Molecular Authentication of *Dendrobium* Species by Multiplex Polymerase Chain Reaction and Amplification Refractory Mutation System Analysis

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**ABSTRACT.** The genus *Dendrobium* is important in traditional Chinese herbal medicine, and the precise identification of *Dendrobium* species is critical for the treatment and for pharmacological research. In the present study, a ribosomal DNA (rDNA) internal transcribed spacer (ITS) region-based analysis was used to ascertain the phylogenetic relationship among 20 *Dendrobium* species. The lengths of the ITS regions among the 20 species ranged from 636 to 653 bp, and the identities of the rDNA regions among the different species ranged from 75.7% to 99.1%. The results also showed that the ITS1 and ITS2 regions exhibit more variation than the 5.8S rDNA. A phylogenetic tree derived from the ITS sequence indicated that six medicinal *Dendrobium* species, of which five are common medicinal plants in the Taiwan market, were closely related and shared a common clade. Multiplex polymerase chain reaction (PCR) amplification was successfully performed to identify the six medicinal *Dendrobium* species, and amplification refractory mutation system (ARMS) PCR was used to distinguish *D. tosaense* specifically from the 19 other *Dendrobium* species. The established PCR-based (multiplex and ARMS) analyses can be used for the authentication of the raw materials of medicinal *Dendrobium* from other species.

Orchidaceae, commonly referred as the orchid family, is the second largest family of flowering plants, comprising more than 25,000 species distributed in 880 genera (Gutierrez, 2010). *Dendrobium* is one of the largest genera in Orchidaceae and includes ≈1600 species (Bechtel et al., 1992). Seventy-four *Dendrobium* species and two variations are found in China, and at least 12 species have been identified in Taiwan (Su, 2000). The processed dried stems of certain *Dendrobium* species are known as “Shi-hu” [herbal dendrobii (stems of *Dendrobium*)] that contain compounds with biological activities. Therefore, Shi-hu has been used for medical treatment and as a functional health food in many Asian countries for centuries.

According to the Pharmacopoeia of the People’s Republic of China (China Medical Science and Technology Press, 2010), four species, *D. catenatum, D. nobile, D. chrysotoxum,* and *D. fimbriatum,* are used as the authorized sources for herbal dendrobii. However, other *Dendrobium* species are also used as clinical substitutes: in the Taiwan herb market, *D. tosaense, D. linawianum, D. moniliforme, D. hercoglossum,* and *D. nobile* are common medicinal plants used as the sources for Shi-hu. The former three species are found distributed in the mountain areas of Taiwan, whereas *D. hercoglossum* and *D. nobile* are mainly imported from China (Hsieh, 2004). *D. tosaense* has drawn more attention as an important herbal dendrobii in Taiwan as a result of its natural habitat distribution (Tang and Cheng, 1984) and fairly good growth rate in comparison with other *Dendrobium* species. The mass production of *D. tosaense* by tissue culture has also been well established (Lo et al., 2004b). Furthermore, the plant produces a major bioflavonoid, quercetin, which possesses antioxidant (Gutierrez, 2010; Lo et al., 2004a), antihypertensive, anti-inflammatory, and anticarcinogen properties (Middleton and Kandaswami, 1993), making *D. tosaense* a good source of medicinal *Dendrobium*.

