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Development of a SYBR green-based real-time PCR assay for distinguishing the edible plant *Allium microdictyon* from the toxic plant *Convallaria majalis* and its application

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Abstract

Consumption of wild plants is a common practice globally; however, not all plants are safe for human consumption, as some are toxic. Toxic plants often resemble edible species, which makes their identification difficult, particularly for non-experts. *Convallaria majalis*, a poisonous plant containing cardiac glycosides, poses a significant danger if mistakenly ingested. *Allium microdictyon* is a popular edible wild vegetable in East Asia. Owing to their similar appearance, accidental mixing can occur during harvesting or processing. In this study, specific primer pairs were designed to target chloroplast genes to distinguish the edible plant *Allium microdictyon* from the toxic plant *Convallaria majalis*, and their practicality was tested. The specificity, sensitivity, and applicability of the quantitative real-time PCR assay were evaluated using all primer pairs. Six primer sets (three for *A. microdictyon* and three for *C. majalis*) exhibited strong linearity, with correlation coefficients exceeding 0.98 and slopes ranging from −3.22 to −3.56. PCR efficiencies ranged from 90.80% to 97.85%. Cycle threshold (Ct) values corresponding to 0.1% of the binary mixture were used as the cut-off values. Additionally, the specificity of the primer pairs was validated by analyzing 13 non-target plant species and assessed for practicality using 15 commercial samples. The developed primer pairs will aid in preventing the misidentification of toxic wild plants and serve to guide the identification of toxic species, thereby contributing to public health and safety.

Keywords *Allium microdictyon*, *Convallaria majalis*, DNA marker, SYBR-GREEN real-time PCR, Toxic plant

Introduction

The consumption of wild plants, either raw or cooked, is a common dietary practice in many countries, owing to their nutritional value and cultural significance [1]. However, not all wild plants are safe for human consumption because some are toxic and pose serious health risks

[2, 3]. Many toxic plants share morphological similarities with edible plants, making it difficult to distinguish between them [4, 5]. This similarity often leads to accidental ingestion of toxic plants, resulting in natural poisoning or even fatalities. According to the Consumer Injury Surveillance System, 26 cases of poisoning resulting from the misidentification of toxic plants as wild vegetables were reported in Korea between 2020 and 2023 [27]. The consumption of these toxic plants leads to symptoms such as stomach pain and poisoning. These incidents primarily occurred in spring between March and June. For example, *Ambrosia artemisiifolia* is an

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invasive toxic plant in Korea that disrupts ecosystems, whereas *A. annua* is widely used for medicinal and culinary purposes. Owing to their striking morphological similarities, the confusion between wild toxic and edible plants continues to cause poisoning [6].

Allium microdictyon (mountain garlic) is a perennial plant belonging to the Liliaceae family and is a wild vegetable with a strong garlic-like scent. It grows naturally in the mountainous regions of Asia, North America, and the Himalayas. The leaves of *A. microdictyon* are widely consumed in Siberia and Asia and are utilized in traditional Korean, Chinese, and Mongolian cuisines. *A. microdictyon* has long been consumed as a vegetable or wild edible plant, and is gaining recognition as a functional food because of its pharmacological properties, including neuroprotective, antitumor, anti-inflammatory, and skin barrier-enhancing effects [22, 7]. In addition, *A. microdictyon* is a rich source of biologically active compounds such as flavonoids, organic molecules, and saponins [8].

Convallaria majalis (lily of the valley), a species in the Liliaceae family, is a perennial plant that thrives in cool climates across North America, as well as in specific regions of Europe and Asia. Its bell-shaped flowers are valued for their aesthetic appeal and are commonly cultivated as ornamental plants [9]. However, the entire plant is toxic and contains saponins and cardiac glycosides that induce digestive disturbances and render it unsuitable for human consumption. Convallatoxin is the primary toxic cardiac glycoside present in valley lilies [10]. Convallatoxin is a digitalis-like compound (DLC) that induces arrhythmia, anorexia, nausea, vomiting, diarrhea, abdominal pain, visual impairments, headache, weakness, and dizziness [11]. Accidental poisoning has been documented in humans and animals. A woman who consumed lilies from the valley experienced digestive disturbances resembling digitalis toxicity and required hospitalization, whereas a dog exhibited bradycardia, vomiting, and lethargy after ingesting *C. majalis* containing cardiac glycosides [12, 23].

