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Analytical Methods

# DNA barcoding based identification of *Hippophae* species and authentication of commercial products by high resolution melting analysis

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ABSTRACT

Sea buckthorn (*Hippophae*), an ancient crop with modern virtues, is increasingly consumed in source of foods and nutraceuticals. The growing demand leads to the adulteration of commercial sea buckthorn products, which is a common form of food fraud. Herein, a high resolution melting assay, targeting a DNA barcoding region of the internal transcribed spacer 2 (ITS2) (Bar-HRM) was developed to identify the seven native Chinese *Hippophae* species, and to authenticate commercial sea buckthorn products. Melting data from the HRM assay demonstrated that all *Hippophae* species could be clearly distinguished. Then, application to commercial sea buckthorn products indicated the existence of adulterants or contamination, further confirmed using Sanger sequencing results for PCR products from HRM. The Bar-HRM technique proposed in this work could provide a method for regulatory agencies, promoting consumers trust, and raise the quality and safety of sea buckthorn products.

## 1. Introduction

Sea buckthorn (*Hippophae*) is a member of the family Elaeagnaceae and is primarily distributed in cold arid regions throughout Europe and Asia (Suryakumar & Gupta, 2011; Teleszko, Wojdyło, Rudzińska, Oszmiański, & Golis, 2015). Recent studies and clinical trials have shown that the ripe fruit of sea buckthorn is a good source of bioactive substances, including vitamins, carotenoids, phytosterols, organic acids, fatty acids, free amino acids and different flavonoids, which have antioxidant, immunomodulatory, anti-atherogenic, anti-stress, hepatoprotective, radioprotective and tissue repair activities (Bal, Meda, Naik, & Satya, 2011; Suryakumar & Gupta, 2011). Due to these putative health benefits and nutritional value, sea buckthorn berries have gained worldwide attention and are used in about 200 commercial products, such as food, fresh juice, beverages, herbs, nutraceutical products and cosmetics (Bal et al., 2011).

As a commonly used nutritional supplement, sea buckthorn berries have been recorded in State as well as local standards, such as the List of Medicinal and Edible plant in China (Chinese Pharmacopeia Commission, 1995, 2015; Dhyani, Maikhuri, Misra, & Rao, 2010; Food and Drug Administration of Sichuan Province, 2014; Liu, 1999). However, much confusion exists around uses for the different sea buckthorn species, as the standards differ across localities and the same vernacular name is used for a number of *Hippophae* taxa with similar morphology. More importantly, berries of different species, sharing similar morphological characteristics, are often combined in sea buckthorn raw products. Adulterants may vary in their bioactive components, leading to variation in efficacy and possible loss of consumer trust (Liu et al., 2016; Teleszko et al., 2015). Thus, there is a need for rapid and accurate identification and authentication of *Hippophae* species in commercial products.

High resolution melting (HRM) is a post-PCR method for mutation scanning and genotyping utilizing DNA sequence variants, such as single nucleotide polymorphisms (SNPs) and small insertion/deletions, based on the melting behaviors of double-stranded DNA (dsDNA) (Palais, Liew, & Wittwer, 2005; Sakaridis, Ganopoulos, Argiriou, & Tsaftaris, 2013; Schmiderer, Ruzicka, & Novak, 2015; Sun, Li, Xiong, Zhao, & Chen, 2016). Gradual denaturation resulting from incremental heating produces characteristic melting profiles that can be used to discriminate between species and authenticate commercial products (Xanthopoulou et al., 2016).

DNA barcoding was originally developed to identify plant and

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animal species, and relies on a short, standardized sequence of the genome (Hebert, Cywinska, Ball, & Dewaard, 2003; Li et al., 2014). Recent studies indicated that the ITS2 region could be used as a core DNA barcode for the identification and discrimination of a wide range of plant taxa because of its high variability and mutation rate, especially amongst taxa within *Hippophae* that have similar gross morphologies (Liu et al., 2015; Sun et al., 2016; Zhao et al., 2016).

Bar-HRM combines DNA barcoding and HRM analysis, which is often applied to identify and authenticate target species in herbal medicine, food and agricultural products (Duyvejonck et al., 2015; Ganopoulos, Madesis, Darzentas, Argiriou, & Tsaftaris, 2012; Hong et al., 2015; Kalivas et al., 2014). This method allows the identification of adulterants as well as authentication of desirable species in products.

