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Monitoring and Identification of *Cynanchum wilfordii* and *Cynanchum auriculatum* by Using Molecular Markers and Real-Time Polymerase Chain Reaction

Jin Ah Ryuk · Hye Won Lee · Young Seong Ju · Byoung Seob Ko

Abstract *Cynanchum wilfordii* (Asclepiadaceae) is widely distributed throughout Korea, Japan, and China. Dried roots of this plant have been used as a tonic to promote renal function. Due to the morphological similarities of the dried roots of this plant to those of *Cynanchum auriculatum*, which is used as a substitute herbal medicine for *C. wilfordii*, distinguishing these two species is extremely difficult. The present study was conducted to develop molecular markers to distinguish *C. wilfordii* and *C. auriculatum* by using conventional polymerase chain reaction (PCR) and real-time PCR analyses. Comparative analysis based on the sequence of the *trn*L-*trn*F intergenic spacer revealed 4 base-pair variations, and the inter-individual sequences of the 2 species separately showed 100% homology. According to these results, the variations were divided into 2 groups. The 2 species were further distinguished using a sequence-characterized amplified region marker developed based on a randomly amplified polymorphic DNA-PCR product, and then a single nucleotide polymorphism marker was designed based on the *trn*L-*trn*F intergenic spacer for more efficient detection in real-time PCR. The results showed that species-specific molecular markers might allow accurate discrimination of *C. wilfordii* and *C. auriculatum*.

Keywords *Cynanchum auriculatum* · *Cynanchum wilfordii* · identify · molecular marker · real-time polymerase chain reaction · Sequence characterized amplified region marker

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Introduction

Cynanchum wilfordii Radix is listed in the Korea Herbal Pharmacopoeia as the original plant of *Cynanchum wilfordii* Hemsley (Asclepiadaceae; KFDA, 2011). However, *C. wilfordii* is not listed in the Japanese Pharmacopoeia or the Chinese Pharmacopoeia, indicating that this herbal medicine is predominantly used in Korea. In Korea, the roots of *C. wilfordii* are called Becksuo and have been used in folk medicine for the prevention and treatment of various geriatric diseases associated with vascular disorders, diabetes mellitus, and ischemia-induced diseases, as well as to slow the aging progress (Huh, 1613). According to the World Health Organization reports on Korean herbs, *C. wilfordii* has hepatoprotective and antihepatotoxic activities (Kim and Park, 1994). Moreover, a strong antioxidant called gagaminine, isolated from the roots of *C. wilfordii*, has been identified as the active component of the root extract and is known to be effective for alimentation, improving vigor, and nourishing blood (Rahman and Choudhary, 1999).

Cynanchum auriculatum Royle ex Wight is widely distributed in Korea and India. Because the growth condition and appearance of this species is similar to those of *C. wilfordii*, it is used as a substitute herbal medicine for Becksuo in Korea. Due to its low yields, *C. auriculatum* introduced from China is predominantly cultivated in Korea. However, whether *C. auriculatum* can be substituted for *C. wilfordii* has not yet been verified; *C. auriculatum* is being marketed without differentiation from *C. wilfordii*, although they are two different species. Even though these two species can be distinguished by leaf shapes and the presence/absence of sap, there are limitations to their accurate identification based on only the external morphology, because they are marketed as cut and dried roots in the Oriental herbal medicine market. Hence, there is an urgent need to determine a method for the accurate differentiation of these herbal medicines.

