



Barcode High Resolution Melting analysis for forensic uses in nuts: A case study on allergenic hazelnuts (*Corylus avellana*)

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ABSTRACT

Tree nuts are nutritionally important foods, being rich sources of vitamins, minerals, healthy fats and proteins and having cardio protective effects. Despite their healthy attributes, some tree nuts are also known for triggering adverse immune responses to individuals. Thus, an ongoing challenge in molecular biology is to develop rapid, accurate and reliable techniques to identify organisms and their processed foods, particularly those which could be contaminated with allergenic compounds. Lately, in addition to different chemical and immunological methods used, DNA based methods were also applied for this purpose. We describe here a novel, specific and highly sensitive High Resolution Melting analysis based method, using chloroplast barcoding regions (Bar-HRM) in order to a) obtain barcoding information for the major tree nut species (we have also included peanuts and sesame in our study) and b) to quantitatively identify one such allergenic tree nut species, like hazelnut in processed foods. The results show that the proposed method i) is capable of distinguishing among different tree nut species, ii) can be applied to processed tree nut products such as biscuits and iii) can detect a ratio of 0.01% of hazelnut in biscuits. In conclusion, it is demonstrated that BAR-HRM is suitable for the rapid, closed-tube and accurate screening of large numbers of tree nuts and/or of their processed foods and for the identification of allergenic tree nut species contained in processed foods.

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1. Introduction

Tree nuts are defined as dry seed or fruit of certain plants or, in terms of botanical definition, nuts are “simple dry fruit with one seed, in which the seed wall becomes very hard at maturity” (Fielder, Higgs, & Barden, 2009). In this study we have included, besides tree nuts, also peanuts and sesame and for simplicity we will refer to all as nuts hereafter.

Edible nuts are globally popular foods, consumed as dry seeds or used as culinary additives. They are highly nutritional and have important qualities. They are rich sources of proteins, healthy lipids like omega 3 and 6 fatty acids, certain vitamins and minerals in significant amounts (Coates & Howe, 2007). In particular, the US Food and Drug Administration has approved a qualified health claim for nuts for reducing cardiovascular diseases through lowering LDL cholesterol (Mukuddem-Petersen, Oosthuizen, & Jerling, 2005). Yet, some nuts are highly allergenic, like hazelnuts, almonds and peanuts (which is a legume species considered nut) (Etesamifar & Wüthrich, 1998). A hazelnut protein of 17 kD, which has been identified as a pathogen

related protein homologous to birch, has been recognized as the major allergen factor of hazelnuts (Hoffmann-Sommergruber et al., 1996). Therefore, allergy to hazelnut is very frequent to humans with respiratory allergy to the pollen of birch, hazel and alder, causing breathlessness. Food allergy is typically characterized by a variety of clinical symptoms like: anaphylaxis, urticaria-angioedema, flare-up of atopic eczema, rhinitis, asthma, gastrointestinal symptoms and oral allergy syndrome (OAS) (Bruijnzeel-Koomen et al., 1995; Ortolani et al., 2000).

Hazelnuts are widely used in the food industry. They are used in pre-packaged foods, especially for the production of pastry and ice cream. This wide use of hazelnuts in the food industry could pose a considerable risk to people suffering from hazelnut allergy, as food labels might not list foods that are found in small quantities and food might be contaminated by unintended adulteration (Steinman, 1996). This is a real case scenario as there have been reports of people presenting allergic reactions to hazelnut found in minimum quantities in commercial food products (Yman, Eriksson, Everitt, Yman, & Karlsson, 1994).

The regulatory agencies have implemented regulations on food labeling in order to protect the consumers, instructing the compulsory inscription of food allergens and their derivatives when used as food ingredients (Fielder et al., 2009). In EC food allergy is governed by Directive 2000/13/EC, as amended by Directive 2003/89/EC and 2006/142/EC, which set out a list of ingredients, including 14 allergenic foods

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Table 1

List of plant species and commercial food commodities used for HRM analysis. Specificity test for HazeltrnL and *rbcl* primers was performed on genomic DNA of leaves and seeds. Legend: + positive result (corresponding to a peak of predicted Tm); – negative result (corresponding to absence of peak).

