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Full Length Article

From Electrophoresis Detection to Visual Judgment: New Application of SYBR Green I in the Authentication of the Traditional Medicine Rhizoma Paridis

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Abstract

Paris polyphylla Smith var. *chinensis* (Franch.) Hara (PPC) has been used as a traditional Chinese medicine for a long period and is recorded in the Chinese Pharmacopoeia as the certified original plant component of Rhizoma Paridis. However, we discovered that eight other *Paris* species and subspecies are often present act as adulterants of PPC owing to the decrease in wild resources, according to a survey of resources and research into the herb market. In order to prevent fraudulent substitution and ensure clinical safety, this study aimed to devise a specific authentication method for the differentiation of PPC from its adulterants in the form of eight *Paris* species and subspecies. For this purpose, we devised a visual molecular authentication method for the differentiation of PPC from its adulterants, which is based on the diversity of the internal transcribed spacer (ITS) sequence of nuclear ribosomal DNA (nrDNA) and employs the double-stranded DNA-specific dye SYBR Green I. This method consists of three main procedures, namely, analysis of the ITS sequence of nrDNA, a species-specific polymerase chain reaction and a fluorescence reaction. We successfully devised a visual molecular identification method whereby PPC can be accurately differentiated from its adulterants. This study provides a good example of how to reliably, sensitively, and immediately differentiate an authentic medicinal plant from its closely related adulterants using a molecular approach.

Keywords: *Paris* sp.; Species-specific PCR; SYBR Green I; Visual authentication
Abbreviations: PPC: *Paris polyphylla* Smith var. *chinensis* (Franch.) Hara; TCM: traditional Chinese medicine; CP: Chinese Pharmacopoeia; ITS: internal transcribed spacer; PPP: *Paris polyphylla* var. *polyphylla*; PT: *Paris thibetica*; PFB: *Paris fargesii* var. *brevipetalata*; PM: *Paris mairei*; PPS: *Paris polyphylla* var. *stenophylla*; PVA: *Paris vaniotii*; PA: *Paris axialis*; PVI: *Paris vietnamensis*

Introduction

The genus *Paris* (Trilliaceae) is widely distributed in many regions of Eurasia, such as China, India, Vietnam, Myanmar, and Japan, and includes 24 species (Li, 1998). The roots of some *Paris* species have been used as traditional medicines ineastern and southeastern Asian countries owing to their antipyretic, analgesic, anti-inflammatory, antitoxic, antibacterial, and antitumor activities. Owing to differences between the physiological effects and chemical compositions of different *Paris* species, scientists worldwide have conducted systematic studies of these plants and their chemical components, physiological activities and genetic characteristics (Matsuda *et al*., 2003; Deng *et al*., 2008; Lee *et al*., 2008; Nguyen *et al*., 2009).

Paris polyphylla Smith var. *chinensis* (Franch.) Hara (PPC) is a valuable medicinal herb that has historically been regarded as the original plant component of the traditional medicine Rhizoma Paridis in China. It has obvious effects in terms of reducing swelling and relieving pain, bruises and snakebites and sore throats, and is used as an essential raw material in many famous Chinese patent drugs, such as Jidesheng snake tablets, Yunnan Baiyao, and Gongxuening capsules. The *Paris* saponins in PPC are considered to be the active ingredients responsible for its antibacterial, antitumor, hemostatic and analgesic properties (Wen *et al*., 2012). We previously carried out an investigation into the area where Rhizoma Paridis is produced and the markets for medicinal materials and found that, in recent years, eight sympatric species and subspecies of the genus *Paris* (*P. polyphylla* var. *polyphylla* [PPP], *P. thibetica* [PT], *P.fargesii* [PFA], *P. mairei* [PM], *P. polyphylla* var. *stenophylla* [PPS], *P. vaniotii* [PVA], *P. axialis* [PA], and *P. vietnamensis* [PVI]), as well as some *Trillium* species, have been marketed as PPC. However, variations in the origin of medicinal materials can seriously affect their

