Specific PCR Identification between *Peucedanum praeruptorum* and *Angelica decursiva* and Identification between Them and Adulterant Using DNA Barcode

Bang-Xing Han 1,2, Yuan Yuan 2, Lu-Qi Huang 2, Qun Zhao 1,2, Ling-Ling Tan 3, Xiang-Wen Song 1, Xiao-Mei He 1, Tao Xu 4, Feng Liu 1,4, Jian Wang 4

1 College of Biological and Pharmaceutical Engineering, West Anhui University, Lu’an, Anhui Province, 2 State Key Laboratory Breeding Base of Daodi Herbs, China Academy of Chinese Medical Sciences, Beijing, 3 School of life science, Qingdao agricultural university, Qingdao, Shandong Province, 4 Department of Pharmacy, Anhui University of Chinese Medicine, Hefei, China

Submitted: 19-12-2015 Revised: 20-01-2016 Published: 06-01-2017

ABSTRACT

**Background:** The traditional Chinese medicine (TCM) Qianhu and Zhihuaqianhu are the dried roots of *Peucedanum praeruptorum* and *Angelica decursiva*, respectively. Since the plant sources of Qianhu and Zhihuaqianhu are more complex, the chemical compositions of *P. praeruptorum* and *A. decursiva* are significantly different, and many adulterants exist because of the differences in traditional understanding and medication habits. Therefore, the rapid and accurate identification methods are required.

**Objective:** The aim was to study the feasibility of using DNA barcoding to distinguish between Traditional Chinese medicine Qianhu (*Peucedanum praeruptorum*), Zhihuaqianhu (*Angelica decursiva*), and common adulterants, based on the internal transcribed spacer (ITS) sequences, as well as specific PCR identification between *P. praeruptorum* and *A. decursiva*. **Materials and Methods:** The ITS sequences of *P. praeruptorum*, *A. decursiva*, and adulterant were studied, and a phylogenetic tree was constructed. Based on the ITS barcode, the specific PCR primer pairs QH-CP19s/QH-CP19a and ZHOC-P2s/ZHOC-P2a were designed for *P. praeruptorum* and *A. decursiva*, respectively. The amplification conditions were optimized, and specific PCR products were obtained. **Results:** The results showed that the phylogenetic trees constructed using the BI and MP methods were consistent, and the phylogenetic tree formed their own monophyly. The experimental results showed that in PCR products, the target bands appeared in the genuine drug and not in the adulterant, which suggested the high specificity of the two primer pairs. **Conclusion:** The ITS sequence was ideal DNA barcode to identify *P. praeruptorum*, *A. decursiva*, and adulterant. The specific PCR is a quick and effective method to distinguish between *P. praeruptorum* and *A. decursiva*.

**Key words:** Angelica decursiva, DNA barcode, ITS, *Peucedanum praeruptorum*, specific PCR

**SUMMARY**

- *Peucedanum praeruptorum* and *Angelica decursiva* sequence haplotypes formed their own monophyly.

INTRODUCTION

The traditional Chinese medicine (TCM) Qianhu is the dried root of *Peucedanum praeruptorum*, and its primary functions include depressing qi, reducing phlegm, dispelling wind, and clearing heat. Zhihuaqianhu is the dried root of *Angelica decursiva* and has the same efficacy as *P. praeruptorum*. However, the chemical compositions of *P. praeruptorum* and *A. decursiva* are significantly different. The taxonomic status of *A. decursiva* is controversial, vacillating between *Peucedanum* and *Angelica*. The plant sources of Qianhu and Zhihuaqianhu are more complex, and many adulterants exist because of the differences in traditional understanding and medication habits. Moreover, Qianhu and Zhihuaqianhu are easily confused in practical applications, because the morphological characteristics of these two kinds of TCM are quite different.

This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-Share Alike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Cite this article as: Han BX, Yuan Y, Huang LQ, Zhao Q, Tan LL, Song XW, He XM, Xu T, Liu F, Wang J. Specific PCR identification between *Peucedanum praeruptorum* and *Angelica decursiva* and identification between them and adulterant using DNA barcode. Phcog Mag 2017;13:38-45.
similar, and difficult to be identified according to their appearance characteristics. Thus, some rapid and accurate identification methods are required.

The medicinal materials are identified according to the characteristics, microscopic feature, HPLC fingerprint, and GC/MS. Some of the abovementioned methods are complex and time-consuming, and cannot accurately differentiate between genuine drug and adulterant. The application of modern molecular biological techniques, especially ribosomal RNA gene DNA internal transcribed spacer, has been widely performed in interspecific plant identification and determination of genetic relationship. These techniques are new approaches for the identification of medicinal materials. For example, Yongxing Xiong et al. conducted a preliminary study on the identification of *P. praeruptorum*, *A. decursiva*, and adulterant using the internal transcribed spacer (ITS) 2 sequence DNA barcode. The phylogenetic tree of *P. praeruptorum* and *A. decursiva* was constructed only using neighboring-joining method, therefore, the accuracy was found to be deficient. However, this method has many deficiencies, which are, as follows: time-consuming, requiring sequencing, big workload, complex procedure, and high appraiser requirements. Thus, a more accurate and simple method should be developed for large-scale identification of TCM samples.

