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ARTICLE

Development of cpDNA markers for discrimination between *Cynanchum wilfordii* and *Cynanchum auriculatum* and their application in commercial *C. wilfordii* food products

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Abstract *Cynanchum wilfordii* has been used as a health-enhancing food product in Korea. Owing to its morphological similarity to *C. wilfordii* and relative ease of cultivation, *C. auriculatum* has been illegally used as a substitute plant for *C. wilfordii*. In the present study, we developed markers to discriminate between *C. wilfordii* and *C. auriculatum*. Species-specific primer sets for *C. wilfordii* and *C. auriculatum* were designed based on the single nucleotide polymorphisms of the chloroplast *matK* genes for quantitative real-time PCR using SYBR green. The limit of detection of primer sets for each species was assessed by analyzing serially 1/10-diluted DNAs at concentrations of 0.001–100% (=10 ng) and binary mixtures of a flour matrix spiked with decreasing concentrations (10^3–1 mg/g) of non- and heated *C. wilfordii* and *C. auriculatum*, respectively. We found that species-specific primer sets indicated good amplification efficiency and correlation coefficients (R^2) of the standard curves in the extracted DNA. The developed markers were successfully applied to 19 commercial *C. wilfordii* food products and could prove a useful tool for verifying the presence of *C. wilfordii* and *C. auriculatum* in commercial products.

Keywords Commercial products · cpDNA marker · *C. auriculatum* · *Cynanchum wilfordii* · Quantitative real-time PCR

Introduction

Improvement in the quality of human life has led to increased use of health-enhancing food products. The Functional Food Center (Dallas, Texas, USA) has defined "functional food" as natural food product that contains known or unknown bioactive compounds that exert health benefits (Martirosyan and Singh 2015). The physiological benefits of functional food may include reduced risks of chronic diseases. The ingredients of functional foods with plant origins have been effective in preventing diseases in humans. For example, the health benefits of cranberries have been demonstrated in the suppression of urinary tract infections (Avorn et al. 1994), and a meta-analysis has indicated that garlic lowers blood pressure (Silagy and Neil 1994). Despite the many advantages of functional foods, the commercial production of these foods has several problems such as food fraud, including the deliberate mislabeling and illegal substitution of food ingredients. The illegal products produced as a result of food fraud might threaten human health. In particular, food ingredients derived from plants have been used in the form of dried powders to produce the commercial foods; it is therefore difficult to discriminate between authentic and spurious raw materials. To protect consumer from food fraud such as mislabeling and intended or unintended mixture, food inspectors need to develop various techniques for detecting illegal food products.

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Cynanchum is a genus containing about 300 species, belonging to the family Apocynaceae. *Cynanchum wilfordii* is listed in the Korea Herbal Pharmacopoeia as the original plant of *C. wilfordii* Hemsley (KFDA 2011). In addition, *C. wilfordii* is known to have active components in its root extracts, which are known to be effective for alimentation, improving vigor, and nourishing blood in the form of herbal medicines (Atta-ur-Rahman and Choudhary 1999). Additionally, *C. wilfordii* has been often used as a health food supplement in Asia, and its importance as a functional food ingredient has increased in Korea. Recently, the root tissues of *C. auriculatum* Royle ex Wight, which is forbidden to be used as a food ingredient in Korea, has been illegally substituted for those of *C. wilfordii* to produce food products since it is difficult to distinguish between these two species when used as ingredients in commercial food products. The adulteration of *C. wilfordii* commercial products has become a particularly serious social problem in Korea since April 2015 (Cheongju, Korea Consumer Agency, http://www.kca.go.kr/). About 60% of *C. wilfordii* products circulated in the market were detected to contain added *C. auriculatum*.

Chloroplasts are plant organelles that play an important role in photosynthesis. Most chloroplast genomes have a circular structure and are 120–170 kb in size (Clegg et al. 1994; Shaw et al. 2007). Unlike the diploid nuclear genome, the chloroplast genome is haploid, containing approximately 100 genes (McFadden 2001), and exhibits low levels of substitution. For this reason, it has been used as a useful tool for evolutionary studies in several plant species. For example, the MatureasK (*matK*) gene, located within the intron region of *trnK*, is highly conserved in plants and has been used to identify species using methods such as DNA barcoding (Selvaraj et al. 2008). Fuse and Tamura (2000) reported that the variation in the stop codon positions of the *matK* gene is high among the monocotyledons. In many studies, the *matK* gene has been used to resolve family-as well as species-level relationships (Steele and Vilgalys 1994; Koch et al. 2001; Tamura et al. 2004).

