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RESEARCH ARTICLE

Development and application of DNA markers to detect adulteration with *Scopolia japonica* in the medicinal herb *Atractylodes lancea*

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Abstract *Atractylodes lancea* rhizomes are commonly consumed in east Asia as traditional medical herbs. However, in Korea, because of their morphological similarity, *A. lancea* rhizomes can be contaminated with those of *Scopolia japonica* imported from China. To detect adulteration with *S. japonica* in the complex products of *A. lancea*, we developed two PCR-based DNA markers, multiplex PCR and quantitative real-time PCR. The sensitivity of the multiplex PCR primer combinations and real-time PCR was confirmed with a series of DNA concentrations (0.01–10 ng/μL). The specificity of the developed PCR assays was confirmed with 14 other species. In addition, 14 commercial *A. lancea* medicinal herbs and 20 blind samples were tested with the developed PCR assays to demonstrate the reliability. Taken together, the developed multiplex and real-time PCR-based target-specific primer sets may be useful for detecting the target species and have the potential to contribute to food safety and consumer health care.

Keywords *Atractylodes lancea* · Multiplex PCR · SNPs · Species identification · SYBR-GREEN real-time PCR

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Introduction

Medical herbs have been used in traditional medicine practices for a long time. According to the World Health Organization (WHO) reports, about 80% of the population in developing countries relies on medical herbs for primary health care (World Health Organization (WHO, 2007). In addition, 19% of the population in the United States of America uses medicinal herbs for medical treatment (Gardiner et al., 2007). The American botanical council reported that the sales of traditional herbal medicines in the US reached a total of $7.45 billion in 2016 (Smith et al., 2017). The market value of herbal medicine is expected to grow and reach a global value of $129.68 billion by 2023 (Market Research Future, 2018).

A Chinese herbal medicine, *Atractylodes lancea* (Tunb), belongs to the *Compositae* family and has long been used as an important herb in East Asia. In general, *A. lancea* has been used to reduce acute digestive disorders, rheumatic disease, night blindness, and influenza (Qian et al., 2006). The substances extracted from *A. lancea* are effective in exerting anticancer, anti-digestive disorders, anti-obesity, and anti-inflammatory properties (Koonrungsesomboon et al., 2014). Among these properties, the anticancer activities of *A. lancea*, particularly against cholangiocarcinoma cells, have been demonstrated in previous studies, both *in vitro* and *in vivo*. The crude ethanolic extract of *A. lancea* rhizome was shown to reduce potently and selectively the viability of the cholangiocarcinoma cell line (CL-6), with an IC_{50} (concentration that controls cell growth by 50%) of 24.09 g/mL and a selectivity index of 8.6 (Mahavorasirikul et al., 2010). In addition, *A. lancea* extract has been shown to delay gastric emptying and stimulate small intestinal motility. The anti-digestive disorder mechanisms of *A. lancea* extract are related to the

inhibition of dopamine D_2 and 5-HT_3 receptors, the activation of vagal tone, and the inhibition of corticotropin-releasing factor (Zhang et al., 2008).

According to the Ministry of Food and Drug Safety reports, Korea, in 2017, the adulteration of *A. japonica*, which belongs to the same genus as *A. lancea*, with *Scopolia japonica* was reported, as well as adulteration addictive symptoms that were caused by *S. japonica*. *S. japonica* belongs to the *Solanaceae* family and is a highly toxic plant (Okamura et al., 1992). In the Solanaceae family, the two most typical tropane alkaloids, hyoscyamine and scopolamine, have been found (Hashimoto and Yamada, 1987). Hyoscyamine functions as an anticholinergic and antispasmodic agent, while scopolamine (hydrobromide) is an authentic hallucinogen (Heinrich and Gibbons, 2001). The main addictive symptom, hallucination, presents visual, auditory, and tactile symptoms (Magos et al., 1987). However, the morphological similarity between *A. lancea*, *A. japonica*, and *S. japonica* rhizomes can result in adulteration, intentionally or unintentionally. Therefore, the development of a methodology to detect adulteration of *S. japonica* in medicinal herbs, from the genus *Atractylodes*, is required for consumer safety.

Various technologies, such as sensory, physicochemical, chromatographic, spectroscopic, and DNA-based assays, have been used to detect the fraudulent ingredients in complicated mixed foods (Hong et al., 2017). Among them, DNA-based methods, such as quantitative real-time PCR (qPCR), multiplex PCR, and PCR–RFLP have been successfully applied to detect food fraud and adulteration due to their economic and time-saving advantages over other approaches (Kane and Hellberg, 2016; An et al., 2018). In particular, DNA from two or more target species in the same reaction can be reliably detected using multiplex PCR. Multiplex PCR has been successfully used to detect species in complex foods, such as meat (Matsunaga et al., 1999; Rodríguez-Plaza et al., 2006; López-Andreo et al., 2006), fish, and seafood (Trotta et al., 2005) products. Furthermore, multiplex PCR has been used as a rapid and convenient detection assay for GM crops, such as maize, soybean, and canola (James et al., 2003; Germini et al., 2004; Forte et al., 2005). Although it has many advantages, multiplex PCR also has many challenges, such as cross-reactivity among the primer combinations, low sensitivity, and lack of efficiency in various food products for broad applications (Xu et al., 2012).

Another DNA-based assay, quantitative real-time PCR assay (qPCR), can be detect complex foods with high specificity and sensitivity. qPCR methods are divided into probe-based real-time PCR (TaqMan assay) and dye-based real-time PCR (SYBR Green I assay) (Arya et al., 2005). In the probe-based real-time PCR assay, the target sequence is specifically amplified with a gene-specific primer set and a fluorescent reporter probe, which are designed to be complementary to a target sequence (López-Calleja et al., 2007). During the extension stage, the probe is broken apart by DNA-polymerase, and then, the fluorescence signals increase in intensity. However, this approach requires many SNPs or indels to design probes, and it is difficult to optimize real-time PCR conditions (Safdar and Junejo, 2015; Şakalar and Kaynak, 2016). On the other hand, SYBR green-based real-time PCR is a more flexible, convenient, and inexpensive method compared to probe-based real-time PCR methods, because the SYBR green dye interacts with double-stranded DNA in a sequence-independent manner (Safdar and Junejo, 2015). However, the drawback of the SYBR green based real-time PCR is that non-specific PCR products might be amplified as a false-positive signal (David, 2000).

