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Full Length Research Paper

Molecular authentication of the medicinal plant *Paris polyphylla* Smith var. *yunnanensis* (Melanthiaceae) and its related species by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP)

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***Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz. (Melanthiaceae) is a traditional herb widely used by Chinese people, especially in southwestern provinces. Its dried rhizome (medicinal *Paris*) is a traditional Chinese medicine. In the trade and raw drug market, the medicinal *Paris* is available in the mixture form with other *Paris* species, thus making it difficult to distinguish medicinal *Paris* from its related species. Recent studies have shown that the *Paris* spp. possess different chemical constituents, pharmacological activities, and efficiency in clinical application, which may cause a series of inconsistent therapeutic effects and quality control problems in the herbal medicine industry. In this study, the differential identification of *P. polyphylla* var. *yunnanensis* and its 11 congeners were investigated through DNA sequence analysis of nuclear internal transcribed spacer (ITS) regions. Based on sequence alignments, we concluded that the ITS sequence can distinguish *P. polyphylla* var. *yunnanensis* from other *Paris* species. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was applied successfully through the digestion of ITS product by the restriction enzyme *EaeI*. The specific sizes of the two digested products were 440 and 194 bp, which could be investigated in *P. polyphylla* var. *yunnanensis* The PCR-RFLP technique developed in this study can be used to discriminate medicinal *Paris* and its related species.**

Key words: *Paris polyphylla* Smith var. *yunnanensis,* Melanthiaceae, internal transcribed spacer (ITS), molecular authentication, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP).

INTRODUCTION

The genus *Paris*, which comprises 24 species of perennial herbs distributed throughout Europe and Eastern Asia, belongs to the family Melanthiaceae. Twenty-two species are found in East Asia only, mainly in China (19 species), with the Yunnan-Guizhou Plateau as the center of diversity (Li, 1984, 1998). *Paris* is well-known in China for its medicinal values. Species with thick rhizomes are used as traditional Chinese medicines or medicinal herbs for their antitumoral, hemostatic, and anti-inflammatory properties among others (Long et al., 2003; He et al., 2006). Recently, *Paris* has got in much attention because of its significant biological activities, such as anti-tumor, analgesia, anti-inflammatory, and antifungal (Wu et al., 2004). In addition, its effect on inhibit ethanol-induced gastric lesions (Matsuda et al., 2003) and immunostimulating activity (Zhang et al., 2007) has also been reported.

According to the Chinese Pharmacopoeia (The Pharmacopoeia Commission of the PRC, 2005), medicinal *Paris* refers only to the rhizomes of *P. polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz. and *P. polyphylla* Smith var. *chinensis* (Franch.) Hara. The dried rhizomes of medicinal *Paris* are commonly used as a major source of raw material for some

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Table 1. Samples used in this study with voucher information, and GenBank accession numbers.

Taxa	Sample origin	Voucher or source	Accession no.
Paris polyphylla var. *yunnanensis*	Yunnan, China	Y.H. Ji 131 (KUN)	DQ404223
P. dunniana	Yunnan, China	Y.H. Ji 128 (KUN)	DQ404225
P. daliensis	Yunnan, China	Q. Guo s.n. (KUN)	DQ404226
P. vietnamensis	Yunnan, China	Y.H. Ji 139 (KUN)	DQ404212
P. mairei	Yunnan, China	W.Y. Xu s.n. (KUN)	DQ404213
P. cronquistii	Yunnan, China	Y.H. Ji 133 (KUN)	DQ404214
P. delavayi var. delavayi	Yunnan, China	Y.H. Ji 135 (KUN)	DQ404215
P. thibeitca	Yunnan, China	GLGS Exp. 21597 (KUN)	DQ404216
P. fargessi	Yunnan, China	Y.H. Ji 129 (KUN)	DQ404217
P. delavayi var. petiolata	Yunnan, China	S.T. Chen s.n. (KUN)	DQ404220
P. marmomata	Yunnan, China	Y.H. Ji 197 (KUN)	DQ404222
P. axialis	Yunnan, China	Y.H. Ji 149 (KUN)	DQ404210