Plant	Hazelnut (%)	HazeltrnL	rbcl
<i>Prunus dulcis</i> (Almond)	–	–	–
<i>Bertholletia excelsa</i> (Brazilnut)	–	–	–
<i>Anacardium occidentale</i> (Cashew)	–	–	–
<i>Castanea sativa</i> (Chestnut)	–	–	–
<i>Corylus avellana</i> (Hazelnut)	100	–	–
<i>Macadamia</i> spp. (Macadamia)	–	–	–
<i>Carya illinoensis</i> (Pecan)	–	–	–
<i>Arachis hypogaea</i> (Peanut)	–	–	–
<i>Pistacia vera</i> (Pistachio Aigina)	–	–	–
<i>Sesamum indicum</i> (Sesame)	–	–	–
<i>Juglans regia</i> (Walnut)	–	–	–
<i>Hordeum vulgare</i> (Barley)	–	–	–
<i>Triticum aestivum</i> (Wheat)	–	–	–
Biscuit			
With traces of nuts (Commercial1)	Unknown		
With hazelnut and traces of nuts (Commercial2)	Unknown		
With cream and traces of hazelnut (Commercial3)	Unknown		
With eggs without traces of nuts (Commercial4)	Unknown		
With butter without traces of nuts (Commercial5)	Unknown		

(tree nuts are on this list), which must always be identified and listed at the label, when used in the manufacture of pre-packed foods. This applies regardless of how small is the amount used and in the case that is used in the same premises for other food preparation.

Different methods have been developed in order to detect and quantify specific products and in particular allergenic food components, like proteomics, immunochemical tests and DNA-based methods (van Hengel, 2007). These methods could detect either the allergenic protein or the encoding gene (Monaci & Visconti, 2010). Despite their use these methods could identify false positives (Diaz-Amigo & Popping, 2009). PCR-based methods are highly specific and sensitive, although they do not detect directly the allergenic factor, or quantify they could target

“allergy responsible genes” which are located in the genomic DNA or in the plastidial DNA, like the mitochondria or chloroplasts (Diaz-Amigo & Popping, 2009; Watanabe et al., 2012). Recently, taking advantage of the High Resolution Melting analysis (HRM) technology we combined HRM and barcoding technologies to develop the Bar-HRM method for the identification and quantitation of adulterants in plant based foods and products (Bosmalı, Ganopoulos, Madesis, & Tsafaris, 2012; Ganopoulos, Madesis, Darzentas, Argiriou, & Tsafaris, 2012; Madesis, Ganopoulos, Anagnostis, & Tsafaris, 2012).

Herein, we report the development of Bar-HRM method using universal and specific chloroplast primers, for barcoding tree nut species (including peanut and sesame) and, most importantly, for the rapid detection, quantification and adulteration measurements of allergenic hazelnut in commercial processed food products like biscuits. The HRM based method developed was proven capable not only to distinguish among the different nut species but also to detect a ratio of down to 0.01% of hazelnut contamination in biscuits.

2. Materials and methods

2.1. Plant and food material and DNA isolation

The species used in this study are: *Prunus dulcis* (Almond), *Anacardium occidentale* (Cashew nut), *Corylus avellana* (Hazelnut), *Arachis hypogaea* (Peanut), *Juglans regia* (Walnut), *Bertholletia excelsa* (Brazilnut), *Carya illinoensis* (Pecan), *Castanea sativa* (Chestnut), *Pistacia vera* (Pistachio Aigina), *Macadamia* spp. (Macadamia), and *Sesamum indicum* (Sesame). All nut materials and dried seeds as well as commercial samples were purchased at local grocery stores. Moreover, we have used different types of biscuits labeled as containing hazelnuts or not which were purchased from different retailers and stored at room temperature in the dark. In addition, we have prepared biscuit cookies using 130 mL olive oil, 1 egg, 250 g wheat flour, and 150 g sugar, baked for 25 min at 180 °C in a commercial Miele® oven (the temperature was constantly recorded using an analog oven thermometer (Kuchenprofi, Ofen-Thermometer)). Known amounts of hazelnuts were mixed in the dough (from 0.01% up to 50% w/w). The complete list of materials used for DNA extraction and for PCR analyses

