Journal of Food and Drug Analysis, Vol. 17, No. 5, 2009, Pages 380-385 藥物食品分析 第十七卷 第五期

Discriminating Astragali Radix from Hedysarum Radix in Chinese Medicine Preparations Using Nested PCR and DNA Sequencing Methods

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(Received: July 10, 2009; Accepted: September 22, 2009)

ABSTRACT

Astragali Radix are originated from *Astragalus membranaceus* and *A. membranaceus var. mongholicus*. However, Hedysarum Radix, the dried root of *Hedysarum polybotrys*, is often used as a substitute in Taiwan. In Chinese medicinal preparations, authenticity of Astragali Radix is difficult to be certified. The nested PCR and DNA sequencing method was used to identify Astragali Radix and Hedysarum Radix in preparation samples. Primer sets were designed for detecting Astragali Radix and Hedysarum Radix. The total DNA of all samples was extracted. Internal transcribed spacer (ITS) regions of the DNA samples were amplified by nested PCR and the sequences received from an auto-sequencer. We compared ITS sequences of the preparation samples with the standard to check whether Astragali Radix or Hedysarum Radix was in the preparation samples. Results indicated that 20 out of 30 samples contained a mixture of Astragali Radix and Hedysarum Radix, five Hedysarum Radix as substitute and five Astragali Radix. The combination of nested PCR and restriction fragment lengths polymorphism (RFLP) was employed to rapidly differentiate *Astragalus membranaceus*, *A. membranaceus var. mongholicus* and hybrid. The nested PCR and DNA sequencing method successfully identified Astragali Radix and Hedysarum Radix in Chinese medicine preparations.

Key words: Astragali Radix, Hedysarum Radix, internal transcribed spacer (ITS), nested PCR, DNA sequencing, restriction fragment length polymorphism (RFLP)

INTRODUCTION

Astragali Radix, the dried roots of *Astragalus membranaceus* and *A. membranaceus var. mongholicus*, are widely used in Chinese medicine. Ancient Chinese medical books indicate that Astragali Radix is used to invigorate chi (vital energy) and strengthen the body. Modern studies indicate that medical effects of Astragali Radix are immune modulation[(1,2)], immune restoration[(3)] and anti-tumor activity[(4)]. Besides medicinal usage, Astragali Radix is also used in medicinal cuisine for health-maintenance. However, it is often misused with substitution by Hedysarum Radix (the dried root of *Hedysarum polybotrys*) in Taiwan. Hedysarum Radix is used to disperse swelling by external use, and thus efficacy of the two herbs is quite different. The misuse may cause decreased medicinal effects as well as possible health damage.

Methods for identifying Astragali Radix have been developed, such as random amplified polymorphic DNA (RAPD)[(5,6)] and sequence characterized amplified regions[(7)]. However, these methods have only been applied to raw materials and Astragali Radix has not yet been identified in preparations. In modern time, Traditional Chinese medicine (TCM) have always been made as preparation for marketing purposes. For efficacy and safety concerns, it is necessary to certify that herbs used in these preparations are indeed authentic.

The nested PCR and DNA sequencing method is the first molecular biological technique that can identify designated herb components in Chinese medicinal preparations[(8-10)]. The internal transcribed spacer (ITS) was chosen as the DNA marker. Our previous study was successful in identifying Saposhinkoviae radix in

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concentrated Chinese medicinal preparations[8]. In this study, we used the same method to identify Astragali Radix and Hedysarum Radix in TCM preparations. A combination of nested PCR and restriction fragment lengths polymorphism (RFLP) was employed to differentiate *Astragalus membranaceus* from *A. membranaceus var. mongholicus*.

MATERIALS AND METHODS

I. *Samples*

Sixteen specimens of Astragali Radix and four of Hedysarum Radix, deposited at the Chinese herbal drug specimen room of the Bureau of Food and Drug Analysis, had been authenticated by our researchers and used as raw material samples. Thirty samples of Chinese medicinal preparations, with Astragali Radix on the ingredient label, were purchased from local traditional Chinese (herbal) pharmacies.

