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RESEARCH ARTICLE

Development of real-time PCR-based markers for differentiation of *Oplopanax elatus* and *Aralia cordata* in commercial food products

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Abstract

Oplopanax elatus and *Aralia cordata*, commonly referred to as "Dureub" in Korea, are generally used as medicinal or food raw materials. Although *O. elatus*, a rare and endangered plant, is typically sold at high prices, the more abundant *A. cordata* is comparatively inexpensive. Given their common names and morphological root similarities, both plants can easily be confused, thereby providing potential opportunities for fraudulent use in food products. Species-specific molecular markers that can be used for quantitative real-time PCR (qPCR) analysis were developed. Verification of the six primer pairs revealed a correlation coefficient greater than 0.99, with a slope between -3.33 and -3.56. The assay confirmed specificity based on an analysis of 14 non-target plant species and verified its practicality using 10 commercial products with reliability based on a blind test. Thus, qPCR assays can contribute to food safety and protect consumer rights and interests.

Keywords *Aralia cordata* · DNA marker · *Oplopanax elatus* · Species identification · SYBR-GREEN real-time PCR

Introduction

According to a report by the World Health Organization (WHO), a majority of the populations in developing countries are dependent on herbs for medicinal purposes (WHO, 2007). In addition to developing countries, as phytonutrients and dietary supplements, herbal medicines are also used in many developed countries to treat different health problems, and demand is rapidly increasing (WHO, 2004). However, with the increasing demand for these herbal products, there has been a concomitant increase in public health and safety concerns, owing to the lack of quality control or inadequate labeling of these products (Raynor et al., 2011).

Plants in the family Araliaceae, which comprises 43 genera and approximately 1,500 species, have for long been widely used for medicinal purposes or as raw food materials. The bark and leaves of these plants contain saponins, alkaloids, calcium, phosphorus, and vitamin C, which have been demonstrated to have medicinal efficacy in the treatment of a number of adult diseases and disorders, including diabetes, high blood pressure, and rheumatism (Shin, 2006). Among these plants, *Oplopanax elatus* and *Aralia cordata*, which in Korea are commonly referred to "Dureub," are generally used medicinally or as food raw materials.

O. elatus, which is widely, although sparsely, distributed in Korea and China, is known to have physiological activities similar to those of ginseng, and is mainly used to treat fevers, coughs, and inflammation (Kim et al., 2012). In addition, the roots of *O. elatus* contain large amounts of falcarindiol and oplopandiol, both of which exhibit strong anticancer activity (Huang et al., 2010). However, although *O. elatus*, which has diverse pharmacological effects, has been used medicinally as a whole plant, owing to its requirement for specific growth conditions, it is difficult to propagate. Consequently, it has been designated as a rare and endangered plant in Korea (Seong et al., 2019). *A. cordata* is widely distributed in mountainous regions of Asia, including Korea

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and China. Its roots, which are used for medicinal purposes, have been established to have efficacy in the treatment of inflammation, cough, and diabetes (Lee et al., 2015). Unlike the *O. elatus*, the growth of the whole plant is vigorous. In addition, the yield tends to be high due to its strong regenerative ability (Kim et al., 1999).

Given their common name "Dureub" and the morphological similarity of their roots, which are used as medicinal materials, *O. elatus* and *A. cordata* can readily be confused. However, whereas *O. elatus*, a rare and endangered plant, is sold at a high price, *A. cordata* is comparatively inexpensive. Accordingly, to prevent the misleading substitution of *O. elatus* with *A. cordata*, it would be desirable to have a reliable discriminatory assay. To date, however, there have been no such assays developed that could be used to differentiate between these two species.

A diverse range of techniques, including DNA-based, spectroscopic, sensory, and chromatographic analyses, have been developed to detect counterfeit constituents in complex food products (Hong et al., 2017), among which, DNA-based quantitative real-time PCR (qPCR) analysis has high sensitivity and specificity, thereby facilitating the detection of very small amounts of target components (Kane and Hellberg, 2016; Kim et al., 2022). qPCR analysis is generally divided into probe-based TaqMan and fluorescent dye-based SYBR Green assays, which have high accuracy and sensitivity (Arya et al., 2005). Of these two approaches, it is relatively difficult to design probes and primers for genotyping when using probe-based TaqMan assays, as this necessitates the presence of numerous SNPs and indels between the target and non-target species (Navarro et al., 2015, Safdar and Junejo, 2015; Şakalar and Kaynak, 2016). In contrast, the SYBR Green assay-based detection of target species tends to be less expensive and more convenient than probe-based qPCR, as independent fluorescent dyes bind to DNA duplexes and can be used to detect target species with measured fluorescence values. However, false-positive results may occur (Safdar and Junejo, 2015).

