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Authentication of Gardeniae Fructus in Chinese Medicine Preparations by Nested PCR and DNA Sequencing Methods

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ABSTRACT

Chinese medicinal preparations are usually composed of various raw material components, and the complexity of herbal ingredients therein complicates the identification of constituent species. In this study, nested PCR coupled with DNA sequencing methods were established for Gardeniae Fructus in Chinese medicinal preparation. The botanical origin of Gardeniae Fructus is the dried mature fruits of *Gardenia jasminoides* Ells. The morphology of the dried mature fruits of *G. jasminoides* var. *grandiflora* (Lour.) Nakai is similar to that of *G. jasminoides*, which results in misuse. In this study, 28 raw materials and 22 preparation samples were collected. The total DNA of all samples were extracted by CTAB method and purified. Authentication was executed based on the sequence of internal transcribed spacer (ITS) which was amplified by nested PCR followed by sequencing. The ITS sequences of all samples were the same, with the exception of position 153. The nucleotide base of 3 raw material samples, identified as *G. jasminoides* var. *grandiflora* by morhpology, were base "G". The raw material samples, identified as *G. jasminoides* by morphology, had the base "G" or "A" on this position. Gardeniae Fructus could be identified in all preparation samples by nested PCR and DNA sequencing.

Key words: Gardeniae Fructus, *Gardenia jasminoides,* internal transcribed spacer (ITS), nested PCR, DNA sequencing

INTRODUCTION

Chinese medicinal preparations are usually made of many different herbs, which render it difficult to identify the herbs therein, due to appearance destruction. Moreover, the chemical components of the preparations are complex. Therefore, it is difficult to differentiate one herb from the other by physical or chemical analysis methods. Currently, DNA analysis is an increasingly important tool in the identification of Chinese medicine raw materials. This method of analysis is more reliable than some chemical or physical analyses as, unlike phenotypes, a plant's genotype is unaffected by different growth environments or its use in different medical preparations.

According to Chinese Medicine Pharmacopoeia, true Gardeniae Fructus is the dried mature fruits of *Gardenia jasminoides* Ellis. The morphology of the dried mature fruits of *G. jasminoides* var. *grandiflora* (Lour.) Nakai is similar to that of *G. jasminoides*, which causes misuse. Current research suggests that the components extracted from Gardeniae Fructus have antioxidant[1], anti-inflammatory[2], hypoglycemic[3], anti-microbial effects[4], and improve liver function[5].

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Our previous study was successful in identifying Saposhinkoviae radix in Chinese medicinal preparations by nested PCR and the DNA sequencing method[6]. The internal transcribed spacer (ITS) sequence was used as the DNA marker for *G. jasminoides*. Nested PCR and the DNA sequencing techniques were first adopted to identify the designated herbal constituent in Chinese medicinal preparations[6,7]. In this study the same strategy will be employed to identify Gardeniae Fructus in Chinese medicinal preparations.

MATERIALS AND METHODS

I. *Samples*

Twenty-eight samples of raw materials were collected from local traditional Chinese (herbal) pharmacies and mainland China (Table 1). The preliminary identification of raw material samples was preceded by morphology. Twenty-two samples of Chinese medicinal preparations (included 11 formulae as listed in Table 2), with Gardeniae Fructus noted on the ingredient labels, were purchased from local traditional Chinese (herbal) pharmacies.

Journal of Food and Drug Analysis, Vol. 20, No. 4, 2012

Table 1. The base pair in the location 153 of the ITS sequence of raw material samples

Sample No.	Identification by morphology	bp153
GJ01	*G. jasminoides*	G
GJ02	*G. jasminoides*	G
GJ03	*G. jasminoides* var. *grandiflora*	G
GJ08	*G. jasminoides*	G
GJ09	*G. jasminoides*	A
GJ10	*G. jasminoides*	A
GJ11	*G. jasminoides*	A
GJ12	*G. jasminoides*	G
GJ13	*G. jasminoides*	A
GJ14	*G. jasminoides*	A
GJ15	*G. jasminoides*	A
GJ16	*G. jasminoides*	A
GJ17	*G. jasminoides*	G
GJ18	*G. jasminoides*	A
GJ19	*G. jasminoides*	G
GJ20	*G. jasminoides*	A
GJ21	*G. jasminoides*	G
GJ22	*G. jasminoides*	A
GJ23	*G. jasminoides*	A
GJ24	*G. jasminoides*	A
GJ25	*G. jasminoides*	A
GJ26	*G. jasminoides*	A
GJ27	*G. jasminoides*	A
GJ28	*G. jasminoides*	A
GJ29	*G. jasminoides*	A
GJ30	*G. jasminoides*	G
FGJ02	*G. jasminoides* var. *grandiflora*	G
FGJ04	*G. jasminoides* var. *grandiflora*	G

Table 2. Gardeniae Fructus preparation sample list

Formula	Sample No.	base 153
Qing-Fei-Tang	Gje5A	A
	Gje5B	G
Uen-Ching-Tang	Gje6A	G
	Gje6B	G
Dun-Sou-San	Gje7A	G
	Gje7B	G
San-Huang-Shi-Gao-Tang	Gje10A	G
	Gje10B	G
Ping-Gan-Liu-Gi-Yin	Gje11A	G
	Gje11B	G
Shi-Gan-Ming-Muh-San	Gje12A	A
Chai-Hu-Qing-Gan-Tang	Gje13B	G
Ching-Shan-Farng-Feng-Tang	Gje14A	A
	Gje14B	G
Tzy-Shenn-Ming-Muh-Tang	Gje16A	A
Huang-Lian-Jie-Du-Tang	Gje17A	G
	Gje17B	G
Xin-Yi-Qing-Fei-Tang	Gje20A	G
	Gje20C	A
	Gje20D	G
	Gje20I	G
	Gje20K	A

