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ORIGINAL ARTICLE

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

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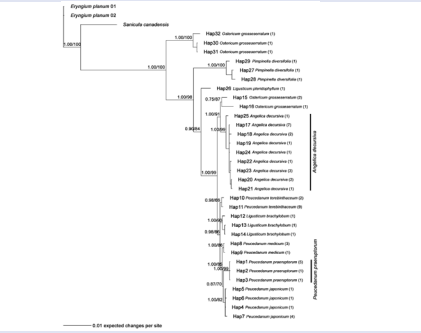
ABSTRACT

Background: The traditional Chinese medicine (TCM) Qianhu and Zihuaqianhu are the dried roots of *Peucedanum praeruptorum* and *Angelica decursiva*, respectively. Since the plant sources of Qianhu and Zihuaqianhu 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*), Zihuaqianhu (*Angelica decursiva*), and common adulterants, based on 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 ZHQH-CP3s/ZHQH-CP3a 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 *P. praeruptorum* and *A. decursiva* sequence haplotypes 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 suggests 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.
- The ITS sequence was ideal DNA barcode to identify *P. praeruptorum*, *A. decursiva*, and adulterant.
- Specific PCR is a quick and effective method to distinguish between *P. praeruptorum* and *A. decursiva*.



Abbreviations used: TCM: The traditional Chinese medicine, *P.: Peucedanum, A.: Angelica*, ITS: The internal transcribed spacer, PCR: Polymerase chain reaction, NCBI: National Center for Biotechnology Information, NI: Number of individuals, HN: Haplotype number; GAN: Gen Bank accession numbers, *L.: Ligusticum, O.: Ostericum, A.: Angelica, P.: Pimpinella,* BI: Bayesian inference, MP: Maximum parsimony, AIC: Akaike Information Criterion, MCMC: Markov Chains Monte Carlo, TBR: Tree bisection-reconnection, LPP: Length of PCR product, PRP: PCR reaction procedure, SNP: Single nucleotide polymorphisms, PP: Posterior probability, BS: Bootstrap.Qun Zhao

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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. Zihuaqianhu is the dried root of *Angelica decursiva* and has the same efficacy as *P. praeruptorum*.[1] However, the chemical compositions of *P. praeruptorum* and *A. decursiva* are significantly different.[2-4] The taxonomic status of *A. decursiva* is controversial, vacillating between *Peucedanum* and *Angelica*.[1,3,5-7] The plant sources of Qianhu and Zihuaqianhu are more complex, and many adulterants exist because of the differences in traditional understanding and medication habits.[8-11] Moreover, Qianhu and Zihuaqianhu are easily confused in practical applications, because the morphological characteristics of these two kinds of TCM are quite

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similar, and difficult to be identified according to their appearance characteristics.[6,11] 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.[1,9,12,13] 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.[14-16] 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.[11] The phylogenetic tree of *P. praeruptorum* and *A. decursiva* was constructed only using neighbor-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 Jinqianbaihuashe. In this paper, the feasibility of differentiating among *P. praeruptorum*, *A. decursiva*, and adulterant using ITS barcode sequences, was investigated.[17-20] 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.); SYQ-DSX-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 Jinzhai County of Anhui Province. The phylogenetic tree reflecting the genetic

