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

Tao Liu¹ and Xiaorong Guo²*

¹Faculty of Agronomy and Biotechnology, Yunnan Agricultural University, Heilongtan, Kunming 650201, P. R. China.  
²College of Life Sciences, Yunnan University, 2 Green Lake Northern Road, Kunming 650091, P. R. China.

Accepted 12 November, 2010

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 *atpB*-rbcL 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), *atpB*-rbcL.

**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 pharmacological 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 is very 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.
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 atpB-rbcL 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 grams 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 atpB-rbcL intergenic spacer was amplified with the universal primers atpB (5'-ACATCKARTACKGGACCAATAA-3') and rbcL (5'-AACACCCAGCTTTAATCCCA-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\(^+\)), 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 94°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 atpB-rbcL 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 rbcL (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\(^+\)), 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 atpB-rbcL 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

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

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Localities</th>
<th>Abbreviate</th>
<th>Voucher</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. yunnanense</td>
<td>Nanchuan, Chongqing</td>
<td>ZJ</td>
<td>GaoM s.n.</td>
<td>HQ221738</td>
</tr>
<tr>
<td>A. yunnanense</td>
<td>Kunming, Yunnan</td>
<td>KM</td>
<td>LiuT 2008212</td>
<td>FJ638618</td>
</tr>
<tr>
<td>A. yunnanense</td>
<td>Fuming, Yunnan</td>
<td>FM</td>
<td>LiuT 2008213</td>
<td>FJ638619</td>
</tr>
<tr>
<td>A. yunnanense</td>
<td>Chuxiong, Yunnan</td>
<td>CX</td>
<td>LiuT 2008214</td>
<td>FJ638620</td>
</tr>
</tbody>
</table>

Table 1. Plant samples used in this study.
Figure 1. Neighbor-Joining tree of \textit{atpB-rbcL} intergenic spacers DNA sequences was constructed with Kimura-2-Parameter distance (scale bar showing the distance).

Figure 2. Allele-specific primers designed for the identification of \textit{P. ternata} and \textit{A. yunnanense} based on variation of \textit{atpB-rbcL} intergenic spacer. PTF: the sense primer for \textit{P. ternata}; AYF: the sense primer for \textit{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 \textit{atpB-rbcL} intergenic spacer of \textit{P. ternata} and \textit{A. yunnanense} (Figure 2). Based on this, two species-specific sense primers, PTF (5'-CAATAATGATGTTTGGACG-AA-3') for \textit{P. ternata} and AYF (5'-TTTGATTGTCGCAAATAC-3') for \textit{A. yunnanense}, were designed. The universal primer \textit{rbcL} (5'-AACACCAGCCTTTAATCCAA-3') was used as the anti-sense (Chiang et al., 1998). With the primer pair PTF and \textit{rbcL}, diagnostic PCR will receive a positive result (a DNA fragment of about 200 bp) for \textit{P. ternata} but a negative result for \textit{A. yunnanense}. Accordingly, the result will be reversal when the primer pair AYF and \textit{rbcL} are used.

To verify whether the designed primers could accurately identify the targeted species, DNA samples extracted from crude drugs of \textit{P. ternata} and \textit{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 \textit{A. yunnanense} that...
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 *atpB*-rbcL 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 the *atpB*-rbcL 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.

**ACKNOWLEDGMENTS**

This research was financially supported by Natural Science Foundation of Yunnan Province (NSFN, 2008C071 to XR Guo), the Education Department of Yunnan Province (08Y0020 to XR Guo; 08Y0165 to T Liu), and the National Natural Science Foundation of China (NSFC, 30800062 to XX Li). We are also grateful to M Gao and CL Li for providing the samples used in this study.
REFERENCES