Polymerase chain reaction (PCR)-based methods are rapid and low cost (Mafra et al. 2008). In particular, quantitative real-time PCR (qRT-PCR) is rapid, accurate, and sensitive, and can be used to detect very low levels of target DNA sequences in foods (Overbergh et al. 2003; Malorny et al. 2008). DNA-based methods are being increasingly used to clarify species-level relationships. In addition, they are used to evaluate food authenticity and safety, owing to their high specificity and sensitivity. For example, many studies have reported successful species identification using these methods in foods such as celery (Pafundo et al. 2009), rice (Hwang et al. 2015), almond (Fuchs et al. 2012), meat (Jonker et al. 2008), and seafood (Herrero et al. 2011). In this study, we developed species-specific molecular markers derived from the *matK* genes of *C. wilfordii* and *C. auriculatum* using single nucleotide polymorphisms (SNPs) and then applied these markers to verify the presence of both species in commercial food products.

Materials and methods

Samples

Standard samples of *C. wilfordii* and *C. auriculatum* were obtained from Herbal Medicine Research Division, National Institute of Food and Drug Safety Evaluation (Kim et al. 2015). Nineteen commercially available *C. wilfordii* products, marketed as health-enhancing food, were purchased from local markets (Table 2).

Genomic DNA extraction

Genomic DNA was extracted from 200 mg each of standard and commercial product sample using the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, Seongnam, Korea), according to the manufacturer's protocol. The quantity of the extracts was measured using a Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, Grand Island, NY, USA) with a Qubit dsDNA BR Assay Kit (Invitrogen™, Life Technologies), according to the manufacturer's protocol. Their purity was confirmed using agarose gel electrophoresis with a Molecular Imager® Gel DOC™ XR+ System (Bio-Rad, Hercules, CA, USA), and BioPhotometer Plus UV/Vis Photometer (Eppendorf, NY, USA). DNA concentrations were measured and determined to be in the range of 101–114 ng/μL, and the ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) of about 1.78 was as expected for pure samples (Glasel et al. 1995).

PCR amplification and DNA sequencing

Chloroplast DNA sequences of *matK* of *C. acutum* were downloaded from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) in order to design primer pairs to amplify these regions of both plants. The *matK* gene in both plants was amplified using appropriate primer pairs (Table 1). The PCR analysis was performed in a final volume of 25 μL using the C1000 Thermal Cycler (Bio-Rad™). The PCR reaction mixture contained 10 ng of DNA, 10× buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, 10 pmol of each primer, and 0.5 U/μL EX *Taq* DNA polymerase (Takara Bio Company, Kusatsu, Shiga, Japan). The amplification conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 30 s each at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. The

Table 1 Information of primers used in this study

Species	Primers	Sequence (5′ → 3′)	Target gene	Fragment length (bp)	Reference
Conventional PCR (Cloning)					
Cynanchum	*Cynanchum_matK_F*[a]	GCT CTT CTT GAA CGA ATC TAT T	*matK*	763	This work
	Cynanchum_matK_R[b]	CCA AAT ACC AAA TCC GAC TTC			
Real-time PCR					
Cynanchum wilfordii	*C. wilfordii_matK_F*	CTT GTT CCA ATT ATT CT	*matK*	151	This work
	C. wilfordii_matK_R	AAT GAG AAA GGT TTC TA			
Cynanchum auriculatum	*C. auriculatum_matK_F*	CTT GTT CCA ATT ATT CC	*matK*	151	This work
	C. auriculatum_matK_R	AAT GAG AAA AGT TTC TG			
Plant system	*18S rRNA_F*	TCT GCC CTA TCA ACT TTC GAT GGT A	*18S rRNA*	137	Allmann et al. (1993)
(Amplification control)	*18S rRNA_R*	AAT TTG CGC GCC TGC TGC CTT CCT T			

[a] Forward primer

[b] Reverse primer

amplifications were confirmed on a 1.5% agarose gel electrophoresis with a 1 kb Plus DNA Ladder (Invitrogen, Life Technologies), and visualized using the Molecular Imager® Gel DOC™ XR+ System (Bio-Rad). The amplicons were cloned using the RBC T&A Cloning Kit (Real Biotech Co., Taipei, Taiwan), according to the manufacturer's protocol and sequenced by a commercial service (Macrogen, Seoul, Korea).