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
Peucedanum	*Peucedanum 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; JF977806; JF977805
	P. medicum	3	Hap8	KF806573; JF977814; JF977811
	P. medicum	1	Hap9	JF977812
	P. terebinthaceum	2	Hap10	JF977822; JF977821
	P. terebinthaceum	9	Hap11	KF806575; KF725035; KF725034; KF725036; KF725037; KF725038; JN603232; KF806576; AY548216
Ligusticum	*Ligusticum brachylobum*	1	Hap12	DQ270205
	L. brachylobum	1	Hap13	KF806583
	L. brachylobum	1	Hap14	EU236173
	L. pteridophyllum	1	Hap26	KF806581
Ostericum	*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	*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	AJ131293
Pimpinella	*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 ClustalX 1.81 software. The sequences were edited using Bio Edit 7.0.9.0 software[22] 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 M rModeltest 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 l 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 public conservative area. This l primer was used in the PCR reaction to test the quality of DNA template. One pair of universal primers 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 $MgCl_2$ (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 ddH_2O. 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 111th 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/C).[28] 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 5.8S 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	GGAATGCGCCAAGG	187 bp	95 °C for 30 min 25 cycles (degeneration at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s) extension at 72 °C for 10 min
TY3a	TGCGTTCAAAGACTCGA		
QH-CP19s	TGGCCACTCCCGGaTT	485 bp	
QH-CP19a	GCCTAAGGGTCCTGAATCTC		
ZHQH-CP3s	CACGCATCGTATTGCaT	252 bp	
ZHQH-CP3a	TAGTCCCGCCTGACCTG		

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 $MgCl_2$, 0.5 μL (10 mmol·L^{-1}) of dNTPs, 0.5 μL (5u/μL) of *Taq* DNA polymerase, and 17.5 μL of ddH_2O.

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, 50, 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 (Biometra 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 was 56.4%, and the average G + C content of *P. praeruptorum* sequence was 54.4%. 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 0.021, far from intraspecific genetic distances. For *A. decursiva*, the maximum intraspecific genetic distance was 0.018, and the minimum interspecific genetic distance was 0.030. 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

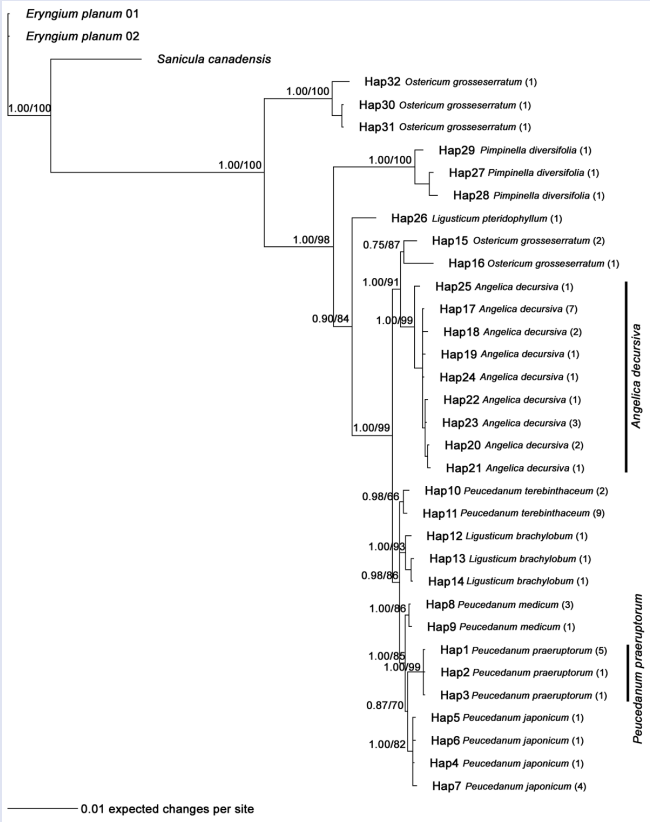

Figure 1: The partitioned Bayesian phylogenetic tree based on partial ITS regions. Numbers above the branches represent Bayesian posterior probabilities (PP) and MP bootstrap (BS) values. Taxa are haplotypes; all haplotype designations are listed in Table 1, followed by the species and numbers of individuals from each species having that haplotype [e.g., *Ostericum grosseserratum* (1)].

