Food Science and Biotechnology (2023) 32:239–247
https://doi.org/10.1007/s10068-022-01176-y

RESEARCH ARTICLE

Establishing DNA markers to differentiate *Agastache rugosa* and *Pogostemon cablin*, which are confusedly used as medicinal herbs, using real-time PCR

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Received: 5 May 2022 / Revised: 7 July 2022 / Accepted: 6 September 2022 / Published online: 1 October 2022
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Abstract
Agastache rugosa and *Pogostemon cablin* are used as medicinal herbs and aromatic plants and belong to the family *Lamiaceae*. Despite differences in composition and physicochemical properties, both plants are frequently sold as the medical substance "Kwakhyang" in some Asian countries. Molecular markers were established to distinguish between the two plants using quantitative real-time PCR. Species-specific primers were designed in the nuclear internal transcribed spacer region of ribosomal DNA and in the chloroplast genes *matK*, *rbcL*, and *rpoB*. Six primer sets were tested, the correlation coefficient was higher than 0.99, and the slope was approximately − 3.36 to − 3.58. Efficiency ranged from 90.13 to 98.52%. The developed real-time PCR assay was validated with 14 off-target species, and its reliability was verified through blind testing of 14 commercial products. The assay developed here may help protect consumer rights, and the designed primers can be used to distinguish between the target species.

Keywords DNA marker · *Lamiaceae* · Medical herb · Species identification · SYBR-GREEN real-time PCR

Introduction

Herbal medicines have been traditionally used to treat and prevent various human diseases. Interest in and acceptance of naturopaths has surged in developing and developed countries in the past, and it is estimated that approximately 4 billion people in developing countries rely on herbal products as a primary means of health care (Ekor, 2014). The value of the growing global herbal medicine market is predicted to reach $129.6 billion by 2023 (Market Research Future, 2018). However, as the use of herbal medicines increases globally, new products are being released, and the safety of herbal medicines is threatened by insufficient quality control and improper labeling (Raynor et al., 2011). Therefore, it is necessary to identify and clearly indicate the exact ingredients of herbal medicines.

Numerous plants belonging to the family *Lamiaceae* (also referred to as mint family) are used as medicinal plants and aromatic crops worldwide. The respective medicinal plants have been reported to exert strong analgesic effects, thus, various *Lamiaceae* crops are promising with respect to novel potential therapeutics with regard to pain treatments (Uritu et al., 2018). Among plants of the *Lamiaceae* family, the dried above-ground part of the annual *Agastache rugosa* (Fisch. & C.A.Mey.) Kuntze is used as an herbal medicine referred to as "Kwakhyang" (Korean designation), whereas dried above-ground parts of the perennial *Pogostemon cablin* Bentham are used as "Gwanggwakhyang" (Korean name; Kim et al., 2003).

Agastache rugosa is known as Korean mint and is cultivated in East Asian countries, including Korea (Shin et al., 2018). This species has been used medicinally to treat common cold, anorexia, cholera, vomiting, poisoning, and other afflictions, and its various biological and pharmacological activities, including antibacterial, antifungal, anti-inflammatory, anticancer, and antioxidant effects, have been documented (Li et al., 2013). *Pogostemon cablin* exerts similar effects to those of *A. rugosa*, however, it is an entirely

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different plant. In the Japanese Pharmacopeia, the generic drug designation of *P. cablin* is "Kwakhyang", thus there is some confusion as to the use of this compound (Ministry of Food and Drug Safety, 2022).

A comparative analysis of the components of essential oils of *A. rugosa* and *P. cablin* (Wu et al., 2013) and a comparative analysis of physicochemical properties through HPLC (Kim et al., 2003) were published; however, no such study has been performed to elucidate differences in DNA sequences.

Various techniques, including spectroscopy and sensory, chromatographic, and DNA-based analyses, have been used to detect inappropriate ingredients in processed foods (Hong et al., 2017). Multiplex PCR and quantitative real-time PCR (qPCR) have been successfully used to detect food fraud because of their timeliness and cost effectiveness, compared to other methods (An et al., 2018; Oh et al., 2022; Kane and Hellberg, 2016). qPCR, which can be used to detect target DNA fragments with high sensitivity and specificity in complex food products, can generally be designed as probe-based qPCR (e.g., TaqMan analysis) and dye-based qPCR (e.g., SYBR Green analysis) (Arya et al., 2005). Probe-based qPCR is used to detect a target sequence through a fluorescent reporter probe and primers (Navarro et al., 2015). However, this method requires many SNPs and indels when designing probes to differentiate between species, thus making it difficult to optimize qPCR conditions (Safdar and Junejo, 2015; Şakalar and Kaynak, 2016). By contrast, SYBR Green-based qPCR can be a more convenient, flexible, and inexpensive method than probe-based qPCR as it is based on DNA intermediate dye that binds to a fluorescent dye independent of double-stranded DNA; however, non-specific PCR products may occur (Safdar and Junejo, 2015).

