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Identification of Ginseng Radix in Chinese Medicine Preparations by Nested PCR-DNA Sequencing Method and Nested PCR-Restriction Fragment Length Polymorphism

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ABSTRACT

Ginseng Radix, the dried root of *Panax ginseng*, is easily confused with other herbs of the Panax species by appearance. In Chinese medicine preparations, it is difficult to identify misuse of Ginseng Radix. In this study, the nested PCR and DNA sequencing methods were established for the identification of Ginseng Radix, whereas the combination of nested PCR and restriction fragment length polymorphism (RFLP) could differentiate Ginseng Radix from other Panax species and thus certify the use of impure raw material of Ginseng Radix in Chinese medicine preparations. Nested PCR and DNA sequencing methods were applied to verify ginseng ingredients in the preparations. Of the 58 samples analyzed, 48 samples contained *P. ginseng*, 8 *P. quinguefolius,* and 2 *P. notoginseng*. Utilizing restriction enzyme *Bst*YI and *Dde*I, nested PCR products of the samples were digested for restriction fragment lengths of polymorphism (RFLP). It was proven that 23 of the 58 samples contained a mixture of Ginseng Radix and Panacis quinquefolii Radix. Twenty-five samples contained pure raw material of Ginseng Radix. Ginseng components of 10 samples were confirmed using a substitute of *P. quinguefolius* or *P. notoginseng*. The RFLP results were consistent with those of the sequencing method results.

Key words: *Panax ginseng*, Ginseng Radix, internal transcribed spacer (ITS), nested PCR, DNA sequencing, Restriction Fragment Length Polymorphism (RFLP)

INTRODUCTION

Ginseng Radix, the dried root of *Panax ginseng,* also known as ginseng, is the most popular Chinese medicinal herb. In an ancient medicinal book, Shen Nong Ben Cao Jing, written before A.D. 100, ginseng was used for repairing the five viscera, quieting the spirit, curbing emotions, stopping agitation, removing noxious influences, brightening the eyes, enlightening the mind, and increasing wisdom. Ginseng extracts have been used as a commercial dietary supplement. Current research suggests that the main components extracted from ginseng, ginsenosides, have anti-cancer, immune modulation[(1)], antioxidant[(2,3)], anti-inflammatory[(4)], anti-apoptotic[(5)], and immunostimulant[(6,7)] effects on human health.

The chemical components and appearance of ginseng differ by habitat. Ginseng is easily confused with Panacis quinquefolii Radix in appearance. Panacis quinquefolii Radix, the dried root of *P. quinguefolius*, is known as American ginseng. Because ginseng and American ginseng have different pharmacological effects[(8)], many chemical and physical methods have been developed to differentiate ginseng from other *Panax* species. For example, molecular spectroscopy, such as near infrared diffuse reflection spectroscopy, Raman spectroscopy and infrared spectroscopy, were used for differentiation among Ginseng Radix, Ginseng Rubra Radix and Panacis Quinquefolii Radix[(9)]. High performance thin-layer chromatography (HPTLC) fingerprint analysis was used for species authentication of *Panax ginseng*, *Panax quinquefolium* and *Panax notoginseng* [(10)]. Molecular biological methods were also useful to authenticate ginseng, such as arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR), direct amplification of length polymorphisms (DALP), and sequencing[(11)].

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Ginseng is prepared according to the formulas of ancient medicinal books published in eastern Asia. Each formula consists of many different herbs. Because that all herbs in the formula have lost their appearance after drying, also the chemical components of the formula are complex in the preparation. Therefore, it is difficult to differentiate ginseng from other herbs by physical and chemical analysis methods.

Because PCR cross-amplifies the DNA of these herbs, molecular biological methods may have difficulties in identifying ginseng. In our previous study[12], we were successful in differentiating Achyranthis Bidentatae Radix from Cyathulae Radix by nested PCR and DNA sequencing methods. Using this method for quality control and medicinal component testing purposes require greater budget for auto-sequencing, other necessary equipments, and materials for sequencing. Therefore, a fast and inexpensive method must be developed. In this study, nested PCR and DNA sequencing methods were applied to identify ginseng ingredients of preparations. Moreover, nested PCR was combined with restriction fragment lengths of polymorphism (RFLP) to identify *P. ginseng* in the preparations, and to determine whether the preparations contain other *Panax* species. The combination methodology reduces time, equipment, and materials costs for the authentication of ginseng, and has more potential applications in pharmaceutical industries and research institutions

