Identification of *Dendrobium* Species Used for Herbal Medicines Based on Ribosomal DNA Internal Transcribed Spacer Sequence

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Stems of genus *Dendrobium* (Orchidaceae) have been traditionally used as an herbal medicine (Dendrobii Herba) in Eastern Asia. Although demand for *Dendrobium* is increasing rapidly, wild resources are decreasing due to over-collection. This study aimed to identify plant sources of Dendrobii Herba on the market based on sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. We constructed an ITS1-5.8S-ITS2 sequence database of 196 *Dendrobium* species, and the database was employed to identify 21 herbal samples. We found that 13 *Dendrobium* species (*D. catenatum*, *D. cucullatum*, *D. denudans*, *D. devonianum*, *D. eriiflorum*, *D. hancockii*, *D. linawianum*, *D. lituiflorum*, *D. loddigesii*, *D. polyanthum*, *D. primulinum*, *D. regium*, and *D. transparens*) were possibly used as plant sources of Dendrobii Herba, and unidentified species allied to *D. denudans*, *D. eriiflorum*, *D. gregulus*, or *D. hemimelanoglossum* were also used as sources. Furthermore, it is clear that *D. catenatum* is one of the most important sources of Dendrobii Herba (5 out of 21 samples).

Key words *Dendrobium*; Orchidaceae; molecular identification; ribosomal DNA; internal transcribed spacer; herbal medicine

Dendrobium is one of the largest genera in the Orchidaceae, of which about 1200 species are distributed widely in the Asia-Pacific region.[1,2] Owing to the enormous number of species and great diversification of morphological characters, many taxonomic and phylogenetic problems of this genus remain unresolved.

Dried stems of specific *Dendrobium* species, so-called Dendrobii Herba, have been used in traditional Chinese medicine for benefiting the stomach, promoting the production of body fluid, nourishing "yin," and antipyresis.[3,4] Stems of some *Dendrobium* species contain compounds that exhibit antioxidant activity and antitumor activity.[5—7] Accordingly, the demand for Dendrobii Herba has increased rapidly, and the wild resources of *Dendrobium* have decreased severely due to over-collection. Seventy-four *Dendrobium* species are distributed in China.[8] Among them, the Pharmacopoeia of the People's Republic of China (2010 edition) lists four species, *D. catenatum* Lindl., *D. nobile* Lindl., *D. chrysotoxum* Lindl., and *D. fimbriatum* Hook. as the authorized plant sources for Dendrobii Herba.[3] Additionally, following 16 species are described in the Chinese Materia Medica Dictionary (2006 edition) as medicinal plants[4]; *D. aduncum* Wall. *ex* Lindl., *D. bellatulum* Rolfe, *D. chrysanthum* Wall. *ex* Lindl., *D. chryseum* Rolfe, *D. crepidatum* Lindl. *ex* Paxt., *D. cucullatum* R. Br., *D. densiflorum* Lindl. *ex* Wall, *D. devonianum* Paxt., *D. hancokii* Rolfe, *D. henryi* Schltr., *D. hercoglossum* Reichb. f., *D. linawianum* Rchb. f., *D. loddigesii* Rolfe, *D. lohohense* Tang *et* Wang, *D. longicornum* Lindl., and *D. moniliforme* (L.) Sw.

However, in practice, *Dendrobium* species that are not described in either of these references are used clinically, and other orchid genera such as *Pholidota* have also been used as adulterants.[9—11] Such a lax situation in commercial distribution could result in the risk of inconsistent therapeutic effect and even be a threat against consumer safety. Therefore, discrimination of Dendrobii Herba is important for quality control. However, it is difficult to identify *Dendrobium* species from dried stems by traditional methods based on morphological, anatomical and/or chemical analyses[12—14] because these characteristics are not always useful markers at the species level and often vary depending on growing conditions.

To date, several molecular analytical methods have been applied to identify herbal medicines.[15] For example, DNA sequencing, DNA microarrays, random amplified polymorphic DNA (RAPD), and DNA fingerprinting have been used for identification of *Dendrobium* species.[16—27] Unfortunately, these methods have only been applied to a limited number of *Dendrobium* species, and molecular markers that can be applied more universally to *Dendrobium* have not been developed. To recognize herbal medicines accurately at the species level, a comprehensive DNA sequence database that covers *Dendrobium* species potentially used for medicine is desired, and to create such a database, the following conditions are required: (1) precise identification of the plant species, (2) comprehensive data banking of reference sequences for the relevant plant group at the species level, and (3) employment of sequence markers that can discriminate interspecific differences.