In this study, molecular markers were developed to distinguish between *A. rugosa* and *P. cablin* through SYBR green-based qPCR. Blind tests and specificity tests showed that this assay is reliable, and it is easily applicable to commercial products.

Materials and methods

Sample preparation

Seeds of *A. rugosa* were kindly provided by the Gangwon-do Agricultural Research and Extension Services (Cheorwon, Gangwondo, Korea). Dried *P. cablin* samples were provided by the Herb Resource Bank of Traditional Korea Medicine (Seoul, Korea). Common non-target plant species were included to estimate marker specificity. The samples used for evaluating specificity and applicability of the developed markers were purchased at a local market. DNA was isolated from fresh or dried leaves.

Genomic DNA extraction

DNA was extracted using a DNeasy Plant Pro Kit (QIGEN, Hilden, Germany) according to the manufacturer's instructions. For large-scale samples, such as in binary mixing tests, DNA was extracted according to a modified large-scale CTAB-based method (Minas et al., 2011). DNA concentration and purity were measured using a SPECTROstar Nano Leader (BMG Labtech, Otenberg, Germany). The DNA purity ratio was 1.7–2.0. At a ratio < 1.7, high-quality DNA was obtained using a Wizard DNA Clean-up System (Promega, Madison, WI, USA).

Binary mixture samples

To test applicability of the developed markers for processed commercial products, a quantitative binary mixture was produced using 2 g of a mixture of *A. rugosa* powder containing *P. cablin* [at 0.1% (2 mg), 1% (20 mg), 10% (200 mg), or 100% (2 g)]. The binary mixture samples were then treated as follows: (1) drying at 55 °C for 48 h in a drying oven, (2) boiling at 100 °C for 15 min, and (3) autoclaving at 121 °C for 20 min at 150 kPa in an autoclave.

Blind samples

A total of 25 blind mixtures were provided by the National Institute of Food and Drug Safety (Cheongju, Korea). The samples were mixtures of *A. rugosa* and *P. cablin* at random proportions. A total of 400 mg of the final mixture was mixed with *A. rugosa* powder or *P. cablin* powder at a concentration of 0–10%, respectively.

Sequence analysis and primer design

Chloroplast DNA sequences of *matK, rbcL,* and *rpoB* of the two target species (*A. rugosa* accession NC_053706.1 and *P. cablin* accession NC_042796.1) and internal transcribed spacer (ITS) sequences of *A. rugosa* (accession MH711397.1) and *P. cablin* (accession KR608755) were downloaded from the National Center for Biotechnology Information (NCBI) database to design specific primers. Nucleotide sequences were aligned using BioEdit 7.2 (Ibis Biosciences, Carlsbad, CA, USA) and ClusteralW2 (EMBL-EBI, Hinston, Cambridgeshire, UK). Species-specific primers were designed using Beacon Designer software (Primer Biosoft, Palo Alto, CA, USA) based on SNP between *A. rugosa* and *P. cablin* and were inspected

manually. Primers were synthesized by a commercial company (Seoul Macrogen, Seoul, Korea).

Cloning of PCR amplicons and sequencing

Conventional PCR was performed using a VeritiPro Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). A mixture of DNA was prepared using TaKaRa Ex Taq DNA Polymerase (TaKaRa, Kusatsu, Shiga, Japan). The PCR conditions were as follows: pre-denaturation for 5 min at 95 °C, 35 cycles of 95 °C for 10 s, 53–61 °C for 30 s (depending on the primers), and 72 °C for 1 min, and a final step of 72 °C for 5 min. The PCR product was cloned using an RBC T&A cloning vector (Real Biotech Corporation, Taipei, Taiwan). Plasmid DNA extracted using the DokDo-Prep Plasmid Mini-Kit (ELPISB) was sequenced by a commercial service (Seoul Macrogen).

