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Full Length Research Paper

Discrimination of the medicinal plant *Fallopia multiflora* and its adulterants by diagnostic polymerase chain reaction (PCR)

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The roots of *Fallopia multiflora* are widely used in Chinese medicine for their anti-inflammatory prosperities. While recently, *F. multiflora* has been found to be adulterated with some species with similar morphologies but little or different biological activity due to its natural resource deficiency. The discrimination of *F. multiflora* from its adulterants is currently limited to methods of morphology and chemical fingerprinting. To distinguish *F. multiflora* from its adulterants efficiently, the chloroplast *atp*B-*rbc*L intergenic spacers of them were sequenced and analyzed, and one pair of diagnostic primers were designed for differentiating *F. multiflora* from its adulterants. This technique provided accurate, effective, and rapid approaches to distinguish *F. multiflora* from the adulterants.

Key words: *Fallopia multiflora, atp*B-*rbc*L intergenic spacers, diagnostic polymerase chain reaction, adulterants, molecular authentication.

INTRODUCTION

Fallopia multiflora Thunb (Polygonaceae) is a traditional Chinese herbal medicine which is distributed in northeast Asia. The dried root of *F. multiflora* and *F. multiflora* var. *angulatum*, namely *Radix Polygoni* Multiflori ("*he-shou-wu*"), is commonly used as counteracting toxicity, curing carbuncles, and relaxing the bowels (The State Pharmacopoeia Committee of China, 2005). However, because of over-harvesting, the natural resources of *Radix Polygoni* have been nearly exhausted in recent years, resulting in some closely related species which have similar morphology but weaker or different biological activity are also used medicinally in some places of China (Chen et al., 2000), which include *Polygonum aubertii*, *Pteroxygonum giraldii*, and *Polygonum ciliinerve* of the family Polygonaceae, *Cynanchum auriculatum* of the family Asclepiadaceae, *Dioscorea bulbifera* of the family Dioscoreaceae, and *Rodgersia aesculifolia* of the family Saxifragceae (Xu et al., 1998; Chen et al., 2000). The dried root of *C. auriculatum*, is bearing the Chinese name"bai-shou-wu", which is often confused with "*he-shou-wu*" in the herbal market. Thus, these two Radixes are some of the most indiscriminately used herbal medicines because of their similar morphologies and names. In addition, the dried roots of *C. auriculatum* have often been indiscriminately used as a substitute and/or a contaminant of "*he-shou-wu*" because it grows more rapidly and is more productive than *F. multiflora*. The three adulterants, namely *P. aubertii*, *P. giraldii* and *P. ciliinerve,* are from the same family as *F. multiflora*, and their roots are the source of the traditional Chinese Medicine "*hong-yao-zi*". Their roots are used as herbal medicine for hemostasia, rheumatism and detoxification etc in China (The State Pharmacopoeia Committee of China, 2005). In addition, some other plants, such as *R. aesculifolia* and *D. bulbifera,* have also been admixed with *F. multiflora* because of the dried root morphological similarity among them (Xu et al., 1998). Inevitably, the practice of mixing these adulterant 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. The precise identification of crude drugs is crucial

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Table 1. Plant materials used in this study.

Taxa	Locality	Voucher
Fallopia multiflora	Guangzhou, Guangdong	HJ Yan, 09001
F. multiflora var. *angulatum*	Guangzhou, Guangdong	HJ Yan, 09002
Dioscorea bulbifera	Guangzhou, Guangdong	HJ Yan, 09003
Polygonum ciliinerve	Bejing	HJ Yan, 09004
Follopia aubertii	Bejing	HJ Yan, 09005
Rodgersia aesculifolia	Bejing	HJ Yan, 09006
Cynanchum auriculatum	Bejing	HJ Yan, 09007
Pteroxygonum giraldii	Funiushan, Henan	HJ Yan, 09008

for the standardization of clinical prescriptions and chemical and pharmacological research of herbal medicines. Traditionally, subjective methods based on the morphological features such as shape, color, texture, and odor, were used for the discrimination of herbal medicines. It is often difficult to accurately identify medicinal plants from wild populations, or to differentiate species within the same genus based on this subjective evaluation.