traditional Chinese medicines, for example, “Yunnan Baiyao”, which is well-known for its analgesic and hemostatic uses (Long et al., 2003; The Pharmacopoeia Commission of the PRC, 2005). The cultivation of medicinal *Paris* began in the 1990s, but there are still some technical bottlenecks involved (He et al., 2006; Li., 1998). Pharmaceutical companies producing *Paris*-based products have to purchase raw materials collected from the wild. However, other *Paris* species that do not conclude in the Chinese Pharmacopoeia are also considered as a source of medicinal *Paris* by most people, especially in southwestern China, the main producing area of medicinal *Paris*. During herbal drug market survey, it was observed that in total of 11 species (varieties) were being sold in mixed form with *P. polyphylla* var. *yunnanensis* in Guizhou, Sichuan, and Yunnan Provinces. All species of the genus *Paris* were confused with each other and the identification of these species possess considerable difficulties due to the similarities in their appearance when these species were available only in fragmentary form. Based on the modern phytochemical and pharmacological studies, the active compounds responsible for the biological activities of medicinal *Paris* are a series of steroidal saponins, especially various diosgenin glycosides and pennogenyl saponins, such as polyphyllins VII and polyphyllins II, which could strongly inhibit gastric lesions induced by ethanol and indomethacin (Matsuda et al., 2003), and polyphyllins I, which was reported as a potent apoptosis inducer in drug-resistant HepG2 cells (Cheung et al., 2005).

However, among species of *Paris*, biologically active compounds have large variations. For example, polyphyllins VII has been found in many species except *P. polyphylla* var. *pseudothibetica* (Huang et al., 2005). Furthermore, quantitative analysis of the chemical components of medicinal *Paris* and its congeners from southwest Sichuan and northwest Yunnan shown that the contents of their chemical compounds significant differs from each other (Yin et al., 2007a, b). Considering the various application of *Paris*, for the sake of medicinal safety, efficacy, and quality control, it is necessary to develop an effective and reliable method to authenticate and distinguish *P. polyphylla* var. *yunnanensis* from its related species.

In this paper, we describe the analysis of the internal transcribed spacer (ITS) nuclear ribosomal DNA of *P. polyphylla* var. *yunnanensis* and its related species. The results show that the alignment of ITS DNA sequences could allow the discrimination of the target plant species from its close relatives. The polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) based on the ITS sequence can uniquely identify *P. polyphylla* var. *yunnanensis* from its adulterants successfully and easily.

MATERIALS AND METHODS

Plant materials

Twelve species (Table 1) were sampled in Yunnan province, southwestern China. The plant specimens in this study were indentified by the authors, and vouchers were deposited at the herbarium of the Kunming Institution of Botany, Chinese Academy of Sciences (KUN).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from silica gel-dried or fresh leaves by using the method of Doyle and Doyle (1987). The total DNA was dissolved in TE buffer and was stored at -20°C for PCR.

Amplification was carried out in the Peltier Thermal Cycler 200 (BioRad Lab, USA). For the whole ITS region amplification, the primers of ITS_4 and ITS_5 (White et al., 1990) were used. The PCR programme was as follows: 94°C for 3 min; 35 cycles of 94°C for

30 s, 55°C for 30 s, 72°C for 45 s; and 72°C for 7 min. The PCR reaction mixture (total volume 25 µl) contained 10 ng of genomic DNA, 2.5 µl 10 × PCR buffer (with Mg^{2+}), 10 pmol/L primers, 5 mmol/L dNTP mix, and 1.5 U Taq DNA polymerase (Biomed; Beijing, China). Each PCR reaction were identified by electrophoresis with 2000 bp DNA ladders (Biomed; Beijing, China) on a 1.5% agarose gel which contained ethidium bromide, and detected under UV-light. Automated DNA sequencing was run on an ABI 3730 Sequencer (Foseter, USA) by United Gene Holdings, Ltd. (Shanghai, China).

Sequence alignment and RFLP analysis

DNA sequences were compared and compiled with Sequencher 4.2 version (Gene Codes Corp., USA), and were aligned using the clustal W multiple alignment tool of the software BioEdit version 7.0. Sequence analysis was performed with the software molecular evolutionary genetics analysis (MEGA) version 4.1. A neighbor-joining tree was constructed based on the Kimura 2-parameter distance method.

Restriction maps of the ITS sequences were done using the software Bioedit 7.0. From the restriction maps, *EaeI* was selected as a suitable candidate for discrimination of *P. polyphylla* var. *yunnanensis* and its congeners. Digestions with *EaeI* were recommended by the manufacturer (37°C for 3 h). The DNA was fractionated by a 2% agarose gel electrophorisis and visualized by ethidium bromide (EB) staining under ultraviolet light. Twelve *Paris* species (varieties) were analyzed with the PCR-RFLP method.

