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Detection of *Valeriana jatamansi* as an Adulterant of Medicinal *Paris* by Length Variation of Chloroplast *psb*A-*trn*H Region

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Key words
- *Paris polyphylla* var. *chinensis*
- *Paris polyphylla* var. *yunnanensis*
- Melanthiaceae
- *Valeriana jatamansi*
- Valerianaceae
- molecular authentication
- *psbA-trn*H

Abstract

Two varieties of *Paris polyphylla* Smith (Melanthiaceae), *P. polyphylla* Smith var. *chinensis* (Franch.) Hara, and *P. polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz., are used as medicinal *Paris* in China. Their dried rhizomes are the major source of raw material for some medicines. In recent years, medicinal *Paris* has been found to be adulterated with *Valeriana jatamansi* Jones (Valerianaceae) due to its high market demand and natural resource deficiency. After the chloroplast *psb*A-*trn*H regions of medicinal *Paris* and *V. jatamansi* were sequenced and analyzed, it was found that their characteristic sizes were > 1000 and around 250 bp, respectively. Based on length variation, medicinal *Paris* and the mixed adulterant were detected and distinguished from each other by amplification and electrophoresis. The amount of *V. jatamansi* that can be identified as an adulterant of medicinal *Paris* was also investigated. A trace amount (1 : 1000) of the adulterant was detected in the sensitivity tests. The established method has been proven to be sensitive and reliable.

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Introduction

The genus *Paris* belongs to the family Melanthiaceae which comprises 24 species of perennial herbs distributed throughout Europe and Eastern Asia. Twenty-two species are found in Eastern Asia only, mainly in China (19 species), with the Yunnan-Guizhou Plateau as the center of diversity [1, 2]. *Paris* is well-known in China for its medicinal values. Species with thick rhizomes are used as traditional Chinese medicines or medicinal herbs because of their antitumoral, hemostatic, and anti-inflammatory properties among others [3, 4]. Two varieties of *Paris polyphylla, P. polyphylla* Smith var. *chinensis* (Franch.) Hara and *P. polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz., are the botanical origins of medicinal *Paris* [5]. The dried rhizomes of *Paris* species are commonly used as a major source of raw material for some traditional Chinese medicines, e.g., "Yunnan Baiyao," which is well-known for its analgesic and hemostatic uses [3, 5]. The cultivation of medicinal *Paris* began in the 1990s, but there are still some technical bottlenecks involved [4, 6]. Pharmaceutical companies producing *Paris*-based products have to purchase raw materials collected from the wild. Furthermore, the natural growth and propagation of medicinal *Paris* are very slow, and most of their natural habitats have been destroyed by human activities. Due to its high market demand, the natural resource of medicinal *Paris* has been nearly exhausted by overharvesting, resulting in a dramatic increase in its price in recent years [4]. To make more profits, local collectors or herbal dealers often resort to fraud by adulterating the rhizomes of medicinal *Paris* with the ones of the cheaper and more abundant *Valeriana jatamansi* Jones (Valerianaceae) (YH Ji, person. observ.).

The chemical components in the rhizomes of *V. jatamansi* and medicinal *Paris* are quite different. They also show distinct medicinal properties. Therefore, the practice of mixing *V. jatamansi* and *Paris* is a serious quality control issue in the herbal industry. This has to be dealt with because the mixture causes inconsistent therapeutic effects and may jeopardize the safety of consumers. However, the rhizomes of *V. jatamansi* and medicinal *Paris* have similar morphologies; hence, distinguishing them based on morphological fea-

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tures alone is difficult. At present, identification of the adulterant in market trading or pharmaceutical production basically relies on people's experiences or is made, for instance, simply by smelling and tasting. Unfortunately, these methods are not reliable because they lack scientific control. Furthermore, only few skilled people can so identify adulterants. Hence, an accurate, sensitive, and simple method for the reliable identification of the adulterant is urgently needed.

