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学位論文題目 **Genetic and chemical polymorphism of medicinally-used  
*Codonopsis* species and its application to evaluate  
Codonopsis Radix  
(薬用 *Codonopsis* 属植物の遺伝的・成分的多様性と「党参」の  
品質評価への応用)**

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## 論文内容の要旨

Codonopsis Radix, called as “Dangshen” in Chinese and “Tojin” in Japanese, has been used in traditional Chinese medicine for replenishing deficiency of qi (vital energy) and blood, strengthening immune system, decreasing blood pressure and remedying poor gastrointestinal function. This crude drug has been in high demand in China, and recently the demand is increasing in Japan. It is prescribed as the roots of *Codonopsis pilosula* (Franch.) Nannf., *C. pilosula* var. *modesta* (Nannf.) L. D. Shen and *C. tangshen* Oliv. in Chinese Pharmacopoeia. Phytochemical researches revealed that Codonopsis Radix contained polyacetylenes, phenylpropanoids, alkaloids and triterpenoids, and pharmacological studies showed that lobetyolin, a polyacetylene component played a protective role in gastric mucosa injury, total alkaloids caused a significant enhancement of nerve growth factor-induced neurite outgrowth in PC12 cells, and total saponins had protective effect on ischemia-reperfusion injury in rats.

Most of the researches used the unidentified crude drug of Codonopsis Radix as experimental materials, because there is no developed method for exact identification. Moreover, the quality evaluation of Codonopsis Radix was limited to quantitation of lobetyolin and HPLC fingerprint, but not simultaneous evaluation of multiple bioactive components. Therefore, this study aims to clarify the genetic and phytochemical 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.

### **1. Genetic polymorphism of medicinally-used *Codonopsis* species in an internal transcribed spacer (ITS) sequence of nuclear ribosomal DNA**

The genetic analysis of ITS sequences which has been widely used for species-level phylogenetic studies was conducted on 96 specimens of three medicinally-used *Codonopsis* taxa collected widely from Gansu Prov., Chongqing city and Hubei Prov. of China, the main producing areas of Codonopsis Radix (Fig. 1). The length of ITS1, 5.8S rDNA and ITS2 regions were 257 bp, 163 bp and 235 bp, respectively, in all *Codonopsis* specimens. Significantly genetic polymorphism was observed, representing by 11 types of ITS sequences in *C. pilosula*, 5 types in *C. pilosula* var. *modesta* and 5 types in *C. tangshen* (Table 1). The informative sites for discriminating the three taxa were detected at the nucleotide positions 122nd, 226th, 441st and 489th from upstream of ITS sequence. For discrimination of the types of *C. tangshen*, the nucleotides at positions 135th, 489th and 500th were informative. The nucleotide additivity detected in ITS sequence indicated hybridization occurred in these species. Among the determined sequences, 1, 1 and 2 types were thought to be of pure lines of *C. pilosula*, *C. pilosula* var. *modesta* and *C. tangshen*, respectively, designated as types **P0**, **PM0**, **T1** and **T3**,

and the rest might be derived from hybridization. Moreover, three pure lines with types **S0**, **Q0** and **T0** sequences were also observed in *Codonopsis* sp., *Codonopsis Radix* sample produced in Gansu Prov. and that produced in Henan Prov., respectively. Hybrids were inferred to be resulted from the combination of the pure lines (Table 1). 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. Among the three taxa, *C. pilosula* had various hybrid lines which were growing widely in all of the cultivation areas of Gansu Prov. Based on the sequences of the 6 informative sites, botanical sources of *Codonopsis Radix* were identified: those produced in a wide range of southeast Gansu Prov. were *C. pilosula*, just those from Wenxian county of Gansu Prov. were *C. pilosula* var. *modesta*, and those produced in Chongqing city and Hubei Prov. were derived from *C. tangshen*.

## **2. Development of HPLC-UV method for analysis of polyacetylenes, phenylpropanoid and pyrrolidine alkaloids**

Large scale methanol extraction of *Codonopsis Radix* (*C. tangshen*) followed by chromatographic separation and semipreparative HPLC, 13 compounds were isolated and identified by comparing their spectral data (MS, <sup>1</sup>H NMR and <sup>13</sup>C NMR) 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.