The similar appearance of the toxic *C. majalis* and edible *A. microdictyon* may have led to accidental poisoning owing to confusion between the two species. However, no analytical method has been established to differentiate between these two species. Quantitative real-time PCR demonstrates high sensitivity and specificity, making it ideal for the precise detection of adulteration in commercially available processed foods [13]. Quantitative real-time PCR analysis is typically categorized into probe-based TaqMan and fluorescent dye-based SYBR Green assays [14]. A probe-based quantitative real-time PCR method, such as TaqMan, offers high specificity in detecting target sequences, as the probes are designed to hybridize exclusively with the target DNA, reducing nonspecific amplification and improving accuracy;

however, optimization of the TaqMan method is more complex than primer and probe design for the SYBR Green method owing to the greater number of single nucleotide polymorphisms (SNPs) and insertion-deletion (InDels) required for precise probe development [15, 24]. The SYBR Green Assay offers a more affordable and convenient alternative to probe-based quantitative real-time PCR because it does not require a sequence-specific probe. Instead, it uses a DNA-binding dye that fluoresces upon binding to double-stranded DNA, enabling the real-time detection of target amplification [16].

In the present study, we developed a SYBR Green-based qPCR to distinguish edible *A. microdictyon* from toxic *C. majalis*. The specificity and sensitivity of each primer set leveraging the SNPs found in the chloroplast genes of the two species were evaluated. The applicability of this method was confirmed using commercial products and was further validated under simulated gastrointestinal conditions. The developed primer set may offer additional utility in forensic and clinical studies of toxic plant ingestion.

Materials and methods

Plant sample preparation

A. microdictyon seedlings were provided by Gangwon Agricultural Research and Extension Services (Pyeongchang, Korea). *C. majalis* seedlings were provided by Gyeongsangbuk-do Agricultural Research and Extension Services (Bonghwa, Korea). The plants were grown in horticultural soil for their leaves in a greenhouse at a constant temperature. Non-target plant materials were purchased from local herbal markets and suppliers in Korea in May, 2024. All commercial products used in this study were bought from local markets in July 2024 and stored at 23 °C.

Genomic DNA extraction

The genomic DNA (gDNA) used to evaluate the efficiency of the assay with the designed primer set was extracted according to the manufacturer's protocol using the DNeasy Plant Pro Kit (Qiagen, Hilden, Germany). Genomic DNA was extracted and used to construct a calibration curve for a reference binary mixture. The majority of the extracted DNA had a purity range of 1.7 to 2.0, and any DNA with a purity below 1.7 was further purified using the Wizard DNA Clean-Up System (Promega, Madison, USA). The DNA concentration and purity were measured using a SPECTROstar Nano reader (BMG Labtech, Ortenberg, Germany). An 18S rRNA primer set was used as a positive control to assess the quality of gDNA [17].

Pretreatment of digested samples in artificial gastric juice

Pulverized plant leaves were digested in artificial gastric juice to evaluate the verification effectiveness. The artificial gastric juice was prepared by dissolving 2.0 g of NaCl and 3.2 g of porcine pepsin in 7.0 mL of 0.2 mol L⁻¹ HCl, then diluting the solution with water to a final volume of 1 L and a pH of 1.2, following the method described [25]. To simulate peristaltic motion, 25 mg of ground leaves were digested in 50 mL of artificial gastric juice at 37 °C in a shaking incubator set to 50 rpm, with samples collected at various time intervals (1, 2, 4, and 6 h). The high acidity of the harvested samples (pH 1.2) negatively affected the DNA extraction. The pH was neutralized using potassium hydroxide, and the samples were washed three times with distilled water.

Target sequence analysis and primer design

Chloroplast DNA sequences of *A. microdictyon* (accession number: OQ183596.1) and *C. majalis* (accession number: NC_063623.1) were obtained from the National Center for Biotechnology Information (NCBI) database as reference sequences. The nucleotide sequences were aligned using BioEdit 7.2 (Ibis Biosciences, Carlsbad, CA, USA) and ClustalW2 (EMBL-EBI, Hinxton, Cambridgeshire, UK). Species-specific primers were designed based on the SNPs identified in the aligned sequences and synthesized by Macrogen (Seoul, Korea).

Blind samples

The Ministry of Food and Drug Safety (Cheongju, Republic of Korea) provided the blinded samples. The mixtures were randomly divided into 25 samples containing different proportions of *A. microdictyon* and *C. majalis*. *C. majalis* was added to *A. microdictyon* at 0–10% (w/w) concentration, and each sample was standardized to a final weight of 2 g.

Binary mixture sample

To validate the developed discrimination method for commercial products, a standard curve was generated using a binary mixture of *A. microdictyon* and *C. majalis* powders. The binary mixture was prepared in ratios of 0.1% (2 mg), 1% (20 mg), 10% (200 mg), and 100% (2 g), respectively, by combining these powders. Before DNA extraction, the binary mixture was dried in an oven at 60 °C for 48 h.