A major problem in Asian medicinal herb markets is the use of imitations and adulterants. Adulterants that are composed of cheaper and common orchids are found on the market (Ma et al., 1995), and the similarity in appearance with the authentic counterparts makes Shi-hu difficult to discriminate. Therefore, the authentication of medicinal *Dendrobium* species is important to ensure the therapeutic quality and safety of this Shi-hu.
Many attempts have been made to identify Dendrobium species. In general, the differentiation relies on the morphology and anatomy of the fresh materials (Carlsward et al., 1997); however, this approach requires an empirical taxonomist for reliable identification. Pharmacognostic and chemical analyses may be used in the identification of medicinal Dendrobium. However, these analytical approaches are not always applied for species identification. In contrast, a molecular genetics approach offers a reliable and effective means of Dendrobium species identification. To date, several molecular analytical strategies have been applied to identify Dendrobium species. A suppression subtraction hybridization array was used to identify five Dendrobium species (Li et al., 2005). The internal transcribed spacer region in the rDNAs encoding nuclear rRNA genes and the plastid and mitochondrial genomes were used as DNA bar codes for the authentication of plant species (Tsai et al., 2004; Yao et al., 2009). Amplified fragment length polymorphism technology was also used to screen for genetic markers within a Dendrobium hybrid that was associated with a phenotype of interest (Hong et al., 2000). Random amplified polymorphic DNA was used to identify the genetic diversity of D. officinale (Ding et al., 2009) and to construct linkage maps of D. officinale and D. hercoglossum (Xue et al., 2010). In addition, adapter ligation-mediated allele-specific amplification was applied to authenticate three Dendrobium species, including D. aphyllum, D. devonianum, and D. officinale (Zhang et al., 2009). A set of polymorphic markers from 10 microsatellite loci were used to analyze the genetic diversity of D. fimbriatum (Fan et al., 2009). A species-specific inter-simple sequence repeat analysis was also used to authenticate several populations of D. officinale (Shen et al., 2006) and to evaluate the genetic diversity of 31 Dendrobium species (Wang et al., 2009).

The ITS region (ITS1-5.8S-ITS2) is highly repeated, up to many thousands of copies, in plant nuclear genomes (Baldwin et al., 1995), and this high copy number and its location between the conserved sequences of 18S and 26S RNA coding regions promote the detection, amplification, cloning, and sequencing of rDNA. Because the ITS1 and ITS2 regions exhibit high divergence among species and populations, they are suitable targets for species identification; in contrast, 18S, 5.8S, and 26S exhibit lower divergence among species and have been explored to study more distant relationships at the order or family level (Yip et al., 2007). The rapid concerted evolution of the ITS region through unequal crossing-over and gene conversion promotes the accurate reconstruction of species relationships according to the ITS sequences (Baldwin et al., 1995; Hillis et al., 1991).

Based on the ITS sequences, the multiplex PCR method can be used to amplify target-containing samples by mixing specific primers in a single PCR. This approach has been widely used to identify microbes and medicinal plants (Jigden et al., 2010). The amplification reagents were prepared using reverse primers for the amplification of the ITS region through unequal crossing-over in Vigna radiata (Shen et al., 2006) and Vigna radiata (Fan et al., 2009). A species-specific inter-simple sequence repeat analysis was also used to authenticate several populations of D. officinale (Shen et al., 2006) and to evaluate the genetic diversity of 31 Dendrobium species (Wang et al., 2009).

PLANT MATERIALS AND GENOMIC DNA ISOLATION. Twenty different species of Dendrobium were collected in Taiwan. The selected specimens were identified by experts and deposited in the School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan. The details of the plant materials are listed in Table 1. Genomic DNA (gDNA) was isolated from 0.08 g of either fresh or dried leaves or stems using a gDNA Purification Kit (Gene Mark Technology, Taichung, Taiwan) according to the manufacturer's instructions. The gDNA quantity and quality were determined by spectrophotometry and electrophoresis. The isolated gDNA was stored at -20°C.

POLYMERASE CHAIN REACTION AMPLIFICATION AND SEQUENCING OF THE INTERNAL TRANScribed SPACER REGION. The universal primers used for the amplification of the ITS region are listed in Table 1. Primers 5′-18S and 3′-26S were designed according to the known rice [Oryza sativa (Takaiwa et al., 1984, 1985), tomato [Solanum lycopersicum (Kiss et al., 1989a, 1989b), mung bean [Vigna radiata var. radiate (Schielbe and Hemleben, 1989)], and melon [Cucumis melo (Kavanagh and Timmis, 1988)] sequences. PCR for ITS from each Dendrobium species was performed in a 50-μL volume consisting of 50 ng of respective gDNA, 0.2 mM dNTP, 0.2 μM each upstream and downstream primer, 1 U of Tag DNA polymerase XL, 1X DNA polymerase buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.1% (w/v) gelatin, and 0.8% Triton X-100) (Protech Technology Enterprise, Taipei, Taiwan) on a thermal cycler (PTC-150; MJ Research, Waltham, MA). The PCR program consisted of an initial denaturation step at 93°C for 4 min followed by 33 cycles of 93°C for 40 s, 50°C for 1 min and 68°C for 1 min, and a final extension step at 68°C for 10 min. The amplified products were electrophoresed on 0.7% agarose gels. The DNA was eluted and cloned into the T&A vector (Yeastern Biotech, Taipei, Taiwan) and transformed in Escherichia coli XL-1. The cloned putative ITS fragments from each Dendrobium species were confirmed by sequencing using both M13 forward and reverse primers.