MATERIALS AND METHODS

I. *Samples*

Fifty-eight samples of Chinese medicinal preparations, with Ginseng noted on the ingredient labels, were purchased from local traditional Chinese (herbal) pharmacies. Ginseng Radix was collected in Jilin, China, in July 2002, and was identified by Dr. Lin-Yu Lin of Chinese Academy of Medical Sciences, Beijing, China. Panacis quinquefolii Radix was purchased from a local importer of Chinese herbal medicines in May 2006 and was authenticated in our laboratory. We deposited the voucher specimens of Ginseng Radix-A-4-B03 and Panacis quinquefolii Radix-950011831 at the Chinese herbal drug specimen room of the Food and Drug Administration, Taipei, Taiwan, R.O.C.

II. *Primers*

We amplified the ITS region of Panax species for preparations by PCR with primers PgF3 (5'-GATCATTGTCGAAACCTGCAT-3') and PgR (5'-CAGGGTCATGAGAGCTTTTC-3') and re-amplified the PCR products by nested PCR with primer PgF1 (5'-CCGCGAACACGTTACAATAC-3') and PgR3 (5'-GGTCACCGCACGACATGAGA-3'). These two primer pairs were designed according to the ITS sequences deposited in Genbank, which included *Panax assamicus* (AY233321), *P. stipuleanatus* (U41696), *P. trifolius* (U41698), *P. notoginseng* (U41685), *P. ginseng* (AJ786235), *P. japonicus* (AF263373), *P. quinquefolius* (U41689), *P. vietnamensis* (AY271924), *P. wangianus* (U41691), *P. zingiberensis* (U41700) and *P. pseudoginseng* (AY233327).

III. *DNA Extraction*

The DNA extraction method was adopted from our previous research[12]. First, the herb samples were ground by grinders. One hundred milligrams of powder of all samples was placed in a 2.0-mL micro-centrifuge tube, digested in 1 mL lysis buffer [100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1% N-lauroyl sarcosine sodium salt (sarcosyl), and 1 mg/mL proteinase K] and incubated at 56°C for 1 hour. The sample solution was extracted with 1 mL of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) mixture solution and centrifuged at 12000 ×g for 5 min. The aqueous layer was mixed with 0.2 mL of 5 M NaCl and 0.15 mL of 10% hexadecyltrimethylammonium bromide (CTAB) in 0.7 M NaCl, and further incubated at 65°C for 15 min. The solution was extracted with 1 mL of chloroform/isoamyl alcohol (24:1, v/v) mixture solution and centrifuged at 12000 ×g for 5 min. The aqueous solution was then transferred into another 2.0-mL microcentrifuge tube. DNA in the solution was precipitated by adding 0.7 mL isopropanol and 0.1 mL of 3 M sodium acetate (pH 5.5). Precipitated DNA was centrifuged at 12000 ×g for 5 min, air-dried, and dissolved in 0.1 mL of sterile distilled water. A PCR purification kit (QIAGEN GmbH, Germany) was used to purify the dissolved DNA, with the silica membrane to absorb DNA through the high concentration of chaotropic salt in solutions. The purified DNA product was reserved for further PCR analysis.

IV. *Polymerase Chain Reaction and ITS Fragment Amplification*

Purified DNA was used as the templates for PCR amplification. Amplification was performed in 50 μL of solution by incubating 1 μL DNA with 5 μL 10 X Taq buffer, 0.5 μL of 25 μM PCR primer, 1 μL of 10 mM dNTP, 2 units of Taq polymerase, and sterilized distilled water. A control (no template) was included in each reaction. PCR reactions were performed in Astec PC320 (Astec, Fukuoka, Japan) using the following program: 30 sec of denaturation step at 94°C, 30 sec of annealing step at 62°C, and 30 sec of extension step at 72°C for a total of 25 cycles. One microliter of the resultant PCR product was used as a template for nested PCR. Nested PCR was carried out 25 times, each cycle consists of an initial DNA denaturation step at 94°C for 30 sec, an annealing step at 65°C for 30 sec, and an extension step at 72°C for 30 sec. Five microliters of final PCR products

were analyzed by electrophoresis in a 1.8% agarose gel. After electrophoresis in 0.5 X TBE buffer (Tris-Boric acid-EDTA) at 100 volts for 30 min, the gel was stained with ethidium bromide (0.5 mg/mL) before being photographed under ultraviolet (UV) illumination.

