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Molecular authentication by multiplex-PCR of three similar medicinal plant species: *Cynanchum wilfordii*, *Cynanchum auriculatum* and *Polygonum multiflorum* (*Fallopia multiflorum*)

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Polygoni Multiflori radix (Hashuoh in Korean and Heshouwu in Chinese) and Cynanchi Wilfordii Radix (Baekshuoh in Korean and Beishouwu in Chinese) are important oriental medicinal herbs in Korea, Japan, and China. The *trn*L (tRNA-Leu) intron region was targeted for molecular analysis with universal primers to discriminate the morphologically similar to Polygoni multiflori Radix and Cynanchi wilfordii radix from their adulterant, *Cyanchum auriculatum*. Analysis of sequencing data revealed DNA polymorphisms in the three species, including single nucleotide polymorphisms (SNP) sites, insertions, and deletions. Based on these DNA polymorphisms, specific primers were designed for each of the three species. Multiplex polymerase chain reaction (PCR) with specific primers was conducted for molecular authentication of *Polygonum multiflorum*, *Cyanchum wilfordii* and *C. auriculatum*. The established multiplex-PCR system effectively identified Polygoni multiflori Radix and Cynanchi Wilfordii radix. The results indicate that the *trn*L(tRNA-Leu) intron region can be used for inter-specific polymorphic study, and the methods presented in this study can be applied to molecular identification of other medicinal materials.

Key words: Polygoni Multiflori radix, Cynanchi Wilfordii radix, *Polygonum multiflorum*, *Cyanchum wilfordii*, *Cyanchum auriculatum*, *trn*L(tRNA-Leu) intron, polymorphism, multiplex polymerase chain reaction (PCR).

INTRODUCTION

Polygoni Multiflori radix (Hashuoh in Korean and Heshouwuin Chinese) and Cynanchi Wilfordii radix (Baekshuoh in Korean and Beishouwuin Chinese) are important Oriental medicinal herbs in Korea, Japan, and China. Polygoni Multiflori radix (Hashuoh) has been used as a tonic antioxidant (Chiu et al., 2002; Ryuet al., 2002), an anti tumor drug (Kimura et al., 2000), and a neuro protective drug (Um et al., 2006). Cynanchi wilfordii radix has been used to prevent graying hair, to strengthen bones and connective tissue, and to enhance immunity

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(Jiangsu College of New Medicine). These two radixes are often confused with each other in the market place because of their similar names and morphologies (Song et al., 2004). According to the Korean, Japanese and Chinese pharmacopoeias, Polygoni Multiflori radix (Heshuwu) consists of dried root tubers of *Polygonum multiflorum* (*Fallopia multiflorum*) from the family Polygonaceae (Korea Food and Drug Administration, 2008; Ministry of Health, Labour and Welfare, 2006; China Pharmacopoeia Committee, 2005).On the other hand, Cynanchi Wilfordii radix (Baishouwu) is actually an appellative name for the root tubers of *Cyanchum wilfordii* and *Cyanchum auriculatum* (The Health Department and National Chinese Medicine Management office, 1999). The Korean pharmacopoeia (Korea Food and Drug Administration, 2008) mentions only the dried root tuber of *P. multiflorum* and does not include *C. auriculatum* which is considered an adulterant in Korea. *C. auriculatum* grows faster and thus can be harvested more quickly than *C. wilfordii*.

C. auriculatum and *C. wilfordii* are morphologically similar and are often sold dried or in slices, which makes it even more difficult to distinguish between the two. In Korea, Polygoni Multiflori radix and Cynanchi Wilfordii radix can be differentiated by the color of their dried root tubers.Traditionally, medicinal plants have been authenticated by historical and morphological features, but growth stages and environmental conditions can affect this authentication (Zhu et al., 2004). Therefore, more robust and advanced DNA technology such as multiplex polymerase chain reaction (PCR) should be applied when identifying these plants (In et al., 2010; Sun et al., 2010).