Quantitative real-time PCR analysis

The obtained sequences were aligned using ClustalW2 (ftp://ebi.ac.uk/pub/software/clustalw2/) to compare the differences between the sequences of the *matK* genes of both plants. The primer pairs were designed using Beacon Designer™ (PRIMER Biosoft, Palo Alto, CA, USA) and were synthesized by a commercial service (Macrogen,

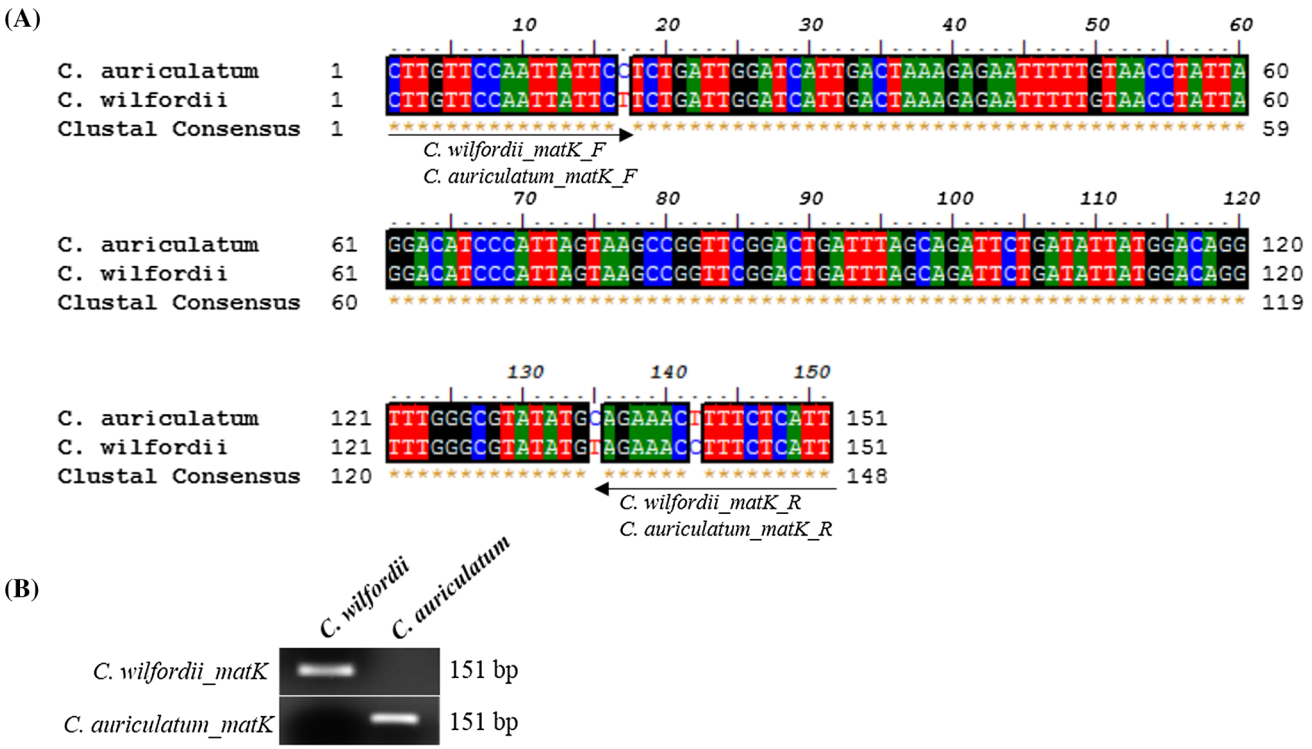

Fig. 1 Sequence alignment of cloned *C. wilfordii* and *C. auriculatum MatK* genes. Species-specific primers were designed on the basis of SNPs of both genes (**A**). Amplified PCR products were electrophoresed in order to confirm cross-reactivity (**B**)

Seoul, Korea), setting up an amplicon size of 151 bp (Fig. 1B).

qRT-PCR was performed in a final volume of 20 µL using a CFX Connect™ Real-Time PCR Detection System (BIO-RAD™). The reaction mixture contained 10 ng of DNA, 10 µL of SYBR® Green TOP real qPCR 2xPreMIX (Enzynomics™, Daejeon, Korea), and 10 pmol of each primer. The amplification conditions were as follows: 10 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, annealing time at the appropriate annealing temperature (T_m) of each primer pair, and 30 s at 70 °C. PCR products were denatured at 95 °C for 10 s and then annealed at 65 °C for 1 min. This step was followed by a melt curve, ranging from 60 to 95 °C, with temperature increments of 0.5 °C every 30 s (Table S1).

