Study on the identification of *Polygonatum kingianum* and its adulterants by PCR amplification of specific alleles

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Abstract. Using the allele-specific diagnostic PCR for the rapid and efficient identification of Chinese herbal medicines and their adulterants. In this paper, we analyze the research object of *P. kingianum* and its adulterants. The total DNA of the samples to be identified was extracted, and the PCR was amplified by using universal primers. After the homology comparison, the specific sites were found out by using BioEdit software, and the specific PCR primers were designed by Premier primer 5.0 software, and the samples were amplified by the primers. The 331bp bands were amplified by *P. kingianum* of the specific primers, but the results of their adulterants were not amplified. The results show that the method can provide guidance for the allele-specific diagnostic PCR for quick and easy to select *P. kingianum*, which is not affected by environmental factors, affecting the growth period of the plant, were identified directly from the molecular level of DNA. This method having important application value in accurate introduction and clinical application can provide a model for other identification.

1 Introduction

According to the flora of Chinese records, *Polygonatum kingianum Coll. et Hemsl.* is Polygonatum plants grown in the forest, shrub or damp grass, sometimes in the rock, elevation of 700-3600 meters. It is distributed mainly over Yunnan, Sichuan, Guangxi and Guizhou provinces and other places [1]. According to Pharmacopoeia of People's Republic of China, *P. kingianum* has beneficial effects on the spleen, lungs and kidney. And it can be used for the treatment of spleen qi deficiency, body tired fatigue, stomach yin deficiency, dry food less, lung deficiency cough dry cough, hemoptysis, blood deficiency, Yaoxisuanruan, premature graying, heat diabetes and other symptoms [2]. In the dictionary of traditional Chinese medicine, there are three original plants included in *P. sibiricum*, and they are classified as *P. sibiricum* in the flora of Chinese records and the Pharmacopoeia[1,3]. But there are significant differences between the different producing areas for the same kind of Chinese medicinal herbs in the contents of main chemical components, such as polysaccharides and saponins. At present, in order to guarantee the quality and clinical effects, people rely mainly on the

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morphological and physiological methods, however *P. kingianum* and its adulterants were highly similar, it was not easy to identify, and traditional morphological and chemical identification is easily affected by man-made and environmental factors. In recent years, with the continuous development of molecular identification technology, more and more researchers will begin to apply molecular identification technology to the identification of Chinese herbal medicines. However, the molecular identification methods reported in the present are often complicated and long time, such as identify *ruthenicum L.* used by ITS [4], using NCBI nucleotide database analysis DNA identification of Zijingpi's adulterant species *Schisandra sphenanthera* [5], using cpDNA RBCL and translated amino acid sequences to distinguish *Dryopteris crassirhizoma* [6]. Site specific PCR requires only a single PCR reaction, and then we can judge the genuine from whether there is amplification, is very simple and fast. Today, the site-specific PCR technology has been applied in *Panax ginseng* [7], *Humulus lupulus L.* [8], *Sarcandra glabra* and *Chloranthus* [9], *Amomum villosum Lour.* [10], *Mentha haplocalyx* and *Mentha spicata* [11].

Today, people often mix *P. kingianum* up with *P. odoratum* and *P. uncinatum* on the market. While, the method can overcome obstacles of Large sample size and Strong subjectivity, provide a set of PCR primers and identification of Polygonatum and its adulterants, only PCR amplification reaction in a reaction system. One can quickly and accurately identify *P. kingianum* and its adulterants.

2 Materials and methods

2.1 Plant materials and extraction of the total dna

The experimental materials include *P. kingianum*, *P. odoratum*, Ku Huang Jing, *P. uncinatum Diels*, *Solanum tuberosum*, *Ipomoea batatas*, *Dioscorea polystachya Turcz.*, *Zingiber officinale* and *Colocasia esculenta.* Which was collected and acquired from Yunnan, China in 2015 (Table 1). *P. kingianum*, *P. odoratum*, Ku Huang Jing and *P. uncinatum Diels* were wild, the remaining samples for sale in the market. Wild samples were taken from leaves 1g while the samples for sale were taken from the tuber 1g, using the modified CTAB method to extract the total DNA, and the DNA was dissolved into the sterile ddH_2O, which was preserved in -20°C.

