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Identification of Three Chinese Herbal Medicines and Their Adulterants Based on Allele-Specific Diagnostic PCR

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Abstract: Owing to the high pharmacological and economic value, the Chinese medicinal herbs: *Paris polyphylla* var. *yunnanensis*, *Polygonatum kingianu*m and *Bletilla striata*, whose quality has been damaged by their adulterants. In this study, the allele-specific diagnostic PCR method was used to identify three herbal medicines and their adulterants. And the nuclear gene ITS2 sequence and chloroplast gene psbA-trnH sequence, as international common barcode sequences, are widely used in the molecular identification of medicinal plants. Therefore, three pair of specific PCR primers were respectively designed according to the sequence of *Paris polyphylla* var. *yunnanensis* on the ITS2, *Polygonatum kingianum* and *Bletilla striata* on the psbA-trnH, using Primer 5.0. And these specific identification primers could amplify special bands respectively at 130bp, 331bp and 508bp, while adulterants could not amplify the bands. The results showed that this method could respectively distinguish three herbal medicines and their adulterants. And after many experiments, the method had good repeatability. The allele-specific PCR identification method established in this study is easy to operate and fast in identification. This method can distinguish three kinds of traditional Chinese medicines and their adulterants, which provides a reference for the identification of other traditional Chinese medicines, so as to further protect the Chinese herbal medicine industry from economically motivated adulteration.

Keywords: Paris Polyphylla var. Yunnanensis, Polygonatum Kingianum, Bletilla Striata, Adulterants, Allele-Specific Diagnostic PCR, Identification

1. Introduction

At present, in the actual production and marketing of Chinese herbal medicines, many economically motivated adulterations have occurred. Traditional methods for identification of medicinal herbs rely mainly on morphological and histological characteristics. However, most traditional Chinese medicinal materials are very similar to their original plants, and are difficult to be identified. Moreover, traditional morphological and histological identification methods are susceptible to human factors and environmental factors, and inevitably exist errors. Therefore, finding an accurate identification method becomes the key to solve the problem. In recent years, with the development of molecular identification technology, more and more researchers have applied molecular identification technology in the authentication of Chinese herbal medicines. Nevertheless, currently reported molecular identification methods are often complex and time-consuming, such as using cpDNA rbcl and translated amino acid sequences to distinguish *Dryopteris crassirhizoma* and adulterant species [1], using DNA barcodes for the identification of *Anoectochilus roxburghii* and its adulterants [2], using species-unique single nucleotide polymorphism markers to develop an authentication system of *Panax* [3]. Allele-specific

PCR only needs a PCR reaction, and then we can judge the authenticity from whether there is an amplification, which is simple and fast. Nowadays, the allele-specific PCR technology has been applied in *Panax ginseng* [4, 5], *Humulus lupulus* L. [6], *Sarcandra glabra* and *Chloranthus* [7], *Amomum villosum* Lour. [8], *Mentha haplocalyx* and *Mentha spicata* [9], *Pericarpium citri reticulatae* and *Citriunshius pericarpium* [10], *Ligusticum tenuissimum* [11].

P. polyphylla var. *yunnanensis* is one of original plants of Chinese medicinal "Chong Lou". *P. kingianum* is one of original plants of Chinese medicinal "Huang Jing". And *B. striata* is the original plant of Chinese medicinal "Bai Ji" [12]. The rhizomes of them are used as Chinese herbal pieces. They all are perennial herbs, among them, *P. polyphylla* var. *yunnanensis*, is mainly distributed in southwest China. *P. kingianum* is mainly distributed in Yunnan, Sichuan, Guizhou and Guangxi provinces of China. And *B. striata* is found mainly in China, Japan, and northern Myanmar [13]. *P. polyphylla* var. *yunnanensis* is widely reported to have antibacterial activity [14], cytotoxic [15], antioxidant activity [16], antitumor activity [17]. *P. kingianum* is widely used to treat hyperlipidemia [18], nonalcoholic fatty liver [19] and diabetes [20]. And *B. striata* is widely used to treat enterocutaneous fistula [21], ulcerative colitis [22] and bleeding [23] in East Asian countries.

