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Development of a species-specific real-time PCR assay for identifying the medicinal plant *Artemisia princeps* and the toxic plant *Corydalis speciosa*

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Abstract
The medicinal herb *Artemisia princeps* and the poisonous plant *Corydalis speciosa* have a high degree of morphological similarity, which may contribute to unintentional contamination. Indeed, cases of acute toxicity have been reported in which *C. speciosa* was mistakenly ingested in place of *A. princeps*. To ensure accurate identification, a species-specific quantitative real-time PCR (qPCR) assay was developed using six primer pairs targeting single-nucleotide polymorphisms or insertion/deletion regions within chloroplast genes. The respective limits of detection were validated, and a contamination-based cutoff Cq value (0.1%) was established for assessing authenticity. All six primer pairs showed excellent performance, with correlation coefficients > 0.99, slopes of -3.12 to -3.28, and efficiencies of 101.82%–109.47%. The developed qPCR assay was verified based on blind and commercial tests for reproducibility and specificity, respectively. This qPCR assay represents a sensitive and reliable method for distinguishing similar medicinal and toxic plants, thereby contributing to improved product quality and safety.

Keywords *Artemisia princeps*, *Corydalis speciosa*, Identification, qPCR, SYBR® Green

Introduction

Medicinal herbs have long been used for health promotion and therapeutic purposes, whereas in contrast, certain poisonous plants can pose serious health risks, even when ingested in small quantities. Given that many of these herbs grow commonly in the wild and are being foraged by an increasing number of people [23], confusion between morphologically similar medicinal and toxic plants has become a growing public health concern, as this can lead to accidental ingestion and potential toxicity from consumption of unidentified plant material [6, 26].

A notable example of such mistaken identity concerns the plants *Artemisia princeps* and *Corydalis speciosa*, which are characterized by a similar leaf morphology. Morphological differentiation between *A. princeps* and *C. speciosa* is particularly challenging during the early stages of growth and becomes even more difficult when plant materials have been processed or mixed. Indeed, several cases of acute toxic hepatitis have been reported in individuals who have accidentally ingested *C. speciosa*, in the mistaken assumption that they were consuming *A. princeps* [8, 12]. *A. princeps* is a medicinally safe botanical used extensively for its anti-inflammatory, antioxidant,

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and antidiabetic activity, whereas *C. speciosa* contains toxic alkaloids that may cause severe liver injury upon ingestion [3, 10, 27, 28], thereby, emphasizing the importance of accurate identification of these two species to ensure consumer safety and prevent toxic exposure.

A number of molecular markers have been reported to facilitate differentiation between medicinal herbs and poisonous plants [21], and although species-specific markers have been developed for several species within the genus *Artemisia*, including *A. annua*, *A. capillaris*, and *A. iwayomogi*, there have to date been no studies that have focused specifically on the development of markers to distinguish *A. princeps* from the toxic plant *C. speciosa* [14, 16, 17], thus highlighting the need to develop molecular markers for distinguishing between these two species. DNA-based markers are widely used for detecting and identifying specific biological targets by employing molecular techniques such as quantitative real-time PCR (qPCR), digital PCR, and next-generation sequencing in food identification and in vitro diagnostics [4, 15]. Among these, qPCR offers notable advantages with respect to speed and cost-effectiveness [18]. In particular, SYBR Green-based qPCR facilitates simpler primer design and reduced costs compared with others similar techniques, such as the use of TaqMan probes, as it does not require the preliminary synthesis of specific probes [24, 25]. However, given that SYBR Green binds non-specifically to all double-stranded DNA, it is prone to non-specific amplification. Thus, melt curve analysis is required to confirm the specificity of amplification. Accordingly, despite the aforementioned limitations, SYBR Green-based qPCR can serve as an effective tool for species identification when specificity is validated by performing supplementary melt curve analysis [2, 15].

In this study, for the purposes of marker development, we selected conserved chloroplast genes to enable stable and sensitive species-specific detection [1, 19]. To enhance primer specificity, we developed SYBR Green-based molecular markers using primers targeting single-nucleotide polymorphism (SNP) and insertion/deletion (INDEL) regions and evaluated their applicability for the identification of *A. princeps* and *C. speciosa*. The specificity and limits of detection of the primers thus developed were confirmed. A standard curve was generated based on binary mixture analysis, and detection was determined using a Cq cutoff value of 0.1%. The reliability and applicability of the assay were further evaluated by performing blind tests and analyses of commercially available products. In addition, the potential for forensic application was evaluated by assessing the detectability of DNA in samples treated with artificial gastric juice [11, 20]. On the basis of this work, we succeeded in developing a reliable molecular tool for morphologically similar, thereby contributing to consumer safety and rights protection.

Materials and methods

Sample preparation and extraction of genomic DNA

Samples of *A. princeps* and *C. speciosa* were provided by the National Institute of Biological Resources (Incheon, Korea), and commercial products were purchased from a local market. All samples were initially ground in liquid nitrogen using a pestle and mortar and stored at -70 °C until further use. Extraction of the genomic DNA (gDNA) of samples used in all experiments was performed using a DNeasy Plant Pro Kit (Qiagen, Hilden, Germany). The purity and concentration of the extracted DNA were determined using a SPECTROStar Nano Leader (BMG Labtech, Ortenberg, Germany), which indicated purity ratios of 1.7 to 2.0. To verify the efficiency of amplification, the extracted gDNA was evaluated using 18 S rRNA as a positive control.