The target bands were detected and amplified with highly specific PCR using a high-efficiency correctly-matched primer. Only a small amount of amplified product was produced using the mismatched primer. The target bands were not detected after a certain number of PCR cycles. The genuine drug and adulterant can be differentiated. At present, a high-specificity PCR method has been successfully applied in the identification of Chuanmingshen, Lubian, and Jingjianbaihuashe. In this paper, the feasibility of differentiating among *P. praeruptorum*, *A. decursiva*, and adulterant using ITS barcode sequences, was investigated. Based on this, a specific PCR method was established to identify *P. praeruptorum* and *A. decursiva* to provide the basis for rapid and accurate identification of these two kinds of TCM in this study.

**MATERIALS AND METHODS**

**Instruments and reagents**

PCR instrument: MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad company), TProfessional standard Thermocycler (Biometra company) and Light Cycler® 96 System (Roche company); Mikro 120 type microcentrifuge (Hettich company); BG-Power 600k electrophoresis apparatus (Beijing Baijing Biotechnology Co., Ltd.); automatic gel imaging analyzer (Beijing Baijing biotechnology limited company); UV-2102 PCS type ultraviolet visible spectrophotometer [Unique (Shanghai) Instrument Co. Ltd.]; WH-3 type vortex oscillator (Shanghai Huxi analytical instrument Factory Co. Ltd.); SYG-DXSZ-280B type portable stainless steel autoclave (Shanghai Shenan medical instrument factory).

2×CTAB extract; ethidium bromide (EB); Taq DNA Polymerases (Shanghai Sangon bioengineering Co., Ltd.); Hot start Taq DNA polymerase, Taq DNA Polymerase, and Taq Plus DNA Polymerase; dNTPs. DNA marker (100bp to 600bp); and agarose (Shanghai Sangon bioengineering Co., Ltd.). Other related reagents were molecular biology grade or analytically pure.

**Experimental sample**

The ITS sequences of *P. praeruptorum*, *A. decursiva*, and adulterant used for DNA barcode identification and ITS sequences, used as outgroup taxa for the construction of the phylogenetic tree, were derived from National Center for Biotechnology Information (NCBI) GenBank [Table 1]. *P. praeruptorum* and *A. decursiva* samples used for specific PCR identification were collected from Ningguo City and Jinhua County of Anhui Province. The phylogenetic tree reflecting the genetic similarity between these two kinds of TCM are shown in Table 1.

**Table 1:** Specimens and GenBank accession numbers for species used in this study. The following abbreviations are applied: NI, number of individuals; HN, Haplotype number; GAN, GenBank accession numbers.

| Genus        | Species                        | NI   | HN               | GAN                        |
|--------------|--------------------------------|------|------------------|---------------------------|
| *Pseudostem* | *Praeptorum praeruptorum*      | 5    | Hap1             | EU418383; KF806580; KF806578; EU592009; DQ132871 |
|              | *P. praeruptorum*              | 1    | Hap2             | KF806577                  |
|              | *P. praeruptorum*              | 1    | Hap3             | KF806579                  |
|              | *P. japonicum*                 | 1    | Hap4             | KP058321                  |
|              | *P. japonicum*                 | 1    | Hap5             | AB697612                  |
|              | *P. japonicum*                 | 1    | Hap6             | KF806570                  |
|              | *P. japonicum*                 | 4    | Hap7             | JF977807; JN603231; JF9777806; JF9777805 |
|              | *P. medicus*                   | 3    | Hap8             | KF806573; JF977814; JF977811 |
|              | *P. medicus*                   | 1    | Hap9             | JF977812                  |
|              | *P. terebinthaceae*            | 2    | Hap10            | KF806575; KF725035; KF725034; KF725036; KF725037; KF725038; JN603232; KF806576; AY548216 |
|              | *P. terebinthaceae*            | 9    | Hap11            | JF977822; JF977821; KF806575; KF725035; KF725034; KF725036; KF725037; KF725038; JN603232; KF806576; AY548216 |
| *Ligusticum* | *brachylobum*                  | 1    | Hap12            | DQ270205                  |
|              | *L. brachylobum*               | 1    | Hap13            | KF806583                  |
|              | *L. brachylobum*               | 1    | Hap14            | EU236173                  |
|              | *L. pteridophyllum*            | 1    | Hap26            | KF806581                  |
| *Ostericum*  | *Grosseserratum*               | 2    | Hap15            | AY548212; AY534622        |
|              | *O. grosseserratum*            | 1    | Hap16            | AF455749                  |
|              | *O. grosseserratum*            | 1    | Hap30            | KF806562                  |
|              | *O. grosseserratum*            | 1    | Hap31            | GU390409                  |
|              | *O. grosseserratum*            | 1    | Hap32            | DQ270199                  |
| *Angelica*   | *Decursiva*                    | 7    | Hap17            | JX022912; JX022911; KF806566; KF806564; DQ263563; KF806563; GU395153 |
|              | *A. decursiva*                 | 2    | Hap18            | EU592012; DQ132872        |
|              | *A. decursiva*                 | 1    | Hap19            | JN603216                  |
|              | *A. decursiva*                 | 2    | Hap20            | JN603217; JN603215        |
|              | *A. decursiva*                 | 1    | Hap21            | AY548220                  |
|              | *A. decursiva*                 | 1    | Hap22            | EU592007                  |
|              | *A. decursiva*                 | 3    | Hap23            | DQ263579; DQ263574; HQ256684 |
|              | *A. decursiva*                 | 1    | Hap24            | KF806565                  |
|              | *A. decursiva*                 | 1    | Hap25            | A131293                   |
| *Pimpinella* | *Diversifolia*                 | 1    | Hap27            | KF806585                  |
|              | *P. diversifolia*              | 1    | Hap28            | JF831517                  |
|              | *P. diversifolia*              | 1    | Hap29            | DQ516369                  |
relationships among *P. praeruptorum*, *A. decursiva*, and adulterant was constructed. The two ITS sequences of *Eryngium planum* (GenBank accession numbers EU169002 and EU070696) and one ITS sequence of *Canada Sanicula canadensis* (EU070746) were used as the outgroup, whereas the ingroup included ITS sequences of *P. praeruptorum*, *A. decursiva*, and adulterant.