Reported methods of distinguishing oriental herbal medicines include the traditional visual and organoleptic examination that still plays an important role in practical differentiation by utilizing all five senses (Lee et al., 2011) and differentiation by internal morphology (Kim et al., 2009), physicochemical reactions (Toh, 1989), liquid chromatography-electrospray ionization mass spectrometry (Zhang et al., 2009), and genetic analysis (Lim et al., 1993; Mizukami et al., 1997). In particular, differentiation by genetic analysis involves the analysis of the *trn*L-*trn*F intergenic spacer of chloroplast DNA, which is transmitted to offsprings mainly through maternal inheritance. In terms of phylogenetic analysis, chloroplast DNA has a more stable structure, because it has a lower copy number; thus limiting the chances of mutations, but it is also advantageous, because it can be used to simultaneously analyze various taxonomic groups (Lang, 2006). Sequence-characterized amplified region (SCAR) analysis, which can select higher numbers of DNA markers, by producing the primer following sequence analysis of the DNA clone and by using the polymorphism of each plant, are frequently used in genetic differentiation to analyze base sequences of polymerase chain reaction (PCR) products from randomly amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism, followed by the selection of markers (Paran and Michelmore, 1993; Schilling, 1996; Hermosa, 2001; Castrillo, 2003; Lardner et al., 2005). In real-time PCR analysis, real-time monitoring of PCR amplification of the markers being analyzed is achieved through fluorescence staining, resulting in a rapid and more accurate genetic differentiation unlike that afforded by existing conventional PCR methods (Ryuk et al., 2010). Moon et al. (2010) reported the SCAR marker for differentiating *Polygonum multiflorum* from *C. wilfordii* and *C. auriculatum*; however, we intended to distinguish *Cynanchum* species by using rapid and easy methods.

The present study aimed to analyze the sequence of the *trn*L-*trn*F intergenic spacer in the chloroplast DNA in order to develop a species-specific primer that can distinguish *C. wilfordii* from *C. auriculatum* by using SCAR analysis based on RAPD-PCR. In addition, genotype analysis involving real-time PCR amplification was used to distinguish the two species in a considerably easy, fast, and accurate manner.

Materials and Methods

Materials. Fresh leaves and dried roots were purchased from commercial suppliers in Korea and China (Table 1). The primary identification of the collected fresh leaves was conducted by Prof. Young SeongJu (Department of Oriental Medicine, Woosuk University, Korea; Fig. 1). The samples (Nos. 1–33 in Table 1) were deposited in the herbarium of the Korea Institute of Oriental Medicine. Samples for monitoring were randomly collected from the Korean herbal markets of each region (Yeongju, Yeongcheon, Seoul, Cheongsong, and Jeju), and 40 samples were used in the present study.

Preparation of genomic DNA. The genomic DNA of each sample was extracted according to the manual for NucleoSpin® Plant II (Macherey-Nagel, Germany). DNA concentration and purity were determined by spectrophotometry (Nanodrop ND-1000;Nanodrop, USA) as well as by electrophoresis on a 1.5% agarose gel with a DNA size marker (TaKaRa, Shiga, Japan). Each sample was diluted to the concentration of approximately 20 ng/mL with diethyl pyrocarbonate (DEPC)-distilled water for PCR amplification.

PCR amplification and DNA sequencing. Primers for *trn*L and *trn*F described by Taberlet et al. (1991; Table 2) were used to amplify the chloroplast DNA. The 30-mL reaction volume contained 1mM of each primer, EmeraldAmp PCR Master Mix (2×multi-premix), Taq polymerase (TaKaRa), and 20 ng/mL DNA template. Reactions were performed using the C-1000 Thermal Cycler (Bio-Rad, USA). The PCR conditions were as follows: 1 cycle of 5 min at 98ºC, followed by 30 cycles of 30 s at 98ºC, 30 s at 55ºC, 1 min at 72ºC, and 1 cycle of 4 min at 72ºC. PCR products with loading star (DyneBio, Korea) were electrophoresed on a 1.5% agarose gel, with a 100-bp DNA size marker (TaKaRa) in Tris borate-EDTA buffer. The amplified DNA fragments (*trn*L and *trn*F) were separated by gel electrophoresis and extracted from the agarose gel by using the Gel Extraction Kit (Promega, USA). The fragments were then subcloned into the pGEM-Teasy vector (Promega). The nucleotide sequences of the resulting inserted DNA fragments were determined using an automatic DNA sequencer (ABI3730; Applied Biosystems, USA).

Development of molecular markers. Sequence alignment analysis was performed using an automatic sequence program (BioEdit;Ibis Biosciences, USA). The species-specific forward (CW-F09, CW-R10) and reverse (CAW-F, CAW-R) primers were designed within the *trn*L-*trn*F region by comparing chloroplast DNA sequences (Table 2). The total reaction volume of 30 mL contained 10 pmol/mL of each primer, 2×multi-premix Taq (Solgent, Korea), and 20 ng/mL DNA template. Reactions were carried out using the C-1000 Thermal Cycler (Bio-Rad). The temperature program was as follows: 1 cycle of 5 min at 95ºC, followed by 28 cycles of 20 s at 95ºC, 30 s at 60ºC, 60 s at 72ºC, and 1 cycle of 5 min at 72ºC. The PCR products with a loading star (DyneBio) were separated on 2.0% agarose gels.