Sequence analysis and primer design

The sequences of reference chloroplast genomes for *A. princeps* (accession number NC_071927) and *C. speciosa* (accession number NC_072193) were obtained from the National Center for Biotechnology Information (NCBI) database. The reference sequences were aligned using ClustalW2 (EMBL-EBI, Hinxton, Cambridgeshire, UK) and then compared with sequences of the samples of *A. princeps*, *C. speciosa* and other plant species used for the specificity test. All species-specific primer pairs were designed based on SNPs and INDELs between the two species using BioEdit v.7.2 (Ibis Biosciences, Carlsbad, CA, USA) and Beacon DesignerTM (PRIMER Biosoft, Palo Alto, CA, USA) and were commercially synthesized by Macrogen (Seoul, Korea). The sequences of the chloroplast gene regions selected for species-specific primer design were confirmed by cloning. Conventional PCR was performed using a VeritiPro Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA), with DNA mixtures prepared using TaKaRa Ex Taq DNA Polymerase (TaKaRa, Kusatsu, Shiga, Japan). The PCR cycling protocol was as follows: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 10 s, 53.5–59.7 °C for 30 s (depending on the annealing temperature of the primers), and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were cloned into RBC T&A cloning vectors (Real Biotech Corporation, Taipei, Taiwan). Plasmid DNA was extracted using a DokDo-Prep Plasmid Mini Kit (ELPISB), with subsequent sequencing being performed by Macrogen.

Quantitative real-time PCR assay

qPCR was performed using AccuPowerR 2× GreenStar qPCR Master Mix (Bioneer, Daejeon, Korea). The

reaction mixtures comprised 10 µL of 2× GreenStar Master Mix, 0.5 µL of each primer (10 pmol), 1 µL of 10 ng μL^{-1} gDNA, and 0.25 µL of ROX Dye, made up to a final volume of 20 µL with distilled water. qPCR reactions were performed using a Quantstudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: pre-denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 59–60.5 °C for 30 s (depending on the annealing temperature of each primer), and 72 °C for 30 s. All qPCRs were performed in triplicate.

Construction of qPCR standard curves

The extracted gDNAs were diluted to 10 ng, from which we prepared standard curves by obtaining 10-fold serial dilutions down to 0.001 ng. Correlations between Cq values and diluted the DNA were estimated using the default parameters. The standard curves were determined as $y = -ax + b$, where "a" is the slope of the curve and "b" is the y-intercept. The amplification efficiency was calculated as $E\ (\%) = (10^{-1/a} - 1) \times 100$.

Specificity test

To assess the cross-reactivity of the developed primer pairs, we performed a specificity test using 12 non-target plant species, *A. princeps*, and *C. speciosa*, among which there were samples from eight species (including *A. princeps*) in the family Asteraceae and three species (including *C. speciosa*) in the family Fumariaceae, along with *Allium monanthum* and *Capsella bursa-pastoris*.

Binary test

To evaluate the applicability of the developed primer pairs, Binary tests were performed using mixtures of *A. princeps* and *C. speciosa*. Leaf samples were dried in an oven at 65 °C for 48 h and then finely ground using a blender. Binary mixture samples were prepared using 200 mg of the target species sample with 0.1% (0.2 mg), 1% (2 mg), 10% (20 mg), and 100% (200 mg) of the non-target species.

Blind mixture samples

Twenty-seven blind samples were provided by an independent laboratory (Ministry of Food and Drug Safety, Cheongju, Republic of Korea). These samples were randomly mixed with powdered *A. princeps* and *C. speciosa* to adjust the final sample weight to 200 mg. In addition, to assess the detectability of the toxic plant *C. speciosa*, 200 mg blind samples were prepared containing 0% to 20% *C. speciosa* and 80% to 100% *A. princeps*.

Preparation of artificial gastric juice

Artificial gastric juice was prepared by dissolving 2.0 g of NaCl and 3.2 g of porcine pepsin in 7.0 mL of HCl, with the final volume being made up to 1 L using distilled water [20]. The pH of the solution was maintained at 1.2. To simulate peristaltic movement, 50 mg of ground leaf tissue was incubated for 1, 2, 4, and 6 h with 50 mL of the artificial gastric solution at 37 °C in a shaking incubator set at 50 rpm. The collected samples were neutralized with KOH and washed three times with distilled water to remove residual acidity. This step was performed to prevent the negative effects of low pH on DNA extraction. The extracted DNA was subsequently purified using a Wizard® DNA Clean-Up System (Promega, Madison, WI, USA) to obtain high-purity gDNA.

Phylogenetic analysis based on ITS sequences

To determine the degrees of sequence similarity among the species used in the specificity test, we retrieved internal transcribed spacer (ITS) sequences for four *Artemisia* species (*A. gmelinii*, *A. iwayomogi*, *A. capillaris*, and *A. annua*) and *C. speciosa* from the NCBI GenBank database. Multiple sequence alignments were performed using MAFFT, and phylogenetic trees were generated using IQ-TREE2 and FastTree with 1,000 bootstrap replicates [13, 22]. All analyses were conducted in a Linux environment, and the resulting trees were visualized using FigTree.