RESULTS AND DISCUSSION

For a DNA-based identification of *P. polyphylla* var. *yunnanensis*, the nuclear ribosomal ITS DNA region was submitted to multiple sequence alignment. The alignment of all sequences can be found in Figure 1. The ITS DNA sequence of all samples were successfully amplified and sequenced. These sequences were deposited in GenBank with the accession numbers listed in Table 1. Excluding the primer flanking sites, the sizes of the ITS_1 DNA were from 242 to 247 bp. The similarities among *P. polyphylla* var. *yunnanensis* and other species ranged from 92.7 to 96.8%. The sizes of the ITS_2 region were found to be 226 to 231 bp and the sequence similarities among *P. polyphylla* var. *yunnanensis* and other species were between 90.3 and 94.9%.

The sizes of the 5.8S rDNA region were from 226 to 231 bp and the sequence similarities among *P. polyphylla* var. *yunnanensis* and other species were between 97.6 and 98.8%. The overall ITS regions of all species were 632 to 636 bp in length. The interspecies percentages of nucleotide differences in the sequence of *Paris* species ranged from 0.4 to 5.8%. The sequence divergence among *P. polyphylla* var. *yunnanensis* and its related species were wide enough to differentiate them (Figure 2).

For the purpose of finding a quick, easily used, and accurate system of identification and to determine if the ITS region can be used for species identification, a Neighbor-Joining (NJ) tree was constructed. Base on the NJ tree, the 12 species (varieties) were divided into several clades (Figure 3). Among them, *Paris delavayi* var. *delavayi* and *Paris delavayi* var. *petiolata* was found to be closely related, which is also obvious from their name. *P. polyphylla* var. *yunnanensis* together with *Paris daliensis, Paris mairei*, *Paris thibeitca, Paris marmomata, Paris axialis* was separated with bootstrap support of 96%. Our research shows that the divergence of ITS region can distinguish *P. polyphylla* var. *yunnanensis* from its related species. This was supported by sequence alignment analysis, which revealed the high sequence variation is enough for species identification. Previous studies has also showed the ITS region is fitful for the identification of other medical plants, such as the authentication of *Saussurea lappa* carried out by Chen et al. (2008). Our results support the conclusions that ITS spacer could be a potential molecular maker for species discrimination.

During the analysis of restriction maps in the ITS region among the *Paris* spp., the sequence of *P. polyphylla yunnanensis* had one unique restriction enzyme site (C/GGCCA) for *EaeI* at position 200. The other 11 species did not have this restricted site. It suggested that the ITS regions of *P. polyphylla* var. *yunnanensis* could be digested by *EaeI* into two fragments whereas those from the adulterants could not be. After partial digestion, two new fragments were observed in the *P. polyphylla* var. *yunnanensis* samples and the negative results were found in the electrophoresis patterns of the samples of other *Paris* species (Figure 4). Because of the distinctive sizes of the two digested products, 440 and 194 bp, they could be separated into two fragments by using 1.5% agarose gel electrophoresis.

The RFLP fingerprinting could be easily be detected. The fragments of 634 bp existed with two digested fragments due to the partial digestion of the PCR products of the ITS region. We have tested several reaction conditions such as increased quantities of enzymes and prolonged reaction time, incomplete digestion is most likely a result of nuclear introgression between related species, which we had noted in previous study (Ji et al., 2006). Furthermore, by the analysis of the genotype between *P. polyphylla* var. *yunnanensis* and *P. mairei* (or *P. daliensis*), it suggested that natural hybridization between them occurred in at least some of the samples, which was justified by the fact that *P. polyphylla* Smith var. *yunnanensis* together with *P. daliensis, P. mairei* was separated with the high bootstrap support of 96% in the NJ tree.

Therefore, introgression among related species which resulted from natural hybridization is likely to be the main reason for the incomplete digestion. However, partial digestion did not influence the identification of *P. polyphylla* var. *Yunnanensis* because the PCR products from other species cannot be digested at all. Hence, the medicinal plant *P. polyphylla* var. *yunnanensis* can be distinguished from its related species based on the sequence divergence of ITS DNA regions, and the PCR-RFLP technique developed in the current study.

