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Towards authentication of Korean ginseng-containing foods: Differentiation of five *Panax* species by a novel diagnostic tool

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ABSTRACT

Panax ginseng C.A. Meyer (Korean ginseng) is one of the most valuable medicinal plants, recognised for its neuroprotection and other beneficial health effects. It is present in a wide range of food products, namely plant food supplements (PFS) and herbal infusions. However, other *Panax* species, having distinct therapeutic effects, are also known as ginseng, pointing out the need of authenticating such products. The present work aims at proposing a new high-resolution melting (HRM) method to differentiate five *Panax* species (*P. ginseng*, *P. quinquefolius*, *P. notoginseng*, *P. japonicus* and *P. trifolius*), targeting the gene encoding the dammarenediol synthase, involved in the biosynthesis pathway of ginsenosides. A *Panax*-specific real-time PCR assay was successfully developed with high analytical performance parameters (Efficiency = 100.5 %, R^2 = 0.995, dynamic range 10 ng-1 pg of ginseng DNA). *Panax* DNA was detected in 17 out of 23 ginseng-containing commercial foods, including herbal infusions and PFS. For the first time, HRM analysis differentiated five *Panax* species with high level of confidence (>98 %), which corroborated sequencing data. Fourteen products were successfully clustered, being all except one in accordance with their labelling statements. Therefore, the present work proposes a reliable and high-throughput tool to authenticate ginseng products that could be useful for control laboratories.

1. Introduction

Panax ginseng C.A. Meyer, a perennial plant belonging to the Araliaceae family, is a high value medicinal plant, used for thousands of years for its medicinal purposes. It is the most cultivated, researched and expensive *Panax* species. *P. ginseng* is commonly known as Korean ginseng and can be classified into three types, namely fresh ginseng, white ginseng and red ginseng, depending on the age and how it is processed (Yun, 2001). Each type can be present in a variety of ginseng products in the forms of fresh sliced, powder, juice, liquid extract (tincture or boiled extract), infusion, tablet, capsule, among others (Jung, Kim, Yang, Bang, & Yang, 2014; Yun, 2001). Several therapeutic effects of *P. ginseng* have been reported, namely treatment and/or prevention of some diseases of the central nervous, immune, endocrine and cardiovascular systems, presenting immunomodulatory, antihypertensive, anti-inflammatory, antiallergic, antiatherosclerotic, antidiabetic and anticarcinogenic actions (Christensen, 2008; Kim et al., 2018; Zheng, Wu, & Wu, 2012). Its pharmacological and biological activities are due to the presence of triterpenoid saponins, known as ginsenosides, which are present in all *Panax* species. Ginsenosides have different chemical structures and contents, depending on the species, thus providing distinct therapeutic effects (Christensen, 2008; Christensen, Jensen, & Kidmose, 2006; Peng et al., 2012).

The high demand for *P. ginseng*, its high economic value and the difficulties in the morphological differentiation among *Panax* species owing to their great similarity and processing leads to intentional and/or unintended adulterations. Such practices might be due to the addition and/or substitution of *P. ginseng* with other plant species or to inadvertent misidentification of the other *Panax* species. This might compromise the efficacy and safety of ginseng products and, consequently, the health of the consumers. Therefore, the authentication of such products is of utmost importance, for which chemical and molecular markers have been proposed. Several chromatographic and spectroscopic methods have been developed for *Panax* species differentiation, relying on the ginsenoside profiling (Chan et al., 2000; Sun & Chen, 2011; Yang et al., 2016) and metabolomics (Nguyen et al., 2016; Yuk et al., 2013). However, chemical profiles, such as ginsenosides, can vary with the part/tissue of the plant, age, environmental

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conditions and processing, which restrict their applicability for authentication purposes (Yang et al., 2016). On the other hand, DNA-based approaches take advantage of the higher thermal stability of the DNA molecules and their independence from physiological and external conditions, being considered as powerful tools for botanical identification of plant species in a wide range of foods, including herbal products and plant food supplements (Grazina, Amaral, Costa, & Mafra, 2020; Grazina, Amaral, & Mafra, 2020; Sucher & Carles, 2008; Techen, Parveen, Pan, & Khan, 2014). Among the DNA-based methods, DNA barcoding has shown a remarkable application in the botanical authentication of a wide range of herbal products (de Boer, Ichim, & Newmaster, 2015; Parveen, Gafner, Techen, Murch, & Khan, 2016; Techen et al., 2014), including the identification of *Panax* species (Han et al., 2016; Manzanilla et al., 2018; Wallace et al., 2012; Zuo et al., 2011). However, DNA barcoding might present limited application when analysing samples containing highly processed plant material, such as PFS, which provide degraded DNA, disabling the amplification of the commonly used barcodes (>500 bp). Other shortcomings regard the fact of relying on Sanger sequencing and the lack of available sequences in databases.