**Experimental method**

**DNA extraction**

All individual samples were stored at 80°C. About 50 mg dry sample or 100 mg fresh sample were weighed. The liquid nitrogen was added and the sample was grounded. The total DNA was extracted by CTAB method and preserved at 20°C for use.[21]

**Sequence analysis and phylogenetic tree construction**

The ITS sequences of *P. praeruptorum*, *A. decursiva*, and adulterant were aligned using Clustal X 1.81 software. The sequences were edited using Bio Edit 7.0.9.0 software after alignment. The nucleotide composition and variation sites of all the sequences were counted using MEGA 4.0 software.[23] The interspecific and intraspecific genetic distances of different sequences were calculated by Kimura double parameter method.[24]

Based on all the aligned and edited ITS sequences, the phylogenetic analyses of *P. praeruptorum*, *A. decursiva*, and adulterant were conducted using Bayesian inference (BI) and maximum parsimony (MP), respectively. The BI and MP phylogenetic trees were constructed. The BI tree was constructed using MrBayes 3.1.2 software,[25] and the MP tree was constructed using PAUP*4 beta 10 software.[26] When BI tree was constructed, the optimum data model (GTR + I + G) was selected according to Akaike Information Criterion (AIC) test criterion, using MrModeltest 2.3 software.[27] Markov Chains Monte Carlo (MCMC) was set to four chains and operated for 500000 generations. To confirm the convergence of MCMC runs, two independent runs were performed. The tree was sampled after every 100th generation. A total of 10002 samples were used. After analysis, the two MCMC runs converged into the stationary distribution after 20000 generations. The total residual samples were 9602. These 1 samples were used to construct the phylogenetic tree and estimate the Bayesian posterior probabilities. For the MP analyses, bootstrap analysis using a heuristic search was performed with 1000 bootstrap replications. The algorithm used by branch-swapping was tree bisection-reconnection (TBR).

**Design of universal primers**

The Clustal X 1.81 software was used for alignment ranking and comparison for all ITS sequences. The universal primer of all sequences was designed in this study, named as TY3s/TY3a. Among them, TY3s was the upstream primer and TY3a was the downstream primer. The primer sequence was synthesized by the Shanghai Sangon Biotechnology Co., Ltd., as shown in Table 2.

**DNA template quality detection**

The one pair of primers designed in PCR reaction was used to detect the DNA template quality. The total reaction volume was 25 µL, and the following components were included: 0.5 µL (5 ng to 50 ng) of DNA template, 1 µL of upstream primer and 1 µL of downstream primer (10 pmol), 2.5 µL of 10x PCR Buffer, 1.5 µL of MgCl₂ (25 mmol·L⁻¹), 0.5 µL (10 mmol·L⁻¹) of dNTPs, 0.5 µL of *Taq* DNA polymerase (5 U·µL⁻¹), and 17.5 µL of ddH₂O. The universal primer PCR reaction procedure is shown in Table 2.