II. *Primers*

We amplified the ITS region of raw material samples using PCR with primer 18F1 (5'-GTGAACTGCGGAAGGATC-3') and 28R (5'-CCGCCTGACCTGRGGTC-3'; R = A/G). This primer pair was designed based on the conserved region of the 18S and 26S rRNA genes. For preparation samples, we amplified the ITS region of Astragali Radix by PCR with primers AmF4 (5'-GCCTTACATGCAGACCAACTA-3') and AmR2 (5'-AAAAGACGCATGTGGGTCATC-3'), and then the PCR products were re-amplified by nested PCR with primer AmF3 (5'-TGAATACTTAGGGATGGCTG-3') and AmR6 (5'-GAAGCCATATTATGGGGAG-3'). For Hedysarum Radix component detection, we amplified the ITS region by PCR with primers HpF2 (5'-GTGAATCTGTTCGAATACGTG-3') and HpR (5'-ACTGGAGGCCAAATCTGGTA-3'), and then the PCR products were re-amplified by nested PCR with primer HpF3 (5'-AACTCCTCGACCTCCCTTGA-3') and HpR2 (5'-AACACCCTACCAAGGACTCG -3').

III. *DNA Extraction*

The DNA extraction method was followed as in our previous study[8]. First, the herb samples were grounded by grinders. One hundred miligrams powder of each sample were individually placed in a 2.0 mL microcentrifuge tube, digested in 1 mL of 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/isoamyl alcohol (25: 24: 1; v/v/v) mixture solution and centrifuged at 12,000 × 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 12,000 × g for 5 min. The aqueous solution was then transferred into another 2.0 mL micro-centrifuge tube. DNA in the solution was precipitated by adding 0.1 mL of 3 M sodium acetate and 0.7 mL of isopropanol. Precipitated DNA was centrifuged at 12,000 g for 5 min, air-dried, and dissolved in 0.1 mL of sterile, distilled water. A PCR purification kit (QIAGEN GmbH; Hilden, 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*

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 primers, 1 μL of 10 mM dNTP, 2 units of Taq polymerase, and sterilized distilled water. A blank (no template) was included in each reaction. PCR reactions were performed in Astec PC320 (Astec; Fukuoka, Japan). Mixtures containing the DNA extracted from raw material samples were amplified by 40 cycles of PCR, each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 58°C, and 30 sec of extension at 72°C. For Astragali Radix component detection, the mixtures containing DNA from preparation samples were amplified by 25 cycles of PCR, each cycle consisting of a 30-sec denaturation step at 94°C, a 30-sec annealing step at 67°C, and a 30-sec extension step at 72°C. One microliter of the resultant PCR product was taken as template for nested PCR. Nested PCR conditions were set as 25 cycles of an initial DNA denaturation step at 94°C for 30 sec, an annealing step at 64°C for 30 sec, and an extension step at 72°C for 30 sec. For Hedysarum Radix component, the mixtures were amplified by 25 cycles of PCR with a 30 sec annealing step at 67°C. Nested PCR where 1 μL of the resultant PCR product taken as template were set at 25 cycles, and an annealing step at 64°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 final PCR products were incubated with 2 μL of ExoSAP-IT (USB; Cleveland, Ohio, USA) at 37°C for 15 min to remove the excess dNTP and the primers and then heated at 80°C for 15 min to inactive

the enzymes. The 1 μL aliquot of the resulting mixture was used for the sequencing reaction using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems; Foster, CA, USA). All products of sequencing reaction were purified by BigDye XTerminator purification kit (Applied Biosystems; Foster, CA, USA), and the sequences were determined for both strands using 3130 Genetic Analyzer (Applied Biosystems; Foster, CA, USA). The sequence data were compared with the GenBank database.

VI. *Restriction Fragment Length Polymorphism*

Five microliters of final PCR products were incubated with 2 units of restriction enzyme *Fnu*4HI (New England BioLabs; Ipswich, MA, USA) at 37°C. Five microliters of the resulting mixture 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. Several DNA fragments were recovered by QIAquick Gel Extraction kit (QIAGEN GmbH; Hilden, Germany) from electrophoresis gel and then analyzed by sequencing.

RESULTS AND DISCUSSION

Nested PCR and DNA sequencing methods were applied to identify the Astragali Radix component in Chinese medicine preparations. At first, the standard sequences have been ensured. Astragali Radix is often misused in Taiwan with substitution by Hedysarum Radix. The raw materials of both herbs were collected and identified using morphological and histological methods. The total DNA extracted from sixteen samples of Astragali Radix and four of Hedysarum Radix were amplified by PCR to obtain ITS fragments. The sequences of the ITS fragments were analyzed by autosequencer and compared with the GenBank database to identify these samples. The results of DNA sequencing corresponded to the results of the morphological and histological methods as shown in Table 1.