The application of nuclear genes as target sequences for a DNA barcode has some problems due to their low copy numbers and low discriminatory power between species (Chase et al., 2007). Internal transcribed spacer (ITS) regions of ribosomal DNA have been used as molecular markers of species discrimination in eukaryotes (Vijayan and Tsou, 2010). It is believed that the sequences of the ribosomal DNA genes are well-conserved, while their spacer sequences possess many inter-species variations. For this reason, the ITS region has good potential for designing DNA markers for species identification (Hirao et al., 2009; Pereira et al., 2008).

Chloroplasts are organelles in plants, and their function is to conduct photosynthesis. The chloroplasts genome is generally a single circular molecule, and its size varies among species, ranging from 107 to 208 kb (Daniell et al., 2016). The chloroplasts genes are generally present in hundreds of copies per cell, and the characteristic two membrane layers protect DNA from degradation during food processing, such as heating, fermentation, and boiling (Garino et al., 2016). The chloroplast genome generally contains 120–130 genes. Certain chloroplast genes e.g., *matK*, *ndhF*, *ycf1*, and *ccsA*, exhibit higher frequencies of variations, including single-nucleotide polymorphisms (SNPs) and insertion/deletions (indels), than other chloroplast genes (Daniell et al., 2016). Therefore, these chloroplast genes are usually used for plant species identification in food complexes.

In this study, we developed two PCR-based assays, multiplex PCR and SYBR green-based qPCR, to identify *A. lancea* and *S. japonica*. We subsequently examined their sensitivity, specificity, and reliability, and the two PCR assays clearly detected adulteration with *S. japonica* in the medical herb *A. lancea*. Subsequently, the two developed PCR-based molecular markers were applied to commercial *A. lancea* products.

Materials and methods

Sample preparation

The *A. lancea* dried leaves and rhizomes were kindly provided by the National Institute of Food and Drug Safety Evaluation of the Ministry of Food and Drug Safety (Cheongju, Korea). The *S. japonica* plants and DNAs were kindly provided by the Medical Plant Taxonomy lab from Kangwon National University (Chuncheon, Korea) and the National Institute of Biological Resources (NIBR) (Incheon, Korea), respectively. Both plants' genomic DNA were isolated from leaves or rhizomes.

Binary A. lancea and S. japonica mixtures

Binary ground rhizomes mixtures that contained different amounts (0.1, 1, 10, and 100%, total 2 g) of *A. lancea* were mixed with *S. japonica* (99.9, 99, 90, and 0%). Additionally, to evaluate the applicability of the designed method for processed rhizome products, the reference binary mixtures were treated with three different processing conditions, as follows: (1) dried at 55 °C for 48 h in a dry oven, (2) boiled at 100 °C for 15 min in a water bath, and (3) autoclaved at 121 °C 150 kPa for 20 min.

Blind samples

Blind mixture samples (n = 20) were provided by the National Institute of Food and Drug Safety Evaluation of the Ministry of Food and Drug Safety (Cheongju, Korea). The blind mixture samples were randomly mixed with different percentages of *A. lancea* and *S. japonica* ground rhizomes. The ground *S. japonica* rhizomes were added to those of *A. lancea* at concentrations of 0–10% (w/w), to prepare final mixtures of 2 g.

Genomic DNA extraction

DNA was isolated from the dried leaves using the DNeasy Plant Pro Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. To effectively isolate genomic DNA from large amounts of binary mixture rhizome samples, a modified large-scale CTAB-based method was used (Minas et al., 2011). DNA quantity and purity were checked using a SPECTROstar Nano reader (BMG Labtech, Ortenberg, Germany). The DNA purity was in the range of 1.7–2.0. If the DNA purity was less than 1.7, it was cleaned using the Wizard DNA Clean-Up system (Promega, Madison, USA).

Sequence analysis and primer design

Target gene sequences, such as *matK*, *ycf1*, and *ITS2* of *A. lancea* (NC_037483.1), were downloaded from the National Center for Biotechnology Information (NCBI) and used to design target-specific primer sets. Information on certain target gene sequences, such as *matK*, *ycf1*, and *ITS2* in *S. japonica,* were lacking in the NCBI database. To obtain these sequences, conventional primer sets were designed based on their *S. parviflora* nucleotide sequences (NC_030282.1), which were closely related to those of *S. japonica*. After PCR using DNA from *S. japonica*, which was kindly provided by NIBR, the amplicons were sequenced using a commercial service (Macrogen, Seoul, Korea) and nucleotide sequence alignments of both species were conducted using ClustalW2 (EMBL-EBI, Hinxton, Cambridgeshire, UK) and BioEdit 7.2 (Ibis Biosciences, Carlsbad, CA, USA). Target-specific primer sets were designed based on the variable region between *A. lancea* and *S. japonica* using Beacon DesignerTM (PRIMER Biosoft, Palo Alto, CA, USA) and commercially synthesized (Macrogen, Seoul, Korea).

Multiplex PCR analysis

Multiplex PCR analysis was carried out using TaKaRa Ex Taq DNA polymerase (TaKaRa Bio Company, Kusatsu, Shiga, Japan), with mixtures of 10 ng/μl DNA and different concentrations (10–50 pmol) of each multiplex primer combination using a C1000 Thermal Cycler (BIO-RAD, California, USA) (Supplementary Table 1). To examine the efficiency of multiplex PCR primer sets, ten-fold dilutions (0.01–10 ng/μl) of DNAs from both target species (*A. lancea* and *S. japonica,* 1:1 ratio v/v) were used as templates for multiplex PCR testing. The multiplex PCR amplification conditions were as follows: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 20 s, primer extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. Multiplex PCR products were electrophoresed on a 2% agarose gel and then visualized using a Chemi Doc XRS$^+$ (BIO-RAD, California, USA).