**Design of identification primer**

On the premise that *P. praeruptorum* and *A. decursiva* were monophyletic respectively, two pairs of specific PCR identification primers were designed to distinguish between *P. praeruptorum* and *A. decursiva*. One was specific PCR identification primer with *P. praeruptorum* as the genuine drug, while the other was specific PCR identification primer using *A. decursiva* as the genuine drug. The Clustal X 1.81 software was used for alignment ranking and comparison for all ITS sequences. The differential fragments were determined. The results showed that T was in 11th bp of *P. praeruptorum*, the ITS1 sequence, and G was in the corresponding site of *A. decursiva*. According to the abovementioned specific sites of variation [single nucleotide polymorphisms (SNP) sites], one pair of specific PCR primers was designed by Primer Premier 5.0 software using *P. praeruptorum* as the genuine drug. The forward primer was named QH-CP19s, and the reverse primer was named QH-CP19a. The forward primer QH-CP19s 3’ terminal base must be located at the SNP site. To improve the specificity of *P. praeruptorum* primer, the second mismatched base must be artificially introduced in QH-CP19s 3’ terminal.[28] The 3’ terminal base T was strongly mismatched (T/C), the second mismatched base A was also introduced in the third of its 3’ terminal to form a weak mismatch (A/G).[29] The *P. praeruptorum*-specific identification primers were designed according to the above principles, shown in Table 2.

The results showed that T was in 12th bp of 5.88 rRNA sequence between *A. decursiva* ITS1 and ITS2 by using the same method above, and C was in the corresponding site. One pair of specific primers with *A. decursiva* as the genuine drug, was designed according to the above specific SNP sites. The forward primer was named ZHQH-CP3s, and the reverse primer was named ZHQH-CP3a. The 3’ terminal base of the forward primer ZHQH-CP3s must be located at the SNP site. To improve the specificity of the primer, the second mismatched base was artificially introduced to the 3’ terminal of ZHQH-CP3s. The 3’ terminal base T was weakly mismatched (T/G), so the second mismatched base A was introduced to the second site to form strong mismatches (A/G).

Based on the above mentioned principles, the specific identification primer pairs QH-CP19s/QH-CP19a and ZHQH-CP3s/ZHQH-CP3a designed for *P. praeruptorum* and *A. decursiva*, respectively, were synthesized by Shanghai Sangon Biotechnology Co., Ltd., shown in Table 2.

**Table 2:** Primers and PCR reaction conditions. The lowercase letter in Primer sequence is the second mismatched base artificially introduced. LPP indicates length of PCR product. PRP indicates PCR reaction procedure.

| Primer name | Primer sequence (5’→3’) | LPP | PRP |
|-------------|--------------------------|-----|-----|
| TY3s        | GGAATGCGGCAAGG           | 187 bp | 95°C for 30 min, 25 cycles |
| TY3a        | TGGGTTCAAAAAGACTCGA      |     |     |
| QH-CP19s    | TGGCCACCCGGGATT          | 485 bp | (degeneration at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s) |
| QH-CP19a    | GCTAAGGGTCCTGATCTC       |     |     |
| ZHQH-CP3s   | CAGCGATGCTATGGCAT        | 252 bp | extension at 72°C for 10 min |
| ZHQH-CP3a   | TAGTCCCCGGCCTGACCTG      |     |     |
Determination of specific PCR amplification conditions

The P. praeruptorum and A. decursiva DNA samples were used to determine the specific PCR amplification conditions.

The total reaction volume was 25 µL and comprised the following: 0.5 µL (5 ng to 50 ng) of DNA template, 1 µL (10 pmol) of upstream primer and 1 µL (10 pmol) of downstream primer, 2.5 µL of 10×PCR Buffer, 1.5 µL (25 mmol·L⁻¹) of MgCl₂, 0.5 µL (10 mmol·L⁻¹) of NTPs, 0.5 µL (5u/µL) of Taq DNA polymerase, and 17.5 µL of ddH₂O.

The specific PCR reactions were performed using P. praeruptorum and A. decursiva identification primer pairs QH-CP19s/QH-CP19a and ZHQH-CP3s/ZHQH-CP3a, respectively. The following amplified reaction procedures were investigated and optimized.

Annealing temperatures: 49, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 and 68 C. Cycle number: 20, 25, 30, 35 cycles. DNA template dosage: In the reactions where P. praeruptorum was used as the genuine drug, DNA template dosages were 0.657, 1.313, 2.625, 5.25, 10.5, 21, and 42 ng, respectively, after double-dilution. In the reactions with A. decursiva as the genuine drug, the template DNA dosages were 2.25, 4.5, 9, 18, 36, 72, and 144 ng. The Taq enzyme species are: Hot Start Taq DNA Polymerase, Taq DNA Polymerase, and Taq Plus DNA Polymerase. PCR instruments: MJ research PTC-200 Peltier Thermal Cycler (Bio-Rad company), Professional standard Thermocycler (Biomera company) and Light Cycler® 96 System (Roche company).

PCR product detection

At the end of the PCR runs, 5 µL of amplified reaction products was added to 1 µL of 6×Loading buffer. The sample was detected using 2% agarose gel electrophoresis, stained with EB, and photographed with the full-automatic gel-imaging analyzer. The blank control, negative control, and positive control groups without template DNA were included in the experiment. The experiment was repeated thrice.