Real-time PCR assay. The primers and probe, including the forward primer (5'-TCC CCA TTT GAA TGA TTT ACA ATC CAT ATC A-3'), reverse primer (5'-GTT TGA TCT AGG TAC TGG AAT TTC TTG GA-3'), and probes with the 5'-end labeled with the fluorescent reporter dye (VIC5'-ACG ACT TTT GAA GTT TC-3/FAM 5-AGA CGA CTT TTT AAG TTT C-3'), and the non-fluorescent quencher dye attached to the 3'-end of both probes were selected using the Sequence Detection Software (Ver. 2.0). The primers and probe were purchased from Applied Biosystems. Reactions were set up in a final volume of 10 mL by using a 96-well optical reaction plate (Applied Biosystems). The reaction volume contained 5 mL of 2×Taqman® Genotyping master mix, 0.5 mL of 20×Taqman®primer/probe reagent (CWnCA),

Table 1 List of *Cynanchum wilfordii* and *Cynanchum auriculatum* samples used in the study

No.	Species	Voucher No.	Tissue	Collection Place	Collection Date
1	*C. wilfordii*	16-1-1	Leaf	Yangpyeong, Korea	2002.05.19
2	*C. wilfordii*	16-3-1	Leaf	Suwon, Korea	2002.06.19
3	*C. wilfordii*	16-3-4	Leaf	Jinan, Korea	2006.06.08
4	*C. wilfordii*	16-3-5	Leaf	Jinan, Korea	2006.09.11
5	*C. wilfordii*	16-3-6	Leaf	Jinan, Korea	2010.10.16
6	*C. wilfordii*	16-4-1	Leaf	Suwon, Korea	2010.02.01
7	*C. wilfordii*	H-1-3	Dry root	Yeongcheon, Korea	2002.08.01
8	*C. wilfordii*	10G1011	Dry root	Yeongcheon, Korea	2009.07.24
9	*C. wilfordii*	10G1012	Dry root	Andong, Korea	2009.07.24
10	*C. wilfordii*	11i1001	Dry root	Yeongju, Korea	2011.04.01
11	*C. wilfordii*	11i1003	Dry root	Yeongju, Korea	2011.04.01
12	*C. auriculatum*	11i1004	Dry root	Yeongju, Korea	2011.04.01
13	*C. wilfordii*	11i1006	Dry root	Yeongju, Korea	2011.04.01
14	*C. wilfordii*	11i1008	Dry root	Yeongju, Korea	2011.04.01
15	*C. wilfordii*	11i1009	Dry root	Yeongju, Korea	2011.04.01
16	*C. wilfordii*	11i1012	Dry root	Yeongju, Korea	2011.04.01
17	*C. wilfordii*	11i1013	Dry root	Andong, Korea	2011.04.01
18	*C. wilfordii*	11i1015	Dry root	Yeongcheon, Korea	2011.04.01
19	*C. auriculatum*	K0028	Dry root	Commercial, China	2009.07.24
20	*C. auriculatum*	16-3-3	Leaf	Beijing, China	2003.07.30
21	*C. auriculatum*	16-3-7	Leaf	Yeongju, Korea	2006.10.27
22	*C. auriculatum*	H-1-4	Dry root	Yeongju, Korea	2002.08.01
23	*C. auriculatum*	H-1-10	Dry root	Hwasun, Korea	2002.12.01
24	*C. auriculatum*	H-1-12	Dry root	Yeongju, Korea	2002.12.01
25	*C. auriculatum*	10G1013	Dry root	Yeongju, Korea	2009.07.24
26	*C. auriculatum*	10G1014	Dry root	Commercial, China	2009.07.24
27	*C. auriculatum*	DJKO	Dry root	Daejeon, Korea	2001.06.01
28	*C. auriculatum*	11i1002	Dry root	Yeongju, Korea	2011.04.01
29	*C. auriculatum*	11i1005	Dry root	Yeongju, Korea	2011.04.01
30	*C. auriculatum*	11i1007	Dry root	Commercial, China	2011.04.01
31	*C. auriculatum*	11i1010	Dry root	Yeongju, Korea	2011.04.01
32	*C. auriculatum*	11i1011	Dry root	Yeongcheon, Korea	2011.04.01
33	*C. auriculatum*	11i1014	Dry root	Yeongju, Korea	2011.04.01