Other DNA-based methods targeting single nucleotide polymorphisms (SNP) have been successfully developed to identify *Panax* species by means of multiplex allele-specific PCR (Wang et al., 2011) and PCR followed by digestion with restriction enzymes (Nguyen et al., 2020), having the drawback of requiring further agarose gel electrophoresis analysis. Indel variations have also been exploited as markers to differentiate *P. ginseng* from *P. quinquefolius* by means of high-resolution melting (HRM) analysis (Jung et al., 2014) and multiplex PCR (Tian, Lv, Tian, Wang, & Wang, 2020). HRM analysis targeting the internal transcribed spacer 2 (ITS2) was also successfully applied to discriminate *P. ginseng* from *P. notoginseng* (Osathanunkul & Madesis, 2019). HRM analysis has been suggested as a fast, reliable and cost-effective approach for the identification and discrimination of closely related species (Grazina, Amaral, Costa, & Mafra, 2021). It relies on the detection of small nucleotide variations by the design of one single set of primer without the need of sequencing, contrarily to other methods such as multiplex PCR that needs multiple primer sets and DNA barcoding that requires sequencing and further data analysis. Besides, it enables targeting informative short sequences (<300 bp) as mini-barcodes of nuclear, plastidial or mitochondrial regions with a high potential for species, or even varietal, discrimination (Sun, Li, Xiong, Zhao, & Chen, 2016).

In the present work, a new HRM approach is proposed to differentiate, for the first time, five *Panax* species: *P. ginseng*, *P. quinquefolius*, *P. notoginseng*, *P. japonicus* and *P. trifolius*. The method targeted the gene encoding the dammarenediol synthase (DS), which is an enzyme involved in the biosynthesis pathway of ginsenosides (Wang Zhao, Cao, & Sun, 2014). Considering that ginsenoside profile varies with the *Panax* species, the DS gene was hyphotetised as a potential marker of *Panax* genus. The applicability of the method was assessed using several commercial products, including dried roots, herbal infusions and plant food supplements (PFS) in order to verify their label compliance.

2. Material and methods

2.1. Sampling

Plant materials of *Panax* specimens were used as voucher species for the method development: leaves of *P. ginseng*, *P. quinquefolius*, *P. notoginseng* and *P. japonicus* (Royal Botanic Garden Edinburgh, Scotland, UK) and seeds of *P. trifolius* (Kew gardens, Sussex, UK). A total of 23 commercial products, including 6 dried roots, 7 herbal infusions and 10 PFS were acquired at the retail market, e-commerce and specialised herbal stores. Other plant species, including several medicinal plants, herbs, spices, cereals, legumes, fruits, tree nuts, among others used as food, were tested for specificity assays (Table S1, supplementary material).

Plant material in the form of leaves, roots and herbal infusions were ground using a knife mill (Grindomix GM 200, Retsch, Haan, Germany). The seeds were crushed using a mortar and pestle. The PFS were treated differently, depending on the type: tablets were reduced to powder using a mortar and pestle, hard capsules and soft capsules were opened, and their contents were collected.

2.2. DNA extraction

In this work, two different methods were applied to extract DNA from 50 to 100 mg of each sample. The Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) was used to extract most of the samples, by means of two protocols as described by the manufacturer's instructions. The protocol with lysis buffer PL1 was applied to samples of PFS, following the manufacturer's instructions, without adding RNase. The DNA extraction of herbal infusions, roots, seeds and leaves was performed using the PL2 lysis buffer, according to the manufacturer's instructions with slight modifications, as described by Costa et al. (2016). CTAB-PVP method was also used as described by Costa, Melo, Santos, Oliveira, and Mafra (2015) to extract DNA from some herbal infusion samples that did not provide high quality DNA with none of the Nucleospin Plant II kit protocols.

The determinations of yield and purity of DNA extracts were performed by UV spectrophotometry, using a Take3 micro-volume plate accessory, on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The nucleic acid protocol was set for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA) to determine the DNA content and purity of each extract.

2.3. Target gene selection and oligonucleotide primer design

The primers were designed on the gene encoding for the DS that is involved in the biosynthesis pathway of ginsenosides, the bioactive compounds responsible for the main medicinal effects of *Panax* species (Christensen, 2008; Wang, Zhao, Cao, & Sun, 2014). Sequences available at NCBI database (http://www.ncbi.nlm.nih.gov/) corresponding to the DS encoding gene of *P. ginseng* and *P. quinquefolius* (accession no. KJ939266.1 and KC316048.1, respectively) were chosen to design *Panax*-specific primers to amplify a fragment with 129 bp (Pax1-F/-Pax1-R), suitable to perform the HRM analysis (Table 1). The fragment length plays an essential role in the development of this method because it should be short to enable method application to samples containing degraded DNA, but still possessing enough nucleotide polymorphic variability among the species. A second set of primers was further designed in the same region, encompassing the target HRM fragment for sequencing purposes (Pax5-F/Pax6-R). Basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify regions of local similarity among homologue sequences of different *Panax* species and calculate the statistical significance of the matches. Primer specificity assessment was performed using the Primer-BLAST tool that allows revealing homologies in relation to all sequences available in the NCBI database. The OligoCalc software (http://www.

Table 1
Oligonucleotide primers used in high-resolution melting analysis and sequencing targeting the dammarenediol synthase (DS) gene of *Panax* spp.

Primer	Sequence (5' → 3′)	Amplicon (bp)	GenBank Accession no.
Pax1-F **Pax1-R**	GGCAGACAATATTGGGAGTTTC CAGCATATCACTGCATGGATGA	129	KJ939266.1
Pax5-F **Pax6-R**	AGAATGTGGAAGCTGAAGGTTG CTTTAATAAGCTGCCTGCGCA	215	KC316048.1

basic.northwestern.edu./biotools/oligocalc.html) was used to assess oligonucleotide properties and the absence of hairpins and primer self-hybridisation. All primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.4. End-point PCR

The capacity of amplification of the DNA extracts was assessed by PCR targeting a highly conserved universal eukaryotic region (nuclear 18S rRNA gene), using two sets of primers (18SRG-F/18SRG-R and EG-F/EG-R), as described by Costa, Oliveira, and Mafra (2013) and Villa, Costa, Oliveira, and Mafra (2017), respectively.