MATERIALS AND METHODS

Plant

P. multiflorum, C. wilfordii and *C. auriculatum* were collected from the mountains and herbal farms in Korea. Plant materials were identified by Prof. Woo-Saeng Kwon, expert in plant physiology. Polygoni Multiflori radix and Cynanchi Wilfordii radix samples were purchased from local markets in Korea and China, respectively (Table 1).

DNA extraction and PCR amplification of the *trn*L(tRNA-Leu) intron region

Sample roots were frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was isolated and purified using a G-spin^{TM} genomic DNA extraction kit (iNtRON, Biotechnology, Seoul, Korea), and each dried sample was isolated using a modified cetyltrimethylammonium bromide (CTAB) method (Murray et al., 1980). The primer pairs used for amplification of the chromosomal DNA *trn*L(tRNA-Leu) intron region were *trn*LF-c (5'-CGAAATCGGTAGACGCTA-3') and *trn*LF-f (5'-ATTTGAACTGGTGACACGAG-3') (Taberlet et al., 1991).

PCR amplification was performed in a total volume of 20 µl, and the reaction mixture consisted of each of the primers at a concentration of 0.5 µM, 20 ng of template DNA, and 10 µl of 2× TOPsimle ^{TM}DyeMIX-Tenuto(Genotech, South Korea). The TOPsimle ^{TM}DyeMIX-Tenutocomponents were nTaq-DNA Polymerase 0.2 unit/µl, 4 mM Mg^{2+} and the dNTP mixture 0.4 mM. The amplification profile consisted of one pre-denaturation cycle of 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, and a final extension at 72°C for 7 min. The PCR products were visualized via 1.0% agarose gel electrophoresis with ethidium bromide staining under ultra violet (UV) light.

Sequencing and DNA sequence analysis

The PCR products were purified using a PCR product purification kit (GENEALL PCR SV, General Bio Systems) per the manufacturer's instructions and then sequenced by Genotech, Inc. The DNA sequences of the *trn*L(tRNA-Leu) intron region obtained in sequencing experiments were compiled using SeqMan software, and sequences were edited with the BioEdit program (Hall, 1999). Multiple sequence alignments were performed using the online ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/).

Design of specific primers

Specific primers were designed for *P. multiflorum*, *C. wilfordii* and *C. auriculatum* based on the detected DNA polymorphisms (Figure 1). PmF, CwF and CaF were designed for authentication of *P. multiflorum, C. wilfordii* and *C. auriculatum,* respectively after analysis of specific polymorphismsites. Primer PmF was designed for specific identification of *P. multiflorum* based on its specific 22-bp insertions. Primer CwF was designed for specific identification of *C. wilfordii* based on its specific 2-bpdeletion. Another sense primer of CaF was designed to serve as a positive control for *C. auriculatum*. The sequences and orientations of specific primers and common primers are shown in Table 2 and Figure 2.

Multiplex PCR

Molecular authentication of *P. multiflorum, C. wilfordii* and *C. auriculatum* was performed using multiplex PCR. Five primers (*trn*LF-c, PmF, CwF, CaF and *trn*LF-f) were used simultaneously in multiplex PCR amplification. The reaction mixture was identical to the one described earlier except the concentrations of *trn*LF-c, PmF, CwF, CaF and *trn*LF-f were 0.5, 0.37, 0.25, 0.25 and 0.5 µM, respectively. PCR amplification was performed in a total volume of 20 µl. The amplification profile consisted of one pre-denaturation cycle of 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 61°C, 1 min at 72°C, and a final extension at 72°C for 7 min. The PCR products were visualized on a 3% agarose gel.

RESULTS AND DISCUSSION

Chloroplast *trn*LF regions have proven useful for species-level identification (Olmstead and Palmer, 1994). These regions can be used for authentication of *P. multiflorum*, *C. wilfordii* and *C. auriculatum*. The sequences of introns and intergenic regions are highly variable due to the lack of sequence conservation (Quandtet al., 2004).

Table 1. Plant samples used in this study.

