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ITS Sequence Based Phylogenetic Relationship of Dangshen Radix

TZU-CHAO LIN[1], CHANG-CHI HSIEH[2], DINESH CHANDRA AGRAWAL[3], CHAO-LIN KUO[4], FU-SHIN CHUEH[5] AND HSIN-SHENG TSAY[3]*

[1.] *Institute of Chinese Pharmaceutical Science, China Medical University, Taichung 40402, Taiwan (R.O.C.)*

[2.] *Graduate Institute of Integrated Medicine, China Medical University, Taichung 40402, Taiwan (R.O.C.)*

[3.] *Graduate Institute of Biotechnology, Chaoyang University of Technology, 168, Jifong E. Rd., Wufong, Taichung 41349, Taiwan (R.O.C.)*

[4.] *School of Chinese Medicine Resources, China Medical University, Taichung 40402, Taiwan (R.O.C.)*

[5.] *Department of Applied Life Science, Asia University, Wufong, Taichung 41354, Taiwan (R.O.C.)*

ABSTRACT

Dried root of *Codonopsis* species (Campanulaceae), commonly known as "Dangshen" or "Dangshen Radix" is a multipurpose crude drug used in traditional Chinese medicinal system. The drug is sometimes used as a substitute of much expensive *Panax ginseng*. Due to variation in processing methods and producing areas, Dangshen is known by several trade names. This results in confusion of crude drug administration and application. Accounding to Chinese Pharmacopoeia, 3 species of *Codonopsis*; *C. pilosula*, *C. modesta* and *C. tangshen* have been mentioned as sources of the crude drug "Dangshen". In Taiwan, *C. javanica*, *C. kawakamii* and *Campanumoea lancifolia* known as "Tu-dangshen" are commonly used as "Dangshen Radix", *Codonopsis kawakamii* is indigenous to Taiwan. The morphological features of dried roots of these six plant species are quite similar; hence it is difficult to identify these species on the basis of physical appearance alone. In the present study, Internal Transcribed Spacers (ITS)-based analysis was used to ascertain the phylogenetic relationship among the six species. Results showed that the length of the ITS regions among the six species ranged from 638 to 655 bp and the contents of GC in ITS (ITS1+5.8S+ITS2) regions ranged from 60.31 to 62.52%. The lengths of ITS and GC contents of ITS sequence and molecular phylogenetic trees of six Dangshen sources indicate that *C. javanica* has more homology to *Campanumoea lancifolia* than the other four species of *Codonopsis*.

Key words: Dangshen, *Codonopsis*, *C. pilosula*, *C. modesta*, *C. tangshen*, *C. kawakamii*, *C. javanica*, *Campanumoea lancifolia*, internal transcribed spacer (ITS)

INTRODUCTION

Dried root of *Codonopsis* species (Campanulaceae), commonly known as "Dangshen" or "Dangshen Radix" is used in traditional Chinese medicinal system. Dangshen has a sweet taste and acts on the spleen and lung channels[(1)]. Over three hundred years, dried roots of Dangshen have been used in enhancing vital energy and blood circulation, lowering the blood pressure, and for treatment of watery stool with poor appetite, neurosis, hematopoietic disease, poor gastrointestinal function, gastric ulcer, nephritis[(2)]. The medicine is also used to induce saliva production[(3)] and sometimes used as a substitute of *Panax ginseng*[(4)].

There are more than 60 species of *Codonopsis* mainly growing in Central and Eastern Asia. At least 39 species have been found in Taiwan and Mainland China. Due to the variation in processing methods and producing areas, Dangshen is known by several trade names. This results in confusion of crude drug administration and application. In the Chinese Pharmacopoeia[(5)], 3 species of *Codonopsis*, i.e. *C. pilosula*, *C. modesta* and *C. tangshen* have been mentioned as sources of crude drug "Dangshen". In Taiwan, *C. javanica*, *C. kawakamii* and *Campanumoea lancifolia* known as "Tu-dangshen" are commonly used as "Dangshen Radix". *Codonopsis kawakamii* is indigenous to Taiwan. The species, used to distrute in the central mountain areas, is now adapted to a narrow set of environmental conditions and can be found only at high mountain area (2,300-3,500 m). The morphological features of dried roots of these six plant species are quite similar; hence it is difficult to identify these species on the basis of physical appearance alone.