V. *Sequencing*

Five microliters of the nested PCR products were incubated with 2 μL of ExoSAP-IT (USB, OH, USA) at 37°C for 15 min to remove excess dNTP and residual primers. Afterwards, the mixture was heated at 80°C for 15 min to inactive the enzymes. One microliter of the resulting mixture was used for the sequencing reaction using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA). The sequencing reaction was purified by BigDye XTerminator purification kit (Applied Biosystems, CA, USA), and the sequences were determined for both strands using 3130 Genetic Analyzer (Applied Biosystems, CA, USA) and compared with the sequences in GenBank database.

VI. *Restriction Fragment Length Polymorphism*

Five microliters of the nested PCR products was incubated with 2 units of restriction enzyme *Bst*YI at 60°C and 2 units of *Dde*I (New England BioLabs, MA, USA) at 37°C for 1 hr, respectively. Five microliters of the resulting mixture was analyzed by electrophoresis in a 1.8% agarose gel. After electrophoresis in 0.5 X TBE buffer (Tris-Boric acid-EDTA) at 100 volts for 30 min, the gel was stained with ethidium bromide (0.5 mg/mL) before being photographed under ultraviolet (UV) illumination. DNA fragments recovered from electrophoresis gel were extracted by QIAquick Gel Extraction kit (QIAGEN GmbH, Germany) and analyzed by sequencing.

RESULTS AND DISCUSSION

The most commonly misused herb for Ginseng Radix is Panacis quinquefolii Radix. The ITS sequences of raw material samples of Ginseng Radix and Panacis quinquefolii Radix were analyzed and compared with the sequences in GenBank data to confirm the two samples as *P. ginseng* and *P. quinguefolius,* respectively. The two samples were then used as standard herbs for the following tests. By applying the designed primer sets in nested PCR, the DNA of all preparation samples could be amplified. The electrophoresis pattern is showed in Figure 1. The sequence data of the nested PCR products were obtained to identify which herb of the *Panax* species was in the preparation sample. The results are listed in Table 1. Among the 58 samples, 48 samples contained *P. ginseng*, 8 contained *P. quinguefolius,* and 2 contained *P. notoginseng.*

In this study, we found that Panacis quinquefolii Radix and Notoginseng Radix are misused as Ginseng Radix in these samples. Notoginseng Radix is the dried root of *P. notoginseng* that is used to stop bleeding and has different medicinal effects from Ginseng Radix. The designed primer sets could be used to amplify the DNA of eleven herbs of the *Panax* species at the same time. PCR products may contain two kinds of DNA; however, it was difficult to distinguish the differences by electrophoresis and sequencing results. The results of ginseng ingredient identified by sequencing methods could only prove that the preparation samples contained more than one *Panax* species. Therefore, another method was needed to differentiate the mixed *Panax* species in the samples.

This study analyzed the ITS sequence of eleven *Panax* species collected from GenBank to determine the appropriate recognition sites of restriction enzymes for discriminating *P. ginseng* from other *Panax* species. The restriction enzyme *Bst*YI could cut the amplified ITS fragment of *P. ginseng* and *P. japonicus*, and *Dde*I only cut the one of *P. japonicus* but not the ITS sequences of the other *Panax* species. In other words, if the amplified ITS fragment can be cut by *Bst*YI but not by *Dde*I, it means the sample only contained *P. ginseng.*

Each nested PCR product of the examined samples was tested separately with restriction enzyme *Bst*YI and *Dde*I. RFLP patterns could identify whether the samples contain pure ingredients of Ginseng Radix. The nested PCR products of all preparation samples were first treated with *Dde*I. No PCR products were cut by *Dde*I, indicating that no samples contained *P. japonicus*. The