Table 1. Samples used in this study.

Chinese name	Amounts	Location	Time	Scientific name
Dian Huang Jing	21	Yanjin, Yunnan, China	Mar-15	*Polygonatum kingianum*
Yu Zhu	7	Kunming, Yunnan, China	Mar-15	*Polygonatum odoratum*
Ku Huang Jing	5	Kunming, Yunnan, China	Mar-15	
Xiao Huang Jing	8	Kunming, Yunnan, China	Mar-15	*Polygonatum uncinatum Diels*
Yang yu	6	Kunming, Yunnan, China	Jul-15	*Solanum tuberosum*
Fan shu	6	Kunming, Yunnan, China	Jul-15	*Ipomoea batatas*
Shu Yu	6	Kunming, Yunnan, China	Jul-15	*Dioscorea polystachya Turcz.*
Jiang	6	Kunming, Yunnan, China	Jul-15	*Zingiber officinale*
Yu	6	Kunming, Yunnan, China	Jul-15	*Colocasia esculenta*

2.2 PCR amplification

The amplification of universal primers (psbA-trnH-F/R) was amplified by PreMix (2×PCR MasterMix, Sangon Biotech, Shanghai, China). The simple PCR cycling parameters implemented using one cycle of 5 min at 94 °C, followed by 35 cycles of 1min at 94 °C, 1 min at 56 °C, and 1 min extension at 72 °C with the final extension at 72 °C for 7 min. PCR products were analyzed via 1.5% agarose gel electrophoresis (Promega, Madison, Wisconsin, USA) and visualized by Nucleic acid dye (Dingguo Changsheng, Beijing, China) under UV. (Figure 1(B))

2.3 Propagating sequence analysis and primer design

BioEdit software was used for sequence analysis, collation and comparison, to find out the specific mutation sites in the *P. kingianum.* Using premier 5.0 primer software to design a pair of specific PCR primers (DHJ-F, DHJ-R). (Table.2)

2.4 Specific pcr for authentication of *p. kingianum* and assay

The total volume of the PCR reaction system was 25μL, the reaction system including 2.5μL10 * PCR Taq buffer (Mg2+plus 20mM), 0.5μL (10mM) dNTP, primers and 1μL (10μM), DNA Taq polymerase (Dingguo Changsheng, Beijing, China) 0.5μL (2U/μL), DNA template 1μL (10-100ng), distilled water 18.5μL. Using oscillators to shake evenly after the preparation of PCR reaction solution, put PCR tube into the instrument of PCR reaction, which is in the 4min at 94°C of pre-degeneration, and degeneration of 94°C for 1min, annealing at 56°C for 45s, and 1 min extension at 72 °C with the final extension at 72 °C for 10 min in total 32 cycles, with 4°C to save. After the amplification, the products were amplified by 5μL, and 40min was observed by 1.5% agarose gel electrophoresis, 100V voltage electrophoresis and staining with nucleic acid dyes. (Figure 1(C))

3 Results

3.1 Assay of the total DNA extraction

In experiments, extraction of different samples DNA detected by the concentration of 1% agarose gel electrophoresis, mix sample with the 8L DNA solution and bromophenol blue solution, 0.5 times TBE, 120V DC voltage in the gel electrophoresis of 30min, automatic camera observation imaging system. As show in Figure 1 (A).

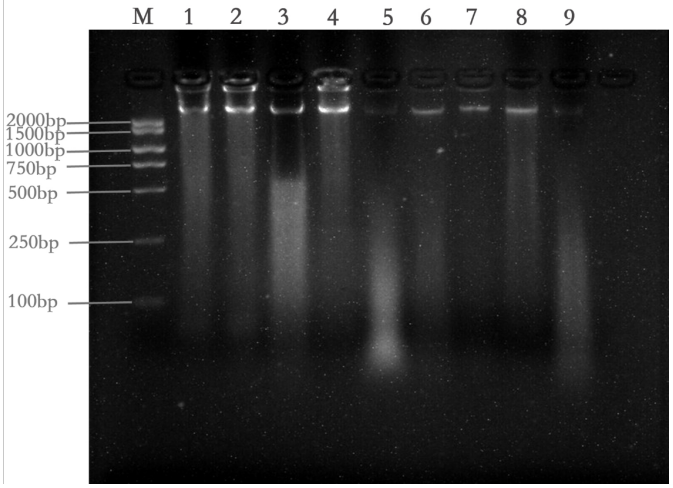

Figure 1(A). Gel electrophoresis of total DNA to *P. kingianum* and its adulterants.