Quantitative real-time PCR assay

Quantitative real-time PCR was carried out in a final reaction volume of 20 µL using the AccuPower® 2×GreenStar qPCR Master Mix containing SYBR Green (Bioneer, Daejeon, Korea). The reaction mixture comprised 10 µL of 2×GreenStar Master Mix, 0.5 µL of each primer (10 pmol), 1 µL of genomic DNA (10 ng/µL), and

0.25 µL of ROX Dye. qPCR amplification was performed using the QuantStudio 3 qPCR System (Applied Biosystems, Foster City, CA, USA). The quantitative real-time PCR conditions included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55–60 °C for 20 s (depending on the primer-specific annealing temperature), and extension at 72 °C for 30 s. Each quantitative real-time PCR was conducted in technical triplicates for three independent biological replicates.

Standard curve analysis for quantitative real-time PCR

Ten-fold serial dilutions of gDNA (10 ng to 1 pg/µL) were used to assess primer pair efficiency and sensitivity. A standard curve was generated by plotting cycle threshold (Ct) values from quantitative real-time PCR experiments at the threshold level of log-based fluorescence across five ten-fold dilution points of extracted gDNA. The correlation between Ct values and DNA concentrations was calculated using default settings. The standard curve equation was defined as $y = -ax + b$, where "a" represents the slope and "b" the y-intercept. Amplification efficiency was calculated as $E = (10^{(-1/a)} - 1)$ (Arya et al. 2005b). Acceptable amplification efficiency ranges from 90 to 110%, equivalent to a slope between -3.1 and -3.6 [18].

Interlaboratory validation

To verify the reproducibility of the developed discrimination method, quantitative real-time PCR was performed in two independent laboratories. These laboratories employed a CFX96™ Deep Well Real-Time System (Bio-Rad, Hercules, CA, USA) and a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A standard curve was generated from three replicates.

Results and discussion

Design and validation of species-specific primers

The cp genome sequences of *A. microdictyon* and *C. majalis* were obtained from the NCBI database. Chloroplast genes *accD*, *atpA*, *ccsA*, and *ndhF* were selected as candidate markers for species identification based on the presence of SNPs between the two species (Supplementary Fig. S1). After cloning and sequence verification against reference sequences in the NCBI database, species-specific primers targeting the identified SNP regions were designed (Supplementary Table S1). Specifically, by comparing the chloroplast gene sequences of the two species, we identified primer-binding regions containing 11, 9, and 11 SNPs in the *accD*, *atpA*, and *ndhF* regions, respectively, for *A. microdictyon*. For *C. majalis*, the selected primer-binding regions included 12, 7, and 9 SNPs in the *atpA*, *ccsA*, and *ndhF* regions, respectively. Primers were designed to amplify short amplicons in the range of 120–186 bp to avoid DNA fragmentation that

may occur because of food processing techniques such as drying, mixing, and heating (Hrnčířová et al., 2008, Lockley and Bardsley, 2000. Therefore, in addition to *A. microdictyon* and *C. majalis*, 13 other species were analyzed to evaluate the specificity of the six primer sets designed for this study (Table 1). These included four Liliaceae species (*Tulipa edulis*, *Tulipa gesneriana*, *Lilium longiflorum*, and *Erythronium japonicum*), two leafy vegetables (*Lactuca sativa* and *Petasites japonicus*), two herbs (*Perilla frutescens* and *Pimpinella brachycarpa*), four Brassicaceae species (*Brassica oleracea*, *Brassica rapa*, *Brassica juncea*, and *Eruca vesicaria*), and one root crop (*Beta vulgaris*). These plants were selected to assess the assay specificity for phylogenetically diverse and commonly consumed edible and medicinal species. None of the six primer sets generated amplicons from the non-target species within 40 amplification cycles. Taken together, these results suggest that the developed DNA markers are highly specific for detecting the target species, thereby minimizing the risk of false-positive amplification.

Assessment of the amplification efficiency of the species-specific primers

Sensitivities of the six primer sets were evaluated separately for each species. For *A. microdictyon*, the primer sets *AM_accD*, *AM_atpA*, and *AM_ndhF* were tested using tenfold serial dilutions of genomic DNA (10–0.001 ng). For *C. majalis*, the primer sets *CM_atpA*, *CM_ccsA*, and *CM_ndhF* were used under identical conditions. The correlation coefficients (R^2) of all six primer

sets were greater than 0.98, the slopes ranged from –3.22 to –3.56, and the amplification efficiencies calculated from these slopes ranged from 90.81 to 99.85% for each target species (Fig. 1). All R^2 values, amplification efficiencies, and slopes complied with the European Network of GMO Laboratories guidelines [26]. The desired amplicon sizes were consistently amplified from DNA amounts ranging from 10 ng to 1 pg while maintaining the same T_m values (Supplementary Fig. S2). The reproducibility of the method was assessed by cross-validation in two laboratories using different quantitative real-time PCR machines. The standard curve based on tenfold serial dilutions of purified genomic DNA was utilized for this interlaboratory validation. The results showed that the R^2 values were greater than 0.98, the slopes ranged from –3.15 to –3.42, and the efficiencies ranged from 95.86 to 102.04% (Supplementary Table S2). These results confirm that the developed primer sets exhibit high reproducibility for detecting the target species. A standard curve with tenfold serial dilutions of purified genomic DNA was used to evaluate the efficiency and reproducibility of each primer set. Generally, the quality of DNA extracted from commercial processed products, such as dried foods, pickles, and sauce, is less than that of fresh leaf samples. To establish the threshold Ct values to discern commercial products, another standard curve was constructed using *A. microdictyon* and *C. majalis* powders.