In this study, we developed a molecular marker system to differentiate between *O. elatus* and *A. cordata* using qPCR analysis. The reliability of the developed assay was validated based on blind assessments and specificity tests, and its practical applicability was examined using commercial products.

Materials and methods

Sample preparation and genomic DNA extraction

The leaves and stems of *O. elatus* were provided by the Bioactive Natural Product Chemistry Laboratory of Kangwon National University (Chuncheon, Gangwon-do, Korea). The leaves and stems of *A. cordata* were kindly provided by the Gangwon-do Agricultural Promotion Agency (Chuncheon, Gangwon-do, Korea). Both samples were stored in a -70 °C deep freezer until use. All commercial plant-based products used in this study were purchased from a local market and stored at room temperature (23–24 °C).

Genomic DNA was extracted from samples using a DNeasy Plant Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were measured using a SPECTROstar Nano Leader microplate reader (BMG Labtech, Otenberg, Germany), and if the measured purity had a value of less than 1.7, a Wizard DNA Clean-up System (Promega, Madison, WI, USA) was used to obtain higher quality DNA. The purity of all gDNA used in this study was between 1.7 and 2.0.

Preparation of binary mixtures of *O. elatus* and *A. cordata*

Reference binary mixtures containing different amounts (0.1, 1, 10, and 100%, total 2 g) of *O. elatus* were mixed with *A. cordata* powder (99.9, 99, 90, and 0%). In addition, to evaluate the applicability of the developed markers in detecting differently processed medicinal herbs, binary mixture samples were treated as follows: (1) drying in an oven at 55 °C for 48 h, (2) boiling at 100 °C for 15 min, and (3) autoclaving at 120 °C for 15 min at 150 kPa. The gDNA of the reference binary mixtures was then extracted according to a modified large-scale CTAB-based method (Minas et al., 2011).

Sequence analysis and species-specific primer design

The sequence of the chloroplast genome of *A. cordata* (accession number NC_040964) was downloaded from the National Center for Biotechnology Information (NCBI) database, and the chloroplast gene sequence of *O. elatus* was obtained from the National Instrumentation Center for Environmental Management database (NICEM, Seoul, Korea; http://nicem.snu.ac.kr). To develop species-specific primer pairs based on sequences of the chloroplast genes of the two species, the nucleotide sequences were aligned using ClusteralW2 (EMBL-EBI, Hinston, Cambridgeshire, UK) and primers were designed on the basis of SNPs between the two species using BioEdit v.7.2 (Ibis Biosciences, Carlsbad, CA, USA). The designed primers were synthesized commercially by Macrogen (Seoul, Korea).

Optimization of the qPCR assay

Real-time PCR analysis was performed using an AccuPower® 2×GreenStar qPCR Master Mix (Bioneer, Daejeon,

Korea). The reaction mixture for this assay contained 1 µL of 10 ng μL^{-1} genomic DNA, 0.5 µL of each primer (10 µM), 0.25 µL of ROX Dye, 10 µL of 2 × GreenStar Master Mix, and distilled water to a final volume of 20 µL. The resulting mixture was subjected to qPCR using a Quantstudio 3 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The qPCR conditions were as follows: an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 56–59 °C (melting temperature of each primer) for 30 s, and extension at 72 °C for 30 s. All qPCR experiments were performed in triplicate.

Construction of a qPCR standard curve and data analysis

The efficiency of the developed primer pairs was confirmed using five concentrations (10 ng/µL to 1 pg/µL) obtained by tenfold dilutions of the target DNA. The threshold level of logarithmic fluorescence was defined as the cycle threshold (Ct) in the qPCR analysis. Default parameters were used to determine the correlation between the Ct standard curve and diluted DNA. The standard curve was determined as $y = -ax + b$, where a is the standard curve slope and b is the y-intercept. The reaction efficiency was calculated using the following formula: $E = (10^{-1/a} - 1) \times 100$. The permissible range of amplification efficiency should be between 110 and 90%, which corresponds to a slope of between − 3.1 and − 3.6 (ENGL, 2015).

Inter-laboratory validation

To confirm the applicability of the developed qPCR assay, inter-laboratory validation was performed by two independent laboratories. The instruments used in this assessment experiment were a CFX Connect Real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems), respectively. qPCR was performed in triplicate under the same conditions as those used for the developed assay.