Currently, Internal Transcribed Spacers (ITS) is widely used in taxonomy and molecular phylogenetics[(6,7)]. ITS is a sequence that has two internal transcribed spacers, ITS1 and ITS2. The location of ITS1 is between the 18S and 5.8S genes, while, ITS2 between the 5.8S and 28S genes. These two ITS regions are useful markers for authentication as they are hyper-variable among plant species. Since ITS can be amplified by conserved primers in the flanking regions[(8)], this make

* Author for correspondence. Tel: +886-4-2330-4920; Fax: +886-4-2330-4921; E-mail: hstsay@cyut.edu.tw

Journal of Food and Drug Analysis, Vol. 15, No. 4, 2007

ITS analysis become easy and reliable. The method has several advantages including simplicity of operation, lower costs, and need of minute quantities of DNA samples[9].

In the present study, ITS based analysis was used to ascertain the genetic relationship among the six species, *Codonopsis pilosula* (Franch.) Nannf, *C.* Nannf. var. *modesta* (Nannf.) L. T. Shen and *C. tangshen* Oliver, *C. javanica*(Blune) Miq. subsp. *Japonica*, *C. kawakamii* Hayata and *Campanumoea lancifolia* (Roxb.) Merr.

MATERIALS AND METHODS

I. *Plant Materials*

Plant materials (whole plant, leaves or roots) of 6 species (*Codonopsis pilosula* (Franch.) Nannf, *C.* Nannf. var. *modesta* (Nannf.) L. T. Shen and *C. tangshen* Oliver, *C. javanica*(Blune) Miq. subsp. *Japonica*, *C. kawakamii* Hayata and *Campanumoea lancifolia* (Roxb.) Merr) were collected from Taiwan and China, as listed in Table 1. The voucher specimens were deposited in the Herbarium of China Medical University (CMU), Taichung, Taiwan. The species were identified by Prof. C.C. Chen and Prof. C.L. Kuo of CMU.

II. *DNA Extraction*

Samples of fresh leaves collected in liquid nitrogen were ground into powder. Dried roots were first debarked with a sharp knife, made into thin slices and then ground into powder with liquid nitrogen. Genomic DNA extraction procedure for both the samples was followed as reported by Dellaporta *et al.* 1983[10]. The extraction buffer consisted of 100 mM Tris-HCl (pH 8.0), 50 mM EDTA-Na, 500 mM NaCl, 10 mM β-mercaptoethanol, 0.5% sodium dodecyl sulfate and 1% polyvinyl polypyrrolidone. The mixture was incubated for 10 min at 65°C and then 5 M potassium acetate (400 µL) was added and kept on ice for 30 min for cell lysis. After that, the mixture was centrifuged at 12,000 rpm for 10 min. Equal volume of isopropanol was added to the supernatant for precipitation. After centrifugation at 12,000 rpm for 15 min, pellet formed was dissolved in 500 µL of TE buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and extracted by equal volumes of Tris-HCl (pH 8.0) saturated phenol. The aqueous phase was collected and re-extracted by equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). To the aqueous phase was added an equal volume of chloroform/isoamyl alcohol (24:1). The extracted aqueous phase was precipitated by two volume of absolute ethanol and 150 µL of 7.5 M ammonium acetate. After centrifugation at 12,000 rpm for 15 min at 4°C, the resulting pellet was dissolved in 200 µL of TE buffer.

III. *Polymerase Chain Reaction (PCR) Amplification*

Each PCR reaction (25 µL) consisted of 2.5 µL of 10X reaction buffer, 200 µM each of the four dNTPs stock, 1.0 µM of forward and reverse primers (synthesized by Protech Technology Enterprise Co., Ltd., Taiwan), and 1 unit of Klen*Taq* polymerase (Protech Technology Enterprise Co., Ltd., Taiwan). The primers

Table 1. Particulars of materials used as Dangshen

Code	Folk name	Locality	Species	Region
P1	Bai-Taio-Dang	Weiyuan, China	*Codonopsis pilosula*	Leaves
P2	Bai-Taio-Dang	Weiyuan, China	*C. pilosula*	Dried roots
P3	Bai-Taio-Dang	Wudou, China	*C. pilosula*	Dried roots
P4	Lu-Dang	Hong Kong	*C. pilosula*	Dried roots
M1	Wun-Dang	Wudou, China	*C. modesta*	Leaves
M2	Wun-Dang	Taichung, Taiwan	*C. modesta*	Dried roots
M3	Wun-Dang	Taichung, Taiwam	*C. modesta*	Dried roots
T1	Su-Dang	Sinjhu, Taiwan	*C. tangshen*	Dried roots
T2	Ban-Chao-Dang	Taichung, Taiwan	*C. tangshen*	Dried roots
T3	Chuang-Dang	Chengdu, China	*C. tangshen*	Dried roots
K1	Taiwan-Dangshen	Kunyang, Taiwan	*C. kawakamii*	Leaves
K2	Taiwan-Dangshen	Yuanfong, Taiwan	*C. kawakamii*	Leaves
K3	Taiwan-Dangshen	Mt.Lingjhi, Taiwan	*C. kawakamii*	Leaves
J1	To-Dangshen	Caoling, Taiwan	*C. javanica*	Leaves
J2	To-Dangshen	Sitou, Taiwan	*C. javanica*	Leaves
L1	Taiwan-Dangshen	Sitou, Taiwan	*Campanumoea lancifolia*	Leaves
L2	Taiwan-Dangshen	Jhonglin, Taiwan	*Campanumoea lancifolia*	Leaves