Table 1 Specificity for non-target plants using quantitative real-time PCR primer pairs

NO.	Order	Family	Species	18s rRNA	<i>A. microdictyon</i>			<i>C. majalis</i>		
					<i>accD28.51</i>	<i>atpA</i> 27.76	<i>ndhF</i> 29.05	<i>atpA</i> 30.71	<i>ccsA</i> 29.38	<i>ndhF</i> 30.70
1	Liliales	Liliaceae	<i>Allium microdictyon</i>	^a ++	++	++	++	-	-	-
2			<i>Convallaria majalis</i>	++	^c -	-	-	++	++	++
3			<i>Tulipa edulis</i>	++	-	-	-	-	-	-
4			<i>Tulipa gesneriana</i>	++	-	-	-	-	-	-
5			<i>Lilium longiflorum</i>	++	-	-	-	-	-	-
6			<i>Erythronium japonicum</i>	++	-	-	-	-	-	-
7	Asterales	Asterales	<i>Petasites japonicus</i>	++	-	-	-	-	-	-
8			<i>Lactuca sativa</i>	++	-	-	-	-	-	-
9	Zingiberales	Brassicaceae	<i>Brassica oleracea</i>	++	-	-	-	-	-	-
10			<i>Brassica rapa</i>	++	-	-	-	-	-	-
11	Caryophyllales	Chenopodiaceae	<i>Beta vulgaris</i>	++	-	-	-	-	-	-
12	Papaverales	Cruciferae	<i>Brassica juncea</i>	++	-	-	-	-	-	-
13	Lamiales	Lamiaceae	<i>Perilla frutescens</i>	++	-	-	-	-	-	-
14	Brassicales	Brassicaceae	<i>Eruca vesicaria</i>	++	-	-	-	-	-	-
15	Apiales	Apiaceae	<i>Pimpinella brachycarpa</i>	++	-	-	-	-	-	-

^aCycles, cut-off Ct values of each specific primer sets

^b++, indicates that the sample was amplified before target species cycles

^c-, indicates no amplification before cut-off Ct cycles

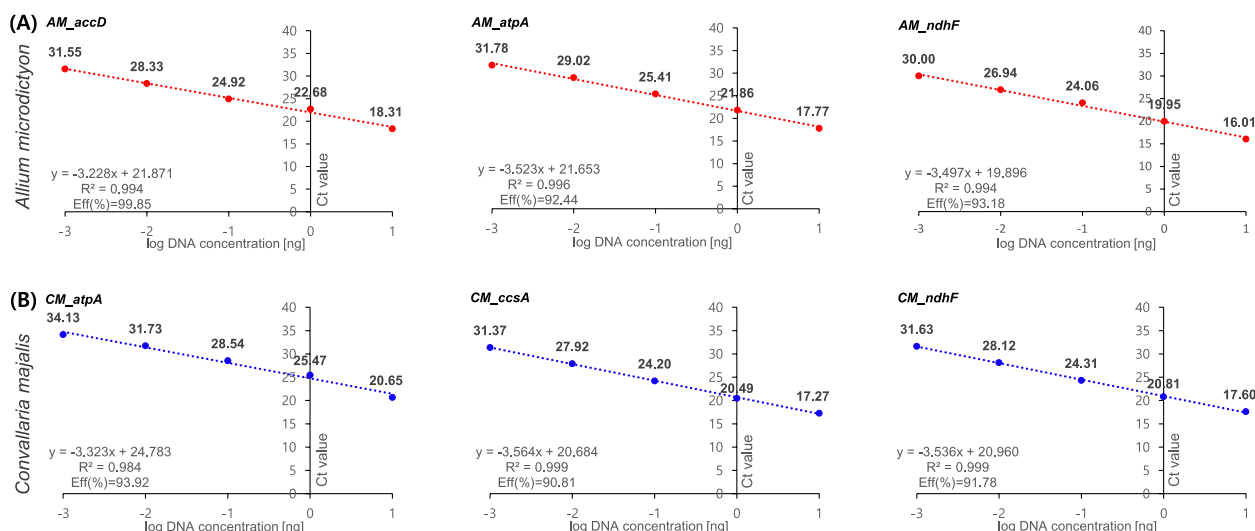


Fig. 1 Standard curve were obtained using six primers pairs targeting *A. microdictyon* or *C. majalis*. These curves were constructed based on the efficiency and correlation of counts (R^2) with tenfold serially diluted genomic DNAs. The x-axis represents the log DNA concentration (ng) and the y-axis represents the mean of Ct values $\pm$ SD. **(A)** *A. microdictyon* targeting primer sets (AM_accD, AM_atpA, and AM_ndhF). **(B)** *C. majalis* targeting primer sets (CM_atpA, CM_ccsA, and CM_ndhF). quantitative real-time PCR was performed in triplicate ($n=3$)