RESULTS

Sequence analysis and phylogenetic tree construction

Sequence analysis

The partial ITS homologous fragment sequences of P. praeruptorum, A. decursiva, and related adulterants and outgroup were compared and edited. The length of the sequence fragment was 632 bp (including Gap). A total of 290 variants were obtained. Of these variants, 231 were simple informative sites. The average G + C content of P. praeruptorum sequence was 56.4%, and the average G + C content of A. decursiva was 56.6%. Therefore, there was little difference in the average G + C content of P. praeruptorum and A. decursiva ITS sequences. The intraspecific genetic distance of P. praeruptorum was 0.000 to 0.000, and the interspecific genetic distance between P. praeruptorum and other species was 0.021 to 0.324. The intraspecific genetic distance of A. decursiva was 0.000 to 0.018, and interspecific genetic distance was 0.030 to 0.329. The intraspecific genetic distance of P. praeruptorum was 0.000. The minimum interspecific genetic distance was larger than intraspecific genetic distance. Therefore, the intraspecific genetic distances of P. praeruptorum and A. decursiva were smaller than the interspecific genetic distances of other adulterants.

Phylogenetic tree of P. praeruptorum, A. decursiva, and adulterant

The results show that the phylogenetic trees constructed using the BI and MP methods were consistent [Figure 1]. The posterior probability (PP) of BI tree and Bootstrap (BS) of MP tree were labeled in the nodes of pedigree branches. The results also showed that P. praeruptorum and A. decursiva sequence haplotypes formed their own monophyly.
QUN ZHAO, et al.: Identification between two kinds of TCM and adulterant

(P. praeruptorum: PP = 1.00, BS = 99; A. decursiva: PP = 1.00, BS = 99). Therefore, P. praeruptorum and A. decursiva, and adulterant could be obviously distinguished using the BI tree and MP tree based on P. praeruptorum and A. decursiva. In addition, P. praeruptorum and other Peucedanum species (including P. japonicum, P. medicum, and P. terebinthaceum) and Ligusticum short fragment clustered in a larger branch (PP = 0.98, BS = 86). The A. decursiva and Ostericum grosseserratum were clustered in another larger branch (PP = 1.00, BS = 91), thereby suggesting that P. praeruptorum and A. decursiva does not belong to the same genus.

**Determination of template DNA quality by universal primer**

The P. praeruptorum and A. decursiva DNA were amplified using the universal primer TY3s/TY3a designed in the study. The DNA template quality of the sample was detected. The Agarose gel electrophoresis results showed that target bands, at around 187 bp, were amplified from P. praeruptorum and A. decursiva samples DNA [Figure 2], indicating that the template DNA quality of the sample was in accordance with the requirements of PCR reaction in the experiment.

**Validation of specific PCR identification primer**

The PCR amplifications of P. praeruptorum and A. decursiva samples DNA were performed using the differential primers QH-CP19s/QH-CP19a with P. praeruptorum as the genuine drug and ZHQH-CP3s/ZHQH-CP3a with A. decursiva as the genuine drug. Results showed that a band ~485 bp in length was amplified from P. praeruptorum using the identification primer with P. praeruptorum as the genuine drug. However, the band was not amplified from the adulterant A. decursiva [Figure 3a]. A band with the size of 252 bp was amplified from A. decursiva using the identification primer and was not amplified from adulterant P. praeruptorum [Figure 3a]. The band was not detected in the blank control group, suggesting that no interference occurred. The abovementioned experimental results showed that the primer pairs QH-CP19s/QH-CP19a and ZHQH-CP3s/ZHQH-CP3a could be used as specific PCR identification primers for P. praeruptorum and A. decursiva, respectively. Therefore, the identification primers were used to optimize the specific PCR reaction conditions of P. praeruptorum and A. decursiva.

**Optimization of PCR reaction conditions**

**Specific PCR reaction condition optimization of genuine P. praeruptorum**

When the annealing temperature increased from 48°C to 61°C, the sample DNA could be effectively amplified. The luminance of the amplified product target bands was stronger in agarose gel electrophoresis. When the annealing temperature increased from 48°C to 61°C, the sample DNA was performed using the differential primers QH-CP19s/QH-CP19a and ZHQH-CP3s/ZHQH-CP3a with A. decursiva as the genuine drug and ZHQH-CP3s/ZHQH-CP3a with A. decursiva as the genuine drug. Results showed that a band ~485 bp in length was amplified from P. praeruptorum using the identification primer with P. praeruptorum as the genuine drug. However, the band was not amplified from the adulterant A. decursiva [Figure 3a]. A band with the size of 252 bp was amplified from A. decursiva using the identification primer and was not amplified from adulterant P. praeruptorum [Figure 3a]. The band was not detected in the blank control group, suggesting that no interference occurred. The abovementioned experimental results showed that the primer pairs QH-CP19s/QH-CP19a and ZHQH-CP3s/ZHQH-CP3a could be used as specific PCR identification primers for P. praeruptorum and A. decursiva, respectively. Therefore, the identification primers were used to optimize the specific PCR reaction conditions of P. praeruptorum and A. decursiva.