Specificity test

To verify the cross-reactivity of the six developed primer pairs, specificity assays were conducted for 14 non-target plant species (Table 2). Plant samples of the 14 species were purchased from a local market and used in the experiments. Samples were homogeneously ground using liquid nitrogen and a mortar for subsequent DNA extraction. The extracted DNA was used to assess and verify the amplification efficiency using 18S rRNA as a positive control.

Cloning and sequencing of PCR products

Conventional PCR for sequencing the target species was performed in a VeritiPro Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) using TaKaRa Ex Taq™ DNA polymerase (TaKaRa Bio Company, Kusatsu, Shiga, Japan). The PCR conditions were pre-denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 10 s, 53–58 °C for 30 s (depending on the primer), and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The PCR products thus obtained were processed as previously described (Uh and Jang, 2023).

Results and discussion

Design of DNA markers based on sequence variation

Primers for distinguishing *A. cordata* from *O. elatus* were designed based on the chloroplast genes, *acetyl-CoA carboxylase subunit D (accD), β" subunit of RNA polymerase* (*rpoC2*), *ATP synthase beta subunit* (*atpB*), and *Cytochrome* b_6 *subunit* (*petB*), which have frequently been used for species identification in other studies. Following alignment of the sequences of *A. cordata* and *O. elatus*, primers were designed based on SNPs detected between the respective genes in the two species (Supplementary Fig. 1). Processing of different medicinal materials, such as drying, heating, and autoclaving, may reduce the quality of the DNAs contained in complex food products (Lo and Shaw, 2018). Primer pairs were designed to amplify short sequences of 92–188 bp (Table 1), given that low-quality DNA (e.g., that attributable to fragmentation) reduces the efficiency of qPCR.

Amplification efficiency and reproducibility of the developed primers

To assess the sensitivity of the six developed primer pairs (*AC_rpoC2, AC_atpB, AC_accD, OE_accD, OE_rpoC2,* and *OE_petB*), we initially performed regression analysis using a tenfold serial dilution of DNA samples (10–0.001 ng/µL). The correlation coefficient (R^2) of the six primer pairs was higher than 0.99, with slopes ranging from -3.3 to -3.56. In addition, on the basis of the slope, we also assessed the amplification efficiency, which was estimated to be between 90.86% and 101.13% (Fig. 1). According to the guidelines issued by European Network of GMO Laboratories (ENGL), amplification efficiencies should be within the range between 90 and 110%, corresponding to a slope of between − 3.1 and − 3.6, with a correlation coefficient (R^2) of 0.98 or more. Consequently, our results were within the acceptable range. Inter-laboratory validation was conducted using different qPCR systems in two different laboratories to assess the

Table 1 The primer pairs developed for target species in this study and universal primers for all plants

Species	Target gene	Primer	Length(bp)	Sequence (5′→3′)	Amplicon size (bp)	Tm (°C)
Oplopanax elatus	*accD*	*accD_F*	16	CGTCAAGGACATTAGG	112	56
		accD_R	20	GTCAATCTCGAAAATATGAT		
	rpoC2	*rpoC2_F*	18	CCATTCTCCACGCAAATT	188	59
		rpoC2_R	20	TCTGTATCGAGGATCATTGA		
	petB	*petB_F*	17	CTCAGTCGTCTTTAGCT	169	57
		petB_R	20	GAATCTGAGGTTTTAATTGA		
Aralia cordata	*accD*	*accD_F*	19	ATTGAGACTTACATTGGAA	125	56
		accD_R	19	GTTAAATCACTACCAGCC		
	rpoC2	*rpoC2_F*	15	GTCTCCACGCAACTT	95	57
		rpoC2_R	18	CATTAGTTCGTTCTCGTG		
	atpB	*atpB_F*	20	CGTTTCTTATCACAACCTTT	92	58.5
		atpB_R	17	TGAAACCCCCTAATGGT		
All plants	*18 s rRNA region*	*18srRNA_F*	25	TCTGCCCTATCAACTTTCGATGGTA	137	58
		18srRNA_R	25	AATTTGCGCGCCTGCTGCCTTCCTT		