Binary mixture analysis for sensitivity evaluation

In addition to genomic DNA-based curves, we created binary mixture-based standard curves to discern commercial products. Sensitivities of the six primer sets were validated using a binary mixture test. *A. microdictyon* powder and *C. majalis* powder were serially diluted tenfold (0.1%, 1%, 10%, and 100%) by weight to achieve a final mass of 2 g. For the *A. microdictyon* primers, *A. microdictyon* powder was serially diluted (0.1%–100%) to a final mass of 2 g, with the remaining weight supplemented with *C. majalis* powder. The same dilution method was used for the *C. majalis* primers, using *C. majalis* powder with *A. microdictyon* powder as a supplement. The R^2 values for all primers exceeded 0.993, with slopes ranging from -3.112 to -3.519 , and efficiencies ranging from 93.58% to 109.59% (Fig. 2). Impurity levels below 0.1% are generally not considered intentional adulteration. Ct values associated with the 0.1% limit of quantitation may serve as a reference standard for evaluating authenticity [19]. This approach allowed us to assess whether the processed sample matrix acted as a PCR inhibitor and evaluate detection sensitivity at the 0.1% threshold, which is typically seen as the cutoff for intentional contamination.

Blind sample analysis using the developed assay

A blinding test was conducted to verify the reliability of six primer pairs developed in this study. The test was conducted using 25 randomly prepared blinded samples. An independent research group prepared 25 blinded samples and did not disclose information on the proportions of *A. microdictyon* and *C. majalis* powders in the mixtures. The quality of the extracted DNA was assessed using 18 s rRNA as the positive control. All samples yielded Ct

values (13.81–16.88), confirming that the DNA was suitable for analysis. The samples were examined using the cut-off Ct values of the six primer sets (Table 2). All 25 samples exhibited Ct values lower than the cut-off for the *A. microdictyon* primer sets, indicating the presence of *A. microdictyon* powder in all samples. In contrast, five samples (sample numbers 7, 11, 12, 17, and 19) showed Ct values higher than the cut-off for the *C. majalis* primer sets, suggesting the absence of *C. majalis* powder in these samples. The Ct values of the remaining 20 samples were lower than the cut-off of *C. majalis* primer sets, indicating that these samples were mixtures of *A. microdictyon* and *C. majalis* powders. These results confirmed that the developed primer pairs were effective in determining the authenticity of *A. microdictyon* and *C. majalis* products in blind samples.

Application of the developed assay for the analysis of commercial products

To evaluate the applicability of the six developed primers to commercial products, 15 commercial *A. microdictyon* samples were analyzed (Table 3). The 18 s rRNA primer pair was used to ensure that the DNA quality was sufficient for quantitative real-time PCR analysis. The presence of both species in commercial products was determined using a cut-off Ct value (0.1% threshold Ct value for a specific primer set in a binary mixture). Among the 15 products labeled as *A. microdictyon*, amplification with AM-specific primers (AM_accD, AM_atpA, and AM_ndhF) yielded Ct values below the cut-off, whereas amplification with CM-specific primers (CM_atpA, CM_ccsA, and CM_ndhF) produced CT values above the cut-off. These findings indicate that *C.*