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quality and clinical safety. Because of the high similarities between the morphological characteristics of the different *Paris* species and subspecies mentioned above (Fig. 1A and B), adulterations and substitutions of PPC usually cannot be correctly identified or distinguished by morphological techniques. Moreover, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been employed to identify four kinds of saponins in Rhizoma Paridis (*Paris* saponins, I，II,VI, and VII; Fig. 1C), which are used as markers in HPLC methods described in the Chinese Pharmacopoeia (2015). These fourmarkers can be detected in most of the other eight *Paris* species and subspecies. Furthermore, there are significant differences between the saponin constituents of PPC from different distribution regions and at different stages of growth (Table 1). Hence the results of the above-mentioned identification methods can be misleading.

It is widely accepted that DNA from different species is suitable for the accurate identification of plant species. This is because DNA present in any tissues is not affected by external conditions or physiological states (Sucher and Carles, 2008; Wallace *et al*., 2012; Verma and Goswami, 2014; Ferri *et al*., 2015; Li *et al*., 2015; Lin *et al.*, 2019). In recent years, DNA barcoding technology has been widely used for the authentication of traditional Chinese herbal medicines, especially in the case of adulterants and substituted and/or mixed species (Ji *et al*., 2006). Numerous studies have proven that the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) is a suitable DNA barcode for the identification of most angiosperm species (Chase. *et al*., 2003; Chase. *et al.,* 2005; Kress *et al*., 2005). A series of reports have demonstrated that a species-specific polymerase chain reaction (PCR) has been successfully used for the authentication of some medicinal herbs and their adulterants, such as the detection of gecko, the bulb of *Fritillaria pallidiflora* and Pseudostellariae Radix (Liu *et al*., 2001; Li *et al*., 2003; Wang *et al*., 2005; Zhao *et al*., 2014). In our previous research, several molecular identification systems for herbal medicines were devised. For example, Chen *et al.* (2012) successfully developed a DNA-based molecular identification method for ginseng ingredients present in some Chinese patent medicines. Even though molecular authentication methods have been used on traditional Chinese medicines widely and effectively, there have been virtually no reports on the development of visual molecular markers for the identification of congeneric species.

Traditional medicines play an important role in health care, in particular in industrialized countries. Current quality assessment and authentication methods mainly rely on morphology and analytical phytochemistry-based methods, which are detailed in pharmacopoeias. However, because of the high similarities between the morphological characteristics of allied species, it has become difficult to identify or distinguish such species by traditional authentication methods, which has seriously affected the quality of medicinal materials and their clinical safety.

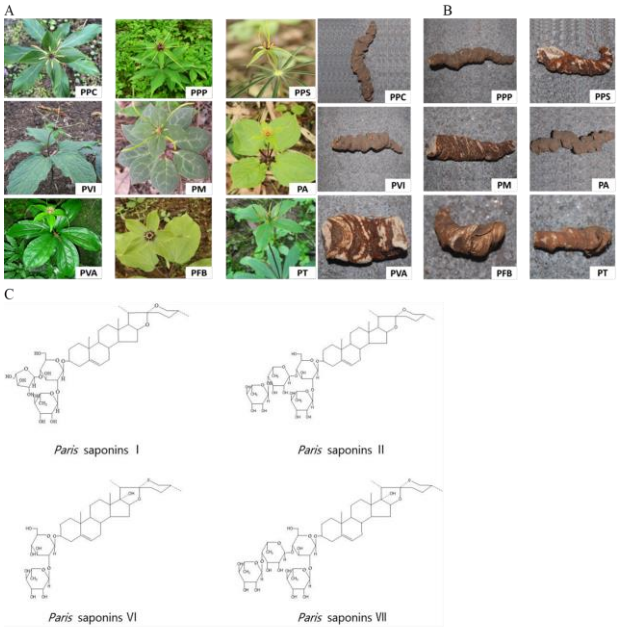

Fig. 1: Whole plants (A) and medicinal parts (B) of PPC and its common adulterants. Chemical structures of the four saponins (C)

SYBR Green I is one of the most sensitive stains available for detecting double-stranded DNA (dsDNA) and has been widely used as a reagent in quantitative real-time fluorescence PCR. Because of its exceptionally low background fluorescence and high sensitivity, SYBR Green I can be an ideal reagent for detecting trace amounts of samples. We hypothesized that this system can easily and rapidly differentiate PPC from its common adulterants because the green fluorescence generated by a positive reaction is significantly stronger than that produced by a negative reaction. In view of this, in this study, a PCR amplification system comprising species-specific primers and SYBR Green I was devised. The visual molecular identification method we reported does not require agarose gel electrophoresis or DNA sequencing.