Determination of amplification efficiency, correlation coefficient, and limit of detection (LOD)

To evaluate the correlation between C_t values and DNA concentration, standard curves were obtained using tenfold serially diluted DNA samples of *C. wilfordii* and *C. auriculatum* at concentrations of 0.0001, 0.001, 0.01, 0.1, 1, and 10 ng. Correlation coefficient (R^2) was determined using the liner regression method ($R^2 \geq 0.98$) (Ramakers and Ruijuter 2003). The slope of the standard curve ranges from −3.6 to −3.1 (Mazzara et al. 2011). Amplification efficiency was calculated based on the standard curve using the equation: $E = 10^{-1/\text{slope}}$ and efficiency (%) = (E–1) × 100. The LOD was regarded as the analytical concentration at which the methods detected the presence of a target nucleic acid in at least 95% of true positive biological samples (less than 5% of false negative results) (Ferreira et al. 2016).

Results and discussion

Development of cpDNA markers

We have attempted a qRT-PCR assay to detect *C. wilfordii* and *C. auriculatum* cpDNA using species-specific primer pairs. In previous studies, cpDNA markers derived from *matK* have been used to analyze phylogenetic relationships and to perform plant identifications (Steele and

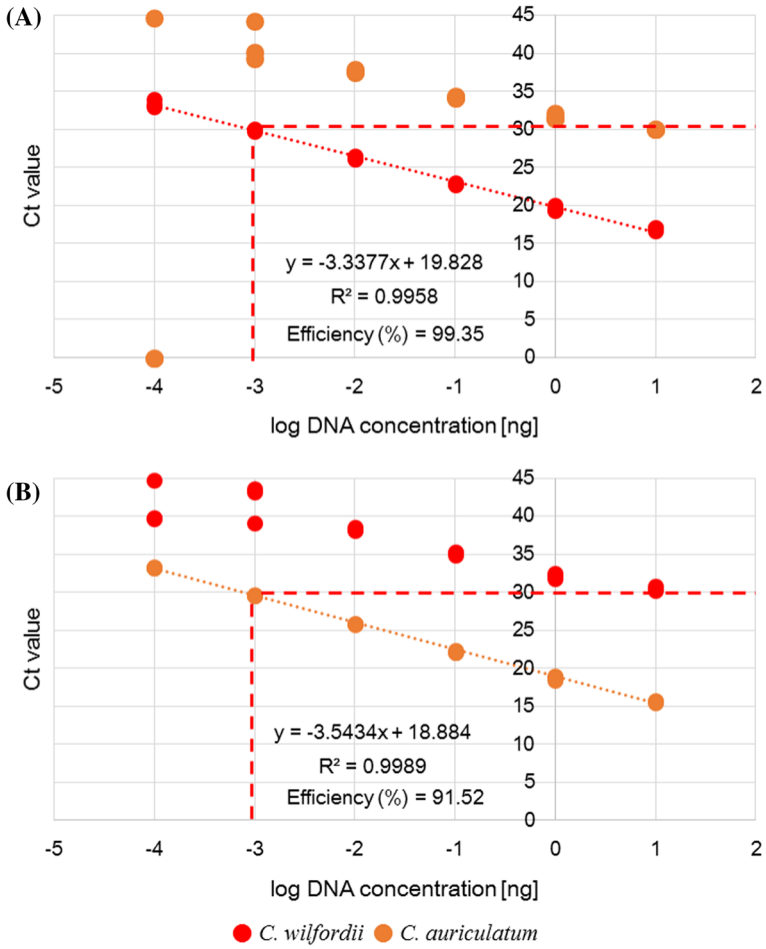

Fig. 2 Standard curves obtained by analyzing serially diluted DNAs of *C. wilfordii* (**A**) and *C. auriculatum* (**B**), respectively. The *standard curves* obtained are based on efficiency and correlation of coefficient (R^2) of DNA extracted from the plant roots. The *x*-axis represents log DNA concentration, and the *y*-axis represents C_t value

