Genetic and chemical polymorphism of medicinally-used *Codonopsis* species and its application to evaluate Codonopsis Radix

Jing-Yu HE

Division of Pharmacognosy

Institute of Natural Medicine

University of Toyama

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Introduction

Codonopsis Radix, called “Dangshen” in Chinese and “Tojin” in Japanese, has been used in traditional Chinese medicine for replenishing qi (vital energy) deficiency, strengthening the immune system, improving poor gastrointestinal function, gastric ulcer and appetite, decreasing blood pressure, etc. (Nanjing University of Chinese Medicine, 2006). This crude drug has been in high demand in China, which is included in several formulations such as Guipi Wan (帰脾丸), Sijunzi Wan (四君子丸), Shiquan Dabu Wan (十全大補丸), etc. (Chinese Pharmacopoeia Commission, 2010), and sometimes is used as a substitute for Ginseng because it has antifatigue and immunomodulatory activities similar to *Panax ginseng* (Wang et al., 1996). In the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), Codonopsis Radix is prescribed as the dried roots of *Codonopsis pilosula* (Franch.) Nannf., *C. pilosula* Nannf. var. *modesta* (Nannf.) L. D. Shen and *C. tangshen* Oliv. of family Campanulaceae. Recently, the demand of Codonopsis Radix as a health food has been increasing in Japan, and those cultivated in Gansu and Guizhou Provinces, China have been imported and available in Japanese markets. However, the botanical sources of crude drug samples are uncertain due to morphological similarity of the three taxa. To authenticate Codonopsis Radix, the morphological and histological studies have been carried out on the roots of seven *Codonopsis* species, including the above three taxa (Namba et al., 1992a, b). However, the histological characters such as the shape of parenchyma cells of the phloem and xylem, the percentage value of the diameter of the xylem to that of the root may be varied in different growing stage of plants and drying condition of the roots. Thus, it is difficult to elucidate the botanical origin of the

Codonopsis Radix based on morphological inspection and histological characters. The other accurate identification methods are required because the botanical source is one of the important factors which affect the efficacy of crude drug.

Recently, molecular approaches are widely used not only in plant taxonomy but also for identification of crude drugs. Lin et al. (2007) reported the phylogenetic relationship among 5 *Codonopsis* species and *Campanumoea lancifolia* (Roxb.) Merr. based on the internal transcribed spacer sequence (ITS) of nuclear ribosomal DNA (nrDNA). On the other hand, Guo et al. (2007) reported the abundant genetic diversity in cultivated *C. pilosula* populations from Longxi County, Gansu Prov. based on randomly amplified polymorphic DNA (RAPD) analysis. Therefore, after the genetic polymorphism of medicinally-used *Codonopsis* species, the molecular identification method should be developed.

Phytochemically, the roots of *C. pilosula* or *C. tangshen*, and Codonopsis Radix have been reported to contain polysaccharides, polyacetylenes, phenylpropanoids, alkaloids, triterpenoids, etc. (Ishimaru, et al., 1991; Ishimaru, et al., 1992; Wang and Wang, 1996; Zhu et al., 2001; He et al., 2006; Song et al., 2008a; Tsai and Lin, 2008; Wakana et al., 2011). Pharmacological studies showed that lobetyolin, a polyacetylene component played a protective role in gastric mucosa injury (Song et al., 2008b); total saponins from Codonopsis Radix had protective effect on ischemia-reperfusion injury in rats after kidney transplantation (He et al., 2011); total alkaloids from Codonopsis Radix caused a significant enhancement of nerve growth factor-induced neurite outgrowth in PC12 cells as well as increase of the phosphorylation of mitogen-activated protein kinase (MAPK) (Liu et al., 2003); polysaccharides had protective effect against renal ischemia/reperfusion injury in rats (Li et al., 2012), and inhibitory effect on tumor

growth and metastasis *in vitro* (Xin et al., 2012). These findings indicate such constituents contribute much to therapeutic effects of Codonopsis Radix. However, for the quality assurance of Codonopsis Radix relating to chemical constituents, only lobetyolin was used as a marker compound in Chinese Pharmacopoeia. There is still no suitable method to assess the quality of Codonopsis Radix relating to multiple components having potential bioactivities. Moreover, pharmacological reports did not mention which *Codonopsis* species contained the effective constituents. The characteristic chemical profiles of the three original plants of Codonopsis Radix are unclear.

Of the three taxa, *C. pilosula* and *C. pilosula* var. *modesta* mainly distributed in western and northern parts of China, such as Gansu, Sichuan, Guizhou, Shaanxi and Shanxi Provinces, etc. (Hong et al., 2011). Gansu Prov. is the main producing area of Codonopsis Radix. *C. tangshen* mainly distributed in Chongqing city, Sichuan and Hubei Provinces (Hong et al., 2011). Thus, field investigations in Gansu Prov., Chongqing city and Hubei Prov. were carried out to understand the status of resources of Codonopsis Radix and to collect a number of plant specimens of *Codonopsis* species between 2008 and 2010. At the same time, the crude drug samples of Codonopsis Radix were purchased from the markets of China, Korea and Japan.

This study aims to clarify the genetic and chemical polymorphism of the three medicinally-used *Codonopsis* taxa, and further to find out the genetic and chemical markers for identification and standardization of Codonopsis Radix. First, internal transcribed spacer sequence (ITS) of nuclear ribosomal DNA (nrDNA) was determined to reveal genetic polymorphism of three *Codonopsis* taxa and provide useful dataset to allow identification of the three taxa and authentication of Codonopsis Radix (Chapter

I). Second, standard compounds were isolated from a Codonopsis Radix, and an efficient and simple HPLC method was developed for simultaneously quantitative analysis of seven marker compounds (Chapter II). Then a comparative study on three *Codonopsis* species and Codonopsis Radix by the developed HPLC-UV method was carried out to show the interspecies variation of chemical constitution and to evaluate the quality of Codonopsis Radix (Chapter III).

Chapter I

Genetic polymorphism of medicinally-used *Codonopsis* species in the internal transcribed spacer sequence of nuclear ribosomal DNA and its application to authenticate Codonopsis Radix

1.1 Introduction

Codonopsis Radix is prescribed as the dried roots of *Codonopsis pilosula* (Franch.) Nannf., *C. pilosula* Nannf. var. *modesta* (Nannf.) L. D. Shen and *C. tangshen* Oliv. in Chinese Pharmacopoeia. For methodologies on research and evaluation of traditional medicines, the first step in assuring quality, safety and efficacy of traditional medicines is correct identification (World Health Organization 2000). However, botanical origin of Codonopsis Radix was difficult to elucidate just using morphological inspection and histological identification. DNA-based markers have now become a popular tool for the identification of plants because genetic composition is unique for each individual irrespective of the physical form and is less affected by age, physiological condition, environmental factors, harvest, storage and processing (Balasubramani et al., 2011). In addition, molecular approaches are widely used not only in plant taxonomy but also for identification of crude drugs. Guo et al. (2007) reported the abundant genetic diversity in cultivated *C. pilosula* populations from Longxi County, Gansu Prov. based on randomly amplified polymorphic DNA (RAPD) analysis. Lin et al. (2007) reported the phylogenetic relationship among 5 *Codonopsis* species and *Campanumoea lancifolia* (Roxb.) Merr. based on the internal transcribed spacer sequence (ITS) of nuclear ribosomal DNA (nrDNA), in which *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen* had quite similar ITS sequences, because only 1 or 2 nucleotide substitutions were observed among them. However, in a preliminary experiment, we found a considerable intraspecies polymorphism within the ITS sequences of *Codonopsis* plants. Therefroe in the first part of study, in order to clarify not only interspecies but also intra-species polymorphism of medicinally-used *Codonopsis* species in ITS sequence and apply the result to establish an accurate identification method for Codonopsis Radix,

we collected a number of specimens of *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen* which are cultivated mainly in Gansu Prov., Chongqing city (previously belonged to Sichuan Prov.) and Hubei Prov. of China, besides crude drug samples, and analyzed their ITS sequences.

1.2 Materials and Methods

1.2.1 Materials

Ninety-six plant specimens of 3 *Codonopsis* taxa, which were carefully identified as *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen* by authors, and 4 unidentified specimens of genus *Codonopsis* were analyzed. Most specimens were collected from the cultivation fields of Gansu Prov., Chongqing city and Hubei Prov., China during our field investigation in 2008 ~ 2010 (Table 1.1).

Forty-four, seven, five and one samples of Codonopsis Radix were purchased from the markets of mainland China, Hongkong, Japan and Korea, respectively, which are called Dangshen, Baitiaodangshen (Baitiaodang), Tiaodang, Wendangshen (Wendang), Fengdang, etc. in China, Tojin in Japan and Man Sham in Korea (Table 1.2). Two analytes for every crude drug sample were analyzed.

For a confirmation test in the judgement of the double peak on electrophoretogram, GS98 specimens of *C. pilosula* from Minxian County, Gansu Prov., China and commercial Dangshen sample TMPW no. 26991 derived from *C. tangshen* in Japanese market were used.

All of the plant specimens and crude drug samples were stored in the Museum of

Table 1.1 Plant specimens of *Codonopsis* species used in this study, and their types of ITS sequences

Voucher no.	Idensification based on morphology	Wild/Cult.[3]	Locality (Altitude)	Locality no.[4]	Date of collection	Result
						Sequence type (ITS)[5]
GS138	*C. pilosula*	C	Gaotai, Longxi, Dingxi, Gansu, China (2153m)	1	2010.7.28	P7
GS139	*C. pilosula*	C				P3
GS140	*C. pilosula*	C				P3
GS141	*C. pilosula*	C				P1
GS142	*C. pilosula*	C				P3
GS143	*C. pilosula*	C				P7
GS144	*C. pilosula*	C				P3
GS114	*C. pilosula*	W	Longxi, Dingxi, Gansu, China (1870m)	2	2010.7.27	P3
GS132	*Codonopsis* sp.[1]	W	Longxi, Dingxi, Gansu, China (1905m)	2	2010.7.27	S0
GS97	*C. pilosula*	C	Chabu, Minxian, Dingxi, Gansu, China (2280m)	3	2010.7.26	P0
GS98	*C. pilosula*	C				P0
GS99	*C. pilosula*	C				P0
GS100	*C. pilosula*	C				P3
GS101	*C. pilosula*	C				P3
GS87	*C. pilosula*	C	Hadapu, Tanchang, Longnan, Gansu, China (2201m)	4	2010.7.26	P9
GS88	*C. pilosula*	C				P0
GS89	*C. pilosula*	C				P9
GS62	*C. pilosula*	C	Nanyang, Tanchang, Longnan, Gansu, China (1929m)	5	2010.7.26	P3
GS64	*Codonopsis* sp.[1]	C				S0
GS65	*C. pilosula*	C				P1
GS68	*C. pilosula*	C				P10
GS69	*C. pilosula*	C				P6
GS70	*C. pilosula*[2]	C				PM0
GS71	*C. pilosula*	C				P5
GS84	*C. pilosula*	C	Nanyang, Tanchang, Longnan, Gansu, China (1871m)	5	2010.7.26	P9
GS85	*C. pilosula*	C				P8
GS86	*C. pilosula*	C				P1
Cgs7	*C. pilosula*	C	Longxing, Wudu, Longnan, Gansu, China	6	2009.8.01	P3
Cgs8	*C. pilosula*	C				P1
Cgs4	*C. pilosula*	C	Gaolou Mountain, Wenxian, Longnan, Gansu, China	7	2009.7.30	P7
Cgs5	*C. pilosula*	C				P6'
Cgs6-1	*C. pilosula*	C	Gaolou Mountain, Wenxian, Longnan, Gansu, China	7	2009.7.30	P3
Cgs6-2	*C. pilosula*	C				P4
GS35	*C. pilosula* var. *modesta*	W	Gaojiashan, Wenxian, Longnan, Gansu, China (1914m)	8	2010.7.25	PM0'
GS36	*C. pilosula*	C			2010.7.25	P7
GS37	*C. pilosula* var. *modesta*	C			2010.7.25	PM3
GS38	*C. pilosula* var. *modesta*	C			2010.7.25	PM0
GS39	*C. pilosula* var. *modesta*	C			2010.7.25	PM4
GS42	*C. pilosula*	C			2010.7.25	P6
GS43	*C. pilosula* var. *modesta*	C			2010.7.25	PM2
GS44	*C. pilosula*	C	Gaojiashan, Wenxian, Longnan, Gansu, China (1909m)	8	2010.7.25	P2
GS45	*C. pilosula* var. *modesta*	C			2010.7.25	PM0
GS46	*C. pilosula* var. *modesta*	C			2010.7.25	PM0
GS47	*C. pilosula* var. *modesta*	C			2010.7.25	PM0
GS49	*C. pilosula*	C			2010.7.25	P2
GS50	*C. pilosula*	C	Gaojiashan, Wenxian, Longnan, Gansu, China (1905m)	8	2010.7.25	P7
GS51	*C. pilosula*	C			2010.7.25	P7
GS52	*C. pilosula*	C			2010.7.25	P1
GS53	*C. pilosula* var. *modesta*	C			2010.7.25	PM0
GS54	*C. pilosula* var. *modesta*	C			2010.7.25	PM3
Cgs1	*C. pilosula* var. *modesta*	W	Huangtuping, Baoziba, Wenxian, Longnan, Gansu, China	9	2009.7.30	PM0
Cgs2	*C. pilosula* var. *modesta*	W	Huangtuping, Baoziba, Wenxian, Longnan, Gansu, China	9	2009.7.30	PM2
Cgs3	*C. pilosula* var. *modesta*	W	Huangtuping, Baoziba, Wenxian, Longnan, Gansu, China	9	2009.7.30	PM1

Table 1.1 Plant specimens of *Codonopsis* species used in this study, and their types of ITS sequences (continued)

CJZ10	*C. tangshen*	C	Dahe, Huangying, Chongqing, China (1235m)	10	2009.7.19	T4'
CJZ11	*C. tangshen*	C	Dahe, Huangying, Chongqing, China (1279m)	10	2009.7.19	T4'
CJZ14	*C. tangshen*	C	Xinshu, Huangying, Chongqing, China (1462m)	11	2009.7.19	T3
CJZ16	*C. tangshen*	C			2009.7.19	T3
CJZ17	*C. tangshen*	C			2009.7.19	T3
CJZ58	*C. tangshen*	C	GAP Base, Jianshan, Wuxi, Chongqing, China (1700m)	12	2009.7.24	T2
CJZ59	*C. tangshen*	C			2009.7.24	T4
CJZ61	*C. tangshen*	C			2009.7.24	T3
CJZ62	*C. tangshen*	C			2009.7.24	T5
CJZ72	*C. tangshen*	W	Hongchiba, Wenfeng, Wuxi, Chongqing, China (1835m)	13	2009.7.25	T3
CJZ73	*C. tangshen*	W			2009.7.25	T3
CJZ74	*C. tangshen*	W			2009.7.25	T3
ZS01	*C. tangshen*	C	Zhoujiaping, Niuzhuang, Wufeng, Hubei, China (1730m)	14	2010.7.28	T1
ZS04	*Codonopsis* sp.[1]	C	（Seeds purchased from Sichuan Prov.: Former cultivation		2010.7.28	S0
ZS05	*Codonopsis* sp.[1]	C	field)		2010.7.28	S0
ZS08	*C. pilosula*	C			2010.7.28	P0
CF10	*C. tangshen*	C	Shennongjia, Hubei, China	15	2008.8	T3
CF13	*C. tangshen*	W			2008.6	T1
CF16	*C. tangshen*	W			2008.8	T4
ZS18	*C. tangshen*	W	Honghecun, Hongping, Shennongjia, Hubei, China	16	2010.7.31	T4
ZS20	*C. tangshen*	W			2010.7.31	T1
ZS21	*C. tangshen*	W			2010.7.31	T4
ZS23	*C. tangshen*	W			2010.7.31	T1
ZS24	*C. tangshen*	W			2010.7.31	T1
ZS25	*C. tangshen*	W			2010.7.31	T1
CF5	*C. tangshen*	C	Xingshan, Yichang, Hubei, China	17	2008.7	T4
CF11	*C. tangshen*	W	Xingshan, Yichang, Hubei China	17	2008.7	T1
CF12	*C. tangshen*	W	Xingshan, Yichang, Hubei China	17	2008.7	T1
CF1	*C. pilosula*	W	Changyang, Yichang, Hubei, China	18	2008.9	P3
CJZ47	*C. tangshen*	C	Laoguashi, Enshi, Hubei, China	19	2009.7.23	T5
CJZ48	*C. tangshen*	C			2009.7.23	T5
CJZ91	*C. tangshen*	W	Liziping, Wufeng, Yichang, Hubei, China	20	2009.7.28	T3
CJZ92	*C. tangshen*	W			2009.7.28	T3
CJZ93	*C. tangshen*	W			2009.7.28	T3
CJZ94	*C. tangshen*	W			2009.7.28	T1
CJZ95	*C. tangshen*	W			2009.7.28	T4
CJZ96	*C. tangshen*	W			2009.7.28	T4
CJZ97	*C. tangshen*	W			2009.7.28	T5
ZS10	*C. pilosula*	C	Erlongping, Caihua, Wufeng, Yichang, Hubei, China	21	2010.7.28	P7
ZS12	*C. tangshen*	W	Hejialing, Liziping, Wufeng, Yichang, Hubei, China	22	2010.7.29	T3
ZS15	*C. tangshen*	W			2010.7.29	T5
ZS16	*C. tangshen*	W			2010.7.29	T3
ZS17	*C. tangshen*	W	Gualiangwan, Liziping, Yichang, Hubei, China	23	2010.7.29	T2

[1] The shape of flowers with half-inferior ovary is similar to that of *C. pilosula*, while that of leaves is similar to that of *C. tangshen*.

[2] not exactly identified because only stem present.

[3] W, wild; C, cultivation

[4] Collection localities are shown in Fig. 1.3.

[5] The sequence type is indicated in Table 1.4.

Table 1.2 Crude drug samples used in this study, and types of ITS sequences

Code no.	Drug name	Producing area	Purchased from	Date of collection	TMPW no.[1]	Length x Diameter (cm)	Result	
							Sequence type (ITS)[2]	
							Analyte a	Analyte b
	Mainland China's Market							
C1	Dangshen	Lixian, Gansu, China	Chuntian Pharmacy, Lixian, Gansu	2010.7.28	27040	cut pieces	PM0	P8
C2	Baitiaodang	Longxi, Gansu, China	Shouyang Crude Drug Market, Longxi, Gansu	2010.7.27	27027	10-18 ×0.5-0.7	-	PM0
C3	Dangshen	Gansu, China	Longxi Zhongtian Pharmaceutical Private Co., Ltd., Gansu	2010.7.27	27030	cut pieces	PM0	S0
C4	Dangshen	Gansu, China	Hadapu, Tanchang, Longnan, Gansu	2010.7.26	27025	13-23 ×0.6-1.4	P3	PM1
C5	Baitiaodang	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26	27014	24-27 ×0.7-1.0	P3	-
C6	Dangshen	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26	27016	11-14 ×1.4-1.7	P3	PM1
C7	Dangshen	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26	27017	23 ×1.6	PM1	/
C8	Dangshen	Wenxian, Gansu, China	Longnan Mingyue Chinese Herbal Slices Company, Longnan, Gansu	2010.7.25	27007	20-34 ×0.6-1.2	-	P0
C9	Wendang	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26	27015	19-22 ×1.5-2.2	PM0	T1
C10	Wendangshen	Gansu, China	Baoziba, Wenxian, Longnan, Gansu	2009.7.30	26655	23-32 ×1.3-1.7	PM0	-
C11	Wendangshen	Gansu, China	Baoziba, Wenxian, Longnan, Gansu	2009.7.30	26669	17-19 ×0.8-1.2	T1	PM1
C12	Tiaodang	Minxian, Dingxi, Gansu, China	Minxian, Dingxi, Gansu	2009.8.6	26671	18-26 ×0.8-1.1	S0	PM0
C13	Dangshen	Gansu, China	Hehuachi Crude Drug Market, Chengdu, Sichuan	2009.8.3	26657	26-34 ×0.7-0.9	PM0	CC1
C14	Dangshen	Gansu, China	Qinghua Pharmacy, Nanchang, Jiangxi	2009.7.11	26666	cut pieces	PM0	PM0
C15	Dangshen	Gansu, China	Xi'an Crude Drug Market, Shaanxi	2010.8.1	27056	cut pieces	P0	P1
C16	Tiaodang	Longxi, Gansu, China	Xi'an Huakang Crude Drug Store, Shaanxi	2009.7.7	26674	cut pieces	P1	P1
C17	Baitiaodangshen	Gansu, China	Qingping Crude Drug Market, Guangzhou, Guangdong	2009.8.18	26659	27-34 ×1.2-1.8	P8	Q0
C18	Yedangshen	Gansu, China	Qingping Crude Drug Market, Guangzhou, Guangdong	2009.8.18	26660	23-40 ×1.0-2.1	P0	P3
C19	Huangdangshen	Gansu, China	Qingping Crude Drug Market, Guangzhou, Guangdong	2009.11.19	26662	26-32 ×0.8-1.2	P3	CC2
C20	Dangshen	Gansu, China	Shanghai Yiyao Huangshanhuashi Private Co., Ltd., Shanghai	2009.8.3	26598	cut pieces	P3	P2
C21	Dangshen		Hanzhong Pharmacy, Shaanxi	2010.7.22	26937	cut pieces	P10	P1
C22	Dangshen		Xianyang, Shaanxi	2009.12	26716	cut pieces	P5	P0
C23	Fengdang	Fengxian, Baoji, Shaanxi, China	Fengxian, Baoji, Shaanxi	2009.8.6	26670	18-27 ×1.5-2.0	T4	P6
C24	Dangshen	Chongqing, China	Huangshui, Shizhu, Chongqing	2009.7.19	26542	15-29 ×0.6-1.4	T2	-
C25	Dangshen	Sichuan, China	Chongqing Crude Drug Market, Chongqing	2009.7.17	26538	16-31 ×0.5-0.7	P3	-
C26	Dangshen		Chongqing	2009.12	26723	cut pieces	P8	P3
C27	Dangshen	Shanxi, China	Wulin Parmacy, Hangzhou, Zhejiang	2009.8.7	26626	21-27 ×0.8-1.6	P3	P6
C28	Dangshen		Datong, Shanxi	2009.12	26713	cut pieces	P0	P0
C29	Dangshen		Shanxi	2009.12	26714	cut pieces	P5	P3
C30	Dangshen		Taiyuan, Shanxi	2009.12	26715	cut pieces	P5	P3
C31	Dangshen	Enshi, Hubei, China	Enshi Fenglan Banqiaodangshen Co., Ltd., Hubei	2009.7.22	26560	21-34 ×0.8-1.3	T5	-
C32	Dangshen	Yichang, Hubei, China	Wantan Crude Drug Store, Wufeng, Yichang, Hubei	2009.7.29	26589	cut pieces	T4	P7
C33	Yedangshen	Shennongjia, Hubei, China	Shennongjia Juneng, Pharmaceutical Co., Ltd., Hubei	2011. 2.1	27168	20-34 ×0.6-1.4	T1	T3

Table 1.2 Crude drug samples used in this study, and types of ITS sequences (continued)

C34	Dangshen		Wuhan, Hubei	2009.12	26718	cut pieces	P0	P3
C35	Dangshen		Liying Chinese Medicinal Clinic, Changchun, Jilin	2009.5.2	26501	cut pieces	PM0	P5
C36	Dangshen		Beiyang, Henan	2009.12	26717	cut pieces	P3	P1
C37	Dangshen		Xuzhou, Jiangsu	2009.12	26720	cut pieces	P3	S0
C38	Dangshen		Fuzhou, Fujian	2009.12	26722	cut pieces	P8	PM1
C39	Dangshen		Kunming, Yunnan	2009.12	26724	cut pieces	PM0	P5
C40	Dangshen		Qiannan, Guizhou	2009.12	26725	cut pieces	P0	P0
C41	Dangshen		Nanning, Guangxi	2009.12	26726	cut pieces	P5	P1
C42	Dangshen		Nanning, Guangxi	2009.12	26727	cut pieces	P3	P0
C43	Dangshen		The People's Hospital of Guangxi, Nanning, Guangxi	2009.12	26728	cut pieces	P7	P1
C44	Dangshen		Guangzhou, Guangdong	2009.12	26729	cut pieces	P5	CC3
	Hongkong's Market							
H1	Wendangshen	Gansu, China	Hongkong Liyuanfeng Trading Co.	2010.8.11	26820	12-20 × 1.7-2.2	-	HC1
H2	Wendangshen	Gansu, China	Hongkong Liyuanfeng Trading Co.	2010.8.11	26821	12-19 × 1.4-1.7	HC1	-
H3	Dangshen		Hongkong Yongsheng Wholesale Private Co., Ltd.	2010.8.11	26827	12-18 × 1.6-2.0	PM0	PM0
H4	Dangshen		Hongkong Runfengshenrong Private Co., Ltd.	2010.8.11	26812	14-22 × 1.0-1.4	-	PM0
H5	Dangshenpian		Hongkong Longxi Zhongtian Pharmaceutical Private Co., Ltd.	2010.8.12	26850	cut pieces	P3	P3
H6	Dangshenwang	Minxian, Gansu, China	Gansu Minxian Tianrong Indigenous Products Private Co., Ltd.	2010.8.12	26853	16-18 × 0.7-1.2	P0	-
H7	Dangshen		Hongkong Gansu Longmai Medicinal Materials Private Co., Ltd.	2010.8.12	26849	17 × 1.4	HC2	/
	Japanese Market							
J1	Tojin	Gansu, China	Tochimoto Tenkaido, Co., Ltd., Osaka	2009.1	26865	cut pieces	P8	P5
J2	Tojin	Guizhou, China	Uchida Wakanyaku, Co., Ltd., Tokyo	2009.1	26864	9-18 × 0.5-0.9	PM0	P1
J3	Tojin	Henan, China	National Institute of Health Sciences, Japan (To-HS-001)	2006.4	26991	cut pieces	T0	T2
J4	Tojin	Guizhou, China	National Institute of Health Sciences, Japan (To-HS-002)	2007.5	26992	cut pieces	JC1	P1
J5	Tojin	Gansu, China	National Institute of Health Sciences, Japan (To-HS-003)	2008.3	26993	cut pieces	JC2	-
	Korean market							
K1	Man Sham	China	Seoul	2010.9.28	26928	cut pieces	P8	P1

[1] The registration number of the Museum of Material Medica, Institute of Natural Medicine, University of Toyama (TMPW).