SEQUENCE ANALYSIS AND PHYLOGENETIC TREE CONSTRUCTION. The ITS DNA sequences of Dendrobium species were aligned and compared using the Clustal X program (Thompson et al., 1997). The pairwise evolutionary distances for the nucleotide sequences were estimated using the DNADIST program of PHYLIP software package Version 3.69 (Felsenstein, 2009) and the F84 method as the substitution model. Relationship dendrograms were drawn using the program TreeView (Page, 1996) with the neighbor-joining (NJ) and maximum parsimony (pars) analyses keeping the bootstrap value of 1000 replicates.
MULTIPLEX POLYMERASE CHAIN REACTION. The nucleotide sequences from the ITS1 region of six medicinal *Dendrobium* species (*D. tosaense*, *D. nobile*, *D. linawianum*, *D. hercoglossum*, *D. moniliforme*, and *D. huoshanense*) were used to design a specific primer, 5′-Herb. This primer was especially designed for multiplex PCR to distinguish the medicinal *Dendrobium* species from the other 14 *Dendrobium* species used in this experiment. The multiplex PCR was performed with the 5′-5.8S, 3′-26S, and 5′-Herb-specific primers (Table 2). The PCR conditions were the same as described previously with the exception of the concentrations of the primers (0.4 μM for 5′-Herb and 3′-26S, 0.1 μM for 5′-5.8S) and the annealing temperatures (40 to 68 °C).

AMPLIFICATION REFRATORY MUTATION SYSTEM POLYMERASE CHAIN REACTION ANALYSIS. Based on the alignment results of the ITS region, two SNPs were identified in *D. tosaense* at the nucleotide positions 44 and 614 with adenine (A) bases at both positions. In *D. goldschmidtianum*, the position 44 had a cytosine (C), whereas in *D. victoriae-reginae*, the positions 44 and 614 had a cytosine and adenine, respectively (Table 3). However, in all the other 17 *Dendrobium* species, these positions had either thymine (T) or guanine (G). According to the SNP sites, a pair of non-mutation ARMS primers, 5′-Tosae and 3′-Tosae, were designed to authenticate *D. tosaense* (Table 2). To increase the specificity, a pair of mutation ARMS primers, 5′-Tosae-5m and 3′-Tosae-5m, was designed by mismatching at the fifth nucleotide from the 3′ terminus of each primer (Table 2). The bases were mutated by the substitution at position 40 of A to G and 618 of G to C. Amplification of the fragment was performed in a 30-μL reaction volume containing 1 U of Taq DNA polymerase, 3 μL 10X DNA polymerase buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, and 25 mM MgCl2] (Protech Technology Enterprise), 0.2 mM dNTP, 0.2 μM of each primer (5′-Tosae-5m and 3′-Tosae-5m), and 50 ng of total DNA. The mixture was denatured at 93 °C for 4 min and subjected to 33 cycles at 93 °C for 40 s, 55 °C for 40 s, 72 °C for 1 min followed by a final extension at 72 °C for 10 min. Annealing temperatures ranging from 40 to 68 °C were tested in the experiment.

**Results**

The ITS regions (≈750 bp) between the 18S and 26S rDNA were amplified from 20 *Dendrobium* species. The amplified fragment comprised of a portion of the 18S rDNA, ITS1, 5.8S rDNA, ITS2, and a portion of the 26S rDNA. The sequences were submitted to the National Center for Biotechnology Information GenBank, and their assigned accession numbers are listed in Table 1. The lengths of the ITS regions in the 20 *Dendrobium* species varied from 636 to 653 bp, and the lengths of ITS1 (231 to 236 bp) were shorter than the ITS2 (241 to 254 bp). The length of the 5.8S rDNA was 163 bp, and it was conserved among all of the 20 *Dendrobium* species.