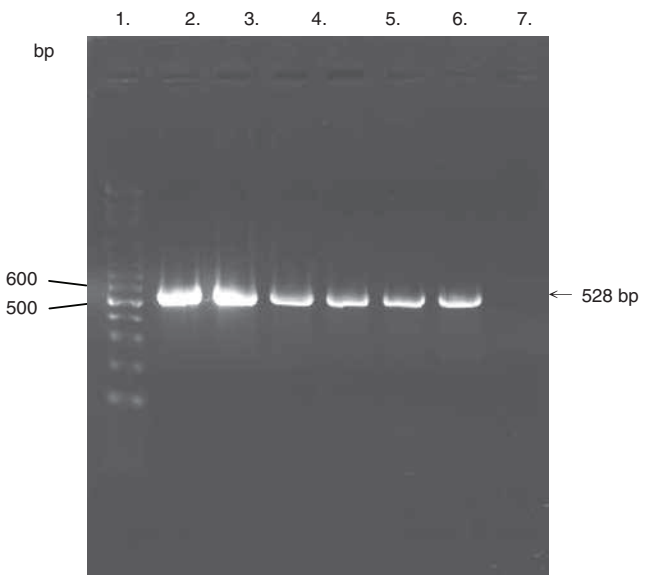

Figure 1. Nested PCR products of Ginseng Radix and preparation samples.
Lane 1: 100 bp ladder marker; Lane 2: Ginseng Radix; Lane 3: Pg1A; Lane 4: Pg2B; Lane 5: Pg9E; Lane 6: TC-C; Lane 7: blank (no template).

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Table 1. The results of ginseng ingredient identification and RFLP from sample preparations

Preparation	Sample Number	Identified Ingredient by DNA sequencing method	Cut number of RFLP*
Jen-Shen-Bai-Du-Tang	Pg1A	*P. ginseng*	2
Liu-Ho-Tang	Pg2A	*P. ginseng*	2
	Pg2B	*P. ginseng*	2
Pan-Hsia-Pai-Chu-Tien-Ma-Tang	Pg3A	*P. ginseng*	2
	Pg3B	*P. ginseng*	1
	Pg3C	*P. ginseng*	1
	Pg3E	*P. quinguefolius*	0
	Pg3F	*P. ginseng*	1
Chai-Ham-Tang	Pg4A	*P. ginseng*	2
Ching-Tsao-Chiu-Fei-Tang	Pg5B	*P. ginseng*	1
Sheng-Su-Yin	Pg6A	*P. ginseng*	2
	Pg6B	*P. ginseng*	1
Pu-Ji-Xiao-Du-Yin	Pg7A	*P. ginseng*	2
Wen-Jing-Tang	Pg8B	*P. ginseng*	1
Pu-Chung-I-Chi-Tang	Pg9A	*P. ginseng*	2
	Pg9B	*P. ginseng*	1
	Pg9C	*P. ginseng*	1
	Pg9E	*P. quinguefolius*	0
	Pg9F	*P. notoginseng*	0
Tu-Huo-Chi-Sheng-Tang	Pg10B	*P. ginseng*	1
Jen-Shen-Yang-Rong-Tang	Pg11A	*P. ginseng*	2
	Pg11B	*P. ginseng*	1
	Pg11C	*P. ginseng*	1
	Pg11E	*P. ginseng*	2
	Pg11F	*P. quinguefolius*	0
San-Bi-Tang	Pg12A	*P. ginseng*	2
	Pg12B	*P. ginseng*	1
	Pg12C	*P. quinguefolius*	0
	Pg12D	*P. ginseng*	1
	Pg12E	*P. ginseng*	2
	Pg12F	*P. quinguefolius*	0
Hsiao-Hsu-Ming-Tang	Pg13B	*P. ginseng*	1
Ten-Wang-Pu-Hsin-Tan	Pg14A	*P. ginseng*	2
	Pg14B	*P. ginseng*	1
	Pg14D	*P. ginseng*	1
Sheng-Mai-San	Pg15A	*P. ginseng*	2
	Pg15B	*P. ginseng*	2

Table 1. Continued

Preparation	Sample Number	Identified Ingredient by DNA sequencing method	Cut number of RFLP*
Ding-Zhi-Wan	Pg16A	*P. ginseng*	2
Fu-Tzu-Li-Chung-Tang	Pg17A	*P. ginseng*	2
	Pg17B	*P. ginseng*	2
	Pg17D	*P. ginseng*	1
Sang-Piao-Hsiao-San	Pg18A	*P. ginseng*	2
	Pg18B	*P. ginseng*	1
	Pg18C	*P. ginseng*	1
	Pg18D	*P. ginseng*	1
Ching-Chu-I-Chi-Tang	Pg19A	*P. ginseng*	2
	Pg19B	*P. ginseng*	2
	Pg19C	*P. quinguefolius*	0
	Pg19D	*P. ginseng*	1
Tzu-Wen-Tang	Pg20A	*P. ginseng*	2
	Pg20B	*P. ginseng*	1
Yang-Hsin-Tang	Pg21A	*P. ginseng*	2
	Pg21B	*P. ginseng*	1
	Pg21C	*P. quinguefolius*	0
Kuei-Lu-Erh-Hsien-Chiao	TC-B	*P. ginseng*	1
	TC-C	*P. notoginseng*	0
	TC-E	*P. ginseng*	1
	TC-F	*P. quinguefolius*	0