Quantitative real-time PCR assay

qPCR was performed using AccuPower® 2X GreenStar qPCR Master Mix (Bioneer, Daejeon, Korea). The reaction mixture comprised 10 µL 2X GreenStar Master Mix, 0.5 µL of each primer (10 pmol), 1 µL 10 ng μL^{-1} genomic DNA, 0.25 µL ROX Dye, and distilled water to 20 µL final volume. The qPCR reaction was performed using a Quantstudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermocycling protocol: 10 min of 95 °C, followed by 40 cycles of 95 °C for 30 s, 58–60 °C for 20 s (depending on the melting temperature of the respective primers), and 72 °C for 30 s. All qPCRs were performed in triplicates.

qPCR standard curve analysis

A standard curve for evaluating the efficiency and sensitivity of the developed primer set was produced by applying a tenfold diluted genomic DNA mix at five concentrations (10 ng/µL to 1 pg/µL) of target gDNA and non-target gDNA. The extracted DNA was diluted to a final concentration of 10 ng/µL, and the thresholds and baselines were set for further experiments (Yuan et al., 2006). The correlation between the cycle threshold (Ct) standard curve and diluted DNA was estimated using basic parameters. The standard curve was calculated as $y = -ax + b$ (where a is the standard curve slope and b is the y-intercept). The percentage efficiency was evaluated as $(E - 1) \times 100\%$, and the reaction efficiency (E) was calculated as $E = (10^{-1/a})$ (European Network of GMO Laboratories, ENGL, 2015; Lo and Shaw, 2018).

Interlaboratory validation

Interlaboratory validation was performed using two laboratories to confirm the reproducibility and accuracy of the developed assay. Interlaboratory validation was performed using the Applied Biosystems 7500 Fast Real-Time PCR Instrument System (Applied Biosystems) and the Step 1 Plus Real-Time PCR System (Applied Biosystems) under the same qPCR conditions.

Results and discussion

DNA markers in variable regions

Primer sets for distinguishing *A. rugosa* and *P. cablin* were designed in the nuclear ITS region of ribosomal DNA and in chloroplast genes such as *matK, rbcL*, and *rpoB*. The ITS region of the rRNA gene, which is a ribosomal gene essential for protein synthesis, is indispensable for organisms and is well conserved in plants. Therefore, because ITS fragments occur in almost all taxa, such primers are well established and can be used for a wide range of organisms (Baldwin et al., 1995). Chloroplast genes such as *matK*, *rbcL,* and *rpoB* have also been used for species identification in other studies (CBOL Plant Working, 2009). Four genes (*matK*, *rbcL*, *rpoB*, and *ITS*) of both species were downloaded from the NCBI database to design species-specific primer sets. Subsequently, the genes were cloned using conventional PCR-amplicon from both plants and sequenced to verify the downloaded sequences. There was no difference in the sequences between the NCBI data and the cloned genes. Species-specific primer sets were designed based on various SNPs between the aligned sequences of nuclear DNA and chloroplast genomes of *A. rugosa* and *P. cablin* (Supplementary Fig. 1). Food processing may affect DNA quality as DNA may be broken down into short pieces during drying, heating, and blending (Lo and Shaw, 2018). Therefore, we developed species-specific primers to detect target species in commercial products by amplifying relatively short amplicons (109–180 bp; Table 1).

Amplification efficiency of the designed primer sets

The sensitivity of six primers pairs (AR_matK, AR_rbcL, AR_ITS, PC_matK, PC_rbcL, and PC_rpoB) was evaluated by constructing a standard curve using tenfold serially diluted DNA extracted from leaves (10–0.001 ng/µL) and performing regression analyses. The correlation coefficients of the six primer sets exceeded 0.99, and the slopes were between − 3.36 and − 3.58. Also, the efficiency, according to the slope, was estimated to range from 90.13 to 98.52% (Fig. 1). According to the guidelines of the

Table 1 Developed primer sets and established universal 18S rRNA primers for all plants