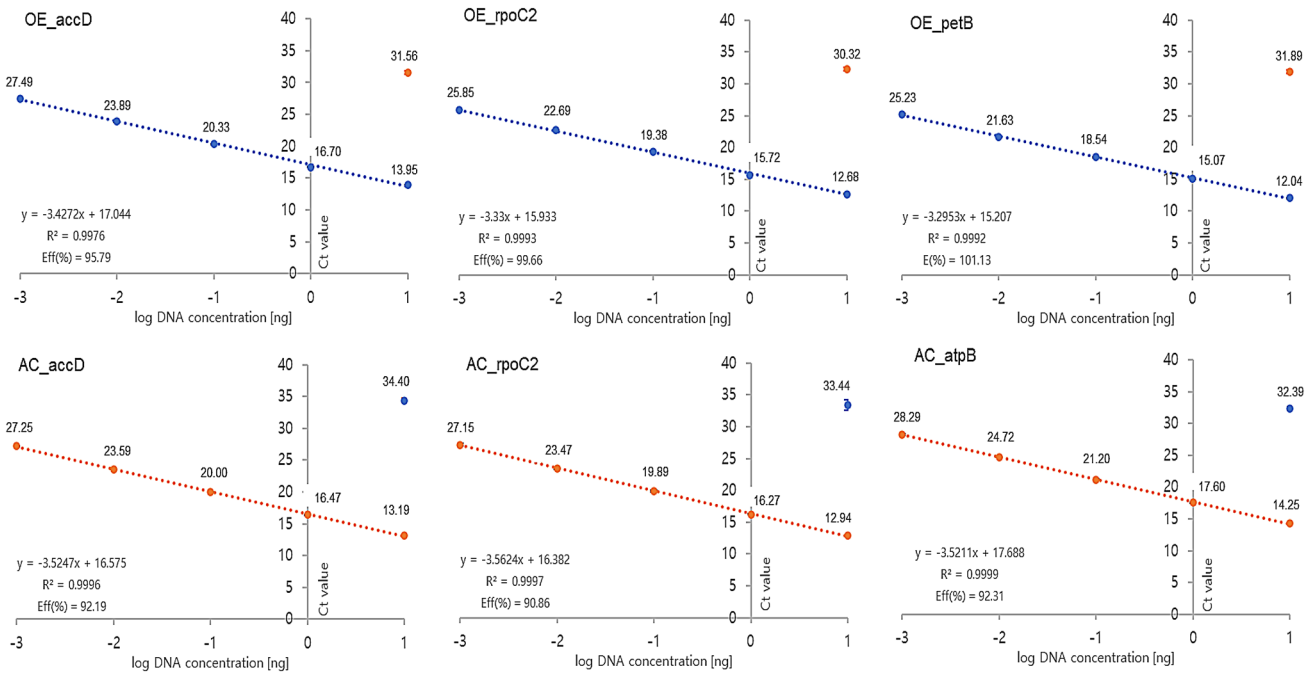

Fig. 1 Threshold cycle (Ct) values were plotted against the logarithm of DNA concentration (ng) obtained from a series of tenfold serial dilutions of gDNA using primers targeting either *Oplopanax elatus* or *Aralia cordata*. The x-axis represents the log DNA concentration (ng), and the y-axis represents the mean of Ct values ± SD. Blue and orange dots indicate target and non-target species, respectively. Quantitative real-time PCR was performed in triplicate (n = 3)

reproducibility of the standard curve of the six primer pairs, inter-laboratory validations were conducted using different real-time PCR systems in two different laboratories. The results, thus obtained indicated an R^2 value higher than 0.99, a slope of between − 3.14 and − 3.59, and a PCR efficiency of between 90.05% and 108.27% (Supplementary Table 1). The amplification efficiency and reproducibility of all six primer pairs were accordingly considered acceptable for further study.

Specificity validation of the developed primers

To examine the specificity of the six primer pairs, qPCR analyses were performed using 14 other plants that have

Table 2 Specificity test results for non-target plants using the real-time PCR primer pairs