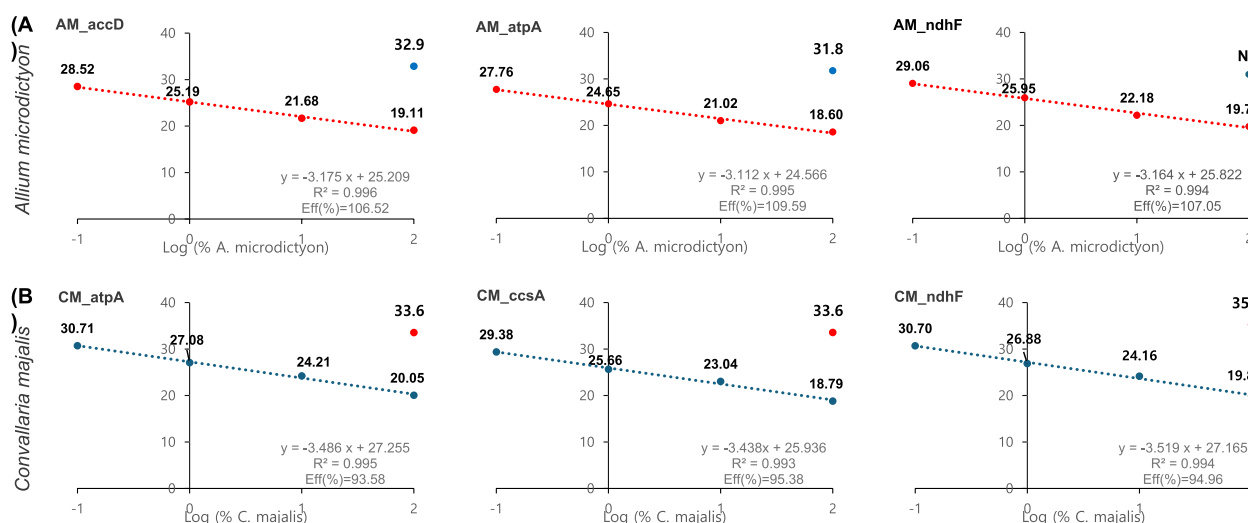


Fig. 2 Standard curves of threshold cycle (Ct) were obtained based on the efficiency and correlation of coefficients (R^2) by binary mixtures. The x-axis represents the log percentage (%) of target species, and the y-axis represents the mean Ct value $\pm$ SD. It was plotted against the log of target species concentration (100%, 10%, 1%, and 0.1%). **(A)** *A. microdictyon* powder was mixed with *C. majalis* powder, and tenfold serial dilutions (0.1%, 1%, 10%, and 100%, a total mass of 2 g) were prepared. Each gDNA mixture (10 ng/ μ L) was amplified using a species-specific primer set for *A. microdictyon* (AM_accD, AM_atpA, AM_ndhF). The red dashed line represents the Ct value of the 0.1% binary mixture amplified using the *A. microdictyon*-targeting primer set. **(B)** *C. majalis* powder was mixed with *A. microdictyon* powder and tenfold serial dilutions (0.1%, 1%, 10%, 100%, a total mass of 2 g) were prepared. Each gDNA mixture (10 ng/ μ L) was amplified using the species-specific primer set for *C. majalis* (CM_atpA, CM_ccsA, and CM_ndhF). The blue dotted line indicates the Ct value of the 0.1% binary mixture amplified using the *C. majalis*-targeting primer set. ND indicates not detected at less than 40 times. All reactions were performed in triplicate ($n = 3$)

majalis was absent from commercial products. Thus, the primer pairs developed in this study were confirmed to be applicable to commercial products.

Validation analysis in simulated gastric content

Rapid and convenient identification is crucial for forensic investigations of poisoning caused by toxic plants. We also added a validation step using simulated gastric fluid to evaluate the robustness of the analysis under physiologically relevant conditions. Therefore, we conducted an applicability test to determine whether the developed primers could amplify the target plant DNA following digestion in simulated gastric fluid (Table 4). Simulated gastric fluid samples were prepared from both plants after 1 h, 2 h, 4 h, and 6 h of raw digestion. All the developed primer sets effectively amplified the products after at least 2 h of digestion. Conversely, all six primer sets at 4–6 h exhibited Ct values higher than the cut-off, indicating that the sensitivity of each primer set diminished as the digestion time increased to 4 h. These limitations indicate that this method can be used for early detection within a typical gastric emptying timeframe. These results indicate that the high acidity of the simulated gastric fluid causes DNA fragmentation [20]. Given that the normal gastric emptying time is less than 2 h, the developed primer sets may serve as tools for forensic investigations and patient care [21]. However, in this study, digestion was conducted under a single pH condition (pH 1.2), which reflects that of standard gastric fluid.

Gastric pH varies depending on individual differences and clinical conditions. Further testing at different pH levels will enhance the applicability of this method. As a result, the quantitative real-time PCR assay developed in this study can be effectively applied to forensic investigations of patients who have ingested toxic plants.

This study utilized quantitative real-time PCR as a highly sensitive and rapid approach for distinguishing between *A. microdictyon* and *C. majalis*. Six primer sets were designed to target chloroplast DNA from both species. To confirm the specificity of the designed primers, 13 non-target plant species were tested; none showed amplification within 40 cycles, demonstrating high target specificity. Marker sensitivity was assessed through standard curve generation and reproducibility was evaluated via cross-validation performed in two independent laboratories using different quantitative real-time PCR devices. Analysis of 25 blind samples confirmed that *C. majalis* was not detected in 5 samples based on the cut-off Ct values, demonstrating that the assay effectively differentiates between the two species. The practical applicability of the primers was validated by detecting the target species in commercial food products and simulated gastric fluid. The quantitative real-time PCR system established in this study is an efficient tool for identifying toxic plants and for forensic and clinical investigations related to the consumption of toxic plants, food safety, and the protection of consumer rights.