The available supply of these herbs is facing an increasing shortage because its growth period needs more than 3 years, and the consumption of these herbs in the pharmaceutical industry has increased dramatically in recent years. The price of *P. polyphylla* var. *yunnanensis* exceeded 800 yuan/kg in 2018, so its mixed adulterants appeared in the sales market, and the same as *P. kingianum* and *B. striata*. The adulterants have affected the quality of three Chinese herbal medicines seriously. In this study, our aim is to use the allele-specific diagnostic PCR of ITS2 and non-coding region of chloroplast gene to identify *P. polyphylla* var. *yunnanensis*, *P. kingianum* and *B. striata*, and their adulterants respectively.

2. Materials and Methods

2.1. Plant Materials and Genomic DNA Extraction

There are three groups of materials, *P. polyphylla* var. *yunnanensis* and its adulterants (Table 1); *P. kingianum* and its adulterants (Table 2); *B. striata* and its adulterants (Table 3). Samples were collected from Yunnan province, China, with the exception of *Solanum tuberosum*, *Ipomoea batatas*, *Dioscorea polystachya Turcz*, *Zingiber officinale* and *Colocasia esculenta* purchased from the local market. Genomic DNA was extracted from leaves or roots using the improved CTAB method, dissolved in sterile ddH_2O, and stored at -20°C. The quality and the concentration of extracted genomic DNA were measured using an ultraviolet spectrophotometer and agarose gel electrophoresis.

Table 1. *Samples of P. polyphylla var. yunnanensis and its adulterants used in this study.*

Chinese name	Amounts	Location	Scientific name
Dian Chong Lou	13	Kunming, Yunnan, China	*P. polyphylla* var. *yunnanensis*
Mao Chong Lou	7	Wenshan, Yunnan, China	*Paris mairei*
Hua Ye Chong Lou	9	Kunming, Yunnan, China	*Paris marmorata*
Xia Ye Chong Lou	6	Wenshan, Yunnan, China	*Paris polyphylla* var. *stenophylla*
Da Li Chong Lou	17	Wenshan, Yunnan, China	*Paris daliensis*
Nan Chong Lou	20	Wenshan, Yunnan, China	*Paris vietnamensis*
Chang Zhu Chong Lou	11	Kunming, Yunnan, China	*Paris forrestii*
Qi Ye Yi Zhi Hua	15	Kunming, Yunnan, China	*Paris polyphylla*
Wu Zhi Lian Chong Lou	4	Kunming, Yunnan, China	*Paris axialis*

Table 2. *Samples of P. kingianum and its adulterants used in this study.*

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

Table 3. *Samples of B. striata and its adulterants used in this study.*

Chinese name	Amounts	Location	Scientific name
Bai Ji	30	Kunming, Yunnan, China	*B. striata*
Shan Lan	20	Wenshan, Yunnan, China	*Oreorchis patens*
Jian Xue Qing	20	Kunming, Yunnan, China	*Liparis nervosa*
Du Suan Lan	11	Wenshan, Yunnan, China	*Pleione bulbocodioides*
Tong Ban Lan	14	Wenshan, Yunnan, China	*Anthogonium gracile*
Yue Nan Bai Ji	7	Wenshan, Yunnan, China	Unidentify