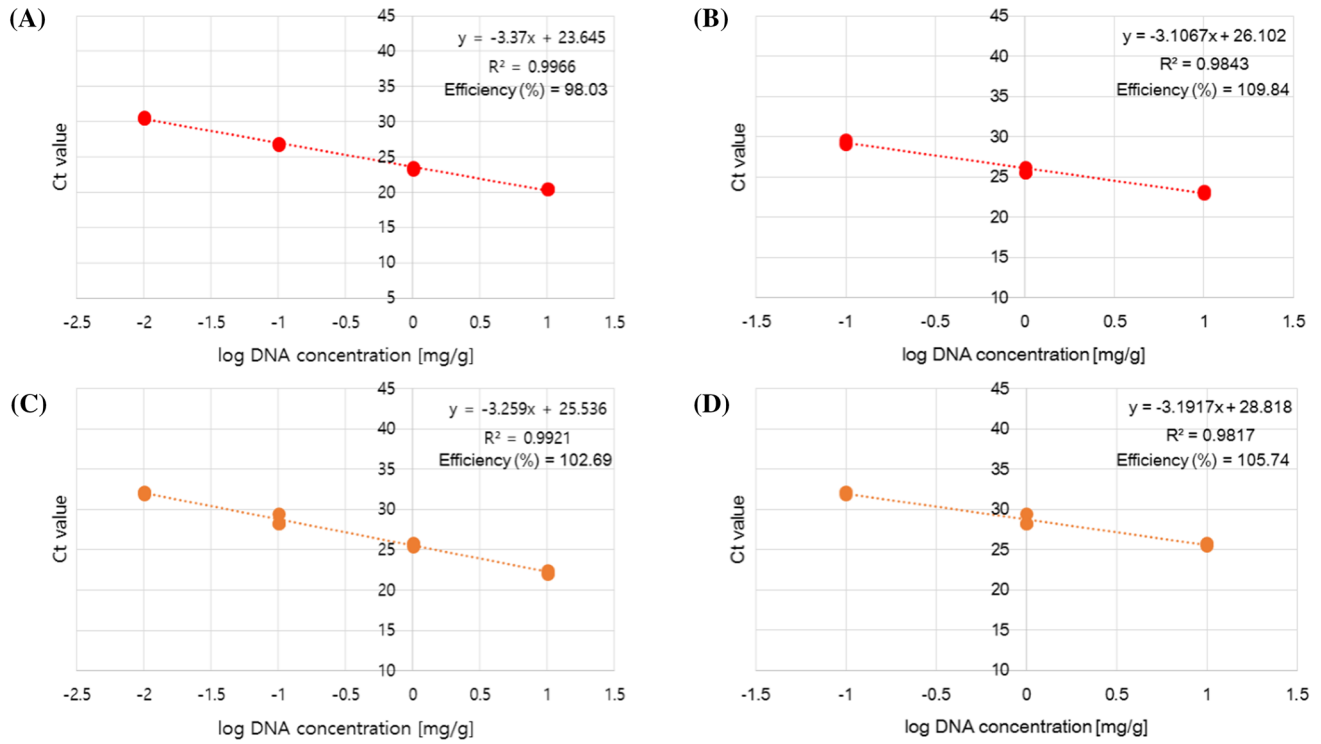

Fig. 3 Standard curves of LOQ, based on triplicate analysis of binary mixtures of flour matrices, spiked with decreasing concentrations of *C. wilfordii* and *C. auriculatum*. Non-heated *C. wilfordii*, heated *C. wilfordii*, non-heated *C. auriculatum,* and heat-treated *C. auriculatum* (130 °C for 15 min), shown in (**A**–**D**), respectively. The *standard curves* obtained are based on efficiency and correlation of coefficient (R^2) of DNAs extracted from both genotypes of roots. The *x*-axis represents a log DNA concentration, and the *y*-axis represents the C_t value