Furthermore, the use of chromatographic techniques and marker compounds to standardize herbal medicines is also limited because of variable chemical complexity, which is affected by growth, storage conditions, harvest times, and variable sources (Joshi et al., 2004; Zhang et al., 2007). Genetic tools that use hybridization, polymerase chain reaction (PCR), and sequencing techniques provide more objective and reliable methods for authenticating herbal medicines (Zhang et al., 2007; Shcher and Carles, 2008). The methods have recently proven to be useful for the identification and standardization of traditional Chinese medicines (Yang et al., 2001). Maternally inherited chloroplast DNA (cpDNA) bears the advantage of stability in generation transmission and hence has the potential as reliable marker for the identification of non-hybridized species (Palme et al., 2003).

In the present study, we used the DNA sequences of chloroplast *atp*B-*rbc*L intergenic spacer to examine the differences among *F. multiflora* and its adulterants. On this basic, a method of PCR amplification with allele-specific primers to discriminate them was developed based on the sequence divergence.

MATERIALS AND METHODS

Plant materials

Silica gel dried leaves of *F. multiflora* and its adulterants were collected from different localities of China (Table 1). 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).

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using the modified CTAB methods (Doyle et al., 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, 2.5 µl 10 x PCR buffer (with Mg^{2+}), 10 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 *F. multiflora* and its adulterants 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 *F. multiflora* and its adulterants samples, the species-specific sense primers were designed (Figure 1). The primers were synthesized by Sangon Corp. (Shanghai, China). The universal primer *rbc*L was used as the anti-sense.

The diagnostic PCR reaction mixture (total volume 25 µl) contained less than 10 ng of genomic DNA, 2.5 µl 10 x PCR buffer (with Mg^{2+}), 10 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 54°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.

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PRV                                  5'-TCTATCG CATTGGCTCT CGCA-3'
Fallopia multiflora                  ATTTCTATCG CATTGGCTCT CGCATT--GG
Fallopia multiflora var angulatum    .......... .......... ......--..
Dioscorea bulbifera                  GA.CTC..T. GT.ATTG.T. T--------T
Polygonum ciliinerve                 .......... .......C.. T---..--TT
Follopia aubertii                    .......... .......C.. T---..--TT
Rodgersia aesculifolia               GCCCT...TT ....TTT.A. TTTC..ATTT
Cynanchum auriculatum                TAG..C..T. GC..TTT.T. T.T...--T-
Pteroxygonum giraldii                .......... ...C...C.. T-TT..--TT
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Figure 1. Allele-specific primers designed for the identification of *F. multiflora* and its adulterants samples based on variation of *atp*B-*rbc*L intergenic spacer. PRV: the sense primer for *F. multiflora*.

RESULTS AND DISCUSSION

In order to distinguish *F. multiflora* and *F. multiflora* var. *angulatum* from the adulterants, we sequence the *atp*B-*rbc*L region of all them. From the sequences alignment, we found the *atp*B-*rbc*L sequences of *F. multiflora* and *F. multiflora* var. *angulatum* were divergent remarkably from those of its adulterants. Therefore, this DNA region itself could be used as an effective molecular marker for distinguishing *F. multiflora* from its adulterants. By MEGA 4.1 (Tamura et al., 2007), a neighbor-joining tree was constructed based on Kimura-2-Parameter distance. The result also showed that *F. multiflora* and *F. multiflora* var. *angulatum* differed significantly from the other closely related species (Figure 2).

We used a PCR-based method to find species-specific PCR primer of *F. multiflora* and *F. multiflora* var. *angulatum* at the genomic level. To control for individual variations, we examined three samples per species. In this study, we used *atp*B-*rbc*L sequences to analyze polymorphic patterns. The result showed the lengths range from 620 to 757 bp among all the samples. After a sequence alignment of the studied species by clustal W, we found the shortest *atp*B-*rbc*L sequence came from *F. multiflora* Thunb*,* because there was a 143 bp sequence lost between site 383 and site 525. In addition, we found that there were some variable sites on the aligned positions 416 to 437 among *F. multiflora*, *F. multiflora* var. *angulatum* and their adulterants. In order to distinguish them from the adulterants by a simple PCR, we designed one species-specific sense primers, PRV (5'-TCTATCGCATTGGCTCTCGCA -3') for *F. multiflora* and *F. multiflora* var. *angulatum*. The universal primer *rbc*L (5'-AACACCAGCTTTRAATCCAA-3') was used as the anti-sense. To be sure our sample DNA is good for the diagnostic PCR, we verified them again using the universal primer atpB and rbcL mentioned above. Then, with the primer pair PRV and *rbc*L, disgnostic PCR received a positive result for *F. multiflora* (a DNA fragment of about 250 bp for *F. multiflora*, and 400 bp for *F. multiflora* var. *angulatum*), but a negative result for its adulterants were detected Figure 3.

