A Novel High-Resolution Melting Method for Detection of Adulteration on Pistachio (Pistacia vera L.)

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Research Article

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Abstract

Background

The pistachio nut (*Pistacia vera* L.) is a popular nut which is beneficial for health. As a result of its high price, adulteration of pistachio products with other foods is common. The green pea (*Pisum sativum* L.) is similar to pistachio in colour and texture when both are in a fine powder form, therefore powdered pistachio products are often adulterated with powdered green pea. In this study, we aimed to develop a novel method based on high resolution melting (HRM) to detect green pea adulteration in powdered pistachio products as low as in ratio.

Methods and Results

Novel primers that targeted the chloroplastidial sequence *atpA*, *rpoC*1 and *ycf3* were tested on admixture ratios of 90:10, 99:1, 499:1 and 999:1 of pistachio:green pea. The melting temperatures (T_m) were found to detect the adulteration for the targets *rpoC*1 and *ycf3*. Since the *atpA* amplicon has identical guanine-cytosine content in both pistachio and green pea, it failed to distinguish the admixtures from the positive control samples. Differential plots and genotyping confidence percentages clearly distinguished the admixtures from positive controls down to a 0.1% ratio of pistachio:green pea. Sequencing of the amplicons supported the use of HRM to distinguish between pistachio and green pea.

Conclusions

The use of HRM with novel rpoC1 and ycf3 primer sets was found to be useful for rapid, cost-effective and reliable detection of green pea adulteration of pistachio down to a ratio of 0.1%.

1. Introduction

Food safety, especially for value-added and high-value products, has always been a critical issue. Due to rising market demand and limited production because of a loss of available cultivated land, consumers often purchase food items which have been adulterated with other products. Therefore, species identification of plant-based food products is critical for to be able to trace the composition of their food, which has implications on allergy and disease information for the consumer.

Pistachio (*Pistacia vera* L.) has high economical value and is ranked fifth in total level of global production. Pistachios have been consumed in human diets since prehistoric times. Pistachio nuts are rich in lipids, protein, potassium, phenols, lutein [1]. As a result of the mineral and vitamin content of pistachios, it is recommended to consume them for their health benefits to aid against diseases such as cancer [2]. As our understanding of the nutritional properties of pistachios increases, the amount of pistachio nuts consumed increases. Pistachios are used in several diverse ways, such as direct consumption of the nuts, or as a flavouring in bakery and deli products. Ground nuts are also used to produce pistachio paste. The health properties and multiple usages make pistachios an expensive nut, meaning that pistachio products are susceptible to adulteration. Green pea (*Pisum sativum* L.) is the most common and widely used adulterant of pistachio products due to its similar colour and texture when grounded. Moreover, green pea is six to seven times cheaper than pistachio in the market. It is not easily possible for consumers to identify green pea adulteration in a pistachio product, especially grounded products. It is also difficult for researchers to detect green pea adulteration of pistachio products in the lab, for example by using spectroscopic methods [3, 4].

Because of these issues, a rapid, robust and cost-effective method is needed to detect green pea adulteration of pistachio products. Identification of the adulterants at the level of DNA is more dependable since DNA is a stable molecule and exists in all tissues. Although a novel polymerase chain reaction (PCR)-based method targeting the *trnL-trnF* intergenic spacer with capillary electrophoresis (CE) was developed and successfully applied to detect green pea at the ratio of 5% [5], a more sensitive, faster and simpler method, which does not require post-PCR processing and electrophoresis, is still missing. A novel DNA hybridisation-based method DNAFoil was described in the literature [6] which is practical and easy to in field usage

product which offers 30 min in field analysis time for detection of the target organism. However, it has some drawbacks compared to in lab techniques such as at least 1000 mg of sample required, gives only one (±) answer is provided and being a single use product. Since it is a commercial product with limited production, it is difficult to reach every researcher or consumer.

High resolution melting (HRM) is a DNA-based method that distinguishes DNA variants from one another by single nucleotide polymorphisms (SNP) and insertions and deletions. The differences between amplicons generate different melting patterns and distinguish between genotypes or species, which makes the method extremely sensitive. Since it is an add-on step implemented to the end of the PCR reaction, it does not require extra laboratory duties or extensive bioinformatics knowledge. It is also not necessary to sequence the PCR products or hybridisation [7]. Moreover, since HRM is a closed-tube method, there is no contamination risk compared with other post-PCR methods.