The nucleotide identity of the complete ITS region among the 20 *Dendrobium* species ranged from 75.7% to 99.1% and the variation in ITS1 and ITS2 was high with nucleotide identities ranging from 65.5% to 99.1% and 70.2% to 99.6%, respectively. The 5.8S region was highly conserved in all 20 of the *Dendrobium* species with the sequence identity ranging from 90.2% to 100%.
A phylogenetic tree was established based on the ITS sequences of the 20 Dendrobium species and Epigeneium nakaharaei (an Orchidaceae family member) was used as an outgroup. The maximum parsimony tree shown in Figure 1 was similar to the trees generated by the NJ method (data not shown). As shown in Figure 1, the common clade shared by all the six medicinal Dendrobium species (D. tosaense, D. nobile, D. linawianum, D. hercoglossum, D. moniliforme, and D. huoshanense) indicates their close relationship. It is interesting that D. leptoclandum, which is not considered a medicinal Dendrobium, was grouped together with the six medicinal Dendrobium species. Other Dendrobium species (D. crumenatum, D. equitans, D. goldschmidtianum, and D. victoriae-reginae) formed a separate clade and displayed distance from the other medicinally important Dendrobium species. As an outgroup, E. nakaharaei solely comprised a separate clade.

Multiplex PCR was performed using three primers (5’-5.8S, 3’-26S, and 5’-Herb-specific) for amplifying the ITS region (Fig. 2). The expected amplicons were detected in all the six medicinal Dendrobium species. The primers 5’-5.8S and 3’-26S amplified a product of 439 bp, whereas the primers 5’-Herb and 3’-26S amplified a product of 597 bp. However, from the other 14 Dendrobium species, as expected, no product was amplified by the primers 5’-Herb and 3’-26S primers, whereas the primers 5’-5.8S and 3’-26S amplified a 439-bp product (Fig. 2).

ARMS-PCR was performed using non-mutated and mutated ARMS primers (Table 2) to discriminate D. tosaense from the other Dendrobium species. When the annealing temperatures ranged between 62.2 and 64.4 °C, the expected 610-bp amplicon was detected for D. tosaense using the non-mutated ARMS primers. However, a band at the same position was also observed from D. nobile when the annealing temperature was below 62.2 °C (data not shown). A clear amplicon of 610 bp was exclusively detected for D. tosaense using the mutated ARMS primers (5’-Tosae-5m and 3’-Tosae-5m), whereas there was no amplicon detected for the other 19 Dendrobium species using annealing temperatures ranging between 51.2 and 62.2 °C (Fig. 3). However, a higher molecular weight product of ≈700 bp was weakly amplified from D. victoriae-reginae [Fig. 3 (lane 19)]. The results indicate that the accuracy of the Dendrobium species authentication can be improved by using mutated ARMS primers.

Discussion

The present study was performed to analyze the ITS regions in 20 Dendrobium species. The ITS regions were analyzed to locate specific nucleotide sequences to identify Dendrobium species and to study the phylogenetic relationships between the species. The phylogenetic analysis revealed that all six of the
Dendrobium species shared the same clade (Fig. 1). The nucleotide sequences from the ITS regions of these six species were aligned, and a species-specific primer was designed from the conserved regions among these species. This primer was then used in multiplex PCR along with two universal primers to discriminate the six Dendrobium species from other 19 Dendrobium species using a mixture of three primers. The universal primer pairs 5′-5.8S and 3′-26S designed for identifying Dendrobium species produced a DNA fragment of 439 bp. The primer pair 5′-Herb (designed based on the conserved sequence of six herbal Dendrobium species) and 3′-26S produced a specific DNA fragment of 597 bp. Lanes 1 to 20 correspond to the Dendrobium species listed in Table 1. Details of the primers are provided in Table 2. Lane M = DNA ladder (GM100; Gene Mark Technology, Taichung, Taiwan).