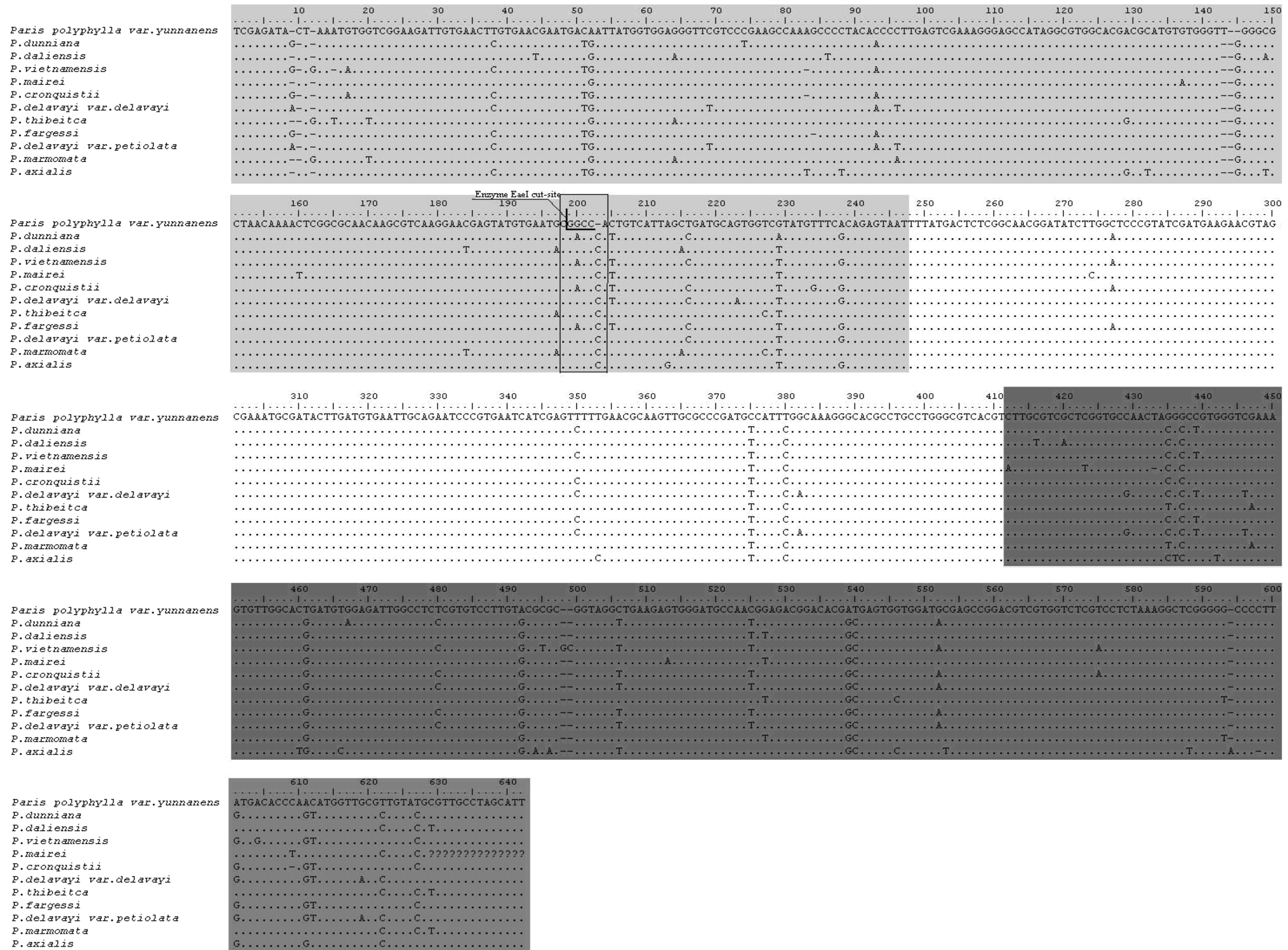

Figure 1. Alignment of ITS regions of *Paris* by Clustal W, showing ITS1 (shadows), 5.8s and ITS2 (black shadow). The "·" represents the same base as *P. polyphylla var.yunnanensis* in the same site. Box characters represent the enzyme *EaeI* cut-site.