2.2. PCR amplification Conditions

The total volume of the PCR reaction system was 25μL, the reaction system including 2.5μL10 * PCR Taq buffer (Mg^{2+}plus 20mM), 0.5μL (10mM) dNTP, 1μL (10μM) of each primer, 0.5μL (2U/μL) DNA Taq polymerase (Dingguo Changsheng, Beijing, China), 1μL (10-100ng) DNA template and 18.5μL ddH_2O. And the amplification of universal primers were amplified by PreMix (2×PCR Master Mix, SangonBiotech, Shanghai, China). The PCR cycling program included an initial step at 94°C for 5 min followed by 35 cycles at 94°C for 1min, at 56°C for 1min, and at 72°C for 1min, and a final extension at 72°C for 7 min. Finally, PCR products were examined on 1.5% agarose gels. The universal primers ITS2-F and ITS2-R were used to amplify *P. polyphylla* var. *yunnanensis* and its adulterants (Figure 1). The universal primers psbA-trnH-F and psbA-trnH-R were not only used to amplify *P. kingianum* and its adulterants (Figure 2), but also used to amplify *B. striata* and its adulterants (Figure 3).

2.3. Design of Identification Primer

BioEdit software was used for sequence analysis, collation and comparison, to find out the specific mutation sites of the samples (*P. polyphylla var. yunnanensis*; *P. kingianum*; *B. striata*). And Premier 5.0 was used to design three pairs of specific PCR primers (DCL-F, DCL-R; DHJ-F, DHJ-R; BJ-F, BJ-R). (Tables 4-6)

2.4. Specific PCR Reaction Conditions

The following reaction procedure can effectively distinguish the above mentioned authentic Chinese herbal medicine from its adulterants. After repeated tests, the effect is ideal. And the samples that participated in the amplification reaction were P. polyphylla var. yunnanensis and its adulterants (pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30s, annealing at 56°C for 45s, refolding at 72°C for 1 min, extension at 72°C for 10 min, at 4°C for storage, 35 cycles) (Figure 4); P. kingianum and its adulterants (pre-denaturation at 94°C for 4 min, denaturation at 94°C for 1min, annealing at 56°C for 45s, refolding at 72°C for 1 min, extension at 72°C for 10 min, at 4°C for storage, 32 cycles) (Figure 5); B. striata and its adulterants (pre-denaturation at 94°C for 4 min, denaturation at 94°C for 45s, annealing at 58°C for 1 min, refolding at 72°C for 1 min, extension at 72°C for 7 min, at 4°C for storage, 35 cycles) (Figure 6).

3. Results

3.1. Assay of Universal Primer Amplification

Select universal primers (ITS2; psbA-trnH) to amplify authentic herbs (P. polyphylla var. yunnanensis; P. kingianum; B. striata) and their adulterants, and conduct electrophoresis detection, as shown in Figures 1-3.