The length of the chloroplast *psb*A-*trn*H region varies enormously from 198 to 1077 bp in angiosperms [7]. In previous studies it has been observed that this length ranges from 1078 to 1103 bp among species of *Paris* [8], which was thought to be atypical [7]. Comparatively, the length is much shorter (174 to 334 bp) among species of the genus *Valeriana* [9,10]. Based on conserved *psb*A and *trn*H genomic sequences, the universal primer pair of *psb*A and *trn*H were developed [11]. Hence, the region could be amplified in almost all flowering plants [6,12,13]. Therefore, the length variation of the *psb*A-*trn*H region between species of *Paris* and *Valeriana* is likely to be a useful marker to distinguish between medicinal *Paris* and the adulterant. Correspondingly, the present study aims (1) to determine whether the length variation of the *psb*A-*trn*H region of medicinal *Paris* and *V. jatamansi* can be a good tool for the rapid identification of the genus *Paris* and (2) to investigate the detection level of *V. jatamansi* as an adulterant of medicinal *Paris* using *psb*A-*trn*H region length variation.

Materials and Methods

Plant material

Silica gel dried leaves of *V. jatamansi* and medicinal *Paris* were collected from different localities in China (**Table 1**). All samples were identified by Dr. Yunheng Ji, and vouchers were deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN). Commercially prepared crude drugs of medicinal *Paris* and *V. jatamansi* (dried rhizomes) were purchased from the market.

Preparation of the medicinal *Paris* / *V. jatamansi* mixtures

To determine what amount of *V. jatamansi* tissue can be detected as an adulterant of medicinal *Paris,* medicinal *Paris* / *V. jatamansi* mixtures were prepared. Crude drugs of *V. jatamansi* and medicinal *Paris* were ground to powder with liquid nitrogen, and mixed in 8 grades in quantity ratios of 1 : 1, 1 : 1.5, 1 : 20, 1 : 100, 1 : 200, 1 : 500, 1 : 750, and 1 : 1000. Fifty mg powder from each mixture was used for DNA extraction.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from silica dried leaves following modified CTAB methods [14], and from crude drugs and medicinal *Paris* / *Valeriana jatamansi* mixtures using the Takara Universal Genomic DNA Extraction Kit Ver.3.0 (Takara) according to the manufacturer's protocol. The chloroplast *psb*A-*trn*H region was amplified with the primer pair *psb*A (5′-GTTATGCATGAACGTAATGCTC-3′) and *trn*H (5′-CGCGCATGGTGGATTCACAATCC-3′) [11]. The PCR reaction mixture (total volume 25 µL) contained less than 10 ng of genomic DNA, 2.5 µL 10X PCR buffer (Mg^{2+}), 10 pmol/L primers, 5 mmol/L dNTP mix, and 1.5 U Taq DNA polymerase (Biomed). PCR was performed with a thermal cycler as follows: 92 °C for 4 min initially, 35 cycles at 94 °C for 1 min, annealing at 52 °C for 1 min, 64 °C for 1 min (extension), and one final cycle at 64 °C for 8 min. Each PCR reaction was analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized under UV light. The sizes of the PCR products were compared to molecular standards 100 and 2000 bp DNA ladders (Biomed) and detected under UV-light.

Amplified fragments of medicinal *Paris* and *V. jatamansi* (leaves and crude drugs) were purified and sequenced at Sunbiotech Corp. The forward and reverse strands of all medicinal *Paris* samples were sequenced. Comparatively, samples of *V. jatamansi* were determined in one direction, with either the primer *psb*A or *trn*H since the sequences were of sufficient quality, making sequence generation of the reverse strand unnecessary.

Sequence analysis

Sequences were compiled with Sequencher 4.2 version (Gene Codes Corp.) and compared with the *psb*A-*trn*H regions of other

Table 1 Plant materials used in this study.