HRM has previously been successfully applied to various food products. In a previous study, the method was applied to detect bovine, ovine and caprine adulterants in Greek Protected Designation of Origin (PDO) feta cheese [8]. HRM accurately identified the presence of bovine milk, which is not allowed in feta cheese, at a concentration of 0.1%. HRM has also been used to identify another PDO food product that contains a type of Turkish apricot (*Prunus armeniaca* cv. Şalak) [9], where a simple sequence repeat (SSR)-HRM assay identified the PDO apricot from other closely related cultivars. In another study, microsatellite-based HRM analysis was applied to verify grapevine and olive cultivars [10].

HRM is also used to identify the components of medicinal plants and products. Liquorice is a traditional herbal medicine which is susceptible to substitution and adulteration. Researchers applied SSR-HRM to identify true liquorice from another species [11]. Results discovered that SSR markers generated distinct melting profiles for different liquorice species. DNA barcoding regions can also be used as HRM markers. Researchers used the internal transcribed spacer-2 (ITS2) barcode region coupled with HRM (Bar-HRM), to differentiate *Psammosilene tunicoides*, which is an important herb using in traditional Chinese medicine, from other ingredients [12]. This novel approach both detected the adulterant *Silene viscidula* and quantified the most common admixture.

Another successful usage of HRM is to differentiate edible plants from poisonous plants for food safety. Poisonous *Urobotrya siamensis* Hiepko can cause death when consumed due to misidentification by consumers. It has young leaves that are similar to vegetables such as *Melientha suavis* Pierre and *Sauropus androgynus* (L.) Merr. Bar-HRM can successfully identify seedlings of those species at amounts of 0.001 ng of DNA [13].

There are many studies that demonstrate how HRM is useful for genotyping [14–17], identifying medicinal plants [12, 18, 19] and identifying species of fish [20–22].

Herein, we developed and evaluated a novel HRM-based method using specific chloroplastidial primers for the rapid, dependable, closed-tube and minimum sample handling detection of green pea adulteration of pistachio. HRM was proven to be capable of detecting the presence of green pea down to concentrations of 0.1% in pistachio.

2. Materials And Methods

2.1. Sampling, Preparing the admixtures and DNA extraction

We obtained rough pistachio nuts (*Pistacia vera* cv. Siirt) from a local store and stored them at − 80°C until DNA extraction. Fresh green pea (*Pisum sativum* cv. Sativum) was purchased frozen from local retail stores.

To prepare the admixtures, we first separated the pistachio nuts from the shell and peeled off the pericarp, then ground the fruits until a fine powder was obtained using a porcelain grinder. We then used another identical grinder to obtain the powder of green pea. We prepared a series of admixtures at ratios of 999:1, 499:1, 99:1 and 90:10 (pistachio:green pea) each, adjusted to 50 g.

Cetyltrimethylammonium bromide (CTAB)-based DNA extraction of each sample was performed with ~ 100 mg of powdered starting material, in according with the literature (Aydin et al., 2018). DNA concentration and integrity were checked by Qubit 2.0 using a dsDNA BR assay kit (Invitrogen, USA) and agarose gel electrophoresis.

2.2. Data mining and designing of the primers

We retrieved the complete chloroplast genomes of *P. vera* (NC_034998) and *P. sativum* (MT120808) from the GenBank (National Center for Biotechnology Information) and imported the sequences to the Geneious R8 software [24]. Then, the sequences were aligned (Geneious Alignment Tool with default settings). The primer sets, targeting *atpA*, *rpoC* 1 and *ycf3* sequences, were designed by considering the variable positions, guanine-cytosine (GC) content (~ 50%), expected amplicon size (90–120 bp) and melting temperature (~ 60°C) in the same software (Table 1). The designed primers were then confirmed in-silico for their specificity using the Primer-BLAST tool on the NCBI website and by end-point PCR with the extracted DNA.

Table 1
Primers' information

Primer Name	Target Gene	Sequence (Forward and Reverse, 5′→3′)	T_m (°C)	Expected (Detected) Amplicon Size (bp)	Variation	GC Content of the Amplicon (%)	Temperature Optimisation for HRM	
							Lower Limit	Upper Limit
HRM-atpa	*atpA*	CAATAAGTCCTGTTTGAAGAGGC AAACCTATTGACGGTCGAGG	61.1 58.4	~ 118 (130)	10 (7.7%)	*P. vera*: 43.1 *P. sativum*: 43.1	78.54	82.86
HRM-rpoc1	*rpoC*1	AGCAACTCCTATGTTCGAAGC CATTACATCGATGTGGATTGCC	60.3 61.1	~ 107 (118)	13 (11.0%)	*P. vera*: 39.8 *P. sativum*: 38.1	77.17	81.41
HRM-ycf3	*ycf* 3	GAAACTCTCAAGTACGGTTCTAAGG TTCAGAATCTCCCTGTCGAATGG	64.2 62.9	~ 87 (100)	9 (9.0%)	*P. vera*: 51 *P. sativum*: 47	79.00	84.39

T_m: Melting temperature.

