Journal of Food and Drug Analysis

Volume 12 | Issue 4 Article 4

2004

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Journal of Food and Drug Analysis, Vol. 12, No. 4, 2004, Pages 367-369 藥物食品分析 第十二卷 第四期

The rDNA Sequence Analysis of Three *Dendrobium* Species

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(Received: April 14 , 2003; Accepted: September 22, 2004)

ABSTRACT

Positive molecular identification of medicinal plants is necessary to their misuse. Dendrobii Caulis is a famous Chinese herbal medicine traditionally used in ophthalmology. However, the identification of the species based on morphology is difficult. In this study, three *Dendrobium* species were collected and differentiated by the sequences of partial rDNA (ribosomal DNA) sections which contain the ITS 1, 5.8S, and ITS 2 regions. The *Dendrobium* species used in this reseach included *Dendrobium tosaense*, *Dendrobium officinale*, and *Dendrobium moniliforme*. The length of PCR products are 632 nt for *D. tosaense*, 627 nt for *D. officinale*, and 628 nt for *D. moniliform*, respectively. The similarities of the rDNA region between the species pairs shown range from 91% to 95%. The results we present here may be helpful in identifying other species of *Dendrobium*.

Key words: *Dendrobium*, phylogeny, rDNA, ITS

INTRODUCTION

The genus of *Dendrobiuma*, the largest in the Orchidaceae family, contains approximately 1,500 species. The major distribution of these species is in Southeast Asia, extending from Japan, Korea, and China through to the Indomalayan region and Indonesia to Australia(1). More than 80 species are found in China. These plants have been used as Chinese medicines to nourish vital essence, to clear up evil heat and improve secretion by administering drugs sweet in flavor and cold in property. They have also been found effective in moistening the lung and relieving cough symptoms(2). In addition, 15 species of *Dendrbium* found in Taiwan have been reported(1), but no anatomic descriptions were published. No significant deviations among the species were found when microscopic identification was performed, except for the needle-shaped calcium oxalate crystal found in some target species.

To investigate the variations resulting from the different ecological habitats among the species of *Dendrobium*, Jones *et al*.(3) determined the nuclear DNA contents of the 37 species of *Dendrobim* collected. Their results revealed a large diversity among the samples. Their DNA content values ranged from 1.53 pg $2C^{-1}$ to 4.23 pg $2C^{-1}$. Lau *et al*.(4) differentiated 16 species of Dendrobium using the ITS 2 (internal transcribed spacer 2) data. They found that the variation among the *Dendrobium* species was about 12.4% on average, while only 1% variations were revealed within the different *Dendrobium* species.

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MATERIALS AND METHODS

I. *Materials*

The plants of the three *Dendrobium* species, *Dendrobium tosaense*, *Dendrobium officinale*, *Dendrobium moniliforme*, collected from China and Taiwan, were transplanted in the green house of Taiwan Agricultural Research Institute. The identification of these species was performed according to the morphological characteristics of their flowers(1). The fresh leaves of the samples were ground into powder with mortar and pestle in liquid nitrogen and stored at -70˚C. All voucher specimens (Accession No. SP 010418) were prepared and deposited at the herbarium of Taipei Medical University.

II. *DNA Extraction*

Total DNA was extracted from 100 mg of powdered sample using a modified CTAB (cetyltrimethylammonium bromide) procedure(5). To this was added 1.2 mL of 2X CTAB extraction buffer (2% CTAB; 100 mM Tris-HCI, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 0.2% 2-mercaptoethanol). The suspension was incubated with RNase A (1 mg/ 1 mL) in a water bath at 50˚C for 30 min with occasional shaking, then cooled to room temperature and extracted with one vol. aqueous phenol/chloroform/isoamyl alcohol (25:24:1) twice. After centrifugation at 12,000 × g for 10 min, 0.1 vol. 65˚C 10% CTAB buffer (10% CTAB; 0.7 M NaCl) was added to the upper aqueous layer. The mixture was extracted by phenol/chloroform/isoamyl alcohol extraction one more time. The two phases were separated by centrifugation and one vol. CTAB precipitate buffer (1% CTAB;

50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) was added to the aqueous phase. The mixture was centrifuged at 12,000 × g for 20 min. The pellet was washed with 70% ethanol twice and prepared for genetic analysis.