Ultrasound-assisted methanol extracts of samples were analyzed using reversed phase HPLC on a YMC-Pack Pro-C<sub>18</sub> 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. Quality evaluation of medicinally-used *Codonopsis* species and *Codonopsis Radix* based on 7 components**

A comparative study on 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 7 components (**1-7**). The quantitative results indicated that the contents of these 7 compounds were considerably variable among the samples not only inter-species but also intra-species. *C. pilosula* and *C. pilosula* var. *modesta* showed similar chemical composition, while *C. tangshen* considerably differed from these two in chemical composition (Fig. 2). Codonopyrrolidium B (**1**) was the main component in the roots of *C. pilosula* and *C. pilosula* var. *modesta*, while tangshenoside I (**3**) and

codonopyrrolidium A (**2**) were with relatively high contents in the roots of *C. tangshen*. The crude drugs derived from *C. pilosula* and *C. pilosula* var. *modesta* showed relatively high content of **1** among the 7 components, consisting with the result obtained from the plant specimens. In addition, most of crude drugs derived from *C. tangshen* showed characteristic chemical composition as *C. tangshen*. The results of principal component analysis (PCA) indicated that two main groups were classified (Fig. 3); one group mainly included *C. pilosula*, *C. pilosula* var. *modesta* and the crude drugs derived from these two species; the other group was composed of *C. tangshen* and its derived crude drugs. Therefore, **3**, **2** and **1** could be the chemical markers to differentiate *C. tangshen* from *C. pilosula* and *C. pilosula* var. *modesta*. In addition, lobetyolin (**6**) was widely existed in the roots of the three *Codonopsis* taxa as well as *Codonopsis Radix*.

## Conclusion

This study assessed the quality of the three medicinally-used *Codonopsis* taxa and crude drugs of *Codonopsis Radix* based on genetic and chemical analyses. The results of genetic analysis indicated that the ITS sequences were useful markers allowing identification of the three taxa and authentication of *Codonopsis Radix*. Significant genetic polymorphism in the ITS sequences of the three medicinally-used *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. The chemical profiles of the three medicinally-used *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 to differentiate *C. tangshen* from *C. pilosula* and *C. pilosula* var. *modesta*. This study provided the fundamental information benefiting not only identification and standardization but also efficient use of *Codonopsis Radix*.

## References

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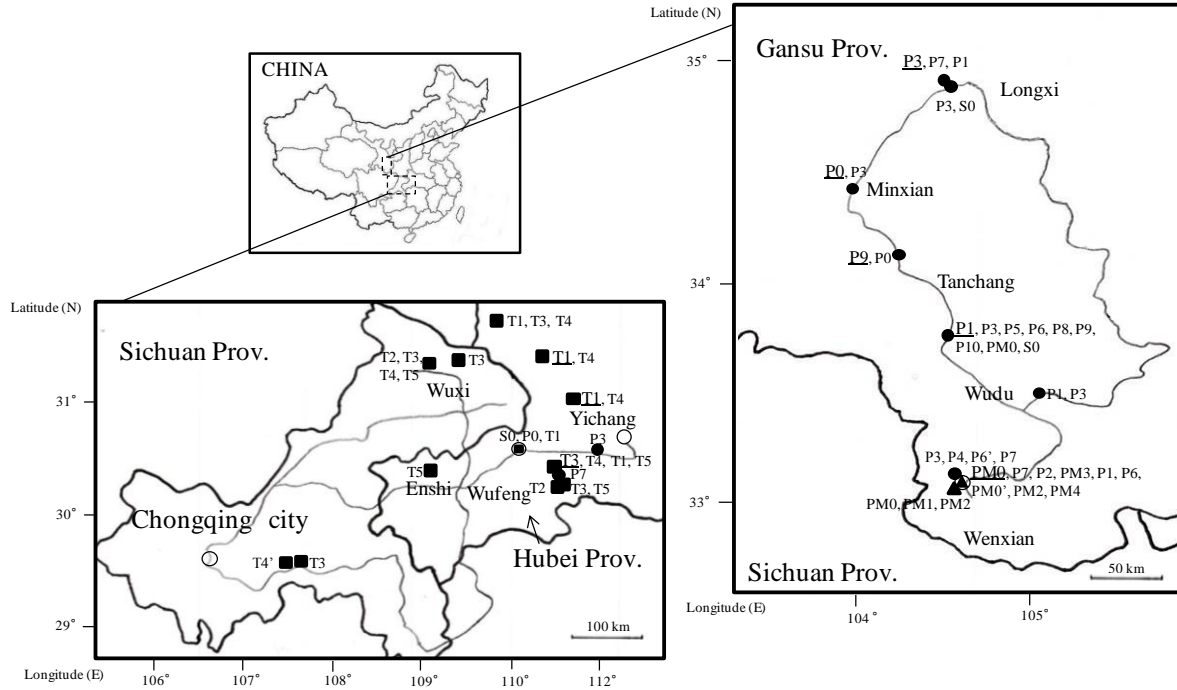