and 1 mL of 5–20 ng/mL template DNA. DEPC-treated water was used as the negative control. Each amplification cycle consisted of a denaturation step of 10 min at 95ºC, followed by 40 cycles of denaturation for 15 s at 95ºC, annealing for 1 min, and extension at 60ºC. The result was evaluated using 7500 software v.2.0.1 (Applied Biosystems).

Results and Discussion

Analysis of cpDNA *trn*L-*trn*F region. Total length of the *trn*L-*trn*F region of the chloroplast DNAs of *C. wilfordii* and *C. auriculatum* was found to be 789 base pairs (bp). The alignment gap data encompassed 229 nucleotide positions and revealed nucleotide variations at positions 653, 707, and 710 in the aligned sequences. A deletion of 1 bp was detected in *C. wilfordii*. Therefore, 4 nucleotide variations were noted between *C. wilfordii*

and *C. auriculatum* (Fig. 2). The *trnL-trnF* regions region has a stable structure than the nucleotide ribosomal DNA due to its lower copy number of mutations percentage; thus this region was used to analyze the two species. The nucleotide variations could be divided into two groups, and the percentage of sequence variation within each group was 0% (data not shown). These results confirmed that the homology percentage of inter-species nucleotide sequences exceeded 100%. In addition, the *trn*L-*trn*F sequence results were compared by performing a nucleotide basic local alignment search tool search of the National Center for Biotechnology Information database. However, no information on *trn*L-*trn*F sequences of *C. wilfordii* and *C. auriculatum* has yet been listed in this database. Nucleotide sequencing was performed using 25 specimens obtained from the pharmaceutical market. The results were identical to the nucleotide sequencing of the bio-specimens; 11 specimens were identified as *C. wilfordii*, and 14 specimens were identified as *C. auriculatum*. Accordingly, the

Fig. 1 Photographs of dried *Cynanchum wilfordii* and *Cynanchum auriculatum.*(A), Uncut *C. wilfordii* sample; (B), Cut *C. wilfordii* sample; (C), Uncut *C. auriculatum* sample; and (D), Cut *C. auriculatum* sample.

Fig. 2 Sequence alignment of the *trn*L-*trn*F intergenic space of *Cynanchum wilfordii* (Nos. 1–6 of Table 1) and *Cynanchum auriculatum* (Nos. 20–21 of Table 1). The arrows were indicated the designed primer (CAW-F, CAW-R, RT-F, and RT-R) separately and boxes were shown the probe-VIC and probe-FAM positions in the sequences of the 2 species. Dots denote identical nucleotides, and middle bars denote deletion of bases in the sequence.

analysis of the nucleotide sequences forming the *trn*L-*trn*F region was found to be useful for confirming the differences between *C. wilfordii* and *C. auriculatum*.