Variation percentages were calculated regarding the detected amplicon lengths.

2.3. HRM-PCR amplification and data analysis

Prior to HRM, the concentrations of all the DNA samples were normalised to 10 ng mL^{-1}. HRM-PCR amplification was performed using a Rotor-Gene-Q 5 Plex HRM instrument (Qiagen, USA). The reaction mixture prepared with 15 ng of DNA, 5 mL of Luminaris Color HRM Master Mix (Thermo Scientific, USA), 0.5 mL of 10 mmol L^{-1} of both forward and reverse primers and nuclease-free water to 10 mL final volume. The following conditions were used for HRM-PCR amplification: an initial denaturation step at 95°C 10 min followed by 40 cycles of 95°C denaturation for 10 s, 60°C annealing for 30 s and 72°C elongation for 30 s. Fluorescence data was acquiesced following each elongation step. We added 95°C for 30 s and 50°C for 30 s steps for heteroduplex formation to the end of the reaction. HRM was performed immediately after the amplification in increments of 0.1°C s^{-1} hold time from 65°C to 95°C and the fluorescence data was acquiesced continuously. We performed all the reactions in triplicate and added no template control (NTC) to each reaction.

The HRM data was analysed using Rotor-Gene-Q 2.3.5 software. The HRM curves were normalised by removing the background fluorescence, then difference plots were drawn against pistachio for each primer. The genotyping confidence percentages (GCPs) for each sample were calculated by the software. We set the confidence threshold to 90% for more reliable results.

2.4. Validation of the primers and HRM results

To validate the specificity of the designed primers and lengths of the amplicons, we used CE (Qiaxcel Advanced, USA) following end-point PCR. 20 ml of PCR reaction included a 2X Reaction Buffer (Thermo Scientific, USA), 0.1 mM dNTP, 0.2 mM of both primers, 1 U Taq DNA polymerase (Thermo Scientific, USA), 1 mM Mg^{+2}, 10 ng DNA and nuclease free water. The thermal cycling (Applied Biosystems, USA) conditions were 95°C for 3 min in the first denaturation, followed by 35 cycles of denaturation at95°C for 30 s, 60°C for 30 s annealing, and 72°C for 1 min elongation. Thermal cycling was finalised by 72°C for 10 min as a final extension step. CE was performed using a Qiaxcel DNA High Resolution Kit (Qiagen, USA) and the software settings were used as follows: process profile: default high res v2.0; method: 0H1200; size marker run beside the samples: Gene Ruler 100 bp Plus (Thermo Scientific, USA); alignment marker: QX15bp-3kb. The results were visualised and analysed using the ScreenGel 1.6 (Qiagen, USA) software.

To validate HRM results and to visualise the nucleotide polymorphisms, we sequenced each amplicon generated by three primer sets of pistachio and green pea. The Sanger sequencing was performed by Macrogen Inc. (Netherlands) using the same primers on both directions used for HRM-PCR. The *ab1* files were checked for sequencing quality, trimmed and assembled to generate consensus sequences in the Geneious R8 software. The consensus sequences were aligned by the Geneious Aligner algorithm in the same software. We also calculated the nucleotide variation count and percentage.

2.5. Statistical analysis

Descriptive statistical analyses of the quantification cycle (C_q), melting point (T_m) and HRM GCPs were calculated and presented as mean ± standard deviation (SD) with triplicate data. One-way ANOVA and post-hoc analyses (LSD) were used for mean comparison of the positive controls and the admixtures at the significant level of $P < 0.0001$. Linear regression was also performed to calculate the correlation coefficient of the GCPs using XLSTAT Basic 2021 (Addinsoft) software.

3. Results

3.1. DNA extraction and CE validation of the primers

We obtained DNA concentrations between 10.7–40.7 ng mL^{-1} and the A260/A230 ratio ranged from 1.704–1.880, and the A260/A280 ratio ranged between 1.755–1.908 which is sufficient for HRM analysis. Each primer set clearly amplified the specific target region for positive controls and admixtures. The CE validation of amplicons revealed the exact amplicon lengths (Fig. 1) which were slightly longer than the expected sizes (Table 1). There was no amplicon difference in length between pistachio and green pea on each target region.