Quantitative real-time PCR analysis (qPCR)

qPCR analysis was performed using the GoTaq® qPCR Master Mix kit (Promega, Madison, Wisconsin, USA). The real-time PCR reaction mixture consisted of 10 μL 2X GoTaq® qPCR Master Mix; 0.5 μl of 10 pmol each primer; 1 μl of 10 ng/μl DNA; 0.2 μl of the CXR reference dye; and free water, up to a final volume of 20 μl. Quant studio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform real-time PCR

amplification as follows: pre-denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 55.5–65.0 °C for 60 s according to each primer annealing temperature, and primer extension at 60 °C for 60 s. All qPCR reactions were performed in triplicate.

Construction of real-time PCR standard curve and data analysis

The efficiency of the designed real-time primer sets was confirmed using target and non-target DNA that was diluted ten-fold into five series (10 ng–1 pg/μl). Isolated DNA from each binary mixture was diluted to a final concentration of 10 ng/μL, and a baseline and threshold were set for further analysis. The cycle numbers at the threshold level of log-based fluorescence were defined as the cycle threshold (Ct) numbers, which represented the observed value in the real-time PCR experiments (Yuan et al., 2006). Correlations between diluted DNAs and cycle threshold (Ct) standard curves were confirmed using a default parameter. According to the ENGL documents, the standard curve was calculated as $y = -ax + b$ (a indicates the standard curve slope and b indicates the y-intercept) (ENGL, 2015). The efficiency of the reaction (E) was calculated as $E = (10^{-1/a})$, and the efficiency of the primer sets was evaluated as $(E - 1) \times 100\%$ (ENGL, 2015; Lo and Shaw, 2018). To evaluate the amplification sensitivity of the designed real-time PCR primer sets, two criteria should be confirmed to define an acceptable real-time PCR assay, based on previous reports (Bustin et al., 2009): linear dynamic range and amplification efficiency. The linear dynamic range should ideally extend over four $\log_{10}$ concentrations, with the coefficient of determination (R^2) being greater than 0.98. Additionally, the amplification efficiency should be in the range of 110–90%, which corresponds to a slope between − 3.1 and − 3.6 (ENGL, 2015).

To validate the accuracy and sensitivity of the designed target-specific qPCR primer sets, an interlaboratory experiment was performed in two different laboratories. Validation was performed in two laboratories using the same qPCR conditions, with either an Applied Biosystems 7500 Fast Real-Time PCR Instrument System (Applied Biosystems, Foster City, CA, USA) or a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Results and discussion

Design of the variable region-based DNA markers

First, the *A. lancea* and *S. japonica* specific multiplex PCR primer combinations were designed from chloroplast genes, including *matK* and *ycf1*. The variable sequences in the chloroplast gene (*matK* and *ycf1*) between two species were detected by aligning the target gene sequences. These genes (*matK* and *ycf1*) showed high sequence variability between *A. lancea* and *S. japonica*. As shown in Supplementary Fig. 1, the target-specific multiplex PCR primers were designed with one common forward primer shared between the two species, and target-specific reverse primers were designed based on target sequences that had polymorphisms and differences in amplicon sizes between the two species (Supplementary Fig. 1 and Supplementary Table 1). The multiplex PCR amplification product size ranged from 279 to 499 (Supplementary Table 1).

Secondly, the target-specific qPCR primer sets were designed from the nuclear ITS region of ribosomal DNA and chloroplast genes, *matK* and *ycf1*. Sequence alignments between *A. lancea* and *S. japonica* were performed, and we designed the qPCR primer sets based on variable sequences between both plants (Supplementary Fig. 2 and Supplementary Table 1). Food processing, such as drying, heating, and mixing, is known to be harmful and reduces the quality of DNA (Lo et al., 2018). If the PCR amplicon length is long, the efficiency of qPCR with low quality DNA decreases. For these reasons, based on variable sequences between both species, target-specific qPCR primers were designed to amplify short amplicons ranging from 78 to 180 bp (Supplementary Fig. 2 and Supplementary Table 1).

Evaluation and validation of designed DNA markers

Evaluation of the sensitivity with target specific multiplex PCR primer combinations

The multiplex PCRs were carried out with DNA from each species and combined DNA of both species. For the single-species DNA, we found one clear PCR amplicon with multiplex primer combinations. In the combined DNA, two clear PCR amplicons were found with expected sizes and identical sequences to the target genes (Fig. 1A). To determine the optimum combinations of both primer concentrations, each of the reverse target-specific multiplex PCR primer concentrations were diluted to 50–10 pmol, and then, both specific primers were combined to perform the multiplex PCRs. The optimal reverse primer concentrations are shown in Supplementary Table 1.