PCR assays were carried out in a total reaction volume of 25 μL, containing 2 μL (20 ng) of DNA extract, buffer (67 mmol/L Tris-HCl, pH 8.8, 16 mmol/L $(NH_4)_2SO_4$, 0.1 g/L Tween 20), 3.0 mmol/L of $MgCl_2$, 5.6×10^{-12} kat (1 U) of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Uml, Germany), 200 μmol/L of each dNTP (Grisp, Porto, Portugal) and 200 nmol/L of each primer (Table 2). The reactions were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), using the following temperature programs: initial denaturation at 95 °C for 5 min; 40 cycles of amplification at 95 °C for 30 s, 62 °C or 59 °C (for primers Pax1-F/Pax1-R and Pax5-F/Pax6-R (Table 2), respectively) for 30 s, extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min.

PCR products were verified by electrophoresis in a 15 g/L agarose gel stained with GelRed 1 × (Biotium, Inc., Hayward, CA, USA) and carried out in 1 × SGTB (Grisp, Porto, Portugal) for 25–30 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was recorded using Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Real-time PCR coupled to HRM analysis

The real-time PCR assays were carried out in 20 μL of total reaction volume, containing 2 μL of DNA (20 ng), 1x of SsoFast® Evagreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 300 nmol/L of each primer (Pax1-F/Pax1-R) (Table 1). A fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used to perform the reactions, using the following conditions: 95 °C for 5 min; 50 cycles at 95 °C for 20 s and at 63 °C for 50 s, with collection of fluorescence signal at the end of each cycle. Data were processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR trials were repeated in two independent assays using four replicates in each one.

Table 2
Results of qualitative polymerase chain reaction (PCR) and real-time PCR coupled to high-resolution melting (HRM) analysis of *Panax* reference species.

Species	Qualitative PCR		Real-time PCR	HRM analysis	
	18S rRNA gene	DS gene[a]	Ct (mean ± SD)[b]	Cluster	Level of confidence (%, mean ± SD)
Panax ginseng	+	+	26.20 ± 0.21	1	99.6 ± 0.2
P. quinquefolius	+	+	26.25 ± 0.17	2	99.6 ± 0.5
P. notoginseng	+	+	27.35 ± 0.11	3	98.7 ± 0.8
P. japonicus	+	+	26.71 ± 0.19	4	98.1 ± 0.8
P. trifolius	+	+	25.98 ± 0.20	5	99.6 ± 0.5

[a] DS, dammarenediol synthase;
[b] Mean cycle threshold (Ct) values ± standard deviation (SD) of $n = 8$ replicates.

For HRM analysis, PCR products were denatured at 95 °C for 1 min and then annealed at 65 °C for 5 min in order to allow the correct formation of the DNA duplexes. These two steps were followed by melting curve ranging from 65 °C to 95 °C with temperature increments of 0.2 °C every 10 s, with fluorescence data collection at the end of each cycle. The fluorescence data were further processed using the Precision Melt Analysis 1.2 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves as a function of temperature and difference curves for visual identification of clusters, in order to discriminate different species of *Panax* genus. Cluster detection settings were defined targeting high sensitivity and threshold yields for more heterozygote clusters. The confidence value indicates the relative probability of the sample being included in a specific cluster (Bio-Rad, 2012Bio-Rad). Therefore, the melting curve shape sensitivity parameter was adjusted as a default value between 40 % and 50 % and Tm difference threshold was set around 0.20.

2.6. PCR products sequencing

Sequencing analysis with primers Pax5-F/Pax6-R targeting the DS gene, encompassing the HRM fragment (Pax1-F/Pax1-R), was performed for the *Panax* species (*P. ginseng, P. quinquefolius, P. notoginseng* and *P. japonicus*) in order to validate the HRM results. Since *P. trifolius* was not possible to amplify with primers Pax5-F/Pax6-R, the primers Pax1-F/Pax1-R were used for sequencing this species. PCR products were purified with GRS PCR & Gel Band Purification Kit (GRISP, Porto, Portugal) to remove any possible interfering components and sent to a specialised research facility (Eurofins Genomics, Ebersberg, Germany) for sequencing.

Direct sequencing of both strands in opposite directions was performed, allowing the production of two complementary sequences for each species. To ensure sequencing results with high quality, each species was sequenced at least twice in both directions. The sequencing data were analysed using the software BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and FinchTV (Geospiza, Seattle, WA, USA).

