the *matK* and *rbcL* regions were not suitable for the identification of *Tripterygium*. However, for the ITS2 and *psbA–trnH* indi-

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**Table 4. Authenticating of 11 Batches Commercial *Tripterygium* Based on BLAST**

| Practical sample | Locality of collection | Market label                        | BLAST               |
|-----------------|------------------------|-------------------------------------|---------------------|
| 1               | Kunming                | *T. hypoglauccum*                   | Tripterygium       |
| 2               | Kunming                | *T. hypoglauccum*                   | *C. angulatus*     |
| 3               | Yunnan                 | *T. hypoglauccum*                   | *C. angulatus*     |
| 4               | Yunnan                 | *T. hypoglauccum*                   | *T. hypoglauccum*  |
| 5               | Yunnan                 | *T. hypoglauccum*                   | *T. hypoglauccum*  |
| 6               | Guangxi                | *T. wilfordii*                      | *Flemingia macrophylla* |
| 7               | Hubei                  | *T. wilfordii*                      | *C. angulatus*     |
| 8               | Guangxi                | *T. wilfordii*                      | *C. angulatus*     |
| 9               | Henan                  | *T. wilfordii*                      | *T. hypoglauccum*  |
| 10              | Jiangxi                | *T. wilfordii*                      | *T. hypoglauccum*  |
| 11              | Hubei                  | *T. wilfordii*                      | *T. hypoglauccum*  |

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Fig. 3. NJ Tree Based on ITS2+psbA–trnH Sequences from *Tripterygium* Species
vidual regions and their combination, *T. regelii* clustered into its own clade, supported with a bootstrap support of 99% (Fig. 3). However, *T. hypoglaucum* was not distinct from *T. wilfordii*, indicating that *T. hypoglaucum* and *T. wilfordii* are potentially conspecific, or the limited barcode did not provide enough variation for their differentiation. These results were consistent with previous reports.\(^5,7\) In addition, among the four regions and their combinations, ITS2+psbA–trnH had a relatively high success of species identification. The combination of ITS2+psbA–trnH+matK (Supplementary Fig. S11), ITS2+psbA–trnH+rcbL (Supplementary Fig. S12), and ITS2+psbA–trnH+matK+rcbL (Supplementary Fig. S14) decreased the bootstrap support to 90, 98, or 94%, respectively. Therefore, the combination of ITS2+psbA–trnH is a good candidate barcode for the identification of *Tripterygium* and its adulterants, and can be prospectively applied in monitoring *Tripterygium* crude drugs in the market.

**Authenticating of Commercial *Tripterygium* and Its Adulterants Based on NCBI BLAST and Tmbarcode**

Fourteen batches of commercial Leigongteng (*T. wilfordii*) and Kunming shanhaitang (*T. hypoglaucum*) were surveyed using ITS2 and psbA–trnH markers. Of the 14 samples, 11 samples of *Tripterygium* were positively amplified and qualified for further analysis. Identification of 11 commercial *Tripterygium* samples was conducted using BLAST against GenBank and Species Identification System for TCM (http://www.tcmbarcode.cn) for selected taxa with a minimum BLAST cut off of 99% identity for a top match. As shown in Table 4, samples one, four, five, nine, and eleven were similar to *Celastrus orbiculatus* and *Flemingia* specimen. These results indicate that *Tripterygium* could be distinguished unambiguously from its adulterants. The commercial *T. hypoglaucum* and *T. wilfordii* are usually substituted by *Celastrus angulatus*, *Celastrus orbiculatus* and *Celastrus scandens*, or even by *Flemingia macrophylla*. However, the DNA barcodes were unable to distinguish *T. wilfordii* from *T. hypoglaucum*. Further study should be concentrated on designing the specific PCR primers for each *Tripterygium* plant species and additional molecular markers should be researched and developed for the identification of *T. wilfordii* and *T. hypoglaucum*.

**CONCLUSION**

In this work, a total of 25 individuals representing three species of *Tripterygium* were successfully amplified and sequenced using four DNA barcodes (ITS2, matK, rbcL, and psbA–trnH). Compared with the other DNA barcodes applied individually or in combination, ITS2+psbA–trnH is the most suitable, accurate, and applicable for the identification of *Tripterygium* and its adulterants. Our results indicated that *T. hypoglaucum* and *T. wilfordii* can be considered as conspecific, while *T. regelii* was recognized as a separate species. Furthermore, the ITS2+psbA–trnH barcode combination was successfully used to distinguish commercial *Tripterygium*, providing an accurate, commonly, convenient, and stability method to identify and distinguish *Tripterygium* and its adulterants, which will be beneficial to the quality control of *Tripterygium* medicinal preparations and to the management of medicinal herb trade in markets.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.

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