Development of genetic markers. *C. wilfordii* and *C. auriculatum* can be differentiated by nucleotide sequencing of the *trn*L-*trn*F region, but using DNA markers is a simpler and more convenient method to analyze the differences. Therefore, 4 primers that can differentiate *C. wilfordii* and *C. auriculatum* were designed. First, the specific region of *C. wilfordii* was identified using RAPD-PCR analysis in order to design primers specific to *C. wilfordii.* After the analysis was performed using 10 universal University of British Columbia (UBC) primers, specific bands of *C. wilfordii* were confirmed in a ~200-bp sequence of the UBC 481 primers (Fig. 3C). A molecular marker that can only be amplified in *C. wilfordii* was designed based on the SCAR analysis. The difference in the nucleotide sequences from the SCAR analysis of *C. wilfordii* and *C. auriculatum* was used to design a DNA marker by which the two species could be
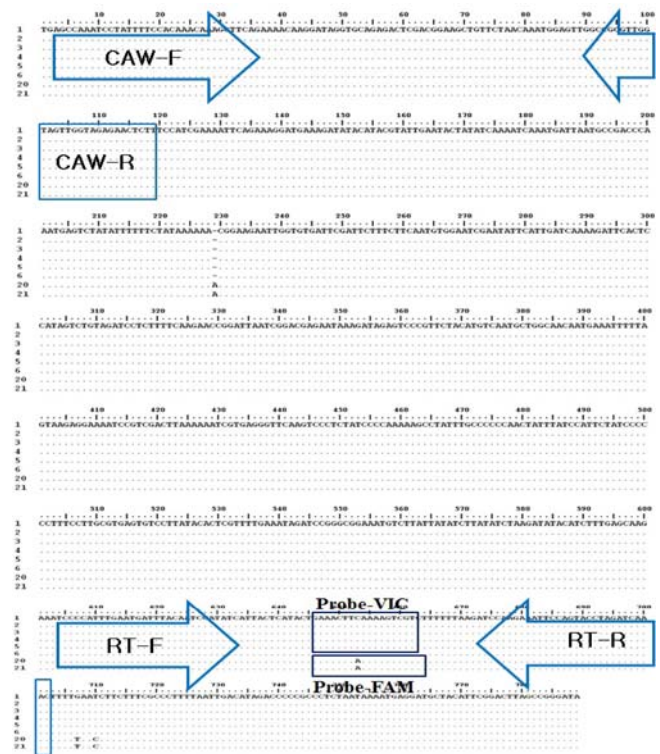
accurately differentiated (Fig. 3A). A forward 20-mer primer and a reverse 21-mer primer were used to design a 170-bp genetic marker that was specific to *C. wilfordii* (Fig. 3D). A prepared BLAST search of each primer sequence was used to compare the homology that was registered with NCBI. In addition, a positive control marker that would be amplified in both the species was also designed using the *trn*L-*trn*F sequence in order to confirm

Table 2 Primer sequences used

| Primer Name	Sequence (5'-3')	Reference
CW-F09	GGG CAG CAT CAG TTT AAG CA	present study for SCAR marker
CW-R10	AAT TGC GCA TAG GAA CTG TGA	present study for SCAR marker
CAW-F	AGC CAA ATC CTA TTT TCC ACA AAC AAA GGT TCA G	present study for SCAR marker
CAW-R	AAG AGT TCT CTA CCA ACT ACC AAC GCG GCC	present study for SCAR marker
RT-F	TCC CCA TTT GAA TGA TTT ACA ATC CAT ATC A	present study for real time PCR
RT-R	GTT TGA TCT AGG TAC TGG AAT TTC TTG GA	present study for real time PCR
Probe-VIC	ACG ACT TTT GAA GTT TC	present study for real time PCR
Probe-FAM	AGA CGA CTT TTT AAG TTT C	present study for real time PCR
trnL	GGT TCA GTT CCC TCT ATC CC	Taberlet et al.
trnF	ATT TGA ACT GGC GAC ACG AG	Taberlet et al.
UBC-481	GTA ATT GCG C	University of British Columbia
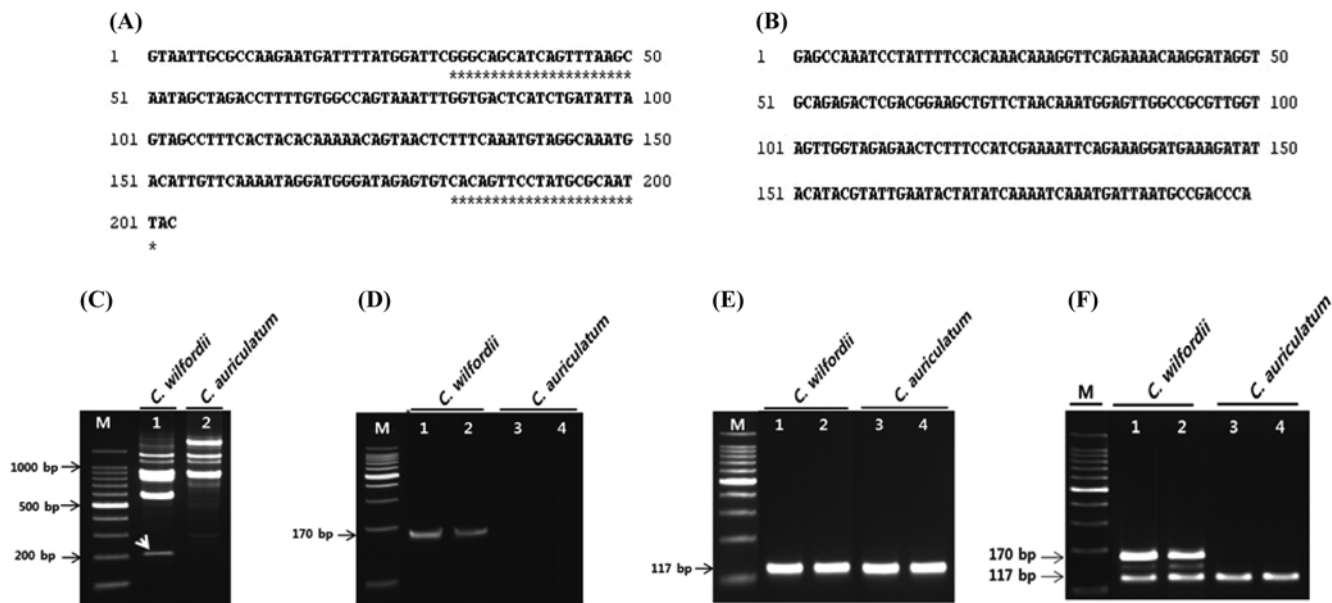
Fig. 3 Polymerase chain reaction analysis of *Cynanchum wilfordii* and *Cynanchum auriculatum* by using designed primers. Lanes 1 and 2, *C. wilfordii*; Lanes 3 and 4, *C. auriculatum*; M, 100-bp DNA ladder. (A) The nucleotide sequence of *C. wilfordii* yields a 203 bp amplicon. The designed CW-F09 and CW-R10 primer site is denoted by asterisks. (B) Nucleotide sequences of the *trn*L-*trn*F intergenic spacer from *C. wilfordii* and *C. auriculatum*. The designed CAW-F and CAW-R primer site is denoted by asterisks. (C) The arrow indicates the UBC 481 primer used in randomly amplified polymorphic DNA (RAPD). The amplification band is specific for the purposes of a sequence-characterized amplified region (SCAR) marker of *C. wilfordii*. (D) The 170-bp fragment was amplified using the CW primer designed based on the RAPD product. (E) The 117-bp fragment was amplified using the CAW primer designed based on the *trn*L-*trn*F region. (F) Multiplex polymerase chain reaction amplification products obtained using combinations of the designed primer pair (CW, CAW).