Statistical analysis

Statistical analysis was conducted for the results obtained in the artificial gastric digestion experiment, which are expressed as the mean ± standard deviation (SD) of three independent biological replicates ($n = 3$); each replicate represents an independently prepared sample that underwent separate artificial gastric juice digestion and DNA extraction. One-way analysis of variance (ANOVA) was performed to compare Cq values at different incubation times (0, 1, 2, 4, and 6 h). For cases in which the overall ANOVA values indicated significance, Dunnett's post hoc test was applied to perform multiple comparisons between values obtained at each incubation time point and the 0 h control value, with adjustment for multiple testing. Values for basic descriptive statistics were calculated using Microsoft Excel 2018 (Microsoft), and post hoc analyses were performed in R. Statistical significance was set at the $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ levels. ns signifies non-significance.

Results and discussion

Species-specific primer design

Using BLAST, nucleotide sequences downloaded from the NCBI database were aligned to search for candidate gene regions containing either SNPs or INDELs. Given their high levels of conservation in species, we selected regions of chloroplast genes for the design of species-specific primers. To ensure primer specificity, we initially

aligned the chloroplast sequences of *A. princeps* and the closely related *Artemisia* species (*A. annua*, *A. capillaris*, and *A. iwayomogi*) (Supplementary Fig. 1). Owing to the high degree of sequence similarity, some candidate gene regions were deemed unsuitable for the design of species-specific primers. Consistent with this, ITS-based phylogenetic analysis revealed a dense clustering, short branch lengths, and low bootstrap support among *Artemisia* species (Supplementary Fig. 2). Among the other assessed regions, *matK*, *ndhF*, and *psaA* showed sufficient variation to differentiate *A. princeps* from non-target species. For *C. speciosa*, the gene regions *atpA*, *rpoC2*, and *ycf2* were selected based on their sequence divergence from other non-target species, including other members of the Fumariaceae family. On the basis of these findings, we thus selected the six chloroplast genes *atpA*, *matK*, *rpoC2*, *ycf2*, *psaA*, and *ndhF* for the design of species-specific primers to discriminate between *A. princeps* and *C. speciosa* (Table 1 and Supplementary Fig. 1). In addition, to account for potential DNA fragmentation during food processing (e.g., heating), the primers were designed to generate short amplicons ranging from 83 to 173 bp. All six of these target genes were cloned and sequenced to verify the correspondence between the NCBI reference sequences and those from the authenticated reference samples. In all cases, these sequence comparisons confirmed 100% identity, and the sequences thus validated were used in subsequent experiments.

Evaluation of the sensitivity, reproducibility, and efficiency of the designed primer pairs

To verify the sensitivity of the primer pairs developed for the genes *AP_matK*, *AP_ndhF*, and *AP_psaA and CS_atpA*, *CS_rpoC2*, and *CS_ycf2*, we used 10-fold serial dilutions of DNA samples (10–0.001 ng μL^{-1}) and accordingly established that all primer pairs were effective in detecting DNA concentrations as low as 0.001 ng. The correlation coefficient (R^2) values obtained for the six primer pairs exceeded 0.99 with slopes of the standard curves ranging from 3.11 to 3.27, and amplification efficiencies ranging from 102.35% to 109.83% (Fig. 1). Among the assessed NTC samples, we observed no amplification (no Cq values within 40 cycles) using any of the selected primer pairs. For all standard curve concentrations, melt-curve analysis revealed a single peak at the expected melting temperature (Tm) for each primer pair, with no additional peaks indicative of primer–dimer formation or non-specific amplification (Supplementary Fig. 3).These findings thus indicated the high sensitivity and specificity of the qPCR assay. In addition, to confirm reproducibility, the six primer pairs were evaluated in two independent laboratories using different qPCR instruments (Supplementary Table 1). The results showed slopes ranging from – 3.36 to -3.56, R^2 values from 0.99 to 1.00, and amplification efficiencies of 90.6% to 98.4%. All values were within the acceptance criteria of the European Network of GMO Laboratories (ENGL), thus confirming that the primer pairs satisfied the requisite amplification efficiency and reproducibility standards (ENGL [7]), .

Validation of the qPCR assay for species-specific detection in commercial products

According to the ENGL guidelines, levels of impurities in commercial products of less than 0.1% are not considered indicative of intentional mixing (ENGL [7]), . Consequently, to assess the presence of the target species in commercial samples, the cutoff Cq value was established based on the Cq corresponding to 0.1% of the target DNA in binary mixtures, as determined from qPCR analysis. Given that in commercial markets, *A. princeps* is commonly sold in powdered form and is typically produced via heat-drying, it is assumed that DNA fragmentation occurs during food processing (Chen et al., 5). Thus, to

Table 1 Information on primer sets developed in this study and universal plant primers