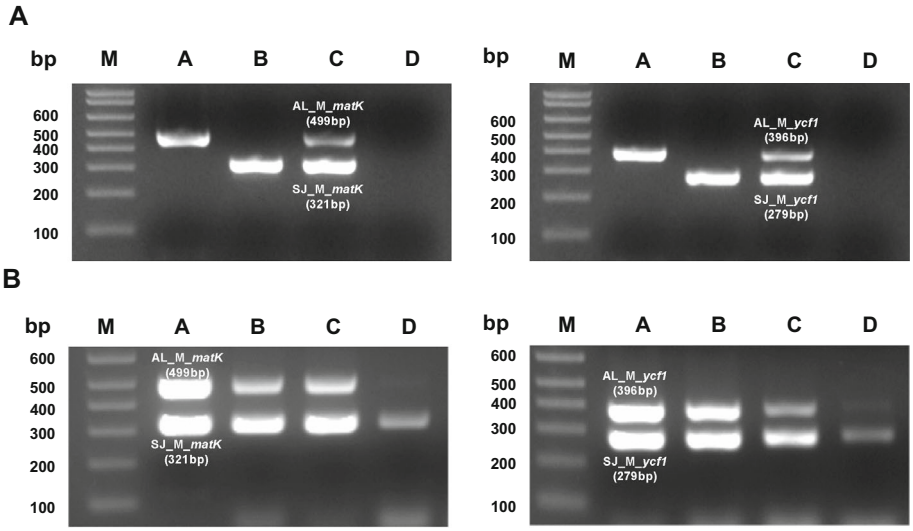

Fig. 1 **A** Specificity of the target specific multiplex PCR primer sets (AL_M_matK, SJ_M_matK, AL_M_ycf1 and SJ_M_ycf1) used for identification of *A. lancea* and *S. japonica.* The PCR amplicons of 499, 321, 396, and 279 bp correspond to the *A. lancea* and *S. japonica*. Line M, l kb DNA ladder marker, Line A, DNA of the *A. lancea* with multiplex primer sets, Line B, DNA of the *S. japonica* with multiplex primer sets, Line C, DNA of the both species with multiplex primer sets, Line D, NTC. **B** Sensitivity of target target-specific multiplex primer sets (AL_multi_matK, SJ_multi_matK, and AL_multi_ycf1 and SJ_multi_ycf1). The multiplex PCRs were performed with ten-fold serially diluted DNAs of both target species (10 -0.1 ng/ul). Line M, l kb DNA ladder marker; Line A, 10 ng; Line B, 1 ng; Line C, 0.1 ng; Line D, 0.01 ng

In addition, to validate the sensitivity of the multiplex primer combinations, we conducted multiplex PCR with ten-fold diluted (10–0.01 ng/µl) DNA templates of both species. As a result, both multiplex primer combinations (AL_M_matK and SJ_M_matk, and AL_M_ycf1 and AL_M_ycf1) can clearly detect both target species DNA until 0.1 ng/µl of both species; however, the combined DNA of 0.01 ng/µl was not clearly detected (Fig. 1B).

Evaluation of the sensitivity with target specific real-time PCR primer sets

The sensitivity of the six primer sets (AL_R_ITS2, AL_R_matK, AL_R_ycf1, SJ_R_ITS2, SJ_R_matK, and SJ_R_ycf1) was evaluated by analyzing standard curves using the tenfold serially diluted DNAs isolated from rhizomes of target species (10–0.001 ng/µl), and regression analysis was performed (Fig. 2). As a result, the slopes the primer sets ranged from − 3.31 to − 3.55, and the correlation coefficients (R^2) were higher than 0.99 (Table 1). In addition, the efficiency values ranged from 91.11–100.32% (Table 1), and all criteria satisfied the ENGL guidelines (ENGL, 2015). All *A. lancea* specific real-time PCR primer sets (AL_R_ITS2, AL_R_matK, and AL_R_ycf1) showed *S. japonica* DNA amplicons greater than 33 Cts, while all *the S. japonica* specific real-time PCR primer sets (SJ_R_ITS2, SJ_R_matK, and SJ_R_ycf1) exhibited *A. lancea* gDNA amplicons greater than 32 Cts (Fig. 2). In addition, the interlaboratory experiments were conducted to evaluate the adaptability of six real-time PCR primer sets with different real-time PCR machines in two independent laboratories. As shown in Supplementary Table 3, the real-time PCR primer sets were found to meet the ENGL criteria ($R^2 > 0.98$ and efficiency ranges of 90.39 – 100.62) (Supplementary Table 3).

Based on the evaluation of amplification sensitivity of the two PCR assays, multiplex and real-time PCR primer sets, the designed primer sets were found to be suitable for amplifying the target species DNA in the combined DNA samples of the target species with low DNA concentrations (0.1 ng for multiplex PCR and 0.01 ng for real-time PCR).

Species specificity test of the designed PCR assays

To confirm the cross-reactivity of the designed DNA markers, we conducted a specificity test using multiplex and real-time PCR markers. A total of 16 species, including *A. lancea* and *S. japonica*, were used in the specificity test. 18S plant rRNA primer sets were used as a positive control (Allmann et al., 1993), and the PCR products were amplified for less than 20 cycles in the multiplex PCR and before 20 Cts in the real-time PCR. Multiplex PCRs were performed with target-specific multiplex PCR primer combinations (AL_M_matK, AL_M_ycf1, SJ_M_matK, and SJ_M_ycf1) and various PCR cycles, such as 25, 28, 31, 34, and 40 cycles, respectively (Table 1). As a result, the cut-off cycles (28 cycles for AL_M_matK, AL_M_ycf1, and SJ_M_matK and 31 cycles for