PCR amplification (Fig. 3B). A forward 34-mer primer and a reverse 30-mer primer were used to design a 117-bp genetic marker that was universal to *C. wilfordii* and *C. auriculatum* (Fig. 3E). The specificity to the CW primer of *C. wilfordii* yielding the 170-bp product was then tested (Fig. 3F). An internal primer that was present in both *C. wilfordii* and *C. auriculatum* was prepared, yielding a 117-bp product. Thus, in *C. wilfordii*, amplified products were observed at 117 bp and 170 bp on the gel. In *C. auriculatum*, only a 117-bp product was amplified (Fig. 3F). In addition, the optimal concentration of the multiplex PCR conditions of the prepared primer was confirmed by performing the experiment using different primer concentrations. Optimal amplification results were obtained for the CW-F09 and CW-R10 primers at 2 pmol/mL and for the CAW-F and CAW-R primers at 10 pmol/mL (Fig. 3F). PCR was performed using the dried herb under the established PCR conditions. This confirmed the presence of the species-specific 170-bp amplification product in *C. wilfordii*. When this molecular marker was applied to all samples, they were found to be identical to the results of the nucleotide sequencing (Fig. 4). Therefore, it was suggested that the two species could be efficiently differentiated using a genetic marker. The analysis of the constituents revealed that *C. wilfordii* and *C. auriculatum* could be differentiated as previously described. The results of the analysis of nucleotide sequences forming the SCAR revealed that an inter-species difference could also be detected. Thus, nucleotide sequence differences between *C.* *wilfordii* and *C. auriculatum* were present, and these inter-species genetic differences were confirmed to be detectable.