Species	Target gene	Primer	Length(bp)	Sequence (5′ → 3′)	Amplicon size (bp)	Tm(°C)	Reference
Artemisia princeps	*matK*	*matK_F*	20	ATATGAATCTGGCTTCCTCT	120	60	This study
		matK_R	18	GACCTGACAAAGACATGC			
	ndhF	*ndhF_F*	22	CTTGATTTCTAATAATGAGGTC	157	59	
		ndhF_R	18	AGCATGTATAAGAGCTGA			
	psaA	*psaA_F*	20	TCCGAGGAATACAAATAACC	83	59	
		psaA_R	18	GGTACAATAGAGCTGCAA			
Corydalis speciosa	*atpA*	*atpA_F*	20	ATACAAGAAGGAAGTTCGGT	159	60.5	
		atpA_R	18	AGGAGCGGGAGATTCTAT			
	rpoC2	*rpoC2_F*	19	TTCTCAGTGACTACTGGTC	173	59.5	
		rpoC2_R	20	CTCTTTGCCACTAAATCTGA			
	ycf2	*ycf2_F*	19	GGATCGTGAAAAAGAGTGA	102	59.5	
		ycf2_R	22	GAAACTCCAGATATTGGATCTC			
All plants	*18s rRNA*	*18s rRNA_F*	25	TCTGCCCTATCAACTTTCGATGGTA	137	58	Zhang et al. (29)
		18s rRNA_R	25	AATTTGCGCGCCTGCTGCCTTCCTT			

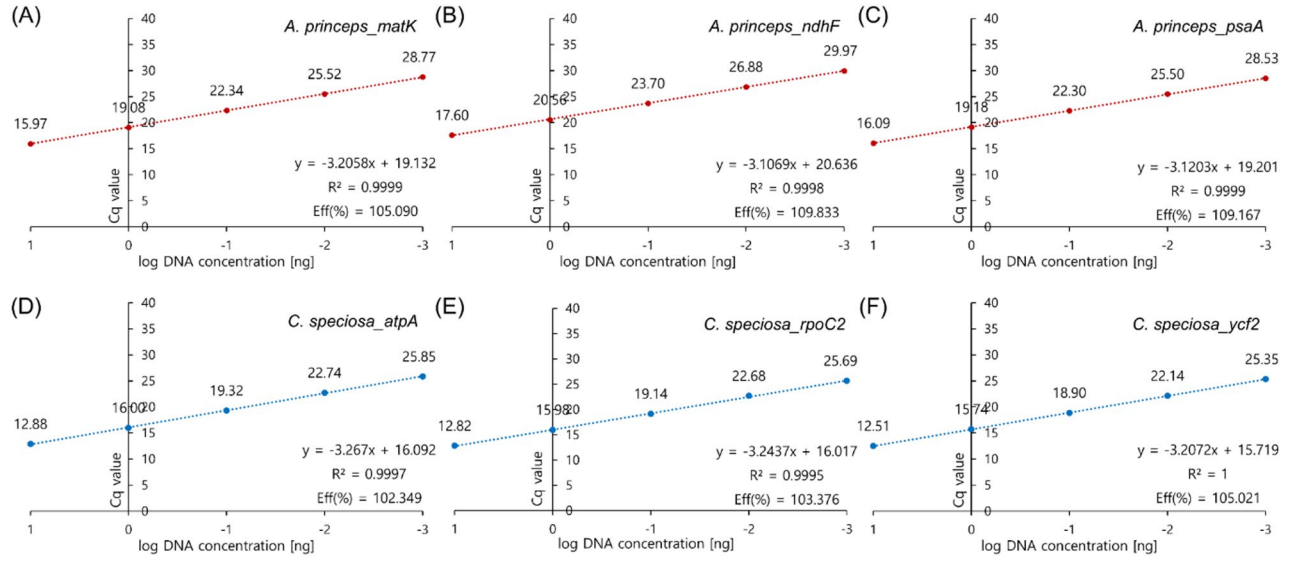

Fig. 1 Standard curves obtained using the developed primer pairs targeting *Artemisia princeps* and *Corydalis speciosa*. Curves were generated based on amplification efficiency and correlation coefficients (R^2) using 10-fold serial dilutions of genomic DNA from 10 to 0.001 ng. The x-axis represents log-transformed DNA concentrations (ng), and the y-axis shows the mean ± SD of Cq values. (**A-C**). Primer pairs targeting *A. princeps* (*AP_matK*, *AP_ndhF*, and *AP_psaA*); (**D-F**) Primer pairs targeting *C. speciosa* (*CS_atpA*, *CS_rpoC2*, and *CS_ycf2*). qPCR for each primer pair was performed in triplicate ($n = 3$)