This study was designed to produce a rapid genetic test for identification of *F. multiflora* and *F. multiflora* var. *angulatum* and their potential adulterant. Thus, to prevent individual variations, we examined three samples per species and found the constancy among each species. When using the universal primer atpB and rbcL for *atp*B-*rbc*L sequences, we could found The *atp*B-*rbc*L sequences of *F. multiflora* is shorter than other samples, which could be used to discriminate *F. multiflora* from the other species. However, in order to distinguish *F. multiflora* Thun and *F. multiflora* var. *angulatum* from the adulterants, we sequence the *atp*B-*rbc*L region of all them, and align the sequences using clustal W. From the alignment, we found the *atp*B-*rbc*L sequences of *F. multiflora* Thun and *F. multiflora* var. *angulatum* differentiated remarkably from those of its adulterants, so the region sequence itself could be used as an effective molecular marker for distinguishing *F. multiflora* from its adulterants. By MEGA 4.1 (Tamura et al., 2007), a neighbor-joining tree was constructed based on Kimura-2-Parameter distance. The result also revealed that *F. multiflora* Thun and *F. multiflora* var. *angulatum* differed significantly from the other closely related species (Figure 2) and this DNA sequence could be used as an effective molecular marker for distinguishing between *F. multiflora* Thun, *F. multiflora* var. *angulatum* and its adulterants. The results demonstrated that the diagnostic PCR primers could help authenticate *F. multiflora* and *F. multiflora* var. *angulatum* from the adulterants easily.

Several discriminative standards based on morphological characteristics were suggested by Xu et al. (1998) to distinguish *F. multiflora* and *P. ciliinerve*. Gang et al. (2008) analyzed the vegetative and reproductive organs to differentiate *P. ciliinerve* from allied species. However,