Species	Target gene	Primer	Length (bp)	Sequence (5'→3')	Amplicon size (bp)	Tm (°C)
Agastache rugosa	*ITS*	*ITS_F*	17	AGTGGTGGTTGAACTCA	149	59
		ITS_R	19	CTTAAACTCAGCGGGTAAT		
	matK	*matK_F*	20	TTCTGGAGTTCTTCTTGAAA	156	59
		matK_R	18	CCCTTGGAAGCCATAATG		
	rbcL	*rbcL_F*	18	AAGATCTGCGAGTTCCTA	122	58.5
		rbcL_R	21	AGGTTTAATAGTACATCCCAG		
Pogostemon cablin	*rpoB*	*rpoB_F*	21	ACTTCAACTCCATTAACAACT	180	58
		rpoB_R	19	ACTAGGATGGATATCTCGG		
	matK	*matK_F*	18	GGTTCAAATCGTTCGCTA	110	59
		matK_R	19	TCGCTTTCTTTGGAGTAAT		
	rbcL	*rbcL_F*	21	GAGGGTAATGTAATTATCCGC	113	60.5
		rbcL_R	20	TCATCCAAAGTATCCACTGG		
All plants	18 s rRNA region	18 s rRNA_F	24	TCGATGGTAGGATAGTGCCCTACT	109	58
		18 s rRNA_R	23	TGCTGCCTTCCTTGGATGTGGTA		

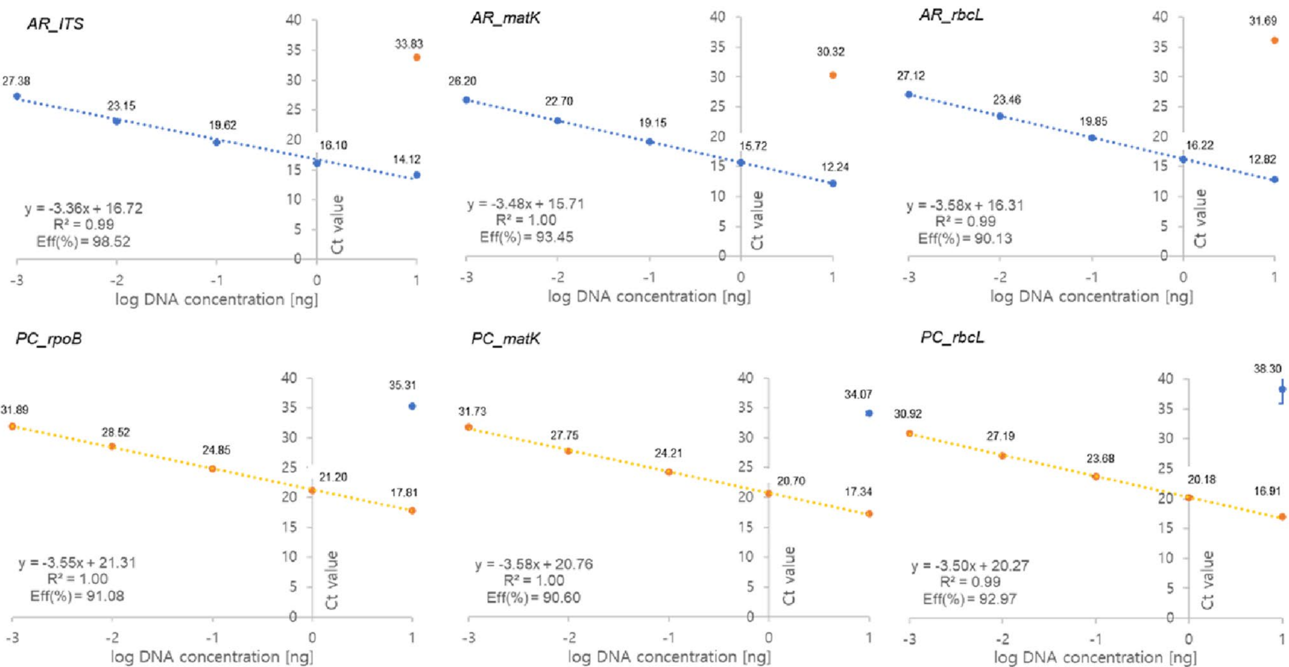

Fig. 1 A standard curve was obtained based on the efficiency and correlation of coefficient (R^2) from genomic DNA serially diluted tenfold using primers targeting *A. rugosa* and *P. cablin*. The *x*-axis indicates the log-transformed DNA concentration (ng) and the *y*-axis represents means of Ct values ± SD. **A** *Agastache rugosa* targeting primer sets (AR_ITS, AR_,matK, and AR_rbcL). **B** *Pogostemon cablin* targeting primer sets (PC_rpoB, PC_matK, and PC_rbcL). Blue and yellow dots indicate *A. rugosa* and *P. cablin*, respectively. qPCRs were carried out in triplicates ($n=3$)