III. *PCR Reaction*

We refer to the primers for rDNA amplification of *Krigia biflora* used by Kim and Jansen[6]. The forward

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                    1                                                  50
D. moniliforme  (1) TCGAGACCGAAACACAACGAGCGATTTTGTGAACCTGTAAAAAGAAGCGG
D. officinale   (1) TCGAGACCGAAACACAACGAGCGATTTTGTGAACCTGTAAAAATAAGCGG
D. tosaense     (1) TCGAGACCGAAACACAACGAGCAATTTTG-GAACCCGTTAAAAAAAGCGG

                   51                                                 100
       (51) TGGCTCTTGCTGCTGCGATAAAATCCACCCGAGTCATTCGCCTCATCCCC
       (51) TGGCTCTTGCTGCTGCGATAAAATTCACCTGAGTCCATCGCCTCATCCCA
       (50) CGGCTCTTGCTGCTGAGATAAAATCCACTGAAGTC-ATCGCCTCATCCCC

                  101                                                 150
      (101) TCTTTGGGATGGGGACGTGATGAAGGATGGATGAACCCTCAAATCGGCGC
      (101) TCTTTGGGGTGGGGACGTGATGAAGGATGGATGAACC-TCAAATCGGCGC
       (99) TCTATGGGGTGTGGACGTGATGAAGGATGGATGAACCCTAAAATCGGCGC

                  151                                                 200
      (151) AGCGTAGCGCCAAGGGAATATT-GAAACACAAGCCTATAAATGGGCTTTG
      (150) AGCGTAGCGCCAAGGGAATCTT-GAAACACAAGCCCATAAATGGGTTTTG
      (149) AGCGTAGCGCCAAGGGAATCTT-GAAACACAAGCCCATAA-TGGGTTTTG

                  201                                                 250
      (200) TGGGATGGGGTGTTGTCGCACGCCATATTGATTGACACGACTCTCGGCAA
      (199) TGGGATGGGGTGCTGTCGCACGCCATATTGATTGACACGACTCTCGGCAA
      (197) TGGGATGGGGTGTTGTCGCACGCCATATCGATTGACACGACTCTCGGCAA

                  251                                                 300
      (250) TGGATATCTCGG-TTCTCGCATCGATGAAAAGCGCAGCGAAATGCGATAT
      (249) TGGATATCTCGG-CTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATAT
      (247) TGGATATCTCGG-CTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATAT

                  301                                                 350
      (299) GTGGTGCGAATTGCAAAATCCCGCGAACCATCGAGTCTTTGAACGCAAGT
      (298) GTGGTGCGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAAGT
      (296) GTGGTGCGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAAGT

                  351                                                 400
      (349) TGCGCCTGAGGCCAACTGGCTGAGGGCACGTCCGCCTGGGCGTCAAGCAT
      (348) TGCGCCTGAGGCCAACCGGCTGAAGGCACGTCCGCCTGGGCGTCAAGCAT
      (346) TGCGCCCAAGGCCAACCGGCTAAGGGCACGTCCGCCTGGGCGTCAAGCAT

                  401                                                 450
      (399) TTTATCACTCTGTGCCTAGTCTCACATCCATGGATGTGTTGCCAAGGCTC
      (398) TTTATCACTCCGTGCCTAGTCTCCCATCCATGGATGTGTTGCCAAGGCTC
      (396) TTTATCTCTCCGTGCCTAATCTCCCATCCATGGATGTGTTACTAAGGCTC

                  451                                                 500
      (449) GGGATGTGCAC-GGTGGCTCGTCGTGCCCCTTGGTGCGGCGGGC-TGAAG
      (448) GGGATGTGCAC-GGTGGCTCGTCGTGCCCCTTGGTGCGTCGGGC-TGAAG
      (446) GG-ATGTGCAATGGTGGCTCCTCGTGCCCCTTGGTGCGGCGGGC-TGAAG