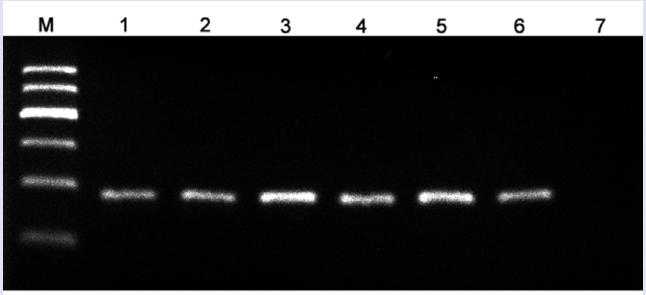

Figure 2: Amplification result of *P. praeruptorum* and *A. decursiva* by universal primer. **M**: DNA Marker (containing a mix of 6 individual DNA fragments from top to bottom denoting 600, 500, 400, 300, 200, and 100 bp); 1, 2, 3: *P. praeruptorum*; 4, 5, 6: *A. decursiva*; 7: negative control without DNA template.

(*P. praeruptorum*: PP = 1.00, BS = 99; *A. decursiva*: PP = 1.00, BS = 99). Therefore, *P. praeruptorum*, *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*s 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 4a]. 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 was increased from 62°C to 64°C, the luminance of the amplified product bands was very weak. When the annealing temperature increased from 65°C to 66°C, the target bands appeared. However, the luminance of the amplified product bands was very weak and difficult to observe. But when the annealing temperature was increased from 67°C to 68°C, the amplified bands did not appear [Figure 3b] and also it did not appear at various given temperatures for the luminance. In the present paper, the annealing temperature was set to 55°C.

At 20 cycles, the band was not amplified from *P. praeruptorum* and Theluminance. At 25 cycles, a clear band with strong luminance was amplified from *P. praeruptorum*. The band was not amplified from *A. decursiva*. When the cycle index was 30, the target band of *P. praeruptorum* was brighter, but the target band of *A. decursiva* was very weak, and no amplified band was observed. At 35 cycles, the band was amplified from *P. praeruptorum* and *A. decursiva*. The *P. praeruptorum* had higher band specificity, and only one band was observed. The various non-specific bands were amplified from *A. decursiva*. Therefore, the genuine *P. praeruptorum* and *A. decursiva* could be distinguished after 25 and 30 cycles, respectively. The best results were obtained after 25 cycles. To shorten the reaction time and improve the detection efficiency and reliability of results, 25 cycles were adopted in the PCR reaction [Figure 3c].

When the DNA template amount was increased from 0.657 ng to 2.625 ng, the amplified band did not appear in *P. praeruptorum* and *A. decursiva*. When the amount of template was increased from 5.25 ng to 42 ng, the target band was amplified in *P. praeruptorum*, but not in *A. decursiva*. The highest intensity of the target band was obtained when the amount of DNA template was 42 ng, whereas the bands amplified from the remaining three templates were very weak. As for PCR amplification effect, the amount of template DNA was set to ~42 ng [Figure 3d].

The PCR reaction was performed under the abovementioned optimized reaction conditions using three different *Taq* DNA polymerases. The same 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 PCR instruments. The same identification results could be achieved with different PCR instruments. The target band was amplified from genuine *P. praeruptorum* and was not amplified from *A. decursiva* [Figure 3f].

Specific PCR reaction conditions optimization of genuine 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 three different *Taq* DNA polymerases [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 $MgCl_2$, 0.5 μL (10 mmol·L^{-1}) of dNTPs, 0.5 μL (5 u/μL) of *Taq* DNA polymerase, and 17.5 μL of ddH_2O. 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 30s, 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, 2.5 μL of 10 PCR buffer, 1.5 μL (25 mmol·L^{-1}) of $MgCl_2$, 0.5 μL (10 mmol·L^{-1}) of dNTPs, 0.5 μL (5 u/μL) of *Taq* DNA polymerase, and 17.5 μL of ddH_2O.The initial denaturation occured 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, and 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.[7] The ITS is the