The present study aimed to identify different *Hippophae* species, using the ITS2 DNA barcode with the HRM. Additionally, the Bar-HRM served as a method for rapid detection and measurement of adulterants in commercial sea buckthorn products.

## 2. Materials and methods

### 2.1. Plant material and DNA isolation

Reference samples (Table 1) of different *Hippophae* species were provided by the College of Ethnic Medicine, Chengdu University of Traditional Chinese Medicine. Ten commercial sea buckthorn products were gathered from major herb markets and stores in China (Table 1, Supplementary Fig. 1). All of the corresponding voucher samples were deposited at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

The dried plant tissues were first ground with liquid nitrogen and 60 mg of the resulting fine powder used for DNA extraction with the Plant Genomic DNA Kit (Tiangen Biotech Co., Beijing, China) following the manufacturer's instructions. DNA concentrations from all the samples were adjusted to a final concentration of 50 ng/μL. The DNA was stored at −20 °C for further use.

### 2.2. PCR amplification

PCR amplification, DNA melting, and fluorescence measurements were performed in a total reaction volume of 25 μL using a Rotor-Gene Q MDx (QIAGEN Co., Hilden, Germany). The reaction mixture contained 1 μL genomic DNA, 12.5 μL 2 × HRM PCR master mix (Type-it® HRM™ PCR Kit, QIAGEN Co., Hilden, Germany) and 1 μL of 10 μM forward and reverse primers (ITS2F: 5′-ATGCGATACTTGGTGTGAAT-3′, ITS3R: 5′-GACGCTTCTCCAGACTACAAT-3′), and the total volume was adjusted to 25 μL with RNase-Free water (Keller et al., 2009; Sun et al., 2016). The reaction conditions were: an initial denaturing step at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s. Fluorescent data for PCR amplification was recorded during the extension step in the green channel. HRM analysis was conducted immediately after PCR amplification. In this experiment, the temperature was ramped from 70 °C to 95 °C at 0.15 °C increments with a 2 s holding time for each increment. Fluorescence data were acquired at each increment.

### 2.3. Data analysis

Rotor-Gene Q MDx v 2.3.1 (QIAGEN GmbH, Hilden, Germany) was used to genotype the *Hippophae* species. Several curves were used to identify different species, 1) negative derivative of fluorescence (F) over temperature (T) (dF/dt) curve, which primarily displays the melting temperature ($T_m$), 2) normalized raw curve, which depicts the decreasing fluorescence vs. increasing temperature, and 3) difference curves, which visualizes small differences between individual melting curve. (Kalivas et al., 2014). Principal component analysis (PCA) was conducted on $T_m$ for each sample to distinguish and classify different species.

### 2.4. Sequence confirmation

PCR products were sequenced directly, with the same primers used for PCR, in a 3730XL sequencer (Applied Biosystems, Foster, California, USA). Proofreading and contig assembly of sequencing peak diagrams were performed using CondonCode Aligner V 3.7.1 (CodonCode Co., Centreville, MA, USA). All the sequences were submitted to a DNA barcoding system (http://www.tcmbarcode.cn/en/) and GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to verify the HRM results and identify unknown adulterants using BLAST (Basic Local Alignment Search Tool).

## 3. Results

### 3.1. Hippophae species analysis using Bar-HRM

The melting characteristics of *Hippophae* species were assessed by plotting two informative melting curves (Fig. 1). Analysis of the normalized HRM melting curves with the barcode ITS2 (Fig. 1a) indicated that the majority of *Hippophae* species samples were distinguishable with different genotypes exhibiting unique transitions. This can be seen in the HRM profile plots, such as melting curve shapes. However, the curve profiles for some species were similar and could not be discriminated visually. By assigning *H. rhamnoides* ssp. *sinensis* as the reference genotype, the remaining species could be easily discriminated (Fig. 1b), except for *H. goniocarpa* and *H. tibetana*. In order to differentiate these, a separate data subset was generated using their HRM difference melting curves (Supplementary Fig. 2). Results from this analysis revealed HRM curves for *H. goniocarpa* and *H. tibetana* that were distinct. Thus, all the *Hippophae* species tested were clearly identifiable.