NO	Family	Species	Whole plant systems	*Oplopanax elatus*			*Aralia cordata*		
			18 s rRNA	*accD*	*rpoC2*	*petB*	*accD*	*rpoC2*	*atpB*
				31.63[a]	29.57	30.98	24.34	26.70	25.22
1	*Araliaceae*	*Oplopanax elatus*	++[b]	++	++	++	−	−	−
2		*Aralia cordata*	++	−[d]	−	−	++	++	++
3		*Eleutherococcus sessiliflorus* (Stalkless-flower eleuthero)	++	−	−	−	−	−	−
4		*Hedera helix* (Common ivy)	++	−	−	−	−	−	−
5		*Dendropanax trifidus* (Korean dendropanax)	++	−	−	−	−	−	−
6		*Fatsia japonica* (Paperplant)	++	−	−	−	−	−	−
7		*Panax ginseng* (Ginseng)	++	−	−	−	−	−	−
8	*Apiaceae*	*Oenanthe javanica* (Dropwort)	++	−	−	−	+[c]	+	−
9		*Coriandrum sativum* (Coriander)	++	−	−	−	−	−	−
10		*Angelica gigas* (Korean angelica)	++	−	−	−	−	−	−
11		*Daucus carota* (Carrot)	++	−	−	−	−	−	−
12		*Petroselinum crispum* (Pasley)	++	−	−	−	−	−	−
13	*Compositae*	*Ligularia fischeri* (Fischers ragwort)	++	−	−	−	−	−	−
14		*Petasites japonicus* (Butterbur)	++	−	−	−	−	−	−
15		*Aster scaber* (Wild greens)	++	−	−	−	−	−	−
16		*Chrysanthemum coronarium* (Crown daisy)	++	−	−	−	−	−	−

[a]Cycles, cut-off Ct values of each specific primer sets (Ct values of 0.1% binary mixture)

[b]++, Highly amplified (like targer species cycle)

[c]+, Slightly amplified (less than target species cycle, but higher than non-target species cycle)

[d]−, Not detected (like less than non-target species cycle)

been used as medicinal herbs, as well as *O. elatus* and *A. cordata*. Among these non-target plants, five Araliaceae plants, stalkless-flower eleuthero, ivy, Korean dendropanax, paperplant, and ginseng, were included on account of their close phylogenetic relationship with *O. elatus* and *A. cordata*. Furthermore, given that *A. cordata* is widely consumed as a vegetable plant, we also included a further five Apiaceae plants, namely, water dropwort, coriander, Korean angelica, wild carota, and parsley, which are commonly consumed as vegetable plants. In addition, as an outgroup, we examined four species from the family Compositae, which are generally used as medicinal herbs in Korea. The 18S rRNA primer pairs developed as a whole plant system (Allmann et al., 1993) were used as a positive control, and we confirmed that all amplifications were achieved prior to the 20th cycle. Thereafter, using the six newly developed primer pairs, we performed qPCR analyses of the 14 species, along with *O. elatus* and *A. cordata* (Table 2). Except for the primer pairs *AC_accD* and *AC_rpoC2*, the PCR products for the 14 test species were not amplified prior to the 40th cycles. In the case of these two primer pairs, samples of *Oenanthe javanica* were found to have been amplified within 40 cycles, albeit later than the designated cut-off Ct value, which was sufficient to enable an distinction of this species from the target species (*A. cordata*). In conclusion, all primers developed in this study showed specificity for detection of the two target species without appreciable cross-reactivity with any of the other assessed plant species.

Application of the developed qPCR assay using binary samples subjected to drying, heating, and autoclaving

Generally, commercial products of *A. cordata* and *O. elatus* undergo rigorous processing such as heating, drying, and high-pressure treatment. These processes can cause damage to the DNA of pharmaceutical constituents and complex food products. Heat treatment is known to cause particularly severe DNA damage and is accordingly unsuitable for use in PCR-based marker assays for complex food authentication (Hwang et al., 2015). Consequently, we used the developed real-time PCR assay to evaluate the applicability for commercially available herbal medicines by examining processed food samples. Initially, to assess the applicability of the designed method for commercial herbal medicines and foods, we examined the slope, R^2, and efficiency using

reference binary mixtures that had been subjected to drying, heating, and autoclaving. The leaves of both plants were mixed to prepare binary mixtures (0.1–100% w/w). DNA extracted from the binary mixtures was diluted to 10 ng/μL and used for the developed qPCR assay. All slopes evaluated were between − 3.23 and − 3.55, and the R^2 values were higher than 0.99 for each of the six designed primer pairs. Using further dried, heated, and autoclaved leaf binary mixtures (0.1%–100% w/w), we examined the slope efficiencies for target species (Supplementary Fig. 2). Ct values were obtained for each sample treated with the three methods (Supplementary Table 2). Binary mixtures containing dried leaves had higher Ct values than untreated samples, whereas heated and autoclaved samples had higher Ct values than dried samples, which is consistent with the findings of previous studies indicating that drying leaves causes less DNA damage than heating or autoclaving. This accordingly indicates the necessity of applying different Ct values, depending on the food processing conditions, for complex food authentication (Uh and Jang, 2023). The designed real-time qPCR primer pairs showed slopes of − 3.13 to − 3.58, R^2 values greater than 0.99, and efficiency values of between 90.28% and 108.78% for the dried, heated, and autoclaved binary mixtures (Table 3). An impurity of less than 0.1% in mixtures generally indicates that it cannot be acceptable an intentional mixture for economic benefit in commercial markers. Therefore, in the qPCR analysis, the target species Ct value corresponding to 0.1% in the dried binary mixture was designated as the cutoff cycle, and was considered to be sufficient for the detection of target species in commercial dry-processed products.