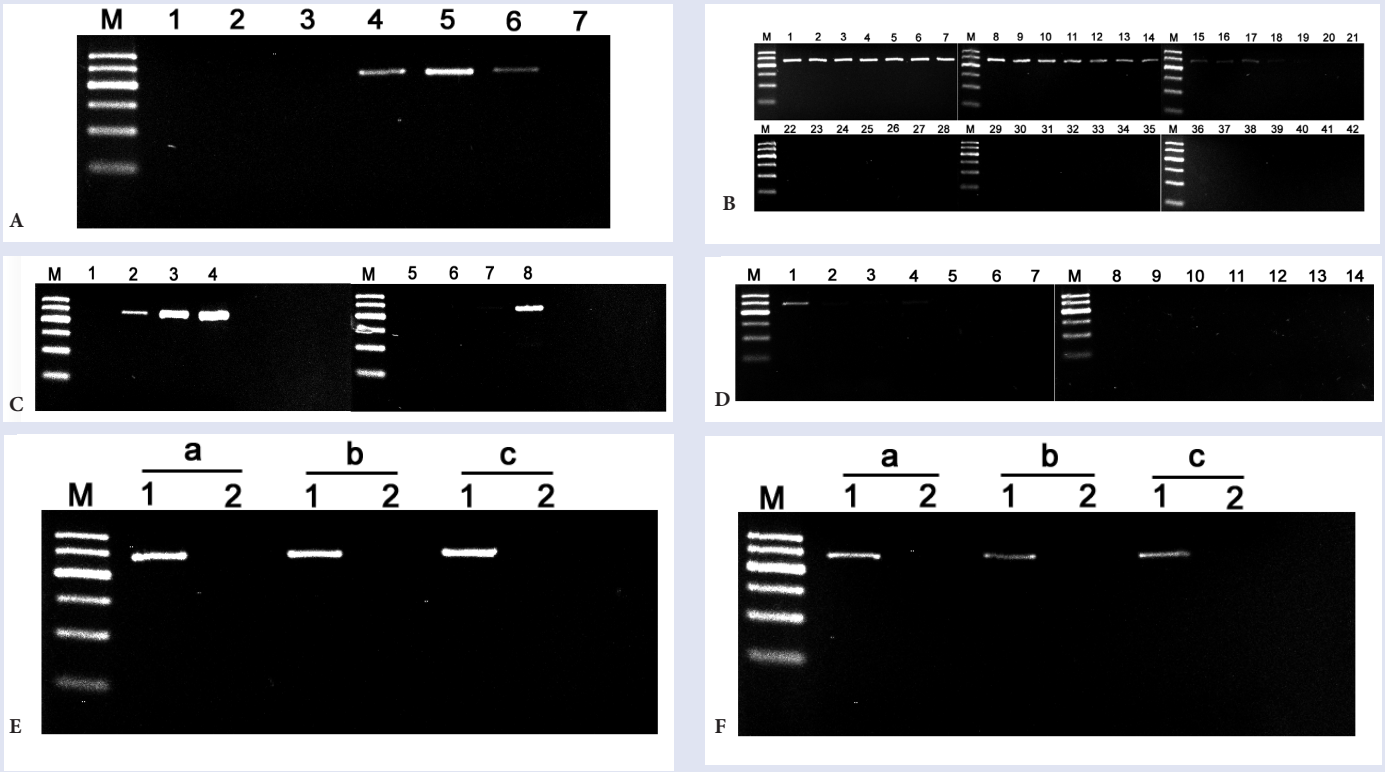

Figure 3: Optimization of different influence factors in specific PCR of *P. praeruptorum*.