### 3.2. Detection of commercial sea buckthorn products through Bar-HRM analysis

The same approach was applied for the identification of 10 batches of commercial sea buckthorn products. A preliminary comparison and analyses showed that *H. rhamnoides* ssp. *sinensis* and *H. rhamnoides* ssp. *turkestanica* were the most similar. Due to this, only these two were utilized as references for the visual authentication of 10 commercial products (Fig. 2). The normalized HRM curves for these two species and 10 commercial sea buckthorn products are shown in Fig. 2a. Four of the commercial products tested produced dramatically different melting plots that were distinct from the reference *Hippophae* species. Of the remaining six samples, four commercial products grouped with *H. rhamnoides* ssp. *turkestanica*, and one with *H. rhamnoides* ssp. *sinensis*. Interestingly, one commercial product produced a unique melting plot (Species 7) and remained ungrouped. Thus all samples were successfully classified, with five samples assigned to species origin genotypes. These groupings were supported by the analysis of the difference graphs for the two species origin genotypes samples (*H. rhamnoides* ssp. *sinensis* as reference genotype) and 10 commercial sea buckthorn products, and revealed the identification of any others present (Fig. 2b).

Furthermore, unique plots for each species were produced using derivative melting curve for the two botanical genotypes and the commercial products (Fig. 2c), which could be characterized using different peaks (Table 2). Further corroboration of the sample classifications was provided by PCA score plots (Fig. 2d) which illustrated that five commercial products belonged to *Hippophae* species but the remainder were unknown.

**Table 1**
Samples (fruits) used in this study.

| Samples No. | Species | Voucher No. | GenBank No. | Collection area |
|---|---|---|---|---|
| OP[a] 1 | *H. rhamnoides* subsp. *sinensis* | YC0546MT02 | KJ843998 | Sichuan China |
| | | YC0546MT08 | KJ844004 | Qianghai China |
| | | YC0546MT17 | KJ844013 | Sichuan China |
| OP 2 | *H. rhamnoides* subsp. *mongolica* | YC0547MT01 | KJ843986 | Xinjiang China |
| | | YC0547MT02 | KJ843987 | Xinjiang China |
| | | YC0547MT03 | KJ843988 | Xinjiang China |
| OP 3 | *H. rhamnoides* subsp. *yunnanensis* | YC0548MT03 | KJ817425 | Tibet China |
| | | YC0548MT04 | KJ939408 | Yunnan China |
| | | YC0548MT05 | KJ939409 | Yunnan China |
| OP 4 | *H. rhamnoides* subsp. *turkestanica* | YC0549MT01 | KJ844038 | Xinjiang China |
| | | YC0549MT02 | KJ844039 | Xinjiang China |
| | | YC0549MT03 | KJ844040 | Tibet China |
| OP 5 | *H. rhamnoides* subsp. *wolongensis* | YC0550MT01 | KJ844024 | Sichuan China |
| | | YC0550MT02 | KJ844025 | Sichuan China |
| | | YC0550MT03 | KJ844026 | Sichuan China |
| OP 6 | *H. goniocarpa* | YC0551MT01 | KJ844018 | Sichuan China |
| | | YC0551MT02 | KJ844019 | Sichuan China |
| | | YC0551MT03 | KJ844020 | Sichuan China |
| OP 7 | *H. litangensis* | YC0552MT01 | KJ844015 | Sichuan China |
| | | YC0552MT02 | KJ844016 | Sichuan China |
| | | YC0552MT03 | KJ844017 | Sichuan China |
| OP 8 | *H. neurocarpa* subsp. *neurocarpa* | YC0553MT01 | KJ844042 | Qinghai China |
| | | YC0553MT02 | KJ844043 | Sichuan China |
| | | YC0553MT03 | KJ844044 | Sichuan China |
| OP 9 | *H. neurocarpa* subsp. *stellatopilosa* | YC0554MT01 | KJ844027 | Sichuan China |
| | | YC0554MT02 | KJ844028 | Sichuan China |
| | | YC0554MT03 | KJ844029 | Sichuan China |
| OP 10 | *H. salicifolia* | YC0653MT01 | KJ844021 | Tibet China |
| | | YC0653MT02 | KJ844022 | Tibet China |
| | | YC0653MT03 | KJ844023 | Tibet China |
| OP 11 | *H. gyantsensis* | YC0654MT04 | KJ843992 | Tibet China |
| | | YC0654MT05 | KJ843993 | Tibet China |
| | | YC0654MT08 | KJ843996 | Tibet China |
| OP 12 | *H. tibetana* | YC0655MT02 | KJ844031 | Sichuan China |
| | | YC0655MT06 | KJ844035 | Sichuan China |
| | | YC0655MT08 | KJ844037 | Sichuan China |
| CP[b] 1 | –[c] | SSYC1 | | Anguo herb market |
| CP 2 | – | SSYC2 | | Akesu Xinjiang |
| CP 3 | – | SSYC3 | | Bozhou herb market |
| CP 4 | – | SSYC4 | | Hehuachi herb market |
| CP 5 | – | SSYC5 | | Jinghe Xiangjiang |
| CP 6 | – | SSYC6 | | Chaoyang Liaoning |
| CP 7 | – | SSYC7 | | Hohhot Inner Mongolia |
| CP 8 | – | SSYC8 | | Xining Qinghai |
| CP 9 | – | SSYC9 | | Urumqi Xinjiang |
| CP 10 | – | SSYC10 | | Kunming Yunnan |