Taxon	Collected locality	Voucher No	Assession Number
Paris polyphylla var. *chinensis*	Jinping, Yunnan (JP)	X Gong *s. n.*	GU477652
P. polyphylla var. *chinensis*	Nanchuan, Chongqing (NC)	YH Ji, 03107	GU477653–GU477654
P. polyphylla var. *chinensis*	Hefeng, Hubei (HF)	YH Ji, 02089	GU477655–GU477657
P. polyphylla var. *chinensis*	Zhangjiajie, Hunnan (ZJJ)	YH Ji, 02078	GU477658–GU477660
P. polyphylla var. *yunnanensis*	Lijiang, Yunnan (LJ)	YH Ji, 08032	GU477661–GU477663
P. polyphylla var. *yunnanensis*	Songming, Yunnan (SM)	YH Ji, 07232	GU477664–GU477666
P. polyphylla var. *yunnanensis*	Shizong, Yunnan (SZ)	YH Ji, 07228	GU477667–GU477668
P. polyphylla var. *yunnanensis*	Tengchong, Yunnan (TC)	GLGS EXP, 23448	GU477669–GU477671
P. polyphylla var. *yunnanensis*	Luquan, Yunnan (LQ)	YH Ji, 03121	GU477672–GU477674
P. polyphylla var. *yunnanensis*	Huili, Sichuan (HL)	YH Ji, 04045	GU477675–GU477677
P. polyphylla var. *yunnanensis*	Yanyuan, Sichan, (YY)	YH Ji, 07043	GU477678–GU477679
Valeriana jatamansi	Xichang, Sichan (XC)	YH Ji 07191	GU477680
V. jatamansi	Muli, Sichan (ML)	YH Ji 07182	GU477681
V. jatamansi	Wuding, Yunnan (WD)	YH Ji 08243	GU477684
V. jatamansi	Gongshan, Yunnan (GS)	M Gao *s. n.*	GU477682
V. jatamansi	Huize, Yunnan (HZ)	YH Ji 08187	GU477686
V. jatamansi	Songming, Yunnan (SM)	TZ Zhao *s. n.*	GU477685
V. jatamansi	Heqing, Yunnan (HQ)	R Yue *s. n.*	GU477683
V. jatamansi	Luquan, Yunnan (LQ)	XM Li *s. n.*	GU477687

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species of *Paris* and *Valeriana* using the Basic Local Alignment Search Tool (BLAST) [15]. This is to ensure that the correct region and species were amplified. The sequences were aligned by BioEdit version 7.0.4 [16] and analyzed using MEGA 4.0 [17].

Supporting information

The aligned sequences of chloroplast *psb*A-*trn*H from *Paris polyphylla* var. *chinensis, P. polyphylla* var. *yunnanensis*, and *Valeriana jatamansi* are available as Supporting Information.

Results

In total, 41 DNA fragments were amplified and sequenced in the present study. Among them, 9, 19, and 8 were from the silica dried leaves of *P. polyphylla* var. *chinensis, P. polyphylla* var. *yunnanensis*, and *V. jatamansi*, respectively. In addition, 5 sequences were from commercially available crude drugs of medicinal *Paris* (3 sequences) and *V. jatamansi* (2 sequences). Comparison of these obtained sequences with those derived from GenBank showed that the similarities to *P. polyphylla* var. *chinensis* are 99.3%; to *P. polyphylla* var. *yunnanensis*, 92.9–99.4%; and to *V. jatamansi*, 67.7–72.6%. The size of the amplified *psb*A-*trn*H region was conserved in both medicinal *Paris* and *V. jatamansi* samples. It ranged from 1077 to 1084 among samples of *P. polyphylla* var. *yunnanensis* (**Fig. 1S**), and 263 to 267 bp among samples of *V. jatamansi* (**Fig. 2S**).