The raw material samples of Astragali Radix were identified as *A. membranaceus*, *A. membranaceus var. mongholicus,* and a hybrid. The length of the ITS fragment is 636 bps. At the nucleotide position 487, base T is in *A. membranaceus* and C in *A. membranaceus var. mongholicus*. Mixed bases of T/C were present in some samples that were hybrid crossbreed between *A. membranaceus* and *A. membranaceus var. mongholicus*. Guo *et al.*[11] also reported that the sequence of Atractylodes lancea rhizome hybrids contains mixed bases. It may mean that some medicinal plants have been hybridized in the cultivation process.

By utilizing the RFLP method, restriction enzyme *Fnu*4HI could be used to discriminate between *A. membranaceus*, *A. membranaceus var. mongholicus* and the hybrid. F*nu*4HI recognized the difference in the ITS sequences. In its electrophoresis pattern, we found that the PCR products are uncut, one band (636 bps) in *A. membranaceus*, completely cut to two bands (149 bps and 487 bps) in *A. membranaceus var. mongholicus*, and partially cut to display three bands (149 bps, 487 bps and 636 bps) in hybrid (Figure 1 and Table 1). The results of RFLP corresponded well with the results of the DNA sequencing method.

Chinese medicinal preparations always contain a variety of herbal ingredients and a trace of herbal DNA. The ITS sequences of these herbs may be similar. A single-run PCR process cannot amplify sufficient and pure ITS fragments from an individual herb. We applied nested PCR to improve the specificity and efficiency of PCR to obtain ITS fragments of the target herb.

The sequences from raw material samples were required to be identical with GenBank to serve as standards. Two primer sets based on the standards were designed for nested PCR to amplify the ITS fragment. The primer sets for detecting Astragali Radix and Hedysarum Radix were examined and exhibited no cross-reaction with each other. Every preparation sample was then examined. The lengths of nested PCR products were approximately 534 bps for detecting Astragali Radix and 451 bps for Hedysarum Radix. The electrophoresis patterns are shown in Figure 2 and 3. The results can be

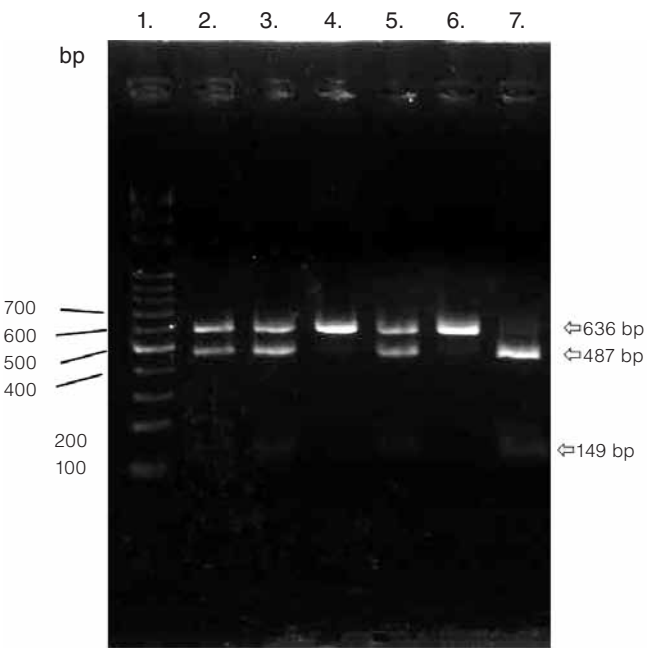

Figure 1. RFLP from the PCR products of Astragali Radix.
Lane 1: 100 bp ladder marker, Lane 2: hybrid (AR-1), Lane 3: hybrid (Am31-5), Lane 4: *A. membranaceus* (Am31-6-2), Lane 5: hybrid (Am31-7), Lane 6: *A. membranaceus* (Am31-17), Lane 7: *A. membranaceus var. mongholicus* (Am31-24).

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Table 1. Identification of raw material sample of Astragali Radix and Hedysarum Radix using Nested PCR-DNA sequencing methods and RFLP.