[2] The sequence type is indicated in Table 1.4: Q0, CC1, CC2, CC3, HC1, HC2, JC1 and JC2: There are no same DNA sequences as plant specimens.
-: indicates failure in determining sequence due to serious DNA degradation in sample. /: indicates only one sample for test.

Materia Medica, Institute of Natural Medicine, University of Toyama, Japan.

1.2.2 Genomic DNA extraction

Total DNA was extracted from 40-50 mg of dried leaves or 80-90 mg of roots by DNeasy Plant Mini Kit (Qiagen, Germany) with several modifications to the protocol provided by manufacturer, i.e., the incubation time at 65 ℃ was extended from 10 min to 2 h for dried leaves or 4 h for roots, and the incubation time on ice was extended from 5 min to 1 h. Extracted DNA was detected by electrophoresis on 1% agarose gel stained by ethidium bromide. The extracted total DNA was stored at -20 °C before using and was further used as template in the following PCR amplification.

1.2.3 PCR amplification

The primers used for PCR amplification of approximately 700 bp fragment including ITS1-5.8S-ITS2 region were oligonucleotide ITS-1F (forward primer: 5′-TCC ACT GAA CCT TAT CAT TTA G-3′) and 18S-25S-3′R (reverse primer: 5′-CCA TGC TTA AAC TCA GCG GGT-3′) (Sukrong et al., 2007) (Fig. 1.1). PCR amplification was performed using 10-100 ng of total DNA as a template in 25 μL of reaction mixture consisting of 1 × PCR Buffer for KOD-Plus, 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 U KOD-Plus DNA polymerase (Toyobo, Japan), and 0.25 μM of each primer. A Takara thermal cycler TP-600 (Takara, Japan) was used to carry out PCR amplification under the cycling condition: initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 52 ℃ for 30 s, extension at 68 ℃ for 50 s, and then final extension at 68 ℃ for 10 min. The 2 μL of PCR product was examined by

1.0% agarose gel electrophoresis and then remaining part was purified using Millipore montage-PCR column (Millipore, U.S.A).

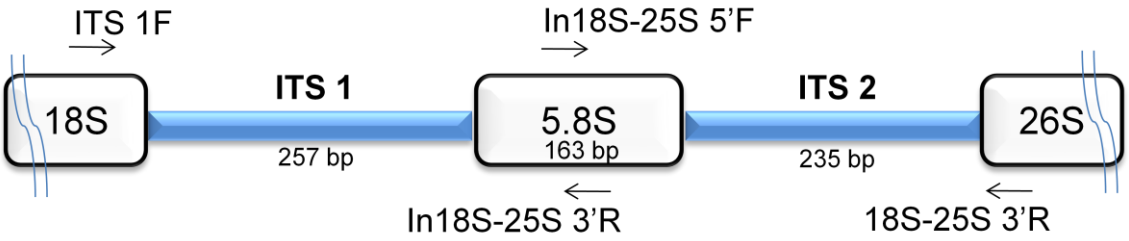


Fig. 1.1 Structure of ITS region

The position of primers used in PCR amplification and sequence determination of ITS region are indicated by arrows.

1.2.4 Sequencing and Sequencing analysis

Sequencing reaction using the purified PCR products as template was carried out using ABI PRISM Bigdye Terminator v3.1 Cycle sequencing kits (Applied Biosystems, U.S.A.) with each of the 4 primers, ITS-1F and In-18S-25S-3′R (5′-GAC TCG ATG GTT CAC GGG ATT CT-3′) and In-18S-25S-5′F (5′-TCT CGC ATC GAT GAA GAA CG-3′) and 18S-25S-3′R (Fig. 1.1). Sequence was determined directly by ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, U.S.A.) and analyzed by sequencing analysis Software (v5.3 Patch 1, Applied Biosystems, U.S.A.). The analyzed sequences were assembled, and consensus sequence of each sample as finally constructed. Then the obtained DNA sequences were aligned and compared by Multalin software (http://multalin.toulouse.inra.fr/multalin/). The ITS sequences obtained in this study were recorded in DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers AB769260- AB769284.

As double peaks which indicate additive nucleotides at the same sites were observed

in the resulting electrophoretogram, judgement of the double peaks was carried out in accordance with our previous study (Kitani et al., 2011). A confirmation test was conducted using experimental mixture of two PCR products [one product from GS98 specimen (**P0**) and one product from TMPW no. 26991 (**T0)**] which showed a clear peak signal of pure cytosine (C) or thymine (T) at position 122nd, and guanine (G) or adenine (A) at position 500th, respectively. The two PCR products were mixed at a series of ratios: 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 95% of one PCR product and then used as a template to do a sequencing reaction. The *S*-value (2nd peak intensity/sum of main peak intensity and 2nd peak intensity) of the additive site was calculated. The level of background noise was calculated as an *N*-value: noise peak intensity (which absolutely did not derive from additive nucleotide)/sum of the main peak intensity and noise peak intensity. The results of experimental mixture test (Fig. 1.2) showed that the relative intensity of the 2nd peak was too low to be differentiated from the noise signal in the mixture solution with the ratios of 5% (G and A) and 10% (C and T). The additivity could not be discriminated in such case. However, when the *S*-value is more than 20%, the 2nd peak could be clearly detected and affirmatively differentiated from the noise signal (average *N*-value < 10%). In the present study, the *S*-values of additive peaks in all samples were almost higher than 20%, while the noise level was less than 10%. The sites of additive peak were very clear and easy to be detected.

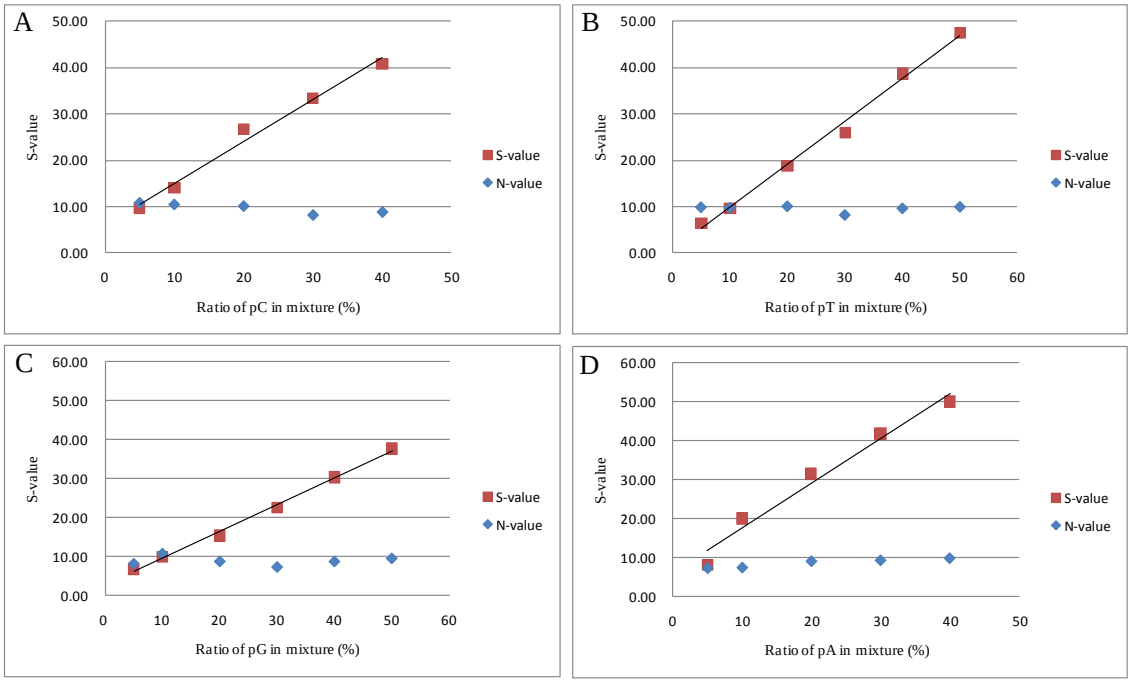


Fig. 1.2 Results of *S*-value from experimental mixture test by using two PCR products with different types of sequences (type P0: C at position 122nd, G at position 500th; type T0: T at position 122nd, A at position 500th).

A) *S*-value (C as the 2nd peak) of the additive peak (C and T) at the position 122nd and average *N*-value.

B) *S*-value (T as the 2nd peak) of the additive peak (C and T) at the position 122nd and average *N*-value.

C) *S*-value (G as the 2nd peak) of the additive peak (G and A) at the position 500th and average *N*-value.

D) *S*-value (A as the 2nd peak) of the additive peak (G and A) at the position 500th and average *N*-value.

1.2.5 Cloning of PCR products

As the ITS region is of biparental inheritance, the nucleotide additivity detected in ITS sequence indicated hybridization occurred in the three *Codonopsis* taxa. Cloning analysis of the samples with additive nucleotides could provide benefit information for assuming lineages related to hybridization. Specimens GS100, GS42, GS89 ZS15 and ZS17 were selected for cloning. The ITS region was amplified from total DNA using a primer set of ITS-1F and 18S-25S-3′R. After purification using the QIA Quick PCR Purification Kit (QIAGEN), the ligation reaction of the PCR products was performed

following the manufacturer's protocol (TArget Clone-Plus, Toyobo). First, the A-attachment mixture consisting with 9 μL of the PCR products (100-150 ng/μL) and 1 μL of 10 × A-attachment Mix was incubated at 60 ℃ for 1 h. Secondly, 10 μL of the mixture containing 5 μL of 2 × ligation Buffer, 1 μL of pTA2 Vector (50 ng/μL), 1 μL of T4 DNA Ligase, 2 μL of A-attachment PCR products and 1 μL of distilled water were prepared and subsequently incubated at 4 ℃ overnight. The ligation solution was mixed with competent cells (Competent high DH5a, Toyobo) at the ratio of 1: 100 for transformation under condition of: initial incubation at 42 ℃ for 30s, followed by incubation on ice for 4 min, after adding 1 mL of liquid LB/Amp medium (1% polypeptone, 0.5% yeast extract, 1% NaCl and 100 μg/mL ampicillin) further incubation on ice for 4 min, then incubation at 37 ℃ for 4 h. 100 μL of the transformed cells was spread on a LB/Amp plate (1% polypeptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 100 μg/mL ampicillin, 40 μg/mL X-gal and 71.5 μg/mL isopropyl β-D-1-thiogalactopyranoside) and incubated at 37 ℃ for 22 h. White colonies were picked up, and sub-cultured separately in 2 mL of liquid LB/Amp medium. After incubation at 37 ℃ for 22 h, the bacteria were collected by centrifugation at 4000 rpm for 10 min and plasmids were obtained by using QIAprep Spin Miniprep Kit (Germany) following the manufacturer's protocol. The plasmid was subjected to direct sequencing.

1.3 Results

1.3.1 Morphological characters of *Codonopsis* plants

According to the morphological description in Flora of China (Editorial board of Flora of China, 1983), *C. pilosula* has ovate or narrowly ovate leaves with cordate base

and half-inferior ovary to calyx, whereas *C. tangshen* has ovate, narrowly ovate or lanceolate leaves with obtuse or rotundate base and superior ovary to calyx. Compared with *C. pilosula*, the whole plant of *C. pilosula* var. *modesta* is glabrous and the calyx lobe is comparably small (Table 1.3). Moreover, during our field investigation, we observed that the inner surface of corolla of *C. tangshen* was with obvious reddish brown pattern in lower half part, but that of *C. pilosula* was not. Among 53 *Codonopsis* specimens collected in Gansu Prov., most of them were identified as *C. pilosula*, excluding those from Wenxian County (7-9 in Fig. 1.3) which were identified as either *C. pilosula* var. *modesta* or *C. pilosula* on the basis of the above key characters. Moreover, a wild specimen from Longxi County (2 in Fig. 1.3) showed an intermediate feature between *C. pilosula* and *C. tangshen*, that is, the ovary was half-inferior to calyx, similar to that of *C. pilosula*, while the shape of leaves was similar to that of *C. tangshen*. Therefore, we treated such the specimen as *Codonopsis* sp. On the other hand, all specimens collected in Chongqing city (10-13 in Fig. 1.3) were morphologically identified as *C. tangshen*. Four specimens collected in Zhoujiaping, Wufeng County, Hubei Prov. (14 in Fig. 1.3), where the cultivation had been formerly carried out using seeds purchased from Sichuan Prov., had different morphology to each other, therefore, were identified as *C. pilosula*, *C. tangshen* and *Codonopsis* sp., respectively. Most of the specimens collected in Hubei Prov. were morphologically identified as *C. tangshen*, except two specimens collected in Yichang, Hubei Prov. that were *C. pilosula* (18, 21 and 23 in Fig. 1.3).

Table 1.3 Comparison of morphological characters of three *Codonopsis* taxa

		Species		
		C. pilosula	*C. pilosula* var. *modesta*	*C. tangshen*
Leaf	shape of whole leaf	ovate or narrowly ovate	ovate or narrowly ovate	ovate, narrowly ovate or lanceolate
	shape of base	cordate	cordate	obtuse or rotundate
	shape of margin	crenate	crenate	obscurely serrate
	hairs	sparsely or densely villous	glabrous or sparsely villous	glabrous
Ovary	position to calyx	half-inferior	half-inferior	superior
Calyx lobe	length (mm)	10-20	<10	14-17
Corolla	color of inner surface	yellow-green, rarely reddish brown or purple dotted line in the middle part	yellow-green, purple dotted line in the middle part	yellow-green, reddish brown pattern in lower half

1.3.2 ITS sequences of *Codonopsis* plants

The length of ITS1-5.8S-ITS2 region was of 655 bp in all *Codonopsis* specimens. The boundaries of ITS1, 5.8S rDNA and ITS2 regions were defined by comparison with the sequences of rice and mung bean (Takaiwa et al., 1985; Schiebel and Hemleben, 1989), and the length of each region was 257 bp, 163 bp and 235 bp, respectively. After sequencing analyses and BLAST search in GenBank, ITS sequences of *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen* were found to be with high homology to sequences with accession numbers EF190460, EF190461 and EF190462, respectively, which had been reported by Lin et al. (2007). The ITS sequences of *C. pilosula* specimens had the variable sites at the nucleotide positions, 122nd, 130th and 226th in ITS1 region, and 441st, 489th and 519th in ITS2 region. The additive nucleotides of C and T [double peaks of C and T (Y) in electrophoretogram] were observed at position 122nd, 226th, 441st and 489th frequently. Within *C. pilosula* specimens, 11 types (designated as **P0** – **P10**) of ITS sequences were detected (Table 1.4). Among them, type **P0** was a sequence of putative pure line, which was identical to the sequence of accession number EF190460, and other 10 types possessed Y at least at one of 4 informative sites (122nd, 226th, 441st and 489th). Types **P1** - **P6** and **P7** - **P10**

possessed Y and T at position 122nd, respectively. Among 11 types, type **P3** was detected most frequently in specimens, subsequently types **P7**, **P0** and **P1** were found.

There were 5 types of ITS sequences in *C. pilosula* var. *modesta*. The pure line of *C. pilosula* var. *modesta*, designated as **PM0,** had the nucleotides T, C, T and T at the above 4 informative sites, which is identical to the sequence with an accession number EF190461 and different from the P0 sequence at nucleotide position 122nd (C-to-T transition). Other 4 types (**PM1 - PM4**) possessed the same nucleotide T as the type PM0 at position 122nd, however, differed by C-to-Y, T-to-Y and T-to-Y substitutions at positions 226th, 441st and 489th, respectively (Table 1.4).

More than 2 sequence types were detected in the specimens from the same cultivation field of *C. pilosula* or *C. pilosula* var. *modesta*. The specimens with type **P3** sequence were observed in a wide range of Gansu Prov. and those with type **P0** were detected in limited area like Minxian County (3 in Fig. 1.3). The specimens with type **PM0** or other **PM** types of sequences were mainly found in the cultivation fields of Wenxian County, Gansu Prov. (8 in Fig. 1.3), where specimens with sequences of types **P7**, **P6**, **P2** or **P1** were also observed. On the other hand, the specimen with type **PM0** sequence was detected in the cultivation field of *C. pilosula* in Nanyang, Longnan city (5 in Fig. 1.3) near Wenxian County.

The ITS sequence of *C. tangshen* recorded in GenBank (EF190462; designated as **T0**) is different from the type **PM0** sequence of *C. pilosula* var. *modesta* by only one nucleotide at position 500th. However, the type **T0** sequence was not found in *C. tangshen* specimens we examined. Two pure line sequences, types **T1** and **T3** which showed a different nucleotide G or A at position 135th were observed. Totally 5 types of ITS sequences were found in *C. tangshen*. Their ITS sequences had 3 variable sites at

the nucleotide positions 135th, 489th and 500th. The nucleotide C at position 489th was characteristic in *C. tangshen*. Most of *C. tangshen* specimens including wild specimens from Hongchiba, Chongqing city (13 in Fig. 1.3) and those obtained from cultivation field of Xinshu village (11 in Fig. 1.3) were of the type **T3** sequence. However, the specimens from the cultivation site, Jianshan, Chongqing city (12 in Fig. 1.3) where is a Good Agricultural Practice (GAP) base for Codonopsis Radix, showed 4 types of sequences. In Hubei Prov., the specimens with types **T1**, **T3** and **T4** sequences were found in Shennongjia (15, 16 in Fig. 1.3), those with types **T1** and **T4** sequences in Xingshan, Yichang (17 in Fig. 1.3), and those with types **T1**, **T3**, **T4** and **T5** sequences in Wufeng, Yichang (20, 22 in Fig. 1.3). While, the specimens obtained from the cultivation field of Enshi, Hubei Prov. where is the producing area of "Banqiaodanshen" had type **T5** sequence (19 in Fig. 1.3). Among 5 types, type **T3** was detected most frequently in specimens, subsequently types **T1** and **T4** were found.

Codonopsis sp., morphologically unidentified specimens, showed a different sequence from the above 3 taxa at the 4 informative sites (122nd, 226th, 441st and 489th) (designated as **S0**). The nucleotide T at position 226th was characteristic in this pure line. One specimen from medicinal plant garden of Longxi County, Gansu Prov. (2 in Fig. 1.3), one specimen from cultivation field of Nanyang, Gansu Prov. (5 in Fig. 1.3) and two specimens from a former cultivation field, Hubei Prov. (14 in Fig. 1.3) had this type of sequence.

Table 1.4 Types of ITS sequences of *Codonopsis* species and the assumed lineages related to hybridization

Species	Sequence type (ITS)	Accession number in GenBank	Nucleotide position																			Sequence type of supposed parental lineages (nucleotides at 122, 226, 441 and 489)	Number of plant specimens
			ITS1											ITS2									
			52	55	122	130	135	199	206	226	234	253	257	441	483	489	500	509	519	533		
C. pilosula	P0	AB769260	C	G	C	G	G	C	C	C	T	A	C	T	C	T	G	C	A	G		5
	P1	AB769261	*	*	Y	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	P0 (C C T T) × PM0 (T C T T)	5
	P2	AB769262	*	*	Y	R	*	*	*	*	*	*	*	*	*	*	*	*	*	*		2
	P3	AB769263	*	*	Y	*	*	*	*	Y	*	*	*	*	*	*	*	*	*	*	P0 (C C T T) × S0 (T T T T)	11
	P4	AB769264	*	*	Y	R	*	*	*	Y	*	*	*	*	*	*	*	*	*	*		1
	P5	AB769265	*	*	Y	*	*	*	*	*	*	*	*	Y	*	*	*	*	*	*	P0 (C C T T) × Q0 (T C C T)	1
	P6	AB769266	*	*	Y	*	*	*	*	*	*	*	*	*	*	Y	*	*	*	*	P0 (C C T T) × T1 (T C T C)	2
	P6'	AB769267	*	*	Y	*	*	*	*	*	*	*	*	*	*	Y	*	*	R	*		1
	P7	AB769268	*	*	T	*	*	*	*	Y	*	*	*	*	*	*	*	*	*	*	S0 (T T T T) × PM0 (T C T T)	7
	P8	AB769269	*	*	T	*	*	*	*	*	*	*	*	Y	*	*	*	*	*	*	Q0 (T C C T) × PM0 (T C T T)	1
	P9	AB769270	*	*	T	*	*	*	*	Y	*	*	*	Y	*	*	*	*	*	*	S0 (T T T T) × Q0 (T C C T)	3
	P10	AB769271	*	*	T	*	*	*	*	Y	*	*	*	*	*	Y	*	*	*	*	S0 (T T T T) × T1 (T C T C)	1
Codonopsis sp.	S0	AB769272	*	*	T	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*		4
C. pilosula var. *modesta*	PM0	AB769273	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		7
	PM0'	AB769274	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	R		1
	PM1	AB769275	*	*	T	*	*	*	*	*	*	*	*	*	*	Y	*	*	*	*	PM0 (T C T T) × T1 (T C T C)	1
	PM2	AB769276	*	*	T	R	*	*	*	*	*	*	*	*	*	*	*	*	*	*		2
	PM3	AB769277	*	*	T	R	*	*	*	Y	*	*	*	*	*	*	*	*	*	*	PM2 (T C T T) × S0 (T T T T)	2
	PM4	AB769278	*	*	T	R	*	*	*	*	*	*	*	Y	*	*	*	Y	*	*		1
																					Sequence type of supposed parental lineages (nucleotides at 135, 489 and 500)	
C. tangshen	T0	EF190462	*	*	T	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*		0
	T1	AB769279	*	*	T	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*		9
	T2	AB769280	*	*	T	*	*	*	*	*	*	*	*	*	*	Y	R	*	*	*	T0 (G T A) × T1 (G C G)	2
	T3	AB769281	*	*	T	*	A	*	*	*	*	*	*	*	*	C	*	*	*	*		13
	T4	AB769282	*	*	T	*	R	*	*	*	*	*	*	*	*	C	*	*	*	*	T1 (G C G) × T3 (A C G)	7
	T4'	AB769283	*	*	T	*	R	Y	*	*	*	*	*	*	*	C	*	*	*	*		2
	T5	AB769284	*	*	T	*	R	*	*	*	*	*	*	*	*	Y	R	*	*	*	T0 (G T A) × T3 (A C G)	5
Crude drug samples[*1]																						
Baitiaodangshen (no.26659b)	Q0		*	*	T	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*		
Tojin (no. 26991a)	T0		*	*	T	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*		
Dangshen (no.26657b)	CC1		Y	*	Y	*	*	*	*	*	Y	*	*	*	*	*	*	*	*	*		
Huangdangshen (no.26662b)	CC2		*	*	T	*	*	*	*	Y	*	M	*	*	*	*	*	*	*	*		
Dangshen (no.26729b)	CC3		*	*	Y	*	*	*	M	*	*	*	*	*	*	*	*	*	*	*		
Wendangshen (no.26820b, no.26821a)	HC1		*	R	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		
Dangshen (no.26849a)	HC2		*	*	T	*	*	*	*	*	*	*	*	*	Y	C	*	*	*	*		
Tojin (no. 26992a)	JC1		*	*	*	*	*	*	*	*	*	*	M	*	*	*	*	*	*	*		
Tojin (no. 26993a)	JC2		*	*	Y	*	*	*	*	*	*	*	*	*	*	*	*	*	*	R		

Numerals above sequence are aligned nucleotide positions of *C. pilosula* which correspond to all other species.

Asterisk indicates the identical nucleotide to which of *C. pilosula* (accession no. AB769260). Y = C and T, R = A and G, M = A and C

*[1] The nucleotide sequence is only found in crude drug samples. Parenthesis numerals show specimen reference number of Museum of Material Medica, Institute of Natural Medicine, University of Toyama (TMPW no.).

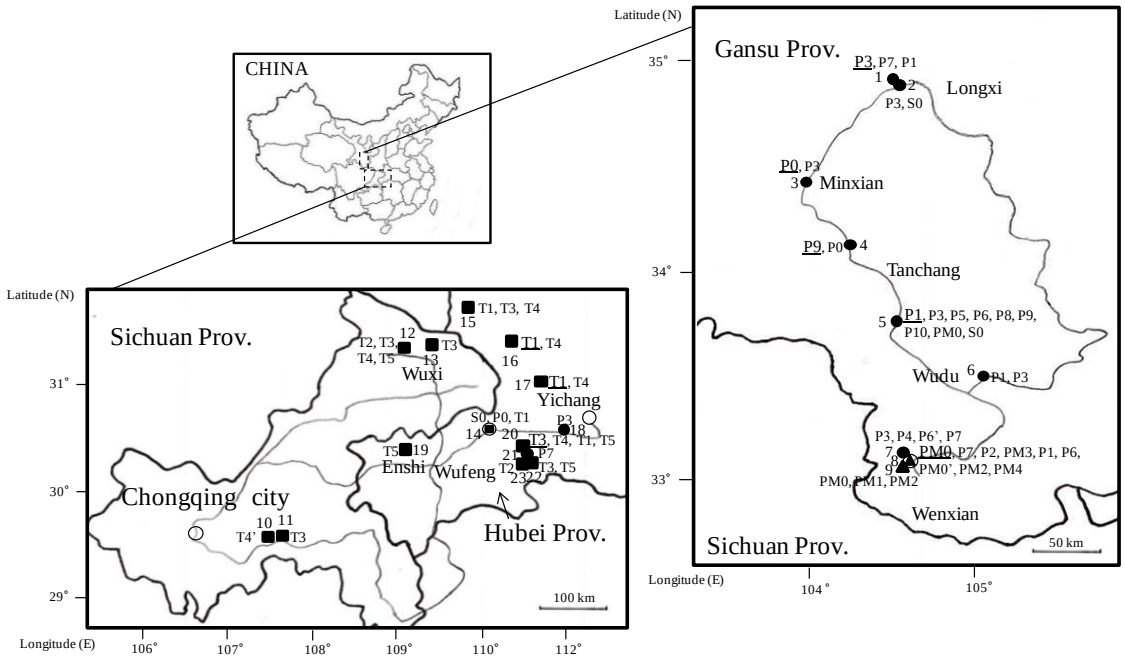


Fig. 1.3 Collection localities of *Codonopsis* speciemens in China (2008-2010)

The marks in the collection localities indicate the collected species, together with ITS sequence types: solid circle, *C. pilosula*; solid triangle, *C. pilosula* var. *modesta*; solid square, *C. tangshen*; solid triangle in circle, *C. pilosula* and *C. pilosula* var. *modesta*; solid square in circle, *C. pilosula* and *C. tangshen.* The numerals next the collection localities are indicated in Table 1.1.