We constructed a sequence database of the internal transcribed spacer (ITS)1-5.8S-ITS2 region for 196 *Dendrobium* species, all of which were identified accurately based on the morphological characters of vegetative and reproductive organs. The database covers most of the *Dendrobium* species from China and adjacent regions. The sequence readout of ITS1 and ITS2 regions of nuclear ribosomal DNA appeared to contain sufficient variation to discriminate *Dendrobium* at the interspecific level.[20,23] In this study, ITS DNA sequenc-

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ing was applied to identify Dendrobii Herba samples obtained in Chinese markets.

MATERIALS AND METHODS

Sequence Database of ITS Regions of *Dendrobium* Many *Dendrobium* species have synonyms (different scientific names used for a single species). Our taxonomic interpretation is based on Flora of China (http://flora.huh.harvard.edu/china/), and we used the currently accepted names defined by Flora of China. Plant materials were obtained from the living collection of Tsukuba Botanical Garden, National Museum of Nature and Science (TNS). All voucher specimens were deposited at TNS. Genomic DNA was isolated from the fresh leaves or flowers by a DNeasy Plant Mini Kit (QIAGEN, Hamburg, Germany) following the manufacturer's instructions and was used as a template for polymerase chain reaction (PCR) amplification. ITS1-5.8S-ITS2 regions were amplified as described by Hidayat *et al.*[28] using the primer sets 17SE (ACGAATTCATGGTCCGGTGAAGTGTTCG) and 26SE (TAGAATTCCCCGGTTCGCTCGCCGTTAC)[29] (Fig. 1). Our database contains 205 sequences of 196 species. Multiple individuals were analyzed for *D. hercoglossum*, *D. moniliforme*, *D. palpebrae*, *D. sanderae*, and *D. thyrsiflorum*. These 205 sequence data were registered in the DNA Data Bank of Japan (DDBJ).

DNA Extraction and Sequencing Analysis of Dendrobii Herba Samples In total, 21 samples of Dendrobii Herba were collected in several herbal markets (Table 1). Photographs of representative samples are shown in Fig. 2. Genomic DNA from Dendrobii Herba was isolated by a DNeasy Plant Mini Kit (QIAGEN). The DNA extracted from herbal medicine often is fragmented and difficult to amplify as long deoxynucleotides by PCR. Therefore, we designed an internal primer, DR2 (TCTTCATCGATGCGAGAGCC), located upstream of 26SE (Fig. 1). Using 1 ng genomic DNA extracted from each sample of Dendrobii Herba as a template, PCR was carried out with 1 U Nova *Taq* polymerase (SHIMAZU, Tokyo, Japan), 1.5 μl 17SE primer (10 pmol/μl), 1.5 μl DR2 primer (10 pmol/μl), 10 μl Ampdirect Plus (SHIMAZU), and 4.9 μl distilled water (total volume 20 μl). PCR conditions were 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min. By using the primer sets of 17SE and DR2, approximately 400 bp fragments of ITS1-5.8S region were amplified. The PCR products were purified with a High Pure PCR Product Purification Kit (Roche Diagnostics, Tokyo, Japan), and sequenced using a CEQ 8000 system (Beckman Coulter, Fullerton, CA, U.S.A.).

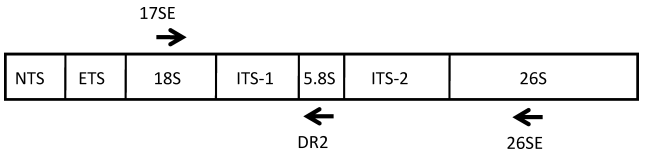

Fig. 1. Sequence Diagram of rDNA Region with Location of Primers Used in This Study

The primer sets 17SE and 26SE were used to sequence the ITS1-5.8S-ITS2 region of 196 *Dendrobium* species. The primer sets 17SE and DR2 were used for sequence analysis of herbal samples.

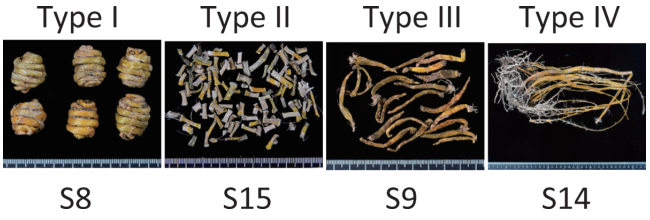

Fig. 2. Representative Photographs of Dendrobii Herba Samples

There were four typical shapes. Type I: rolled stems, Type II: chopped stems shorter than 1 cm, Type III: chopped stems approximately 2—3 cm, Type IV: entire stems with roots.