Materials and Methods

Plant Materials and DNA Extraction

Plant materials: Thirty-five samples of nine *Paris* species and subspecies (*P. polyphylla* var. chinensis, *P. polyphylla* var. *polyphylla*, *P. polyphylla* var. *stenophylla*, *P. mairei*, *P. vietnamensis*, *P. axialis*, *P. vaniotii*, *P. fargesii* var. *brevipetalata*, *and P. thibetica*), including 28 samples of leaves and seven samples of rhizomes, were collected during the flowering and fruiting periods each year from different localities in China. Five commercial samples were purchased from five local pharmacystores in Chengdu, China. All the samples were stored in the Chengdu

Table 1: Contents of the four saponins in different *Paris* species

Species name	Ps I (%)	Ps II (%)	Ps VI (%)	Ps VII (%)	Growth site	Growth years
PPC	nd	nd	nd	0.54±0.01	Pengzhou, Shichuan	2
PPC	nd	0.50±0.01	nd	nd	Pengzhou, Shichuan	5
PPC	0.35±0.01	0.48±0.01	nd	0.37±0.01	Pengzhou, Shichuan	8
PPC	0.67±0.01	0.30±0.01	nd	0.58±0.01	Yaan, Shichuan	-
PPC	0.57±0.01	nd	nd	0.08±0.01	Pengzhou, Shichuan	-
PPP	0.13±0.01	nd	nd	nd	Yichang, Hubei	-
PPS	0.22±0.01	nd	nd	nd	Luzhou, Shichuan	-
PT	0.15±0.01	nd	nd	nd	Pengzhou, Shichuan	-
PFB	0.15±0.01	0.21±0.01	1.60±0.01	1.52±0.01	Pengzhou, Shichuan	-
PA	0.16±0.01	nd	0.14±0.01	nd	Luzhou, Shichuan	-
PVA	nd	1.58±0.01	nd	2.06±0.01	Luzhou, Shichuan	-
PM	nd	nd	nd	0.09±0.01	Liangshan,Shichuan	
PVI	0.12±0.01	nd	0.31±0.01	nd	Dali, Yunnan	

"n.d": not detected; "Ps": *Paris* saponin;"-": unknown

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DNA extraction: The leaves of the *Paris* species were kept at -20°C with silica gel. The rhizomes were washed with clear water, dried in an oven at a moderate temperature, and then ground into a powder to simulate the primary processing of the medicinal material. In this study, in the classical cetyltrimethylammonium bromide (CTAB) method, a plant DNA isolation kit (Foregene Biotech Co., Chengdu, China) and a cell lysis buffer were used for the extraction of DNA. In the CTAB method, a 30 mg powders sample was suspended in 600 μL CTAB buffer and then incubated at 65°C for 1.5 h with occasional shaking. The lysate was extracted with 600 μL chlorophorm: isoamyl alcohol (v/v, 24:1). DNA was precipitated with an equal volume of 100% isopropanol (1 h at -20°C, followed by centrifugationat 4°C and 10000 g for 10 min) and then washed twice with 70% ethanol, vacuum-dried, and resuspended in 50 μL ddH2O. The plant DNA isolation mini kit was used according to the producer's instructions with minor adjustments. In brief, DNA extraction adhered to the protocol using 80 μL ddH_2O (pre-heated to 65°C). A 20 mg sample of the material was suspended incell lysis buffer (20 μL 75% α-amylase [Siker Biological, Japan] with 20 μL protease [Foregene, Chengdu, China] or 10 μL proteinase K [Thermo Scientific, USA] and 250 μL Lysis 20 [Geneseen Biotech Co, Chengdu, China], and the mixture was incubated for 10 min at 95°C with occasional shaking. The supernatant was then extracted for further analysis.