When the annealing temperature increased from 48°C to 61°C, the sample DNA could be effectively amplified. The luminance of the amplified product target bands was stronger in agarose gel electrophoresis. When the annealing temperature increased from 48°C to 61°C, the sample DNA was performed using the differential primers QH-CP19s/QH-CP19a and ZHQH-CP3s/ZHQH-CP3a with A. decursiva as the genuine drug and ZHQH-CP3s/ZHQH-CP3a with A. decursiva as the genuine drug. Results showed that a band ~485 bp in length was amplified from P. praeruptorum using the identification primer with P. praeruptorum as the genuine drug. However, the band was not amplified from the adulterant A. decursiva [Figure 3a]. A band with the size of 252 bp was amplified from A. decursiva using the identification primer and was not amplified from adulterant P. praeruptorum [Figure 3a]. The band was not detected in the blank control group, suggesting that no interference occurred. The abovementioned experimental results showed that the primer pairs QH-CP19s/QH-CP19a and ZHQH-CP3s/ZHQH-CP3a could be used as specific PCR identification primers for P. praeruptorum and A. decursiva, respectively. Therefore, the identification primers were used to optimize the specific PCR reaction conditions of P. praeruptorum and A. decursiva.

The PCR reaction conditions of QH-CP19s/QH-CP19a were optimized according to 2.4.1 method. Optimized conditions were as follows: annealing temperature was 55°C [Figure 4b], a total of 25 cycles [Figure 4c], and the amount of template DNA was 36 ng to 72 ng [Figure 4d]. The same identification results could be achieved with different Taq DNA polymerases. The target band was amplified from genuine P. praeruptorum and was not amplified from A. decursiva [Figure 3e]. The sample was amplified under the abovementioned optimized reaction conditions with three different Taq DNA polymerases. The same identification results could be achieved with different PCR instruments [Figure 4e] and three different PCR instruments [Figure 4f].

**Establishment of specific PCR identification method**

The specific PCR identification method for P. praeruptorum is as follows: The total reaction volume was 25 µL, which includes the following components: 1 µL of (~42 ng) DNA template, 1 µL (of 10 pmol) QH-CP19s primer and 1 µL (10 pmol) of QH-CP19a primer, 2.5 µL of 10 PCR buffer, 1.5 µL (25 mmol·L⁻¹) of MgCl₂, 0.5 µL (10 mmol·L⁻¹) of dNTPs, 0.5 µL (5 µmol/L) of Taq DNA polymerase, and 17.5 µL of ddH₂O. The reaction conditions were as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 min, annealing at 55°C for 30 s, extension at 72°C for 30 s, 25 cycles; extension at 72°C for 10 min. The specific PCR identification procedure for A. decursiva is as follows: 25 µL of total reaction system, including 1 µL of DNA template (about 36 ng to 72 ng), 1 µL (10 pmol) of QH-CP19s primer and 1 µL (10 pmol) of QH-CP19a primer, 2.5 µL of 10 PCR buffer, 1.5 µL (25 mmol·L⁻¹) of MgCl₂, 0.5 µL (10 mmol·L⁻¹) of dNTPs, 0.5 µL (5 µmol/L) of Taq DNA polymerase, and 17.5 µL of ddH₂O. The reaction cycle was as follows: initial denaturation occurred at 95°C for 5 min, degeneration at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, 25 cycles; extension at 72°C for 10 min. The sample was identified according to whether the target band could be amplified using their respective identification primers under the optimized PCR reaction conditions.

**DISCUSSION**

The G + C contents of ITS sequences in P. praeruptorum and A. decursiva were very similar and were higher than that the A + T contents, which was consistent with the conclusion of Huajie Xue. The ITS is the
QUN ZHAO, et al.: Identification between two kinds of TCM and adulterant

During PCR, the sequence was extended along 5’–3’. Optimization of different influence factors in specific PCR of

Pharmacognosy Magazine, 2017, Vol 13, Issue 49

Based on the DNA barcodes and the sequence characteristics of the genuine drug and adulterant, the specific PCR identification primers were designed to quickly distinguish genuine drug and adulterant, the specific PCR identification primers were designed based on ITS sequences with MP and BI methods showed.

Figure 3: Optimization of different influence factors in specific PCR of P. praeruptorum.

