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Full Length Research Paper

Detection of *Arisaema yunnanense* as adulterant of traditional medicine *Pinellia ternata* using allele-specific diagnostic polymerase chain reaction (PCR)

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The dried tubers of *Pinellia ternata* (Araceae) have been widely used in traditional Chinese, Japanese and Korean medicine. Recently, *Pinellia* tuber has been found to be adulterated with *Arisaema yunnanense* in south West China due to its natural resource deficiency. To distinguish between *Pinellia* tuber and *A. yunnanense*, the chloroplast *atp*B-*rbc*L intergenic spacers of *P. ternata* and *A. yunnanense* were sequenced and analyzed, and two pairs of diagnostic primers were designed for differentiating *P. ternata* from *A. yunnanense*. The amount of *A. yunnanense* that can be identified as an adulterant of *Pinellia* tuber was also investigated. A minimum amount (1:200) of the adulterant was detected in the sensitivity tests. The present study provides a simple, reliable and sensitive method to authenticate *Pinellia* tuber, and to detect whether it has been adulterated with *A. yunnanense*.

Key words: *Pinellia ternata*, *Arisaema yunnanense,* Araceae, molecular authentication, diagnostic polymeras chain reaction (PCR), *atp*B-*rbc*L.

INTRODUCTION

Pinellia ternata (Thunb.) Breit. distributed in most provinces in China, is a perennial herb of the monocot family Araceae (The State Pharmacopoeia Commission of the PRC., 2005). The herb is a famous and widely used traditional medicine in East Asia countries. The dried tubers (*Pinellia* tuber) have been used as an antiemetic and an analgesic for about 1,500 years (Luo et al., 2000; He et al., 2007). It is an important ingredient of some well-known multi-herb remedies or prescriptions, example, Chinese “Xiaochaihu-Tang” (Chen et al., 2006), Japanese “Sho-Saiko-To” (Ohtake et al., 2004), and Korean “So-Pung-Tang” (Kim et al., 2007). Recent studies have reported that *Pinellia* tuber has anti-anxiety, anti-cancer, anti-virus, and anti-inflammation effects as well as the ability to induce abortion in early pregnancy (He et al., 2007; Han et al., 2006; Nagai et al., 2002). Modern phytochemical and phamarcological investigations indicated that the major effective constituents responsible for the biological activities are a series of alkaloids (Wu et al., 1998; Wu et al., 2003).

Pinellia tuber is among the top ten most commonly used medicinal herbs in China (Han et al., 2006). Because of over-harvesting, the natural resources have been nearly exhausted in recent years (Zheng et al., 1991). It has been adulterated with dried tubers of *Arisaema yunnanense* Buchet (Araceae) in southwestern China. *A. yunnanense* is chiefly distributed in Yuannan, western Sichan and Guizhou, and so similar with *P. ternata* in the features of aerial part and tuber that it isvery difficult to distinguish them just based on morphological characters. Hence, tubers of *A. yunnanense* were commonly collected and sold in mixed form with *Pinellia* tubers in these areas (Zheng et al., 1991). The chemical components in the tubers of *P. ternata* and *A. yunnanense* are quite different (Ma et al., 1991), and they also show distinct medicinal properties (Zheng et al., 1991). Inevitably, the practice of mixing them would cause inconsistent therapeutic effects and jeopardize the safety

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Abbreviations: CTAB, Cetyl trimethylammonium bromide; **PCR,** polymerase chain reaction**; MEGA,** molecular evolutionary genetics analysis.

Table 1. Plant samples used in this study.

Taxa	Locality	Abbreviate	Voucher	Accession No.
P. ternata	Kunming, Yunnan	KM	LiuT, 2008211	FJ638617
P. ternata	Qiaojia, Yunnan	QJ	Ji&Li 03081	HQ221740
P. ternata	Zhaojue, Sichuan	ZJ	GaoM *s.n.*	HQ221738
P. ternata	Nanchuan, Chongqing	NC	LCL *s.n.*	HQ221739
P. ternata	Tianshui, Gansu	TS	JSY 114	HQ221737
A. yunnanense	Kunming, Yunnan	KM	LiuT 2008212	FJ638618
A. yunnanense	Fuming, Yunnan	FM	LiuT 2008213	FJ638619
A. yunnanense	Chuxiong, Yunnan	CX	LiuT 2008214	FJ638620

of consumers. Therefore, an accurate and sensitive method for the reliable authentication of the adulterant is urgent.