3.2. HRM analysis

Three sets of designed primers (*atpA*, *rpoC*1 and *ycf3*) were used to amplify the target sequences of positive controls and the admixtures using HRM. The T_m values for pistachio and green pea at each target sequence were significantly different ($P < 0.0001$) and could be used to identify the positive controls from each other. In addition, the T_m value itself successfully detected green pea adulteration at the *rpoC*1 and *ycf3* targets, although it failed for pistachio concentrations of 99:1 and 999:1 with *atpA* (Table 2). The GCPs, calculated by the software using melting profiles, successfully validated both the positive controls with > 98.88% confidence, and adulteration on each target region.

Table 2
HRM analysis of positive controls and the admixes.

	Target Region								
	atpA			*rpoC*1			*ycf3*		
Sample	Cq ± SD	T_m ± SD	GCP	Cq ± SD	T_m ± SD	GCP	Cq ± SD	T_m ± SD	GCP
P. vera	12.68 ± 0.08^{a}	80.70 ± 0.04^{cd}	98.93 ± 0.89	12.80 ± 0.27^{a}	79.60 ± 0.00^{a}	99.61 ± 0.23	13.79 ± 0.19^{e}	81.40 ± 0.02^{a}	98.88 ± 0.64
P. sativum	14.66 ± 0.21^{b}	81.04 ± 0.04^{a}	99.54 ± 0.54	14.81 ± 0.06^{b}	79.00 ± 0.04^{d}	99.51 ± 0.00	15.62 ± 0.18^{d}	80.80 ± 0.02^{d}	99.54 ± 0.41
90:10	22.40 ± 0.10^{c}	80.46 ± 0.10^{e}	18.12 ± 0.03	22.00 ± 0.23^{c}	79.41 ± 0.07^{b}	39.9 ± 1.36	20.94 ± 1.16^{b}	81.00 ± 0.07^{c}	58.90 ± 0.03
99:1	28.80 ± 0.07^{d}	80.55 ± 0.05^{de}	0.29 ± 0.03	20.86 ± 0.22^{d}	79.50 ± 0.13^{b}	28.05 ± 1.21	18.28 ± 0.06^{c}	81.04 ± 0.00^{c}	57.56 ± 0.51
499:1	19.50 ± 0.30^{e}	80.93 ± 0.03^{ab}	68.00 ± 0.25	25.62 ± 0.23^{e}	79.45 ± 0.05^{b}	49.21 ± 1.78	23.24 ± 0.02^{a}	81.20 ± 0.05^{b}	61.56 ± 1.32
999:1	23.92 ± 0.39^{f}	80.90 ± 0.19^{bc}	0.15 ± 0.19	30.34 ± 0.08^{f}	79.25 ± 0.01^{c}	56.12 ± 0.90	23.82 ± 0.09a	80.99 ± 0.03^{c}	70.13 ± 1.43
Cq: Cycle threshold									
T_m: Melting temperature									
GCP: Genotyping confidence percentage									
Superscript letters: The mean difference is significant at $P < 0.0001$ level.									

The melting profiles of HRM analysis distinguished the sequences from each other at a higher resolution. The difference plots for *atpA* (Fig. 2), *rpoC*1 (Fig. 3) and *ycf3* (Fig. 4) sequences using pistachio as a reference genotype clearly identified each admixture from pistachio. The GCPs also supported the identification of both positive controls and the admixtures for each target region (Table 2). The efficacy of the method was confirmed by its standard curve with a very high correlation coefficient values 0.998 for atpA, 0.982 for rpoC1 and 0.963 for ycf3 primer sets.

3.3. Validation of HRM results by DNA sequencing

We validated the results of the HRM analysis by sequencing the target amplicons for pistachio and green pea positive controls. The alignment results of each target region allowed us to calculate the nucleotide variation percentages, which allowed us to identify the resolution of distinguishing pistachio from green pea to HRM (Fig. 5). There was no insertion or deletion (indel) between the sequences, but nucleotide transitions and transversions were observed. The basic sequence statistics showed that each target region had enough sequence variation for HRM analysis to work. The *rpoC*1 region had the highest nucleotide variation percentage (11%), followed by *ycf3* (9%) and *atpA* (7.7%) (Table 1). GC content percentages varied for the amplicons, except *atpA* (Table 1).

4. Discussion

4.1. Necessity of sensitive method for pistachio authentication

Pistachio is a popular nut due to its taste and benefits to health. However, adulteration of pistachio products with green pea is common due to the expensiveness of pistachios. It is also very easy to adulterate pistachio with green pea due to their similar

colour and texture when both are in fine powder form. Thus, the authenticity of pistachio products must be validated to prevent consumers from buying adulterated products. There are several ways to detect adulteration of pistachio, such Raman hyperspectral imaging [4], or DNA genotyping using the *trnL-trnF* intergenic spacer marker [5]. Nevertheless, a closed-tube method, which is more sensitive, faster and cheaper, would be more useful to detect adulteration at lower ratios and prevent contamination during sample handling.