Identification of Dendrobii Herba Samples A homology search using Local BLASTN program in GENETXY software (ver. 10) (Genetyx, Tokyo, Japan) was conducted to identify the samples of Dendrobii Herba.

RESULTS AND DISCUSSION

The results of the basic local alignment search tool (BLAST) search for 21 Dendrobii Herba samples are shown in Table 1. We defined a sequence identity value of 97% as the minimum value of conspecificity because Xu *et al.* reported that the intraspecific sequence divergence of *Dendrobium* is less than 3% in ITS1 and less than 4% in ITS2.[23] For example, the sequence of sample S1 had sequence identity of 99% to *D. catenatum*, and the species was regarded as the plant source of the sample S1. Sometimes we obtained multiple hits: for example, *D. denudans* and *D. eriiflorum* were both possible plant sources for the sample S8. When no entry in the database indicated sequence identity larger than 97%, we selected the sequence of the highest identity as the allied species. For example, three species had sequence identity of 96% to the sample S10. They were considered to be allied species of the sample S10.

For the samples S1—S7, S11, S14—S16, S18, S19, and S21, a single species had high sequence identity and low *E*-values (Table 1). For the samples S8, S9, S17, and S20, multiple species had high sequence identity and low *E*-values. For these, it was necessary to sequence other regions (*e.g.*, the maturase-encoding gene (*mat*K) of the chloroplast genome) to discriminate the candidate species.

The samples S12 and S13 showed sequence identity of 97% and 91%, respectively, to *D. gregulus*. Sample S10 showed sequence identity of 96% to *D. eriiflorum*, *D. denudans*, and *D. hemimelanoglossum*. For those samples with a lower identity value than the threshold (97%), we searched further in the GenBank database but found no ITS sequence that exceeded the threshold. We provisionally identified samples S12 and S13 as an allied species to *D. gregulus*, and sample S10 was an allied species to *D. eriiflorum*, *D. denudans*, or *D. hemimelanoglossum*.

In sample S20, we found it was a mixture of at least three different plant sequences. One was a *Dendrobium* assigned to *D. primulinum* or *D. polyanthum* (100%). One was identified as *Hedera* (97%), which belongs to Araliaceae. We could not identify the last sequence in either our database or in GenBank. Sample S20 was thus a mixture of *Dendrobium*, *Hedera*, and an unidentified plant.

In this study, we found that 13 species, *D. catenatum*, *D. cucullatum*, *D. denudans*, *D. devonianum*, *D. eriiflorum*, *D. hancockii*, *D. linawianum*, *D. lituiflorum*, *D. loddigesii*, *D.*