In the present study, we used the DNA sequences of chloroplast *atp*B-*rbc*L intergenic spacer to examine the differences between *P. ternata* and *A. yunnanense*. A method of PCR amplification with allele-specific primers to distinguish them was developed based on the sequence divergence, and the sensitivity of the proposed method was tested.

MATERIALS AND METHODS

Plant materials

Silica gel dried leaves of *A. yunnanense* and *P. ternata* were collected from different localities of China (Table 1). Commercially prepared crude drugs of medicinal *P. ternata* and *A. yunnanense* (dried tubers) were purchased on the market. All samples were identified by specialist from Kunming Institute of Botany, Chinese Academy of Sciences, and vouchers were deposited in the herbarium of the institute (KUN).

Preparation of the *A. yunnanense* and *P. ternata* mixtures

To determine the least amount of *A. yunnanense* which could be detected as an adulterant of medicinal *P. ternata*, *A. yunnanense* / *P. ternata* mixtures were prepared. Crude drugs of *A. yunnanenses* and *P. ternata* were grounded to powders with liquid nitrogen, and then mixed at 5 grads on the quantity ratios of 1:10, 1:50, 1:100, 1:150 and 1:200. Fifty mg (50mg) powder for each mixture was quantified for DNA extraction.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Genomic DNA was extracted using the modified cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Chloroplast *atp*B-*rbc*L intergenic spacer was amplified with the universal primers *atp*B (5'-ACATCKARTACKGGACCAATAA-3') and *rbc*L (5'-AACACCAGCTTTRAATCCAA-3') (Chiang et al., 1998). The PCR reaction mixture (total volume 25 µl) contained *c.*10 ng of genomic DNA, 2.5µL 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). PCR was performed with a thermal cycler as follows: 94°C for 4 min initially; 35 cycles at 95°C for 30 s, annealing at 54°C for 30 s, 72°C for 1.15 min (extension); and one final cycle at 72°C for 7 min. Amplified fragments of *A. yunnanense* and *P. ternata* were purified and sequenced at Sunbiotech Corp. (Beijing, China). The forward and reverse strands of all samples were sequenced.

Data analysis

DNA sequences were compared and compiled with Sequencher 4.2 version (Gene Codes Corp., Ann Arbor, Michigan, USA), and were aligned using the clustal W multiple alignment tool of the software BioEdit version 7.0.4 (Hall, 1999). Sequence analysis was performed with molecular evolutionary genetics analysis (MEGA) software version 4.1 (Tamura et al., 2007). A neighbor-joining tree was constructed based on the Kimura 2-parameter distance method.

Diagnostic PCR amplification

Based on the variation between the *atp*B-*rbc*L intergenic spacer of *P. ternata* and *A. yunnanense* samples, two species-specific sense primers were designed. Both primers were synthesized by Sangon Corp. (Shanghai, China). The universal primer *rbc*L (Chiang et al., 1998) was used as the anti-sense. The diagnostic PCR reaction mixture (total volume 25 µl) contained less than 10 ng of genomic DNA, 2.5µl 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). PCR was performed with a thermal cycler as follows: 95°C for 4 min initially; 35 cycles at 94°C for 30 s, annealing at 52°C for 30 s, 72°C for 45 s (extension); and one final cycle at 72°C for 7 min. 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.

RESULTS AND DISCUSSION

Sequence divergence of *atp*B-*rbc*L intergenic spacers between *P. ternata* and *A. yunnanense*

The present investigation shows a range of 799 to 805 bp among *P. ternata* samples, and from 831 to 842 bp among *A. yunnanense* samples. The homologies among *P. ternata* and *A. yunnanense* samples are 99.13 to 100% and 98.58 to 100%, respectively. Comparatively, the divergence between the two species is distinctive. From