ITS Sequencing of PPC and its Adulterants

The universal primer sequences for the ITS region and the general PCR conditions were selected according to previous studies (Jiang, 2013). The PCR products of *Paris* species amplified with universal primers were purified with a column DNA purification kit according to the manufacturer's instructions. The purified products were sequenced by Tsingke Biotech Co. (Chengdu, China). Each target DNA fragment was sequenced twice. Direct sequencing of both strands in opposite directions was performed, which produced two complementary sequences of high quality.

PCR Amplification and Color Reaction

The sequences were aligned by ClustalW software with ClustalX 2.0 and redundant sequences were removed with Mega 5.0. Then, species-specific primers were designed according to the ITS region of PPC nrDNA with Primer Premier 5.0 software. The sense primer (PPC-F) was 5'-GCTAGGCAACGCGTACAGCAT-3', and the antisense primer (PPC-R) was 3'-TAGGGTGTCCGCATCAAGCG-5'. The universal primers were in the 5.8S region; the sense primer was 5'-CGTGACGCCCAGGCAGGC-3', and the antisense primer was 3'-TCCCGTATCGATGAAGAACGTAGCGA-5'. PCR amplification was performed in a 25 μL reaction mixture containing 1 μL DNA template (approximately 30 ng), 10 × Taq buffer (200 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, and 20 mM $MgSO_4$; pH 8.4), 0.2 mM dNTPs, 0.25 μM of each primer, and 2.5 U EasyTaq polymerase. Template denaturation was conducted at 94°C for 30 s. with annealing and extension at 66°C for 30 s. The final cycle included extension at 72°C for 3 min. in order to ensure full extension of the target fragment. The PCR product was added to 1 μL 1000×SYBR Green I, and the color change was examined under UV light by naked eyes.

Results

Results of Color Reaction of the PCR Product

The results of the color reaction showed that all the PPC samples yielded positive results when compared with the other eight species and subspecies in the case of both leaves and rhizomes. Moreover, there were no significant differences between the colorimetric results for the same species (Fig. 2 AB), which indicated the good reproducibility of this method.

Design of Primers for DNA Sequence Amplification

In order to avoid false negative results (negative results resulting from failures in DNA extraction or PCR), a pair of universal primers were designed in this study.