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Paris polyphylla var.yunnanens   GTGGA TGCGAGCCGG ACGTCGTGGT CTCGTCCTCT AAAGGCTCGG GGG-CCCCTT ATGACACCCA ACATGGTTGC GTTGTAT [627]
P.dunniana                       ..... .A........ .......... .......... .......... ...-...... G......... GT........ .C....C [627]
P.daliensis                      ..... .......... .......... .......... .......... ...-...... .......... .......... .C....C [627]
P.vietnamensis                   ..... .A........ .......... ....A..... .......... ...-...... G..G...... GT........ ......C [627]
P.mairei                         ..... .......... .......... .......... .......... ...-...... ........T. .......... .C....C [627]
P.cronquistii                    ..... .A........ .......... ....A..... .......... ...-...... G.......-. GT........ ......C [627]
P.delavayi var.delavayi          ..... .A........ .......... .......... .......... ...-...... G......... GT......A. .C..... [627]
P.thibeitca                      C.... .......... .......... .......... .......... ..T-...... .......... .......... .C....C [627]
P.fargessi                       ..... .A........ .......... .......... .......... ...-...... G......... GT........ ......C [627]
P.delavayi var.petiolata         ..... .A........ .......... .......... .......... ...-...... G......... GT......A. .C....C [627]
P.marmomata                      ..... .......... .......... .......... .......... ..T-...... .......... .......... .C....C [627]
P.axialis                        C.... ..T....... .......... .......... .......T.. ...A...-.. G......... G......... .C..... [627]
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Figure 2. Representative sequence alignment of the ITS barcode region of 12 different *Paris* samples. Dots indicate idential nucleotide, dashes indicate gaps.

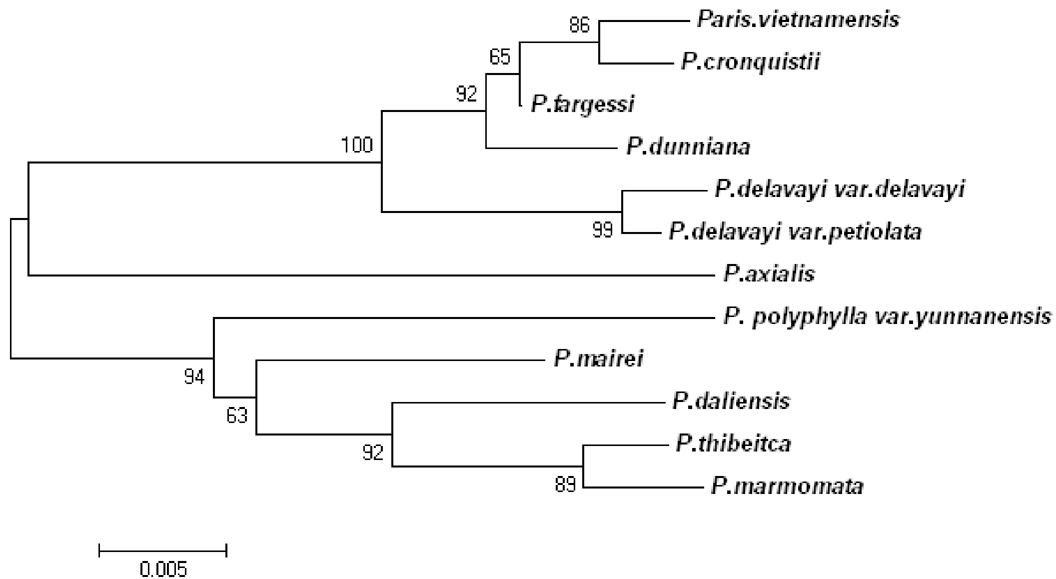

Figure 3. Classification tree of ITS region using the NJ method. Branch length was calculated by Kimura's 2-parameters method. Bootstrap (1000 replication) analysis was performed to estimate the confidence of the topology of the consensus tree. Bootstrap support values are show above branches.

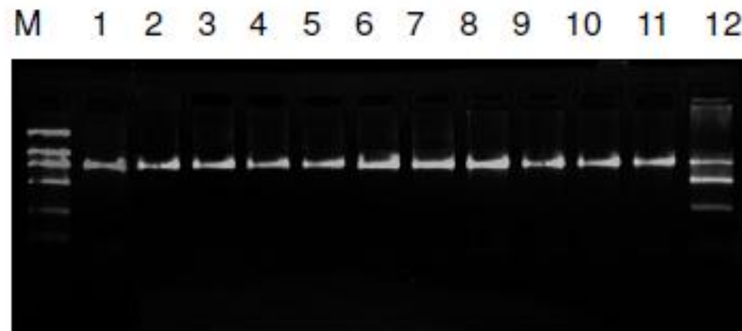

Figure 4. PCR-RFLP assay for *EaeI* digestion of the PCR products which was amplified with primer ITS4 and ITS5. 1, *Paris daliensis*; 2. *P. vietnamensis*; 3. *P. mairei*; 4. *P. cronquistii*; 5. *P. delavayi var.delavayi*; 6. *P. thibeitca*; *7. P. fargessi*; 8. *P. delavayi var. petiolata*; 9. *P. marmomata*; 10. *P. axialis*; 11. *P. dunniana*; 12. *P. polyphylla* var. *yunnanensis*. DNA markers (DL2000) in bp are indicated on left.

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