1.3.3 Cloning of PCR products originated from *Codonopsis* specimens

As the ITS sequences had two or three additive nucleotides at informative sites in direct sequencing (122nd, 226th, 441st, 489th in *C. pilosula* and *C. pilosula* var. *modesta*, 135th, 489th and 500th in *C. tangshen*), which were commonly detected in the tested specimens, the PCR products originated from specimens GS100, GS42 and GS89 of *C. pilosula*, and specimens ZS15 and ZS17 of *C. tangshen* were submitted to cloning. Sequence of each clone was determined. As the results shown in Table 1.5, two, four and three types of ITS sequences were detected in the clones derived from *C. pilosula* specimens GS100, GS42 and GS89**,** respectively, which showed types **P3**, **P6** and **P9** sequences in direct sequencing result. Sequences of types **P0** (C at 122nd and 226th)

and **S0** (T at 122nd and 226th), both were inferred to be the sequence types of the supposed parental lineages of type **P3**, were detected in the clones derived from GS100. The clones from two specimens GS42 and GS89 showed each two known types of ITS sequence which was consistent with the sequence types of supposed parental lineages, types **P0** and **T1** sequences for GS42 and types **S0** and **Q0** sequences for GS89. However, in addition to known sequence, two sequence types which were not detected in our specimens were found in the clones derived from specimens GS42, and one unknown sequence type was detected in those from GS89 (type **P9**). The clones derived from *C. tangshen* specimen ZS17 contained two types of ITS sequences, types **T0** and **T1**, which are the sequence types of supposed parental lineages of type **T2**. While, the clones from specimen ZS15 had three types of ITS sequences, besides types **T0** and **T3** sequences which were supposed to be parental lineages, type **T1** was also detected (Table 1.5).

Table 1.5 ITS sequences of clones derived from *C. pilosula* and *C. tangshen*

Sample	Method	Numbers of clones	Nucleotide position										Sequence type (ITS)
			ITS1							ITS2			
			17	61	122	130	135	226	441	489	500	521	
C. pilosula			A	G	C	G	G	C	T	T	G	G	P0
GS100	Direct sequencing		*	*	Y	*	*	Y	*	*	*	*	P3
	Cloning	4	*	*	*	*	*	*	*	*	*	*	P0
	(9 clones)	5	*	*	T	*	*	T	*	*	*	*	S0
GS42	Direct sequencing		*	*	Y	*	*	*	*	Y	*	*	P6
	Cloning	4	*	*	*	*	*	*	*	*	*	*	P0
	(10 clones)	2	*	*	T	*	*	*	*	C	*	*	T1
		2	G	*	T	*	*	*	*	C	*	*	
		2	*	*	T	*	*	*	*	*	*	A	
GS89	Direct sequencing		*	*	T	*	*	Y	Y	*	*	*	P9
	Cloning	4	*	*	T	*	*	T	*	*	*	*	S0
	(9 clones)	3	*	*	T	*	*	*	C	*	*	*	Q0
		2	G	*	T	*	*	*	*	C	*	*	
ZS15	Direct sequencing		*	*	T	*	R	*	*	Y	R	*	T5
	Cloning	2	*	*	T	*	*	*	*	*	A	*	T0
	(10 clones)	6	*	*	T	*	*	*	*	C	*	*	T1
		2	*	*	T	*	A	*	*	C	*	*	T3
ZS17	Direct sequencing		*	*	T	*	*	*	*	Y	R	*	T2
	Cloning	3	*	*	T	*	*	*	*	*	A	*	T0
	(10 clones)	7	*	*	T	*	*	*	*	C	*	*	T1

Numerals above sequence are aligned nucleotide positions of *C. pilosula* which correspond to all other samples.

Asterisk indicates the identical nucleotide to which of *C. pilosula* (accession no. AB769260).

Y = C and T, R = A and G

1.3.4 ITS sequences of crude drug samples (Codonopsis Radix)

Fifty-seven crude drug samples collected from Chinese, Japanese and Korean markets were analyzed to determine their ITS sequences and then compared with those obtained from the plant specimens. Two or one analyte of every sample was successfully determined the ITS sequences. Among the 43 samples of which two analytes were successfully determined, 37 samples were composed of individuals with different types of sequences. Besides types **P0**, **P1**, **P2**, **P3**, **P5**, **P6**, **P7**, **P8** and **P10** of *C. pilosula*, types **PM0** and **PM1** of *C. pilosula* var. *modesta*, types **T0**, **T1**, **T2**, **T3**, **T4** and **T5** of *C. tangshen*, and type **S0** of *Codonopsis* sp., eight new types of sequences (designated as types **Q0**, **CC1**, **CC2**, **CC3**, **HC1**, **HC2**, **JC1** and **JC2**) were detected (Table 1.6). Among the eight new types, type **Q0** with the nucleotides T, C, C and T at positions 122nd, 226th, 441st and 489th, respectively, was a pure line sequence, which was detected in a sample "Baitiaodangshen" produced in Gansu Prov. (C17 in Table 1.2). The ITS sequence of type **CC1** was similar to that of type **P1**, except for observing C-to-Y and T-to-Y substitutions at nucleotide positions 52th and 234th, respectively. The sequences of types **CC2**, **CC3**, **HC1**, **JC1** and **JC2** were very similar to those of types **P7**, **P1**, **P0**, **P0** and **P1** of *C. pilosula*, respectively (Table 1.4). The sequences of types **HC2** was very similar to that of type **T1** of *C. tangshen*

Of the 20 samples produced in Gansu Prov. (C1-C20 in Table 1.2), analytes with types **PM0**, **P3**, **PM1**, **P1** and **P0** sequences were dominant and those with types **P8**, **P2**, **S0**, **T1**, **Q0**, **CC1** and **CC2** sequences were also observed. As for "Wendangshen" collected in our field investigation in 2009 and 2010, analytes of the 3 samples (C9-C11) showed types **PM0**, **PM1** and **T1** sequences. Within 4 samples produced in or purchased from Shanxi Prov. (C27-C30), analytes with types **P3**, **P5**, **P0** and **P6**

sequences of *C. pilosula* were observed. A few crude drug samples were derived from *C. tangshen*. The analyte with types **T2** or **T4** sequences of *C. tangshen* was found in the sample produced in Chongqing city or Shaanxi Prov. (C23, C24), respectively, and those with types **T1**, **T3**, **T4** and **T5** sequences were detected in the samples produced in Hubei Prov. (C31-C33).

In Hongkong's market, crude drug samples composed of thick roots are usually available. The 5 samples called "Wendangshen" and "Dangshen" (H1-H4, H7), of which the roots were more than 1.0 cm in diameter, were composed of analytes with types **PM0**, **HC1** and **HC2** sequences. The analytes of the other 2 samples (H5, H6) were of types **P3** and **P0** sequences.

Five "Tojin" samples obtained from Japanese market were the crude drugs imported from China. The 2 samples produced in Gansu Prov. (J1, J5) were of types **P8**, **P5** and **JC2** sequences, and the 2 samples in Guizhou Prov. (J2, J4) were of types **PM0**, **P1** and **JC1** sequences. One "Tojin" sample produced in Henan Prov. had types **T0** and **T2** sequences of *C. tangshen*. One sample, "Man Sham" obtained from Korean market (K1) was composed of analytes with types **P8** and **P1** sequences of *C. pilosula*. Although the roots of *C. lanceolata* (Sieb. et Zucc.) Traut. were used as Korean "Man Sham" nearly two decades ago (Namba et al., 1992b), nowadays *C. pilosula* is used. The ITS sequence of *C. lanceolata* was also determined (accession number AB775467), which differed from the sequence of type **P0** of *C. pilosula* by 19 nucleotides.

1.4 **Discussion**

The ITS sequence which has been demonstrated to have a high level variation and a high discriminative power to differentiate closely related species (Chinese Plant BOL

Group, 2011), has been widely used not only for species-level phylogenetic studies but also for identification of crude drugs (Wen and Zimmer., 1996; Sukrong et al., 2007; Balasubramani et al., 2010). Our results also indicated that the ITS sequences were informative for identification of the 3 medicinally-used *Codonopsis* taxa as well as Codonpsis Radix. In addition, the nrDNA ITS region is of biparental inheritance, therefore, nucleotide additivity detected in its sequence provide helpful information to infer involved progenitors or lineages (Sang et al., 1995).

On the basis of ITS sequences of 96 plant specimens, the 4 nucleotides at positions 122nd, 226th, 441st and 489th were found to be important for discrimination of the 3 *Codonopsis* taxa. The pure lines of *C. pilosula* (with type **P0** sequence) and *C. pilosula* var. *modesta* (with type **PM0** sequence) showed the nucleotides of C, C, T and T and those of T, C, T and T at the above 4 informative sites, respectively. On the other hand, *C. tangshen* and its derivative crude drug we examined had three pure lines, two from plant specimens with types **T1** and **T3** sequences and one from crude drug samples with **T0** sequence. The former two types had the nucleotides of T, C, T and C at the 4 informative sites, but differed by the nucleotide at position 135th (G or A). The latter had the nucleotides of T, C, T and T at the 4 informative sites and A at position 500th. Moreover, two pure lines with types **S0** (T, T, T and T) and **Q0** (T, C, C and T) sequences were also observed in an unidentified *Codonopsis* sp. and in one crude drug sample “Baitiaodangshen” produced in Gansu Prov., respectively. Totally, seven pure lines were detected in *Codonopsis* specimens and Codonopsis Radix.

C. pilosula specimens showed 11 types (**P0** – **P10**) of ITS sequences. Except for 5 specimens with type **P0** sequence, additive nucleotides C/T (Y) were frequently detected at the informative substitution sites in most specimens. Therefore, we assumed

that most of them might be originated from hybrid between two pure lines, and further deduced the sequences of their progenitors (Table 1.4). Based on the results of cloning, the additive nucleotides C/T (Y) were resulted from the presence of two or more pure lines having C or T at position 122nd, 226th, 441st or 489th. The results suggested that hybrid plants were growing widely in all of cultivation areas in Gansu Prov.

Although 4 specimens with type **S0** sequence were treated as unidentified samples, they had the identical morphological characters such as half-inferior ovary as *C. pilosula* specimens. Although type **Q0** sequence was not detected in the plant specimens analyzed in this study, it was detected in "Baitiaodangshen" produced in Gansu Prov. Moreover, this pure line might be involved in the formation of the plants with types **P5** and **P8** sequences of *C. pilosula*. Based on the above observation, we supposed that plants with types **S0** and **Q0** sequences might belong to *C. pilosula*.

Among 5 types (**PM0 – PM4**) of *C. pilosula* var. *modesta*, type **PM0** as a pure line sequence was dominant both in plant specimens and crude drug samples. Subsequently type **PM1** which might be derived from hybridization between lineages with types **PM0** and **T1** sequences, was observed mainly in the crude drug samples.

In the cultivation area in Gaojiashan, Wenxian County, Gansu Prov. (8 in Fig. 1), not only types **PM0**, **PM2-PM4** of *C. pilosula* var. *modesta*, but also types **P1**, **P2**, **P6**, **P7** of *C. pilosula* were detected. In the formation of plants with types **P1** or **P2** sequences, those with types **PM0** or **PM2** sequences might be involved. It could be assumed that in Wenxian County the pure lineage of *C. pilosula* var. *modesta* (**PM0**) were mainly cultivated, however, ingression of the lineages with types **P0** and **S0** sequences resulted in arising of the hybrid plants with types **P1** and **P7** sequences, respectively. On the other hand, in wide range of southeastern part of Gansu Prov. excluding special area of

Wenxian County, besides the pure lineage with type **P0** sequence, hybrids with types **P3** and **P1** sequences were widely cultivated, which might be resulted from ingression of lineages with types **S0** and **PM0** sequences, respectively.

Six types of sequences were detected in *C. tangshen*, among which type **T0** , **T1** and **T3** were the sequences of pure lines. In cultivation fields of *C. tangshen* in Chongqing city, not only genetically homogenous lineage with type **T3** sequence (11 in Fig. 1.3), but also heterogenous plants with types **T2**, **T4** and **T5** sequences (12 in Fig. 1.3) were cultivated. In the formation of plants with types **T2**, **T4** and **T5** sequences, those with types **T0**, **T1** and **T3** sequences might be involved (Tables 1.4 and 1.5). Wild *C. tangshen* in Shennongjia, Hubei Prov. had types **T1** and **T4** sequences, that in Xingshan Yichang, Hubei Prov. had type **T1** and that in Wufeng, Yichang, Hubei Prov. had types **T3** and **T5** besides types **T1** and **T4**. The hybrid lines with types **T4** and **T5** sequences tended to spread. The crude drug samples produced in Chongqing city and Fengxian, Shaanxi Prov. (called Fengdang) were found to have types **T2** and **T4** sequences, respectively. Three samples produced in Hubei Prov. showed types **T1**, **T3**, **T4** and **T5** sequences. Such results indicated that Codonopsis Radix derived from *C. tangshen* was available in relatively limited areas neighbor to its producing areas (Chongqing city, and adjacent Hubei and Shaanxi Provinces).

Type **T1** sequence was not detected in the specimens of *C. tangshen* collected from Chongqing city, but was a popular and representative sequence in the specimens of *C. tangshen* collected from Hubei Prov. Plants with type **T1** sequence might be involved in the formation of plants with types **P6**, **P10** and **PM1** sequences, which were observed in cultivation fields of Gansu Prov. On the other hand, analytes of crude drug samples which had type **T1** sequence was detected in 2 samples of “Wendangshen” produced in

Gansu Prov. Therefore, a distribution of *C. tangshen* with type **T1** sequence was not limited to Hubei Prov. As the morphological differences in leaves, flower and ovary between *C. tangshen* and *C. pilosula* were tiny, a new combination as *C. pilosula* subsp. *tangshen* (Oliv.) D. Y. Hong has been advocated (Hong 2010; Hong et al., 2011). The similarity of ITS sequence supported this classification.

"Wendangshen" produced in Wenxian County is composed of thick cylindrical roots, which has been believed to be superior in quality. Our molecular study revealed that the botanical sources of commercial "Wendangshen" were not limited to *C. pilosula* var. *modesta*, which were different from a previous paper (Namba et al., 1992a, b). Codonopsis Radix including "Wendangshen" , which are composed of thick roots over 1.0 cm in diameter, also showed seve1ral types of sequences such as types **P3**, **P6**, **P8**, **P0** and **Q0** of *C. pilosula*, and **T4** and **HC2** of *C. tangshen*. Codonopsis Radix has been graded according to producing areas and sizes which are related to their botanical origin and growth period, respectively (Xu et al., 1994). We further investigated chemical composition of these clearly identified materials to clarify chemical differences according to the botanical source, growth period and the cultivation area. Quality evaluation of Codonopsis Radix based on chemical constituents will be reported in Chapter III.

Lin et al. (2007) reported that only two variable sites in ITS sequences were found among 3 *Codonopsis* taxa by using only 3 or 4 specimens of each taxon. In our study, more variable sites in ITS sequences were detected among 96 plant specimens of the 3 *Codonopsis* taxa collected from Gansu Prov., Chongqing city and Hubei Prov., China, which did provide a more precise and accurate dataset for identification of the 3 *Codonopsis* taxa. Guo et al. (2007) revealed the genetic diversity in the cultivated *C.*

pilosula populations collected from Longxi county of Gansu Prov. by RAPD analysis. In the present study, we widely collected a number of plant specimens of the 3 medicinally-used *Codonopsis* taxa in their main producing areas, and dozens of commercial Codonopsis Radix from markets of mainland China, Hongkong, Japan and Korea. Based on the analysis of ITS sequences, we clarified the pure lines of *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen*, which were the bases of diversity. The sequence data suggested that the significant genetic polymorphism might be induced by a wide range of hybridization among the pure lines, and from their sequences the lineages involved in hybridization could be further inferred. Cloning analysis of the samples with additive nucleotides supported such inference, in which each pure line sequence was clearly separated and detected in respective clones. In addition, Guo et al. (2007) mentioned that many medicinal plants were directly domesticated from local wild resources by farmers. The farmers collected mature seeds randomly and mixed them to plant in the field. Sometimes, the farmers exchanged seeds easily among friends or relatives. Such traditional and irregular agricultural approach might be the main cause for the high level of genetic diversity within the cultivated populations and might improve germnoplastic hybridization. The significant sequence polymorphism observed in this study might be also attributed to such agricultural approach.

Summary of Chapter I

In order to find out genetic markers for identifying the 3 taxa, *Codonopsis pilosula*, *C. pilosula* var. *modesta* and *C. tangshen* and to authenticate Codonopsis Radix, the molecular analysis of the internal transcribed spacer sequence of nuclear ribosomal DNA was conducted on *Codonopsis* plants collected widely from Gansu Prov., Chongqing city and Hubei Prov. of China, the main producing areas of Codonopsis Radix.

1) Significant genetic polymorphism was observed, represented by 11 types of ITS sequences in *C. pilosula*, 5 types in *C. pilosula* var. *modesta* and 5 types in *C. tangshen*.
2) Among the determined sequences, 1, 1 and 2 types were thought to be of pure lines of each taxon, respectively, designated as types **P0**, **PM0**, **T1** and **T3**. Moreover, 3 pure lines with types **S0**, **Q0** and **T0** sequences were also obtained in *Codonopsis* sp. and Codonopsis Radix samples. Types **S0** and **Q0** were supposed to be of *C. pilosula* and type **T0** was of *C. tangshen*.
3) The rest ITS sequence types might be derived from hybridization. Hybrid lines were inferred to be resulting from the combination of these pure lines. Cloning analysis of the specimens with additive nucleotides supported such inference and provided detailed information of parental lineages.
4) The informative sites for discriminating the 3 taxa were detected at the nucleotide positions 122nd, 226th, 441st and 489th from upstream of the ITS sequence. For discrimination of the 6 types of *C. tangshen*, the nucleotides at positions 135th, 489th and 500th were informative.
5) Botanical sources of the crude drugs produced in a wide range of the southeast Gansu

Prov. were *C. pilosula*, just those from Wenxian of Gansu Prov. were *C. pilosula* var. *modesta*. The crude drugs produced in Chongqing city and Hubei Prov. were derived from *C. tangshen*.

Chapter II

Development of HPLC-UV method for analysis of polyacetylenes, phenylpropanoid and pyrrolidine alkaloids

2.1 Introduction

Codonopsis Radix commonly used as a tonic in traditional Chinese medicine, is prescribed as the roots of *Codonopsis pilosula* (Franch.) Nannf., *C. pilosula* Nannf. var. *modesta* (Nannf.) L. D. Shen, and *C. tangshen* Oliv. in Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010). The roots of *C. pilosula* or *C. tangshen*, and Codonopsis Radix have been reported to contain polysaccharides, polyacetylenes, phenylpropanoids, alkaloids, triterpenoids (Wang and Wang, 1996; Zhu et al., 2001; He et al., 2006; Song et al., 2008a; Tsai and Lin, 2008; Wakana et al., 2011). Among the chemical constituents, lobetyolin of polyacetylene was reported to play a protective role in gastric mucosa injury (Song et al., 2008b), total alkaloids to cause a significant enhancement of nerve growth factor-induced neurite outgrowth in PC12 cells as well as an increase of the phosphorylation of mitogen-activated protein kinase (MAPK) (Liu et al., 2003), and total saponins to have protective effect on ischemia-reperfusion injury in rats after kidney transplantation (He et al., 2011). In Chinese Pharmacopoeia only lobetyolin was used as a marker compound for qualitative identification of Codonopsis Radix. However, Qiao et al. (2007) have reported that lobetyolin is widely found not only in the three *Codonopsis* taxa used as Codonopsis Radix, but also in other species of the same genus and even in species of other genera from the family Campanulaceae, indicating it is not characteristic for Codonopsis Radix. Moreover, to assess the quality of Codonopsis Radix it is necessary to develop a suitable method on the basis of multiple components having potential bioactivities.

Several analytical methods to evaluate the quality of Codonopsis Radix have been reported, including quantitative analysis of lobetyolin by HPLC-UV (Song et al., 2008c) or LC-MS (Ong and Len, 2003); comparative analysis by HPLC-UV fingerprints (Qiao

et al., 2007; Song et al., 2008d); and detection of tangshenoside I by TLC-UV (Mizutani et al., 1988; Han et al., 1990). Recently, pyrrolidine alkaloids have been analyzed by quantitative NMR (Li et al., 2009). These methods focus on only one type or limited chemical components, which are considered to be unsatisfied to evaluate the quality of Codonopsis Radix.

In this chapter, a HPLC-UV method to simultaneously detect and quantitate seven analytes (Fig. 2.1), including two pyrrolidine alkaloids (codonopyrrolidiums A, B), a phenylpropanoid (tangshenoside I), and four polyacetylenes (lobetyol, lobetyolin, lobetyolinin and cordifolioidyne B) in Codonopsis Radix was developed and validated.

2.2 Materials and Methods

2.2.1 Materials

A commercial sample of Codonopsis Radix (TMPW no. 26991) which purchased from Japanese market, authenticated to be *C. tangshen*, was used for isolation of standard compounds and also for method validation. The crude drug sample was stored in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan (TMPW).

2.2.2 Reagents, Apparatus and HPLC conditions

HPLC grade acetonitrile and methanol, ultrapure water, analytical grade acetic acid and phosphoric acid were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Column chromatography (CC) was performed using Diaion HP 21 (Mitsubishi Chemical Corporation, Japan), YMC GEL ODS-A (YMC Co., Ltd., Japan), Sephadex LH-20 (GE Healthcare Life Sciences, Sweden) and silica gel (Wako Pure Chemical

Industries, Ltd., Japan). Semipreparative HPLC (Waters 600) was performed on a YMC-Pack R&D ODS-A column (20 mm i. d. × 250 mm, S-5 μm, 12 nm, YMC Co., Ltd. Japan). Mass spectra were measured using JEOL JMS-GC mate II mass spectrometer, JEOL JMS-AX505HAD mass spectrometer (JEOL Ltd., Japan) and Thermo LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific Inc., USA). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) were recorded on a JEOL JNM-ECX400P/TIM spectrometer. HPLC system (Shimadzu Co., Japan) was composed of a LC-10AD pump, a DGU-20A degasser, a SIL-10AD auto-injector, a CTO-10-AS column oven and a SPD-M10A diode array detector. Ultrasonic extraction was performed by sonicator (US-4R, AS ONE Co., Japan).

2.2.3 Isolation and identification of standard compounds

2.2.3.1 Isolation of compounds

Dried and powdered Codonopsis Radix (1.0 kg) (TMPW No. 26991) was extracted by steeping in methanol for three times (10 L, 48h) at room temperature. The combined methanol extracts were evaporated under reduced pressure and then lyophilized to yield a brown solid residue (303.0 g). The residue redissolved in distilled water was applied to a Diaion HP 21 column (9 cm × 55 cm) and successively eluted with H_2O, 20%, 40%, 60%, 80% MeOH (v/v) and MeOH (5.0 L of each solvent). After detecting each eluent by TLC, the eluents with similar chemical compositions were combined and dried by reduced evaporation and lyophilisation to give fractions A (289.0 g), B (5.1 g), C (3.1 g), D (2.7 g), E (2.6 g) and F (2.1 g), respectively. In experimental procedure, fractions were monitored by TLC and HPLC-UV method to guide further isolation and

purification. Fraction A contained saccharides rather than target compounds. Fraction B was subjected to an ODS-A column (4.5 cm × 50 cm) and eluted with H_2O/MeOH (8:2-4:6, v/v; 1.5 L of each ratio) to obtained four fractions. Fraction B-2 (0.7 g) were subjected to a Sephadex LH-20 column (2.5 cm × 70 cm) and eluted with H_2O/MeOH (6:4, v/v; 5.0 L) to give thirteen fractions. Fraction B-2-7 was further purified by semipreparative HPLC [solvent: MeOH/0.1% trifluoroacetic acid (TFA) water solution, 15:85 v/v, flow rate: 6 mL/min] to yield compound **1**. Fraction B-4 (0.5 g) was subjected to ODS-A column (4.5 cm × 50 cm) and eluted with H_2O/MeOH (7:3-6:4, v/v; 1.5 L of each ratio) to yield four factions. Fraction B-4-2 was subjected to ODS-A column (1.5 cm × 50 cm) and eluted with H_2O/MeOH (7:3, v/v; 3.0 L) to yield compounds **8** (10.4 mg) and **9** (2.4 mg). Fraction C was subjected to an ODS-A column (4.5 cm × 50 cm) and eluted with H_2O/MeOH (7:3-3:7, v/v; 1.0 L of each ratio) to give five fractions, and Fr. C-3 (0.5 g) was purified by semipreparative HPLC (solvent: MeOH/0.1% TFA water solution, 30:70-45:55 v/v, linear gradient in 45 min, flow rate: 6 mL/min) to yield compound **2**. To exclude TFA from the obtained compounds, compounds **1** and **2** were dissolved in 0.5% hydrochloric acid and then subjected to an ODS-A column (1.5 cm × 20 cm), respectively. After elution with MeOH/0.5% HCl water solution (0:100, 50:50, v/v; 300 mL of each ratio), the chlorides of compound **1** (22.3 mg) and compound **2** (15.2 mg) were obtained from the 50% MeOH (v/v). Fraction C-4 (0.4 g) was subjected to an ODS-A column (4.5 cm × 50 cm) and eluted with H_2O/MeOH (8:2-5:5, v/v; 1.0 L of each ratio) to yield eleven fractions. Fraction C-4-5 was subjected to a Sephadex LH-20 column (2.5 cm × 70 cm) and eluted with H_2O/MeOH (5:5, v/v; 5.0 L) to yield compounds **10** (5.4 mg) and **11** (14.0 mg). Fraction C-4-8 was subjected to an ODS-A column (1.5 cm × 50 cm) and eluted with

H_2O/MeOH (7:3, v/v; 1.2 L) to yield compound **12** (4.4 mg). Fraction D was chromatographed on an ODS-A column (4.5 cm × 50 cm) and eluted with H_2O/MeOH (6:4-2:8, v/v; 1.0 L of each ratio) to give three fractions. Fraction D-2 (0.6 g) was purified by semipreparative HPLC (solvent: MeOH/0.1% TFA water solution, 30:70-55:45 v/v, linear gradient in 50 min, flow rate: 6 mL/min) to afford compounds **3** (9.8 mg) and **4** (3.5 mg). Fraction E was subjected to a silica gel column (100-200 mesh; 4.5 cm × 50 cm) and eluted with $CHCl_3$/MeOH (9:1-1:1, v/v; 1.5 L of each ratio) to obtain nine fractions. Fr. E-2 (0.2 g) was chromatographed on a silica gel column (100-200 mesh; 1.5 cm × 45 cm) and eluted with $CHCl_3$/MeOH (8:2, v/v; 1.0 L) to give eight fractions, and Fraction E-2-8 was subsequently chromatographed on a Sephadex LH-20 column (1.5 cm × 50 cm) and eluted with H_2O/MeOH (1:1, v/v; 3.0 L) to compounds **7** (3.8 mg) and **13** (9.0 mg). Fraction E-4 (0.3 g) was repeatedly applied to a Sephadex LH-20 column (2.5 cm × 70 cm) and eluted with H_2O/MeOH (1:1, v/v; 5.0 L) to give compound **6** (14.1 mg). Fraction E-6 (0.2 g) was subjected to an ODS-A column (2.5 cm × 50 cm) and eluted with H_2O/MeOH (6:4, v/v; 1.0 L) to give eight fractions. Fraction E-6-5 was subjected to a Sephadex LH-20 column (2.5 cm × 70 cm) and eluted with H_2O/MeOH (1:1, v/v; 4.0 L) to give compound **5** (7.0 mg).