Vilgalys 1994; Tamura et al. 2004). As of September 2015, the complete *matK* gene sequences of *C. wilfordii* and *C. auriculatum* have not been reported, whereas the *matK* gene sequence of *C. acutum* has been deposited in the NCBI public sequence database. To identify the *matK* sequences of *C. wilfordii* and *C. auriculatum*, we designed potential genus-consensus primer pairs (Table 1), on the basis of the gene sequences of *C. acutum*. We successfully amplified 763-bp products in both species (Fig S1). The gene-specific primers were designed on the basis of SNPs of the gene between *C. wilfordii* and *C. auriculatum* (Fig. 1A). Primer sets of cpDNA markers for *C. wilfordii* and *C. auriculatum* were designed based on SNPs in the fragments. To confirm the allele-specific amplification of each cpDNA marker, we performed PCR on the genomic DNA of the two species with cpDNA markers and visualized the PCR products via gel electrophoresis (Fig. 1B); we determined that each primer set could amplify specific PCR products in one species but not the other. We then sequenced the 151-bp amplicons (Fig. 1B) and confirmed the expected sequences (data not shown). These results suggest that the cpDNA markers could be used for discrimination in both species.

Standard curves and amplification efficiency of SYBR Green PCR systems

To estimate the efficiency and sensitivity of primer sets, we carried out a qRT-PCR assay using tenfold serially diluted total DNA (including chloroplast DNA) and then analyzed the statistical measures of each cpDNA marker using the regression test (Fig. 2). A strong linear correlation ($R^2 > 0.99$) was obtained between the crossing point values and log DNA concentration for *C. wilfordii* and *C. auriculatum*. Linearity was observed (range 10 ng–0.1 pg of total DNA). The slopes of the linear equations were −3.34 for *C. wilfordii* DNA (Fig. 2A) and −3.54 for *C. auriculatum* DNA (Fig. 2B), with appropriate amplification efficiencies of 99.35 and 91.52%, respectively. However, the cpDNAs of the counterpart samples were amplified with C_t value higher than 30. These results suggest that the developed markers are able to identify *C. wilfordii* and *C. auriculatum* samples under appropriate amplification conditions with DNA concentrations of more than 1 pg and C_t value of less than 30.

Heat treatment is one of the necessary conditions for the manufacturing process. Therefore, the efficiency and

Table 2 qRT-PCR results on *C. wilfordii* and/or *C. auriculatum* with the primer sets in 19 commercial food products containing *C. wilfordii*

Samples	CWS[a]	CV[c] (%)	CAS[b]	CV (%)	18 s rRNA
Pill (pellet)					
1	ND[d]	ND	17.54 ± 0.07	0.52	12.94 ± 0.01
2	ND	ND	18.53 ± 0.03	0.23	11.51 ± 0.14
3	ND	ND	24.07 ± 0.13	0.76	10.76 ± 0.27
4	26.31 ± 0.20	1.05	ND	ND	10.33 ± 0.05
5	18.31 ± 0.23	1.82	25.27 ± 0.18	0.98	9.35 ± 0.17
6	22.97 ± 0.03	0.18	15.32 ± 0.09	0.83	9.60 ± 0.16
7	ND	ND	21.35 ± 0.10	0.66	10.82 ± 0.24
8	20.10 ± 0.03	0.21	19.45 ± 0.02	0.18	10.15 ± 0.27
9	23.18 ± 0.20	1.19	24.54 ± 0.45	1.82	9.22 ± 0.19
10	ND	ND	21.31 ± 0.27	1.83	10.77 ± 0.28
11	25.79 ± 0.04	0.19	18.57 ± 0.33	2.55	13.11 ± 0.09
12	ND	ND	22.18 ± 0.04	0.22	11.64 ± 0.20
13	ND	ND	ND	ND	16.44 ± 0.10
Crude drug (dry matter)					
14	18.33 ± 0.10	0.73	28.83 ± 0.23	1.10	12.09 ± 0.06
15	18.27 ± 0.14	1.08	27.93 ± 0.14	0.71	12.39 ± 0.06
Crude drug (powder)					
16	17.68 ± 0.21	1.64	21.65 ± 0.07	0.46	9.34 ± 0.07
17	23.96 ± 0.24	1.45	15.99 ± 0.13	1.11	11.85 ± 0.11
18	ND		ND	ND	13.88 ± 0.23
Granula					
19	16.67 ± 0.14	1.15	22.65 ± 0.02	0.12	9.71 ± 0.13