Application of real-time detection. The methods that used a genetic marker and those that used real-time PCR for differentiation were compared by conducting a real-time PCR assay using the same samples. The four single nucleotide polymorphisms (SNPs) in *trn*L-*trn*F nucleotide sequences from *C. wilfordii* and *C. auriculatum* were used to design the primer and probe by using FAM and VIC dye-labeled TaqMan minor groove binder probe sets selected from the Applied Biosystems Assays. A genotyping assay showed that specimen numbers 1–6 in Table 1 (representing *C. wilfordii* isolates) were concentrated along the X axis, and specimen numbers 20–1 in Table 1 (representing *C. auriculatum* isolates) were concentrated along the Y axis. These results suggested that group A (Table 1, samples 1–11, 13–18) were *C. wilfordii*, and group B (Table 1, samples 12, 19–33) were *C. auriculatum*. Group C, which served as the control, was labeled as the No Template Control (NTC), and it showed a cluster that was close to approximately 0 (Fig. 5). These results confirm that discrimination between *C. wilfordii* and *C. auriculatum* is possible. Each allele probe was used to assess a genotype cluster. The sequence variations in the *trn*L-*trn*F region could be used to classify *C. wilfordii* and *C. auriculatum* samples into two groups (A and B) described above. These results directly confirmed those of genetic amplification without additional processing and are therefore advantageous in shortening the analysis time unlike the
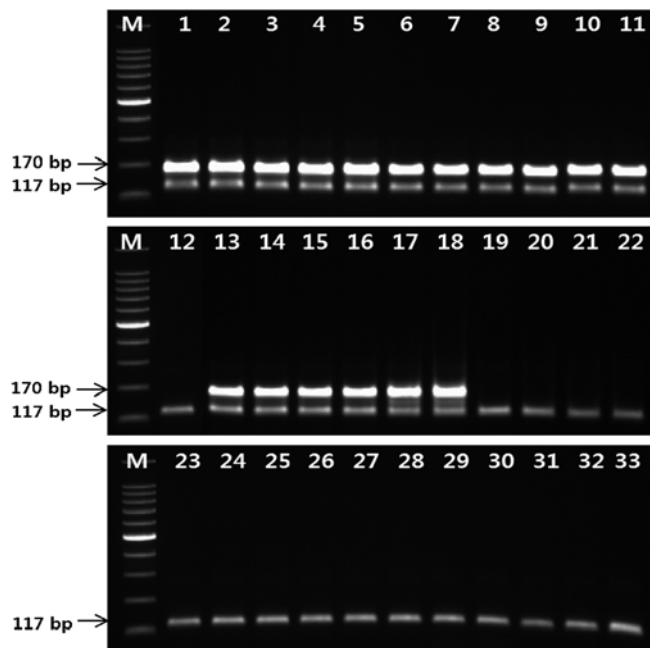
Fig. 4 Polymerase chain reaction amplification products obtained from collected samples by using the designed CW and CAW primers. Lanes 1–33, sample nos. 1–33 from Table 1; M, 100-bp DNA ladder.
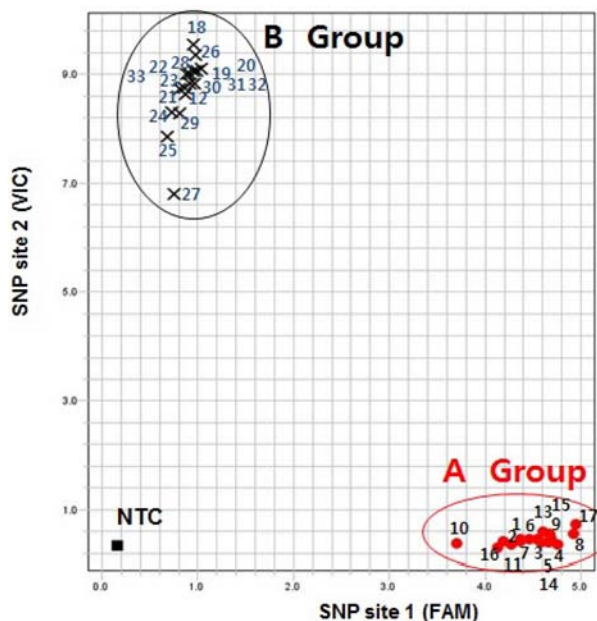

Fig. 5 Real-time polymerase chain reaction to test single nucleotide polymorphism sites in *Cynanchum wilfordii* (sample nos. 1–11, 13–8; Table 1) and *Cynanchum auriculatum* (sample nos. 12, 19–33; Table 1). Group A, *C. wilfordii*; Group B, *C. auriculatum*; NTC, No Template Control.

one that required predetermined methods. In addition, the curve patterns of fluorescent materials present in *C. wilfordii* and *C. auriculatum* revealed a match to the genetic marker, following

genotype analysis by using an automatic analysis program.