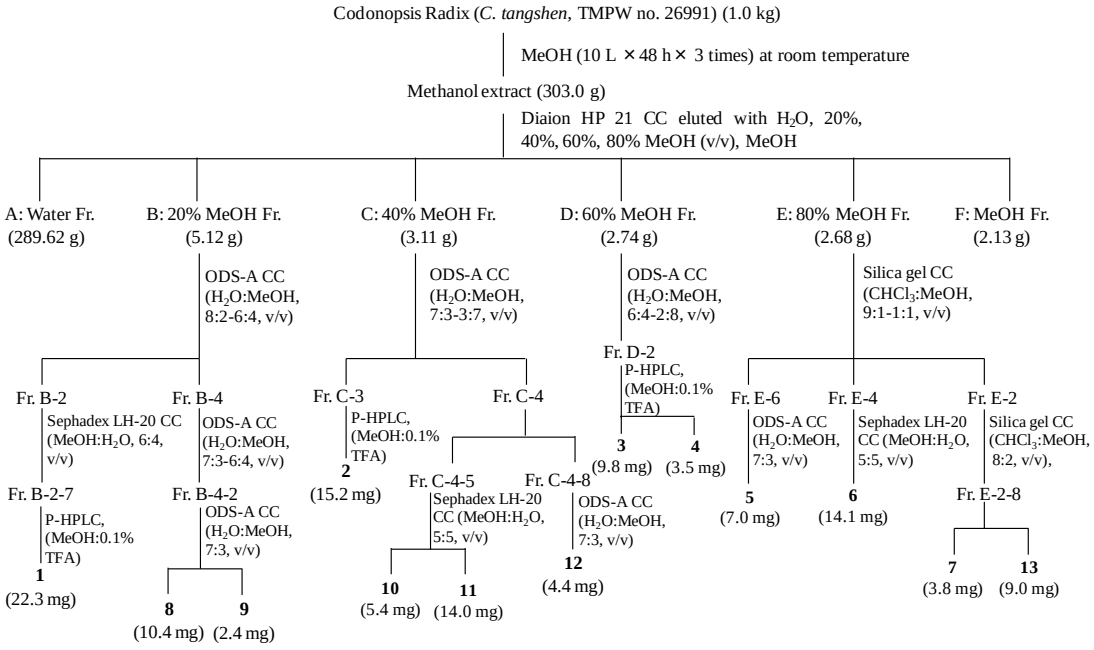
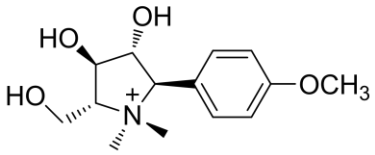
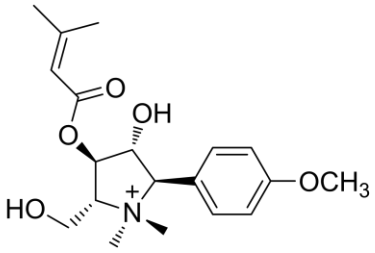


Fig. 2.1 Isolation and purification procedure of Codonopsis Radix

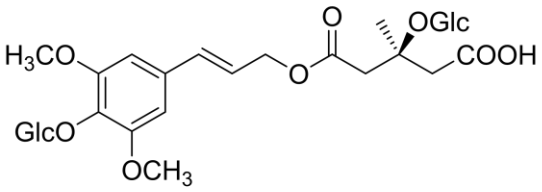
The compounds **1-12** were identified as codonopyrrolidium B (**1**) (Tsai and Lin, 2008), codonopyrrolidium A (**2**) (Tsai and Lin, 2008), tangshenoside I (**3**) (Mizutani, et al., 1988; Cuendet et al., 2001), cordifolioidyne B (**4**) (Mei et al., 2008), lobetyolinin (**5**) (Ishimaru, et al., 1992), lobetyolin (**6**) (Ishimaru, et al., 1991), lobetyol (**7**) (Ishimaru, et al., 1991), vanillic acid (**8**) (Scott, 1972), 5-(hydroxymethyl)-2-furaldehyde (**9**) (Shimizu et al., 1993), catechin (**10**) (Seto et al., 1997), adenosine (**11**) (Feng et al., 2012) and 3, 4-dihydroxybenzoic acid (**12**) (Scott, 1972), comparing their spectral data (MS, ^{1}H NMR and ^{13}C NMR). The purity of each compound for quantitaion was confirmed to be higher than 97% by HPLC-DAD.



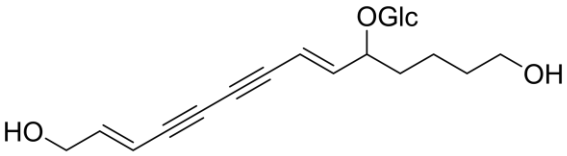
Codonopyrrolidium B (**1**)



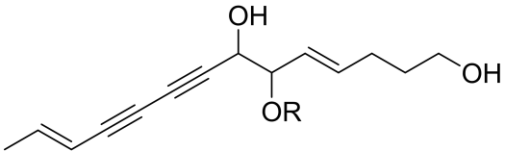
Codonopyrrolidium A (**2**)



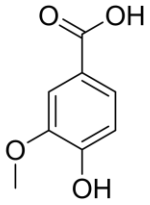
Tangshenoside I (**3**)



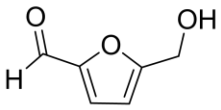
Cordifolioidyne B (**4**)



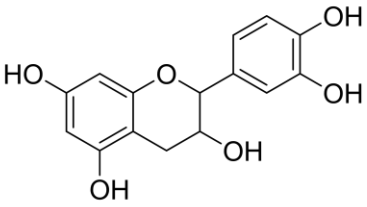
Lobetyolinin (**5**) R=*β*-Glc (6→1)-*β*-Glc
Lobetyolin (**6**) R=*β*-Glc
Lobetyol (**7**) R=H



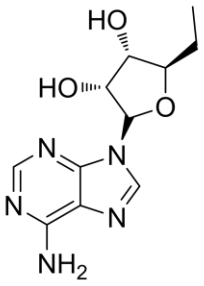
Vanillic acid (**8**)



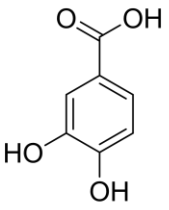
5-(Hydroxymethyl)-2-furaldehyde(**9**)



Catechin (**10**)



Adenosine (**11**)

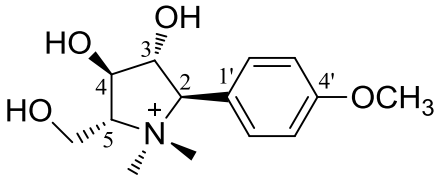


3, 4-Dihydroxybenzoic acid (**12**)

Fig. 2.2 Structures of isolated compounds

2.2.3.2 Spectra data of isolated compounds

Codonopyrrolidium B (**1**)

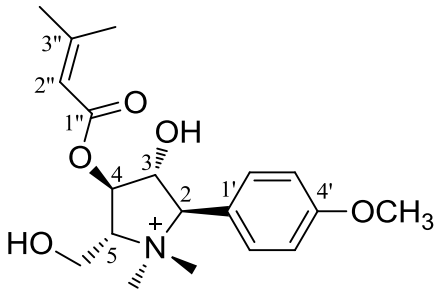


FTMS *m/z*, 268.1512 $[M]^+$.

^{1}H-NMR (CD_3OD, 400 MHz) δ: 7.56 (2H, *d*, *J* = 8.4, H-2' and H-6'), 7.08 (2H, *d*, *J* = 8.4, H-3', H-5'), 4.71 (1H, *dd*, *J* = 9.2, 5.2, H-3), 4.64 (1H, *d*, *J* = 9.2, H-2), 4.30 (1H, *t*, *J* = 5.6, H-4), 4.18 (1H, *d*, *J* = 13.2, H-6), 4.10 (1H, *d*, *J* = 5.6, H-6), 3.85 (3H, *s*, O$C\underline{H}_3$), 3.67 (1H, *m*, H-5), 3.17 (3H, *s*, N-$C\underline{H}_3$), 2.80 (3H, *s*, N-$C\underline{H}_3$).

^{13}C-NMR (CD_3OD, 100 MHz) δ: 163.3 (C-4'), 134.4 (C-2' and C-6'), 121.0 (C-1'), 115.8 (C-3' and C-5'), 83.1 (C-2), 81.7 (C-5), 77.6 (C-3), 76.3 (C-4), 59.3 (C-6), 56.0 (O$\underline{C}H_3$), 52.0 (N-$\underline{C}H_3$), 48.9 (N-$\underline{C}H_3$).

Codonopyrrolidium A (**2**)

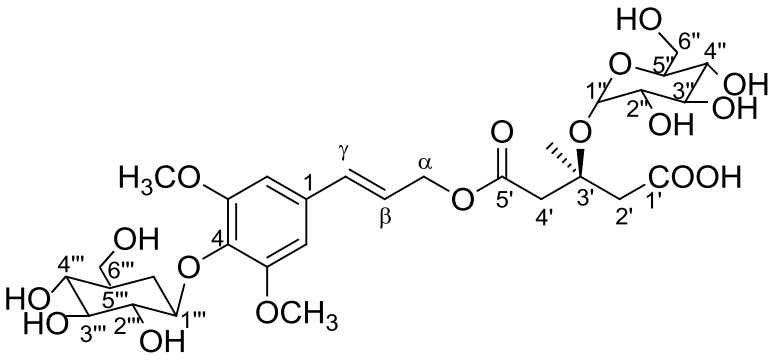


FTMS *m/z*, 350.1925 $[M]^+$.

^{1}H-NMR (CD_3OD, 400 MHz) δ: 7.59 (2H, *d*, *J* = 8.8, H-2', H-6'), 7.11 (2H, *d*, *J* = 8.8, H-3', H-5'), 5.87 (1H, *s*, H-2''), 5.41 (1H, *m*, H-4), 5.07 (1H, *dd*, *J* = 10.4, 4.8, H-3), 4.94 (1H, *d*, *J* = 10.4, H-2), 4.27 (2H, *brs*, H-6), 3.88 (1H, *brs*, H-5), 3.86 (3H, *s*, O$C\underline{H}_3$), 3.10 (3H, *s*, N-$C\underline{H}_3$), 2.88 (3H, *s*, N-$C\underline{H}_3$), 2.22 (3H, *s*, H-5''), 1.98 (3H, *s*, H-4'').

^{13}C-NMR (CD_3OD, 100 MHz) δ: 167.5 (C-1''), 163.7 (C-4'), 162.0 (C-3''), 134.8 (C-2', C-6'), 120.0 (C-1'), 116.0 (C-3', C-5'), 115.6 (C-2''), 81.8 (C-2), 81.4 (C-5), 79.3 (C-4), 75.5 (C-3), 60.2 (C-6), 56.2 (O$\underline{C}H_3$), 50.6 (N-$\underline{C}H_3$), 48.3 (N-$\underline{C}H_3$), 27.8 (C-4''), 20.7 (C-5'').

Tangshenoside I (**3**)

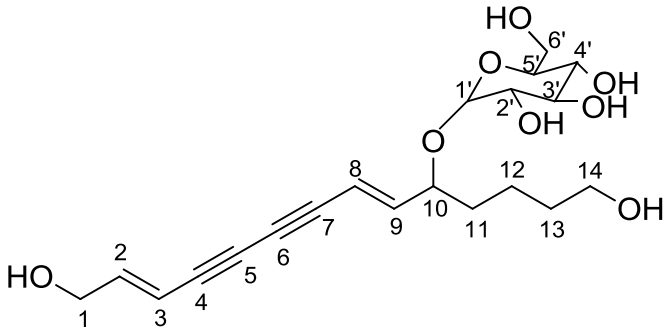


FTMS *m/z*, 701.2195 $[M+Na]^+$.

^{1}H-NMR (CD_3OD, 400 MHz) δ: 6.78 (2H, *s*, H-2, H-6), 6.62 (1H, *d*, *J* = 16.0, H-γ), 6.29 (1H, *dd*, *J* = 16.0, 6.4, H-β), 4.73 (2H, *d*, *J* = 6.4, H-α), 3.86 (6H, *s*, O$\underline{C}H_3$×2), 3.00 (1H, *d*, *J* = 15.6, H-2'), 2.92 (1H, *d*, *J* = 15.6, H-4'), 2.90 (1H, *d*, *J* = 15.6, H-2'), 2.83 (1H, *d*, *J* = 15.6, H-4'), 1.51 (3H, *s*, H-6').

^{13}C-NMR (CD_3OD, 100 MHz) δ: 174.4 (C-1'), 172.4(C-5'), 154.3 (C-3), 154.3 (C-5), 136.1 (C-4), 134.9 (C-1), 134.4 (C-γ), 124.2 (C-β), 105.6 (C-2), 105.6 (C-6), 105.2 (C-1'''), 98.2 (C-1''), 78.2 (C-3'), 77.8 (C-5'''), 77.7 (C-5''), 77.6 (C-3'''), 77.4 (C-3''), 75.6 (C-2'''), 75.0 (C-2''), 71.4 (C-4''), 71.2 (C-4'''), 66.1 (C-α), 62.7 (C-6''), 62.5 (C-6'''), 57.0 (O$\underline{C}H_3$×2), 44.2 (C-2'), 44.2 (C-4'), 24.8 (C-6').

Cordifolioidyne B (**4**)



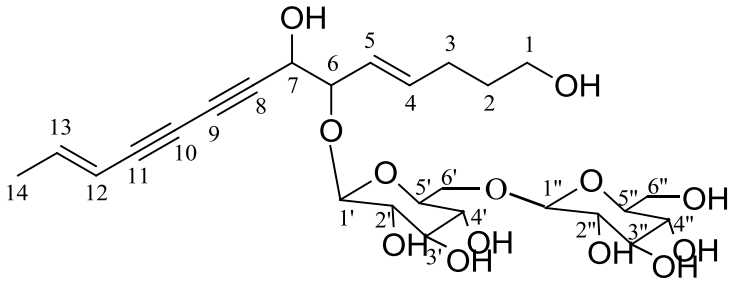
FTMS *m/z*, 419.1657 $[M+Na]^+$.

^{1}H-NMR (CD_3OD, 400 MHz) δ: 6.40 (1H, *dt*, *J* = 16.0, 4.4, H-2), 6.18 (1H, *dd*, *J* = 16.0, 6.8, H-9), 5.97 (1H, *d*, *J* = 16.0, H-8), 5.85

(1H, *d*, *J* = 16.0, H-3), 4.38 (1H, *dt*, *J* = 12.8, 6.4, H-10), 4.14 (2H, *dd*, *J* = 4.4, 2.4, H-1), 3.54 (2H, *t*, *J* = 6.4, H-14), 4.23 (1H, *d*, *J* = 8.0, H-1'), 3.86 (1H, *d*, *J* = 12.0, 2.0, H-6'), 3.64, (1H, *d*, *J* = 12.0, 6.0, H-6').

^{13}C-NMR (CD_3OD, 100 MHz) δ: 148.1 (C-9), 148.0 (C-2), 112.0 (C-8), 108.8 (C-3), 101.6 (C-1'), 80.7 (C-4), 80.1 (C-7), 78.2 (C-5), 78.1 (C-10), 78.1 (C-3'), 78.0 (C-5'), 75.2 (C-2'), 74.6 (C-6), 71.8 (C-4'), 62.9 (C-14), 62.7 (C-1), 62.7 (C-6'), 36.2 (C-11), 33.4 (C-13), 22.6 (C-12).

Lobetyolinin (**5**)

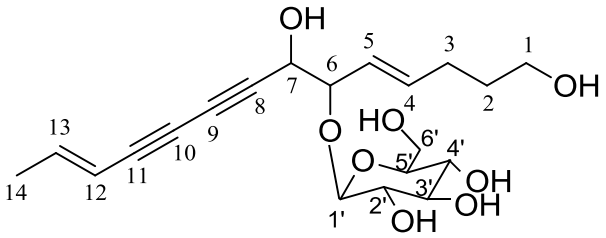


FAB-MS *m/z*, 581 $[M+Na]^+$.

^{1}H-NMR (CD_3OD, 400 MHz) δ: 6.33 (1H, *dq*, *J* = 15.6, 7.2, H-13), 5.93 (1H, *dt*, *J* = 15.6, 8.0, H-4), 5.57 (1H, *dd*, *J* = 15.6, 2.0, H-12), 5.48 (1H, *dd*, *J* = 15.6, 8.0, H-5), 4.45 (1H, *d*, *J* = 6.0, H-7), 4.27 (1H, *t*, *J* = 7.6, H-6), 3.67 (2H, *t*, *J* = 7.2, H-1), 2.18 (2H, *dd*, *J* = 14.0, 7.2, H-3), 1.80 (3H, *dd*, *J* = 6.8, 1.6, H-14), 1.65 (2H, *quin*, *J* = 7.2, H-2), 4.41 (1H, *d*, *J* = 7.6, H-1''), 4.32 (1H, *d*, *J* = 8.0, H-1'), 4.13 (1H, *d*, *J* = 12.0, H-6''), 3.86 (1H, *d*, *J* = 7.6, H-6'), 3.78 (1H, *dd*, *J* = 12.0, 6.0, H-6''), 3.78 (1H, *dd*, *J* = 7.6, 4.4, H-6').

^{13}C-NMR (CD_3OD, 100 MHz) δ: 145.3 (C-13), 138.7 (C-4), 126.5 (C-5), 110.5 (C-12), 104.5 (C-1''), 101.2 (C-1'), 82.4 (C-6), 81.2 (C-11), 78.1 (C-10), 78.0 (C-5''), 77.9 (C-3'), 77.9 (C-3''), 77.9 (C-5''), 77.2 (C-2''), 75.2 (C-2'), 74.8 (C-4'), 72.6 (C-9), 71.6 (C-8), 71.1 (C-4''), 69.7 (C-6'), 66.7 (C-7), 62.7 (C-6''), 62.3 (C-1), 32.9 (C-2), 29.8 (C-3), 18.9 (C-14).

Lobetyolin (**6**)

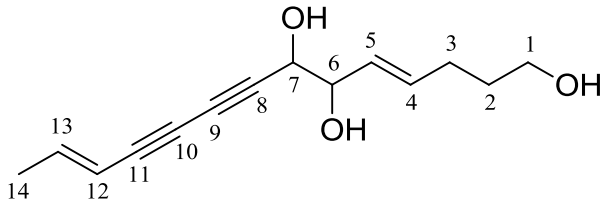


FAB-MS *m/z*, 419 $[M+Na]^+$.

^{1}H-NMR (CD_3OD, 400 MHz) δ: 6.34 (1H, *dq*, *J* = 14.4, 6.8, H-13), 5.92 (1H, *dt*, *J* = 14.4, 6.8, H-4), 5.59 (1H, *dd*, *J* = 14.4, 2.0, H-12), 5.40 (1H, *dd*, *J* = 14.4, 6.8, H-5), 4.42 (1H, *d*, *J* = 6.4, H-7) , 4.31 (1H, *d*, *J* = 7.6, H-1'), 4.27 (1H, *t*, *J* = 6.4, H-6), 3.85 (1H, *dd*, *J* = 12.0, 2.0, H-6'), 3.65 (1H, *dd*, *J* = 12.0, 6.0, H-6'), 3.59 (2H, *t*, *J* = 6.8, H-1), 2.17 (2H, *dd*, *J* = 14.4, 6.8, H-3), 1.80 (3H, *dd*, *J* = 6.8, 2.0, H-14), 1.65 (2H, *quin*, *J* = 6.8, H-2).

^{13}C-NMR (CD_3OD, 100 MHz) δ: 145.3 (C-13), 138.9 (C-4), 126.5 (C-5), 110.5 (C-12), 100.7 (C-1'), 81.8 (C-11), 81.1 (C-6), 78.1 (C-10), 78.0 (C-3'), 77.9 (C-5'), 74.8 (C-2'), 72.4 (C-9), 71.6 (C-4'), 71.2 (C-8), 66.5 (C-7), 62.7 (C-6'), 62.2 (C-1), 32.9 (C-2), 29.8 (C-3), 18.9 (C-14).

Lobetyol (**7**)

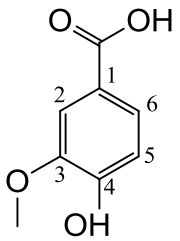


EI-MS *m/z*, 234 $[M]^+$.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 6.35 (1H, *dq*, *J* = 15.6, 6.8, H-13), 5.89 (1H, *dt*, *J* = 15.6, 6.8, H-4), 5.57 (1H, *dd*, *J* = 15.6, 6.8, H-5), 5.53 (1H, *dd*, *J* = 15.6, 1.6, H-12), 4.31(1H, *d*, *J* = 6.4, H-7), 4.15 (1H, *t*, *J* = 6.4, H-6), 3.68 (2H, *t*, *J* = 6.4, H-1), 2.19 (2H, *dd*, *J* = 13.6, 6.4, H-3), 1.82 (3H, *dd*, *J* = 6.8, 1.6, H-14), 1.69 (2H, *quin*, *J* = 6.4, H-2).

^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 144.6 (C-13), 135.2 (C-4), 127.4 (C-5), 109.4 (C-12), 79.0 (C-11), 78.1 (C-10), 75.4 (C-6), 71.4 (C-9), 71.3 (C-8), 66.8 (C-7), 62.3 (C-1), 31.7 (C-2), 28.9 (C-3), 18.9 (C-14).

Vanillic acid (**8**)

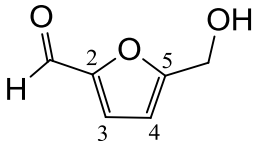


EI-MS *m/z*, 168 $[M]^+$.

^{1}H NMR (CD_3OD, 400 MHz) δ: 7.44 (1H, *d*, *J* = 2.0, H-2), 7.43 (1H, *J* = 7.6, 2.0, H-6), 6.83 (1H, *d*, *J* = 7.6, H-5).

^{13}C NMR (CD_3OD, 100 MHz) δ: 167.2 (C<u></u>OOH), 151.1 (C-4), 147.2 (C-3), 123.5 (C-6), 121.6 (C-1), 115.0 (C-2), 112.7 (C-5), 55.5 (OC<u></u>H_3).

5-(hydroxymethyl)-2-furaldehyde (**9**)

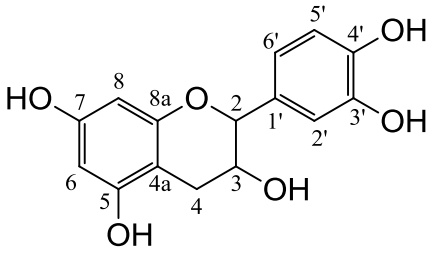


EI-MS *m/z*, 126 $[M]^+$.

^{1}H NMR (CD_3OD, 400 MHz) δ: 9.53 (1H, *s*, C<u>H</u>O), 7.38 (1H, *d*, *J* = 3.6, H-3), 6.58 (1H, *d*, *J* = 3.6, H-4), 4.61 (2H, *s*, -C<u>H_2</u>OH).

^{13}C NMR (CD_3OD, 100 MHz) δ: 179.4 (C<u></u>HO), 163.2 (C-5), 152.3 (C-2), 124.8 (C-3), 110.9 (C-4), 57.6 (C<u></u>H_2OH).

Catechin (**10**)



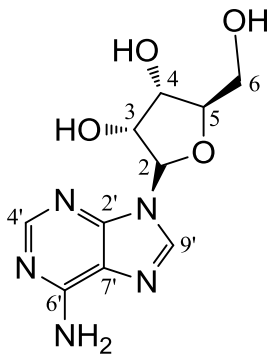
EI-MS *m/z*, 290 $[M]^+$.