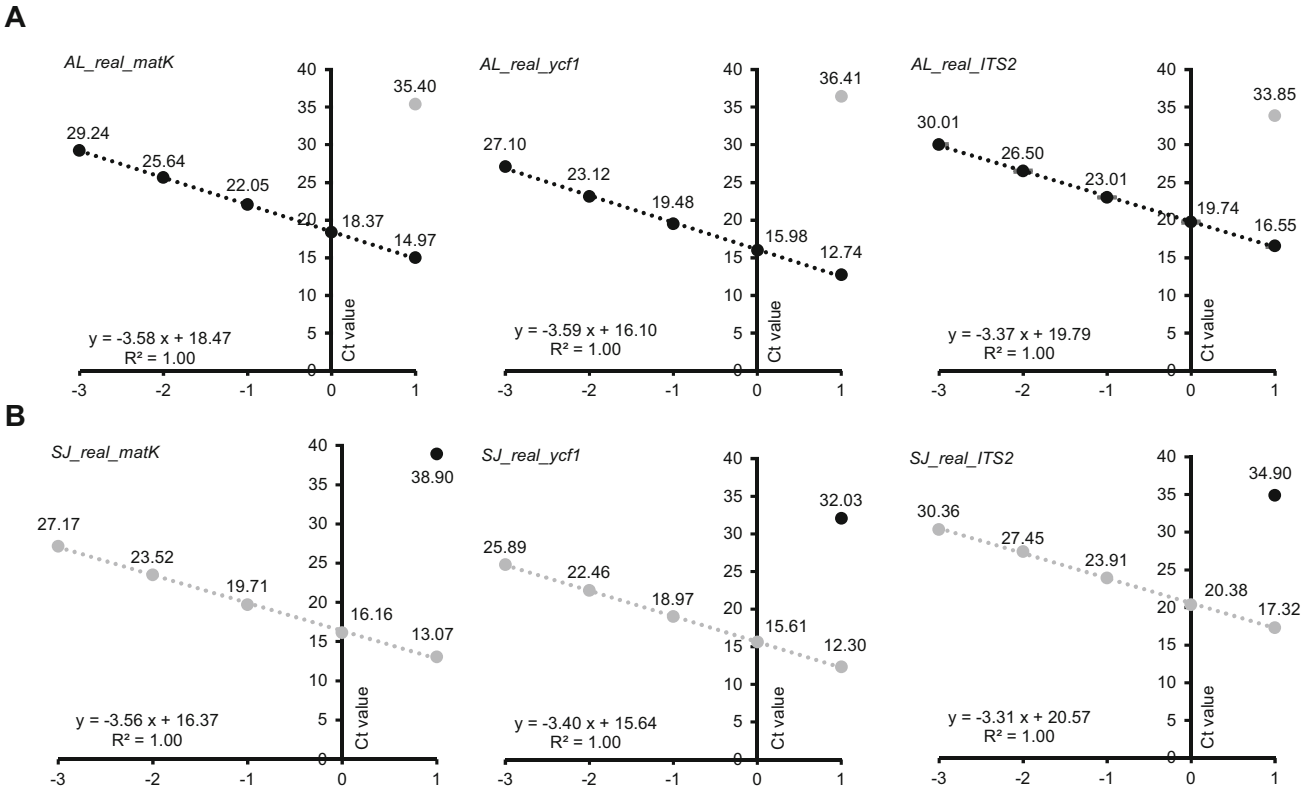

Fig. 2 Sensitivity of target target-specific real-time PCR primers. Standard curve of cycle threshold (Ct) values was obtained on the basis of efficiency and correlation of coefficient (R^2) in serial dilution series DNA (*A. lancea* and *S. japonica*) using target-specific primer sets. The DNA concentration (log numbers) was represented in the x-axis and the means of Ct value ± SD were represented in the y-axis. **A** *A. lancea* specific primer sets (AL_real_ITS2, AL_real_-matK, and AL_real_ycf1). Black dots represent ten-fold dilution series of genomic DNA in *A. lancea* leaves (10 ng–1 pg) and Gray dots represent genomic DNA of *S. japonica* (10 ng/ul); **B** *S. japonica* specific primer sets (SJ_real_ITS2, SJ_real_matK, and SJ_real_ycf1). Gray dots represent ten-fold dilution series of genomic DNA in *S. japonica* (10 ng–1 pg) and black dot represents the gDNA of *A. lancea* (10 ng). All real-time PCRs were carried out in triplicate (n = 3)

SJ_M_ycf1) were identified for multiplex PCR. Before the cut-off cycles, PCR products were amplified in target species, that is, *A. lancea* and *S. japonica*, but not in the other 15 species. The AL_M_matK primer sets could amplify PCR products from six species that belonged to the Compositae family after more than the cut-off cycles but less than 40 cycles, while the AL M_ycf1 could amplify products from two species of the family. In contrast, the SJ_M_matK could amplify PCR products from two species (*I. batatas* and *Z. mays*) after more than the cut-off cycles but less than 40 cycles. However, SJ_M_ycf1 could amplify products only from *C. longa* after more than the cut-off cycles (Table 2).

Subsequently, real-time PCR was performed with the 16 species using the six designed real-time PCR primer sets (Table 1). Five primer sets, except AL_R_ycf1, were not able to amplify PCR products from the other non-targeted 14 species before 40 cycles; however, amplicons were produced with the AL_R_ycf1 from the three species that belonged to the *Compositae* family after more than the cut-off cycles and before 40 cycles. Collectively, the specificity of the two types of DNA marker tests suggests that all designed primer sets could be useful for detecting the target species without cross-reactivity with at least 14 species, which could be used as medicinal herbs or addition agents in food complexes.

Application of designed real-time PCR assays in samples treated with dry, heat, and autoclave

Generally, the rhizomes of *A. lancea* undergo three types of food processing, namely dried, heated, and autoclaved food complexes. The food processing processes could cause severe DNA degradation of medical herbs. For example, heat treatment is known to cause strong DNA fragmentation and thus renders the PCR-based marker assays inefficient and unsuitable in verifying the authenticity of food complexes (Hwang et al., 2015). Therefore, the developed real-time PCR assays would be examined in samples treated with the food processing processes to confirm their

Table 1 Evaluation of Slope, R^2, and efficiency obtained by real-time PCR system with different material conditions using the designed primer sets threshold cycle (Ct) values obtain from three conditions (dried, heat and autoclaved) with reference binary mixtures

DNA standard curve					Binary mixture standard curve of dried materials				
Target species	Primer	Y (Slope)	R^2	Efficiency (%)	Target species	Primer	Y (Slope)	R^2	Efficiency (%)
A. lancea	AL_R_ITS2	− 3.31	1.00	100.32	*A. lancea*	AL_R_ITS2	− 3.25	0.99	103.12
	AL_R_matK	− 3.53	1.00	92.17		AL_R_matK	− 3.29	1.00	101.53
	AL_R_ycf1	− 3.55	1.00	91.21		AL_R_ycf1	− 3.18	0.99	106.27
S. japonica	SJ_R_ITS2	− 3.37	1.00	98.09	*S. japonica*	SJ_R_ITS2	− 3.45	1.00	95.09
	SJ_R_matK	− 3.56	1.00	91.11		SJ_R_matK	− 3.21	1.00	104.99
	SJ_R_ycf1	− 3.40	1.00	96.75		SJ_R_ycf1	− 3.31	1.00	100.43
Binary mixture standard curve of heated materials					Binary mixture standard curve of autoclaved materials				
Target species	Primer	Y (Slope)	R2	Efficiency (%)	Target species	Primer	Y (Slope)	R2	Efficiency (%)
A. lancea	AL_R_ITS2	− 3.50	0.99	92.97	*A. lancea*	AL_R_ITS2	− 3.49	0.99	93.92
	AL_R_matK	− 3.20	0.98	105.01		AL_R_matK	− 3.32	1.00	100.00
	AL_R_ycf1	− 3.19	0.99	106.05		AL_R_ycf1	− 3.16	1.00	107.06
S. japonica	SJ_R_ITS2	− 3.28	1.00	101.80	*S. japonica*	SJ_R_ITS2	− 3.31	1.00	100.32
	SJ_R_matK	− 3.25	1.00	102.88		SJ_R_matK	− 3.22	1.00	104.61
	SJ_R_ycf1	− 3.32	1.00	100.21		SJ_R_ycf1	− 3.12	0.99	109.05