3. Results and discussion

3.1. *Panax*-specific PCR assay

Several regions have been proposed as DNA markers for *Panax* species identification, such as the chloroplast genes *trnH-psbA, matK* and *rbcL,* the *rps2-rpoC2* intergenic spacer or the internal transcribed spacer (ITS) of nuclear ribosomal DNA (ITS1-5.8S-ITS2 regions), either as single (Han et al., 2016; Jung et al., 2014; Osathanunkul & Madesis, 2019) or in combined loci (Wallace et al., 2012; Zuo et al., 2011). In the present work, the gene encoding the dammarenediol synthase that is involved in the biosynthesis pathway of ginsenosides was the targeted region. Ginsenosides are thought to be the main bioactive compounds responsible for the main medicinal properties of ginseng, being almost exclusively present in plant species of *Panax* genus (Christensen, 2008; Wang et al., 2014). They are a special group of triterpenoid saponins that can be classified into two groups by the skeleton of their aglycones, whose profile varies with the *Panax* species, among other factors (Christensen, 2008). Therefore, the use dammarenediol synthase gene was exploited as a candidate marker of *Panax* genus.

The developed *Panax*-specific PCR assay with primers Pax1-F/Pax1-R (Table 2) produced the expected fragment of 129 bp for all the tested *Panax* species (Table S1, supplementary material), namely, *P. ginseng, P. quinquefolius*, *P. notoginseng, P. japonicus* and *P. trifolius*. Besides the *in silico* demonstrated primer specificity, the experimental potential reactivity of primers with 71 non-target plant species, including several medicinal plants, fruits and spices was confirmed (Table S1, supplementary material). The absence of amplification with all the tested non-*Panax* species confirms the specificity of the assay, while the PCR amplification of all DNA extracts with universal primers (eukaryotic)

targeting a nuclear 18S rRNA region (Costa et al., 2013; Villa et al., 2017) ensures the presence of amplifiable DNA and no false negative results (Table S1, supplementary material).

Before proceeding to the application of the *Panax*-specific PCR assay to commercial products, it was important to assess the yield, purity and amplification capacity of the DNA extracts, especially from complex and highly processed matrices, such as PFS. Generally, samples of herbal products (roots and infusions) provided extracts with variable DNA yields (5.1–159.1 ng/μL), but adequate purities (1.6–2.1). The results of PCR amplification targeting a universal eukaryotic region (18S rRNA gene) showed that all DNA extracts provided amplifiable DNA, except for sample #13. This exception might be due to the excess of inhibiting compounds, inferred by the obtained strong yellow colour (Table 3). Similarly, the DNA extracts from PFS presented highly variable yields (3.0–242.8 ng/μL), with generally adequate purities (1.4–2.1), but, most importantly, all providing amplifiable DNA (Table 3). The application results of the proposed *Panax*-specific PCR assay demonstrated that most commercial products of roots, herbal infusions and PFS provided the expected 129 bp amplicon (Table 3).

3.2. Real-time PCR coupled to HRM analysis

3.2.1. Method development

A real-time PCR approach coupled to HRM analysis, using EvaGreen dye and the *Panax*-specific primers, was then developed to allow species discrimination. Fig. 1 presents the real-time PCR amplification curves (Fig. 1A) and respective calibration curve (Fig. 1B) using 10-fold serially diluted DNA of *P. ginseng*. The results show that the assay exhibited a high performance since the analytical parameters were within the acceptable criteria (Bustin et al., 2009; ENGL, 2015), namely the PCR efficiency of 100.5 % (90–110 %), the correlation coefficient (R^2) of 0.995 (≥0.98) and the slope of −3.310 (−3.6 to −3.1) (Fig. 1B). The dynamic range covered 4 orders of magnitude of the target analyte (10 ng–1 pg of ginseng DNA) and the absolute LOD can be established as 1 pg of ginseng DNA, considering that for the lowest concentration level all replicates were amplified ($n = 8$ from two independent assays). The LOQ was set as 1 pg since the LOD value was within the linear dynamic range of the calibration curve (Bustin et al., 2009; ENGL, 2015). The resultant melting curve analysis show almost overlaid profiles with melt peaks of 78.7 ± 0.1 °C, suggesting the absence of unintended amplicons (Fig. 1C).

The five species, using the same initial DNA amount (20 ng), were successfully amplified with close cycle threshold values, ranging between 25.98 and 27.35 (Fig. 2A, Table 2). The conventional melt curve analysis shows close profiles for the five species (Fig. 2B), with melt peaks at 79.0 ± 0.2 °C for four species and a slightly higher value for *P. trifolius* (79.4 °C). This finding emphasises the high nucleotide similarity of the amplicons, which disables their differentiation by this approach. Therefore, the application of HRM analysis, as a technique able to discriminate amplicons with small nucleotide differences, was further performed. The obtained normalised curves allowed discriminating the five species in five different clusters with high levels confidence (≥98 %) (Fig. 2C, Table 2): *P. ginseng* (cluster 1 and reference cluster), *P. quinquefolius* (cluster 2), *P. notoginseng* (cluster 3), *P. japonicus* (cluster 4) and *P. trifolius* (cluster 5). These results were further emphasised by the respective difference curves (Fig. 2D).

HRM analysis was already successfully proposed to differentiate species of *Panax* genus, namely *P. ginseng* from *P. quinquefolius* (Jung et al., 2014) and *P. ginseng* from *P. notoginseng* (Osathanunkul & Madesis, 2019) targeting Indel variations of the chloroplast *rps2-rpoC2* intergenic spacer and ITS2 markers, respectively. However, both HRM approaches were limited to the differentiation of two *Panax* species, both excluding *P. japonicus*, which is a particular relevant species, and also *P. trifolius*. Besides, the method developed by Osathanunkul and Madesis (2019) was not effectivelly applied to authenticate commercial herbal products. Comparing with those two reports, the herein developed HRM method

Table 3
Results of qualitative polymerase chain reaction (PCR) and real-time PCR coupled to high-resolution melting (HRM) analysis of commercial products used in this work.