used for ITS1 were 18D (5'-CAC ACC GCC CGT CGC TCC TAC CGA-3') and 1858 (5'-TTG CGT TCA AAG ACT CGA TG-3'), and for ITS2 were 2858 (5'-CAA CGG ATA TCT CGG CTC TC-3') and 28CC (5'-ACT CGC CGT TAC TAG GTG AA-3')[11]. The positions of ITS regions relative to 18S, 5.8S and 28S rDNA, and corresponding positions of primers used for PCR and sequencing are illustrated in Figure 1. Genomic DNA (50 ng) was used as a template for each PCR reaction carried out in a thermocycler (PC-818, AZTEC Co. Ltd., Fukuoka Japan). PCR programme consisted of denaturation step at 94°C for 5 min followed by 50°C for 1.5 min and 72°C for 3 min for first template amplification, and then 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min, and final extension step at 72°C for 10 min. The PCR products were electrophoresed in 2.0% agarose with ethidium bromide staining and visualized under UV.

IV. *Cycle Sequencing*

Sequencing was carried out by Tri-I Biotech, Inc., Taiwan. Briefly, the PCR products were purified by ethanol precipitation. One microliter (~50 ng) of purified product was added into a mixture containing 4 μL terminator mix including A-Dye terminator labeled with dichloro-R6G, C-Dye terminator labeled with dichloro-TAMRA, G-Dye terminator labeled with dichloro-R110, T-Dye terminator labeled with dichloro-ROX, dexoynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), AmpliTaq DNA polymerase, FS with thermally stable pyrophosphatase, $MgCl_2$, and Tris-HCl buffer, pH 9.0, 1.0 μL of 3.2 μM primer (18D, 28CC) and 5.0 μL deionized water. The mixture was subjected to 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min each cycle. The product for cycle sequencing was again purified by ethanol precipitation and then loaded into an ABI PRISM 3730 Genetic Analyzer for sample electrophoresis. The ITS region of each individual was sequenced in both 5' and 3' direction at least 3 times as to define the ITS sequence.

V. *Sequence Alignments and Phylogenetic Trees*

The DNA sequences were compared and aligned by the programs BioEdit (version 7.0.5.2) and MEGA and further verified by comparing with the sequences of other species i.e. *Platycodon grandiflorum* by BLAST search in the web site of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Comparisons of entire sequences of the tested samples were aligned by a software ClustalW (version 1.83) (http://www.ebi.ac.uk/clustalw/index.html) with a gap opening of 10 and gap extension of 0.05. Phylogenetic tree were based on the hierarchical clustering of the alignments of ITS1, 5.8SrDNA and ITS2 and produced by Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods using the Phylip (version.3.6) and MEGA (version 3.1) software of the bootstrap values (1000 replicates). For outgroup sequences, *Platycodon grandiflorum*, a plant species from same family Campanulaceae was chosen.

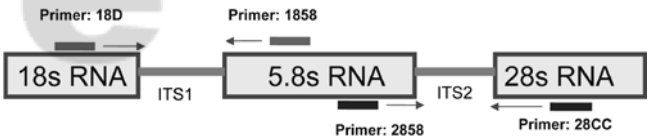

Figure 1. The structure of ribosomal DNA of higher plants.

RESULTS AND DISCUSSION

Out of 17 samples (codes in Table 1) of Dangshen belonging to 6 species (*C. pilosula*, *C. modesta*, *C. tangshen*, *C. kawakamii*, *C. javanica* and *Campanumoea lancifolia*), the sequences of samples P1, P2, P3 and P4 had the variable site in 122 (C/T transition) of ITS, while the sequences of M1, M2 and M3 had the variable site in 489 (C/T transition) of ITS. The sequences of the other 10 respective samples (T1-L2) had no variable sites. There was no variation at all in the Dangshen samples from Taiwan (Data not shown).