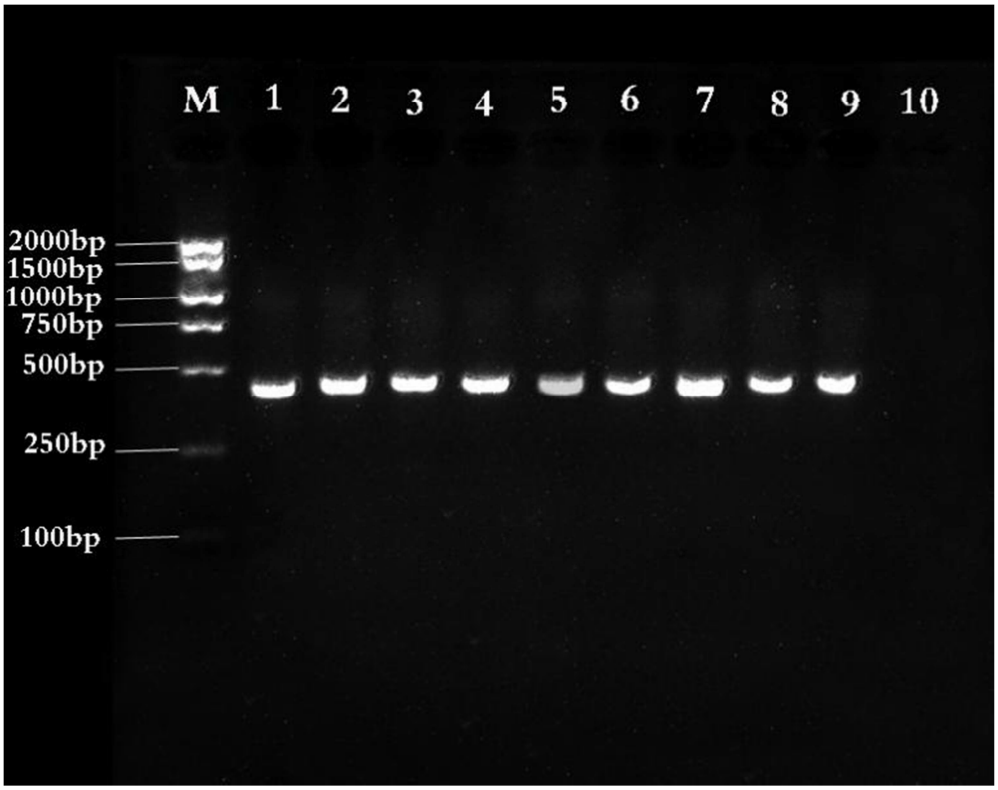

Figure 1. *Gel electrophoresis of ITS2 amplification for P. polyphylla var. yunnanensis and its adulterants. (1. P. polyphylla var. yunnanensis. 2. Paris mairei. 3. Paris marmorata. 4. Paris polyphylla var. stenophylla. 5. Paris daliensis. 6. Paris vietnamensis. 7. Paris forrestii. 8. Paris polyphylla. 9. Paris axialis. 10. Negative control.).*

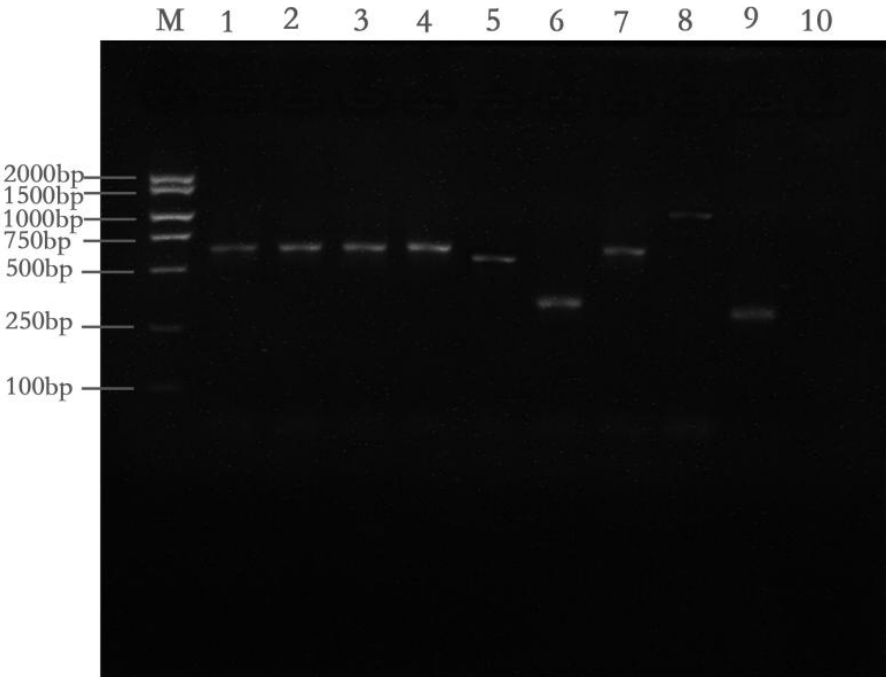

Figure 2. *Gel electrophoresis of psbA-trnH amplification for P. kingianum and its adulterants. (1. P. kingianum 2. P. odoratum. 3. Ku Huang Jing. 4. P. uncinatum Diels. 5. Solanum tuberosum. 6. Ipomoea batatas. 7. Dioscorea polystachya Turcz. 8. Zingiber officinale 9. Colocasia esculenta. 10. Negative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