applicability in the processed food complexes. We examined the slope, R^2, and efficiency of the designed real-time PCR assay using dried, heated (100 °C), and autoclaved rhizome reference binary mixtures, to determine their applicability in food complexes. First, standard curves were constructed using tenfold diluted DNA series of each of *A. lancea* and *S. japonica* with six real-time PCR primer sets (Table 2). All slopes that were evaluated ranged from − 3.31 to − 3.57, and the six correlation coefficients (R^2) were 1.00 for the designed primer sets. The slope efficiencies were estimated to be 91.11% to 100.32% for each target species. Subsequently, DNAs extracted from dried rhizome binary mixtures (0.1–100% w/w) were diluted to the 10 ng/uL and used for the real-time PCR assay. In addition, DNAs of heated and autoclaved rhizome binary mixtures (0.1–100% w/w) were extracted after treatment, respectively. Ct values were obtained from each of the treated samples of three food processing types (Supplementary Table 2). Dried and heated rhizome binary mixtures showed similar low Ct values; however, higher Ct values were found for autoclaved samples than for the dried and heated samples. These results suggest that more severe DNA degradation is present in the autoclaved rhizomes than in the dried and heated rhizomes, which suggests that the application of different Ct values to discern adulteration depends on food processing conditions. For the dried, heated, and autoclaved rhizome binary mixtures, the designed 18 real-time PCR primer sets showed slopes of − 3.18 to − 3.49, $R^2 > 0.99$, and efficiency values of 92.97–109.05 (Table 2). Generally, adulteration of less than 0.1% would not be intended for illegal economic profit. Therefore, in the application of the real-time PCR assay, Ct values of 0.1% target species in all binary mixtures were determined as cut-off cycles for discerning between genuine products and adulteration.

Application of the designed PCR assays for commercial A. lancea products

To screen the adulteration of *S. japonica* in commercial *A. lancea* products, the two designed PCR assays, multiplex PCR and real-time PCR, were performed on three types (dried rhizomes, pill, and dried rhizome powder) of 14 *A. lancea* commercial products purchased from a local market (Supplementary Table 4). First, the 18 s rRNA primer set was used as a positive control to check the amplification ability of the DNA isolated from the commercial products (Allmann et al., 1993). As shown in Table 3, the 18 s rRNA primer sets were amplified with all commercial products, at low Cts that ranged from 13.97 to 16.30. These findings indicate that the DNA from all the commercial products was sufficiently amplifiable. Second, multiplex PCRs with the target-specific multiplex PCR primer combinations were performed until the cut-off cycles. In all of the 14 *A. lancea* commercial products, *A. lancea* specific multiplex PCR primer combinations clearly amplified the PCR products, whereas *S. japonica* specific multiplex PCR primer combinations did not amplify any products before

Table 2 The results of the species specificity test using the designed multiplex PCR and real-time PCR primer sets

No	Family	Species	Whole plants	*A. lancea*					*S. japonica*				
			18 s rRNA	M_matK	M_ycf1	R_ITS2	R_matK	R_ycf1	M_matK	M_ycf1	R_ITS2	R_matK	R_ycf1
		Curt-off cycles	20	28	31	28	28	29	28	31	26	27	27
1	*Compositae*	*Artemisia capillaris*	+ +[a]	+[b]	−[c]	−	−	−	−	−	−	−	−
2		*Artemisia gmelinii*	+ +	+	−	−	−	−	−	−	−	−	−
3		*Dendranthema indicum*	+ +	+	−	−	−	+	−	−	−	−	−
4		*Cirsium japonicum*	+ +	−	+	−	−	+	−	−	−	−	−
5		*Taraxacum platycarpum*	+ +	+	−	−	−	−	−	−	−	−	−
6		*Dendranthema zawadskii*	+ +	+	+	−	−	+	−	−	−	−	−
7		*Aster tataricus*	+ +	−	−	−	−	−	−	−	−	−	−
8		*Artemisia annua*	+ +	+	−	−	−	−	−	−	−	−	−
9		*Atractylodes lancea*	+ +	+ +	+ +	+ +	+ +	+ +	−	−	−	−	−
10	*Liliaceae*	*Veratrum maackii*	+ +	−	−	−	−	−	−	−	−	−	−
11		*Hemerocallis fulva*	+ +	−	−	−	−	−	−	−	−	−	−
12	*Solanaceae*	*Scopolia japonica*	+ +	−	−	−	−	−	+ +	+ +	+ +	+ +	+ +
13	*Convolvulaceae*	*Ipomoea batatas*	+ +	−	−	−	−	−	+	−	−	−	−
14	*Euphorbiaceae*	*Manihot esculenta*	+ +	−	−	−	−	−	−	−	−	−	−
15	*Zingiberaceae*	*Curcuma longa*	+ +	−	−	−	−	−	−	+	−	−	−
16	*Graminae*	*Zea mays*	+ +	−	−	−	−	−	+	−	−	−	−

[a] + +, means that was amplified before cut-off Ct cycles

[b] +, means that was amplified between more than cut-off Ct cycles and less than 40 cycles