ENGL, qPCR assays were considered acceptable based on the linear dynamic range and amplification efficiency. The linear operating range should ideally extend to a four $\log_{10}$ concentration with a coefficient of correlation (R^2) > 0.98, and the amplification efficiency should be in the range of 110%–90%, corresponding to a slope of − 3.1 to − 3.6 (ENGL, 2015). Our results corresponded to the guidelines with a coefficient of determination of ≥ 0.98 and a slope range of − 3.36 to − 3.58 (Fig. 1). To test assay reproducibility, different qPCR devices were used at different laboratories. The correlation coefficient exceeded 0.99, the slope was − 3.38 to − 3.58, and PCR efficiency was 90.16–97.69%

(Supplementary Table 1). Therefore, amplification efficiency and reproducibility of the assay were considered acceptable.

Applicability and specificity of the developed real-time PCR assay

In general, commercial products of medicinal herbs, including *A. rugosa* and *P. cablin*, can be processed by heat, dry, and autoclave treatments. Food processing can cause severe DNA degradation in herbs. For example, heat treatment is known to cause severe DNA fragmentation, rendering PCR-based analysis inefficient (Hwang et al., 2015). The slope, correlation coefficient and efficiency of the designed assay were investigated by drying, heating (100 °C), and autoclaving samples in a reference binary mixture to confirm assay applicability for commercial herbal medicine foods. Six standard curves of *A. rugosa* and *P. cablin* in non-treated, dried, heated, and autoclaved samples were produced (Table 2). All slopes ranged between − 3.10 and − 3.58, and the correlation coefficient was ≥ 0.99 for the six developed primer sets. The slope efficiencies were estimated to range from 90.13 to 109.63%. These results suggested that the developed markers can detect target species in processed food products. Subsequently, DNA extracted from dried, heated, and autoclaved leaf binary mixtures (0.1–100% w/w) were diluted to 10 ng/μL and were used for qPCR analysis. Ct values were obtained for each sample and processing method (Supplementary Table 2). Binary mixtures using dried leaves showed higher Ct values than those of the untreated samples. However, higher Ct values were observed in heated and autoclaved samples than in the dried samples. These results indicated less DNA degradation in dried than in heated and autoclaved samples, suggesting the use of different Ct values, depending on the food processing method. In general, impurities of < 0.1% are not considered illegal mixtures for economic gain. Therefore, the target species Ct values of 0.1% of all binary mixtures with dried samples were used as the cut-off for detecting target DNA in commercial food products. The obtained cut-off Ct values ranged from 22.99 to 23.75 for the three developed primer sets targeting *A. rugosa* and 28.00 to 29.17 for the three sets targeting *P. cablin* (Supplementary Fig. 2).

Fourteen plant species were used to confirm primer specificity. In particular, *Lamiaceae* herbal medicine crops such as peppermint (*Mentha* × *piperita*), apple mint (*M. suaveolens*), and spearmint (*M. spicata*) were included because of their intended or non-intended use as spice ingredients in processed foods containing *A. rugosa* from *P. cablin*. The 18S rRNA primer set was used as a positive control, and qPCR amplification was observed with all tested species at < 20 cycles. qPCR analysis with the six developed primers was performed on 16 species including *A. rugosa* and *P. cablin* (Table 3). Except for the *AR_ITS* and *PC_rbcL* primers, no PCR products were amplified at < 40 cycles. The *AR_ITS* and *PC_rbcL* primers amplified *Mentha* species with the high Ct (22.96) and *Lavandula stoechas* with the high Ct (24.34) respectively, but less than 40 cycles. Taken together, all developed primer sets can be used to

Table 2 Evaluation of slope, R^2, and efficiency obtained with three processing methods (dried, heated, and autoclaved) of a reference binary mixture using the developed primers