In summary, the analysis of nucleotide sequences forming the *trn*L-*trn*F region led to the determination of different nucleotide

Table 3 Monitoring results of the 40 samples for the identification of *Cynanchum wilfordii* and *Cynanchum auriculatum* using real-time polymerase chain reaction analysis

No.	Voucher No.	Identification Results	No.	Voucher No.	Identification Results
1	10G2010	*C. auriculatum*	21	10G2032	*C. wilfordii*
2	10G2011	*C. auriculatum*	22	10G2033	*C. wilfordii*
3	10G2013	*C. wilfordii*	23	10G2034	*C. auriculatum*
4	10G2014	*C. wilfordii*	24	10G2040	*C. auriculatum*
5	10G2015	*C. auriculatum*	25	10G2041	*C. auriculatum*
6	10G2016	*C. auriculatum*	26	10G2043	*C. auriculatum*
7	10G2017	*C. auriculatum*	27	10G2044	*C. auriculatum*
8	10G2019	*C. wilfordii*	28	10G2047	*C. auriculatum*
9	10G2020	*C. wilfordii*	29	10G2048	*C. wilfordii*
10	10G2021	*C. wilfordii*	30	10G2049	*C. auriculatum*
11	10G2022	*C. wilfordii*	31	10G2050	*C. auriculatum*
12	10G2023	*C. wilfordii*	32	10G2051	*C. auriculatum*
13	10G2024	*C. wilfordii*	33	10G2052	*C. auriculatum*
14	10G2025	*C. wilfordii*	34	10G2053	*C. wilfordii*
15	10G2026	*C. auriculatum*	35	10G2054	*C. wilfordii*
16	10G2027	*C. auriculatum*	36	10G2055	*C. wilfordii*
17	10G2028	*C. wilfordii*	37	10G2056	*C. auriculatum*
18	10G2029	*C. wilfordii*	38	10G2060	*C. auriculatum*
19	10G2030	*C. wilfordii*	39	10G2061	*C. auriculatum*
20	10G2031	*C. wilfordii*	40	10G2062	*C. auriculatum*

sequences comprising *C. wilfordii* and *C. auriculatum*. Thus, a genetic marker for *C. wilfordii* was developed. In addition, real-time PCR confirmed the differences between *C. wilfordii* and *C. auriculatum*. This suggests that real-time PCR methods can detect the differences in *C. wilfordii* and *C. auriculatum* easily and promptly. Therefore, they can be used to screen a large number of samples. In conclusion, SCAR-PCR and *trn*L-*trn*F sequence comparison enabled the differentiation of *C. wilfordii* from *C. auriculatum*. The developed molecular marker can be applied to the differentiation of products obtained from mixed species, including *C. wilfordii*, because such differentiation can be difficult morphologically due to the similarities in external characteristics of the products. Further studies are warranted to trace the distribution of these species in the field by using the results of our study.

Monitoring. The developed marker was used to monitor the roots currently marketed as *C. wilfordii* in the Korean herbal market. The monitoring was performed using real-time PCR (TaqMan probe). Analysis of 40 samples revealed that 21 of the roots were *C. auriculatum* and 19 were *C. wilfordii* (Table 3). These findings indirectly suggest that both *C. wilfordii* and *C. auriculatum* are sold together in the pharmaceutical market. These results alone do not conclusively indicate a defect in the overall distribution of *C. wilfordii*; however, we believe that factors other than morphology need to be assessed for accurately differentiating between herbal ingredients of Korean traditional medicine.

In the present study, we developed a specific genetic marker for *C. wilfordii* by using real-time PCR methods. A highly sensitive real-time PCR method has been developed for the detection of misused herbs (Ryuk et al., 2010). However, this method cannot differentiate between *C. wilfordii* and *C. auriculatum*. Thus, our developed molecular marker can be used to efficiently differentiate *C. auriculatum* and *C. wilfordii* in the pharmaceutical market.

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