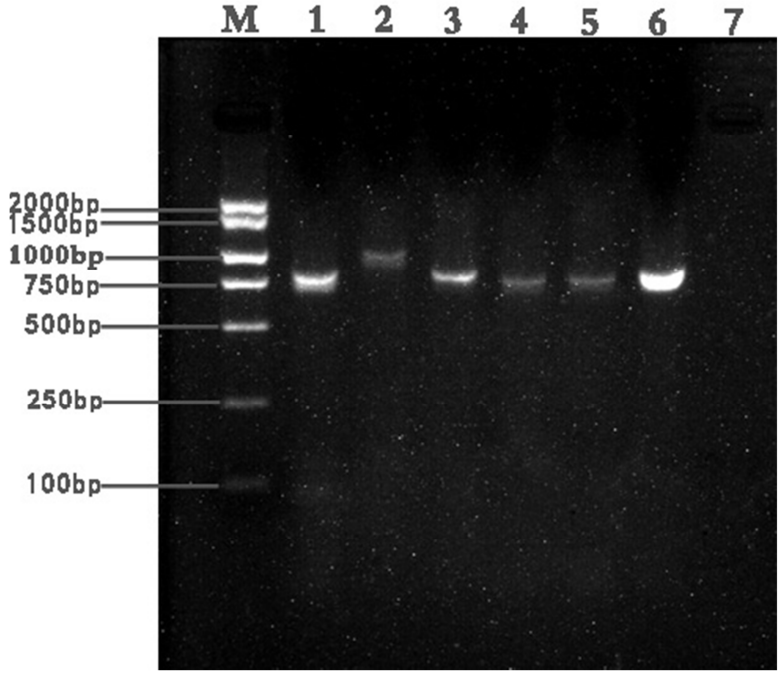

Figure 3. *Gel electrophoresis of psbA-trnH amplification for B. striata and its adulterants. (1. B. striata. 2. Oreorchis patens. 3. Liparis nervosa. 4. Pleione bulbocodioides. 5. Anthogonium gracile. 6. Yue Nan Bai Ji. 7. Negative control.).*

3.2. Specific Primer Design

The primers were used to amplify the samples, and the amplified products were sent to Sangon Biotech Company for sequencing. GenBank was used to search the identification primers for authentic herbs and their adulterants. The sequencing results were analyzed and compared by BioEdit software to find out the unique mutation sites of authentic herbs. And three pairs of specific PCR primers (*P. polyphylla* var. *yunnanensis*: DCL-F, DCL-R; *P. kingianum*: DHJ-F, DHJ-R; *B. striata*: BJ-F, BJ-R) were designed using Primer Premier 5.0 software. The nucleic acid sequence is shown in Tables 4-6.

The mutation sites of *P. polyphylla* var. *yunnanensis* are numerous, it is A in 190bp, *Paris polyphylla* var. *stenophylla* is T in 190bp, while others are C; it is C in

203bp while others are T; it is G in 206bp while others are C; it is A in 275bp while others are C; it is G in 276bp while others are C; it is A in 320bp, others are G; it is A in 331bp, others are T; it is A in 363bp, others are C; it is T in 389bp, others are C; it is T in 391bp, others are C, and specific primers (DCL-F/R) are designed with 203 bp and 331 bp loci, respectively. The mutation site of *P. kingianum* in 71bp is A, *and Polygonatum odoratum* and *Polygonatum uncinatum Diels* are T, while others are none; it is C in 402bp, while Ku Huang Jing is A, and *Colocasia esculenta* is T. The mutation site of *B. striata* in 111bp is A, and *Oreorchis patens* is C, while others are T.