^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.17 (7-O<u>H</u>), 8.93 (5-O<u>H</u>), 8.86 (3'-O<u>H</u>), 8.81

(4'-O<u>H</u>), 6.71 (1H, *d*, *J* = 2.0, H-2'), 6.68 (1H, *d*, *J* = 8.0, H-5'), 6.59 (1H, *dd*, *J* = 6.8, 2.0, H-6'), 5.88 (1H, *d*, *J* = 3.0, H-6), 5.68 (1H, *d*, *J* = 3.0, H-8), 4.86 (1H, *d*, *J* = 5.2), 4.47 (1H, *d*, *J* = 7.6, H-2), 3.80 (1H, *m*, H-3), 2.65 (1H, *dd*, *J* = 16.0, 5.6, H-4a), 2.34 (1H, *dd*, *J* = 16.0, 8.0, H-4b).

^{13}C NMR (DMSO-d_6, 100 MHz) δ: 156.3 (C-5), 156.0 (C-7), 155.2 (C-8a), 144.7 (C-3' and C-4'), 130.4 (C-1'), 118.3 (C-6'), 114.9 (C-2'), 114.4 (C-5'), 98.9 (C-4a), 94.9 (C-6), 93.7 (C-8), 80.0 (C-2), 66.2 (C-3), 27.8 (C-4).

Adenosine (**11**)

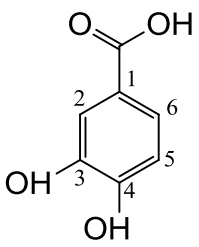


EI-MS *m/z*, 252 $[M]^+$.

^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.35 (1H, s, H-4'), 8.14 (1H, *s*, H-9'), 7.35 (2H, *s*, N<u>H</u>$_2$), 5.87 (1H, *d*, *J* = 6.0, H-2), 4.61 (1H, *s*, H-3), 4.14 (1H, *s*, H-4), 3.96 (1H, *d*, *J* = 3.2, H-5), 3.68 (1H, *d*, *J* = 12.0, H-6), 3.55 (1H, *d*, *J* = 12.0, H-6).

^{13}C NMR (DMSO-d_6, 100 MHz) δ: 156.1 (C-6'), 152.3 (C-4'), 148.9 (C-2'), 139.8 (C-9'), 119.2 (C-7'), 87.8 (C-2), 85.8 (C-5), 73.3 (C-3), 70.5 (C-4), 61.6 (C-6).

3, 4-Dihydroxybenzoic acid (**12**)



EI-MS *m/z*, 154 $[M]^+$.

^{1}H NMR (CD_3OD, 400 MHz) δ: 7.42 (1H, *d*, *J* = 2.4, H-2), 7.39 (1H, *dd*, *J* = 7.6, 2.4, H-6), 6.80 (1H, *d*, *J* = 7.6, H-5).

^{13}C NMR (CD_3OD, 100 MHz) δ: 170.7 (<u>C</u>OOH), 151.2 (C-4), 145.9 (C-3), 123.7 (C-6), 123.7 (C-1), 117.7 (C-2), 115.7 (C-5).

Compound **13** ($C_{18}H_{34}O_5$)

FTMS *m/z*, 353.2248 $[M+Na]^+$.

^{1}H NMR (CD_3OD, 400 MHz) δ: 5.68 (2H, *t*, *J* = 5.2), 4.04 (1H, *dd*, *J* = 12.0, 6.4), 3.90 (1H, *t*, *J* = 5.2), 3.40 (1H, *m*), 2.27 (2H, *t*, *J* =7.2), 1.53 (6H, *m*), 0.90 (3H, *t*, *J* =7.2).

^{13}C NMR (CD_3OD, 100 MHz) δ: 177.7 (COOH), 136.5, 131.1, 76.5, 75.8, 73.0, 38.3, 35.0, 33.5, 33.1, 30.6, 30.4, 30.2, 26.6, 26.5, 26.1, 23.7, 14.4.

2.2.4 Optimized conditions for HPLC analysis

Quantitative analysis was carried out using a YMC-Pack Pro-C_{18} column (4.6 mm i. d. × 250 mm, 5 μm, YMC Co., Ltd.) with column temperature at 30℃. The mobile phase was a binary eluent of acetonitrile (A) and 0.1% (v/v) phosphoric acid (B) with gradient conditions as follows: 0-10 min, 98-92% B; 10-35 min, 92-80% B; 35-50 min, 80-70% B; 50-60 min, 70-50% B; 60-65 min, 50-10% B. Flow rate was 1.0 mL/min and detection wavelength was 215 nm.

2.2.5 Sample preparation

Dried sample was pulverized and then sieved through 300 μm mesh. An aliquot of the powder (ca 1.0 g) was accurately weighed and extracted with methanol (15 mL) by ultrasonication for 20 min. After centrifugation at 3000 rpm for 5 min, the supernatant was separated. The extraction was repeated for three times, and the combined supernatant was evaporated under vacuum. The residue dissolved with methanol was transferred into a 5 mL volumetric flask and made up to volume with methanol. After

filtration through 0.20 μm Millipore filter unit, 20 μL of sample solution was injected into HPLC for analysis.

2.2.6 Preparation of standard solution

Each of the seven standard compounds, codonopyrrolidium B, codonopyrrolidium A, tangshenoside I, cordifolioidyne B, lobetyolinin, lobetyolin and lobetyol, was accurately weighted and dissolved in HPLC grade methanol to obtain the stock standard solution (0.95 mg/mL, 0.92 mg/mL, 0.98 mg/mL, 1.00 mg/mL, 1.05 mg/mL, 1.00 mg/mL, 0.95 mg/mL, respectively). Then a series of dilute working solutions were prepared for drawing calibration curve and for method validation. As for codonopyrrolidium B and codonopyrrolidium A, the chlorides were used.

2.2.7 Method validation

Linearity, LOD and LOQ The standard working solutions at six different concentrations were injected into HPLC system under the optimized chromatographic conditions. UV absorptions of the peak of seven compounds were detected at 215 nm. The calibration curves were constructed by plotting the peak area (y) versus the concentration of each compound (x). The standard working solution of the lowest concentration was diluted with methanol to yield a series of concentration for determination of the detection limit (LOD) and the quantitation limit (LOQ), at a signal to noise ratio (s/n) of 3:1 and 10:1, respectively. The noise of baseline was evaluated by three replicate injection of 20 μL of methanol (blank).

Precision and stability Intraday and interday precisions were evaluated by replicate

injection of a mixture solution containing seven standards. Five injections per day were conducted for three days after preparation. Intraday and interday precisions of sample solution of a commercial Codonopsis Radix was tested in the same way.

Accuracy The recovery test was performed to evaluate the accuracy of the established method. Three different amounts of seven standards (approximately 50%, 100% and 150% of original amount, three replicates each) were added to the weighted sample powder of Codonopsis Radix and then extraction procedure and analysis were performed by the proposed method.

2.3 Results

2.3.1 Optimization of extraction procedure

Selection of a proper solvent for efficient extraction is crucial in quantitative analysis. Song et al. (2008c) investigated different kinds of solvents, including water-methanol (30:70-0:100), methanol-0.1% hydrochloric acid (1:1, 2:1, 1:2) and methanol-chloroform (1:1), and found that methanol was the suitable solvent for quantitative analysis of the lobetyolin in Codonopsis Radix. Methanol was also commonly used as solvent for sample preparation in quantitative and fingerprint analyses of Codonopsis Radix (Qiao et al., 2007; Song et al., 2008d). In addition, Codonopsis Radix has a high content of sugar, which could be an obstacle to analyze the other components (Li et al., 2009). Therefore, methanol, ethanol and acetone but not the solvent composed of water were tested as solvents for extraction. The results (Fig. 2.3A) showed that the concentrations of seven target compounds in methanol extract, especially those of compounds **1-3**, were distinctly higher than in other two solvents,

indicating methanol was efficient and suitable as solvent for extraction. Furthermore, different extraction methods for quantitative analysis of Codonopsis Radix were investigated. Song et al. (2008c) compared four extraction methods, including ultrasonic, refluxing, soaking and Soxhlet extractions, and then selected Soxhlet extraction for sample preparation in quantitative analysis of the lobetyolin in Codonopsis Radix. In this study, three different methods were compared, including the ultrasonic extraction with 15 mL methanol for 20 min, refluxing extraction with 50 mL methanol for 2 h and Soxhlet extraction with 50 mL methanol for 3 h. In Soxhlet extraction, the content of compound **4** was little higher than that in ultrasonic extraction. However, the content of compound **3**, an important marker compound of Codonopsis Radix was lower in Soxhlet extraction than that in ultrasonic extraction. As for the contents of the other target compounds, no notable difference was found among the three different extraction methods (Fig. 2.3B). Therefore, ultrasonic extraction was selected for sample preparation because it was convenient and time saving. In addition, extraction period (10, 15, 20 min each time) was investigated. Unlike the period of 10 min indicating inadequate efficiency during extraction, no obvious difference between the period of 15 and 20 min was observed (Fig. 2.3C). Finally, the period of 20 min was employed for complete extraction.

2.3.2 Optimization of HPLC chromatographic conditions

In our preliminary experiment, three different kinds of columns [Agilent ZORBAX SB-C_{18} (4.6 mm i. d. × 250 mm, 5 μm), YMC-Pack Pro-C_{18} (4.6 mm i. d. × 250 mm, 5 μm) and Inertsil ODS (4.6 mm i. d. × 250 mm, 5 μm)] were compared. A gradient of acetonitrile-water system showed good resolution of the seven analytes. Because the

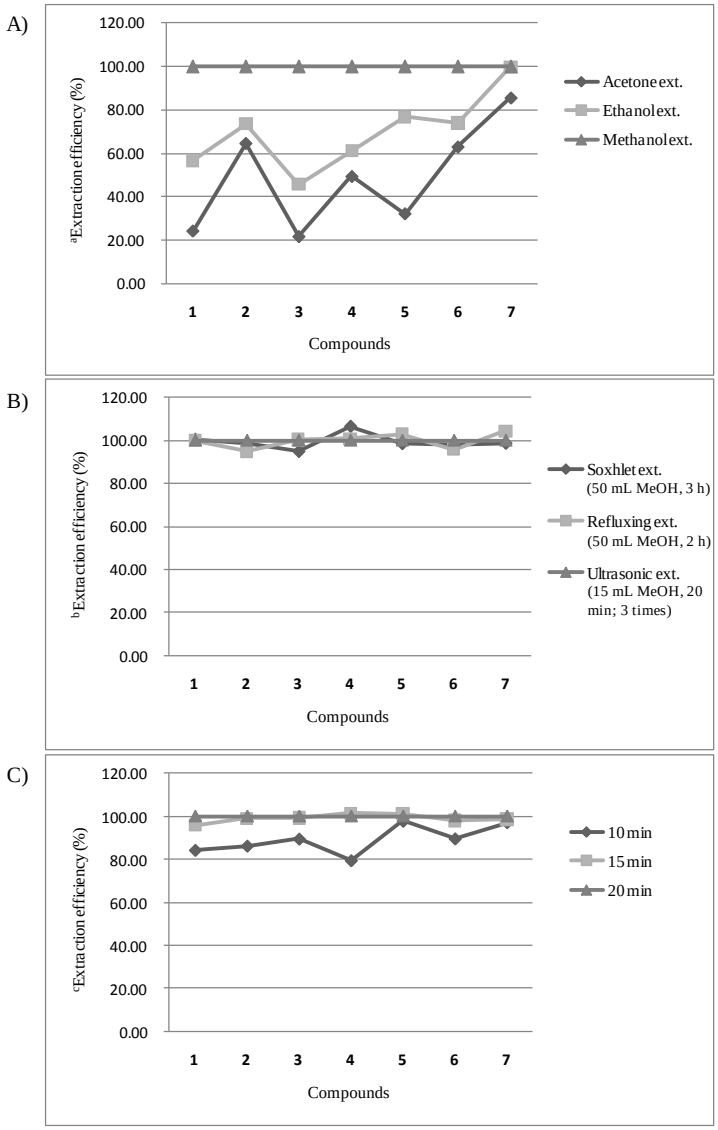


Fig. 2.3 Comparison of extraction efficiency for optimization of extraction procedure using a commercial sample of Codonopsis Radix (TMPW no. 26991)

A) Comparison of different solvents by ultrasonic extraction (20 min; 3 times) [[a] Extraction efficiency (%) = the peak area of compound in respective solvent extract/the peak area of compound in methanol extract ×100]; B) Comparison of different extraction methods with methanol [[b] Extraction efficiency (%) = the peak area of compound in extract by respective extraction method/the peak area of compound in extract by ultrasonic extraction ×100]; C) Comparison of different extraction periods with 15 mL methanol for three times [[c] Extraction efficiency (%) = the peak area of compound in extract with respective extraction period/ the peak area of compound in extract with 20 min extraction ×100]; compounds **1**-**7**: **1**, codonopyrrolidium B; **2**, codonopyrrolidium A; **3**, tangshenoside I; **4**, cordifolioidyne B; **5**, lobetyolinin; **6**, lobetyolin; **7**, lobetyol.

presence of acid may improve the peak shape, especially for compounds **1**-**3**, two different kinds of acids, phosphoric acid and acetic acid, in concentration of 0.1 and 0.5% (v/v) were tested. Finally, the HPLC condition allowed efficient separation of the seven analytes was achieved on the YMC-Pack Pro-C_{18} column with a gradient elution using acetonitrile and 0.1% (v/v) phosphoric acid at 1.0 mL/min.

Currently, detection wavelength of 267 or 268 nm was widely used in quantitative analysis of lobetyolin in Codonopsis Radix. To select suitable wavelength for simultaneous quantitation of seven analytes with different skeletons, UV absorption of the analytes were checked online by diode array detector (DAD). As shown in Fig. 2.4, the maximum absorptions of compounds **1**, **2** and **3** were at 232, 230 and 220 nm, respectively. As for the four polyacetylene components, **5**, **6** and **7** had the specific UV spectra with palm-like shape between 225-300 nm; while, **4** with palm-like shape between 250-325 nm. Compared the HPLC chromatograms at three wavelengths (215, 230 and 267 nm), 215 nm was selected because at this wavelength, compounds **5**, **6** and **7** exhibited strongest signals, and other compounds, **1**, **2**, **3** and **4** also exhibited intensive signals (Fig. 2.4A-C).

2.3.3 Method validation

The linear ranges of compounds **1**-**7** were 1.59-317.50, 0.61-152.50, 1.23-490.00, 1.50-150.00, 0.88-175.00, 1.00-100.00 and 1.19-59.38 μg/mL, respectively, with a correlation coefficient (r) more than 0.9993 for each compound (Table 2.1), which showed good linearity between the compound concentration and the peak area in a wide range of concentrations. The LOD and LOQ were estimated to be 0.10-0.32 μg/mL and

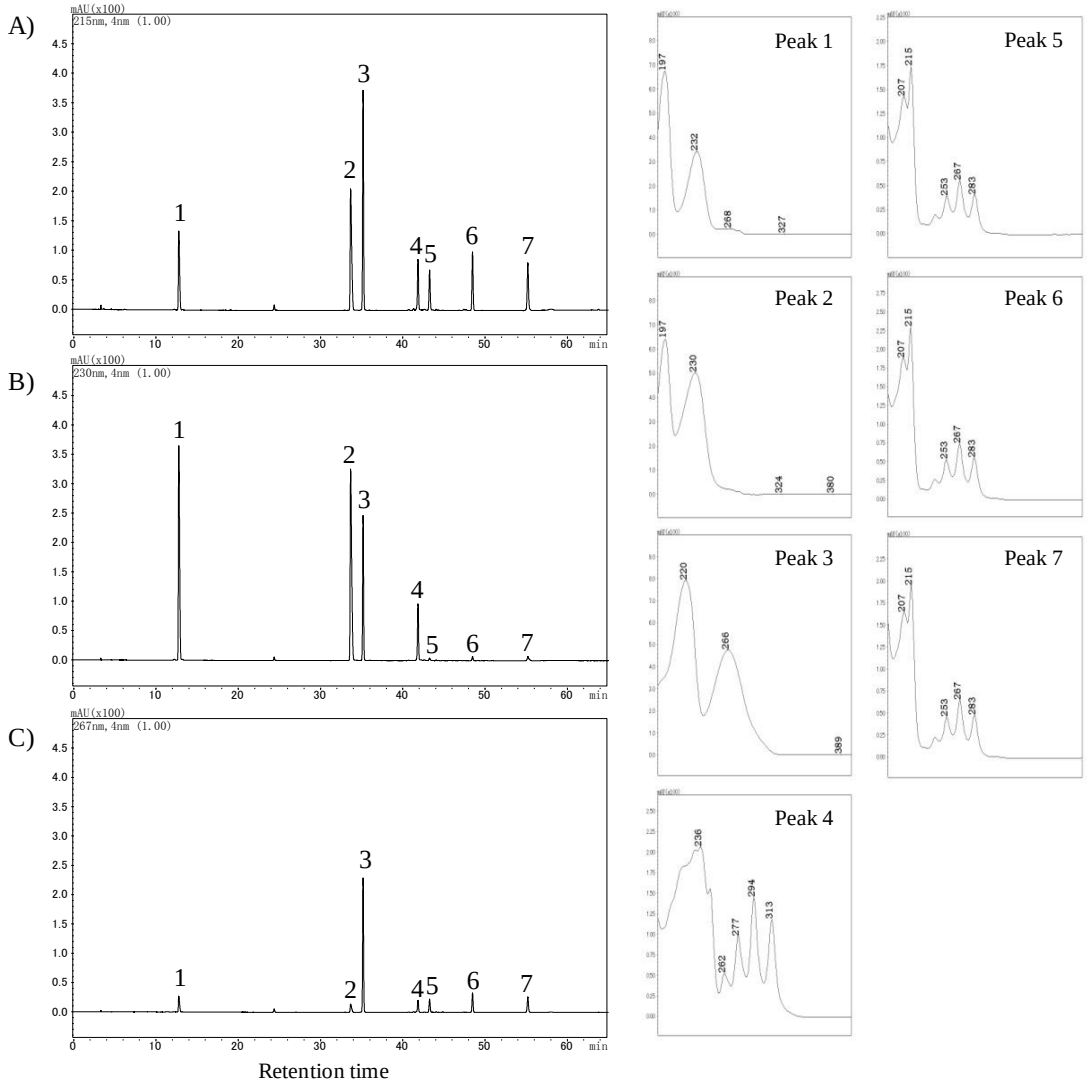


Fig. 2.4 HPLC chromatograms of a mixture of seven standard compounds (left panel) and UV spectrum of the respective compounds (right panel)

A) detected at 215 nm; B) detected at 230 nm; C) detected at 267 nm

Peaks 1-7 are as follows: **1**, codonopyrrolidium B; **2**, codonopyrrolidium A; **3**, tangshenoside I; **4**, cordifolioidyne B; **5**, lobetyolinin; **6**, lobetyolin; **7**, lobetyol.

0.35-1.07 μg/mL, respectively, indicating the method was adequately sensitive for detecting the target compounds (Table 2.1). The precisions of a mixture solution of seven standards were acceptable with RSD less than 2.33% for intraday and 2.84% for interday (Table 2.2). The intraday and interday precisions of a sample solution were 0.85-3.65% and 0.96-3.33%, respectively (Table 2.2). The results indicated that the standard and sample solutions were stable within three days at least, when stored at 4℃. The recoveries of added standards were obtained in the range of 95.8-104.7% with RSD of less than 3.22% (Table 2.3). The precisions and accuracies indicated that the developed method was highly reproducible.

Table 2.1 Linear regression, LOD and LOQ of seven compounds

Compound	Regression equation[a]	Correlation coefficient (r)	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Codonopyrrolidium B (**1**)	y=13707x+34940	0.9997	1.59-317.50	0.32	1.07
Codonopyrrolidium A (**2**)	y=36495x-20742	0.9999	0.61-152.50	0.15	0.51
Tangshenoside I (**3**)	y=17891x+51480	0.9995	1.23-490.00	0.25	0.82
Cordifolioidyne B (**4**)	y=61108x+58113	0.9997	1.50-150.00	0.15	0.50
Lobetyolinin (**5**)	y=43139x+71850	0.9993	0.88-175.00	0.18	0.59
Lobetyolin (**6**)	y=62243x+59441	0.9997	1.00-100.00	0.10	0.35
Lobetyol (**7**)	y=180912x-76713	0.9993	1.19-59.38	0.24	0.80

[a] x is the concentraction of each compound in μg/mL; y is the peak area of respective compound detected at 215 nm.

Table 2.2 Intraday and interday precisions of a mixture solution of seven standards and a sample solution

Compound	Precision			
	Standard mixture solution		Sample solution	
	Intraday (n=5)	Interday (n=15)	Intraday (n=5)	Interday (n=15)
Codonopyrrolidium B (**1**)	1.46	2.84	1.18	1.30
Codonopyrrolidium A (**2**)	0.73	2.13	3.24	0.97
Tangshenoside I (**3**)	1.11	1.78	1.11	1.01
Cordifolioidyne B (**4**)	1.80	2.00	2.90	1.57
Lobetyolinin (**5**)	1.95	1.52	3.65	3.33
Lobetyolin (**6**)	1.35	0.97	0.85	0.96
Lobetyol (**7**)	2.33	1.30	2.12	1.70

The sample solution was prepared from a commercial Codonopsis Radix (TMPW no. 26991).

Results were showed as RSD (%).

Table 2.3 Recoveries of seven compounds (n=3)

Compound	Original (μg)	Added (μg)	Determinated (μg)	Recovery (%)	RSD (%)
Codonopyrrolidium B (**1**)	471	230	693	96.6	0.55
		450	909	97.3	0.83
		800	1302	104.0	1.05
Codonopyrrolidium A (**2**)	469	200	666	98.3	2.44
		400	857	97.0	1.07
		600	1048	96.7	0.72
Tangshenoside I (**3**)	186	100	287	101.3	1.20
		180	361	97.1	2.52
		280	470	101.4	0.88
Cordifolioidyne B (**4**)	18	10	28	99.1	3.22
		18	36	99.2	2.09
		30	46	96.0	1.50
Lobetyolinin (**5**)	30	15	46	104.7	1.31
		30	59	95.8	0.83
		45	74	99.4	1.39
Lobetyolin (**6**)	195	95	290	100.2	1.73
		160	357	101.3	2.44
		300	472	97.6	3.18
Lobetyol (**7**)	22	10	32	99.7	1.53
		20	42	98.3	0.32
		30	50	96.4	1.07

Summary of Chapter II

1) Large scale methanol extraction of Codonopsis Radix (*C. tangshen*) followed by chromatographic separation and semipreparative HPLC, 13 compounds were isolated and 12 compounds were identified by comparing their spectral data with those reported in the literatures. Among them, 7 compounds, codonopyrrolidium B (**1**), codonopyrrolidium A (**2**), tangshenoside I (**3**), cordifolioidyne B (**4**), lobetyolinin (**5**), lobetyolin (**6**) and lobetyol (**7**) were selected as standards for quantitation.
2) Ultrasound-assisted methanol extracts of samples were analyzed using reversed phase HPLC on a YMC-Pack Pro-C_{18} column with a gradient eluent of acetonitrile and 0.1% (v/v) phosphoric acid and monitoring at 215 nm. The developed HPLC-UV method allowed efficient separation of the 7 compounds. All calibration curves showed good linearities ($r > 0.9993$) within the test ranges, and the detection and quantitation limits of the 7 compounds were 0.10-0.32 μg/mL and 0.35-1.07 μg/mL, respectively. Intraday and interday precisions were good with RSD less than 2.84%. The recoveries of all compounds ranged from 95.8 to 104.7%.
3) HPLC-UV is an efficient and accurate method of analysis for simultaneous quantitation of 7 components.

Chapter III

Quality evaluation of medicinally-used *Codonopsis* species and Codonopsis Radix based on the contents of pyrrolidine alkaloids, phenylpropanoid and polyacetylenes

3.1 Introduction

In chapter I, the specimens of three medicinally-used *Codonopsis* taxa widely collected from Gansu Prov., Hubei Prov. and Chongqing city of China and Codonopsis Radix purchased from various Asian markets have been clearly identified using sequences of internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA). In chapter II, a HPLC-UV method has been developed to detect codonopyrrolidium B, codonopyrrolidium A (pyrrolidine alkaloids), tangshenoside I (phenylpropanoid), cordifolioidyne B, lobetyolinin, lobetyolin and lobetyol (polyacetylenes) in three *Codonopsis* taxa. In this study, quantitative analysis of the seven target compounds in a series of identified specimens of three *Codonopsis* taxa and Codonopsis Radix was carried out using the well-established HPLC-UV method to elucidate the characteristic chemical composition of the three medicinally-used *Codonopsis* taxa.

3.2 **Materials and Methods**

3.2.1 Materials

Fifty-six specimens of the three medicinally-used *Codonopsis* taxa, *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen* were collected from Gansu Prov., Hubei Prov. and Chongqing city of China during our field investigation between 2008 and 2010 (Table 3.1). Fifty-four commercial samples of Codonopsis Radix were purchased from markets of mainland China, Hongkong, Korea and Japan (Table 3.2). Two analytes of each commercial sample were extracted and then used for quantitative analysis. All the samples were exactly identified on the basis of genetic analysis on ITS sequences. All vouchers were stored in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan (TMPW).