Blind test assessment of the developed real-time qPCR assay

To evaluate the reliability of the six developed primer pairs, we conducted a blind test using 25 randomly mixed samples, which had been prepared by an independent research group, who withheld information regarding the contents of *A. cordata* and *O. elatus* within these samples. The 18S rRNA primer set was used as a positive amplification control, resulting in low Ct values (13.31–14.27 cycles) (Table 4). The presence of *O. elatus* powder in the blind samples was determined based on the cut-off Ct value for the developed primer pairs (0.1% of *O. elatus* in a dried binary mixture). We accordingly failed to detect the cut-off Ct values of the *O. elatus* primer pairs in five of the 25 samples, thereby indicating that these sample comprised pure *A. cordata* powder and no *O. elatus*. Among the remaining 20 samples, we detected Ct values that were lower than the cutoff values of the primer pairs for both *O. elatus* and *A. cordata*, indicating that the blind samples contained a mixture of *A. cordata* and *O. elatus*. Thus, qPCR assays can be accurately used to authenticate the presence of *A. cordata* and *O. elatus* in complex food samples.

Application of the developed system for analysis of commercial products

To assess the applicability of the developed real-time PCR procedure for the analysis of commercial products, we analyzed nine commercially available herbal medicines labeled as containing *A. cordata* and one product indicated to contain *O. elatus*. Given its rare and endangered status, it is

Table 3 Evaluation of the slope, R^2, and efficiency obtained for reference binary mixtures

DNA standard curve					Dry-treated binary mixture standard curve				
Target species	Primer	Y (Slope)	R^2	Efficiency (%)	Target species	Primer	Y (Slope)	R^2	Efficiency (%)
O. elatus	*OE_accD*	− 3.43	1.00	95.79	*O. elatus*	*OE_accD*	− 3.37	0.99	98.04
	OE_rpoC2	− 3.33	1.00	99.66		*OE_rpoC2*	− 3.24	0.99	103.60
	OE_petB	− 3.30	1.00	101.13		*OE_petB*	− 3.55	1.00	91.18
A. cordata	*AC_accD*	− 3.52	0.99	92.19	*A. cordata*	*AC_accD*	− 3.43	0.99	95.57
	AC_rpoC2	− 3.56	0.99	90.86		*AC_rpoC2*	− 3.51	1.00	93.16
	AC_atpB	− 3.52	0.99	92.31		*AC_atpB*	− 3.42	1.00	102.64
Heat-treated binary mixture standard curve					Autoclave-treated binary mixture standard curve				
O. elatus	*OE_accD*	− 3.50	1.00	93.13	*O. elatus*	*OE_accD*	− 3.45	0.99	94.82
	OE_rpoC2	− 3.47	0.99	94.20		*OE_rpoC2*	− 3.24	1.00	103.50
	OE_petB	− 3.30	1.00	100.75		*OE_petB*	− 3.13	1.00	108.78
A. cordata	*AC_accD*	− 3.44	0.99	95.21	*A. cordata*	*AC_accD*	− 3.23	0.99	103.97
	AC_rpoC2	− 3.58	0.99	90.28		*AC_rpoC2*	− 3.48	0.99	93.71
	AC_atpB	− 3.37	0.99	97.71		*AC_atpB*	− 3.50	0.99	92.91

Table 4 Blind test results for measuring the reliability of the developed primer pairs