Multiple alignment of the *psb*A-*trn*H sequences of medicinal *Paris* and *V. jatamansi* samples was performed. Of the 1086 aligned positions of medicinal *Paris* samples (partial *psb*A gene: 1–53, intergenic spacer: 54–1086), 25 were variable. The similarities among *P. polyphylla* var. *chinensis* and *P. polyphylla* var. *yunnanensis* samples were 100% and 96.8–100%, respectively. The divergence of *psb*A-*trn*H sequences among *P. polyphylla* var. *yunnanensis* samples was unexpectedly high and included an inversion of 16 bp in sites 107–122, an insertion of 8 bp in sites 123–130, and 3 transitions, from which a total of 5 haplotypes were observed (**Table 2**). We carried out a sequence comparison of these haplotypes in *Paris* species. It showed that haplotype-A and -B, haplotype-C and -D, and haplotype-E had the highest similarity (99%) to *P. polyphylla* var. *yunnanensis, P. mairei,* and *P. daliensis*, respectively. The divergence of *psb*A-*trn*H sequences (partial *psb*A gene: 1–70, intergenic spacer: 71–267) among *V. jatamansi* samples was much lower. Only an inversion of 6 bp was observed in aligned sites 134–139 from the XC sample (**Fig. 2S**). The lengths of the PCR products of the *psb*A-*trn*H sequences for medicinal *Paris* were above 1000 bp, whereas those for *V. jatamansi* were around 250 bp. This difference could be obviously seen by electrophoresis on 1% agarose gel (**Fig. 1**). The experiments were repeated thrice, and the same results were obtained. We also investigated the amount of *V. jatamansi* tissue that can be detected as the adulterant of medicinal *Paris* tissue. We extracted DNA from mixtures consisting of *V. jatamansi* and medicinal *Paris* with ratios from 1 : 1 to 1 : 1000. This mixed DNA served as the template in PCR amplification using the primers *psb*A and *trn*H [10]. The PCR protocol was the same as that used for DNA templates extracted from leaf samples and crude drugs. The PCR products were run on 1% agarose gel. Two clear electrophoresis fragments with lengths of around 250 and > 1100 bp in every lane were identified (**Fig. 2**), which accordingly correspond to the amplified *psb*A-*trn*H regions of *V. jatamansi* and medicinal *Paris*, respectively. The experiments were also repeated thrice to verify the results.

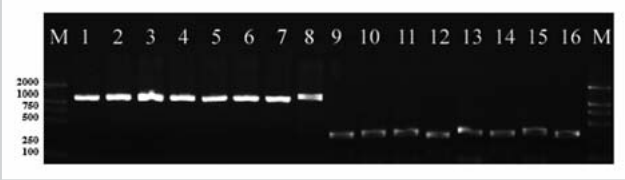

Fig. 1 Agarose gel electrophoresis of PCR products of medicinal *Paris* and *V. jatamansi* collected from different locations. Lane 1–3: *P. polyphylla* var. *chinensis* JP, NC and ZJJ. Lane 4–8: *P. polyphylla* var. *yunnanensis* HL, LJ, LQ, SZ, TC. Lane 9: crude drug of medicinal *Paris*. Lane 10–16: *V. jatamansi* GS, HZ, LQ, ML, SM, WD, XC and crude drug. M: DL2000 DNA marker.

Fig. 2 Agarose gel electrophoresis of PCR products from *V. jatamansi*/medicinal *Paris* mixtures in different ratios. Lane 1–8: 1 : 1000, 1 : 750, 1 : 500, 1 : 200, 1 : 100, 1 : 20, 1 : 5 and 1 : 1. Lane 9: crude drug of *jatamansi*. Lane 10: crude drug of medicinal *Paris*. M: DL2000 DNA marker.

Discussion

Previous studies on *P. polyphylla* var. *yunnanensis* indicated that not only the morphologies but also the content of biologically active components among geographical populations is diversified [2, 18]. Natural hybridization between *P. polyphylla* var. *yunnanensis* and sympatric *Paris* species was also found based on molecular evidence [8]. The divergence of *psb*A-*trn*H sequences among *P. polyphylla* var. *yunnanensis* samples is remarkably high, which is an unexpected result. Homology searches with the five *psb*A-*trn*H haplotypes showed that three of them (haplotype-C,

Table 2 Variable sites of aligned *psb*A-*trn*H region in five haplotypes of *Paris polyphylla* var. *yunnanensis* samples.