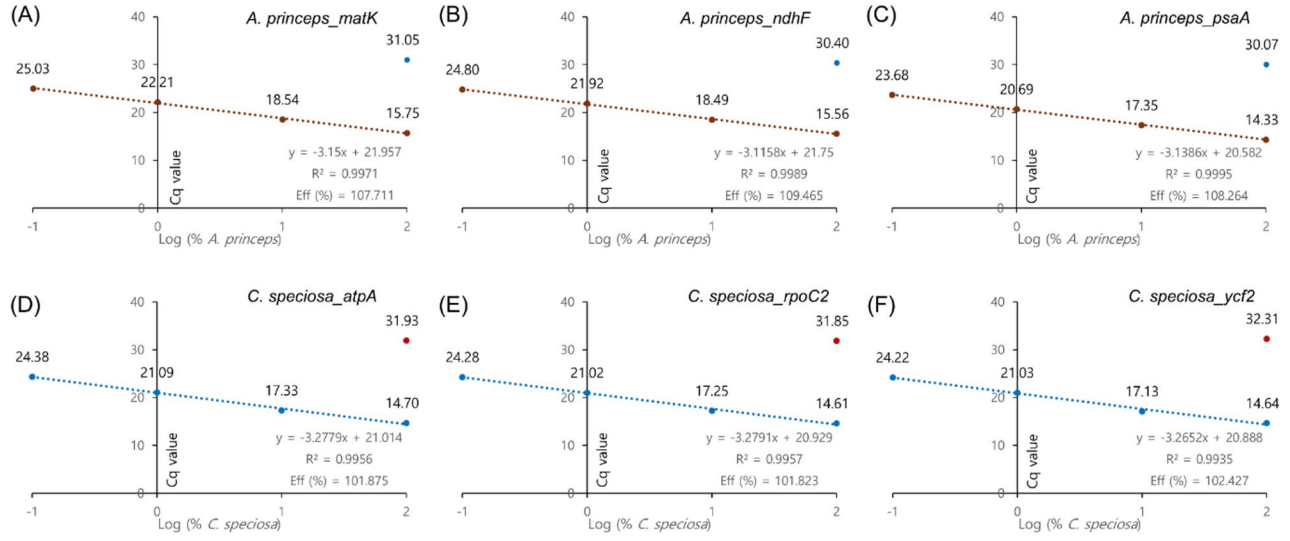

Fig. 2 Standard curves of Cq values generated from binary mixtures of *Artemisia princeps* and *Corydalis speciosa*, based on amplification efficiency and correlation coefficients (R^2). These curves are plotted against the log-transformed concentration of the target species (100%, 10%, 1%, and 0.1%). The x-axis represents the log percentage of the target species and the y-axis represents the mean ± SD of the Cq values. (**A-C**) The left-most dots represent the Cq values of the 0.1% binary mixtures amplified using the *A. princeps*-specific primer pairs. (**D-F**) The left-most dots represent the Cq values of the 0.1% binary mixtures amplified using the *C. speciosa* specific primer pairs. qPCR for each primer pair was performed in triplicate ($n = 3$)

verify whether the developed primer pairs can be used detect target species subsequent to heat treatment, we conducted a binary test using heat-treated samples, which were mixed with non-target samples in defined ratios (100:0; 10:90; 1:99; 0.1:99.9) to prepare binary mixtures. Following DNA extraction, qPCR was performed to evaluate detectability, which accordingly revealed that all the assessed primer pairs had amplification efficiencies within the acceptable range (101.82%–109.47%) (Fig. 2). Thus, each of the target species was successfully detected in binary mixtures, with the cutoff Cq value for each primer being defined based on the Cq value corresponding to the 0.1% mixture. In addition, on the basis of the established cutoff Cq values, we sought to determine the presence of the target species in commercial products. DNAs extracted from the samples of 12 *A. princeps* commercial products (fresh leaf, dried, powder, pill, rice cake, and tea), along with the fresh leaves and seeds of *C.*

speciosa were initially subjected to qPCR, and the DNA qualities of PCR products were assessed using 18 S rRNA as a positive control. Notably, the tea and rice cake samples were found to have relatively higher Cq values for 18 S rRNA, which we speculate could be attributable to the lower content of plant material in these products. All *A. princeps* samples yielded Cq values below the cutoff for the *A. princeps*-specific primers, whereas the values for non-target samples exceeded this threshold (Table 2). Similarly, *C. speciosa* samples showed amplification below the cutoff for the *C. speciosa* primers, whereas all non-target samples remaining undetected. These results confirmed that each of the assessed commercial products contained only the labeled target species, with no evidence to indicate cross-contamination. Consequently, on the basis of these findings, we were able to establish the applicability of the assessed primer pairs for detecting the presence or absence of target DNA in a range of products prepared using different processing techniques when present at different concentrations.

Suitability of the qPCR assay for blinded target species detection

To evaluate the suitability of the developed primers for determining the presence or absence of target species, we prepared blind mixtures containing 0% to 100% of the toxic plant *C. speciosa* and 0% to 100% of *A. princeps*. In addition, an independent group provided a further 25 randomly prepared blinded samples, for which details of the mixing ratios were not disclosed to the researchers. The extracted DNA was analyzed using the six species-specific primer pairs, and the presence (≥ 0.1%) or absence (< 0.1%) of target DNA was determined based on the cutoff Cq value for each primer pair. The quality of DNA in all samples was confirmed using the 18 S rRNA primers, yielding Cq values of 14.55–16.72. For samples 1, 2, 3, 8, and 25, Cq values exceeded the designated cutoff, thus indicating the absence of *C. speciosa*, whereas the remaining samples tested positive. In contrast, sample 26 showed the presence of *C. speciosa* and the absence of *A. princeps* (Table 3). These results were fully consistent with the mixing ratio data provided by the independent research team. Notably, consistent results were obtained using all the assessed primer pairs, thereby confirming their suitability for detecting *A. princeps* and *C. speciosa*.

Specificity of the developed qPCR assay

The specificity of the primers was evaluated based on qPCR analysis using DNA obtained from 15 plant species, among which there were nine species from the Asteraceae family (including *A. princeps*), four from the Fumariaceae family (including *C. speciosa*), and single species from the families Liliaceae and Brassicaceae. The quality of the DNA extracted from all samples was verified using the 18 S rRNA gene as a positive control. In specificity assessments, for each primer pair, Cq values exceeding the cutoff threshold were obtained for all non-target species, indicating that the assay primers can be used to specifically detect the intended target species (Fig. 3A and Supplementary Table 2).