[c] −, means that was not amplified before 40 cycles

Table 3 Results of the designed PCR assays using *A. lancea* commercial products

Primer sets		*A. lancea* commercial products test					
		Dried rhizomes					
		1	2	3	4	5	6
18S rRNA primer		13.97 ± 0.06	14.01 ± 0.04	13.94 ± 0.01	14.29 ± 0.09	14.33 ± 0.07	14.55 ± 0.04
A. lancea	M_matK28 cycles[a]	+	+	+	+	+	+
	M_ycf128 cycles	+	+	+	+	+	+
	R_ITS228 cycles	16.18 ± 0.01	16.53 ± 0.01	15.88 ± 0.02	16.22 ± 0.05	15.39 ± 0.02	15.74 ± 0.02
	R_matK28 cycles	15.89 ± 0.06	15.9 ± 0.09	15.32 ± 0.18	15.45 ± 0.09	16.07 ± 0.12	18.91 ± 0.12
	R_ycf129 cycles	16.13 ± 0.06	16.1 ± 0.02	15.84 ± 0.04	16.04 ± 0.08	16.77 ± 0.01	18.96 ± 0.02
S. Japonica	M_matK28 cycles	−	−	−	−	−	−
	M_ycf131 cycles	−	−	−	−	−	−
	R_ITS226 cycles	28.89 ± 0.80	29.1 ± 0.73	29.66 ± 0.73	29.31 ± 0.81	28.24 ± 1.06	29.91 ± 0.52
	R_matK27 cycles	31.57 ± 1.48	30.48 ± 0.32	30.92 ± 0.08	32.25 ± 0.27	31.59 ± 0.17	31.87 ± 0.44
	R_ycf127 cycles	28.49 ± 0.20	30.02 ± 0.16	30.62 ± 0.27	31.47 ± 0.44	31.9 ± 0.92	31.93 ± 0.70

Primer sets		*A. lancea* commercial products test							
		Dried rhizomes						Pill	Dried Powder
		7	8	9	10	11	12	13	14
18S rRNA primer		14.04 ± 0.02	16.3 ± 0.26	14.76 ± 0.17	15.5 ± 0.14	15.16 ± 0.1	14.68 ± 0.09	14.95 ± 0.14	15.02 ± 0.55
A. lancea	M_matK28 cycles[a]	+	+	+	+	+	+	+	+
	M_ycf128 cycles	+	+	+	+	+	+	+	+
	R_ITS228 cycles	15.17 ± 0.04	16.81 ± 0.08	15.96 ± 0.01	16.74 ± 0.09	16.21 ± 0.13	16.05 ± 0.08	17.37 ± 0.12	17.52 ± 0.16
	R_matK28 cycles	16.1 ± 0.02	16.67 ± 0.07	13.99 ± 0.12	14.72 ± 0.12	14.51 ± 0.14	13.83 ± 0.12	18.48 ± 0.14	13.68 ± 0.08
	R_ycf129 cycles	16.11 ± 0.03	16.82 ± 0.03	14.33 ± 0.03	14.87 ± 0.03	14.94 ± 0.03	14.26 ± 0.03	18.72 ± 0.04	14.13 ± 0.06
S. Japonica	M_matK28 cycles	−	−	−	−	−	−	−	−
	M_ycf131 cycles	−	−	−	−	−	−	−	−
	R_ITS226 cycles	29.06 ± 0.63	29.59 ± 0.06	28.21 ± 0.14	28.21 ± 0.1	29.02 ± 0.12	28.46 ± 0.11	27.91 ± 0.05	27.65 ± 0.1
	R_matK27 cycles	32.56 ± 0.31	30.79 ± 0.20	33.07 ± 0.34	33.31 ± 0.14	32.51 ± 0.24	32.45 ± 0.43	27.96 ± 0.08	29.58 ± 0.17
	R_ycf127 cycles	32.92 ± 0.27	30.26 ± 0.12	30.07 ± 0.07	30.74 ± 0.14	30.14 ± 0.09	29.73 ± 0.18	28.22 ± 0.03	29.42 ± 0.13

[a] the cut-off cycles of each designed DNA markers

Table 4 Results of the blind test for confirming the reliability of the designed PCR assays

Primer sets		*A. lancea* and *S. japonica* blind test								
Sample number		1	2	3	4	5	6	7	8	9
18S rRNA primer		13.93 ± 0.08	13.69 ± 0.33	13.65 ± 0.17	13.57 ± 0.26	13.60 ± 0.11	13.76 ± 0.26	13.76 ± 0.17	13.82 ± 0.01	13.52 ± 0.14
A. lancea	M_matK 28 cycles[a]	+	+	+	+	+	+	+	+	+
	M_ycf1 31 cycles	+	+	+	+	+	+	+	+	+
	R_ITS2 28 cycles	13.93 ± 0.09	14.24 ± 0.22	13.91 ± 0.15	13.89 ± 0.33	13.89 ± 0.06	14.05 ± 0.13	14.05 ± 0.21	14.10 ± 0.06	13.92 ± 0.12
	R_matK 28 cycles	15.14 ± 0.14	16.41 ± 0.08	15.48 ± 0.12	16.84 ± 0.09	14.82 ± 0.11	15.21 ± 0.07	14.72 ± 0.21	15.26 ± 0.07	15.87 ± 0.12
	R_ycf1 29 cycles	17.21 ± 0.04	17.20 ± 0.36	17.10 ± 0.49	16.89 ± 0.34	16.59 ± 0.41	16.62 ± 0.10	16.7 ± 0.11	16.68 ± 0.02	17.21 ± 0.04
S. japonica	M_matK 28 cycles	−	+	+	+	−	+	+	+	+
	M_ycf1 31 cycles	−	+	+	+	−	+	+	+	+
	R_ITS2 26 cycles	26.58 ± 0.12	20.05 ± 0.20	19.66 ± 0.01	19.77 ± 0.09	27.07 ± 0.02	21.37 ± 0.02	20.78 ± 0.10	20.72 ± 0.14	22.43 ± 0.12
	R_matK 27 cycles	28.01 ± 0.02	21.41 ± 0.26	21.01 ± 0.19	21.04 ± 0.27	29.34 ± 0.32	22.85 ± 0.12	22.28 ± 0.09	22.17 ± 0.03	23.70 ± 0.15
	R_ycf1 27 cycles	27.93 ± 0.08	21.53 ± 0.05	21.05 ± 0.01	21.00 ± 0.04	26.72 ± 0.04	22.65 ± 0.02	22.27 ± 0.10	22.21 ± 0.14	24.10 ± 0.13