1. *P. kingianum* 2. *P. odoratum* 3. Ku Huang Jing 4. *P. uncinatum Diels* 5. *Solanum tuberosum* 6. *Ipomoea batatas* 7. *Dioscorea polystachya Turcz.* 8. *Zingiber officinale* 9. *Colocasia esculenta*

3.2 Assay of psbA-trnH amplification

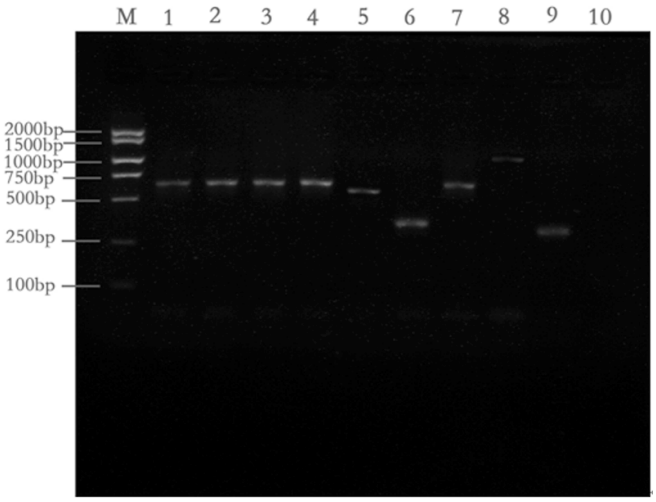

Figure 1(B). Gel electrophoresis of psbA-trnH amplification to *P. kingianum* and its adulterants.
1. *P. kingianum* 2. *P. odoratum* 3. Ku Huang Jing 4. *P. uncinatum Diels*. 5. *Solanum tuberosum* 6. *Ipomoea batatas* 7. *Dioscorea polystachya Turcz.* 8. *Zingiber officinale* 9. *Colocasia esculenta* 10. the negative control

The selection of universal primer psbA-trnH for the amplification of *P. kingianum* and its adulterants, and electrophoresis detection, as shown in the figure 1 (B).

3.3 Specific primer design

Selection of universal primers psbA-trnH for the amplification of *P. kingianum* and its adulterants, the amplified samples were sent to the Shanghai biotechnology company to sequence. The results of

psbA-trnH sequencing to *P. kingianum* and its adulterants using BioEdit software for sequence analysis, proofreading and contrasting. It is find out that the specific mutation of *P. kingianum* is A, while is T of *P. odoratum* and *P. uncinatum Diels* in 71bp, and other adulterants is missing. At 402bp of *P. kingianum* is C, Ku Huang Jing is A, *Colocasia esculenta* is T. Based on the difference, it is designed a couple of specific PCR primers (DHJ-F, DHJ-R) using premier 5.0 primer software, the nucleic acid sequence as shown in Table 2.

Table 2. Primers and PCR reaction conditions used in this study

Primer name	Sequence	Reaction condition
psbA-trnH-F	5'-GTTATGCATGAACGTAA TGCTC-3'	94℃5min, 94℃1min, 56℃1min, 72℃1min, 72℃7min, 32cycles
psbA-trnH-R	5'-CGCGCATGGTGGATTCA CAATCC-3'	94℃5min, 94℃1min, 56℃1min, 72℃1min, 72℃7min, 32cycles
DHJ-F	5'-ATGTATTAAGAATCGTT GAAGGAGC-3'	94℃4min, 94℃1min, 56℃45s, 72℃1min, 72℃10min, 32cycles
DHJ-R	5'-AGCTAATCATTTATCGA GAAAAATG-3'	94℃4min, 94℃1min, 56℃45s, 72℃1min, 72℃10min, 32cycles

3.4 Specific PCR amplification

Put 5μL PCR amplification products in 1.5% agarose gel with nucleic acid dyes, 100V stable voltage electrophoresis 40min, observe the UV Gel imaging system. The results are shown in Figure 1 (C), it reflects that the sample produce band at 331bp, the remaining samples did not produce bands. The 331bp bands were amplified of *P. kingianum* by the specific primers, while that were not amplified of their adulterants.