M: DNA Marker (containing a mix of 6 individual DNA fragments from top to bottom denoting 600, 500, 400, 300, 200, and 100 bp). A Amplification results using P. praeruptorum identification primer pairs QH-CP19α/QH-CP19a: 1, 2, and 3 indicate A. decursiva; 4, 5, and 6 indicate P. praeruptorum; 7 indicates negative control without DNA template. B. Annealing temperatures: numbers from 1 to 21 indicate annealing temperatures of P. praeruptorum with 48, 49, 51, 50, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 and 68 °C; numbers from 22 to 42 indicate annealing temperatures of A. decursiva with 48, 49, 51, 50, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 and 68 °C. C. Cycle numbers: numbers from 1 to 4 indicate cycle numbers of P. praeruptorum with 20, 25, 30, and 35 cycles; numbers from 5 to 8 indicate cycle numbers of A. decursiva with 20, 25, 30, and 35 cycles. D. DNA template dosages: numbers from 1 to 7 indicate DNA template dosages of P. praeruptorum with 42, 21, 10.5, 5.25, 2.625, 1.313, and 0.657 ng; numbers from 8 to 14 indicate DNA template dosages of A. decursiva with 42, 21, 10.5, 5.25, 2.625, 1.313, and 0.657 ng. E. Taq enzyme species: a indicates Taq DNA Polymerase, b indicates Taq Plus DNA Polymerase, c indicates Hot Start Taq DNA Polymerase, 1 indicates P. praeruptorum, and 2 indicates A. decursiva. F. PCR instruments: a indicates LightCycler ®96 System (Roche company), b indicates PCR instruments such as LightCycler ®96 System (Roche company), b indicates Professional standard Thermocycler (Biometra company), c indicates MJ research PTC-200 Peltier Thermal Cycler (Bio-Rad company), 1 indicates P. praeruptorum, and 2 indicates A. decursiva.

The specific PCR identification primers were designed based on the differential bases in the ITS sequence of P. praeruptorum and A. decursiva. During PCR, the sequence was extended along 5’–3’ direction of the primer. The Taq DNA polymerase lacks 3’–5’excision activity, so if the primer was mismatched at 3’ terminal, the amount of amplified product would be lower than the efficient extension of paring primers at the normal 3’ terminal. Thus, the target band can be amplified from the high-efficiency correct primer pairs at the appropriate number of PCR cycles. The amount of amplified product was lower when the mismatched primers were used, and the target band, to distinguish between genuine and adulterant samples, was not detected after a certain number of PCR cycles. Therefore, the primer for P. praeruptorum should be designed such that the 3’ terminal correctly matches the bases of P. praeruptorum and is mismatched with A. decursiva. On the contrary, the 3’ terminal of the identification primer for the A. decursiva should be correctly matched with A. decursiva bases and mismatched with P. praeruptorum bases. To increase the specificity of the identification primer, the second mismatched base was artificially introduced into 3’ terminal of the primer to improve PCR results. Therefore, the identification primers were used to amplify the corresponding genuine drug and adulterant. After 25 and
Figure 4: Optimization of different influence factors in specific PCR of A. decursiva. 
M: DNA Marker (containing a mix of 6 individual DNA fragments from top to bottom denoting 600, 500, 400, 300, 200, and 100 bp). A. Amplification results using A. decursiva identification primer pairs ZHQH-CP3s/ZHQH-CP3a: 1, 2, and 3 indicate A. decursiva; 4, 5, and 6 indicate P. praeruptorum; 7 indicates negative control without DNA template. B. Annealing temperatures: numbers from 1 to 21 indicate annealing temperatures of A. decursiva with 48, 49, 51, 50, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 and 68 °C; numbers from 22 to 42 indicate annealing temperatures of P. praeruptorum with 48, 49, 51, 50, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 and 68 °C. C. Cycle numbers: numbers from 1 to 4 indicate cycle numbers of A. decursiva with 20, 25, 30, and 35 cycles; numbers from 5 to 8 indicate cycle numbers of P. praeruptorum with 20, 25, 30, and 35 cycles. D. DNA template dosages: numbers from 1 to 7 indicate DNA template dosages of A. decursiva with 144, 72, 36, 18, 9, 4.5 and 2.25 ng; numbers from 8 to 14 indicate DNA template dosages of A. decursiva with 144, 72, 36, 18, 9, 4.5 and 2.25 ng. E. Taq enzyme species: a indicates Taq DNA Polymerase, b indicates Taq Plus DNA Polymerase, 1 indicates Hot Start Taq DNA Polymerase, 1 indicates A. decursiva, and 2 indicates P. praeruptorum. F. PCR instruments: a indicates LightCycler® 96 System (Roche company), b indicates Professional standard Thermocycler (Biometra company), c indicates M research PTC-200 Peltier Thermal Cycler (Bio-Rad company), 1 indicates A. decursiva, and 2 indicates P. praeruptorum

30 cycles, the target bands were amplified from the genuine drugs and not from the adulterant because of decreased amplified efficiency caused by the mismatch. When the number of cycles was set to 35, the amplification efficiency of the mismatched primer was reduced, but the target bands appeared in the adulterants because the number of cycles was sufficient for amplification. Therefore, to ensure the accuracy of specific PCR identification, PCR reaction cycles should be optimized. The specific PCR procedures were obtained by designing PCR primer pairs specific for P. praeruptorum and A. decursiva and by optimizing the amplification conditions. The procedure allowed rapid optimizing the amplification conditions. The procedure allowed rapid

Conflicts of interest
There are no conflicts of interest.