4.2. Novel HRM primers needed for pistachio identification

A DNA sequence used for DNA barcoding to identify species must be a standardised, short sequence of DNA (usually 400–800 bp), which should be universal for that group [25]. Nonetheless, the target region used for HRM has extra requirements which must be heeded. The HRM target region must not be longer than 300 bp, like standard DNA barcoding regions, since a longer amplicon risks not recognising nucleotide variation and avoiding primer dimer formation due to the influence of the HRM result [26]. Regarding these issues, universal barcoding regions such the internal transcribed spacer (ITS), maturase K (*matK*), ribulose bisphosphate carboxylase large chain (*rbcL*), etc., are not suitable for HRM analysis. Moreover, bioinformatic analysis performed while designing the primers showed that, since there were too many nucleotide variations at the ITS1 and ITS2 regions, we could not find conserved regions for primers to bind. Another issue with the ITS 5.8S coding region was that there were only five nucleotide variations and the expected amplicon was too short (83 bp) for HRM. Similar to 5.8S, the *matK* (with no variable sites suitable for HRM) and *rbcL* (97 bp amplicon length, 4 nucleotide variations) gene sequences did not have enough variation to be detected by HRM. Considering all these limitations, we decided to design novel primers which are suitable for HRM analysis of pistachio and green pea, targeting other gene regions, namely *atpA*, *rpoC*1 and *ycf3*.

4.3. DNA sequencing validated the HRM discrimination

The melting temperature and denaturation kinetics are dependent on both the length and GC content of the amplicon since G and C are bound by three hydrogen bonds, whereas adenine-thymine (AT) is bound by two. Hence, the T_m and melting kinetics of the amplicons differ on high and low GC containing fragments. HRM analysis is only reliable when the correct PCR product is amplified. Thus, to validate the HRM results while a new method is being employed, sequencing of the target PCR products is needed [27]. Otherwise, it is not possible to verify whether the HRM results are because of sequence differences or technical issues. The sequence alignments of the pistachio and green pea sequences for each target region clearly demonstrated the nucleotide variations that resulted in different melting kinetics of the sequences after HRM. The sequencing results also revealed the GC content of the amplicons. The GC content differed on pistachio and green pea amplicons for *rpoC*1 and *ycf3* PCR products, but were identical for *atpA*.

4.4. *rpoC*1 and *ycf3* as a suitable marker for HRM

HRM, which is a post-PCR process, enables rapid and high-throughput identification of DNA variants, without requiring sequencing [26]. The method overcomes the cost and time compared to DNA barcoding only [28]. Both CE and sequencing results demonstrated that the designed primer sets targeting *atpA*, *rpoC*1 and *ycf3* chloroplast regions were very useful for HRM analysis due to their optimal amplicon length (130, 118, 100 bp, respectively), annealing temperature (~ 60°C) and nucleotide variations (11, 9, 7.7%). Melting temperature is defined as the temperature at which 50% of the DNA is single-stranded [26]. According to the results we obtained from the HRM analysis regarding only the T_m values, the *rpoC*1 and *ycf3* targets could be used to detect the adulteration of pistachio down to concentrations of 0.1% (Table 2), while *atpA* failed to distinguish green pea from pistachio at 99:1 and 999:1 admixtures. This is likely due to the identical GC content in the pistachio and green pea *atpA* sequences (both 43.1%). However, HRM not only considers T_m, but also the melting kinetics of the denaturising DNA [29]. The differential plots of the denaturation process demonstrated a clear distinction of the admixtures. The software Rotor-Gene-Q calculates the GCPs that are used to assign a genotype for each target region with a threshold of 90% using denaturation kinetics. With this approach, each target region used in this study clearly distinguished every admixture from the positive controls for each target region, down to 0.1% level of adulteration.

5. Conclusions

After designing and testing three potential targets for employing HRM to detect green pea adulteration of pistachio, analysis of *rpoC*1 and *ycf3* successfully detected the adulteration of pistachio down to 0.1% green pea content. Therefore, the designed primer sets and protocol could easily be reproduced to detect the adulteration of pistachio in a cost-effective, rapid and contamination-free way. HRM method is applicable technique for various food products which have adulteration issues considering studiously designed and validated target specific primer sets.

Declarations

AUTHOR CONTRIBUTIONS

NA: Investigation, Formal Analysis, writing original draft; KH: Conceptualization, methodology, validation, writing – review & edit.