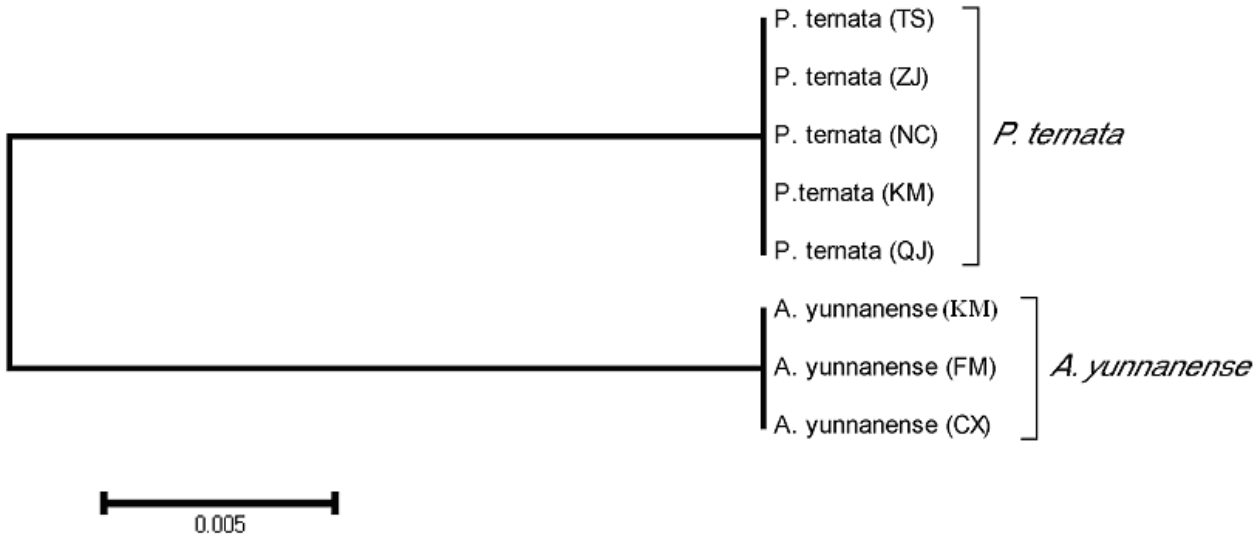

Figure 1. Neighbor-Joining tree of *atp*B-*rbc*L intergenic spacers DNA sequences was constructed with Kimura-2-Parameter distance (scale bar showing the distance).

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PTF                 5'-CAATAATGAT GTATTTGACG AA-3'
P. ternata (TS)        CAATAATGAT GTATTTGACG AATCAAATAC
P. ternata (ZJ)        .......... .......... ..........
P. ternata (NC)        .......... .......... ..........
P. ternata (KM)        .......... .......... ..........
P. ternata (QJ)        .......... .......... ..........
A. yunnanense (KM)     .......... ........TT CG........
A. yunnanense (FM)     .......... ........TT CG........
A. yunnanense (CX)     .......... ........TT CG........
AYF                                5'-TTTGATT CGTCAAATAC-3'
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Figure 2. Allele-specific primers designed for the identification of *P. ternata* and *A. yunnanense* based on variation of *atp*B-*rbc*L intergenic spacer. PTF: the sense primer for *P. ternata*; AYF: the sense primer for *A. yunnanense*.

the 862 aligned sites, totally 26 transitions/transversions and 16 deletions/insertions were observed. A neighbor-joining tree was constructed based on Kimura-2-Parameter distance with MEGA 4.1 version (Tamura et al., 2007). The result indicated that *P. ternata* differs remarkably from *A. yunnanense* (Figure 1), and this DNA sequence could be used as an effective molecular marker for distinguishing between *P. ternata* and *A. yunnanense*.

Reliability and sensitivity of diagnostic PCR using allele-specific primers designed

There is an inversion of four bp (CGAA *vs.* TTCG) on the aligned positions 690 to 693 between the *atp*B-*rbc*L intergenic spacer of *P. ternata* and *A. yunnanense* (Figure 2). Based on this, two species-specific sense primers, PTF (5'-CAATAATGATGTATTTGACGAA-3') for *P. ternata* and AYF (5'-TTTGATTCGTCAAATAC-3') for *A. yunnanense*, were designed. The universal primer *rbc*L (5'-AACACCAGCTTTRAATCCAA-3') was used as the anti-sense (Chiang et al., 1998). With the primer pair PTF and *rbc*L, disgnostic PCR will receive a positive result (a DNA fragment of about 200 bp) for *P. ternata* but a negative result for *A. yunnanense*. Accordingly, the result will be reversal when the primer pair AYF and *rbc*L are used.

To verify whether the designed primers could accurately identify the targeted species, DNA samples extracted from crude drugs of *P. ternata* and *A. yunnanense* were analyzed by PCR amplification using the designed species-specific primers. Although the genomic DNA was severely degraded, the expected results were achieved in both positive and negative control tests (Figure 3). The sensitivity of the proposed method was also tested by investigating the least amount of *A. yunnanense* that