Species	Voucher	Localities of collection	GenBank accessions of *trn*L-F
Polygonum multiflorum	PM101	Dae-jeon, Korea	JX028241
	PM102	Busan, Korea	
	PM103	Chungju, Korea	
	PM104	Seoul, Korea	
Cynanchum wilfordii	CW201	Dae-jeon, Korea	JX028243
	CW202	Seoul, Korea	
	CW203	Suwon, Korea	
	CW204	Yantai, China	
Cynanchum auriculatum	CA301	Suwon,Korea	JX028242
	CA302	Seoul, Korea	
	CA303	Busan, Korea	
	CA304	Dae-jeon, Korea	
	CA305	Jangheung, Korea	
	CA306	Jeju, Korea	
	CA307	Yantai, China	

Table 2. Primers used in this study.

Primer name	Nucleotide sequences (5'→3')	Location
*trn*LF-c	CGAAATCGGTAGACGCTA	Intron
*trn*LF-f	ATTTGAACTGGTGACACGAG	Intron
PmF	TGAAAAAATATTGATGACGACCCGAAT	354-380
CwF	ATATCATTACTCGTACTGAAGCTTC	854-880
CaF	CAGTACCTAGATCAGACTGTTTAAC	917-942

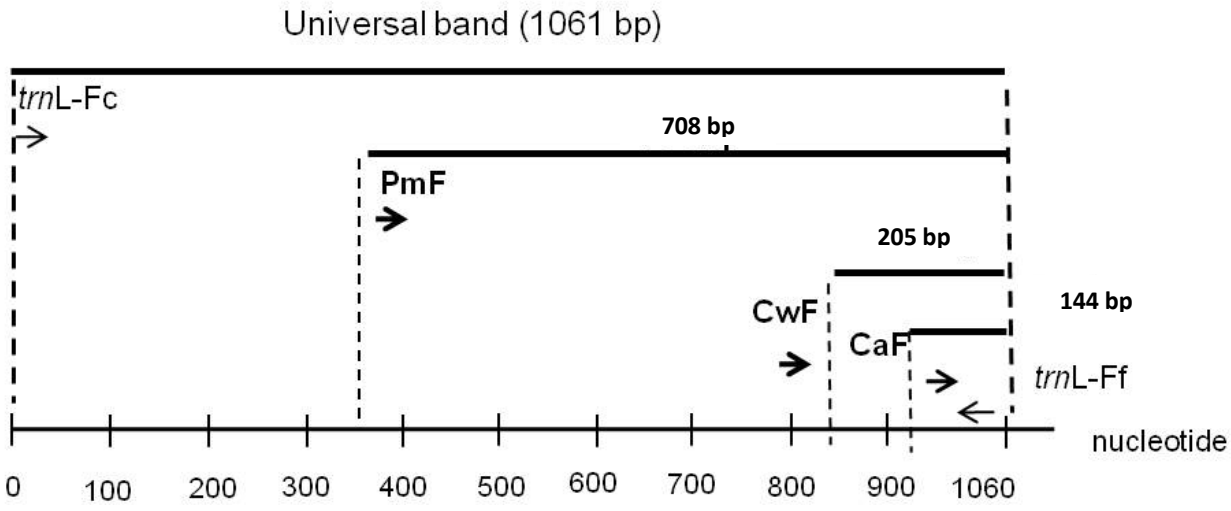

Figure 1. Gel image of multiplex-PCR products.