[a] OP indicates original plant.
[b] CP refers to a commercial product.
[c] – commercial products labeled as sea buckthorn as products without species identified.

### 3.3. Dubious samples

Commercial product SSYC3 (Species 7) exhibited a melting curve with the front half similar to that of *H. rhamnoides* ssp. *sinensis*, but the latter portion of the melting curve diverged from known genotypes (Fig. 2a and b). In a morphological comparison of the vegetative material with *H. rhamnoides* ssp. *sinesis*, it appeared to comprise of *H. rhamnoides* ssp. *sinensis* with adulterants of a similar morphological appearance. To confirm this, TA-cloning (Perez-Tris & Bensch, 2005) was conducted on PCR products from this sample. Sequencing of the cloned products confirmed that DNA from two different species were present. The two separate ITS2 sequences were submitted to the DNA barcoding system and GenBank for identification of the unknown adulterants.

### 3.4. Results validation

To validate the detection of commercial sea buckthorn products by Bar-HRM, and investigate the botanical origins of the unknown samples, all the PCR products were sequenced and submitted to the online DNA barcoding identification systems. The results obtained confirmed that five commercial sea buckthorn products belonged to *Hippophae* species but others were adulterants from different genera (Table 3).

To identify the adulterants, sequences were searched against known DNA repositories and one commercial product SSYC4 (Species 4) was identified as *C. songaricum*, which is known to parasitize the roots of *Nitraria* species (Liu, Chen, Li, & Li, 2013; Zhou et al., 2009). While *C. songaricum* is morphologically different from *Hippophae* taxa, *Nitraria* resembles sea buckthorn quite closely (Supplementary Fig. 1). Subsequently, 10 samples were selected randomly from SSYC4 for further testing. DNA extraction, PCR amplification, sequencing and BLAST results from these samples identified the material as *N. tangutorum*. Based on this testing, it appears that, during the harvesting of raw materials parts of *C. songaricum* were unintentionally mixed with the fruits of *N. tangutorum*. It is important to note that Bar-HRM was able to detect two types of adulterants in the raw material: one morphologically similar and one morphologically divergent from the sea buckthorn.