Table 2: Samples used in this study and the corresponding localities

Species	Abbreviation	Accession no	Locality	Collected parts
P. Polyphylla var. *chinensis*	PPC	KX146530	Dayi, Shichuan	leaf
P. polyphylla var. *chinensis*	PPC	KX146531	Shimian, Shichuan	leaf
P. polyphylla var. *chinensis*	PPC	KX146532	Pingwuxiangyan, Shichuan	leaf
P. polyphylla var. *chinensis*	PPC	KX146533	Pingwuxiangyan, Shichuan	leaf
P. polyphylla var. *chinensis*	PPC	KX146534	Pengzhou, Shichuan	leaf
P. polyphylla var. chinensis	PPC	KX146535	Yaan, Shichuan	rhizome
P. polyphylla var. *chinensis*	PPC	KX146536	Hubei	rhizome
P. polyphylla var. *polyphylla*	PPP-A	KX146537	Chongzhou, Shichuan	leaf
P. polyphylla var. *polyphylla*	PPP-B	KX146538	Hanyuan, Shichuan	leaf
P. polyphylla var. *polyphylla*	PPP-C	KX146539	Puge, Shichuan	leaf
P. polyphylla var. *polyphylla*	PPP-D	KX146540	PingwuXiangyan, Shichuan	leaf
P. polyphylla var. *polyphylla*	PPP-E	KX146541	WenxianTielou, Gansu	leaf
P. thibetica	PT	KX146542	Dayi Xiling, Shichuan	leaf
P. thibetica	PT	KX146543	PingwuMupi, Shichuan	leaf
P. thibetica	PT	KX146544	WenxianTielou, Gansu	leaf
P. fargesii var. *brevipetalata*	PFB	KX146545	ChongzhouYulang, Shichuan	leaf
P. fargesii var. *brevipetalata*	PFB	KX146546	ChongzhouGoujia,Shichuan	leaf
P. fargesii var. *brevipetalata*	PFB	KX146547	PingwuMupi, Shichuan	leaf
P. fargesii var. *brevipetalata*	PFB	KX146548	Dayi Huashuiwan, Shichuan	leaf
P. fargesii var. *brevipetalata*	PFB	KX146549	PengzhouShihcuan	leaf
P. fargesii var. *brevipetalata*	PFB	KX146550	Hubei	rhizome
Paris mairei	PM	KX146551	Shuicheng, Guizhou	leaf
Paris mairei	PM	KX146552	Shimian, Shichuan	leaf
Paris mairei	PM	KX146553	Puge, Shichuan	leaf
Paris mairei	PM	KX146554	Liangshan Huili, Shichuan	rhizome
P. polyphylla var. *stenophylla*	PPS-A	KX146555	Pengzhou, Shichuan	leaf
P. polyphylla var. *stenophylla*	PPS-B	KX146556	Dayi Xiling, Shichuan	leaf
P. polyphylla var. *stenophylla*	PPS-A	KX146557	Chongzhou, Shichuan	rhizome
P. polyphylla var. *stenophylla*	PPS-B	KX146558	YaanTianquan, Shichuan	rhizome
P. vaniotii	PVA	KX146559	Chongzhougoujia, Shichuan	leaf
P. vaniotii	PVA	KX146560	Dayi Huashuiwan, Shichuan	leaf
P. vaniotii	PVA	KX146561	Shimian, Shichuan	leaf
P. axialis	PA	KX146562	Dayi Xiling, Shichuan	leaf
P. axialis	PA	KX146563	yaan, Shichuan	rhizome
P. vietnamensis	PVI	KX146564	Jinping, Shichuan	leaf

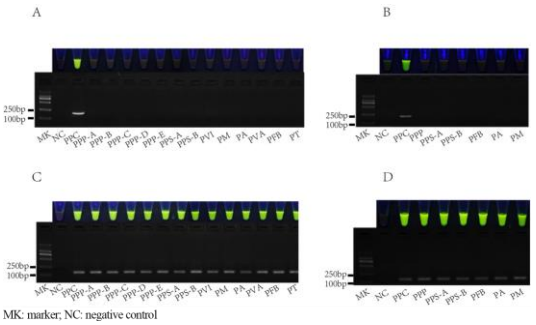

Fig. 2: Results of agarose gel electrophoresis and color reaction using SYBR Green I. (A) Results of amplification by species-specific primers of DNA from samples of leaves from *Paris* species. (B) Results of amplification by species-specific primers of DNA from samples of rhizomes from *Paris* species. (C) Results of amplification by universal primers of DNA from samples of leaves from *Paris* species. (D) Results of amplification by universal primers of DNA from samples of rhizomes from *Paris* species

The universal primers could amplify DNA from all the *Paris* species and most monocotyledons. The results of the amplification are shown in (Fig. 2CD). In order to simplify the procedure, the amplification conditions in species-specific PCR were the same as those in universal PCR.

The ITS sequences of all nine *Paris* species and subspecies were amplified sequenced and submitted to GenBank with accession numbers from KX146530 to KX146564 (Table 2). According to the alignment of these sequences with ClustalX 2.0 and the analysis with Mega 5.0, the species-specific primers designed using Primer Premier 5.0 for the identification of PPC is shown in (Fig. 3). There were variations at sites in the primer binding region between four PPS samples and five PPP samples, Therefore, in this study the sequence of the ITS region from PPS was divided into two types (PPS-A and PPS-B), and that from PPP was divided into five types (PPP-A, PPP-B, PPP-C, PPP-D and PPP-E) according to the intraspecific variations in terms of sites (Fig. 3).