Primer sets		*O. elatus* and *A. cordata* blind test														
Sample number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Positive control	18 s rRNA	14.161	13.894	13.309	13.636	13.746	13.442	13.987	13.846	13.880	13.422	13.603	14.091	13.421	13.500	13.590
		±0.032	±0.074	±0.009	±0.092	±0.025	±0.092	±0.147	±0.036	±0.037	±0.082	±0.132	±0.111	±0.112	±0.089	±0.120
Oplopanax elatus	*accD Region*	24.074	24.698	22.806	35.197	37.120	25.973	25.206	26.520	23.959	28.195	25.245	26.034	23.493	26.225	27.027
	31.63 cycle[a]	±0.124	±0.171	±0.130	±0.774	±0.246	±0.212	±0.067	±0.135	±0.216	±0.039	±0.054	±0.095	±0.336	±0.057	±0.108
	rpoC2 Region	20.564	20.905	19.162	34.732	35.058	22.004	21.125	22.840	20.327	23.922	21.151	22.079	20.013	22.347	22.976
	29.57 cycle	±0.015	±0.030	±0.013	±0.401	±0.430	±0.042	±0.039	±0.028	±0.099	±0.081	±0.039	±0.100	±0.117	±0.041	±0.010
	petB Region	21.329	21.708	19.298	35.185	35.652	22.721	21.627	23.625	24.821	24.897	21.987	23.105	20.979	22.995	23.785
	30.98 cycle	±0.092	±0.111	±0.124	±0.000	±0.932	±0.084	±0.065	±0.196	±0.867	±0.001	±0.013	±0.203	±0.227	±0.121	±0.073
Aralia cordata	*accD* Region	15.316	15.367	14.774	15.113	15.175	15.390	15.538	15.200	15.609	15.125	15.178	15.745	15.325	15.168	15.330
	24.34 cycle	±0.036	±0.047	±0.004	±0.145	±0.118	±0.036	±0.069	±0.001	±0.016	±0.094	±0.013	±0.091	±0.134	±0.014	±0.091
	rpoC2 Region	14.508	14.335	13.970	14.261	14.362	14.444	14.773	14.178	14.501	14.284	14.346	14.698	14.173	14.130	14.492
	26.70 cycle	±0.047	±0.050	±0.022	±0.106	±0.073	±0.039	±0.03	±0.05	±0.133	±0.025	±0.043	±0.094	±0.127	±0.004	±0.025
	atpB Region	16.562	16.217	15.387	15.649	16.523	16.120	16.073	15.842	16.445	15.851	15.891	16.199	16.098	15.872	15.894
	25.22 cycle	±0.395	±0.121	±0.808	±0.134	±0.434	±0.282	±0.001	±0.073	±0.256	±0.076	±0.004	±0.070	±0.263	±0.030	±0.003
Ingredient	*O_elatus*	o	o	o	x	x	o	o	o	o	o	o	o	o	o	o
	A_cordata	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

Primer sets		*O. elatus* and *A. cordata* blind test									
Sample number		16	17	18	19	20	21	22	23	24	25
Positive control	18 s rRNA	14.230	14.103	14.170	13.644	14.272	14.228	14.246	14.049	14.269	14.169
		±0.082	±0.006	±0.087	±0.102	±0.017	±0.107	±0.017	±0.043	±0.209	±0.181
Oplopanax elatus	*accD Region*	27.271	23.520	25.293	27.671	37.269	28.437	24.204	25.489	36.593	35.921
	31.63 cycle[a]	±0.208	±0.253	±0.180	±0.068	±0.447	±0.236	±0.244	±0.426	±0.097	±0.756
	rpoC2 Region	23.393	20.109	21.163	23.151	35.392	24.537	20.249	21.979	34.871	35.007
	29.57 cycle	±0.049	±0.134	±0.065	±0.032	±0.200	±0.087	±0.046	±0.001	±0.845	±0.352
	petB Region	24.393	20.852	22.105	24.261	35.681	25.289	20.718	22.253	35.894	ND
	30.98 cycle	±0.244	±0.013	±0.041	±0.000	±0.000	±0.003	±0.038	±0.203	±0.325	±0.000
Aralia cordata	*accD* Region	15.905	15.794	16.262	15.494	15.998	15.955	16.350	15.957	15.922	15.874
	24.34 cycle	±0.018	±0.085	±0.003	±0.015	±0.035	±0.015	±0.045	±0.007	±0.07	±0.169
	rpoC2 Region	14.744	14.869	15.259	14.581	15.112	14.969	15.272	15.054	14.903	14.562
	26.70 cycle	±0.175	±0.063	±0.011	±0.066	±0.058	±0.002	±0.041	±0.099	±0.161	±0.476
	atpB Region	16.381	16.660	16.793	16.062	16.762	16.783	16.878	16.437	16.147	16.215
	25.22 cycle	±0.152	±0.184	±0.111	±0.018	±0.239	±0.134	±0.128	±0.027	±0.373	±0.808
Ingredient	*O_elatus*	o	o	o	o	x	o	o	o	x	x
	A_cordata	o	o	o	o	o	o	o	o	o	o

[a]Value under the name of the primer sets indicates the cut-off cycles of each designed DNA marker