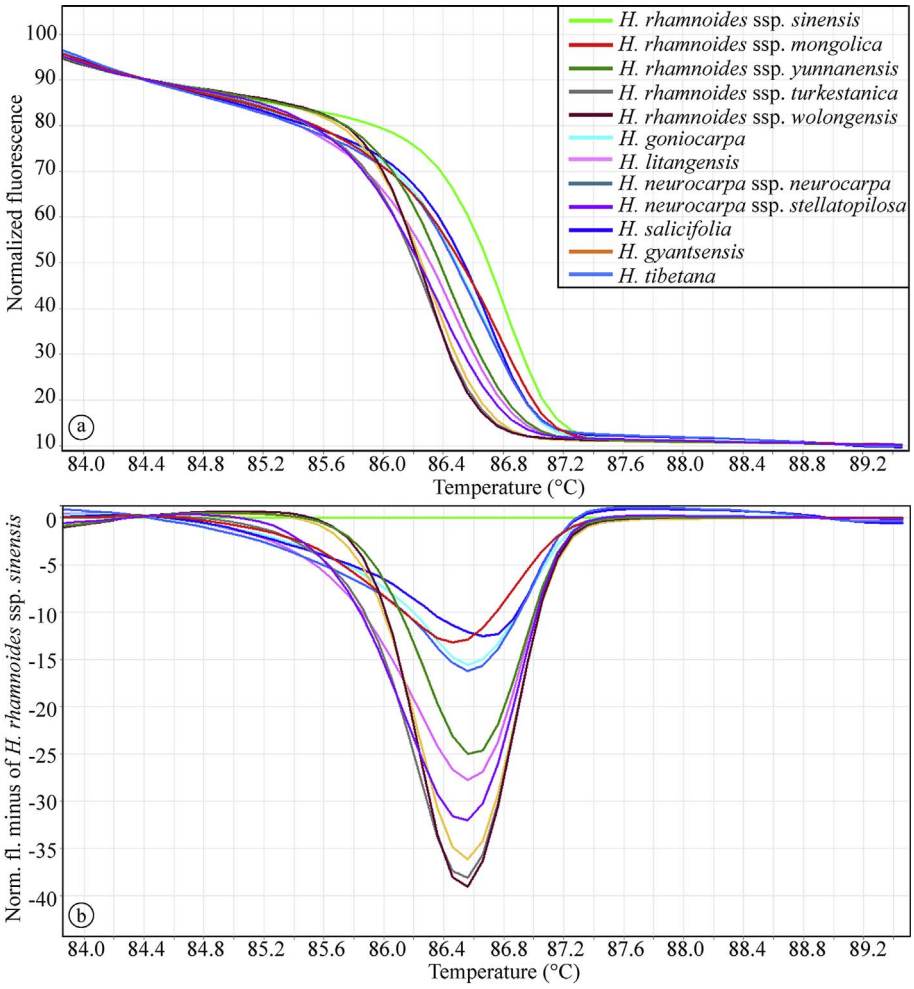


**Fig. 1.** Representative melting curves of *Hippophae* species using HRM analysis with the ITS2 barcode: (a) normalized melting profiles of *Hippophae* species, (b) difference graphs of *Hippophae* species using *H. rhamnoides* ssp. *sinensis* as reference genotype.

## 4. Discussion

Over the last few decades, the global demand for edible plant raw materials has increased due to their potential health benefits (World Health Organization, 2013). As a result, there are large quantities and varieties of raw materials sold in markets, and most of them are in processed or modified forms, such as dried or powdered forms. Thus, the problems of substitution, adulteration and counterfeiting of commercial products have been exacerbated. Thus, development of an accurate, rapid, and reliable method for species identification and adulterant detection is important to ensure quality and efficacy of commercial food and other products.

HRM, a post-PCR method conducted in closed tubes, allows for the rapid analysis of genetic variation in PCR products. The method involves precise monitoring of fluorescence changes caused by the release of an intercalating DNA dye from dsDNA during denaturation as temperature increases. The method allows accurate differentiation of taxa at a variety of nomenclatural levels, (i.e. amongst genera, species, and even below the species boundary), based on the $T_m$ of specific PCR products (Cheng et al., 2006; Ganopoulos et al., 2013).

Previously, we found that some subspecies of *H. rhamnoides* and *H. neurocarpa* could not be discriminated using only DNA barcoding (Liu et al., 2015). Here, with the application of the ITS2 locus, coupled with HRM, *Hippophae* taxa could be identified, especially those targeted for health benefits. This methodology worked well specifically for the five subspecies of *H. rhamnoides* recorded in the Chinese Pharmacopeia (Chinese Pharmacopeia Commission, 2015), and *H. gyantsensis* and *H. tibetana* listed in Chinese local standard in Sichuan, Qianghai and Tibet could be identified unambiguously (Chinese Pharmacopeia Commission, 1995; Food & Drug Administrition of Sichuan Province, 2014). Another important finding was that Bar-HRM detects simultaneously commercial sea buckthorn products as well as substitutions or adulterations. We found that the botanical origins of commercial products could be determined easily using this method. It was notable that half of the commercial products contained adulterant and the results indicated that the botanical origins of these samples included *N. tangutorum, N. sibirica, S. pohuashanensis* and *B. vulgaris*, which are not in the same genus as claimed. These adulterants are morphologically similar, to the point that only trained experts could identify the differences, but these cannot be distinguished when the appearance is altered after processing. For consumers, the threat of fake or substitutions is high. However, Bar-HRM analysis could deliver accurate, unambiguous authentication of sea buckthorn and potential adulterants.