[a] *C. wilfordii*-specific system

[b] *C. auriculatum*-specific system

[c] Coefficients of variation

[d] Not detected

sensitivity of primer sets were assessed using qRT-PCR with binary mixtures containing different concentrations of heated and unheated samples (10^3–10 mg/g) (Fig. 3). The slopes of the linear equations for raw samples were −3.37 for *C. wilfordii* DNA (Fig. 3A) and −3.26 for *C. auriculatum* DNA (Fig. 3C), with appropriate amplification efficiencies of 98.03 and 102.69%, respectively. The slopes of the linear equations for heated samples were −3.11 for *C. wilfordii* DNA (Fig. 3B) and −3.19 for *C. auriculatum* DNA (Fig. 3D), with appropriate amplification efficiencies of 109.84 and 105.74%, respectively. Ferreira et al. (2016) have reported estimated coffee content in commercial products using SYBR green real-time PCR systems and have suggested that the valid standard curves in a regression equation are essential for accurate quantification and identification of different types of potential adulterants. Therefore, the primer pairs designed in the present study would be useful for accurate quantification and identification for both species in commercial food products.

Application of cpDNA markers to commercial *C. wilfordii* products

In order to verify the feasibility of using qRT-PCR systems to detect both species in *C. wilfordii* commercial products, 19 samples were bought from local markets and were tested using this qRT-PCR system. The procedure is crucial not only because an intended (or unintended) mixture of material from both species could adversely affect health, also because *C. wilfordii* is a susceptible target for food fraud owing to its higher price (more than twice that of *C. auriculatum*). Among the samples, 13 were pills, 5 were crude drugs (2 dry matter and 3 powder), and 1 was granular (Table 2). All commercial product samples were amplified using *18S rRNA* genes as a positive control for verifying that DNA was extracted from commercial products. The *C. wilfordii* DNA was detected in 6 pill samples, 2 dry matter samples, and 2 powder sample, but was not detected in 7 pill samples, 1 powder sample, and the 1 granular sample. The *C. auriculatum* DNA was detected in

11 pill samples, 2 dry matter samples, 2 powder sample, and 1 granula. Both *Cynanchum wilfordii* DNA and *C. auriculatum* DNA were both detected in 5 pill samples, and *C. auriculatum* DNA alone was detected in 3 pill samples. However, DNA from neither species was detected in 1 pill and 1 powder sample.

The DNA-based methods for identification of *C. wilfordii* and *C. auriculatum* have been used for species identification in several studies, for example, with sequence-characterized amplified region markers (Moon et al. 2010; Ryuk et al. 2014), single PCR (Kim et al. 2015), multiplex PCR (Moon et al. 2016), and amplification refractory mutation system PCR and high-resolution melt curve analysis (Han et al. 2016). Because sensitivity of the primer sets that were previously developed was not evaluated, it may be difficult to discriminate small amounts of the target species in commercial food products. In addition, few studies showed only application of newly developed primer sets to processed foods. In addition, Li et al. (2013) reported high-performance liquid chromatography (HPLC)-UV analysis for quality assessment and discrimination of the roots of the two plants. Nevertheless, the method requires expensive equipment and experimental skills and entails high operation costs.

Recently, qRT-PCR methods based on SYBR green have proved to be useful tools for species-specific traces and quantification (Pafundo et al. 2009; Sakalar 2013). Many approaches to authenticity verification involve successful qRT-PCR methods with SYBR green in processed foods, such as almond (Pafundo et al. 2009) and coffee (Ferreira et al. 2016). In the present study, we demonstrated sensitivity and accuracy of the primer sets in repeated experiments and in the application to diverse commercial food products containing *C. wilfordii* (Figs. 2, 3). These results suggest that the method developed in this study may help to verify the authenticity of both *Cynanchum* in *C. wilfordii* food products owing to the rapidity and relatively low cost.

In conclusion, we developed primer sets on the basis of SNPs of the *matK* gene of both *C. wilfordii* and *C. auriculatum* for the purpose of authenticity verification of *C. wilfordii* food products. Specificity and sensitivity of the cpDNA markers were examined using SYBR green-based qRT-PCR with diverse samples. Additionally, the markers were applied successfully to 19 commercial *C. wilfordii* food products and could prove a useful tool to detect the presence of *C. wilfordii* and *C. auriculatum* in commercial products.

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