The phylogenetic relationship based on the ITS sequences revealed that the six medicinal Dendrobium species, D. tosaense, D. nobile, D. linawianum, D. moniliforme, D. hercoglossum, and D. huoshanense, are clustered into a common clade of the parsimony tree (Fig. 1). Xue et al. (2006) reported that intraspecific sequence variation in the ITS region of Dendrobium is less than 3% for ITS1 and less than 4% for ITS2. Our results reveal that the intraspecific nucleotide variation of ITS regions from five Dendrobium species (D. tosaense, D. linawianum, D. moniliforme, D. crumenatum, and D. goldschmidtianum) ranged 0% to 3.5% (data not shown). Phylogenetic analysis suggests that the six medicinal Dendrobium are closely related and that the intraspecific genetic diversity does not imply their intimate coevolution (data not shown).

Asahina et al. (2010) constructed a phylogenetic tree according to a plastid maturase-encoding gene (matK), which revealed that D. tosaense, D. nobile, and D. moniliforme were closely related, whereas D. fimbriatum formed a distinct clade. Corroboratively, our results also showed the close relationship of D. tosaense, D. nobile, and D. moniliforme; whereas D. leptoclandum, which is not used medicinally, was located in the same clade as the six medicinal Dendrobium species (Fig. 1). It will be interesting to ascertain whether D. leptoclandum contains certain bioactive ingredients and can possibly be

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**Table 3. Two authenticable sites of ribosomal DNA among 20 Dendrobium species for amplification refractory mutation system (ARMS) primer design to authenticate D. tosaense.**

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*Numbers correspond to those Dendrobium species listed in Table 1.

Authenticable site refer to the position of internal transcribed spacer (ITS) region in D. tosaense (Genbank accession no. EU003113).
considered for use in herbal dendrobii. The pairwise sequence analysis of the *Dendrobium* species with different geographical origins [i.e., *D. goldschmidtianum* (Taiwan) and *D. victoriae-reginae* (Philippines)] showed that they share 99% sequence identity in their ITS regions. The sequence identity between these two species is 99.6%, 99.4%, and 97.6% for the ITS1, 5.8S rDNA, and ITS2 regions, respectively, indicating that they have evolved from the same or a closely related ancestor.

Based on the close genetic relationship and specificity of the nucleotide sequences among the six *Dendrobium* species, the multiplex PCR discriminated the six medicinal *Dendrobium* species from the other 14 *Dendrobium* species (Fig. 2). This approach could be adopted for the detection of multiple therapeutic medicinal plants being used as the sources for Shi-hu materials in the markets. SNPs have been widely used in studies of marker-assisted breeding and herb identification. The ARMS-based PCR method is a simple, time-saving, and effective diagnostic technique to distinguish between adulterants and crude drugs (Cha et al., 1992; Qian et al., 2008). In our study, the ARMS-based PCR technique was successfully used to distinguish *D. tosaense* from 19 other *Dendrobium* species. The non-mutated primer pair contained a complementary 3′ residue that matched to *D. tosaense* but not to the other *Dendrobium* species, whereas the mutated primer pair had an additional mismatch at the fifth nucleotide residue from the 3′ terminus. Previous studies have shown that non-mutated primers with only one mismatch at the terminus may not be specific enough if terminal mismatching has weak destabilizing effects; therefore, mutated ARMS primers are superior to non-mutated ARMS primers (Little, 2000; Qian et al., 2008). A web-based allele-specific PCR assay designing tool to design primers for both SNPs and mutations suggests that a deliberate mismatch at the terminus may not be specific enough if terminal mismatching has weak destabilizing effects; therefore, mutated ARMS primers are superior to non-mutated ARMS primers (Little, 2000; Qian et al., 2008). Our results reveal that a modified ARMS in which an additional mutation was introduced at the fifth residue of the primer produces a dramatic reduction in the non-specific PCR products and has a minor effect on the amplification of the specific allele at wide annealing temperatures, ranging from 51.2 to 62.2 °C.

The established ITS database enables a reliable method to distinguish plant materials at the species level. Using multiplex PCR, we were able to discriminate the six medicinal *Dendrobium* species commonly found on the market in Taiwan from 14 other species. The SNP sites in the rDNA ITS regions were selected to design diagnostic primers to authenticate specific species using ARMS PCR. In this study, the PCR-based multiplex and ARMS methods were successfully used to produce a DNA marker for the routine analysis of medicinal plants. This strategy can potentially be directly applied for the identification of the crude drug Shi-hu for practical use.

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