It has been reported that the power of HRM analysis relies heavily on sequence diversity of the genes tested (Ghorashi, Kanci, & Noormohammadi, 2015). When sequences have little divergence, the melting curve profiles are similar and visual discrimination is difficult. In this study, the original plants belonged to a single genus (*Hippophae*) and, consequently, some of the profiles were very much alike (Fig. 1a). However, by using difference melting profiles with *H. rhamnoides* ssp. *sinensis* as reference genotypes, curves could be distinguished, even at the subspecies level (Fig. 1b).

When examining commercial sea buckthorn products, some specimens showed obvious differences in melting curves, and could be discriminated visually from authentic products. After verification using online DNA barcoding identification systems, these samples were identified and found to belong to other genera. The results demonstrate that difference melting profiles have better visual discrimination

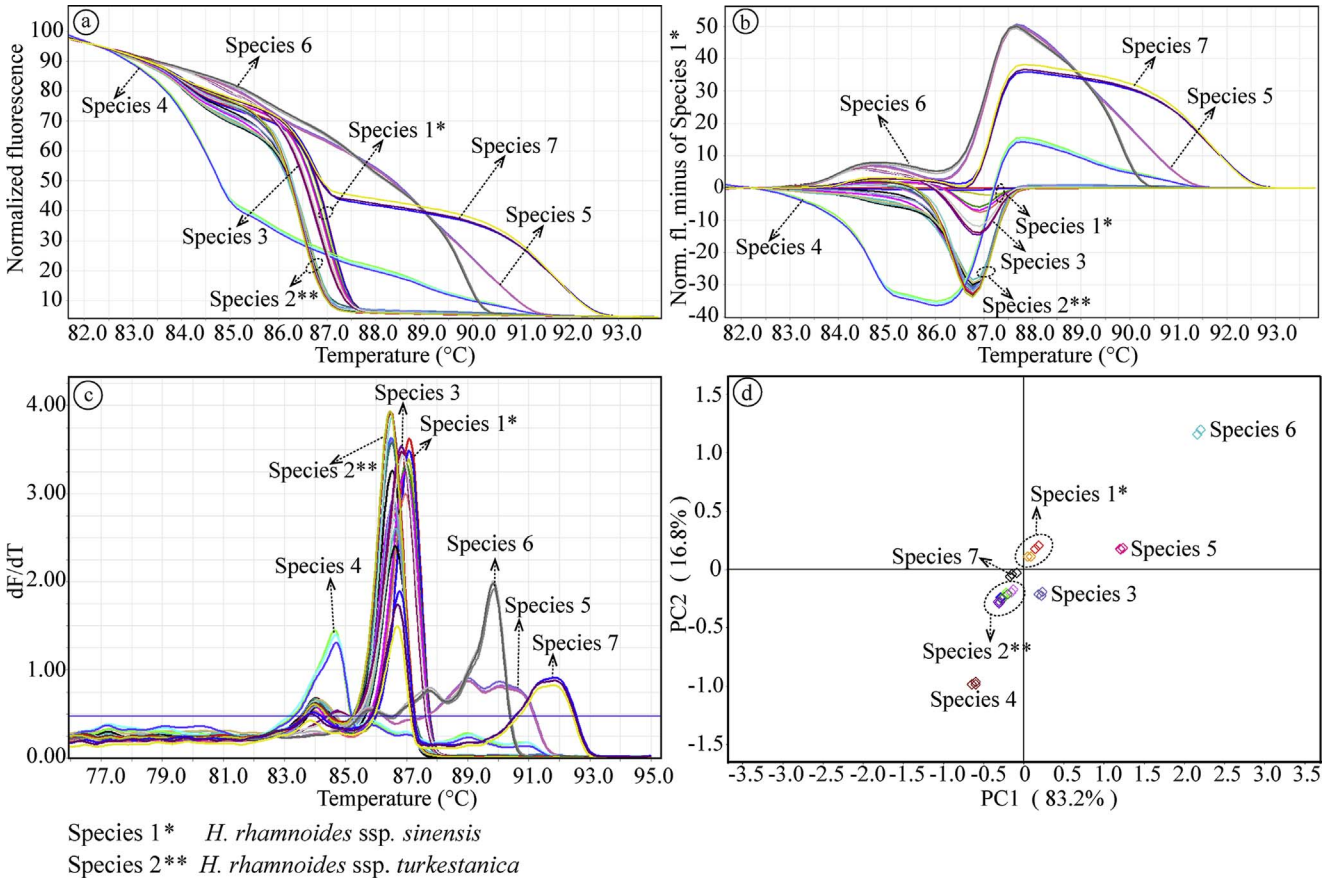


**Fig. 2.** Melting curves of two botanical genotypes (*H. rhamnoides* ssp. *sinensis* and *H. rhamnoides* ssp. *turkestanica*) and tested commercial products using HRM analysis with the ITS2 barcode: (a) normalized melting profiles, (b) difference melting profiles using *H. rhamnoides* ssp. *sinensis* as reference genotype, (c) derivative melting curves of *Hippophae* species and (d) PCA score plots of 10 commercial sea buckthorn products $T_m$ values.

**Table 2**
The melting peaks of different species for commercial products.*

| Species | $T_m$ 1 ± SD* | $T_m$ 2 ± SD* |
|---|---|---|
| Species 1 | 83.98 ± 0.03 | 86.96 ± 0.01 |
| Species 2 | 84.04 ± 0.05 | 86.53 ± 0.06 |
| Species 3 | 84.77 ± 0.03 | 86.85 ± 0.02 |
| Species 4 | 84.67 ± 0.03 | 85.81 ± 0.02 |
| Species 5 | 85.80 ± 0.02 | 87.76 ± 0.01 |
| Species 6 | 85.75 ± 0.00 | 89.04 ± 0.02 |
| Species 7 | 83.90 ± 0.03 | 86.73 ± 0.03 |

* Tm values ± standard deviation (SD).

characteristics for genotypes with high similarity than alternatives, whereas normalized melting profiles differentiated samples more effectively at a genera level.