DNA standard curve					Dry-treated binary mixture standard curve				
Target species	Primer	*Y* (slope)	R^2	Effeciency (%)	Target species	Primer	*Y* (slope)	R^2	Effeciency (%)
A. rugosa	*AR_ITS*	− 3.36	0.99	98.52	*A. rugosa*	*AR_ITS*	− 3.16	1.00	106.90
	AR_matK	− 3.48	1.00	93.45		*AR_matK*	− 3.11	0.99	109.63
	AR_rbcL	− 3.58	0.99	90.13		*AR_rbcL*	− 3.19	0.99	105.49
P. cablin	*PC_rpoB*	− 3.55	1.00	91.08	*P. cablin*	*PC_rpoB*	− 3.19	0.99	105.43
	PC_matK	− 3.58	1.00	90.60		*PC_matK*	− 3.25	0.99	102.99
	PC_rbcL	− 3.50	0.99	92.97		*PC_rbcL*	− 3.10	0.99	110.08
Heat-treated binary mixture standard curve					Autoclave-treated binary mixture standard curve				
Target species	Primer	*Y* (slope)	R^2	Effeciency (%)	Target species	Primer	*Y* (slope)	R^2	Effeciency (%)
A. rugosa	*AR_ITS*	− 3.16	1.00	107.26	*A. rugosa*	*AR_ITS*	− 3.28	0.99	103.67
	AR_matK	− 3.22	1.00	104.54		*AR_matK*	− 3.16	0.99	107.35
	AR_rbcL	− 3.19	1.00	105.97		*AR_rbcL*	− 3.17	0.99	106.80
P. cablin	*PC_rpoB*	− 3.21	0.99	104.99	*P. cablin*	*PC_rpoB*	− 3.5	1.00	93.07
	PC_matK	− 3.25	0.99	103.08		*PC_matK*	− 3.53	1.00	91.97
	PC_rbcL	− 3.51	1.00	92.55		*PC_rbcL*	− 3.16	0.99	107.02

Table 3 Specificity test results using other plants and the developed qPCR primer sets

No.	Family	Species	Whole plant systems	*Agastache rugosa*			*Pogostemon cablin*		
			18 s rRNA	*ITS*	*matK*	*rbcL*	*rpoB*	*matK*	*rbcL*
		Curt-off Ct values		23.75	23.66	22.99	29.17	28.89	28.00
1		*Agastache rugosa*	[a]++	++	++	++	–	–	–
2		*Pogostemon cablin*	++	[b]–	–	–	++	++	++
3		*Mentha canadensis*	++	–	–	–	–	–	–
4		*Rosmarinus officinalis*	++	–	–	–	–	–	–
5	*Lamiaceae*	*Ocimum basilicum*	++	–	–	–	–	–	–
6		*Lavandula stoechas*	++	–	–	–	–	–	+
7		*Mentha suaveolens*	++	–	–	–	–	–	–
8		*Melissa officinalis*	++	–	–	–	–	–	–
9		*Mentha species*	++	[c]+	–	–	–	–	–
10		*Artemisia capillaris*	++	–	–	–	–	–	–
11	*Compositae*	*Artemisia gmelinii*	++	–	–	–	–	–	–
12		*Atractylodes japonica*	++	–	–	–	–	–	–
13	*Poaceae*	*Zea mays*	++	–	–	–	–	–	–
14	*Liliaceae*	*Hemerocallis fulva*	++	–	–	–	–	–	–
15	*Zingiberaceae*	*Curcuma longa*	++	–	–	–	–	–	–
16	*Fabaceae*	*Glycyrrhiza uralensis*	++	–	–	–	–	–	–

[a]++, indicates that the sample was amplified before cut-off Ct cycles

[b]–, indicates no amplification before 40 cycles

[c]+, indicates amplification between more than the cut-off Ct cycles and less than 40 cycles

detect the target species, without cross-reactivity from other plant species.

Application of the developed qPCR assay to blind samples

A blinded test was performed on 25 samples to estimate qPCR assay reliability. An independent study group randomly mixed 25 blind samples and did not provide any information on how much each *A. rugosa* and *P. cablin* powder was mixed. The 18S rRNA primer set used as a positive amplification control showed low Ct values (11.97–13.34 cycles), indicating DNA quality was sufficient for evaluation (Table 4). Whether *P. cablin* powder was present in the *A. rugosa* samples was determined based on the cut-off Ct values of the developed primer sets (PC_rpoB, 29.17; PC_matK, 28.89; and PC_rbcL, 28.00). High Ct values of the *P. cablin* primer set were observed in seven samples, indicating that the *P. cablin* powder was not mixed with the *A. rugosa* powder in these cases. The Ct of the remaining 18 samples was lower than the cu-off value of the *A. rugosa* and *P. cablin* primer sets, indicating that the samples were mixed with *A. rugosa* and *P. cablin* powder. The results from 25 blind samples indicated that the qPCR assay can help confirm authenticity of *A. rugosa* and *P. cablin* products.