Code	Sample	Relevant labelled information	Qualitative PCR		HRM analysis		
			18S rRNA gene	DS[a] gene	Ct (mean ± SD)[b]	Cluster	Level of confidence (%, mean ± SD
#1	Root	*P. quinquefolius*	+	+	25.6 ± 0.5	1	99.3 ± 0.7
#2	Root	*P. ginseng*	+	+	25.4 ± 0.1	1	98.8 ± 0.6
#3	Root	*P. notoginseng*	+	+	27.1 ± 0.5	3	99.8 ± 0.2
#4	Root	*P. ginseng*	+	+	24.5 ± 1.0	6	99.2 ± 0.6
#5	Natural white Korean ginseng root	White Korean ginseng	+	+	25.0 ± 0.1	6	98.9 ± 1.2
#6	Red Korean ginseng root	Red Korean ginseng	+	+	>35		
#7	Green tea with ginseng (sachets)	5 % ginseng (*P. ginseng* roots)	+	+	33.3 ± 0.2	1	99.5 ± 0.4
#8	Green tea with ginseng (sachets)	5 % ginseng (*P. ginseng* roots)	+	+	30.8 ± 0.1	1	99.7 ± 0.3
#9	Herbal infusion with ginseng (sachets)	3 % ginseng root	+	+	34.0 ± 0.2	1	99.2 ± 0.6
#10	Coffee capsule with ginseng	Ginseng	+	+	28.0 ± 0.2	1	99.3 ± 0.3
#11	Tianqui flower tea	Notoginseng flowers	+	+	27.1 ± 0.4	7	99.1 ± 0.9
#12	Green tea with ginseng (sachets)	6 % ginseng (*P. ginseng*)	+	–	NA[c]		
#13	Korean red ginseng tea (sachets)	13.6 % Red Korean ginseng extract	–	–	NA		
#14	Ginseng, ginkgo, gotukola (capsules)	Ginseng	+	+	34.3 ± 0.3	1	99.1 ± 0.6
#15	Ginseng & ginkgo biloba (tablets)	Korean ginseng extract	+	+	34.5 ± 0.9	1	99.0 ± 0.8
#16	Korean ginseng (capsules)	Korean ginseng (ginseng root extract)	+	+	28.8 ± 0.1	1	99.4 ± 0.4
#17	Korean red ginseng powder root (capsules)	Red Korean red ginseng (*P. ginseng*)	+	+	32.1 ± 0.1	1	99.4 ± 0.3
#18	Red ginseng (capsules)	Red Korean ginseng roots (*P. ginseng*)	+	–	NA		
#19	Korean ginseng (capsules)	Korean ginseng powder	+	+	>35		
#20	Korean ginseng (tablets)	Korean ginseng (*P. ginseng*)	+	+	>35		
#21	Korean ginseng dried extract (capsules)	Korean ginseng (*P. ginseng* dry extract)	+	+	30.9 ± 0.1	1	99.1 ± 0.9
#22	Ginseng (capsules)	Ginseng (*P. ginseng*)	+	+	27.5 ± 0.2	1	99.1 ± 0.8
#23	Ginseng & guarana (capsules)	Powder with ginseng leaves dry extract (*P. ginseng*)	+	+	31.4 ± 0.1	1	99.2 ± 0.2

[a] DS, dammarenediol synthase;
[b] Mean cycle threshold (Ct) values ± standard deviation (SD) of $n = 8$ replicates;
[c] NA, no amplification.