Fig. 1 Collection localities of *Codonopsis* specimens in China (2008 – 2010)

The marks in the collection localities indicate the collected species, together with ITS sequence types: ●: *C. pilosula*, ▲: *C. pilosula* var. *modesta*;

■: *C. tangshen*, ⊙: *C. pilosula* and *C. pilosula* var. *modesta*, ⊚: *C. pilosula* and *C. tangshen*.

The sequence type with underline indicates the most detected type in the respective locality.

Table 1 Types of ITS sequences of *Codonopsis* species and the assumed lineages related to hybrid

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			
<i>C. pilosula</i>	P0	AB769260	C	G	C	G	G	C	C	C	T	A	C	T	C	T	G	C	A	G	P0 (C C T T) × PM0 (T C T T)	5	
	P1	AB769261	*	*	Y	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	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	
<i>Codonopsis</i> sp.	S0	AB769272	*	*	T	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*		4	
<i>C. pilosula</i> var. <i>modesta</i>	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	
<i>C. tangshen</i>	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	*	*	*	*	*		7	
	T4'	AB769283	*	*	T	R	Y	*	*	*	*	*	*	*	C	*	*	*	*	*		2	
	T5	AB769284	*	*	T	R	*	*	*	*	*	*	*	Y	R	*	*	*	*		T0 (G T A) × T3 (A C G)	5	
<b>Crude drug samples</b> <sup>*1</sup>																							
Baitiaodangshen (No.26659b)	Q0		*	*	T	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*			
Tojin (No. 26991a)	T0		*	*	T	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*			

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

\*<sup>1</sup> 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



## 学位論文審査の要旨

漢薬「党参」は、薬用人参と同様に強壯補気薬として東アジアで汎用され、日本でもその需要が増加していることから、医薬品として『日本薬局方』に収載し、安全性と有効性を担保することが決まっている。しかし、「党参」の医薬品基準を定めるために必要な基原と品質に関する研究は不十分であり、党参産出地の広域にわたる資源植物の基原種の状況と、日本を含む東アジアに流通する党参市場品の品質に関して、客観的な研究結果が求められていた。

申請者 何 敬愉は、党参の資源並びに市場調査を中国で実施した後、収集品を用いて、薬用 *Codonopsis* 属植物の核 DNA の Internal transcribed spacer (ITS) 領域における遺伝的多様性を明らかにし、同時に基原種の客観的同定法を開発すること、及び胃粘膜保護・NGF 作用増強・がん転移抑制作用などが報告されている化合物群を同時定量する方法を開発して、党参市場品の品質評価を行うことを目的にした研究を行い、次に示すような新たな知見を得た。