Application of the developed assay to commercial herbal medicines

A total of 14 commercial products, including nine samples designated as *A. rugosa* and five designated as *P. cablin*, were purchased for authenticity tests (Table 5). DNA quality was evaluated using the 18S rRNA primer set as the positive control. The 18S rRNA primer set showed low Ct values (12.87–15.30), indicating sufficient DNA quality for examining the developed assay. Therefore, the commercial products were examined using the cut-off Ct values of the six primer sets (Table 5). *A. rugosa* products (samples 1–9) showed lower Ct values (14.29–15.95) than those of the *A. rugosa*-specific primers. However, they showed higher Ct values (29.63–35.79) than those of the *P. cablin*-specific primer sets. The nine examined *A. rugosa* products did not contain *P. cablin*. *P. cablin* products (samples 10–14) showed lower Ct values (16.19–18.62) than the cut-off Ct values of the *P. cablin*-specific primers and were amplified with higher Ct values (28.02–33.35) than the cut-off Ct values of the *A. rugosa*-specific primer sets. Therefore, the five tested *P. cablin* products appeared to not contain *A. rugosa*. These results suggest that the developed qPCR assay can be used to detect both the target species (*A. rugosa* and *P. cablin*) in commercial herbal medicine products.

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

Primer sets		*A. rugosa* and *P. cablin* blind test																								
Sample number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Positive control	18 s rRNA	13.004	12.689	12.803	13.343	12.221	12.812	12.796	12.089	12.020	12.242	12.143	11.970	12.146	12.951	13.006	12.934	12.223	12.741	12.733	12.501	11.933	12.633	12.431	12.503	12.510
		±0.042	±0.033	±0.030	±0.040	±0.014	±0.042	±0.120	±0.131	±0.031	±0.079	±0.028	±0.187	±0.055	±0.023	±0.008	±0.110	±0.062	±0.046	±0.016	±0.045	±0.042	±0.098	±0.007	±0.040	±0.018
	ITS Region	17.553	17.211	16.999	17.859	16.304	17.308	17.413	16.783	16.622	17.108	16.390	16.267	16.764	17.598	17.738	17.733	19.747	17.267	17.228	17.025	16.230	17.146	17.141	17.646	17.062
	23.75cycle[a]	±0.048	±0.086	±0.009	±0.118	±0.046	±0.084	±0.182	±0.120	±0.093	±0.146	±0.077	±0.064	±0.042	±0.030	±0.040	±0.112	±0.362	±0.180	±0.116	±0.080	±0.090	±0.112	±0.111	±0.076	±0.062
A. rugosa	*matK* Region	15.036	14.711	14.835	15.554	14.322	14.693	14.910	14.118	13.998	14.879	14.308	14.303	14.186	15.139	15.142	14.526	17.833	14.916	14.946	14.847	14.197	14.838	14.778	14.821	14.850
	23.66 cycle	±0.039	±0.019	±0.047	±0.018	±0.011	±0.125	±0.009	±0.177	±0.017	±0.080	±0.073	±0.082	±0.035	±0.103	±0.032	±0.653	±0.047	±0.036	±0.031	±0.055	±0.055	±0.076	±0.025	±0.010	±0.003
	rbcL Region	15.210	14.910	14.941	15.707	14.330	15.024	15.101	14.282	14.191	14.893	14.131	14.358	14.535	15.899	15.302	15.128	17.183	14.954	15.035	14.920	14.347	14.894	14.850	14.890	14.931
	22.99 cycle	±0.111	±0.038	±0.001	±0.001	±0.011	±0.035	±0.080	±0.068	±0.148	±0.022	±0.054	±0.128	±0.058	±0.920	±0.017	±0.000	±0.133	±0.024	±0.025	±0.011	±0.001	±0.030	±0.051	±0.088	±0.069
	rpoB Region	36.889	23.007	35.649	26.235	22.743	35.915	ND	24.736	22.340	23.766	24.847	35.553	39.677	24.996	23.843	22.819	22.381	23.050	23.704	ND	22.182	23.790	23.002	23.745	24.851
	29.17 cycle	±0.000	±0.053	±0.000	±0.124	±0.085	±0.000	±0.000	±0.050	±0.029	±0.043	±0.184	±0.000	±0.000	±0.086	±0.057	±0.028	±0.0169	±0.010	±0.009	±0.000	±0.067	±0.031	±0.375	±0.028	±0.108
P. cablin	*matK* Region	[b] ND	22.294	NA	25.628	22.260	ND	35.329	24.101	21.754	23.303	24.271	36.320	36.179	24.185	23.189	22.034	22.687	22.633	23.319	ND	21.784	23.181	22.888	23.335	24.219
	28.89 cycle	±0.000	±0.013	±0.000	±0.046	±0.051	±0.000	±0.000	±0.242	±0.016	±0.058	±0.013	±0.000	±0.000	±0.040	±0.053	±0.064	±0.049	±0.092	±0.063	±0.000	±0.011	±0.087	±0.004	±0.105	±0.089
	rbcL Region	34.903	21.913	34.511	25.225	21.771	34.444	34.958	23.720	21.273	22.724	23.778	34.372	35.208	23.932	22.825	21.883	22.043	22.034	22.696	33.995	21.210	22.706	22.172	22.815	23.808
	28.00 cycle	±0.000	±0.050	±0.000	±0.081	±0.021	±0.000	±0.000	±0.049	±0.039	±0.107	±0.086	±0.272	±0.033	±0.094	±0.008	±0.070	±0.078	±0.009	±0.056	±0.037	±0.078	±0.081	±0.068	±0.058	±0.074
Ingredient	*A_rugosa*	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	P_cablin	X	O	X	O	O	X	X	O	O	O	O	X	X	O	O	O	O	O	O	X	O	O	O	O	O