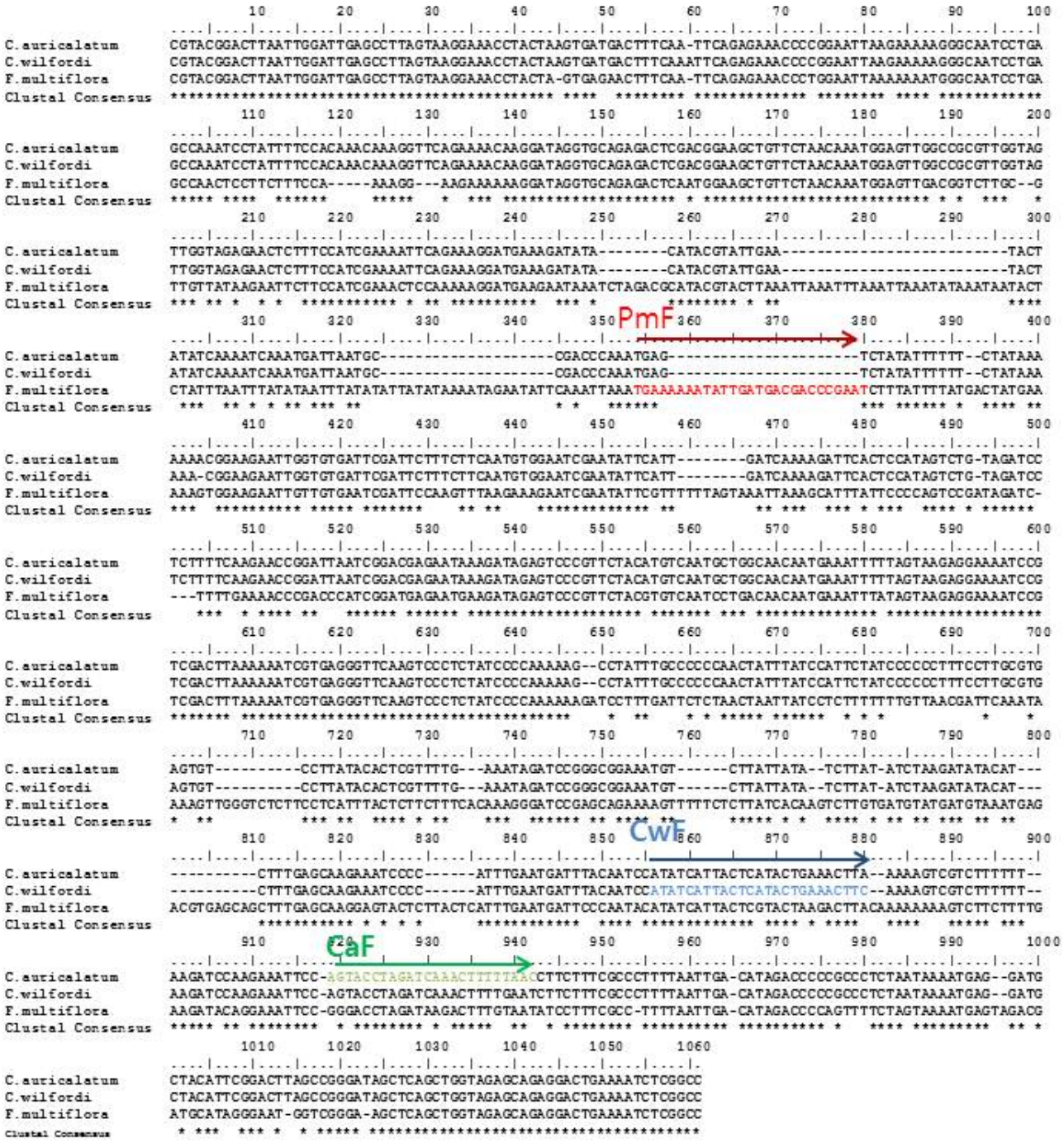

Figure 2. Schematic diagrams of the primers used in multiplex PCR.

Chloroplast *trn*LF region introns are useful for intra-specific polymorphic studies. In this study, the *trn*L(tRNA-Leu) intron region was targeted for molecular analysis and differentiation among *P. multiflorum*, *C. wilfordii* and *C. auriculatum*. The *trn*L(tRNA-Leu) intron regions of three species were PCR-amplified using the '*trn*LF-c'and '*trn*LF-f' universal primer sets. The *trn*L(tRNA-Leu) intron region of *P. multiflorum, C. wilfordii* and *C. auriculatum*