Raw material sample[a]	Sample number	Results of DNA sequencing method	Results of RFLP[b]
Astragali Radix	thin	*A. membranaceus*	uncut
	A-1-4-04	hybrid[c]	2
	C-2-1-08	*A. membranaceus var. mongholicus*	1
	F-3-3-05	*A. membranaceus*	uncut
	X-2-B01	hybrid	2
	Y-2-E01	hybrid	2
	Z-1-A02	hybrid	2
	AR-1	hybrid	2
	Am31-5	hybrid	2
	Am31-6-1	*A. membranaceus*	uncut
	Am31-6-2	*A. membranaceus*	uncut
	Am31-7	hybrid	2
	Am31-17	*A. membranaceus*	uncut
	Am31-19	hybrid	2
	Am31-21	hybrid	2
	Am31-24	*A. membranaceus var. mongholicus*	1
Hedysarum Radix	A-1-3-05	*H. polybotrys*	-
	C-1-5-02	*H. polybotrys*	-
	N-1	*H. polybotrys*	-
	N-2	*H. polybotrys*	-

[a] Authenticity of raw material samples was confirmed by morphological and histological methods.
[b] "1" indicates that the product of nested PCR can be completely cut by restriction enzyme *Fnu*4HI, "2" indicates that the product can be partially cut and "uncut" indicates that the product can not be cut.
[c] "hybrid" indicates that sample is identified as a hybrid of *A. membranaceus* and *A. membranaceus var. mongholicus*.

verified through sequence comparison. By comparing the sequences of nested PCR products with the standard reference, we were able to identify Astragali Radix and Hedysarum Radix in sample contents.

Clear sequence patterns from the nested PCR products were obtained, indicating that our nested PCR method can be used for amplifying DNA fragments of designated herbs from the multi-herb components of the preparation. The results of the identification process are listed in Table 2. Astragali Radix ingredient was found and identified as a hybrid in 25 of the 30 samples. Hedysarum Radix ingredient was found in 25 samples. To summarize the results: 20 preparation samples contained a mixture of Astragali Radix and Hedysarum Radix, five Hedysarum Radix as substitute and five Astragali Radix.

After amplifying ITS by nested PCR, the Astragali Radix component in the preparation samples can be identified by RFLP. By combining nested PCR and RFLP, we can identify which Astragali Radix was included in Chinese medicinal preparations. RFLP is an easy method to use, and the nested PCR provides high specificity and sensitivity. This combination has greater ability to identify Astragali Radix and avoid interference by other herbs.

In this study, Astragali Radix and Hedysarum Radix components were successfully identified through DNA amplification, with nested PCR and sequence comparison. Using nested PCR, we could detect whether Astragali Radix and Hedysarum Radix were in the preparations. Three types of Astragali Radix, *A. membranaceus*, *A. membranaceus var. mongholicus* and hybrid, were identified by sequence comparison and RFLP. These methods were reliable for post-market inspections in official management, quality control in pharmaceutical industries, as well as medical component testing in research.

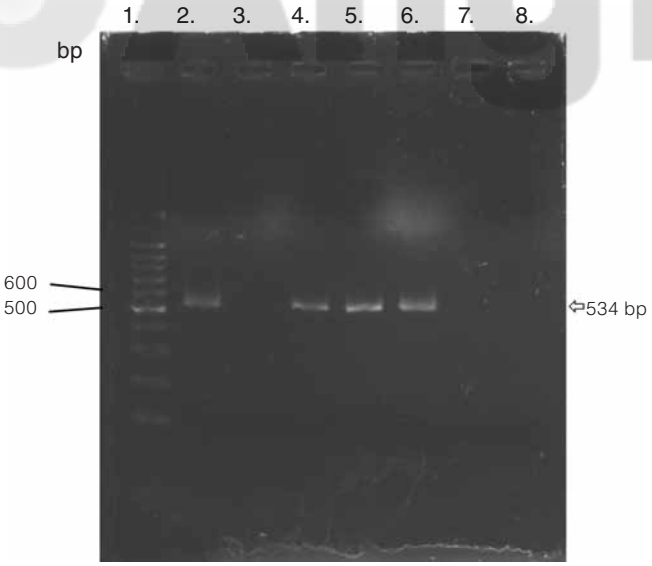

Figure 2. Nested PCR products of Astragali Radix ingredient in preparation samples.
Lane 1: 100 bp ladder marker, Lane 2: Astragali Radix (A-1-4-04), Lane 3: Hedysarum Radix (C-1-5-02), Lane 4: Jen-Shen-Yang-Rong-Tang (Am1E), Lane 5: Yang-Hsin-Tang (Am3B), Lane 6: Tang-Kuei-Yin-Tzu (Am7F), Lane 7: Pu-Chung-I-Chi-Tang (Am6A), Lane 8: blank (no template).