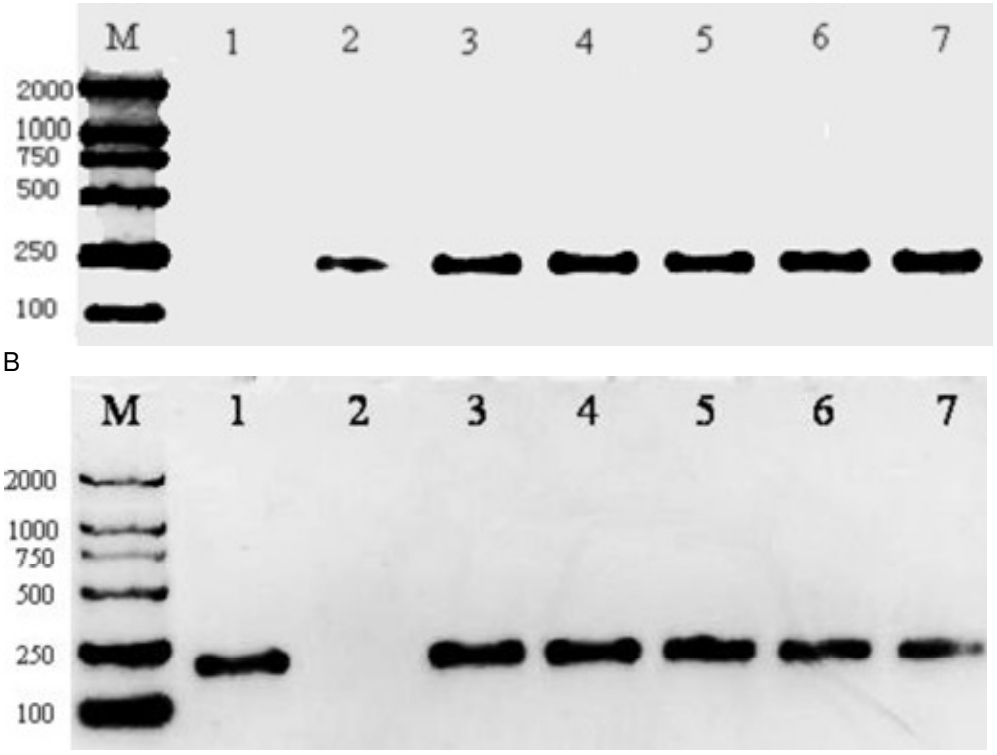

Figure 3. Agarose gel electrophoresis of diagnostic PCR products. M: DL2000 DNA marker; Lane 1, Crude drug of *P. ternate*; Lane 2, crude drug of *A. yunnanense*; Lane 3 to 7, *A. yunnanense* / *P. ternata* mixtures with ratio 1:10, 1:50, 1:100, 1:150, 1:200; A, primers AYF and *rbc*L; B, primers PTF and *rbc*L.

could be detected as the adulterant of *Pinellia* tuber. We extracted DNA from mixtures consisting of crude drugs of *A. yunnanense* and *Pinellia* tuber with ratios from 1:10 to 1:200. The results revealed that as little as 0.5% of *A. yunnanensis* could be detected in the mixture (Figure 3). The experiments mentioned above were repeated thrice for verification.

The main purpose of this study is to determine if the variations of the *atp*B-*rbc*L intergenic spacer can be used to detect *A. yunnanense* as the adulterant of *P. ternata*. Two species-specific primer pairs were designed based on the sequence divergence of this DNA region. With the species-specific primers, diagnostic PCR amplifications received expected results in both positive and negative controlled experiments. The results demonstrated that the sequence variation of t *atp*B-*rbc*L intergenic spacer can help authenticate *P. ternata* and *A. yunnanense.*

Another purpose of this study is to test the sensitivity of the proposed method. The amount of *A. yunnanense* that could be detected as an adulterant of *Pinellia* tuber was determined by analyzing the mixtures of *Pinellia* tuber and the adulterant. Commercially prepared crude drugs of *Pinellia* tuber and *A. yunnanense* purchased from the market were tested. Even though the extracted genomic DNA was partly degraded, the targeted sequences (about 200 bp) were still amplified and recognized as *Pinellia* tuber and *A. yunnanense*. As little as 1:200 of *A. yunnanense* was detected in the mixtures. This proved that the proposed method is sensitive and reliable.

Allele-specific PCR technique has been successfully used for the authentication of a wide range of medicinal plants (Zheng et al., 2009). Unfortunately, only few cases qualify for determining whether a sample has been adulterated (Zheng et al., 2009; Xue et al., 2008). The present study provides a simple, reliable and sensitive method not only for authentication of the medicinal herb *P. ternata*, but also for detection of whether it has been adulterated with *A. yunnanense*. Because products of diagnostic PCR are only about 200 bp, this method can also be used to authenticate crude drugs that have been stored for long periods of time.

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