REFERENCES
1. Pharmacopoeia Committee of the People’s Republic of China. Pharmacopoeia of the People’s Republic of China. Chemical Industry Press, Beijing 2010:1 pp 97–124, 246.
2. Wang CH, Tong OZ. Primary determination of content of coumarin in different species form Radix Peucedani by HPLC. China Pharmacist 2004;7:361–63.
3. Liu CS, Wang PY, Chen ZH, Wang DQ. Study on molecular evidence of revision of taxonomic placement of Peucedanum decursivum. China Journal of Chinese Materia Medica 2006;31:1488–90.
4. Zhou GL, Liu YJ, Ren SL, Shu K, Nie LX, Liu TS, et al. Analysis of volatile oil components of Peucedanum praeruptorum and Peucedanum decursivum. J TCM Univ of Hunan 2010;30:26–28.
5. Committee of the flora of Jiangsu. Flora of Jiangsu. Nanjing: Jiangsu people’s Press. 1982:581.
6. Shan RH, She ML. Flora of China: 55 (3), Angiospermae, Dicotyledoneae, Umbelliferae (3). Science Press 1992.
7. Xue HJ, Yan MH, Wang NH, Wang ML, Lu CM, Wu GR, et al. Relationship Between Angelica and Peucedanum and the Phylogenetic Position of Angelica decursiva / Peucedanum.
Decursivum based on the evidences from ITS sequences. J Nanjing Normal University 2007;30:97-1.

8. Li SF, Zuo XF, Chen QM. Pharmacognostical identification of Radix Peucedani and its forgery-Radix Osterici. The Chinese Pharmaceutical Association 1999;16:23-25.

9. Zhou Y. Identification of Radix Peucedani and its forger-Radix Peucedani Terebinthaceum. China Pharmaceuticals 2008;17:62-63.

10. Qie C, Zuo JZ. SDS-PAGE to identify with Purple Peucedanum and Peucedanum Praeruptorum Dunn and Counterfeit. J HuBei University of Chinese Medicine 2012;14:30-32.

11. Xiong YX, Wu L, Liu YM, Chen QM. Pharmacognostical identification of Radix Peucedani and its forgery-Radix Peucedani. The Chinese Pharmaceutical Association 1999;16:23-25.

12. Zhou Y. Identification of Radix Peucedani and its forger-Radix Peucedani Terebinthaceum. China Pharmaceuticals 2008;17:62-63.

13. Qi C, Chen ZJ. SDS – PAGE to identify with Purple Peucedanum and Peucedanum Praeruptorum Dunn and Counterfeit. J Hubei University of Chinese Medicine 2012;14:30-32.

14. Xiong YX, Wu L, Liu YM, Chen KL. Identification of Peucedani Radix and Its Adulterants by DNA Barcoding Technique. Journal of Chinese Medicinal Materials 2013;36:1782-85.

15. Liu BM, Xu Q. Analysis of dl-Praeruptorin A, dl-Praeruptorin B and Nodakenetin in Qianhu by GC/MS and GC. Guanxi Sciences 2002;9:294-97.

16. Hu TJ, Pan HH, Pu JB, Xu Y, Liang WQ, Zhen JX et al. HPLC Fingerprints of Peucedanum Praeruptorum Dunn. China science and technology of Chinese medicine 2012; 19:437-39.

17. Lee SB, Rasmussen SK. Molecular markers in some medicinal plants of the Apiaceae family. Euphytica 2000;11:43-91.

18. Zhao ZL, Zhou KY, Dong H, Xu LS. Characters of ndDNA ITS region sequences of fruits of Alpinia galangal and their adulterants. Planta Medica 2001;67:381-83.

19. Yuan GJ, Zhang B, Jiang D, Zhang WJ, Lin TV, Wang NH et al. Identification of species and material media within Angelica L. (Umbelliferae) based on phylogeny inferred from DNA barcodes. Molecular Ecology Resources 2015;15:365-71.

20. Tang SY, Fu W, Chen YJ, Wang JY, Jiang X, Zhang YP et al. Research on the Identification of Penis et Testis Cervi with Molecular Taxonomy. China Journal of Chinese Materia Medica 2002;27:573-75.

21. Sun XQ, Wei YL, Guo JL, Zhou YF, Hang YY. Authentication of an Endangered Herb Changium amygdoides from Different Producing Areas Based on rDNA ITS Sequences and Allele-Specific PCR. Archives of Pharmacal Research 2012;35:701-8.

22. Qiang YZ, Tong Q, Wang F, Wu PC, Yang SL, Gong Y et al. Use of a rapid mismatch PCR method to detect gyrA and parC mutations in ciprofloxacin resistant clinical of Escherichia coli. The Journal of Antimicrobial Chemotherapy 2002;49:549-52.