Primer sets		*A. lancea* and *S. japonica* blind test										
Sample number		10	11	12	11	14	15	16	17	18	19	20
18S rRNA primer		13.5 ± 0.08	13.56 ± 0.14	13.45 ± 0.23	13.56 ± 0.14	13.46 ± 0.23	13.49 ± 0.11	13.72 ± 0.19	13.57 ± 0.12	13.47 ± 0.13	13.4 ± 0.25	13.08 ± 0.01
A. lancea	M_matK 28 cycles[a]	+	+	+	+	+	+	+	+	+	+	+
	M_ycf1 31 cycles	+	+	+	+	+	+	+	+	+	+	+
	R_ITS2 28 cycles	13.88 ± 0.25	14.17 ± 0.06	14.14 ± 0.11	14.17 ± 0.06	14.00 ± 0.23	13.95 ± 0.20	13.87 ± 0.08	13.77 ± 0.29	13.85 ± 0.12	13.81 ± 0.12	13.67 ± 0.11
	R_matK 28 cycles	16.21 ± 0.15	17.24 ± 0.09	15.89 ± 0.05	17.24 ± 0.09	15.48 ± 0.06	15.21 ± 0.08	15.78 ± 0.17	16.98 ± 0.04	15.48 ± 0.09	15.23 ± 0.08	15.79 ± 0.12
	R_ycf1 29 cycles	17.04 ± 0.03	16.76 ± 0.06	16.55 ± 0.06	16.76 ± 0.06	16.64 ± 0.07	16.64 ± 0.02	16.96 ± 0.05	17.1 ± 0.2	16.86 ± 0.23	16.8 ± 0.18	16.83 ± 0.12
S. japonica	M_matK 28 cycles	+	+	+	+	+	+	+	−	+	+	+
	M_ycf1 31 cycles	+	+	+	+	+	+	+	−	+	+	+
	R_ITS2 26 cycles	20.23 ± 0.2	20.87 ± 0.16	18.99 ± 0.03	20.87 ± 0.16	22.60 ± 0.07	20.84 ± 0.09	21.76 ± 0.30	27.72 ± 0.02	20.16 ± 0.16	21.19 ± 0.1	22.06 ± 0.14
	R_matK 27 cycles	21.39 ± 0.18	22.13 ± 0.34	20.18 ± 0.04	22.13 ± 0.34	23.95 ± 0.18	22.10 ± 0.20	23.25 ± 0.10	32.72 ± 0.98	21.29 ± 0.13	22.40 ± 0.04	23.43 ± 0.01
	R_ycf1 27 cycles	21.48 ± 0.19	22.10 ± 0.17	20.27 ± 0.19	22.1 ± 0.17	24.04 ± 0.1	22.05 ± 0.09	23.25 ± 0.04	31.11 ± 0.33	21.45 ± 0.33	22.63 ± 0.23	23.33 ± 0.19

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

the cut-off cycles (Supplementary Fig. 3, Table 3). Finally, six specific real-time PCR primer sets were examined for the 14 commercial products. In *A. lancea* real-time PCR primers, Ct values (from 13.83 to 18.96 cycles) lower than the cut-off cycles of each designed primer were found for the 14 commercial products (Table 3). In contrast, the *S. japonica* specific real-time PCR primer sets presented higher Ct values (from 27.91 to 33.31 cycles) than the cut-off cycles (Table 3). As a result, the 14 *A. lancea* commercial products purchased from local markets were not adulterated with *S. japonica* based on the two tested PCR assays. These results indicate that the designed PCR assays can be applied to detect the target DNA (*A. lancea* or *S. japonica*) in commercial complex products.

Application of the designed PCR assays to blind samples

To estimate the reliability of the two PCR assays, a blind test was conducted. Twenty blind samples were mixed randomly by another independent research group with different concentrations of *A. lancea* and *S. japonica*. The 18S rRNA plant primer sets were used as positive amplification controls (Allmann et al., 1993) and amplified PCR products with low Cts (13.27–13.93) (Table 4). Subsequently, multiplex PCR was applied to the 20 blind samples with each primer cut-off cycle (Supplementary Fig. 4, Table 4). *A. lancea* PCR products were amplified in all blind samples; however, *S. japonica* amplicons were found in 17 blind samples, but not in the others (#1, 5, and 17). These results supported that 17 blind samples were adulterated with *S. japonica* (at least more than 0.1%), while three samples were genuine products. Finally, a real-time PCR assay was applied to the 20 blind samples. Three *A. lancea* real-time PCR primer sets could amplify PCR products in less than 18 cycles (13.67 to 17.24), which suggests that all blind samples had *A. lancea* rhizomes. When amplified with three *S. japonica* real-time PCR primers, sets lower than the cut-off cycles were found in 17 blind samples, but higher sets were found in three blind samples (#1, 5, and 17) (Table 4). Collectively, both PCR assays produced identical results in the 20 blind samples and can be used commercially to discern adulteration.

In conclusion, the two PCR assays, multiplex PCR and real-time PCR, that were developed in this study are highly sensitive, fast, and specific methods for detecting *A. lancea* and *S. japonica* target species in processed medical herb complexes. To evaluate the sensitivity of the DNA markers, target-specific multiplex PCR primer combinations were amplified with ten-fold serially diluted (10–0.1 ng) DNA template. Standard curves of real-time PCR primer sets were constructed using ten-fold serially diluted DNAs with three conditions (raw, heated, and autoclaved) of binary DNA mixtures. The specificities of the developed DNA markers were evaluated with 14 other species that can be used as medical herbs or other food additives. The reliability of the developed DNA markers was supported by a blind test and their application to commercial *A. lancea* products. In the above experiments, the DNA markers developed in this study can detect *S. japonica* through a method other than morphological classification in *A. lancea* complex products. Thus, these DNA markers could contribute to human health care to identify adulterated products of *A. lancea* that are mixed with *S. japonica* and protect consumers from food fraud.

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Declarations

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

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