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

[b]Indicates not detected

Table 5 Results of qPCR analysis using 14 commercial herbs

A. Commercial food products declared to contain *Agastache rugosa* in labelling

Species	Sample number	All plants	*AR_ITS*	*AR_matK*	*AR_rbcL*	*PC_rpoB*	*PC_matK*	*PC_rbcL*
		18 s rRNA	23.75 cycle	23.66 cycle	22.99 cycle	29.17 cycle	28.89 cycle	28.00 cycle
A. rugosa	1	14.31 ± 0.05	[a]+	+	+	[c]NA	–	–
	2	13.67 ± 0.03	+	+	+	NA	–	–
	3	14.36 ± 0.02	+	+	+	NA	–	–
	4	14.13 ± 0.04	+	+	+	NA	–	–
	5	13.28 ± 0.01	+	+	+	NA	–	–
	6	13.31 ± 0.09	+	+	+	–	–	–
	7	13.57 ± 0.06	+	+	+	–	–	–
	8	12.90 ± 0.10	+	+	+	–	–	–
	9	12.87 ± 0.00	+	+	+	–	–	–

B. Commercial food products declared to contain *Pogostemon cablin* in labelling

	Sample number	All plants	*AR_ITS*	*AR_matK*	*AR_rbcL*	*PC_rpoB*	*PC_matK*	*PC_rbcL*
		18 s rRNA	23.75 cycle	23.66 cycle	22.99 cycle	29.17 cycle	28.89 cycle	28.00 cycle
P. cablin	10	15.16 ± 0.05	[b]–	–	–	+	+	+
	11	15.01 ± 0.08	–	–	–	+	+	+
	12	15.30 ± 0.01	–	–	–	+	+	+
	13	14.29 ± 0.10	–	–	–	+	+	+
	14	14.45 ± 0.10	–	–	–	+	+	+

[a]+, indicates amplification before cut-off Ct cycles

[b]–, indicates amplification between more than cut-off Ct cycles and less than 40 cycles

[c]NA, indicates no amplification at less than 40 cycles

The qPCR assay developed here is a rapid and sensitive method for detecting target species in processed herbal medicines. To quantify the target species, a standard curve was constructed using binary mixture tests. Additionally, the specificity of the developed primer set was evaluated using 15 other species. Assay reliability was verified by blind testing and application to commercial products. The developed qPCR assay constitutes an effective identification method for distinguishing *A. rugosa* and *P. cablin* and may thus contribute to food safety and the protection of consumer rights.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10068-022-01176-y.

Acknowledgements This research was supported by a Grant (17162MFDS065) from the Ministry of Food and Drug Safety in 2021.

Declarations

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

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