Table 3.1 The plant specimens of *Codonopsis* species used in quantitative analysis

Species	Code no.	Wild/ Cult.	Locality of collection	Cult. period [year(s)]	Date of collection	ITS sequence type[2]
C. pilosula	Cgs4	C	Gaolou Mountain, Wenxian, Longnan, Gansu, China	1	2009.7.30	P7
	Cgs7	C	Longxing, Wudu, Longnan, Gansu, China	2	2009.8.1	P3
	Cgs8	C	Longxing, Wudu, Longnan, Gansu, China	2	2009.8.1	P1
	GS36	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1914 m)	3	2010.7.25	P7
	GS44	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1909 m)	1 or 2	2010.7.25	P2
	GS50	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1905 m)	3	2010.7.25	P7
	GS52	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1905 m)	3	2010.7.25	P1
	GS62	C	Nanyang, Tanchang, Longnan, Gansu, China (Altitude: 1929 m)	1	2010.7.26	P3
	GS65	C	Nanyang, Tanchang, Longnan, Gansu, China (Altitude: 1929 m)	1	2010.7.26	P1
	GS141	C	Gaotai, Longxi, Dingxi, Gansu, China (Altitude: 2153 m)		2010.7.28	P1
	GS144	C	Gaotai, Longxi, Dingxi, Gansu, China (Altitude: 2153 m)		2010.7.28	P3
	CF1	W	Changyang, Yichang, Hubei, China		2008.9	P3
	ZS10	C	Erlongping, Caihua, Wufeng, Yichang, Hubei, China		2010.7.28	P7
Codonopsis sp.[1]	ZS04	C	Zhoujiaping, Niuzhuang, Wufeng, Hubei, China (Altitude: 1730 m)		2010.7.28	S0
C. pilosula	Cgs1	W	Huangtuping, Baoziba, Wenxian, Longnan, Gansu, China		2009.7.30	PM0
var. *modesta*	Cgs2	W	Huangtuping, Baoziba, Wenxian, Longnan, Gansu, China		2009.7.30	PM2
	Cgs3	W	Huangtuping, Baoziba, Wenxian, Longnan, Gansu, China		2009.7.30	PM1
	GS35	W	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1914 m)		2010.7.25	PM0'
	GS37	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1914 m)	3	2010.7.25	PM3
	GS38	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1914 m)	3	2010.7.25	PM0
	GS39	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1914 m)	3	2010.7.25	PM4
	GS45	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1909 m)	1 or 2	2010.7.25	PM0
	GS53	C	Gaojiashan, Wenxian, Longnan, Gansu, China (Altitude: 1905 m)	3	2010.7.25	PM0
C. tangshen	CF10	C	Shennongjia, Hubei, China		2008.8	T3
	CF13	W	Shennongjia, Hubei, China		2008.6	T1
	CF16	W	Shennongjia, Hubei, China		2008.8	T4
	ZS18	W	Honghe, Hongping, Shennongjia, Hubei, China (Altitude: 2000 m)		2010.7.31	T4
	ZS20	W	Honghe, Hongping, Shennongjia, Hubei, China (Altitude: 2000 m)		2010.7.31	T1
	ZS21	W	Honghe, Hongping, Shennongjia, Hubei, China (Altitude: 2000 m)		2010.7.31	T4
	ZS23	W	Honghe, Hongping, Shennongjia, Hubei, China (Altitude: 2000 m)		2010.7.31	T1
	ZS24	W	Honghe, Hongping, Shennongjia, Hubei, China (Altitude: 2000 m)		2010.7.31	T1
	ZS25	W	Honghe, Hongping, Shennongjia, Hubei, China (Altitude: 2000 m)		2010.7.31	T1
	CJZ47	C	Laoguashi, Enshi, Hubei, China		2009.7.23	T5
	CJZ48	C	Laoguashi, Enshi, Hubei, China		2009.7.23	T5
	CF5	C	Xingshan, Yichang, Hubei, China		2008.7	T4
	CF11	W	Xingshan, Yichang, Hubei, China		2008.7	T1
	CF12	W	Xingshan, Yichang, Hubei, China		2008.7	T1
	CJZ91	W	Liziping, Wufeng, Yichang, Hubei, China		2009.7.28	T3
	CJZ92	W	Liziping, Wufeng, Yichang, Hubei, China		2009.7.28	T3
	CJZ93	W	Liziping, Wufeng, Yichang, Hubei, China		2009.7.28	T3
	CJZ94	W	Liziping, Wufeng, Yichang, Hubei, China		2009.7.28	T1
	CJZ95	W	Liziping, Wufeng, Yichang, Hubei, China		2009.7.28	T4
	CJZ96	W	Liziping, Wufeng, Yichang, Hubei, China		2009.7.28	T4
	CJZ97	W	Liziping, Wufeng, Yichang, Hubei, China		2009.7.28	T5
	ZS01	C	Zhoujiaping, Wufeng, Yichang, Hubei, China (Altitude: 1730 m)		2010.7.28	T1
	ZS12	W	Hejialing, Liziping, Wufeng, Yichang, Hubei, China		2010.7.29	T3
	ZS15	W	Hejialing, Liziping, Wufeng, Yichang, Hubei, China		2010.7.29	T5
	ZS16	W	Hejialing, Liziping, Wufeng, Yichang, Hubei, China		2010.7.29	T3
	CJZ14	C	Xinshu, Huangying, Chongqing, China		2009.7.19	T3
	CJZ17	C	Xinshu, Huangying, Chongqing, China		2009.7.19	T3
	CJZ58	C	Jianshan, Wuxi, Chongqing, China (Altitude: 1700m)		2009.7.24	T2
	CJZ61	C	Jianshan, Wuxi, Chongqing, China (Altitude: 1700m)		2009.7.24	T3
	CJZ62	C	Jianshan, Wuxi, Chongqing, China (Altitude: 1700m)		2009.7.24	T5
	CJZ72	W	Hongchiba, Wenfeng, Wuxi, Chongqing, China		2009.7.25	T3
	CJZ73	W	Hongchiba, Wenfeng, Wuxi, Chongqing, China		2009.7.25	T3
	CJZ74	W	Hongchiba, Wenfeng, Wuxi, Chongqing, China		2009.7.25	T3

[1] The shape of flowers with half-inferior ovary is similar to that of *C. pilosula* , while that of leaves is similar to that of *C. tangshen*. This specimen was speculated to be *C. pilosula* based on its ITS sequence.

2 The sequence of respective ITS sequence type can be found in the chapter I.

Botanical origin[1]	Code no.	Drug name	TMPW no.[2]	ITS sequence type[3] Analyte a	Analyte b	Shape of root	Producing area	Purchased from	Date of collection
Mainland China's market									
P	1a	Dangshen	27025	P3	PM1	whole	Gansu, China	Hadapu, Tanchang, Longnan, Gansu	2010.7.26
	2a	Dangshen	27016	P3	PM1	whole	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26
	3b	Dangshen	27007	–	P0	whole	Wenxian, Gansu, China	Longnan Mingyue Herbal Drugs Company, Longnan, Gansu	2010.7.25
	4a, b	Baitiaodangshen	26659	P8	Q0	whole	Gansu, China	Qingping Crude Drug Market, Guangzhou, Guangdong	2009.8.18
	5a, b	Yedangshen	26660	P0	P3	whole	Gansu, China	Qingping Crude Drug Market, Guangzhou, Guangdong	2009.8.18
	6a, b	Huangdangshen	26662	P3	CC2	whole	Gansu, China	Qingping Crude Drug Market, Guangzhou, Guangdong	2009.11.19
	7b	Fengdang	26670	T4	P6	whole	Fengxian, Baoji, Shaanxi	Fengxian, Baoji, Shaanxi	2009.8.6
	8a	Dangshen	26538	P3	–	whole	Sichuan, China	Chongqing Crude Drug Market, Chongqing	2009.7.17
	9a, b	Dangshen	26626	P3	P6	whole	Shanxi, China	Wulin Parmacy, Hangzhou, Zhejiang	2009.8.7
	10	Dangshen	27056	P0	P1	cut pieces	Gansu, China	Xi'an Crude Drug Market, Shaanxi	2010.8.1
	11	Tiaodang	26674	P1	P1	cut pieces	Longxi, Gansu, China	Xi'an Huakang Crude Drug Store, Shaanxi	2009.7.7
	12	Dangshen	26598	P3	P2	cut pieces	Gansu, China	Shanghai Yiyao Huangshanhuashi Co., Ltd., Shanghai	2009.8.3
	13	Dangshen	26937	P10	P1	cut pieces		Hanzhong Pharmacy, Shaanxi	2010.7.22
	14	Dangshen	26716	P5	P0	cut pieces		Xianyan, Shaanxi	2009.12
	15	Dangshen	26723	P8	P3	cut pieces		Chongqing	2009.12
	16	Dangshen	26713	P0	P0	cut pieces		Datong, Shanxi	2009.12
	17	Dangshen	26714	P5	P3	cut pieces		Shanxi	2009.12
	18	Dangshen	26715	P5	P3	cut pieces		Taiyuan, Shanxi	2009.12
	19	Dangshen	26725	P0	P0	cut pieces		Qiannan, Guizhou	2009.12
	20	Dangshen	26717	P3	P1	cut pieces		Beiyang, Henan	2009.12
	21	Dangshen	26718	P0	P3	cut pieces		Wuhan, Hubei	2009.12
	22	Dangshen	26726	P5	P1	cut pieces		Nanning, Guangxi	2009.12
	23	Dangshen	26727	P3	P0	cut pieces		Nanning, Guangxi	2009.12
	24	Dangshen	26728	P7	P1	cut pieces		The People's Hospital of Guangxi, Nanning, Guangxi	2009.12
	25	Dangshen	26729	P5	CC3	cut pieces		Guangzhou, Guangdong	2009.12
	26	Dangshen	26720	P3	S0	cut pieces		Xuzhou, Jiangsu	2009.12
	27a	Tiaodang	26671	S0	–	whole	Minxian, Dingxi, Gansu, China	Minxian, Dingxi, Gansu	2009.8.6
PM	1b	Dangshen	27025	P3	PM1	whole	Gansu, China	Hadapu, Tanchang, Longnan, Gansu	2010.7.26
	2b	Dangshen	27016	P3	PM1	whole	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26
	28a	Dangshen	27017	PM1	–	whole	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26
	29b	Baitiaodang	27027	–	PM0	whole	Longxi, Gansu, China	Shouyang Crude Drug Market, Longxi, Gansu	2010.7.27
	30a	Wendangshen	26655	PM0	–	whole	Gansu, China	Baoziba, Wenxian, Longnan, Gansu	2009.7.30
	31	Dangshen	26666	PM0	PM0	cut pieces	Gansu, China	Qinghua Pharmacy, Nanchang, Jiangxi	2009.7.11
	32b	Wendangshen	26669	T1	PM1	whole	Gansu, China	Baoziba, Wenxian, Longnan, Gansu	2009.7.30
	33a	Wendang	27015	PM0	T1	whole	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26

Table 3.2 Crude drug samples of Codonopsis Radix used in quantitative analysis, summarized by their botanical origin (continued)

P&PM	34	Dangshen	27040	PM0	P8	cut pieces	Lixian, Gansu, China	Chuntian Pharmacy, Lixian, Gansu	2010.7.28
	35	Dangshen	26501	PM0	P5	cut pieces		Liying Chinese Medicinal Clinic, Changchun, Jilin	2009.5.2
	36	Dangshen	26724	PM0	P5	cut pieces		Kunming, Yunnan	2009.12
	37	Dangshen	26722	P8	PM1	cut pieces		Fuzhou, Fujian	2009.12
	38	Dangshen	27030	PM0	S0	cut pieces	Gansu, China	Longxi Zhongtian Pharmaceutical Co., Ltd. Gansu	2010.7.27
T	7a	Fengdang	26670	T4	P6	whole	Fengxian, Baoji, Shaanxi	Fengxian, Baoji, Shaanxi	2009.8.6
	32a	Wendangshen	26669	T1	PM1	whole	Gansu, China	Baoziba, Wenxian, Longnan, Gansu	2009.7.30
	33b	Wendang	27015	PM0	T1	whole	Nanyang, Longnan, Gansu, China	Nanyang, Tanchang, Longnan, Gansu	2010.7.26
	39a, b	Yedangshen	27168	T1	T3	whole	Shennongjia, Hubei, China	Shennongjia Juneng, Pharmaceutical Co., Ltd., Hubei	2011. 2.1
	40a	Dangshen	26560	T5	−	whole	Enshi, Hubei, China	Enshi Fenglan Banqiaodangshen Co., Ltd., Hubei	2009.7.22
	41a	Dangshen	26542	T2	−	whole	Chongqing, China	Huangshui, Shizhu, Chongqing	2009.7.19
T&P	42	Dangshen	26589	T4	P7	cut pieces	Yichang, Hubei, China	Wantan Crude Drug Store, Wufeng, Yichang, Hubei	2009.7.29
Hongkong's market									
P	43b	Wendangshen	26820	−	HC1	whole	Gansu, China	Hongkong Liyuanfeng Trading Co. (1st grade)	2010.8.11
	44a	Wendangshen	26821	HC1	−	whole	Gansu, China	Hongkong Liyuanfeng Trading Co. (2nd grade)	2010.8.11
	45a	Dangshenwang	26853	P0	−	whole	Minxian, Gansu, China	Gansu Minxian Tianrong Indigenous Products Co., Ltd.	2010.8.12
	46	Dangshenpian	26850	P3	P3	cut pieces		Hongkong Longxi Zhongtian Pharmaceutical Co., Ltd.	2010.8.12
PM	47a, b	Dangshen	26827	PM0	PM0	whole		Hongkong Yongsheng Wholesale Co., Ltd.	2010.8.11
	48b	Dangshen	26812	−	PM0	whole		Hongkong Runfengshenrong Co., Ltd.	2010.8.11
T	49a	Dangshen	26849	HC2	−	whole		Hongkong Gansu Longmai Medicinal Materials Co., Ltd.	2010.8.12
Korean market									
P	50	Man Sham	26928	P8	P1	cut pieces	China	Seoul	2010.9.28
Japanese market									
P	51b	Tojin	26864	PM0	P1	whole	Guizhou, China	Uchida Wakanyaku, Co., Ltd., Tokyo	2009.1
	52a	Tojin	26993	JC2	−	cut pieces	Gansu, China	National Institute of Health Sciences, Japan (To-HS-003)	2008.3
	53	Tojin	26992	JC1	P1	cut pieces	Guizhou, China	National Institute of Health Sciences, Japan (To-HS-002)	2007.5
PM	51a	Tojin	26864	PM0	P1	whole	Guizhou, China	Uchida Wakanyaku, Co., Ltd., Tokyo	2009.1
T	54	Tojin	26991	T0	T2	cut pieces	Henan, China	National Institute of Health Sciences, Japan (To-HS-001)	2006.4

[1] Botanical origin: P: *Codonopsis pilosula* , PM: *C. pilosula* var. *modesta* , T: *C. tangshen*

[2] The registation number in Museum of Material Medica, Institute of Natural Medicine, University of Toyama (TMPW).

[3] As a result of the sequence comparison, the samples with ITS sequence types S0, Q0, CC2, CC3, HC1, JC1 and JC2 were supposed to be *C. pilosula* , and that with type HC2 was inferred to be *C. tangshen* .

Two analytes (a, b) in each sample were analyzed genetically for identification. When the two were derived from the whole roots of different taxa, they are indicated repeatly in different lines corresponding to their botanical origin; where the other analyte with different botanical origin is shaded. Quantitative analysis was performed on individual whole roots (a, b) or on the mixture of cut pieces.

3.2.2 Standard compounds and reagents

All of the tested compounds, codonopyrrolidium B (**1**), codonopyrrolidium A (**2**), tangshenoside I (**3**), cordifolioidyne B (**4**), lobetyolinin (**5**), lobetyolin (**6**) and lobetyol (**7**), were isolated from the commercial Codonopsis Radix (TMPW No. 26991) and were exactly identified by comparison of the spectral data (MS, ^{1}H- and ^{13}C-NMR, etc.) with those reported in previous literatures (described in chapter II). The purity of each compound was confirmed to be higher than 97% by HPLC. HPLC grade acetonitrile, ultrapure water and analytical grade methanol and phosphoric acid were purchased from Wako Pure Chemical Industries, Ltd., Japan.

3.2.3 Apparatus and analytical conditions

The HPLC system (Shimadzu Co., Japan) comprised a LC-10AD pump, a DGU-20A degasser, a SIL-10AD auto-injector, a CTO-10-AS column oven and a SPD-M10A diode array detector. Quantitative analysis was carried out using a YMC-Pack Pro-C_{18} column (4.6 mm × 250 mm, 5 μm, YMC Co., Ltd., Japan) with column temperature at 30℃. The mobile phase was a binary eluent of acetonitrile (A) and 0.1% (v/v) phosphoric acid (B) with gradient conditions as follows: 0-10 min, 98-92% B; 10-35 min, 92-80% B; 35-50 min, 80-70% B; 50-60 min, 70-50% B; 60-65 min, 50-10% B. Flow rate was 1.0 mL/min and detection wavelength was 215 nm.

3.2.4 Sample preparation

The whole root of each plant specimen was pulverized. For commercial samples, when the sample was composed of whole root, two individuals used for molecular analysis were pulverized separately; when the sample was composed of cut pieces, it

was pulverized as a mixture. After sieving through 300 μm mesh, 1.0 g fine powder was accurately weighted and extracted with 15 mL methanol by ultrasonic for 20 min. After centrifugation at 3000 rpm for 5 min, the supernatant was separated. The extraction was repeated for three times. The combined supernatant was evaporated under vacuum, and the residue dissolved with methanol was transferred into a 5 mL volumetric flask and made up to volume with methanol. After filtration through 0.20 μm Millipore filter unit, 20 μL of sample solution was injected into HPLC for analysis. As for the twenty samples (specimens CF10, CF11, CF16, CJZ14, CJZ17, CJZ72, CJZ73, CJZ74, CJZ91, CJZ93, CJZ94, CJZ95, CJZ96, ZS01, ZS18, ZS20, ZS21, ZS23 and ZS25, and crude drug samples TMPW no. 27168), the content of tangshenoside I was calculated by decreasing the injection volume to 10 μL because the content of tangshenoside I was too high to be within the linear range if injection of 20 μL.

3.2.5 Statistical analysis

One-way ANOVA followed by the Bonferroni test was used to analyze the difference in contents of the respective compounds among the three medicinally-used *Codonopsis* taxa; $p < 0.05$ was deemed a significant difference. The quantitative analysis data was further subjected to principal component analysis (PCA) to facilitate classification of three medicinally-used *Codonopsis* species. One-way ANOVA and PCA were performed using software IBM SPSS Statistics (version19.0).

3.3 Results

3.3.1 Quantitative analysis of seven compounds in the roots of three *Codonopsis* taxa

The roots of 56 specimens from three *Codonopsis* taxa widely collected from Gansu

Prov., Hubei Prov. and Chongqing city of China were quantitatively analyzed by using the developed HPLC-UV method (Chapter II). The typical HPLC chromatograms of the roots from each taxon with various ITS sequence types and from different collection places were shown in Fig. 3.1. The similarity in chemical profiles of *C. pilosula* and *C. pilosula* var. *modesta*, as well as the difference between these two and *C. tangshen*, were clearly observed. In addition, the wild *C. tangshen* specimens ZS23 and CJZ91 collected from Shennongjia and Wufeng of Hubei Prov., respectively, showed chromatograms with more complicated chemical constituents than those of other plant specimens.

The quantitative data of the respective compounds in each specimen are summarized in Fig. 3.2 and Table 3.3. The contents of two alkaloids, phenylpropanoid and four polyacetylenes varied considerably among the specimens, not only inter-species but also intra-species. The total content of seven target compounds in the roots of 56 specimens from three *Codonopsis* taxa varied from the lowest, 0.659 mg/g, in the root of *C. pilosula* (Code no. Cgs7) to the highest, 9.474 mg/g, in the root of *C. tangshen* (CJZ91), having even more than 10-fold differences. In *C. pilosula* and *C. pilosula* var. *modesta* specimens, the total content of seven compounds was low (0.659-4.437 mg/g), among which codonopyrrolidium B (**1**) was of relatively high content (0.393-2.685 mg/g) (Fig. 3.2). On the other hand, *C. tangshen* specimens had relatively higher total contents of the seven components than *C. pilosula* and *C. pilosula* var. *modesta* specimens. In particular, *C. tangshen* specimens had higher contents of tangshenoside I (**3**) and codonopyrrolidium A (**2**), which were several-fold higher than the contents of the two compounds in *C. pilosula* or *C. pilosula* var. *modesta* specimens. In addition, the wild *C. tangshen* specimens from Shennongjia and Wufeng of Hubei Prov. and Hongchiba,

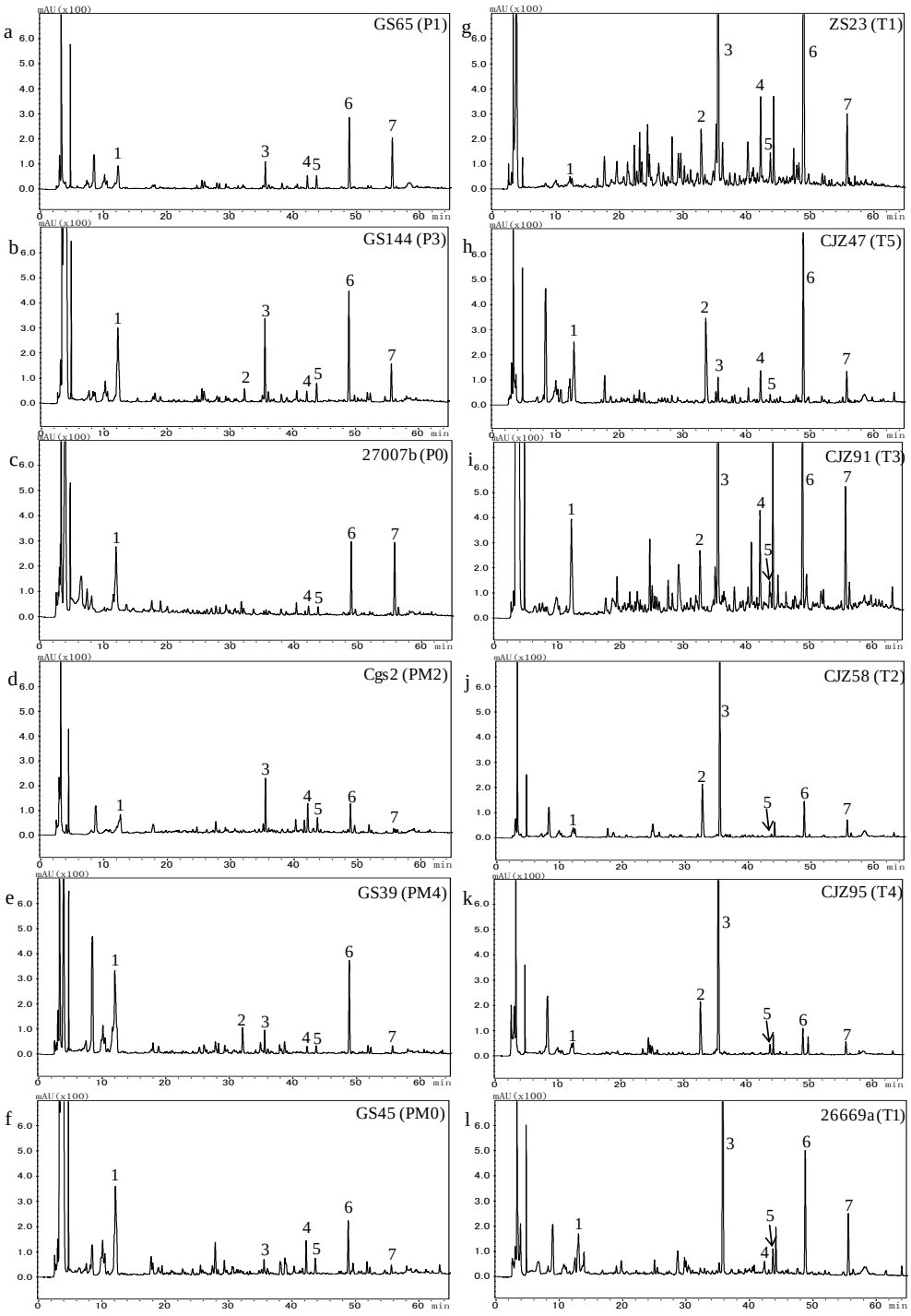


Fig. 3.1 HPLC chromatograms of the roots of *Codonopsis* specimens and commercial samples of Codonopsis Radix detected at 215 nm

Plant specimens (ITS sequence type): a: GS65 (P1), b: GS144 (P3), d: Cgs2 (PM2), e: GS39 (PM4), f: GS45 (PM0), g: ZS23 (T1), h: CJZ47 (T5), i: CJZ91 (T3), j: CJZ58 (T2), k: CJZ95 (T4); crude drug samples: c: TMPW No. 27007b (P0), l: TMPW No. 26669a (T1). Peak: **1**: codonopyrrolidium B, **2**: codonopyrrolidium A, **3**: tangshenoside I, **4**: cordifolioidyne B, **5**: lobetyolinin, **6**: lobetyolin, **7**: lobetyol.