### 1. 薬用 *Codonopsis* 属植物の ITS 領域における遺伝子多型と党参の同定への応用

*Codonopsis* 属 2 種 1 変種の 96 検体について ITS 領域の塩基配列をダイレクトシーケンスで解析した結果、ITS1-5.8S rDNA-ITS2 領域は全て 655bp であったが、種内多型が認められ、*C. pilosula* に 11 タイプ、*C. pilosula* var. *modesta* に 5 タイプ、*C. tangshen* に 6 タイプがあった。上流から 122 番目、226 番目、441 番目及び 489 番目の塩基は、3 分類群を鑑別できるマーカー配列であった。これら 4 箇所及び 130 番目の塩基により *C. pilosula* とその変種が各々タイプ分類され、一方、*C. tangshen* は 135

番目、489番目及び500番目の塩基でタイプ分類された。これらの塩基置換位置には両親由来の塩基の混合による2重ピーク（ヘテロ塩基）が検出される場合が多く、交雑が示唆された。すべてがホモ塩基である植物は、*C. pilosula* とその変種で各1タイプ（P0, PM0）、*C. tangshen* で3タイプ（T0, T1, T3）のみであり、さらに *C. pilosula* の近縁種及び生薬中にも2タイプ（S0, Q0）が存在した。*C. pilosula* の種内多型は主に P0, PM0, S0, Q0, T1 を示す植物間の交雑により、また *C. tangshen* の種内多型は T0, T1, T3 を示す植物間の交雑により生じたものであると考察した。クローニングによる解析もこの結果を支持した。甘粛省南東部の栽培品では *C. pilosula* の交雑タイプが混在しており、同省文県では *C. pilosula* var. *modesta* の純系タイプが主であった。湖北省と重慶市の野生または栽培品は *C. tangshen* の純系または交雑タイプであった。

## 2. Pyrrolidine alkaloids、Phenylpropanoid 及び Polyacetylenes の分析法の開発

*C. tangshen* の根から12成分を単離・同定し、その内 Pyrrolidine alkaloids 2成分（codonopyrrolidium A、codonopyrrolidium B）、Phenylpropanoid 1成分（tangshenoside I）及び Polyacetylenes 4成分（cordifolioidyne B、lobetyolinin、lobetyolin、lobetyol）を同時定量できる HPLC-UV 法を開発した。

## 3. Pyrrolidine alkaloids、Phenylpropanoid 及び Polyacetylenes に基づく薬用 *Codonopsis* 属植物及び党参の品質評価

基原を同定した *Codonopsis* 属植物56検体及び党参市場品54点を HPLC-UV 法で定量分析した結果、*C. pilosula* 及び *C. pilosula* var. *modesta* の根の主な成分は codonopyrrolidium B であり、同種に由来する党参市場品も同様であった。一方、*C. tangshen* の根は tangshenoside I 及び codonopyrrolidium A の含量が他種に比べて有意に



高く、この特徴は野生品で明らかであった。従来、品質評価の指標成分とされた Lobetyolin は 2 種 1 変種に共通して含まれていたが、党参市場品で含量が低く、生薬の保存状態の影響が考えられた。

以上、*Codonopsis* 属 2 種 1 変種について分類群固有の ITS 領域の塩基配列を明らかにし、党参の基原種を同定する方法を開発した。また、2 種の特徴的な成分組成を明らかにした。党参市場品は codonopyrrolidium B を主とする *C. pilosula* が主流品であり、codonopyrrolidium A を含み、tangshenoside I の含量及び 7 成分の総含量が高い *C. tangshen* は限られた市場でのみ流通していた。Lobetyolin 及び Pyrrolidine alkaloids の存在では 2 種に類似性が見られるが、tangshenoside I の多寡では違いがあり、今後この成分の薬理作用の検討が必要である。本研究は、同属植物の遺伝的多様化の現象を証明し、また 2 種 1 変種のカテゴリー的関連性を遺伝子及び成分組成から明らかにした。さらに、党参の標準化に寄与できる重要な知見を見出した。これらの研究成果は、学位論文として十分に評価し得るものである。

主査及び副査は、申請者 何 敬愉 の論文内容について審査を行うとともに面接試験を行い、博士（薬学）を授けるに値するものと判定した。