Table 2 Blind tests for measuring the reliability of the developed primer set

No	Ingredient		aPAC	A.microdiclyon Specific Primer Ct±SD				C.keiskei Specific primer Ct±SD				A/D						
	A.microdiclyon			C.keiskei		AM_accD		AM_atpA		AM_ndhF			CK_atpA		CK_ccsA		CK_ndhF	
						b28.51	27.76	29.05	30.71	29.38	30.70							
1	o	o	14.21	±0.02	±0.06	19.48	±0.03	19.46	±0.07	19.10	±0.03	19.29	±0.05	19.24	±0.00	5A		
2	o	o	14.17	±0.06	±0.06	19.91	±0.02	19.90	±0.07	20.98	±0.31	20.84	±0.11	20.99	±0.07	A		
3	o	o	14.39	±0.13	±0.04	19.90	±0.05	19.83	±0.05	19.65	±0.04	19.56	±0.03	19.75	±0.01	A		
4	o	o	14.83	±0.04	±0.03	20.19	±0.02	20.21	±0.16	19.97	±0.04	19.91	±0.02	20.04	±0.07	A		
5	o	o	14.11	±0.02	±0.09	19.96	±0.02	19.94	±0.04	21.78	±0.04	21.69	±0.03	21.85	±0.07	A		
6	o	o	14.26	±0.09	±0.09	19.70	±0.09	19.45	±0.19	22.24	±0.24	22.34	±0.07	22.30	±0.19	A		
7	o	x	13.83	±0.02	±0.05	19.27	±0.06	18.95	±0.20	33.46	±0.43	34.89	±0.86	32.98	±0.55	A		
8	o	o	13.81	±0.11	±0.03	19.35	±0.03	19.21	±0.07	24.82	±0.13	24.53	±0.02	24.75	±0.08	A		
9	o	o	14.27	±0.05	±0.04	19.77	±0.08	19.74	±0.16	20.53	±0.03	20.43	±0.03	20.65	±0.15	A		
10	o	o	14.24	±0.06	±0.12	19.20	±0.02	19.35	±0.06	21.43	±0.06	21.39	±0.01	21.64	±0.05	A		
11	o	x	14.20	±0.05	±0.10	19.55	±0.04	19.47	±0.05	35.10	±1.98	32.95	±0.47	35.13	±0.39	A		
12	o	x	14.32	±0.14	±0.08	19.74	±0.03	19.68	±0.01	34.29	±1.51	34.68	±0.38	33.48	±0.71	A		
13	o	o	16.88	±0.10	±0.02	22.15	±0.10	22.78	±0.17	28.71	±0.03	28.19	±0.02	28.16	±0.13	A		
14	o	o	16.56	±0.06	±0.01	23.61	±0.06	24.12	±0.06	23.24	±0.08	23.17	±0.01	23.28	±0.03	A		
15	o	o	14.10	±0.09	±0.05	21.06	±0.03	21.45	±0.11	21.24	±0.04	21.42	±0.05	21.15	±0.03	A		
16	o	o	14.03	±0.01	±0.05	21.37	±0.01	21.78	±0.05	20.94	±0.09	21.26	±0.03	21.16	±0.09	A		
17	o	x	16.21	±0.06	±0.08	23.22	±0.01	23.08	±0.10	34.22	±0.50	31.68	±0.27	33.75	±1.50	A		
18	o	o	14.14	±0.08	±0.02	21.58	±0.01	21.96	±0.15	22.04	±0.18	22.59	±0.04	22.40	±0.19	A		
19	o	x	15.46	±0.07	±0.03	21.71	±0.07	21.99	±0.12	33.44	±0.77	31.90	±1.15	32.83	±0.32	A		
20	o	o	14.90	±0.09	±0.04	21.98	±0.12	22.28	±0.01	26.02	±0.05	25.94	±0.06	25.81	±0.10	A		
21	o	o	16.14	±0.07	±0.08	22.15	±0.13	22.75	±0.20	25.20	±0.13	25.21	±0.08	25.28	±0.04	A		
22	o	o	14.69	±0.28	±0.06	22.75	±0.04	23.38	±0.16	22.76	±0.05	22.91	±0.04	22.87	±0.06	A		
23	o	o	15.08	±0.14	±0.14	23.17	±0.04	23.60	±0.07	26.14	±0.02	26.19	±0.08	26.13	±0.09	A		
24	o	o	15.42	±0.09	±0.03	21.31	±0.06	21.81	±0.09	22.89	±0.08	23.01	±0.02	22.87	±0.06	A		
25	o	o	16.24	±0.07	±0.01	21.83	±0.05	22.26	±0.11	26.06	±0.07	26.06	±0.05	25.84	±0.05	A		

^aPositive amplification control (18S rRNA)