                  501                                                 550
      (497) GGCGGGTCA-TCTTCTCGTTGGCTGCCAACAATAAGGGGGTGGATTAAAT
      (496) GGAGGGTCA-TCTTCTCGTTGGCTGCCAACAATAATGGG-TGGATTAAAT
      (494) GGCGGGTCA-TCTTCTCGTTGGTTGCCAACAATAAGGGG-TGGATTAAAA

                  551                                                 600
      (546) AAGGCCTATGCTATTGTGTCAAGCGCGCCTGAGAGATGGGCATACTTTTT
      (544) AAGGCCTATGCTATTGTGTCAAGCAAGCCTGAGAGATGGTCATACTTTTT
      (542) AAGGCCTATGCTATTGTGATAAACGCCCCCGAGAGATGATCATACTTTTT

                  601                 629
      (596) AGGGTGATCCCAATTCATACCGTTGATCC
      (594) AGG-TGATCCCAATTCATGC-GTTGATCC
      (592) AGG-TGATCCCAATCCATGC-GCTAATCC
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Figure 1. The sequence alignment among the three *Dendrobium* species used in the study.

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primer P1 (5'-GGAAGTAAAAGTCGTAACAA-3') is located at the flanking region of the 3' end of 18S and the reverse primer P4 (5'-TCCTCCGCTTATTGATATGC-3') is located at the flanking region of the 5' end of 25S. PCR was performed in a total of 100 μL, which consisted of 1 μM each of the primers, 0.8 mM of dNTP, 50 ng genomic DNA as a template, and 0.625 U *Taq* polymerase (PROTECH, USA) in 1X PCR buffer mixture (PROTECH, USA). Thirty-five reaction cycles were performed at 94˚C, 62˚C, and 72˚C for 1, 1, and 2 min, respectively. PCR products were recovered from the gel and sequenced directly.

IV. *Sequencing*

The sequencing reactions of the PCR products were carried out using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Automatic System according to the manufacturer's protocol. The sequences were aligned manually. The sequence data published in this paper were deposited in the GenBank nucleotide sequence databases with the following accession numbers: AF401488 for *D. tosaense*, AF401490 for *D. officinale*, AF401489 for *D. moniliforme*.

RESULTS

The rDNA regions of the three *Dendrobium* species were amplified by PCR reaction. The length of the fragment of *D. tosaense* was 632 nt (nucleotides). *D. officinale*, and *D. moniliforme* had lengths of 627 nt, 628 nt, respectively. The region contains ITS 1, 5.8S, and ITS 2 sequences. A comparison of the sequence alignments is shown in Figure 1. The similarities of the rDNA sequences among the samples are showed in Table 1.

DISCUSSION

The PCR technique is strongly recommended in identification of Chinese medicines because of the minute amount of DNA needed and the simplicity of the procedure. However, known sequence information must be obtained for PCR primer design. It is difficult to get data on medicinal herbs, so the RAPD technique was considered in the search for more polymorphic fragments using the random primers. RAPD analysis was performed to create several informative markers in herbal differentiation, but the low reproducibility of the technique became the limitation of its application.

The rDNA sequences of plants are widely analyzed for evolution and anatomy studies of plants. The 18S, 5.8S, and 26S are highly conserved in the sequence, making the plants belonging to the same family difficult to distinguish. The ITS 1 and ITS 2 regions were found to be heterologous in sequence and copy number, but low percentage differences were found between the samples of intra-species or inter-species. Fu *et al*.[7] investigated the diversity among the four species of *Codonopsis* by the ITS 1 and ITS 2 sequences. No more than 2% deviation in ITS 1 fragment was found among the samples, and no more than 1% was found in ITS 2.

Table 1. The sequence similarity (%) of the rDNA region between the three *Dendrobium* species pairs used in the study

	D. moniliforme	*D. officinale*	*D. tosaense*
D. moniliforme	100	95	91
D. officinale		100	92
D. tosaense			100

For quality control of Chinese medicines, other more sensitive and accurate biotechniques should be developed. We believe that a lot of herbal chips will be produced for identifying the species of Chinese medicines. More specific fragments able to differentiate between the different genomes are to be aligned on the chips. The testing procedure should to be standardized for routine work in the identification process.

ACKNOWLEDGMENTS

This research was supported by a grant from the National Science Council (NSC91-2317-B-038-001), Taiwan, ROC.

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