Aligned sites						
	107–122	133–130	155	159	395	1074
Haplotype-A	TAGTATATAAAGAGG	–	T	A	G	T
Haplotype-B	TAGTATATAAAGA–	TAAAGAGG	A	A	G	T
Haplotype-C	CCTCTTTATATACTA	–	T	T	G	T
Haplotype-D	CCTCTTTATATACTA	–	T	T	A	–
Haplotype-E	CCTCTTTATATACTA	–	T	A	G	T

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-D, and -E) had the highest similarity to *P. mairei* or *P. daliensis* rather than to *P. polyphylla* var. *yunnanensis*. Voucher specimens of all samples were carefully examined to test if we made a wrong identification. However, no distinctive variation among the samples was detected. Haplotype-C, -D, and -E were present in the samples from central Yunnan and southwestern Sichan where *P. polyphylla* var. *yunnanensis* exists with sympatric *P. mairei* or *P. daliensis*. This suggests that natural hybridization occured in at least some of the samples. Therefore, introgression among related species which resulted from natural hybridization is likely to be the main reason for the unusually high genetic divergence, diversified morphologies, and chemical components among *P. polyphylla* var. *yunnanensi* populations.

The main purpose of this study was to determine if *psb*A-*trn*H length variation can be used to detect *V. jatamansi* as the adulterant of medicinal *Paris*. The *psb*A-*trn*H intergenic region has been successfully amplified from DNA extracted from both leaf and crude drug of *Paris* and *V. jatamansi*. This means that the targeted fragments were successfully amplified with the universal primer pairs. The *psb*A-*trn*H length variation between *V. jatamansi* and medicinal *Paris* is distinctive. The characteristic lengths of the *psb*A-*trn*H regions of *V. jatamansi* and medicinal *Paris* are around 250 bp and > 1000 bp, respectively. Based on this difference, *V. jatamansi* and medicinal *Paris* can easily be detected and distinguished by amplifying this region by PCR and analyzing the products on 1% agarose gel. The results demonstrated that the length variation of the *psb*A-*trn*H region can help to authenticate medicinal *Paris* and its adulterant *V. jatamansi*.

In previous studies, various molecular methods have been developed to authenticate medicinal herbs. These methods include PCR-restriction fragment length polymorphism (PCR-RFLP) [19], amplification refractory mutation system (ARMS) [20], TaqMan real-time PCR [21], and DNA barcoding technology [22]. However, these methods exhibit some clear disadvantages. For instance, in PCR-RFLP, the restriction digestion of PCR products can be incomplete or fail because some components of the PCR reaction hinder the activity of the restriction endonuclease [23]. Meanwhile, in the ARMS method, large amounts of PCR products need to be amplified, sequenced, and analyzed. Finally, TaqMan real-time PCR depends too much on the availability of experts or expensive laboratory equipment. As compared to the above-mentioned methods, the method developed in the current study is simple, cheap, and fast. DNA extraction, PCR amplification, and electrophoresis are the only techniques needed to apply this method; these can all be carried out in an ordinary laboratory by technicians trained in basic molecular biology.

Another purpose of this study was to test the sensitivity of the proposed method. The amount of *V. jatamansi* that can be detected as an adulterant of medicinal *Paris* was determined by analyzing the mixtures of medicinal *Paris* and the adulterant. Commercially prepared crude drugs of medicinal *Paris* and *V. jatamansi* purchased from the market were tested. Even though the extracted genomic DNA was partly degraded, the targeted sequences were still amplified and recognized as medicinal *Paris* and *V. jatamansi*. As little as 1 : 1000 of *V. jatamansi* was detected in the mixtures. This proved that the proposed method is sensitive and reliable.

DNA degradation is inevitable during the harvest, storage, and processing of crude drugs [23]. Some crude drug samples of medicinal *Paris* are unlikely to have the unique long length (> 1000 bp) of the *psb*A-*trn*H region amplified because their DNA has been severely degraded. Nevertheless, this situation does not affect the sensitivity and reliability of the proposed method to detect whether medicinal *Paris* is adulterated with *Valeriana jatamansi*. Due to its relatively short length (approximately 250 bp), amplification of this genomic region from *V. jatamansi* samples can be successful even without the use of high-quality DNA as a template. For instance, amplification of the *psb*A-*trn*H intergenic region with a length of approximately 300 bp can still be successful even if DNA extracted from herbarium specimens collected 50 years ago is used [12].

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