Assessment of qPCR assay performance using simulated gastric contents

As cases of acute toxic hepatitis have been reported following the ingestion of *C. speciosa*, we prepared samples of artificial gastric juice to evaluate the potential applicability of the developed qPCR assay for forensic analysis. To simulate gastric digestion, samples of *C. speciosa*

Table 2 Validation of the developed primer sets in commercial products

Species	No.	Labled as (%)	Sample Type	All plants 18s rRNA	A. princeps primer			C. speciosa primer		
					matK	*ndhF*	*psaA*	*atpA*	*rpoC2*	*ycf2*
					23.68[a]	**25.03**	**24.8**	**24.38**	**24.28**	**24.22**
Artemisia princeps	1	*A. princeps*, 100	fresh leaf	19.3 ± 0.1	16.3 ± 0.1	18.2 ± 0.1	17.4 ± 0.1	33.2 ± 0.4	32.9 ± 0.1	33.8 ± 1
	2	*A. princeps*, 100	fresh leaf	19.8 ± 0.4	16.2 ± 0.4	17.9 ± 0.1	17.2 ± 0.2	32.5 ± 0.5	31.2 ± 0.2	34.8 ± 0.7
	3	*A. princeps*, 100	fresh leaf	18.4 ± 0.2	16.2 ± 0.1	17.2 ± 0	16.5 ± 0.1	33.1 ± 0.2	32.5 ± 0.7	34.5 ± 1.2
	4	*A. princeps*, 100	fresh leaf	18.2 ± 0.2	16.3 ± 0.1	16.2 ± 0.1	15.6 ± 0.1	35.6 ± 0	33.1 ± 0	35.5 ± 0
	5	*A. princeps*, 100	fresh leaf	17.2 ± 0.1	16.9 ± 0	16.7 ± 0	16.2 ± 0.1	32.5 ± 0	35.2 ± 0	33.7 ± 0
	6	*A. princeps*, 100	dry	18.3 ± 0.1	21.2 ± 0	23.2 ± 0.1	20.6 ± 0.1	32.8 ± 0.3	32.9 ± 0.2	33.7 ± 0.8
	7	*A. princeps*, 100	dry	16.2 ± 0.1	20.9 ± 0.1	22.7 ± 0.1	20.4 ± 0.1	31.6 ± 0.3	31.7 ± 0.3	35 ± 0.5
	8	*A. princeps*, 100	powder	16 ± 0.1	18.2 ± 0	18.9 ± 0	17.5 ± 0.1	32.7 ± 0.2	32.3 ± 0.6	35.2 ± 0.2
	9	*A. princeps*, 100	powder	19.1 ± 0.3	20.8 ± 0.1	21.2 ± 0.1	20.6 ± 0.1	32.9 ± 0.2	33 ± 0.7	34.5 ± 1.4
	10	*A. princeps*, 90	pill	17.8 ± 0.5	21 ± 0	22.9 ± 0.2	19.9 ± 0.1	33 ± 0.2	32.8 ± 0.4	34.5 ± 1.1
	11	*A. princeps*, 33	rice cake	22.7 ± 0.4	22.8 ± 0.1	24.3 ± 0.1	20.9 ± 0.2	32.2 ± 0.6	33 ± 0.3	34.3 ± 1
	12	*A. princeps*, 10	tea	25.3 ± 0.8	21.6 ± 0.1	23.1 ± 0.1	20.6 ± 0.1	33 ± 0.1	32.5 ± 0.4	33.7 ± 1.1
Corydalis speciosa	13	*C. speciosa*, 100	seed	14 ± 0.2	33.2 ± 0	37.9 ± 0	32.7 ± 1	15 ± 0	14.3 ± 0.1	13.8 ± 0.1
	14	*C. speciosa*, 100	fresh leaf	19.1 ± 0.1	35.6 ± 0	36.1 ± 1	33.9 ± 0.7	12.9 ± 0	12.3 ± 0	11.8 ± 0.6

[a]The cutoff cycle of developed marker pairs obtained through binary testing (Cq values of 0.1% binary mixture)

Table 3 Evaluation of Cq values from blind samples using the developed primer sets