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-CP19s\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 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*.

ribosomal rDNA gene RNA ITS region. The evolutionary rate of the ITS sequence was faster.[16,29,30] The interspecific variation was larger and the intraspecific conservatism was higher. Thus, the ITS fragment was an ideal DNA barcode to identify the flowering plants. Based on ITS sequence analyses of *P. praeruptorum*, *A. decursiva*, and adulterant, the intraspecific genetic distances of *P. praeruptorum* and *A. decursiva* were less than the interspecific genetic distances between *P. praeruptorum* and adulterant and between *A. decursiva* and adulterant, respectively. The phylogenetic trees of *P. praeruptorum*, *A. decursiva*, and adulterant constructed based on ITS sequences with MP and BI methods showed that all haplotype sequences of *P. praeruptorum* and *A. decursiva* were monophyletic, respectively. Thus the ITS sequence could be used as DNA barcoding for the identification of *P. praeruptorum*, *A. decursiva*, and adulterant. However, when ITS sequence was used as a DNA barcode to distinguish between these two kinds of TCM and their adulterants, DNA extraction and PCR amplification, purification, sequencing, analysis, and construction of phylogenetic trees had to be performed. The entire process was complex and required massive effort.

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 *P. praeruptorum* and *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.[31] 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.[21,32] Therefore, the identification primers were used to amplify the corresponding genuine drug and adulterant. After 25 and

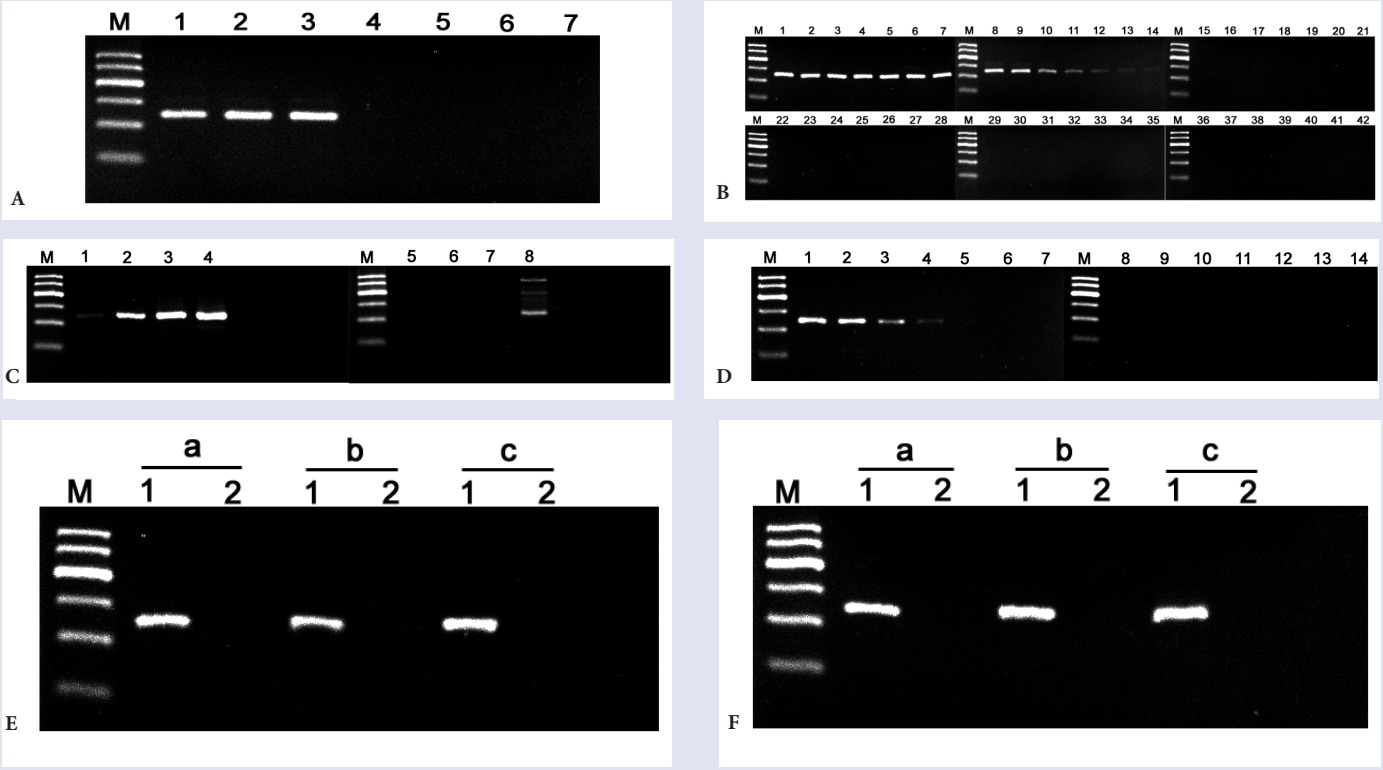

Figure 4: Optimization of different influence factors in specific PCR of *A. decursiva*.