I. *rDNA Sequence Analysis*

The two ITS regions between the 18S and 26S rDNA are useful markers for identification as they are hyper-variable among different plant species but can be amplified by conserved primers in the flanking regions. In the present study, the ITS1-5.8S-ITS2 regions of five *Codonopsis* species: *C. pilosula*, *C. modesta*, *C. tangshen*, *C. javanica*, *C. kawakamii*, and two related adulterants *C. lancifolia* and *P. grandiflorus* were amplified and sequenced. Primers 18d and 1858 were used to amplify the ITS1 region, and primers 2858 and 28CC were used for the ITS2 region of the six species, except for *P. grandiflorus*, which was taken from the GenBank accession number AF134863. Our sequence data had been submitted to GenBank and the GenBank accession numbers of the six ITS1-5.8S-ITS2 sequences are: EF190460 (*C. pilosula*), EF190461 (*C. modesta*), EF190462 (*C. tangshen*), DQ889459 (*C. javanica*), AY322047 (*C. kawakamii*), and EF206701 (*Ca. lancifolia*), respectively.

Phylogenetic analysis of six species of Dangshen was based on ITS sequences in nuclear ribosomal DNA. It is showed that the lengths of the ITS regions among the six species ranged from 638 to 655 bp, the GC contents in ITS (ITS1 +5.8S +ITS2) regions ranged from 60.31 to 62.52%, the lengths of ITSl ranged from 236 to 258 bp and the GC contents in the ITSl regions ranged from 63.57 to 66.95% (Table 2). The lengths of the 5.8S rDNA regions in the six species was 162 bp while those of ITS2 ranged from 231 to 240 bp. The GC contents ranged

Journal of Food and Drug Analysis, Vol. 15, No. 4, 2007

Table 2. Length (bp) and G+C contents of ITS, 5.8SrDNA and ITS2 of seven related of Dangshen

Species	Length (bp)				G+C content (%)				Genbank number
	ITS	ITS1	5.8SrDNA	ITS2	ITS	ITS1	5.8SrDNA	ITS2	
Codonopsis pilosula	655	258	162	235	60.61	63.95	55.56	60.43	EF190460
C. modesta	655	258	162	235	60.46	63.57	55.56	60.43	EF190461
C. tangshen	655	258	162	235	60.31	63.57	55.56	60	EF190462
C. kawakamii	655	258	162	235	60.61	63.95	55.56	60.43	AY322047
C. javanica	643	250	162	231	62.52	65.6	55.56	64.07	DQ889459
Campanumoea lancifolia	638	236	162	240	61.91	66.95	54.94	61.67	EF206701
Platycodon grandiflorus	662	255	162	245	57.55	58.43	54.32	58.78	AF134863

Table 3. Aligned variable site of ITS sequences among *Codonopsis* species of Dangshen

Species	Aligned variable site of ITS sequences																														
	13	79	80	85	86	87	90	94	99	100	101	102	122	131	182	190	192	193	220	225	234	238	250	255	258	432	436	462	467	499	500	599
C. pilosula	C	T	C	C	A	T	A	G	A	C	C	A	C	T	A	C	A	C	T	A	T	C	A	C	A	T	T	C	A	T	G	A
C. modesta	C	T	C	C	A	T	A	G	A	C	C	A	T	T	A	C	A	C	T	A	T	C	A	C	A	T	T	C	A	T	G	A
C. tangshen	C	T	C	C	A	T	A	G	A	C	C	A	T	T	A	C	A	C	T	A	T	C	A	C	A	T	T	C	A	T	A	A
C. kawakamii	C	T	C	C	A	C	A	G	A	C	C	A	T	T	A	C	A	C	T	A	T	C	A	C	A	T	T	C	A	T	G	A
C. javanica	A	C	T	T	G	C	G	A	C	T	T	G	C	G	C	T	T	T	C	G	C	T	G	T	G	C	C	T	C	C	G	C

from 54.94 to 55.56% in the 5.8S rDNA region and 60.0 to 64.07% in the ITS2 region. Among the regions of ITS, ITS1, ITS2, and 5.8S, the most appropriate sequences for genetic relationship analysis in the six species were the ITS regions.

The lengths and GC contents of ITS among 6 Dangshen species indicate that *C. javanica* has more homology to *Campanumoea lancifolia* than to the other 4 species of *Codonopsis* (Table 2). This is in contrast to the new classification (Flora of Taiwan, 1998). It seems that the earlier classification (Flora of Taiwan, 1978), where this species was known as *Campanumoea javanica* is more appropriate. This demonstrates that ITS data might be used to group a species correctly.