No.	Positive control	Ct value							
		Artemisia princeps				*Corydalis speciosa*			
	18S rRNA	*matK*	*ndhF*	*psaA*	Presence(n/3)[b]	*atpA*	*rpoC2*	*ycf2*	Presence(n/3)
		23.68[a]	**25.03**	**24.8**		**24.38**	**24.28**	**24.22**	
1	16.28 ± 0.1	16.08 ± 0.1	17.99 ± 0.1	16.33 ± 0.1	3/3	28.78 ± 0.2	28.91 ± 0	28.82 ± 0.2	0/3
2	15.88 ± 0.1	15.83 ± 0.1	17.31 ± 0.1	15.87 ± 0	3/3	34.09 ± 0.7	33.64 ± 0.8	36.08 ± 1.2	0/3
3	15.88 ± 0.1	15.88 ± 0.1	17.08 ± 0.1	15.97 ± 0	3/3	33.97 ± 0.2	33.35 ± 0.4	34.12 ± 1.3	0/3
4	16.24 ± 0.1	16.25 ± 0	17.72 ± 0.1	16.36 ± 0	3/3	17.07 ± 0	17.16 ± 0.1	17.12 ± 0	3/3
5	15.98 ± 0.1	15.93 ± 0.1	17.25 ± 0.1	16.02 ± 0.1	3/3	17.58 ± 0	17.87 ± 0.1	17.7 ± 0	3/3
6	15.92 ± 0.1	15.76 ± 0.1	17.05 ± 0.1	15.99 ± 0	3/3	18.93 ± 0.1	19.12 ± 0.1	19.15 ± 0	3/3
7	15.99 ± 0.1	15.97 ± 0	17.26 ± 0.1	16.02 ± 0	3/3	18.31 ± 0	18.74 ± 0.1	18.42 ± 0	3/3
8	15.46 ± 0.1	15.17 ± 0	17.04 ± 0.1	15.5 ± 0	3/3	33.28 ± 0.7	32.6 ± 0.8	33.93 ± 0.7	0/3
9	15.83 ± 0	15.75 ± 0.1	17.43 ± 0.1	15.95 ± 0	3/3	17.05 ± 0.1	17.19 ± 0.1	17.13 ± 0	3/3
10	15.31 ± 0.1	15.24 ± 0	16.79 ± 0.1	15.38 ± 0.4	3/3	19.9 ± 0.1	20.17 ± 0.1	19.91 ± 0.1	3/3
11	16.72 ± 0.2	16.63 ± 0.1	16.6 ± 0.1	17.25 ± 0.1	3/3	18.29 ± 0.1	18.45 ± 0.1	18.29 ± 0	3/3
12	14.55 ± 0.1	14.33 ± 0.5	14.41 ± 0.2	15.1 ± 0.1	3/3	19.98 ± 0	20.08 ± 0.1	19.88 ± 0	3/3
13	16.41 ± 0.1	16.25 ± 0.1	16.49 ± 0.1	17.14 ± 0	3/3	22.17 ± 0.2	22.67 ± 0.1	22.48 ± 0.4	3/3
14	16.26 ± 0.1	16.24 ± 0.1	16.37 ± 0.2	16.96 ± 0	3/3	18.42 ± 0.3	18.69 ± 0.1	18.44 ± 0.1	3/3
15	16.29 ± 0.1	16.37 ± 0.1	16.38 ± 0.1	17.18 ± 0	3/3	20.88 ± 0.1	20.97 ± 0	20.76 ± 0.1	3/3
16	16.45 ± 0.1	16.14 ± 0.1	16.37 ± 0.2	17.01 ± 0.1	3/3	20.77 ± 0.2	21.14 ± 0.2	20.89 ± 0.2	3/3
17	16.38 ± 0.2	16.13 ± 0.1	16.25 ± 0.1	17.05 ± 0	3/3	19.34 ± 0.1	19.63 ± 0.1	19.12 ± 0	3/3
18	16 ± 0	15.91 ± 0.1	16.05 ± 0.1	16.74 ± 0.1	3/3	17.94 ± 0	18.11 ± 0.1	17.9 ± 0	3/3
19	16.11 ± 0.1	16.01 ± 0.1	16.16 ± 0.1	16.84 ± 0.1	3/3	18.17 ± 0	18.27 ± 0	18.01 ± 0	3/3
20	16.21 ± 0	16.19 ± 0	16.16 ± 0.1	16.84 ± 0.1	3/3	17.57 ± 0	17.81 ± 0	17.37 ± 0.1	3/3
21	15.46 ± 0.1	15.88 ± 0	17.26 ± 0.2	15.9 ± 0	3/3	16.16 ± 0	16.19 ± 0	16.19 ± 0.1	3/3
22	14.92 ± 0.2	14.89 ± 0.2	16.32 ± 0.1	15.08 ± 0.1	3/3	20.63 ± 0	20.81 ± 0.1	20.31 ± 0.1	3/3
23	14.92 ± 0.1	15 ± 0	16.45 ± 0.2	15.22 ± 0.1	3/3	15.27 ± 0.1	15.35 ± 0.1	15.21 ± 0.1	3/3
24	14.55 ± 0.1	14.82 ± 0.1	16.22 ± 0.1	15.01 ± 0.2	3/3	17.87 ± 0	17.93 ± 0	17.68 ± 0.1	3/3
25	14.58 ± 0.1	14.55 ± 0	16.04 ± 0.1	14.73 ± 0	3/3	29.51 ± 0.2	26.14 ± 0	28.84 ± 0.2	0/3
26	15.82 ± 0.1	35.23 ± 0.7	34.88 ± 0.6	34.33 ± 0.1	0/3	12.49 ± 0	12.83 ± 0.1	12.25 ± 0.2	3/3
27	15.44 ± 0	18.62 ± 0.2	21.23 ± 0.1	17.97 ± 0	3/3	12.13 ± 0.2	12.73 ± 0.2	12.29 ± 0.1	3/3

[a]The cutoff cycle of developed marker pairs obtained through binary testing (Cq values of 0.1% binary mixture)