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, c 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 MJ 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 identification between *P. praeruptorum* and *A. decursiva* ,and provided a reference for the authenticity of the other TCM. Meanwhile, the phylogenetic tree analysis in the study showed that *P. praeruptorum* and *A. decursiva* did not belong to the same genus, supporting the view that *A. decursiva* was different from *Peucedanum*.[6]

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Pharmacopoeia Comittee of the People's Republic of China. Pharmacopoeia of the People's Republic of China. Chemical Industry Press, Bejing.2010;1:pp 97-124, 246.
2. Wang CH, Tong QZ. 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, Zhu 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. 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.
11. 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;36:1762-65.
12. 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.
13. 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.
14. Lee SB, Rasmussen SK. Molecular markers in some medicinal plants of the Apiaceae family. Euphytica 2000;114:87-91.
15. Zhao ZL, Zhou KY, Dong H, Xu LS. Characters of nrDNA ITS region sequences of fruits of *Alpinia galangal* and their adulterants. Planta Medica 2001;67:381-83.
16. Yuan QJ, Zhang B, Jiang D, Zhang WJ, Lin TY, Wang NH, *et al*. Identification of species and *material medica* within *Angelica* L. (Umbelliferae) based on phylogeny inferred from DNA barcodes. Molecular Ecology Resources 2015;15:358-71.
17. 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.
18. Sun XQ, Wei YL, Guo JL, Zhou YF, Hang YY. Authentication of an Endangered Herb *Changium smyrnioides* from Different Producing Areas Based on rDNA ITS Sequences and Allele-Specific PCR. Archives of Pharmacal Research 2012;35:701-8.
19. Zhao D, Zhou T, Jiang WK, Yuan Y, Xiao CH, Zheng W, *et al*. Rapid molecular identification of Pseudostellariae Radix. China Journal of Chinese Materia Medica 2014;39:3690-94.
20. Chen K, Jiang C, Yuan Y, Huang LQ, Li M. Application of rapid PCR to authenticate medicinal snakes. China Journal of Chinese Materia Medica 2014;39:3673-77.
21. Huang LQ, Yuan Operational guidelines molecular identification of Chinese herbs. Shanghai: Shanghai science and technology Press. 2014.
22. Hall TA. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium 1999;41:95-98.
23. Tamura K, Dudley J, Nei M, Kumar S. MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 2007;24:1596-99.
24. Kimura M. Estimation of evolutionary distances between homologous nucleotide sequences. Proceedings of the National Academy of Sciences of the United States of America 1981;78:454-58.
25. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics (Oxford, England) 2003;19:1572-74.
26. Swofford DL. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Associates, Sunderland MA. 2002.
27. Nylander JAA, *MRMODELTEST version 2.1*. Computer program distributed by the author Uppsala University, Uppsala. 2004.
28. Guan F, Ai JT, Yang LG. A new method for detection of SNP: tetra-primer amplification refractory mutation system PCR based on amplification refractory mutation system. Chemistry of Life 2004;24:514-16.
29. Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ, *et al*. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Missouri Bot Garden 1995;82:247-77.
30. Ren BQ, Xiang XG, Chen ZD. Species identification of *Alnus* (Betulaceae) using nrDNA and cpDNA genetic markers. Molecular Ecology Resources 2010;10:594-5.
31. Ye S, Dhillon S, Ke X, Collins AR, Day I. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Research 2010;20:E88.
32. 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.