* "1" indicates that the product of nested PCR can be completely cut by restriction enzyme BstYI, "2" indicates that the product can be cut into three DNA fragments, and "0" indicates that the product can not be cut.

results of the nested PCR products treated with *Bst*YI are shown in Figure 2. The size of the nested PCR products amplified from all samples was about 528 bp. The nested PCR products of Panacis quinquefolii Radix could not be cut by *Bst*YI, while one Ginseng Radix was cut into two fragments of about 90 bp and 438 bp. If a sample contained Ginseng Radix and other *Panax* species, the RFLP pattern showed three DNA fragments of 90 bp, 438 bp, and 528 bp, suggesting that the sample contained both Ginseng Radix and Panacis quinquefolii Radix.

The RFLP results by *Bst*YI are also shown in Table 1. Twenty-three out of 58 samples presented three fragments in the electrophoresis pattern. The uncut fragment was recovered from electrophoresis gel, and then analyzed by sequencing. The sequences of the fragments of all 23 samples were confirmed as *Panax quinguefolius*. The raw materials of Ginseng Radix might be mixed with Panacis quinquefolii Radix in the 23 samples. Ten

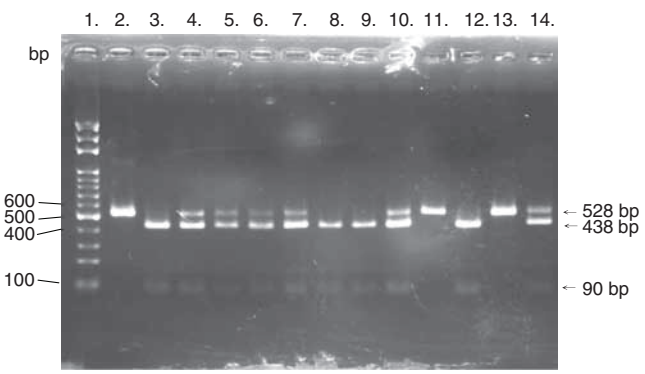

Figure 2. RFLP of the nested PCR products of Ginseng Radix, Panacis quinquefolii Radix and preparation samples digested with *Bst*YI.
Lane 1: 100 bp ladder marker; Lane 2: Panacis quinquefolii Radix; Lane 3: Ginseng Radix; Lane 4: Pg1A; Lane 5: Pg2A; Lane 6: Pg4A; Lane 7: Pg2B; Lane 8: Pg3B; Lane 9: Pg5B; Lane 10: Pg15B; Lane 11: Pg12C; Lane 12: Pg12D; Lane 13: Pg9E, Lane 14: Pg11E.

samples of preparation showed only one fragment after *Bst*YI treatment, and the fragments were recovered and analyzed by sequencing methods to show they belong to *P. quinguefoliu* and *P. notoginseng*. Therefore, we predicted Ginseng Radix was replaced with Panacis quinquefolii Radix or Notoginseng Radix in these preparation samples. The ITS of the remaining 25 samples were cut into two fragments of 90 bp and 438 bp to assure that it contained only Ginseng Radix. The RFLP results corresponded to that of the DNA sequencing method.

The molecular biological methods of nested PCR, DNA sequencing methods, and RFLP are useful techniques for quality control in pharmaceutical industries and medicinal component testing for researchers. These methods can also be applied in post-market inspections for official management. In this study, we combined the methods of nested PCR and RFLP to examine the presence of mixed *Panax* species in herb ingredients of preparation samples and found that Panacis quinquefolii Radix was substituted for Ginseng Radix in 31 out of 58 examined samples. The result was verified by DNA sequencing method, indicating the combination of nested PCR and RFLP methods is a reliable system to differentiate Ginseng Radix from other *Panax* species.

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