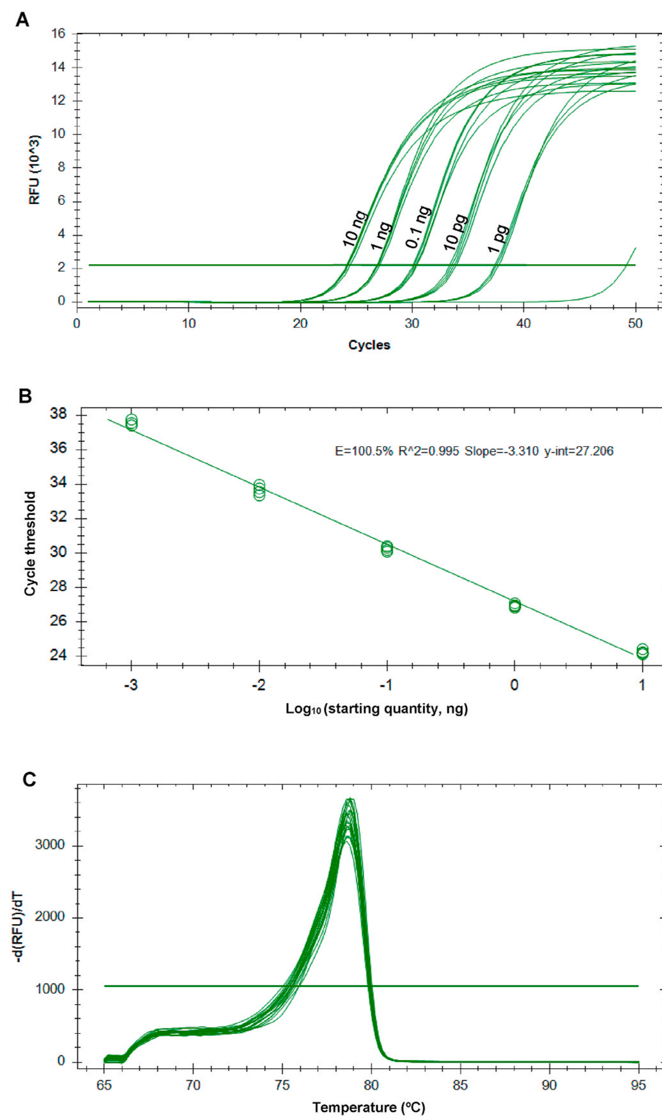

Fig. 1. Amplification (A), calibration (B) and melting (C) curves obtained by real-time polymerase chain reaction (PCR) with EvaGreen® dye targeting the dammarenediol synthase (DS) gene of *Panax* spp. using 10-fold serially diluted *Panax ginseng* DNA from 10 ng to 1 pg ($n = 4$ replicates). Legend: RFU, relative fluorescence units; E, PCR efficiency; R^2, coefient of correlation; y-int, intercept; T, temperature.

has the advantages of discriminating, for the first time, five *Panax* species and being effectively applied. The differentiation of such a high number of *Panax* species was achieved previously only by DNA barcoding. Zuo et al. (2011) identified four to six out of eight considerably divergent *Panax* species using the barcode loci *matK*, *psbK-I*, *psbM-trnD*, *rps16* and *nad1*. The plastome phylogeny and DNA barcoding studies developed by Manzanilla et al. (2018) showed that the markers *trnC-rps16*, *trnS-trnG* and *trnE-trnM* allow unambiguous molecular identification of 7 species in the *Panax* genus. However, the above approaches required the use of several barcode loci, relying on Sanger sequencing (Zuo et al., 2011) or next generation sequencing (Manzanilla et al., 2018) as post PCR analyses, without being effectively applied to authenticate commercial ginseng products.

3.2.2. Sequencing data

In order to validate the HRM method, the five species under study were sequenced. The target amplicon for HRM analysis has 129 bp in length, which is a very short fragment for direct sequencing, considering that the reading of the first 30 nucleotides at the 5′ end of the sequence often does not allow perfect resolution (Binladen, Thomas, Gilbert, Campos, & Willerslev, 2007; Villa, Costa, Meira, Oliveira, & Mafra, 2016). To overcome this problem, other sets of primers were attempted to cover a wider fragment encompassing the target 129-bp amplicon of the five species. From several primer sets, the pair Pax5-F/Pax6-R (Table 3) was selected to amplify a fragment with 217 bp of *P. ginseng*, *P. quinquefolius*, *P. notoginseng* and *P. japonicus*. However, the most distantly related species, *P. trifolius*, was not able to be amplified with the primers Pax5-F/Pax6-R. Therefore, for this species, the primers Pax1-F/Pax1-R had to be used for sequencing. All PCR products were further purified and sent for sequencing in opposite directions, at a specialised research facility. The obtained data allowed confirming the HRM results, covering the target fragment (129 bp). Only electropherograms with high quality and resolution were used in the sequence alignment of each *Panax* species. The sequencing results of the five species were aligned with the consensus *P. ginseng* sequence retrieved from NCBI (accession no. KJ939266.1) and presented in Fig. 3. Sequencing data show that *P. ginseng* is fully aligned with the consensus sequence, having one potential nucleotide mismatch comparing with *P. quinquefolius*, which has a R (A or G) instead of G in *P. ginseng*. This finding justifies grouping these two species into distinct HRM clusters. *P. notoginseng* has three nucleotide mismatches at the positions 66, 93 and 106, comparing with *P. ginseng*, which explains being placed in cluster 3. *P. japonicus* accounts with one mismatch in the forward primer and an insertion of W (A or T) in relation to *P. ginseng*, thus being discriminated in cluster 4. Finally, in *P. trifolius* sequence, five mismatches can be observed comparing with *P. ginseng*, namely A→W (A or T), A→G, A→G, G→A and C→A in positions 108, 129, 141, 159 and 163, respectively, placing this species in a distinct cluster (cluster 5). Therefore, the sequencing results corroborate the HRM species discrimination, thus validating the method. Additionally, the HRM results are in good agreement with phylogenetic studies, in which *P. ginseng* and *P. quinquefolius* are considered as closely related species (Manzanilla et al., 2018), in opposition to *P. trifolius* that is genetically more distant (Zuo et al., 2011).