Supplementary Data

Nucleotide alignments with full sequences of green pea and pistachio amplicons for each primer provided as fasta format in the Supplementary Data 1.

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DECLERATION OF INTEREST

Authors confirm that there is no competing interest.

Compliance with Ethical Standards:

Ethical approval: This article does not contain any studies with human participants performed by any of the authors.

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Figures

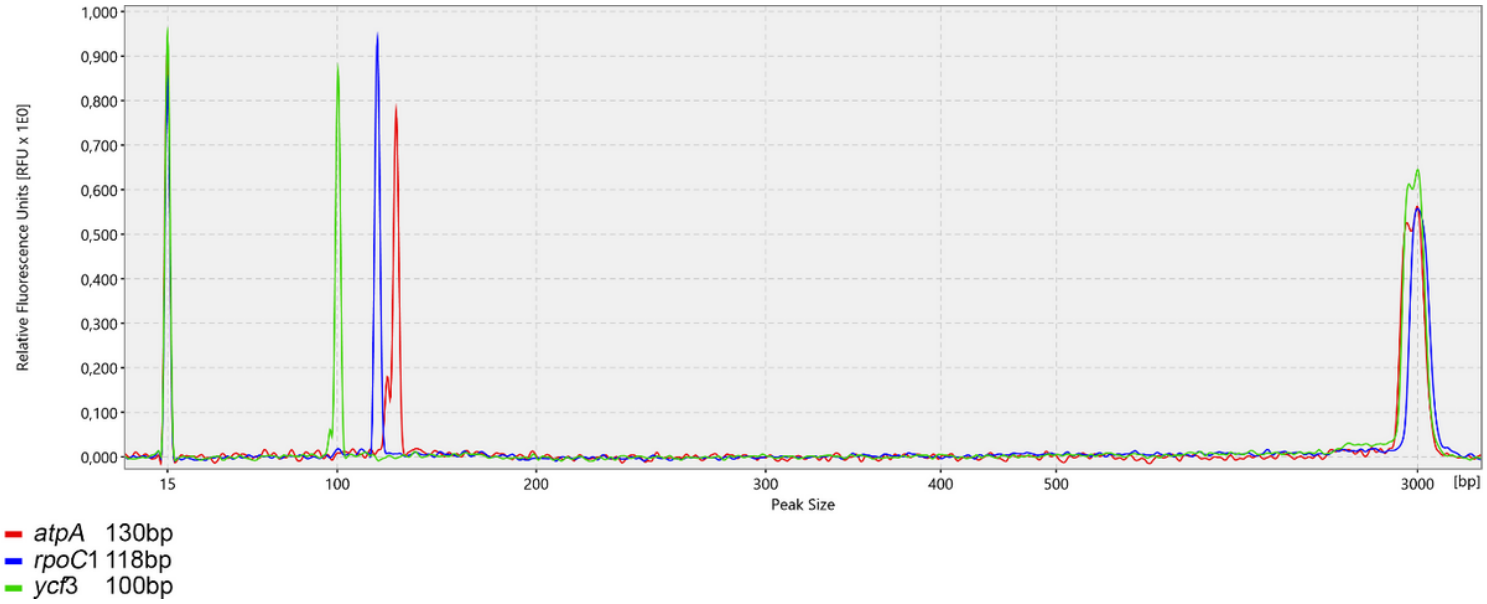

Figure 1

Capillary electropherogram view of each target region amplification profiles for positive controls. Amplicon peaks are colour coded and amplicon lengths of each target region is identical for pistachio (*Pistacia vera* L.) and green pea (*Pisum sativum* L.). Fifteen and 3000 bp peaks represent calibration marker.