Table 5 Results of quantitative real-time PCR analysis using 10 commercial herbs products

Species	Sample number	All plants (18 s rRNA)	*OE_ accD (31.63 cycle)*	*OE_rpoC2 (29.57 cycle)*	*OE_petB (30.98 cycle)*	*AC_accD (24.34 cycle)*	*AC_rpoC2 (26.70 cycle)*	*AC_atpB (25.22 cycle)*
O.elatus	1	14.56 ± 0.03	22.04 ± 0.05	21.27 ± 0.06	21.11 ± 0.08	33.95 ± 0.22	ND	ND
A.cordata	2	16.92 ± 0.03	35.31 ± 0.40	35.27 ± 1.30	33.53 ± 1.40	19.87 ± 0.04	18.63 ± 0.02	22.65 ± 0.10
	3	15.78 ± 0.09	36.85 ± 1.46	34.64 ± 0.01	34.60 ± 1.14	19.48 ± 0.04	17.68 ± 0.01	20.90 ± 0.12
	4	15.24 ± 0.10	34.26 ± 0.45	35.20 ± 0.51	33.85 ± 0.76	17.47 ± 0.12	17.02 ± 0.06	18.41 ± 0.06
	5	15.03 ± 0.12	34.71 ± 3.97	34.89 ± 0.06	34.26 ± 0.21	18.25 ± 0.11	16.07 ± 0.03	18.90 ± 0.10
	6	15.35 ± 0.05	34.08 ± 0.42	32.72 ± 0.20	31.63 ± 0.22	18.84 ± 0.14	17.97 ± 0.01	20.18 ± 0.08
	7	16.01 ± 0.04	35.54 ± 0.00	33.88 ± 1.43	34.47 ± 0.12	19.08 ± 0.07	16.17 ± 0.12	21.15 ± 0.37
	8	15.58 ± 0.05	34.94 ± 0.69	34.02 ± 0.23	34.29 ± 0.96	18.98 ± 0.03	17.77 ± 0.06	20.78 ± 0.48
	9	15.92 ± 0.09	35.65 ± 3.53	34.16 ± 0.44	33.54 ± 0.69	19.33 ± 0.02	17.59 ± 0.02	20.43 ± 0.21
	10	15.13 ± 0.04	33.97 ± 0.68	35.55 ± 0.07	34.81 ± 0.05	18.02 ± 0.08	16.14 ± 0.06	18.81 ± 0.36

[a]ND, indicates not detected at less than 40 cycles

typically uncommon to find commercial products containing *O. elatus* in general food markets, and consequently we were limited to examining this single product. The qualities of the gDNA extracted from these 10 herbal medicines were confirmed using the 18S rRNA primer pair as a positive control (Allmann et al., 1993). The low Ct values (14.56–16.92) obtained using this primer pair indicated that all gDNAs extracted from the 10 commercial products were suitable for further examination (Table 5). On the basis of an evaluation of the cut-off Ct values of each of the six primer pairs, we confirmed that the DNA extracted from the *O. elatus* commercial product (Sample No. 1) was amplified with a lower Ct value than the cut-off of the *O. elatus* species-specific primers (*OE_accD, OE_rpoC2,* and *OE_petB*) at 21.11–22.04 cycles, whereas higher Ct values (or no amplification) were detected when using the *A. cordata* species-specific primers. These findings thus indicate that this product contains no traces of *A. cordata*. DNAs isolated from the purchased *A. cordata* products (sample nos. 2–10) showed Ct values lower than the cut-off values of the *A. cordata* species-specific primers (*AC_rpoC2, AC_atpB,* and *AC_accD*) (16.07 to 22.65 cycles) but higher Ct values than the cut-off value for *O. elatus*. Consequently, we established that none of the *A. cordata* products contained *O. elatus* as a constituent. These findings thus indicate that the newly developed primer pairs could be applied for the authentication of commercial herbal products.

Conclusion

The real-time PCR assay developed in this study provides a highly sensitive, reproducible, and rapid method for detecting the desired target species in processed herbal medicines and complex food products. To detect samples of the target species, we constructed standard curves based on a standard curve test and a two-way mixture test, and evaluated the specificity of the developed primer pairs using 14 different non-target plants. In addition, we verified the reliability of the developed DNA markers by conducting a blind test, and assessed the applicability of the developed assay in the authentication of commercial products. This real-time PCR assay would be a particularly effective method for discriminating *A. cordata* and *O. elatus*, and could accordingly make a valuable contribution to the protection of consumers' rights and interests, as well as ensuring food safety.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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