Table 4. *P. polyphylla var. yunnanensis primers and PCR reaction conditions used in this study.*

Primer name	Sequence	Reaction condition
ITS2-F	5'-ATGCGATACTTGGTGTGAAT-3'	94°C5mins, 94°C30s, 56°C30s, 72°C45s, 40cycles, 72°C7mins,
ITS2-R	5'-GACGCTTCTCCAGACTACAAT-3'	
DCL-F	5'-CGCGTACAACGAAACCATGCTGGGC-3'	94°C3mins, 94°C30s, 56°C45s, 72°C1mins, 35cycles, 72°C10mins,
DCL-R	5'-GAAGAGTGGGATGCCAACTGAGACC-3'	

Table 5. *P. kingianum primers and PCR reaction conditions used in this study.*

Primer name	Sequence	Reaction condition
psbA-trnH-F	5'-GTTATGCATGAACGTAATGCTC-3'	94°C5mins, 94°C1min, 56°C1min, 72°C1min, 32cycles, 72°C7mins
psbA-trnH-R	5'-CGCGCATGGTGGATTCACAATCC-3'	
DHJ-F	5'-ATGTATTAAGAATCGTTGAAGGAGC-3'	94°C4mins, 94°C1min, 56°C45s, 72°C1min, 32cycles, 72°C10mins
DHJ-R	5'-AGCTAATCATTTATCGAGAAAAATG-3'	

Table 6. *B. striata primers and PCR reaction conditions used in this study.*

Primer name	Sequence	Reaction condition
psbA-trnH-F	5'-GTTATGCATGAACGTAATGCTC-3'	94°C5mins, 94°C1min, 56°C1min, 72°C1min, 32cycles, 72°C7mins,
psbA-trnH-R	5'-CGCGCATGGTGGATTCACAATCC-3'	
BJ-F	5'-AGCAATCCCCCAATATCTTGTTCTA-3'	94°C4mins, 94°C45s, 58°C1mins, 72°C1min, 32cycles, 72°C7mins,
BJ-R	5'-GGTCTCGGGCATCTACCA-3'	

3.3. Specific Primer Verification

Finally, the samples were amplified by PCR with specific primers to verify the identification effect. Put 5μL PCR amplification products into 1.5% agarose gel containing nucleic acid dyes, and electrophoresis at 100V for 40 minutes. Results as shown in Figures 4-6, authentic Chinese herbal medicine can obtain PCR product bands of different lengths, while other adulterants cannot produce bands. The samples identified at 130bp were all P. polyphylla var. yunnanensis, and at 331bp were all P. kingianum, and at 508bp were all B. striata.