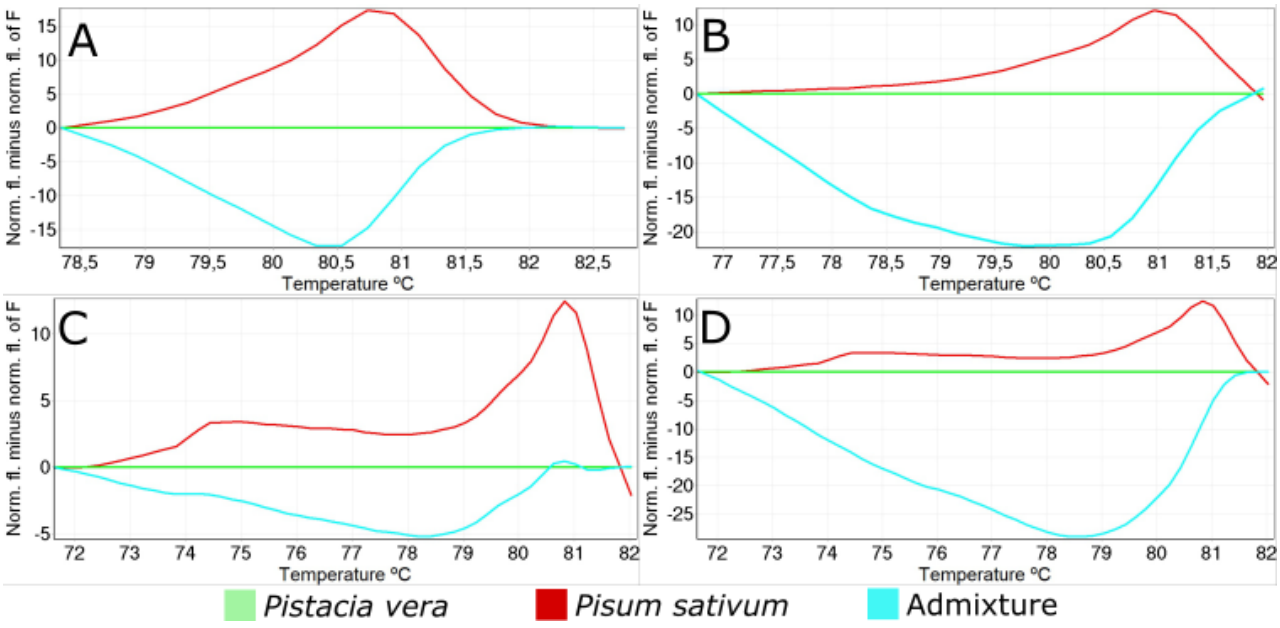

Figure 2

Difference curves obtained by HRM analysis for *atpA* target region of 90:10 (A), 99:1 (B), 499:1 (C), and 999:1 (D) admixtures using *P. vera* as a reference genotype.

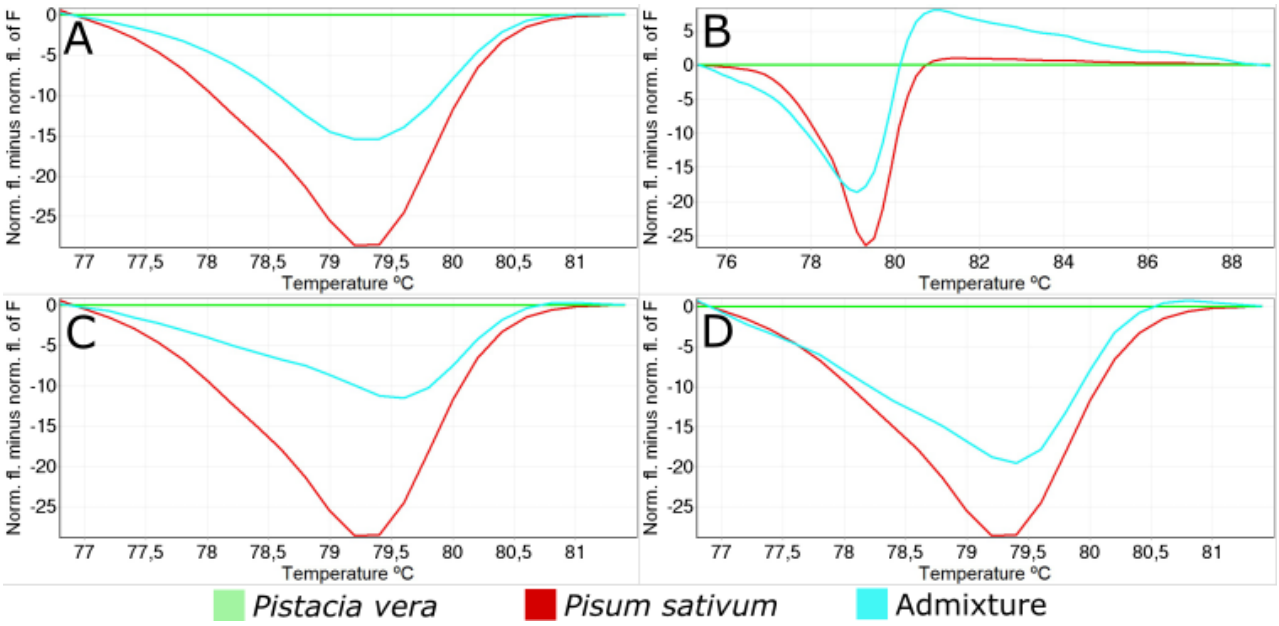

Figure 3

Difference curves obtained by HRM analysis for *rpoC*1 target region of 90:10 (A), 99:1 (B), 499:1 (C), and 999:1 (D) admixtures using *P. vera* as a reference genotype.