3.2.3. Product analysis

Following the method development and validation, the final stage of this work was the method application to analyse commercial food products containing ginseng. Accordingly, 23 ginseng products were amplified by real-time PCR with primers Pax1-F/Pax1-R, followed by HRM analysis. The real-time PCR results confirmed the qualitative PCR findings, showing that *Panax* spp. was not detected in three samples (#12, #13 and #18) and was unreliably amplified at late cycles (>35) in other three samples (#6, #19 and #20), disabling their posterior HRM analysis (Table 3). In samples labelled as containing red Korean ginseng (#6, #13 and #18), the severity of the processing of this type of products (harvested when 6 years old, steamed at 120–130 °C for 2–4 h and dried) (Court, 2000; Yun, 2001) might strongly affect the DNA integrity, making the amplification of 129-bp fragments difficult or even impossible. The other *Panax* negative results (#12, #19 and #20) suggest the absence or only trace amounts of ginseng, particularly in sample #12 of green tea with claimed 6 % of ginseng. In samples #19 and #20, which are PFS in the form of capsules and tables, respectively, the presence of bulking agents, such as lecithin and cellulose, and excipients like titanium dioxide might have hindered the release of ginseng DNA. It is known that the presence of excipients, such as silica, titanium dioxide and iron oxide, might adsorb DNA and hamper its extraction (Costa, Amaral, et al., 2015). In opposition, sample #15 of PFS tablets, although containing silica as excipient, provided amplifiable *Panax* DNA. Therefore, despite the difficulties in analysing such complex and processed samples, 17 out 23 commercial samples confirmed the presence of *Panax* spp. and could be further analysed by HRM.

Fig. 4 presents the normalised (Fig. 4A) and respective difference curves (Fig. 4B), as an example assay of application to commercial

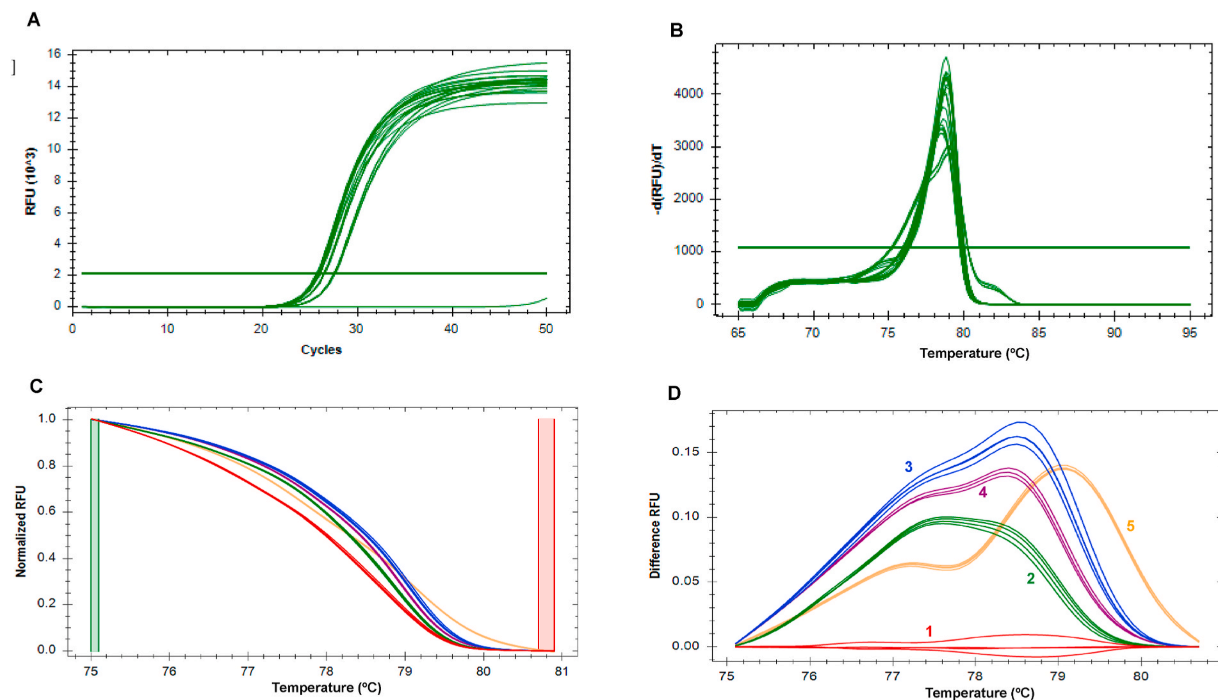

Fig. 2. Amplification (A), melting (B), normalised melt (C) and difference (D) curves obtained by real-time polymerase chain reaction with EvaGreen® dye coupled to high-resolution melting analysis targeting the dammarenediol synthase (DS) gene of *Panax* spp. Legend: Cluster 1, *P. ginseng*; Cluster 2, *P. quinquefolius*; Cluster 3, *P. notoginseng*; Cluster 4, *P. japonicus;* Cluster 5, *P. trifolius* ($n = 4$ replicates). Legend: RFU, relative fluorescence units.

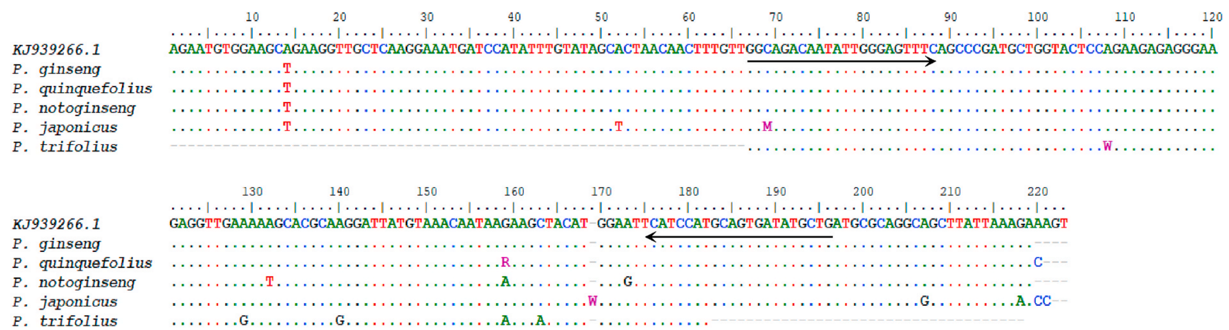

Fig. 3. Alignment of sequencing data of the dammarenediol synthase (DS) gene of *P. ginseng*, *P. quinquefolius*, *P. notoginseng*, *P. japonicus* and *P. trifolius* with the consensus sequence (KJ939266.1) from NCBI database, highlighting the region used in high-resolution melting analysis (arrows represent the forward and reverse primers Pax1-F/Pax1-R).