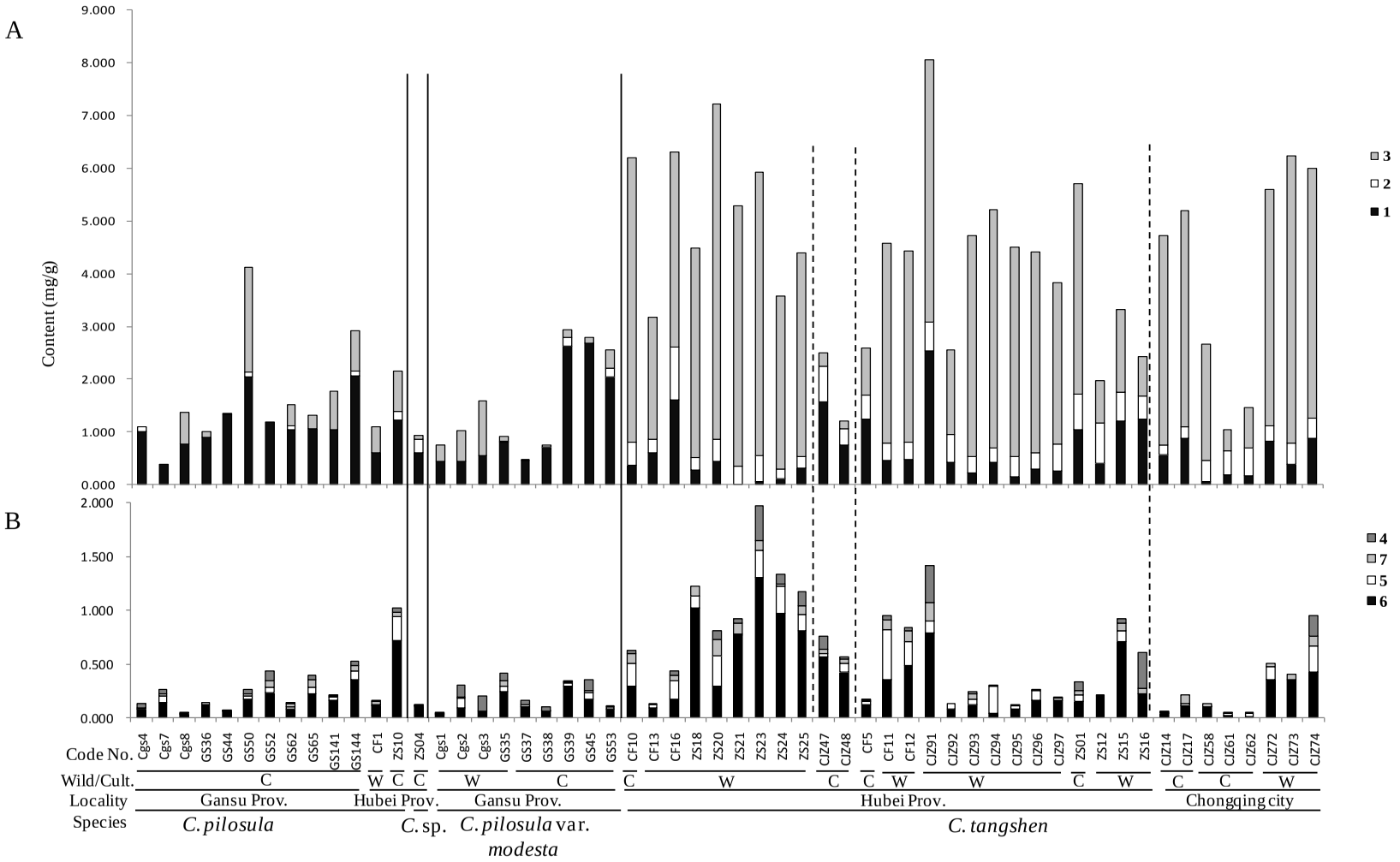


Fig. 3.2 Contents of the seven compounds in the roots of *Codonopsis* specimens

A: Contents of codonopyrrolidium B (**1**), codonopyrrolidium A (**2**) and tangshenoside I (**3**)

B: Contents of lobetyolin (**6**), lobetyolinin (**5**), lobetyol (**7**) and cordifolioidyne B (**4**)

Table 3.3 Contents of seven compounds in the roots of *Codonopsis* specimens

Species	Code No.	Content (mg/g)						
	Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**
C. pilosula	Cgs4	1.001	0.096	trace	0.036	0.019	0.076	trace
	Cgs7	0.393	N. D.	trace	0.045	0.057	0.144	0.020
	Cgs8	0.773	N. D.	0.602	N. D.	0.008	0.034	0.007
	GS36	0.904	N. D.	0.102	trace	trace	0.124	0.016
	GS44	1.325	0.033	trace	0.005	trace	0.058	0.005
	GS50	2.051	0.088	1.986	0.042	0.031	0.172	0.019
	GS52	1.182	trace	trace	0.090	0.048	0.232	0.066
	GS62	1.045	0.075	0.397	0.013	0.015	0.081	0.035
	GS65	1.061	N. D.	0.251	0.041	0.057	0.225	0.067
	GS141	1.034	N. D.	0.742	0.010	0.023	0.166	0.015
	GS144	2.063	0.089	0.762	0.037	0.086	0.351	0.049
	CF1	0.607	N. D.	0.489	0.013	0.011	0.113	0.023
	ZS10	1.217	0.097	0.773	0.034	0.215	0.720	0.049
Codonopsis sp.	ZS04	0.613	0.242	0.074	0.012	0.010	0.097	0.007
C. pilosula var. *modesta*	Cgs1	0.448	N. D.	0.306	0.012	trace	0.032	0.008
	Cgs2	0.450	N. D.	0.568	0.109	0.089	0.096	0.007
	Cgs3	0.546	N. D.	1.050	0.146	trace	0.058	trace
	GS35	0.796	0.036	0.077	0.077	0.051	0.246	0.044
	GS37	0.459	N. D.	0.019	0.038	trace	0.105	0.013
	GS38	0.713	trace	0.041	0.042	trace	0.058	0.004
	GS39	2.622	0.172	0.150	0.016	0.030	0.292	0.010
	GS45	2.685	N. D.	0.100	0.105	0.066	0.171	0.011
	GS53	2.042	0.163	0.351	0.012	N. D.	0.078	0.023
C. tangshen	CF10	0.377	0.420	5.396	0.033	0.209	0.292	0.094
	CF13	0.610	0.243	2.321	N. D.	0.030	0.089	0.008
	CF16	1.598	1.013	3.706	0.037	0.176	0.171	0.045
	ZS18	0.272	0.246	3.976	trace	0.112	1.018	0.096
	ZS20	0.439	0.414	6.372	0.083	0.285	0.288	0.154
	ZS21	trace	0.343	4.941	0.039	0.012	0.766	0.107
	ZS23	0.066	0.491	5.372	0.325	0.252	1.302	0.093
	ZS24	0.105	0.199	3.264	0.088	0.251	0.974	0.019
	ZS25	0.305	0.221	3.879	0.128	0.152	0.807	0.085
	CJZ47	1.565	0.688	0.246	0.121	0.029	0.568	0.040
	CJZ48	0.750	0.316	0.145	0.029	0.082	0.428	0.031
	CF5	1.248	0.449	0.902	0.007	0.030	0.120	0.009
	CF11	0.459	0.320	3.793	0.045	0.467	0.353	0.085
	CF12	0.478	0.320	3.626	0.032	0.220	0.490	0.100
	CJZ91	2.543	0.538	4.974	0.351	0.114	0.788	0.166
	CJZ92	0.424	0.522	1.604	trace	0.044	0.085	trace
	CJZ93	0.217	0.309	4.205	0.025	0.053	0.119	0.048
	CJZ94	0.425	0.264	4.531	trace	0.250	0.039	0.009
	CJZ95	0.156	0.379	3.979	trace	0.031	0.077	0.017
	CJZ96	0.299	0.309	3.806	trace	0.085	0.164	0.019
	CJZ97	0.258	0.518	3.058	trace	0.029	0.157	0.008
	ZS01	1.050	0.667	4.001	0.086	0.053	0.156	0.041
	ZS12	0.408	0.760	0.797	trace	trace	0.200	0.016
	ZS15	1.200	0.545	1.585	0.033	0.108	0.705	0.071
	ZS16	1.246	0.429	0.760	0.338	N. D.	0.219	0.053
	CJZ14	0.563	0.195	3.976	trace	0.014	0.041	0.006
	CJZ17	0.870	0.225	4.101	trace	0.018	0.110	0.087
	CJZ58	0.062	0.391	2.217	N. D.	0.004	0.101	0.021
	CJZ61	0.188	0.446	0.408	N. D.	0.016	0.024	0.010
	CJZ62	0.167	0.523	0.778	N. D.	0.029	0.008	0.007
	CJZ72	0.821	0.300	4.470	trace	0.121	0.357	0.030
	CJZ73	0.394	0.392	5.460	trace	trace	0.357	0.048
	CJZ74	0.871	0.396	4.730	0.193	0.249	0.420	0.087

Compound: 1: codonopyrrolidium B, 2: codonopyrrolidium A, 3: tangshenoside I, 4: cordifolioidyne B, 5: lobetyolinin, 6: lobetyolin, 7: lobetyol

trace: lower than the quantitation limits

N. D.: not detected

Wuxi of Chongqing city had markedly high content of **3** (0.760-6.372 mg/g), whereas the cultivated *C. tangshen* specimens from Laoguashi, Enshi of Hubei Prov. (CJZ47 and CJZ48) and Jianshan, Wuxi of Chongqing city (CJZ61 and CJZ62) had low content of **3**. The average contents of the respective components were compared between the three taxa (Table 3.4). There were significant differences in the contents of **1**, **2** and **3** in the two groups compared, *C. pilosula* vs. *C. tangshen* and *C. pilosula* var. *modesta* vs. *C. tangshen*. The content of **1** was relatively high in the roots of *C. pilosula* (1.127 mg/g) and *C. pilosula* var. *modesta* (1.196 mg/g); in contrast, the contents of **3** and **2** were significantly high in the roots of *C. tangshen* (3.254 and 0.418 mg/g, respectively).

Of the polyacetylene components analyzed, lobetyolin (**6**), which is frequently used as chemical marker for evaluation of Codonopsis Radix, was detected in all the specimens with the contents 0.034-0.720 mg/g in *C. pilosula*, 0.032-0.292 mg/g in *C. pilosula* var. *modesta* and 0.008-1.302 mg/g in *C. tangshen*. Lobetyol (**7**) was also widely present, but with a relatively low content. The contents of cordifolioidyne B (**4**) and lobetyolinin (**5**) were less than 0.351 and 0.467 mg/g, respectively, and could not even be detected in some specimens. Except for the difference in content of **7** found between *C. pilosula* var. *modesta* and *C. tangshen*, no significant difference was detected in the other polyacetylene components among the three *Codonopsis* species (Table 3.4).

Codonopsis sp., to which we could not give a correct scientific name morphologically, was supposed to be *C. pilosula* by comparison of ITS sequence (chapter I). *Codonopsis* sp. ZS04 (type S0 sequence) was consistent with *C. pilosula* in chemical composition, having **1** as its main component among the seven compounds.

As a whole, *C. pilosula* and *C. pilosula* var. *modesta* showed similar chemical

Table 3.4 Contents of seven compounds in the root of three *Codonopsis* Taxa

Compound	*C. pilosula* (n = 13)	*C. pilosula* var. *modesta* (n = 9)	*C. tangshen* (n = 33)
1	1.127 ± 0.483*	1.196 ± 0.964#	0.593 ± 0.544
2	0.042 ± 0.055**	0.041 ± 0.073##	0.418 ± 0.178
3	0.470 ± 0.551**	0.296 ± 0.334##	3.254 ± 1.751
4	0.028 ± 0.025	0.062 ± 0.049	0.060 ± 0.100
5	0.043 ± 0.058	0.027 ± 0.035	0.107 ± 0.112
6	0.192 ± 0.180	0.126 ± 0.091	0.358 ± 0.334
7	0.029 ± 0.023	0.013 ± 0.013#	0.052 ± 0.044

Compound: 1: codonopyrrolidium B, 2: codonopyrrolidium A, 3: tangshenoside I, 4: cordifolioidyne B, 5: lobetyolinin, 6: lobetyolin, 7: lobetyol

Data are shown as mean ± SD (mg/g).

Significant differences were found in *C. pilosula* vs. *C. tangshen* (* $p < 0.05$, ** $p < 0.01$) and in *C. pilosula* var. *modesta* vs. *C. tangshen* (# $p < 0.05$, ## $p < 0.01$).

composition, while *C. tangshen* differed considerably from these two species in chemical composition.

3.3.2 Quantitative analysis of seven compounds in commercial Codonopsis Radix

Fifty-four commercial samples of Codonopsis Radix purchased from markets of mainland China, Hongkong, Japan and Korea were analyzed quantitatively. Codonopsis Radix samples are generally composed of whole root or cut pieces. Two analytes of each sample were used for genetic identification by analysis of ITS sequences. It was found that many samples were mixtures, consisting of individuals with different ITS sequences. Therefore, when the sample was composed of whole root, the two individuals used for genetic analysis are quantitatively analyzed separately; when the sample was composed of cut pieces, it was quantitatively analyzed as a mixture. In the

former case, the quantitative results of the two analytes are shown separately based on the botanical origins; in the latter case, result of the mixture [e. g., T (*C. tangshen*) & P (*C. pilosula*)] is shown (Fig. 3.3).

The quantitative results are summarized in Fig. 3.3 and Table 3.5 according to the collection markets and the botanical origins of the analytes. The genetic analysis showed that most of the commercial samples were derived from *C. pilosula* or *C. pilosula* var. *modesta*, and only a few samples were derived from *C. tangshen*. The samples derived from *C. pilosula* and *C. pilosula* var. *modesta* showed relatively high contents of **1** (0.178-1.848 mg/g) among the seven components, consistent with the result obtained from the plant specimens. However, the contents of the four polyacetylenes varied considerably and **6** was not detected or was trace in several samples. On the other hand, the commercial samples derived from *C. tangshen* were available in limited regions (Table 3.2). The samples no. 39 and no. 40 produced in Hubei Prov. and no. 41 produced in Chongqing city had relatively high contents of **3** and/or **2**, which were the characteristic constituents in *C. tangshen.* Moreover, in the heterogenous samples, such as samples no. 32 and no. 33 produced in Gansu Prov., the analytes identified as *C. tangshen* also showed the characteristic chemical composition of *C. tangshen.* However, the sample no. 7a produced in Shaanxi Prov., which was identified as *C. tangshen*, showed low contents of **3** and **2**.

Among the commercial Codonopsis Radix, the sample no. 49 purchased from Hongkong's market and claimed to be produced in Gansu Prov. showed type HC2 of ITS sequence, which was quite similar to type T1 sequence of *C. tangshen*, but was not detected in the plant specimens. We supposed that it might be originated from *C. tangshen*, according to the ITS sequence (Chapter I). Chemical analysis in the present

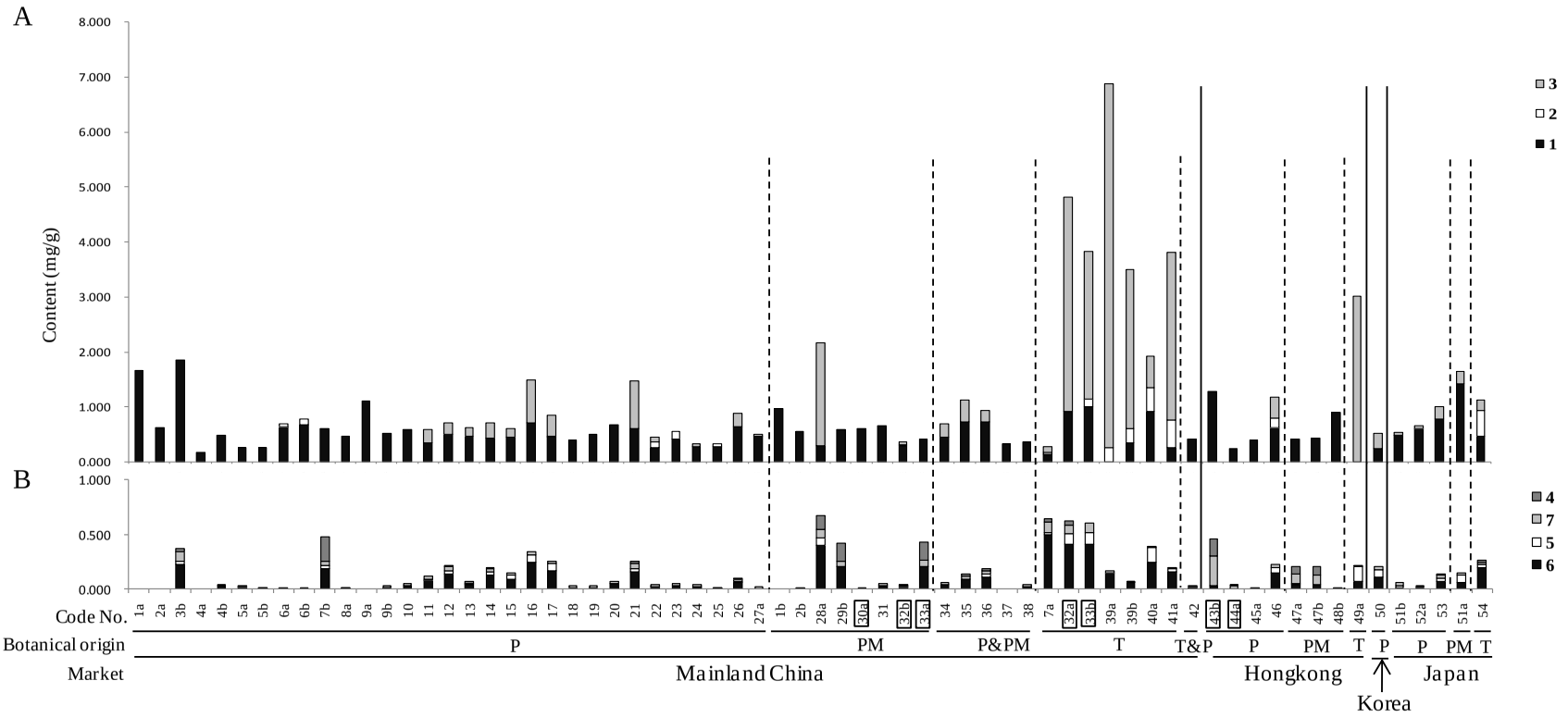


Fig. 3.3 Contents of seven compounds in the commercial samples of Codonopsis Radix

A: Contents of codonopyrrolidium B (**1**), codonopyrrolidium A (**2**) and tangshenoside I (**3**)

B: Contents of lobetyolin (**6**), lobetyolinin (**5**), lobetyol (**7**) and cordifolioidyne B (**4**)

Botanical origin: P: *C. pilosula*, PM: *C. pilosula* var. *modesta*, T: *C. tangshen*.

Boxed numbers indicate the commercial samples labeled as "Wendangshen."

Table 3.5 Contents of seven compounds in the commercial samples of Codonopsis Radix

Botanical origin	Code No.	Compound	Content (mg/g) 1	2	3	4	5	6	7
Mainland China's market									
P	1a		1.671	trace	N. D.	trace	N. D.	trace	N. D.
	2a		0.628	N. D.	N. D.	N. D.	N. D.	N. D.	trace
	3b		1.848	N. D.	N. D.	0.026	0.028	0.226	0.089
	4a		0.178	N. D.	N. D.	N. D.	N. D.	N. D.	trace
	4b		0.487	N. D.	N. D.	trace	N. D.	0.037	0.007
	5a		0.261	N. D.	N. D.	N. D.	N. D.	0.024	0.006
	5b		0.267	trace	N. D.	N. D.	trace	0.006	0.007
	6a		0.641	0.051	N. D.	N. D.	N. D.	trace	0.015
	6b		0.683	0.099	N. D.	N. D.	trace	trace	0.005
	7b		0.607	N. D.	N. D.	0.217	0.026	0.191	0.044
	8a		0.460	trace	trace	trace	N. D.	0.006	0.012
	9a		1.109	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
	9b		0.524	N. D.	N. D.	0.018	trace	0.009	0.009
	10		0.582	N. D.	N. D.	trace	trace	0.034	0.022
	11		0.340	trace	0.253	trace	0.012	0.078	0.030
	12		0.509	trace	0.205	0.012	0.030	0.136	0.041
	13		0.471	N. D.	0.149	0.024	trace	0.041	0.008
	14		0.424	N. D.	0.287	0.014	0.035	0.128	0.022
	15		0.455	trace	0.154	trace	0.033	0.095	0.020
	16		0.714	N. D.	0.776	trace	0.068	0.247	0.025
	17		0.443	0.029	0.381	trace	0.070	0.166	0.023
	18		0.394	N. D.	trace	trace	trace	0.012	0.019
	19		0.500	trace	N. D.	0.006	trace	0.012	0.013
	20		0.669	N. D.	trace	N. D.	N. D.	0.054	0.019
	21		0.602	N. D.	0.877	0.019	0.037	0.155	0.043
	22		0.266	0.102	0.087	trace	0.003	0.023	0.019
	23		0.413	0.143	N. D.	trace	trace	0.029	0.020
	24		0.269	N. D.	0.059	trace	trace	0.025	0.018
	25		0.279	0.053	N. D.	trace	0.009	0.005	trace
	26		0.635	N. D.	0.243	0.007	0.011	0.064	0.017
	27a		0.471	N. D.	0.034	trace	N. D.	0.006	0.015
PM	1b		0.974	N. D.	N. D.	trace	N. D.	trace	trace
	2b		0.551	N. D.	N. D.	trace	trace	0.012	N. D.
	28a		0.293	trace	1.871	0.118	0.074	0.398	0.080
	29b		0.582	trace	trace	0.164	trace	0.207	0.055
	30a		0.614	N. D.	trace	trace	N. D.	0.013	N. D.
	31		0.653	N. D.	N. D.	N. D.	N. D.	0.034	0.023
	32b		0.551	N. D.	trace	N. D.	N. D.	0.012	N. D.
	33a		0.423	N. D.	trace	0.167	trace	0.210	0.052
P&PM	34		0.442	N. D.	0.246	trace	trace	0.040	0.024
	35		0.724	trace	0.402	0.018	trace	0.095	0.024
	36		0.732	N. D.	0.204	0.018	0.026	0.114	0.027
	37		0.326	trace	N. D.	N. D.	N. D.	N. D.	N. D.
	38		0.370	N. D.	N. D.	trace	trace	0.024	0.015
T	7a		0.143	0.027	0.106	0.023	0.022	0.495	0.102
	32a		0.915	N. D.	3.900	0.035	0.103	0.408	0.075
	33b		1.003	0.135	2.684	trace	0.109	0.411	0.088
	39a		N. D.	0.258	6.614	trace	trace	0.149	0.022
	39b		0.341	0.272	2.888	trace	0.002	0.059	0.011
	40a		0.917	0.431	0.566	N. D.	0.136	0.245	0.010
	41a		0.256	0.514	3.035	N. D.	0.029	0.156	0.008
T&P	42		0.423	trace	0.040	trace	trace	0.021	0.016

Table 3.5 Contents of seven compounds in the commercial samples of Codonopsis Radix (continued)

Hongkong's market								
P	43b	1.281	N. D.	N. D.	0.151	trace	0.037	0.271
	44a	0.246	N. D.	N. D.	0.002	N. D.	0.003	0.026
	45a	0.405	N. D.	N. D.	N. D.	N. D.	trace	0.005
	46	0.630	0.164	0.382	trace	0.043	0.152	0.030
PM	47a	0.410	N. D.	N. D.	0.070	0.007	0.044	0.085
	47b	0.429	N. D.	N. D.	0.079	trace	0.046	0.082
	48b	0.896	trace	N. D.	N. D.	N. D.	N. D.	0.005
T	49a	trace	N. D.	3.018	N. D.	0.135	0.076	0.010
Korean market								
P	50	0.234	N. D.	0.287	trace	0.070	0.107	0.027
Japanese market								
P	51b	0.486	N. D.	0.054	trace	0.013	0.016	0.030
	52a	0.603	N. D.	0.063	0.003	N. D.	0.010	0.011
	53	0.780	N. D.	0.233	0.006	0.037	0.069	0.023
PM	51a	1.416	trace	0.222	trace	0.060	0.067	0.021
T	54	0.471	0.469	0.193	0.018	0.030	0.195	0.022

Botanical origin: P: *C. pilosula*, PM: *C. pilosula* var. *modesta*, T: *C. tangshen*

Compound: 1: codonopyrrolidium B, 2: codonopyrrolidium A, 3: tangshenoside I, 4: cordifolioidyne B, 5: lobetyolinin, 6: lobetyolin, 7: lobetyol

trace: lower than the quantitation limits

N. D.: not detected

study indicated that this sample contained considerably high content of **3**, which is the characteristic of *C. tangshen*. In addition, the chemical compositions of the samples no. 43b and no. 44a which were supposed to be *C. pilosula* by their ITS sequence (type HC1), were consistent with that of *C. pilosula*. As for the samples no. 4b (type Q0), no. 6b (type CC2) and no. 25 (type CC3) collected in mainland China's market, as well as no. 52a (type JC2) and no. 53 (type JC1) collected in Japanese markets, which were inferred to be *C. pilosula* by the sequence comparison, the quantitative results also strongly supported the inference mentioned above.

3.3.3 Principal component analysis (PCA)

PCA, an unsupervised method of multivariate data analysis, was further carried out by using the quantitative data of the seven compounds in 56 specimens from the three *Codonopsis* taxa and 54 commercial samples of Codonopsis Radix. The score plots are shown in Fig. 3.4A, where the first and second principal components accounted for 63.0% of the total variance (PC1, 44.2%; PC2, 18.8%). The score plots clearly indicated that two main groups were classified; one group mainly included *C. pilosula*, *C. pilosula* var. *modesta* and the commercial samples derived from these two taxa; the other group was composed of *C. tangshen* and its derived commercial samples. Thus, they were tentatively indicated as P/PM-group and T-group (Fig. 3.4A). From the PCA loading plot shown in Fig. 3.4B, compounds **2**-**7** contributed much to the positive values of PC1, and compound **1** contributed much to the positive value of PC2.

Fifty-five and eight of 66 analytes of Codonopsis Radix were classified into the P/PM-group and T-group, respectively. The results of the PCA score plots were almost in accordance with the results of analysis of ITS sequences. However, several individuals were excluded from the two groups. For example, plant specimens CJZ91, ZS16 and ZS23 were far away from the T-group, because the former two had relatively high contents of **1** and **4**, and the latter one had relatively high contents of **4** and **6.** Plant specimens CJZ47, CF5 and commercial sample no. 7a, having low content of **3**, were located at the mid-point of these two groups. On the other hand, specimen ZS10 and sample no. 28a containing considerably high contents of **6** and **3**, and sample no. 43b with relatively high contents of **1** and **7**, were located outside the P/PM-group.