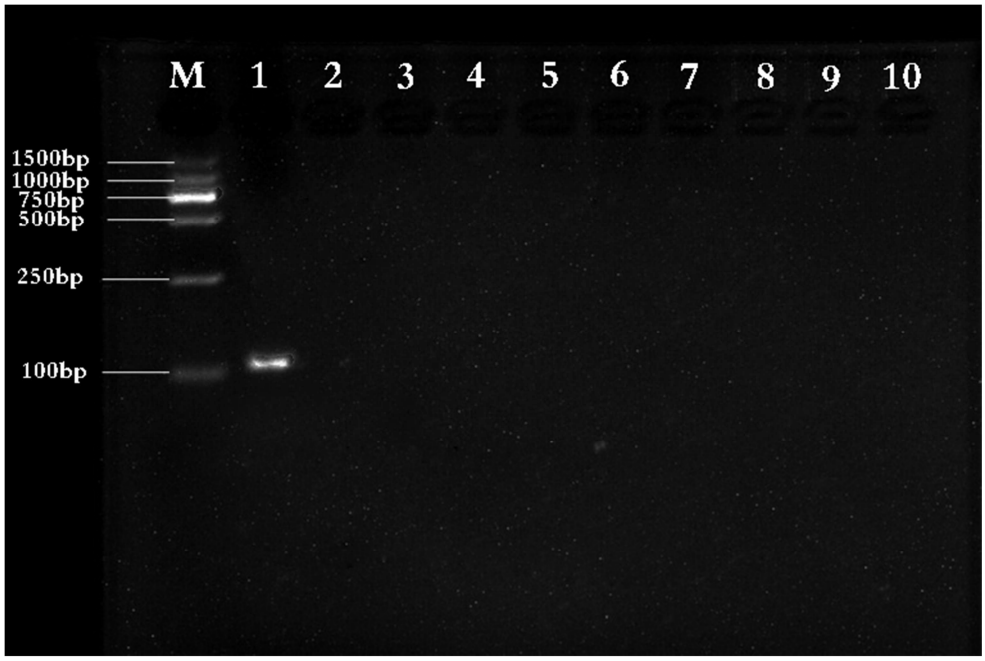

Figure 4. *Gel electrophoresis of specific primer amplification for P. polyphylla var. yunnanensis and its adulterants. (1. P. polyphylla var. yunnanensis. 2. Paris mairei. 3. Paris marmorata. 4. Paris polyphylla var. stenophylla. 5. Paris daliensis. 6. Paris vietnamensis. 7. Paris forrestii. 8. Paris polyphylla. 9. Paris axialis. 10. Negative control.).*

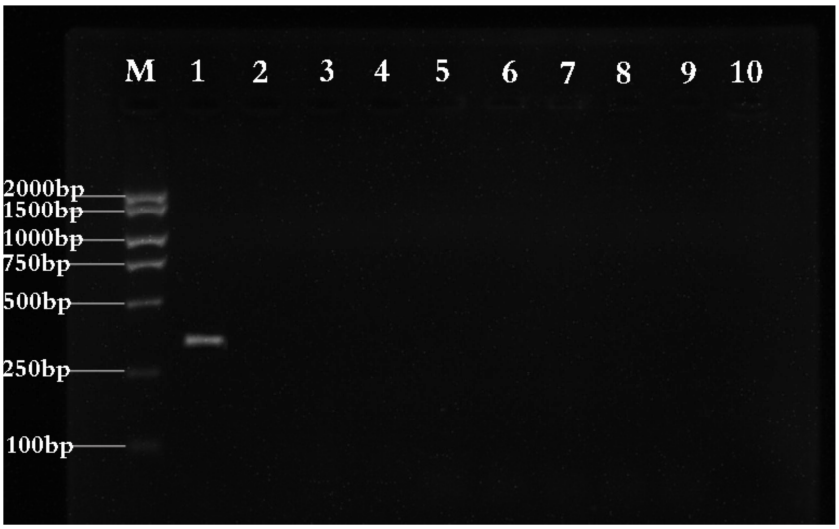

Figure 5. *Gel electrophoresis of specific primer amplification for P. kingianum and its adulterants. (1. P. kingianum. 2. P. odoratum. 3. Ku Huang Jing. 4. P. uncinatum Diels. 5. Solanum tuberosum. 6. Ipomoea batatas. 7. Dioscorea polystachya Turcz. 8. Zingiber officinale 9. Colocasia esculenta 10.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