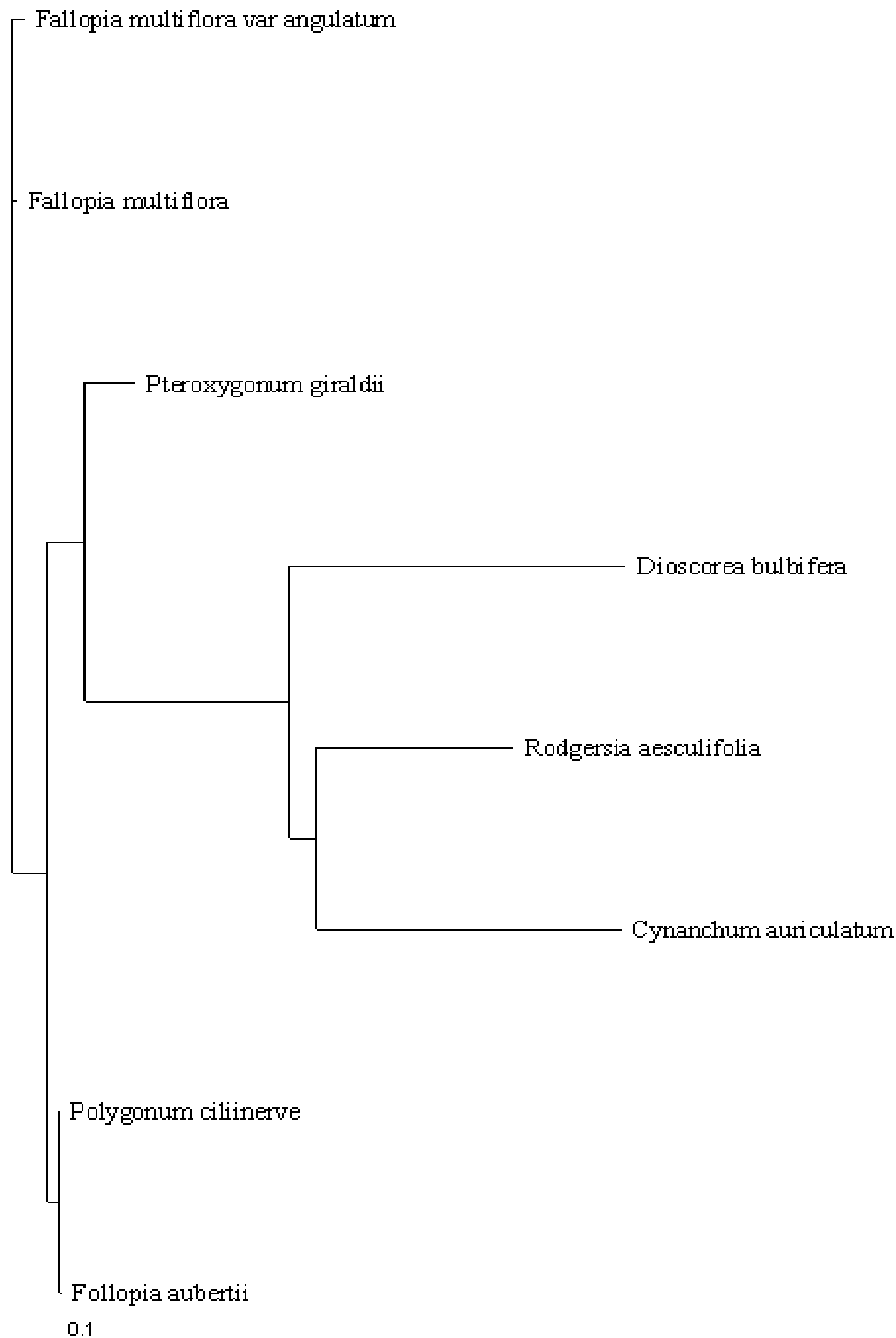

Figure 2. Neighbor-Joining tree of *atp*B-*rbc*L intergenic spacers DNA sequences was constructed with Kimura-2-Parameter distance (scale bar showing the distance).

a clear molecular discrimination marker was not developed to authenticate *F. multiflora* and *F. multiflora* var. *angulatum*. The main purpose of this study was to determine if the variations of the *atp*B-*rbc*L intergenic spacer could be used to detect *F. multiflora* from the adulterants. One species-specific primer pair was designed based on the sequence divergence of this DNA region. With the species-specific primer, diagnostic PCR amplifications received expected results in controlled experiments. The results demonstrated that the

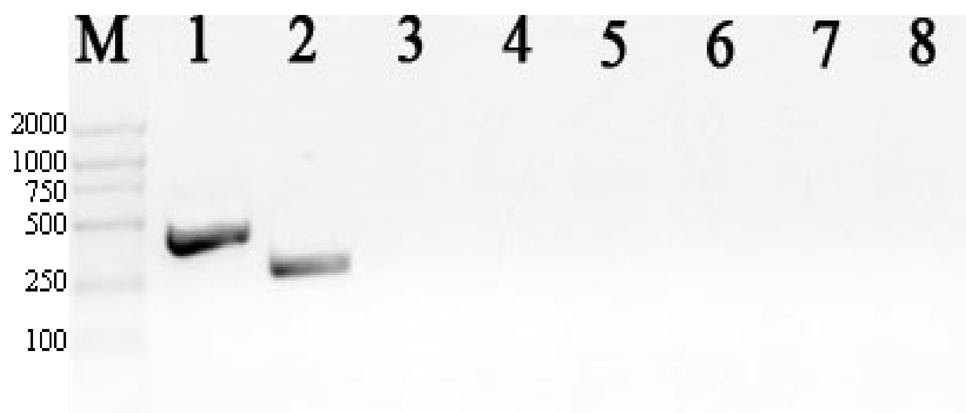

Figure 3. Agarose gel electrophoresis of diagnostic PCR products with primers PRV and *rbc*L.. M: DL2000 DNA marker; Lane 1-7: *F. multiflora*, *F. multiflora* var. *angulatum*, *Pteroxygonum giraldii*, *Polygonum aubertii*, *Cynanchum auriculatum*, *Rodgersia aesculifolia*, *Polygonum ciliinerve* and *Dioscorea bulbifera*.

sequence variation of the *atp*B-*rbc*L intergenic spacer can help authenticate *F. multiflora* and *F. multiflora* var. *angulatum* from the adulterants, and the allele diagnostic-PCR method could be used for the rapid molecular discrimination.

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 approach does not require conventional sequencing of PCR products followed by detailed comparison of individual sequences. They can be routinely performed in medicinal herb inspection laboratories, especially when large numbers of samples have to be analyzed. Allele-specific diagnostic-PCR is more rapid, less expensive, and can also be used to verify medicinal plants that have been stored for long periods of time since the PCR product is only 300 bp or so. The present study provides a simple, reliable and sensitive method not only for authentication of the medicinal herb *F. multiflora* from the adulterants.

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