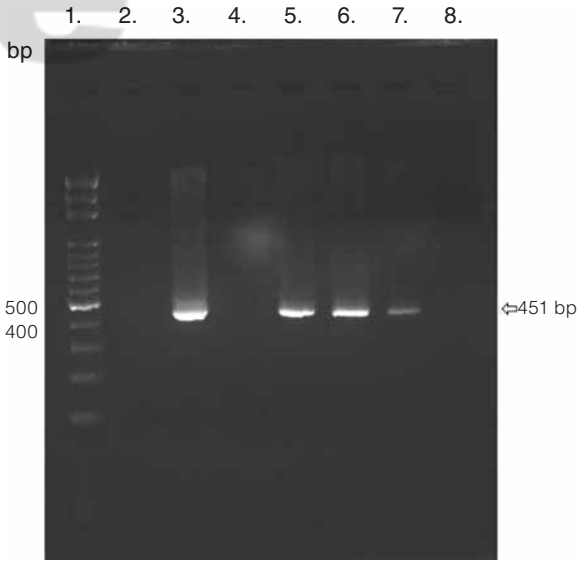

Figure 3. Nested PCR product of Hedysarum Radix ingredient in preparation samples.
Lane 1: 100 bp ladder marker, Lane 2: Astragali Radix (A-1-4-04), Lane 3: Hedysarum Radix (C-1-5-02), Lane 4: Jen-Shen-Yang-Rong-Tang (Am1E), Lane 5: Yang-Hsin-Tang (Am3B), Lane 6: Tang-Kuei-Yin-Tzu (Am7F), Lane 7: Pu-Chung-I-Chi-Tang (Am6A), Lane 8: blank (no template).

Table 2. Ingredient identification from Chinese medicinal preparations.

Preparation sample name	Sample number	Astragali Radix ingredient	Hedysarum Radix ingredient
Jen-Shen-Yang-Rong-Tang	Am1A	hybrid[a]	*H. polybotrys*
	Am1B	hybrid	*H. polybotrys*
	Am1B1	hybrid	*H. polybotrys*
	Am1E	hybrid	ND[b]
	Am1F	hybrid	ND
Ching-Chu-I-Chi-Tang	Am2A	ND	*H. polybotrys*
	Am2B	hybrid	*H. polybotrys*
	Am2C	ND	*H. polybotrys*
	Am2D	hybrid	*H. polybotrys*
Yang-Hsin-Tang	Am3A	ND	*H. polybotrys*
	Am3B	hybrid	*H. polybotrys*
	Am3C	hybrid	*H. polybotrys*
San-Bi-Tang	Am4B	hybrid	*H. polybotrys*
	Am4C	hybrid	ND
	Am4D	hybrid	*H. polybotrys*
	Am4E	hybrid	*H. polybotrys*
	Am4F	hybrid	*H. polybotrys*
Pan-Hsia-Pai-Chu-Tien-Ma-Tang	Am5A	ND	*H. polybotrys*
	Am5B	hybrid	*H. polybotrys*

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Table 2. Continued

Preparation sample name	Sample number	Astragali Radix ingredient	Hedysarum Radix ingredient
	Am5E	hybrid	*H. polybotrys*
	Am5F	hybrid	*H. polybotrys*
Pu-Chung-I-Chi-Tang	Am6A	ND	*H. polybotrys*
	Am6B	hybrid	*H. polybotrys*
	Am6C	hybrid	*H. polybotrys*
	Am6E	hybrid	*H. polybotrys*
	Am6F	hybrid	*H. polybotrys*
Tang-Kuei-Yin-Tzu	Am7B	hybrid	*H. polybotrys*
	Am7C	hybrid	ND
	Am7E	hybrid	ND
	Am7F	hybrid	*H. polybotrys*

[a] "hybrid" indicates that sample is identified as a hybrid of *A. membranaceus* and *A. membranaceus var. mongholicus.*
[b] "ND" : Not detected.

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