samples. The clustering results show 5 reference and 2 additional clusters (Fig. 4B), being the data for all samples presented in Table 1. As expected, most samples were grouped in cluster 1 (*P. ginseng*) with high levels of confidence (≥99 %), thus being in accordance with their labelled information (#2, #7-#11, #14-#17 and #21-#23). Sample #3, labelled as *P. notoginseng* root, was grouped according with the correct species (cluster 3). However, sample #1 labelled as containing *P. quinquefolius*, was grouped in cluster 1 (*P. ginseng*) instead of 2 (*P. quinquefolius*), suggesting the exchange of species. Additionally, samples #4 and #5, which were expected to be grouped with the *P. ginseng* (cluster 1), formed a distinct cluster (6), suggesting the need for further investigation. Similarly, sample #11, labelled as containing *P. notoginseng* flowers, formed the cluster 7.

4. Conclusions

In the present work, two DNA-based methods were proposed to authenticate ginseng-containing foods. A specific PCR approach was developed to detect *Panax* spp. DNA and a real-time PCR assay coupled to HRM analysis was further proposed to differentiate five *Panax* species (*P. ginseng*, *P. quinquefolius*, *P. notoginseng*, *P. japonicus* and *P. trifolius*) for the first time. The *Panax*-specific real-time PCR assay was successfully developed with high analytical performance parameters, achieving a sensitivity down to 1 pg of ginseng DNA. The application of the *Panax*-specific PCR assays (end-point PCR and real-time PCR) to commercial foods of herbal infusions and PFS was successful for the majority of the samples. The HRM method enabled the differentiation of the five referred species with high level of confidence, which was further corroborated with sequencing data. The effective applicability of the HRM method showed that most samples were successfully clustered, being all except one in accordance with their labelling statements. Samples, not grouped in any reference species, suggest their swap with other non-tested *Panax* species or mixed species. Despite the relatively high number of targeted *Panax* species, covering the most relevant ones, there are many others not included, which is a limitation of the method. This finding demands further investigation. The proposed methods proved to be useful for screening the presence of *Panax* DNA in processed ginseng products and further species identification, enabling

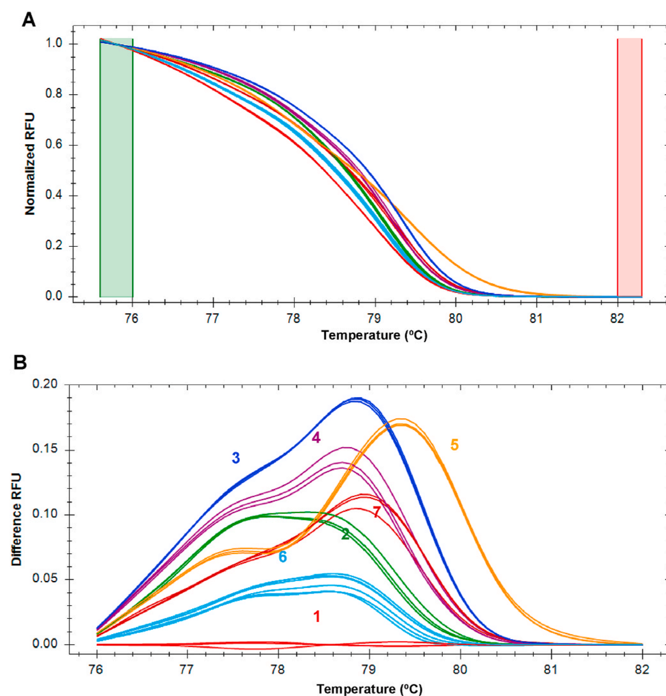

Fig. 4. Normalised melt (A) and difference (B) curves obtained by real-time polymerase chain reaction with EvaGreen® dye coupled to high-resolution melting analysis targeting the DS gene of *Panax* spp. applied to commercial ginseng products. Legend: Cluster 1, *P. ginseng*; Cluster 2, *P. quinquefolius*; Cluster 3, *P. notoginseng*; Cluster 4, *P. japonicus*; Cluster 5, *P. trifolius*; Cluster 6 (undefined) and Cluster 7 (undefined), commercial samples ($n = 4$ replicates). Legend: RFU, relative fluorescence units.

assessing their labelling compliance and detecting eventual fraudulent/mislabelling practices, which can be reliable tools for control laboratories and regulatory agencies.

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CRediT authorship contribution statement

Liliana Grazina: Methodology, Formal analysis, Investigation, Writing – original draft. **Joana S. Amaral:** Conceptualization, Supervision, Writing – review & editing. **Joana Costa:** Formal analysis, Investigation, Writing – review & editing. **Isabel Mafra:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112211.

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