[b]Results of presence or absence of content based on cutoff Cq value. "n/3" indicates how many of the three target primer sets detected the value lower than the cutoff Cq value

were incubated with artificial gastric fluid for 0, 1, 2, 4, and 6 h. Following incubation for the designated periods and subsequent neutralization of the treated sample pH, DNA was extracted and analyzed using the qPCR assay. All primer pairs developed for *C. speciosa* successfully detected the target DNAs for up to at least 2 h after the initiation of treatment based on cutoff Cq values, and among these targets, *ycf2* remained detectable for up to 4 h (Fig. 3B). Specifically, all markers showed a significant increase in Cq values from 2 h onward, consistent with progressive DNA degradation under artificial gastric conditions. *CS_atpA* and *CS_rpoC2* exceeded their respective cutoff Cq values at 4 h, whereas *CS_ycf2* increased more gradually and remained below the cutoff Cq value up to 4 h. These findings confirm that DNA fragmentation occurs during digestion and indicate the temporal sensitivity of the developed primers. From a forensic perspective, given that digestible solids generally remain within the stomach for approximately 2 to 3 h, these results provide evidence for the potential utility of the developed primers for detecting partially degraded plant DNA using the qPCR assay [9]. For the purposes of the present study, *C. speciosa* samples incubated in artificial gastric fluid were initially neutralized using KOH prior to DNA extraction, as low pH can adversely influence the efficiency of DNA recovery. However, for real-world forensic cases, additional processing steps such as pH neutralization are generally avoided in order to preserve the integrity and evidentiary value of the original sample. Consequently, further studies will be needed to evaluate the feasibility of DNA detection without pH adjustment under actual forensic conditions.

In conclusion, in this study, we developed species-specific primer pairs for the detection of *Artemisia princeps* and *Corydalis speciosa* and evaluated their performance in qPCR assays. The SYBR Green-based real-time PCR assay was established to provide a cost-effective method for species detection. In addition, the developed markers

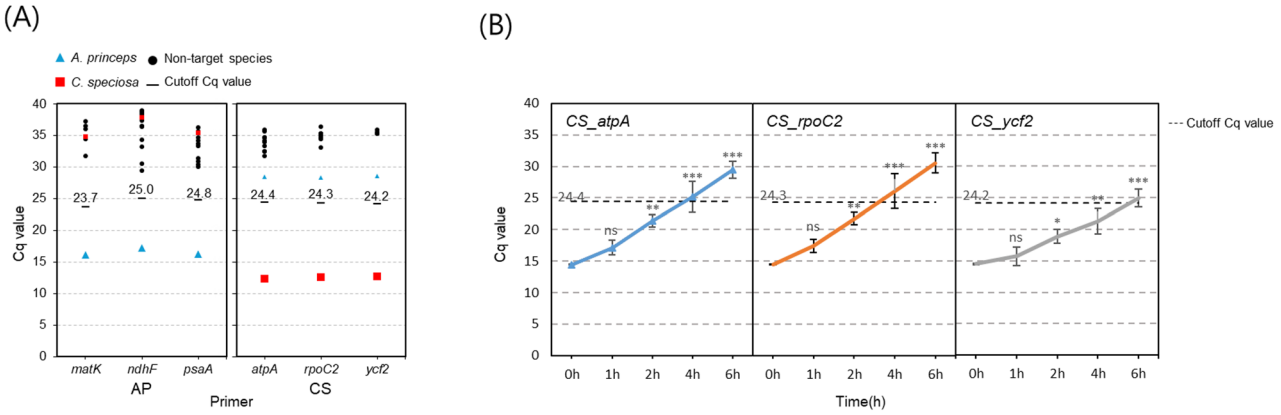

Fig. 3 Specificity of the developed primers and assessment of detection performance following artificial gastric juice treatment. (**A**) Cq values obtained from specificity testing using the qPCR assay. The x-axis shows the targets of the primer pairs (*AP_matK*, *AP_ndhF*, *AP_psaA*, *CS_atpA*, *CS_rpoC2*, and *CS_ycf2*), and the y-axis represents the mean ± SD of Cq values. The blue dots indicate the Cq values of *Artemisia princeps* and the red dots indicate the Cq values of *Corydalis speciosa*. The horizontal line represents the cutoff Cq value for each developed primer pair. qPCR for each primer pair was performed using three technical replicates ($n = 3$). (**B**) Cq values of the three *C. speciosa*-specific primer pairs after treatment with artificial gastric juice for 0, 1, 2, 4, and 6 h. The dashed line indicates the primer-specific cutoff Cq value for each primer pair. qPCR for each primer pair was performed using three independent biological replicates ($n = 3$), each of which was prepared via separate artificial gastric juice digestion and DNA extraction. The asterisks indicate a significant difference based on one-way ANOVA followed by Dunnett's post hoc test (vs. 0 h); ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

were found to show high sensitivity, specificity, and reproducibility. These findings indicate that these markers could serve as robust and practical tools for accurate species identification, and we believe that their application would make a valuable contribution to reducing the risk of unintended contamination, thereby enhancing consumer safety and protecting consumer rights.

Supplementary Information

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Supplementary Material 1.

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Conceptualization, U.C. Park and C.S. Jang; Methodology, U.C. Park; Data curation, U.C. Park; Writing – original draft, U.C. Park; Writing – review & editing, C.S. Jang; Supervision, C.S. Jang; Project administration, C.S. Jang; All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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