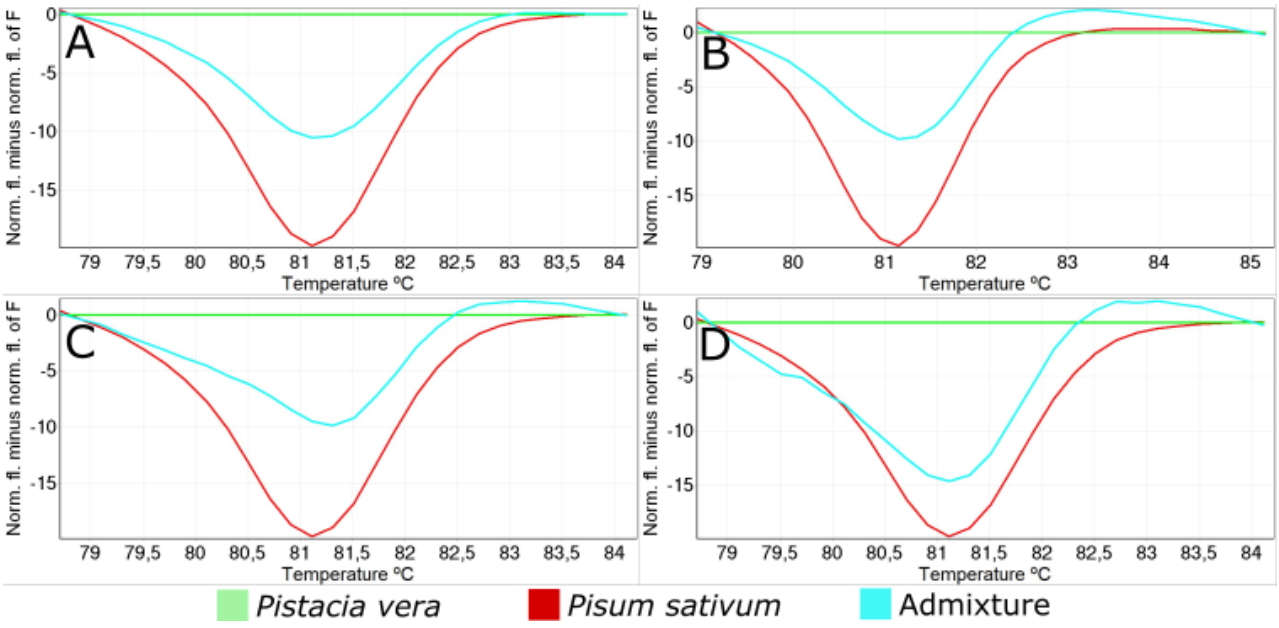

Figure 4

Difference curves obtained by HRM analysis for *ycf3* target region of 90:10 (A), 99:1 (B), 499:1 (C), and 999:1 (D) admixtures using *P. vera* as a reference genotype.

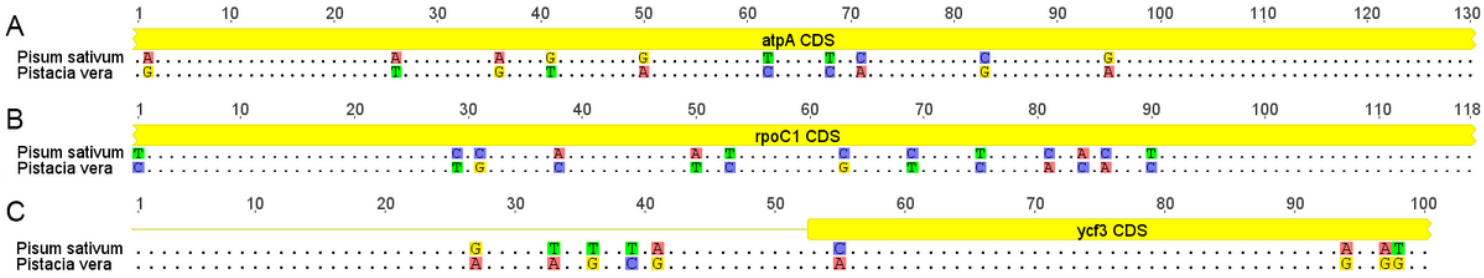

Figure 5

Alignment of *atpA* (A), *rpoC*1 (B), and *ycf3* (C) target regions of HRM analysis. The yellow regions show coding sequences. Nucleotide variation sites were showed in different colours, while identical nucleotides as dots. The numbers over the sequences indicate the positions.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarydata1.txt