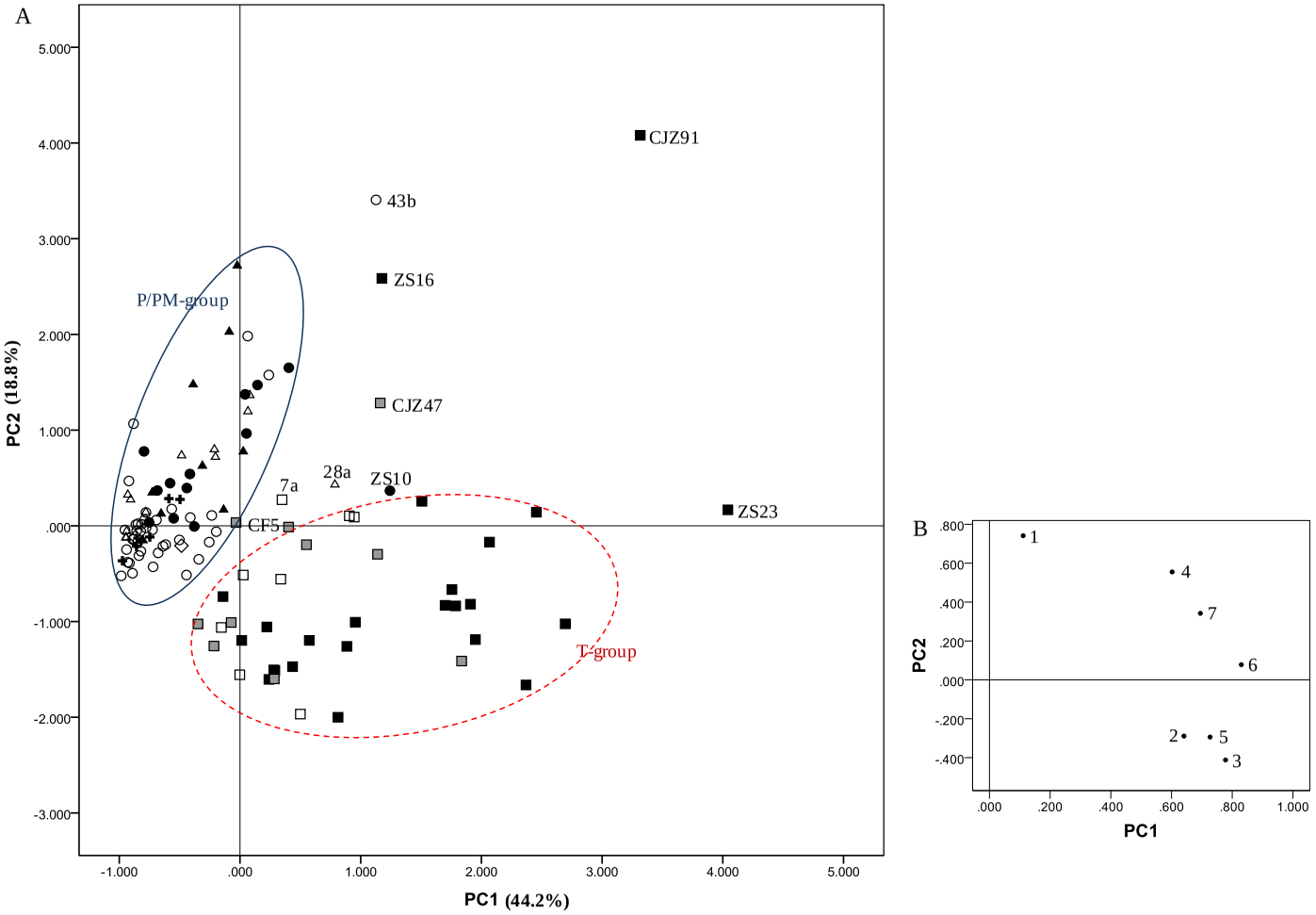


Fig. 3.4 Principal component analysis of chemical component data from 56 *Codonopsis* specimens and 54 Codonopsis Radix samples
A: Score plots, B: Loading plots
Plant specimens: filled circle: *C. pilosula*, filled triangle: *C. pilosula* var. *modesta*, filled square: *C. tangshen* (Wild), gray filled square: *C. tangshen* (cultivated), open diamond: *Codonopsis* sp. (type S0). Crude drug samples were identified as the following taxa: open circle: *C. pilosula*, open triangle: *C. pilosula* var. *modesta*, open square: *C. tangshen*, filled plus: mixture of *C. pilosula* and *C. pilosula* var. *modesta*, times symbol: mixture of *C. tangshen* and *C. pilosula*. The numbers indicate the code nos. in Table 3.1 and 3.2.

3.4 Discussion

Of the seven compounds, lobetyolin (**6**) is usually used as a chemical marker to assess the quality of Codonopsis Radix; however, it is widely found not only in the three *Codonopsis* taxa used as Codonopsis Radix, but also in other *Codonopsis* species and other genera of the Campanulaceae family (Qiao et al., 2007). Song et al. (2008c) reported that the content of **6** in *C. tangshen* was higher than that in *C. pilosula* and *C. pilosula* var. *modesta*. Our results also showed that the average content of **6** in *C. tangshen* was higher than that in *C. pilosula* and *C. pilosula* var. *modesta*, however, the difference was not statistically significant (Table 3.4). In addition, the amounts of polyacetylene compounds including lobetyolin in crude drugs were much less than that in plant specimens, which is due to such compounds are unstable and they might be degraded during storage under different conditions and periods.

Recently, Lin et al. (2013) compared the chemical constituents from saturated *n*-BuOH extracts of 9 commercial Codonopsis Radix and claimed that codonopyrrolidium B (**1**) and codonopyrrolidium A (**2**) existed only in *C. tangshen*-derived samples, which could be used to differentiate *C. tangshen* from *C. pilosula* and *C. pilosula* var. *modesta*. The results of the present study indicated that the presence of **1** and **2** was not limited to *C. tangshen*. The quantitative data from a number of specimens of the three medicinally-used *Codonopsis* taxa and commercial Codonopsis Radix indicated that **1** was the main constituent in the roots of *C. pilosula* and *C. pilosula* var. *modesta*, while tangshenoside I (**3**) and **2** were the characteristic constituents in the roots of *C. tangshen*, which was confirmed by the results of ANOVA and PCA. Therefore, compositions of these three components could serve as chemical markers to differentiate *C. pilosula* and *C. pilosula* var. *modesta* from *C. tangshen*.

However, the cultivated *C. tangshen* specimens, such as CJZ47, CJZ48, CF5, CJZ61 and CJZ62, tended to contain less **3** than the wild *C. tangshen*.

Codonopsis Radix has been graded according to production areas and sizes, which are related to their botanical origin and growth period, respectively (Xu and Xu, 1994). "Wendangshen" as a brand commodity produced in Wenxian County, Gansu Prov., was thought to be derived from *C. pilosula* var. *modesta* and with superior quality (Namba et al., 1992a, b). However, our previous genetic analysis revealed that the botanical sources of commercial "Wendangshen" were not limited to *C. pilosula* var. *modesta*. In this study, five commercial samples named "Wendangshen" or "Wendang" (sample nos. 30a, 32a, 32b, 33a, 33b, 43b and 44a in Fig. 3.3 and Table 3.5) which were purchased from markets of Gansu Prov. and Hongkong, had large variation in total content of the seven compounds. Samples no. 32a and no. 33b, which had type T1 of ITS sequence and were identified as *C. tangshen*, had obviously higher total contents of seven compounds than other samples and with relatively high contents of **3** and **1**, whereas the other samples were characterized by having **1** as the main component, and with no or very low content of **3** and **2**, which were consistent with the characteristics of *C. pilosula* var. *modest* and *C. pilosula*. These results suggested that genetic background (different species) might be the key factor in the formation of chemical composition, having much more effect than the environmental factors.

In addition, the samples no. 43b and no. 44a purchased from the same drug store in Hongkong were marked as first and second grade, respectively, and the roots of sample no. 43b were thicker than those of sample no. 44a. Conventionally, the former was thought to be of superior quality than the latter. Chemical analysis also showed that the contents of the respective compounds in the former were markedly higher than those in

the latter, indicating that the thicker roots (first grade) were of superior quality. The thickness of the roots is generally related to the growth period and environment. During our survey in Gansu Prov., the specimens of *C. pilosula* and *C. pilosula* var. *modesta* with different growth years were collected. As for the cultivated specimens, the contents of chemical constituents in the three-year-growth specimens (GS36, GS50 and GS52 of *C. pilosula*; GS37, GS38, GS39 and GS53 of *C. pilosula* var. *modesta* shown in Table 3.1 and Fig. 3.2) were not obviously higher than those in the one- or two-year-growth specimens (GS44 of *C. pilosula*; GS45 of *C. pilosula* var. *modesta*). Song et al. (2008d) analyzed HPLC fingerprints of Codonopsis Radix from different cultivation regions, and theorized that differences in chemical composition might be attributed to cultivation environment. To investigate the effect of environment and growth period on the formation of chemical compositions, further studies including carefully-designed cultivation experiments are needed.

So far, there are few reports on the pharmacological activities of the pure components from Codonopsis Radix; further studies are desired. From the literatures, radicamines A and B from *Lobelia chinensis* Lour., having similar structure to codonopyrrolidiums A and B, has been reported to be α-glucosidase inhibitor (Shibano et al., 2001). Tangshenoside I is an ester of 3-O-β-D-glucopyranosyl-3-methyl glutaric acid and syringin (eleutheroside B) which has been reported to have anti-oxidant, anti-fatigue, hypoglycemic effects, protective effects against Aβ(25-35)-induced atrophies of axons and dendrites (Takasugi et al., 1985; Lee et al., 2004; Niu et al., 2008; Bai et al., 2011). Such reports may shed light on potential bioactivities of codonopyrrolidiums A and B, and tangshenoside I. This study provided fundamental information which is useful for further pharmacological studies and for standardization and efficient use of Codonopsis

Radix.

Summary of chapter III

A comparative study of 56 specimens of three medicinally-used *Codonopsis* taxa collected from China and 54 commercial samples of Codonopsis Radix available in Chinese, Japanese and Korean markets was carried out by quantitative analysis of seven major components: codonopyrrolidium B (**1**), codonopyrrolidium A (**2**), tangshenoside I (**3**), cordifolioidyne B (**4**), lobetyolinin (**5**), lobetyolin (**6**) and lobetyol (**7**).

1) The quantitative results, based on a well-established HPLC–UV method, indicated that the contents of these seven compounds varied considerably among the samples, not only inter-species but also intra-species. Compound **1** was the main constituent in the roots of *C. pilosula* and *C. pilosula* var. *modesta*, while **3** and **2** had relatively high contents in the roots of *C. tangshen*.
2) The crude drug samples showed characteristic chemical composition similar to their botanical sources. However, the contents of the four polyacetylenes varied considerably and **6** was not detected or was trace in several samples.
3) The results of PCA indicated that two main groups were classified; one group mainly included *C. pilosula*, *C. pilosula* var. *modesta* and the commercial samples derived from these two taxa, while the other group was composed of *C. tangshen* and its derived commercial samples.
4) The composition of **3**, **2** and **1** could be used as chemical markers to differentiate *C. tangshen* from *C. pilosula* and *C. pilosula* var. *modesta*.

Conclusion

The results of genetic analysis indicated that the ITS sequences were useful markers allowing identification of the three medicinally-used *Codonopsis* taxa, *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen*, and Codonopsis Radix. Significant genetic polymorphism in the ITS sequences of the three *Codonopsis* taxa might be induced by a wide range of hybridization among the pure lines, and from their sequences the lineages involved in hybridization could be further inferred. By mainly focusing on the nucleotides at position 122nd, 135th, 226th, 441st, 489th and 500th, almost of crude drugs could be identified.

The chemical profiles of the three *Codonopsis* taxa were elucidated by quantitation of the seven compounds. The quantitative analysis indicated the composition of tangshenoside I, codonopyrrolidium A and codonopyrrolidium B could be applied as chemical markers to differentiate *C. tangshen* from *C. pilosula* and *C. pilosula* var. *modesta*. Characteristic chemical compositions of the two species (*C. pilosula*/*C. pilosula* var. *modesta* and *C. tangshen*) suggested that the botanical origin might be the most important factor affecting the formation of chemical profile, which may result from expressional differences in biosynthetic pathways of such chemical constituents in each species. However, within each species the chemical composition was quite variable in the samples with same genetic type. The results of genetic analysis suggested high frequence of hybridization among the three *Codonopsis* taxa, especially in the cultivation areas. Occurrence of such wide range hybridization must be a factor causing variety of chemical composition. Moreover, many factors, such as growth environment, growth periods, harvesting season, processing method, storage condition and period

might cause variation in chemical compositions of the various samples.

This study assessed the quality of the three medicinally-used *Codonopsis* taxa and crude drugs of Codonopsis Radix based on genetic and chemical analyses. Through genetic and chemical analyses on a number of *Codonopsis* specimens and crude drug samples, this study provided a detailed view on the current status of Codonopsis Radix, including its botanical origins, resource distribution, genetic polymorphism, chemical characteristic of each taxon, quality of crude drugs and market situation. This study also provided the fundamental information benefiting not only identification and standardization but also efficient use of Codonopsis Radix.

References

Bai Y, Tohda C, Zhu S, Hattori M, Komatsu K. 2011. Active components from Siberian ginseng (*Eleutherococcus senticosus*) for protection of amyloid β(25–35)-induced neuritic atrophy in cultured rat cortical neurons. *J Nat Med* **65**: 417-423.

Balasubramani SP, Murugan R, Ravikumar K, Venkatasubramanian P. 2010. Development of ITS sequence based molecular marker to distinguish, *Tribulus terrestris* L. (Zygophyllaceae) from its adulterants. *Fitoterapia* **81**: 503–508.

Balasubramani SP, Goraya GS, Venkatasubramanian P. 2011. Development of ITS sequence-based markers to distinguish *Berberis aristata* DC. from B. *lycium* Royle and B. *asiatica* Roxb. *3 Biotech* **1**: 11–19.

Chen L, Wang TZ. 2008. Content determination of lobetyolin in *Codonopsis* by HPLC. Journal of Military Surgeon in Southwest China **10**: 35-36.

Chinese Pharmacopoeia Commission. 2010. Pharmacopoeia of the People's Republic of China (2010 ed.) Vol I. China Medical Science Press, Beijing, China, pp 264.

Chinese Plant BOL Group. 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proc Nat Acad Sci USA* **108**: 19641–19646.

Cuendet M, Potterat O, Hostettmann K. 2001. Flavonoids and phenylpropanoid derivatives from *Campanula barbata*. *Phytochemistry* **56**: 631-636.

Editorial board of Flora of China, the Chinese Academy of Sciences (ed.). Flora of China, (1983) Vol.73 (2), Science Press, Beijing, China, pp 40-43.

Feng ZF, Chen XF, Di DL. 2012. Online extraction and isolation of highly polar

chemical constituents from *Brassica napus* L. pollen by high shear technique coupled with high-performance counter-current chromatography. *J Sep Sci* **35**: 625-632.

Guo HB, Lu BR, Wu QH, Chen JK, Zhou TS. 2007. Abundant genetic diversity in cultivated *Codonopsis pilosula* populations revealed by RAPD polymorphisms. *Genet Resour Crop Evol* **54**: 917-924.

Han GR, Wang CP, Su XG, He XF, Wang YD, Mizutani K, Tanaka O. 1990. Determination of tangshenoside I in *Codonopsis pilosula* Nannf. by TLC-UV spectrophotometric method. *Chin J Chin Mater Med* **15**: 553-557.

He Q, Zhu EY, Wang ZT, Chou GX, Xu LS, Hu ZB. 2006. Study on Chemical constituents of *Codonopsis pilosula*. *Chin Pharm J* **41**: 10-12.

He B, Zhang Y, Yuan X, Sun J, Wei G, Lin T. 2011. Protective effects of Radix Codonopsis on ischemia–reperfusion injury in rats after kidney transplantation. *Pediatr Surg Int* **27**: 1203-1212.

Hong DY. 2010. Taxonomic Notes on Chinese Campanulaceae. *Novon* **20**: 420-425.

Hong DY, Wu ZY, Raven PH. 2011. Flora of China, Vol.19, Science Press, Beijing, China, Missouri Botanical Garden Press, St. Louis, USA, pp 513-516.

Ishimaru K, Yonemitsu H, Shimomura K. 1991. Lobetyolin and lobetyol from hairy root culture of *Lobelia inflate*. *Phytochemistry* **30**: 2255-2257.

Ishimaru K, Sadoshima S, Neera S, Koyama K, Takahashi K, Shimomura K. 1992. A polyacetylene gentiobioside from hairy roots of *Lobelia inflata*. *Phytochemistry* **31**: 1577-1579.

Kitani Y, Zhu S, Batkhuu J, Sanchir C, Komatsu K. 2011. Genetic diversity of *Ephedra* plants in Mongolia inferred from internal transcribed spacer sequence of nuclear ribosomal DNA. *Biol Pharm Bull* **34**: 717-726.

Lee S, Son D, Ryu J, Lee YS, Jung SH, Kang J, Shin KH. 2004. Anti-oxidant activities of *Acanthopanax senticosus* stems and their lignan components. *Arch Pharm Res* **27**: 106-110.

Li CY, Xu HX, Han QB, Wu TS. 2009. Quality assessment of Radix Codonopsis by quantitative nuclear magnetic resonance. *J Chromatogr A* **1216**: 2124-2129.

Li Z, Zhu L, Zhan, H, Yan, J, Zhao J, Du D, Meng J, Yang F, Zhao Y, Sun, J. 2012. Protective effect of a polysaccharide from stem of *Codonopsis pilosula* against renal ischemia/reperfusion injury in rats. *Carbohydr Polym* **90**: 1739-1743.

Lin LC, Tsai TH, Kuo CL. 2013. Chemical constituents comparison of *Codonopsis tangshen*, *Codonopsis pilosula* var. *modesta* and *Codonopsis pilosula*. *Nat Prod Res* DOI:10.1080/14786419.2013.778849

Lin TC, Hsieh CC, Agrawal DC, Kuo CL, Chueh FS, Tsay HS. 2007. ITS sequence based phylogenetic relationship of Dangshen Radix. *J Food Drug Anal* **15**: 428-432.

Liu JH, Bao YM, Song JJ, An LJ. 2003. *Codonopsis pilosula* (Franch.) Nannf. total alkaloids potentiate neurite outgrowth induced by nerve growth factor in PC12 cells. *Acta Pharmacol Sin* **24**: 913-917.

Mei RQ, Lu Q, Hu YF, Liu HY, Bao FK, Zhang Y, Cheng YX. 2008. Three New Polyyne (=Polyacetylene) Glucosides from the Edible Roots of *Codonopsis cordifolioidea*. *Helv Chim Acta* **91**: 90-96.

Mizutani K, Yuda M, Tanaka O, Saruwatari Y, Jia MR, Ling YK, Pu XF. 1988. Tangshenosides I and II from Chuan-dangshen, the root of *Codonopsis tangshen* Oliv. *Chem Pharm Bull* **36**: 2726-2729.

Namba T, Komatsu K, Iwai M, Xu GJ. 1992a. Pharmacognostical studies on the *Codonopsis* plants (1). On the anatomical characteristics of the root of Sect.

Codonopsis plants. *Shoyakugaku Zasshi* **46**: 156-164.

Namba T, Komatsu K, Iwai M. 1992b. Pharmacognostical studies on the *Codonopsis* plants (2). On the botanical origin of the Chinese crude drug "Dangshen" and its related crude drugs derived from Sect. *Codonopsis* plants. *Shoyakugaku Zasshi* **46**: 165-173.

Nanjing University of Chinese Medicine. 2006. Dictionary of Chinese Traditional Medicine (Zhong Yao Da Ci Dian) (2nd edn.). Shanghai Scientific & Technical Publishers, Shanghai, China; 2578-2579.

Niu HS, Liu I M, Cheng JT, Lin CL, Hsu F L. 2008. Hypoglycemic effect of syringin from *Eleutherococcus senticosus* in streptozotocin-induced diabetic rats. *Planta Med* **74**: 109-113.

Ong ES, Len SM. 2003. Evaluation of surfactant-assisted pressurized hot water extraction for marker compounds in Radix Codonopsis pilosula using liquid chromatography and liquid chromatography/electrospray ionization mass spectrometry *J Sep Sci* **26**: 1533–1540.

Qiao CF, He ZD, Han QB, Xu HX, Jiang RW, Li SL, Zhang YB, But PPH, Shaw PC. 2007. The Use of Lobetyolin and HPLC-UV Fingerprints for Quality Assessment of Radix Codonopsis. *J Food Drug Anal* **15**: 258-264.

Sang T, Crawford DJ, Stuessy TF. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implications for biogeography and concerted evolution. *Proc Natl Acad Sci USA* **92**: 6813-6817.

Schiebel K, Hemleben V. 1989. Nucleotide sequence of the 18S-25S spacer region from rDNA of mung bean. *Nucleic Acids Res* **17**: 2852.

Scott KN. 1972. Carbon-13 nuclear magnetic resonance of biologically important aromatic acids. I. Chemical shifts of benzoic acid and derivatives. *J Am Chem Soc* **94**: 8564–8568.

Seto R, Nakamura H, Nanjo F, Hara Y. 1997. Preparation of epimers of tea catechins by heat treatment. *Biosci Biotechnol Biochem* **61**: 1434–1439.

Shibano M, Tsukamoto D, Masuda A, Tanaka Y, Kusano G. 2001. Two new pyrrolidine alkaloids, radicamines A and B, as inhibitors of α-glucosidase from *Lobelia chinensis* LOUR. *Biol Pharm Bull* **49**: 1362-1365.

Shimizu M, Zenko Y, Tanaka R, Matsuzawa T, Morita N. 1993. Studies on aldose reductase inhibitors from natural products.V. Active components of Hachimi-jio-gan (Kampo Medicine). *Chem Pharm Bull* **41**: 1469–1471.

Song D, Chou GX, Zhong GY, Wang ZT. 2008a. Two New Phenylpropanoid Derivatives from *Codonopsis tangshen* Oliv. *Helv Chim Acta* **91**: 1984-1988.

Song D, Wang ZT, Li LY, Zhong GY. 2008b. Protective effect of Lobetyolin on gastric mucosa of experimental gastric ulcer in rats. *J Emerg Tradit Chin Med* **17**: 963-964.

Song D, Cheng XM, Li LY, Zhong GY, Wang ZT. 2008c. Determination of lobetyolin in root of *Codonopsis tangshen* from various cultivation areas by high performance liquid chromatography. *Chin J Chin Mater Med* **33**: 2133-2135.

Song D, Cheng XM, Li LY, Zhong GY, Wang ZT. 2008d. Study on HPLC Fingerprint of *Codonopsis tangshen* Oliv. *Chin Pharm J* **43**: 1136-1139.

Sukrong S, Zhu S, Ruangrungsi N, Phadungcharoen T, Palanuvej C, Komatsu K. 2007. Molecular analysis of the genus *Mitragyna* existing in Thailand based on rDNA ITS sequences and its Application to identify a narcotic species: *Mitragyna speciosa*. *Biol Pharm Bull* **30**: 1284-1288.

Takaiwa F, Oono K, Iida Y, Sugiura M. 1985. Nucleotide sequence of the 17S-25S spacer region from rice rDNA. *Plant Mol Biology* **4**: 355-364.

Takasugi N, Moriguchi T, Fuwa T, Sanada S, Ida Y, Shoji J, Saito H. 1985. Effect of *Eleutherococcus senticosus* and its components on rectal temperature, body and grip tones, motor coordination, and exploratory and spontaneous movements in acute stressed mice. *Shoyakugaku Zasshi* **39**: 232-237.

Tsai TH, Lin LC. 2008. Phenolic Glycosides and Pyrrolidine Alkaloids from *Codonopsis tangshen*. *Chem Pharm Bull* **56**: 1546-1550.

Xin T, Zhang F, Jiang Q, Chen C, Huang D, Li Y, Shen W, Jin Y, Sui, G. 2012. The inhibitory effect of a polysaccharide from *Codonopsis pilosula* on tumor growth and metastasis in vitro. *Int J Biol Macromol* **51**: 788-793.

Xu GJ, Xu LS. 1994. Species systematization and quality evaluation of Chinese traditional drugs, Vol.1, Fujian Science and Technology Press, Fuzhou, China, pp 5-9.

Wakana D, Kawahara N, Goda Y. 2011. Three new triterpenyl esters, codonopilates A–C, isolated from *Codonopsis pilosula*. *J Nat Med* **65**: 18-23.

Wang JZ, Wang FP. 1996. Study on chemical constituents of *Codonopsis tangshen*. *Nat Prod Res Dev* **8**: 8-12.

Wang ZT, Ng TB, Yeung HW, Xu GJ. 1996. Immunomodulatory effect of a polysaccharide-enriched preparation of Codonopsis pilosula roots. *Gen Pharmacol* **27**: 1347-50.

Wen J, Zimmer EA. 1996. Phylogeny and biogeography of *Panax* L. (the ginseng genus, Araliaceae): inference from ITS sequences of nuclear ribosomal DNA. *Mol Phylogenet Evol* **6**: 166-177.

World Health Organization. 2000. WHO general guidelines for methodologies on

research and evaluation of traditional medicine. Available at: http://whqlibdoc.who.int/hq/2000/WHO_EDM_TRM_2000.1.pdf (Accessed 22 February 2010)

Zhao XH, Liu YQ, Wang RS, Guo JL, Zhao P. 2007. Analysis of lobetyolin in different parts of Codonopsis Radix using RP-HPLC. *Chinese Traditional Patent Medicine* **29**: 1046-1047.

Zhu EY, He Q, Wang ZT, Xu LS, Xu GJ. 2001. Chemical Study on the Root of *Codonopsis pilosula*. *J Chin Pharm Univ* **32**: 94-95.

List of Publications

1. **Jingyu HE**, Shu ZHU, Katsuko KOMATSU, Yukihiro GODA, Shaoqing CAI. Genetic polymorphism of medicinally-used *Codonopsis* species in an internal transcribed spacer sequence of nuclear ribosomal DNA and its application to authenticate Codonopsis Radix. *J Nat Med*, DOI 10.1007/s11418-013-0780-1, 2013
2. **Jingyu HE**, Shu ZHU, Yukihiro GODA, Shaoqing CAI, Katsuko KOMATSU. Quality evaluation of medicinally-used *Codonopsis* species and Codonopsis Radix based on the contents of pyrrolidine alkaloids, phenylpropanoid and polyacetylenes. *J Nat Med*, DOI 10.1007/s11418-013-0801-0, 2013
3. **Jingyu HE**, Shu ZHU, Katsuko KOMATSU. HPLC-UV analysis of polyacetylenes, phenylpropanoid and pyrrolidine alkaloids in medicinally-used *Codonopsis* species. *Phytochem Anal*, 2013 (accepted)

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