^bCycles, cut-off Ct values of each specific primer sets

^cAccordance

Table 3 Quantitative real-time PCR analysis using 15 commercial food products

Sample number	Sample form	All plants 18 s rRNA	<i>A. microdictyon</i>			<i>C. majalis</i>		
			<i>aaccD</i> 28.51	<i>atpA</i> 27.76	<i>ndhF</i> 29.05	<i>atpA</i> 30.71	<i>ccsA</i> 29.38	<i>ndhF</i> 30.70
1	Fresh materials	16.15 ±0.26	16.93 ±0.64	16.98 ±0.59	16.60 ±0.57	bND	37.22 ±0.04	35.17 ±0.08
2	Dry materials	15.87 ±0.26	17.87 ±0.63	16.95 ±0.61	17.19 ±0.09	34.57 ±0.28	35.84 ±0.04	35.31 ±0.09
3		16.75 ±0.30	20.30 ±0.63	19.11 ±0.65	19.80 ±0.58	34.04 ±0.24	33.84 ±0.12	33.86 ±0.11
4		15.72 ±0.31	17.25 ±0.64	16.48 ±0.61	16.95 ±0.58	33.82 ±0.23	37.46 ±0.04	ND
5	Pickles	15.10 ±0.28	17.42 ±0.60	17.38 ±0.56	18.94 ±0.60	34.12 ±0.29	35.20 ±0.51	34.97 ±0.10
6		14.25 ±0.30	17.34 ±0.66	17.17 ±0.52	17.19 ±0.61	32.22 ±0.13	34.09 ±0.12	ND
7		14.04 ±0.32	18.82 ±0.65	18.32 ±0.50	18.16 ±0.53	33.18 ±0.26	35.70 ±0.08	33.94 ±0.09
8		14.37 ±0.32	17.65 ±0.63	17.60 ±0.61	17.25 ±0.54	33.45 ±0.33	33.66 ±0.10	33.83 ±0.09
9		16.89 ±0.32	18.14 ±0.49	18.20 ±0.64	17.51 ±0.55	33.86 ±0.26	35.37 ±0.08	34.91 ±0.09
10		13.76 ±0.31	16.31 ±0.57	16.51 ±0.57	16.18 ±0.59	33.78 ±0.35	34.89 ±0.08	35.09 ±0.08
11		15.01 ±0.32	17.28 ±0.58	17.14 ±0.61	19.07 ±0.61	32.81 ±0.21	34.73 ±0.50	34.93 ±0.08
12		13.35 ±0.27	18.80 ±0.61	18.70 ±0.61	18.45 ±0.54	35.37 ±0.30	36.30 ±0.04	35.46 ±0.07
13		14.13 ±0.26	17.14 ±0.63	16.74 ±0.62	16.88 ±0.57	35.45 ±0.32	34.17 ±0.05	ND
14		14.23 ±0.32	17.74 ±0.62	17.09 ±0.62	17.23 ±0.54	32.51 ±0.08	35.29 ±0.10	34.96 ±0.12
15	Sauce	15.98 ±0.32	19.17 ±0.61	18.14 ±0.58	18.64 ±0.07	34.60 ±0.34	38.49 ±0.04	ND

^aCycles, cut-off Ct values of each specific primer sets

^bbND (non-detected) indicates more than 40 cycles

Table 4 Simulated gastric fluid analysis for forensic applications

Species	Primer	Digested time				
		Non treated	1 h	2 h	4 h	6 h
<i>A. microdictyon</i>	<i>AM_accD</i>	18.25^b	21.18	27.76	35.00	34.92
	^a 28.51	±0.05	±0.15	±0.10	±0.08	±0.07
	<i>AM_atpA</i>	18.28	20.15	24.88	32.19	35.13
	27.76	±0.16	±0.11	±0.20	±0.09	±0.25
	<i>AM_ndhF</i>	19.23	20.70	26.07	33.47	34.22
	29.05	±0.01	±0.10	±0.06	±0.18	±0.03
<i>C. keiskei</i>	<i>CK_atpA</i>	19.05	21.46	29.19	33.02	34.94
	30.71	±0.31	±0.17	±0.13	±0.12	±0.02
	<i>CK_ccsA</i>	18.65	20.33	25.68	31.64	31.64
	29.38	±0.17	±0.13	±0.15	±0.14	±0.10
	<i>CK_ndhF</i>	19.02	20.68	26.81	32.02	32.87
	30.70	±0.10	±0.12	±0.05	±0.14	±0.04

^aCycles, cut-off Ct values of each specific primer sets

^bValues in bold indicate amplification before the cut-off cycle

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13765-025-01032-7>.

Supplementary material 1

Acknowledgements

This study was supported by Grant (22193MFDS471) from the Ministry of Food and Drug Safety in 2024.

Author contributions

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

Funding

Ministry of Food and Drug Safety, 22193MFDS471, Su Yeon Kim.

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 have no competing interests to disclose.

Received: 16 June 2025 / Accepted: 25 August 2025

Published online: 22 September 2025

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