II. *Sequence Alignments / Nucleotide Variation*

Alignment of the ITS1-5.8S-ITS2 sequences from the five species of *Codonopsis* were analyzed by BioEdit (version 7.0.5.2). Thirty two nucleotide variations were found and the frequency of variations was 4.88% of all positions (data not shown). Among these positions, 25 were found in the ITSl region, 0 in the 5.8S region, and 7 in the ITS2 region. Comparative sequence analysis of the ITS1-5.8S-ITS2 rDNA sequences from five species is illustrated in Table 3. The variation in nucleotides in ITS1 region was greater than that in ITS2. On the contrary, the length of 5.8S was very conservative and the average length was 162 bp. There was no nucleotide variation among the five species. This is in consistent with results as reported earlier[12]. In the absence of nucleotide variation in the 5.8S and ITS2 regions, it is difficult to analyze accurately the relatedness among these five species. However our results on the ITS region, (composed of ITS1, ITS2, and 5.8S) did provide a more accurate analysis.

III. *Sequence Coefficients of Identity*

The analysis on the sequence identity matrix using ITS sequences showed that the identity percentages among the six species had a range of 81.6 to 99.8 (Table 4). The pairwise distance in the ITS among the six species was 1 to 58. According to the data, *C. kawakamii* has more relatedness to three species of *Codonopsis* than to the other two species. Similarly, *C. javanica* and *Campanumoea lancifolia* showed higher degree of relatedness (> 81.6%).

IV. *Molecular Phylogenetic Trees*

Phylogenetic trees based on ITS were generated by Neighbor-Joining (NJ) (Figure 2A) and Maximum Parsimony (MP) methods (Figure 2B). It was observed that *C. javanica*, *Campanumoea lancifolia* and *P. grandifloru* were identical in the ITS sequence and *C. kawakamii* was closely related to these three species. The results also showed that *C. javanica* was closely related to *Campanumoea lancifolia*, which is consistent with the results from analysis on length and GC contents of ITS sequence.

Table 4. The percentage identity of ITS (up diagonal) and pairwise distance (Number of differences, down diagonal) of six sources of Dangshen and one out- group species

Species	*Codonopsis pilosula*	*C. modesta*	*C. tangshen*	*C. kawakamii*	*C. javanica*	*Campanumoea lancifolia*	*Platycodon grandiflorum*
C. pilosula	---	99.8	99.6	99.6	83.4	84.7	83.4
C. modesta	1	---	99.8	99.8	83.3	84.5	83.4
C. tangshen	2	1	---	99.6	83.1	84.4	83.3
C. kawakamii	2	1	2	---	83.4	84.7	83.3
C. javanica	51	52	53	51	---	81.6	72.4
Campanumoea lancifolia	56	57	58	56	55	---	73.7
Platycodon grandiflorum	66	66	67	67	102	108	---

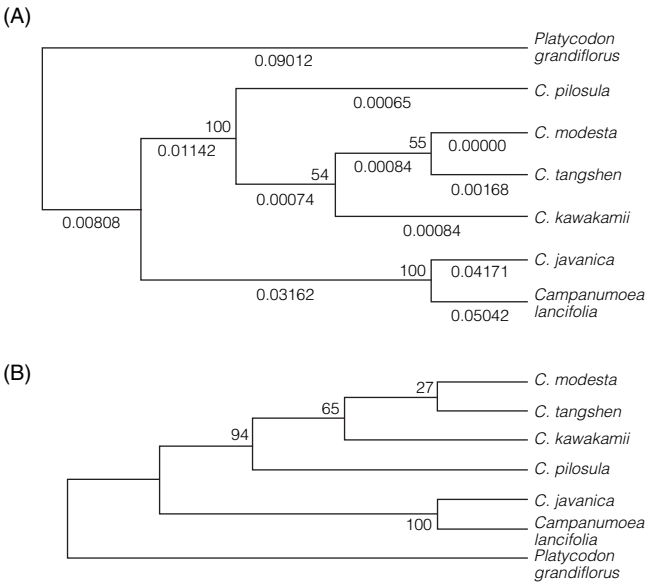

Figure 2. Phyogenetic trees based on the ITS sequence by (A) Neighbor-Joining (NJ) and (B) Maximum Parsimony (MP) methods.

CONCLUSIONS

Results on lengths of ITS, GC contents of ITS sequence and molecular phylogenetic trees of 6 Dangshen species indicated that *C. javanica* has more homology to *Campanumoea lancifolia* than the other 4 species of *Codonopsis*. Therefore the earlier classification (Flora of Taiwan, 1978), where this species was known as *Campanumoea javanica* seems to be more authentic.

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