Key components of food quality and safety are precise labeling information and good manufacturing practices along the commercialization chain (Osathanunkul, Madesis, & de Boer, 2015). Many studies show that substitution, adulteration, and counterfeiting occurs in commercial products (Han et al., 2016; Techen, Parveen, Pan, & Khan, 2014). Thus, an important contribution of this study is the database developed for all seven species and seven subspecies of *Hippophae* native to China using the Bar-HRM method. This database will help recognition and authentication of *Hippophae* species. In addition, the method developed is sufficiently sensitive to identify samples at the subspecies level. We propose that the method offers a basis for authentication and traceability of sea buckthorn products, and overcomes problems associated with visual inspection of morphologically indistinct forms (e.g. powders).

**Table 3**
Identification results of 10 commercial sea buckthorn products.

| CD* No. | Species | Alignment results | GenBank No. of haplotype |
|---|---|---|---|
| SSYC1 | Species 2 | *H. rhamnoides* ssp. *turkestanica* | KX268511 |
| SSYC2 | Species 6 | *Nitraria tangutorum* | KX268512 |
| SSYC3 | Species 7 | *Sorbus pohuashanensis* (SSYC3-1) | KX268513 |
| | Species 1 | *H. rhamnoides* ssp. *sinensis* (SSYC3-2) | KX268514 |
| SSYC4 | Species 4 | *Cynomorium songaricum* (SSYC4-1) | KX268515 |
| | Species 6 | *N. tangutorum* (SSYC4-2) | KX268516 |
| SSYC5 | Species 3 | *Berberis vulgaris* | KX268517 |
| SSYC6 | Species 1 | *H. rhamnoides* ssp. *sinensis* | KX268518 |
| SSYC7 | Species 2 | *H. rhamnoides* ssp. *turkestanica* | KX268519 |
| SSYC8 | Species 2 | *H. rhamnoides* ssp. *turkestanica* | KX268520 |
| SSYC9 | Species 2 | *H. rhamnoides* ssp. *turkestanica* | KX268521 |
| SSYC10 | Species 5 | *N. sibirica* | KX268522 |

* CD indicates commercial product.

In conclusion, Bar-HRM analysis represents an accurate, convenient, and rapid tool for market supervision, and broad application of this approach could provide inspection method for regulatory agencies in the prevention of food fraud.

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## Conflict of interest

The authors declare that no competing interests exist.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.09.040.

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