Table 1. Results of Molecular Identification of Dendrobii Herba

Herbal sample ID	Market	Type	Best match specie	Accession number	Sequence identity, %	*E*-Value	2nd match species	Accession number	Sequence identity, %	*E*-Value
S1 (黃花石斛, 1998.3)	Taiwan	II	*D. catenatum*	AB593517	99	0	*D. scoriarum*	AB593659	98	0
S2 (特級梳唇石斛)	Shenzhen	I	*D. catenatum*	AB593517	100	0	*D. scoriarum*	AB593659	99	0
S3 (龍頭鳳尾石斛)	Shenzhen	I	*D. catenatum*	AB593517	100	0	*D. scoriarum*	AB593659	99	0
S4 (紫皮楓斗竜頭鳳尾)	Xiamen	I	*D. catenatum*	AB593517	100	0	*D. scoriarum*	AB593659	99	0
S5	Guangzhou	I	*D. catenatum*	AB593517	100	0	*D. scoriarum*	AB593659	99	0
S6 (水草楓斗)	Xiamen	I	*D. cucullatum*	AB593539	98	1×10^{-178}	*D. parishii*	AB593630	89	2×10^{-140}
S7	Hong Kong	IV	*D. cucullatum*	AB593539	99	0	*D. parishii*	AB593630	90	8×10^{-144}
S8 (剛節楓斗一等)	Xiamen	I	*D. denudans*	AB593547	99	0	*D. hemimelanoglossum*	AB593578	98	0
			D. eriiflorum	AB593556	99	0				
S9 (紅珠虫条石斛)	Shenzhen	III	*D. denudans*	AB593547	99	0	*D. hemimelanoglossum*	AB593578	98	0
			D. eriiflorum	AB593556	99	0				
S10 (虫草楓斗特等)	Xiamen	I	*D. eriiflorum**	AB593556	96	7×10^{-160}	*D. gregulus*	AB593572	93	5×10^{-143}
			*D. denudans**	AB593547	96	2×10^{-159}				
			*D. hemimelanoglossum**	AB593578	96	3×10^{-158}				
S11 (紫皮楓斗特等)	Xiamen	I	*D. devonianum*	AB593548	99	0	*D. infundibulum*	AB593486	87	2×10^{-133}
S12 (虫草楓斗小真虫)	Xiamen	I	*D. gregulus**	AB593572	97	7×10^{-178}	*D. eserre*	AB593558	96	1×10^{-168}
S13 (紅珠虫石斛)	Shenzhen	I	*D. gregulus**	AB593572	91	2×10^{-153}	*D. eserre*	AB593558	90	8×10^{-146}
S14	Guangzhou	IV	*D. hancockii*	AB593575	99	0	*D. harveyanum*	AB593576	93	5×10^{-167}
S15 (黃櫻石斛, 1998.3))	Taiwan	II	*D. linawianum*	AB593599	99	0	*D. hercoglossum*	AB593580	98	0
S16 (櫻石斛, 1998.3))	Taiwan	II	*D. linawianum*	AB593599	100	0	*D. hercoglossum*	AB593580	99	0
S17	Guangzhou	I	*D. lituiflorum*	AB593602	99	0	*D. wardianum*	AB593686	98	0
			D. transparens	AB593679	99	0				
S18 (細茎石斛)	Guangzhou	IV	*D. loddigesii*	AB593604	99	0	*D. primulinum*	AB593641	89	3×10^{-138}
S19 (小環釵石斛)	Guangzhou	IV	*D. loddigesii*	AB593604	99	0	*D. primulinum*	AB593641	89	1×10^{-137}
S20† (黃草石斛)	Shenzhen	I	*D. primulinum*	AB593641	100	0	*D.* cf. *primulinum*	AB593521	96	6×10^{-166}
			D. polyanthum	AB593636	100	0				
S21 (金釵石斛, 1998.3)	Taiwan	II	*D. regium*	AB593645	99	0	*D. hercoglossum*	AB593580	98	0

BLAST search hits of ITS sequences of 21 Dendrobii Herba samples are shown. The 'Type' column indicates the shapes of samples mentioned in the legend of Fig. 2. The typical threshold for good *E*-value from a BLAST search is 10^{-5} or lower. In this study, the highest *E*-value in 21 samples was 2×10^{-133}. Three herbal samples (S10, S12, S13) had no hits with a sequence identity exceeding the threshold value (97%). Most closely related species to the sample are shown as best match species (marked with *). The herbal sample S20 was contaminated with genus *Hedera*, Araliaceae (marked with †).

polyanthum, *D. primulinum*, *D. regium*, and *D. transparens*, are included in the Dendrobii Herba samples on the market. Among them, seven species, *D. catenatum*, *D. cucullatum*, *D. devonianum*, *D. hancockii*, *D. linawianum*, *D. loddigesii*, and *D. primulinum*, are authorized medical plants listed in the Pharmacopoeia of The People's Republic of China,[3] the Chinese Materia Medica Dictionary,[4] or Zhong Hua Ben Cao.[30,31]

D. denudans, *D. eriiflorum*, *D. lituiflorum*, *D. polyanthum*, *D. regium*, and *D. transparens* were not listed in the abovementioned references. Among them, *D. lituiflorum*, *D. polyanthum*, and *D. transparens* are allied to some medicinal species, for example, *D. chrysanthum*, *D. crepidatum*, *D. cucullatum*, and *D. primulinum*, but there is little in the literature about their pharmacological effects. Little has been reported about the pharmacological effects of *D. denudans*, *D. eriiflorum*, and *D. regium*, either.

Five of 21 samples were identified as *D. catenatum*. It should be noted that *D. officinale*,[32] one of the principal materials of Dendrobii Herba defined by the Pharmacopoeia of The People's Republic of China, has been accepted as a synonym of *D. catenatum* by recent taxonomic studies.[33,34] Thus, we concluded that *D. catenatum* is one of the most important sources of Dendrobii Herba.

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