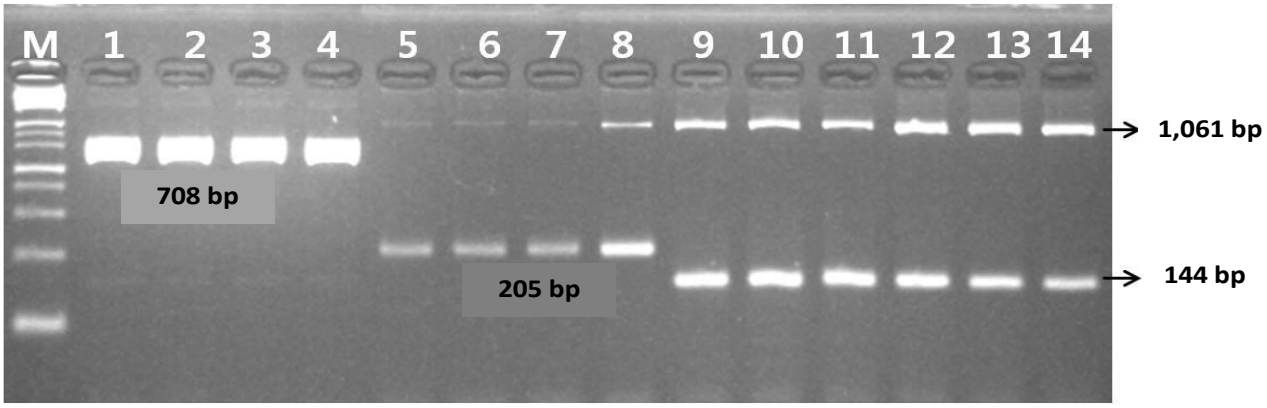

Figure 3. Comparison of the *trn*L(tRNA-Leu) intron regions of *P. multiflorum*, *C. wilfordii* and *C. auriculatum.* Lane M: 1000 bp DNA ladder; lane 1 to 4: *Polygonum multiflorum*; lane 5 to 8: *Cyanchum wilfordii*; lane 9 to 15: *Cyanchum auriculatum*.

was determined to be 1,042, 922 and 922 bp, respectively. Their sequences were deposited in GenBank (JX028241-JX028243).

Multiplex alignments of the *trn*L(tRNA-Leu) intron region of *P. multiflorum*, *C. wilfordii* and *C. auriculatum* were performed with the CLUSTALX program. DNA polymorphisms, including SNP sites, insertions and deletions were detected among these three species. The *P. multiflorum*-specific primer PmF was designed according to the insertions located at the 358th to 379th nucleotide positions. The *C. wilfordii*-specific primer CwF was designed according to the deletions located at the 856th to 880th nucleotide positions. The *C. auriculatum*-specific primer CaF was designed according to DNA polymorphisms located at the 917th-942th nucleotide positions (Figure 1).

Molecular discrimination of *P. multiflorum, C. wilfordii* and *C. auriculatum* was conducted using multiplex PCR with the five primers described. The combination of five specific primers, as shown in Figure 3, yielded expected amplicons for different species. All three species generated a universal band of 1,061 bp with primers *trn*LF-c and *trn*LF-f, which acted as an internal positive control to confirm that the PCR amplification was successful. *P. multiflorum* yielded specific amplicons of 708 bp and *C. wilfordii* and *C. auriculatum* yielded specific amplicons of 205 and144 bp generated by their specific primer sets, PmF, CwF and CaF, respectively. Therefore, *P. multiflorum, C. wilfordii* and *C. auriculatum* can be clearly differentiated from each other by the multiplex PCR system developed in this study.

In the present study, we identified three plant species, *P. multiflorum, C. wilfordii* and *C. auriculatum*, by simultaneously amplifying their specific primer using multiplex PCR. The results indicate that it is possible to identify Polygoni Multiflori radix and Cynanchi wilfordii radix from their adulterant using the chloroplast *trn*L(tRNA-Leu) intron region. Compared to other molecular markers, this method is reliable, efficient, and can be used for numerous repeated tests of many medicinal plants. We strongly recommend multiplex PCR for the identification of medicinal plants, and the methodology presented in this study can be adapted for authentication of other medicinal materials.

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