Although theresults of sequence alignment of ITS region showed that intraspecific genetic distance was less than the interspecific genetic distance between PPC. For the other eight species and subspecies, the primers for PPC were similar to those for the other eight species and subspecies. For instance, the sense primer was the same as that for PA, and the antisense primer, in comparison with that for PA, only differed at two specific sites. So, in order to avoid nonspecific amplification, a mismatch was introduced by the substitution of nucleotide A for G in primer A and A for T in primer Bat the second base from the 3'terminus in both cases (Fig. 3).

Methodological Investigation

In order to confirm the applicability and reliability of this method, five samples of Rhizoma Paridis decoctions were purchased from five local drugstores. Three portions of each sample were removed randomly for the fluorescence reaction. The extracted DNA was analyzed by PCR amplification with the species-specific primers and universal primers described above. The PPC decoctions were identified by the method stated above (Fig. 4). In order to verify the accuracy of results of the color reaction, we amplified the ITS sequences of all the samples and identified them by the basic local alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI). The results indicated the successful practical use of this method in the identification of PPC and its adulterants (Table 3).

Discussion

Recently, many molecular authentication methods for accurately and rapidly differentiating between traditional Chinese medicines have been reported. For example, an ITS2 sequence was successfully used as a barcode to identify the herbal tea ingredient *Plumeria rubra* and its adulterants (Shi *et al*., 2014). Bruni *et al*. (2015) used the *rbcL* and *psbA-trnH* regions to identify the taxonomic composition of multiflower honey, and detected residues from one toxic plant in one sample. Radix Pulsatillae was differentiated on the basis of an ITS2 barcode using PCR-restriction fragment length polymorphism (Zhang *et al*., 2014; Shi *et al*., 2017). Mishra *et al*. (2018) proposed that DNA barcoding coupled with high-resolution melting is an efficient molecular tool for authenticating Senna herbal products.

Our market survey revealed that there were a number of adulterants of Rhizoma Paridis on the market, which would have serious potential health risks for users. Other molecular authentication methods have been proposed for authenticating Rhizoma Paridis, such as inter-simple sequence repeat analysis, amplified fragment length polymorphism, ITS sequencing, and DNA barcoding (Ji *et al*., 2006; He *et al*., 2007; Zhang *et al*., 2008; Jiang *et al*., 2013), which can precisely identify some *Paris* species, but these methods suffer from some technical defects. For example, agarose gel electrophoresis is time-consuming and laborious, and DNA sequencing is costly. In this study, we devised a visualization method for the authentication of PPC and its adulterants, which is based on ITS sequences. As speculated, this method can accurately and rapidly differentiate PPC from common adulterants, even those from species of the same genus, without DNA sequencing. Furthermore, our method successfully differentiated Rhizoma Paridis decoctions and 14 adulterants in samples from local drugstores (Table 3). Therefore, our study indicated that this method is highly specific for *Paris* species and can effectively discriminate them from other species. Moreover, the fluorescence of the PCR product mixture can be observed visually, thus avoiding the use of agarose gel electrophoresis, as well as DNA sequencing or gene scanning.

In order to improve the practicability of the method, a pair of specific primers for PPC was selected, and the length of the PCR products was only 209bp. These are much shorter than other candidate barcodes and can easily be amplified, even in the case of degraded DNA samples that are present in processed or poorly preserved medicinal herbs or medicinal materials that have been stored for a long time.

In order to guarantee the accuracy and reliability of the results, fresh leaves and rhizomes of the original plants were collected via a field investigation. In addition, a pair of universal primers were designed to avoid false negative results, which made the method more scientific and rational. This research will greatly enhance the technical level of the identification of the authenticity of herbal medicines. It can also provide a reference for the investigation of common problems that exist in the determination of the quality of herbal medicines characterized by "various related species of the same genus".

Conclusion

The visual molecular authentication method employing the dsDNA-specific dye "SYBR Green I", which is based on the diversity of ITS sequences of nrDNA, for distinguishing PPC from its adulterants is reliable, sensitive, and quick. It can lay a foundation for the identification of germplasm resources and the determination of the clinical safety of medications containing PPC and provide new ideas and methods for the sustainable utilization of PPC resources.

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