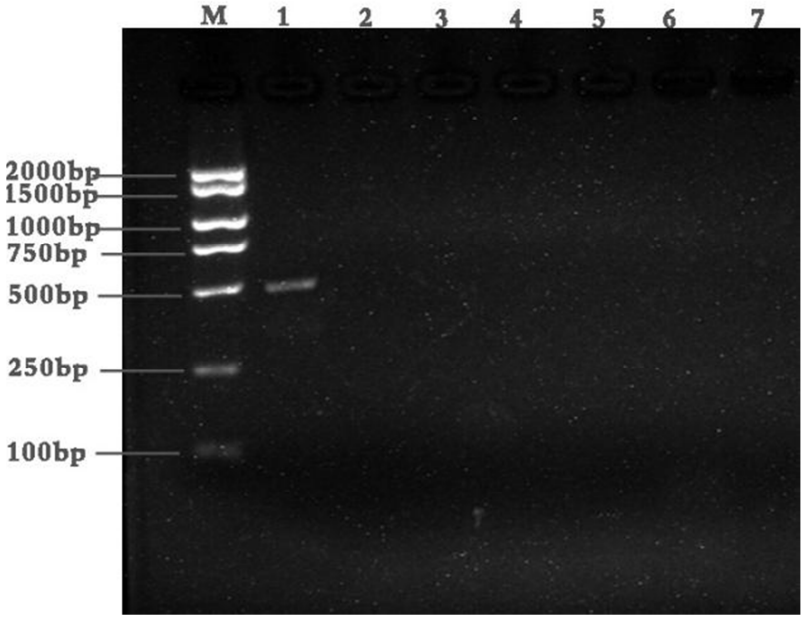

Figure 6. *Gel electrophoresis of specific primer amplification for B. striata and its adulterants. (1. B. striata 2. Oreorchis patens 3. Liparis nervosa. 4. Pleione bulbocodioides. 5. Anthogonium gracile. 6. Yue Nan Bai Ji. 7. Negative control.).*

4. Discussion

The identification of plant species is very significant for the standardization of the herbal medicine industry and the prevention of economically motivated adulteration. Because some Chinese herbal medicines have high pharmacological and economic value, there is ample potential for economically motivated adulteration of Chinese herbal medicine products. Therefore, a simple, reliable and practical method for accurately identifying the authenticity of Chinese herbal medicine plays an important role in the development and protection of the Chinese herbal medicine industry.

There are many identification methods for Chinese medicinal materials, including original plant identification, character identification, microscopic identification and physical and chemical identification, but these identification methods are often affected by human or environmental factors. In contrast, molecular identification technology is stable and not affected by human or environmental factors. Allele specific PCR means that the base mismatch between primer and template can effectively inhibit the PCR reaction, and then achieve the purpose of template differentiation (allelic differentiation). The allele specific PCR is a new technique that is widely used as a biological tool for species identification, breeding, and evolutionary research.

ITS2 sequence is located between 5.8S and 28S nuclear rDNA genes. In plant cells, the secondary structure of ITS2 rDNA is a stem loop structure formed by the self-folding of the primary sequence to form partial base pairing and alternating single strand. The secondary structure of eukaryotic ITS2 is a highly conservative "one ring four arm"

model, which is short in length and easy to amplify, and is suitable for the identification of plant genera and species [24]. The psbA trnH sequence is the spacer sequence between the psbA gene (encoding the D1 protein of the photosynthetic system II reaction center) and the trnH gene (encoding tRNA histidine) in chloroplasts. This sequence only plays the role of linking two genes. It has low selection pressure, relatively many mutant sites, fast evolution rate, and conservative DNA sequences at both ends, facilitating the design of universal primers [25]. Therefore, the nuclear gene ITS2 sequence and chloroplast gene psbA-trnH sequence, as international common barcode sequences, are widely used in the molecular identification of medicinal plants.

5. Conclusion

In this study, based on the original plant ITS2 sequence and psbA-trnH sequence, site specific primers were screened to identify *P. polyphylla* var. *yunnanensis*, *P. kingianum* and *B. striata*, and their adulterants, and a allele specific PCR method was established. By optimizing the reaction conditions, stable and clear identification bands were obtained using this method, which can accurately identify the three kinds of Chinese medicines and their adulterants. The establishment of this method is different from DNA sequence analysis method. The allele specific PCR identification method established in this study is easy to operate and fast in identification. It does not require high technical level for identification personnel and has high application value. This study has successfully identified three kinds of traditional Chinese medicines and their adulterants by site specific PCR, which provides a reference for the identification of the authenticity of traditional Chinese medicines in the future.

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