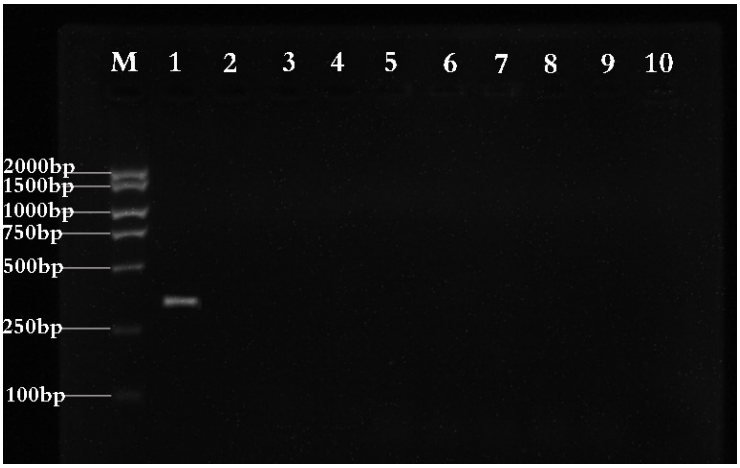

Figure 1(C). Gel electrophoresis of specific primer amplification to *P. kingianum* and its adulterants .
1. *P. kingianum* 2. *P. odoratum* 3. Ku Huang Jing 4. *P. uncinatum Diels* 5. *Solanum tuberosum* 6. *Ipomoea batatas* 7. *Dioscorea polystachya Turcz.* 8. *Zingiber officinale* 9. *Colocasia esculenta* 10. the negative control

4 Discussions

As part of the confusion of Chinese herbal medicines caused great danger to the safe use of traditional Chinese medicine, but the limitations of traditional identification methods affect the safe and effective use of traditional Chinese medicine. The technology of traditional Chinese medicine identification

method runs through every link of the planting, processing and production, with the development of Chinese medicine industry, the traditional identification methods have been difficult to meet the rapid identification and normal needs of hospitals, pharmacies, enterprises, customs and other industries, thus a new methods of identification becoming urgent.

The characteristic of AS-PCR is just should consider in the 3 'end sites when designed primer sequence, while a primer for site specific identification of PCR and ARMS can improve the specificity of amplification not only 3' end sites, but also can be in the 3 'end of the primer close to the second to sixth base position mutation base. This method is widely used in identification of Chinese medicinal materials [12-15].

Allele-specific diagnostic PCR is a type of genotyping based on SNP gene loci, because this method only need a PCR, and PCR products were gel electrophoresis, and then distinguish between wild and mutation type according to the electrophoresis strip size and whether they have, fast and brief, has a unique advantage in the authenticity of products for rapid detection of medicinal materials, be good use of rapid identification of traditional Chinese medicine. There are no reports that identification of *P. kingianum*, *P. odoratum* and *P. uncinatum Diels* by allele-specific diagnostic PCR, for the future interrelated researches or identifications it can play a reference role [16].

5 Conclusions

Early studies indicated that molecular identification of Polygonatum was limited within DNA sequence information, and that the lack of a focus and simplicity needed a great deal of preliminary work of DNA sequence information in Polygonatum cultivars. Overcoming this limitation or improving effected on identification be use of cultivar-specific SNP marker, finding the specific sites and establishing allele specific PCR system to the rapid identification of *P. kingianum*. *P. kingianum* and its adulterants were not easy to identify, and traditional morphological and chemical identification is easily affected by man-made and environmental factors. In this study, the results show that the method can provide guidance for the allele-specific diagnostic PCR for quick and easy to select *P. kingianum*, which is not affected by environmental factors, affecting the growth period of the plant, were identified directly from the molecular level of DNA. This method having important application value in accurate introduction and clinical application can provide a model for other identification.

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