II. *Primers*

The ITS region was amplified for samples by polymerase chain reaction (PCR) using primer pair GjF2 (5'-CGGAAAGCGCCAAGGA-3') and GjR2 (5'-CACGACG ACGACTCGA-3'), and then the PCR products were re-amplified by nested PCR with primer pair GjF3 (5'-GCGCCTGTCGTAACCA-3') and GjR3 (5'-GAGGGACT CAACCACCA-3'). These two primer pairs were designed in accordance with the ITS sequence data of *Gardenia* spp. from GenBank.

III. *DNA Extraction*

The DNA extraction technique was adopted from our previous research[7]. First, the herb samples were ground by grinders. One hundred milligram of powder of sample 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 hr. The sample solution was extracted with 1 mL of phenol/chloroform/isoamylalcohol (25:24:1; v/v/v) mixture solution and centrifuged at 12,000 xg 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) solution and centrifuged at 12,000 xg 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 0.7 mL isopropanol and 0.1 mL of 3 M sodium acetate (pH 5.5). Precipitated DNA was centrifuged at 12,000 xg 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 under 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 30 μL of

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1                                                                    70
GCGCCTGTCG TAACCAAAAC GACTCTCGGC AACGGATATC TCGGCTCTCG CATCGATGAA GAACGTAGCG

71                                                                   140
AAATGCGATA CTTGGTGTGA ATTGCAGAAT CCCGTGAACC ATCAAGTCTT TGAACGCAAG TTGCGCCCGA

141         153                                                      210
AGCCATCAGG CCAAGGGCAC GTCTGCCTGG GCGTCACGCA TCGCGTCGCC ACCCCCCTCC CGCGGGGGCG
         (A or G)
211                                                                  280
GCGGAGACTG GCCTCCCGTG CCCCGGGGCG CGGCCGGCCC AAATGAGAGT TCCTCGGCGA GGGGCGTCAC

281                  303
GACTGGTGGT GGTTGAGTCC CTC
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Figure 1. The ITS sequences of *Gardenia* samples used in the study.

solution by incubating 4 μL DNA with 3 μL 10 X Taq buffer, 0.5 μL 25 μM PCR primer, 0.6 μL 10 mM dNTP, 2 units 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 52°C, and 30 sec of extension step at 72°C for a total of 30 cycles. Four μL of the resultant PCR product was used as a template for nested PCR. Nested PCR was carried out 30 cycles, each cycle consisting of an initial DNA denaturation step at 94°C for 30 sec, an annealing step at 56°C for 30 sec., and an extension step at 72°C for 30 sec. Five μL of final PCR products were analyzed by electrophoresis on 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 μL 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 μL 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 using BigDye XTerminator purification kit (Applied Biosystems, CA, USA), and the sequences were determined for both strands on a 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

RESULTS AND DISCUSSION

There was no sequence data of *G. jasminoides* and *G. jasminoides* var. *grandiflora* in GenBank so that the ITS sequences of *Gardenia* spp. were used to design the primer sets for the nested PCR. Many designed primer sets were applied to amplify ITS fragments of the raw material samples. Only one combination was able to amplify a single DNA fragment, which was subsequently used to obtain clear sequence data. The amplified nested PCR products were all about 303 bp in size, which were analyzed on an auto-sequencer.

The sequences of all raw material samples were identical, with the exception of one nucleotide base at position 153. The results are shown in Table 1, and the sequences in Figure 1. Three samples of *G. jasminoides* var. *grandiflora* have base G in position 153 of ITS sequence, while *G. jasminoides* has nucleotide base G or A. The current identification of Gardeniae Fructus is according to the morphology characteristics, which is based on size, color, and the level of the epidermis longitudinal edge of fruits. These characters would change with the maturity of fruits. The dry fruits of *G. jasminoides* may be misidentified as *G. jasminoides* var. *grandiflora* with different maturity. Comparsion of DNA sequence may be a useful approach for discriminating *G. jasminoides* from *G. jasminoides* var. *grandiflora*. However, the ITS sequence cannot be used to differentiate these two kinds of the sample in the study. Therefore, other distinguishable sequences shall be developed in further study.

Our nested PCR and DNA sequencing method could be applied to the raw material samples. This study attempted to apply this method to the preparation samples in order to detect

Gardeniae Fructus. The presence of complex herbal ingredients in Chinese medicine preparation samples may confound efforts to detect the presence of any single ingredient. All formulae of preparation samples are listed in Table 2. The ITS of Gardeniae Fructus was amplified specifically by nested PCR to obtain an adequate amount of DNA for sequencing. The result of sequence analysis indicates that the sequences of all nested PCR products mirrored those of raw material samples. All preparation samples selected for analysis in this study contained Gardeniae Fructus. Six samples had base A at position 153, while the other samples had base G.

In conclusion, this paper reports the development of a Nested PCR and DNA sequencing method, which was used to identify Gardeniae Fructus in 22 preparations. *G. jasminoides* and *G. jasminoides* var. *grandiflora* could not be differentiated with the sequences of ITS fragments. Therefore, more attempts will be made to search for other DNA markers in future works.

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