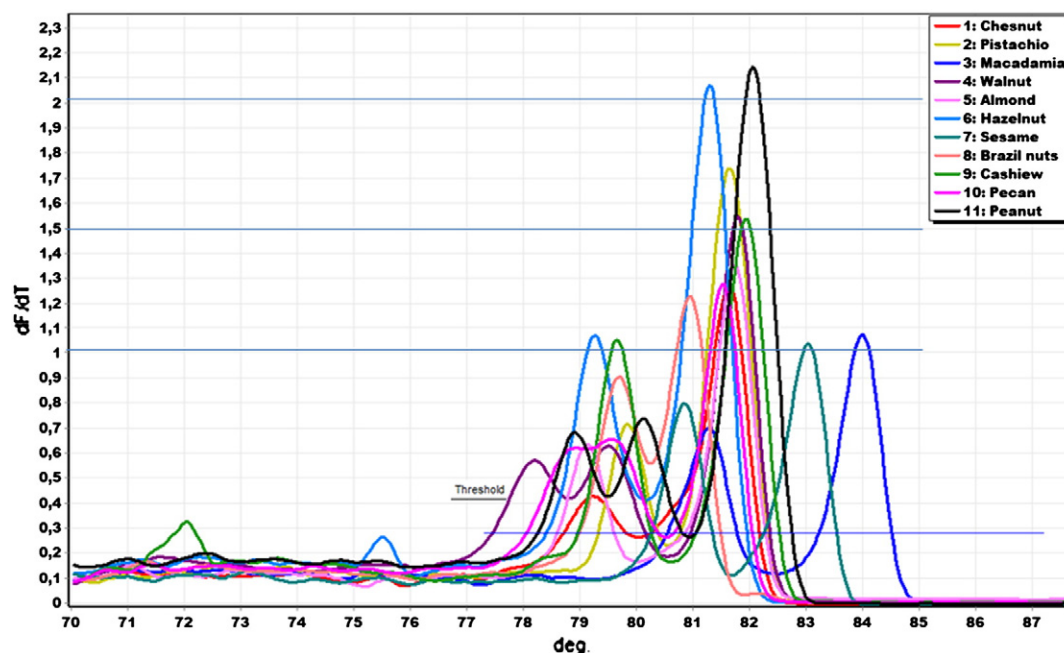


Fig. 1. Conventional melting curves of universal *trnL* marker. Color code table with the species used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Melting peaks temperature of nut species analyzed.

Species	Peak 1 (T _m)	Peak 2 (T _m)	Peak 3 (T _m)
Chestnut	79.23	81.63	
Pistachio Aigina	79.83	81.65	
Macadamia	81.28	83.98	
Walnut	78.2	79.5	81.78
Almond	79.12	81.73	
Hazelnut	79.25	81.3	
Sesame	80.85	83.03	
Brazilnuts	79.7	80.95	
Cashew	79.65	81.93	
Pecan	78.93	79.55	81.53
Peanut	78.9	80.12	82.05

is contained in Table 1. Biscuits without the addition of hazelnut were also used as negative controls.

Seeds of dry nut species were ground to fine powder (particle size approximately of 250 μ m) using a commercial “Waring” blender for 10 min with 15 s intervals. Three independent samples were used per species. For the adulteration detection each sample consisted of at least 50 g of powdered seed, subsamples of 1 g were taken. Three

subsamples were randomly selected, from which 100 mg of seed powder was collected for DNA extraction using the NucleoSpin® Plant II mini kit. Isolation of DNA from processed food products was performed with 100 mg starting material of fine powder, using the NucleoSpin® Food, according to the manufacturer's instructions. The DNA concentration was estimated by standard spectrophotometric methods at 260 nm and 280 nm UV lengths by an Eppendorf BioPhotometer and the integrity by gel electrophoresis in a 0.8% agarose gel. Samples were then diluted to 20 ng/ μ L work concentration.

2.2. PCR amplification and HRM analysis of DNA-barcode region

PCR amplification, DNA melting and end point fluorescence level acquiring PCR amplifications as well as HRM analysis were performed in a total volume of 15 μ L on a real time machine (Rotor-Gene 6000 5P HRM Corbett Research, Sydney, Australia) according to (Ganopoulos, Argiriou, & Tsaftaris, 2011b; Ganopoulos et al., 2012). More specifically the reaction mixture contained 20 ng genomic DNA, 1 $\times$ PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTP, and 300 nM forward and reverse primers. The primers used were the universal primers for the *trnL*UAA intron (forward CGAAATCGGTAGACGCTACG, reverse GGGGATAGAGGGACTTGAAC) (Taberlet et al., 2007). A rapid PCR protocol was conducted in a 36-well

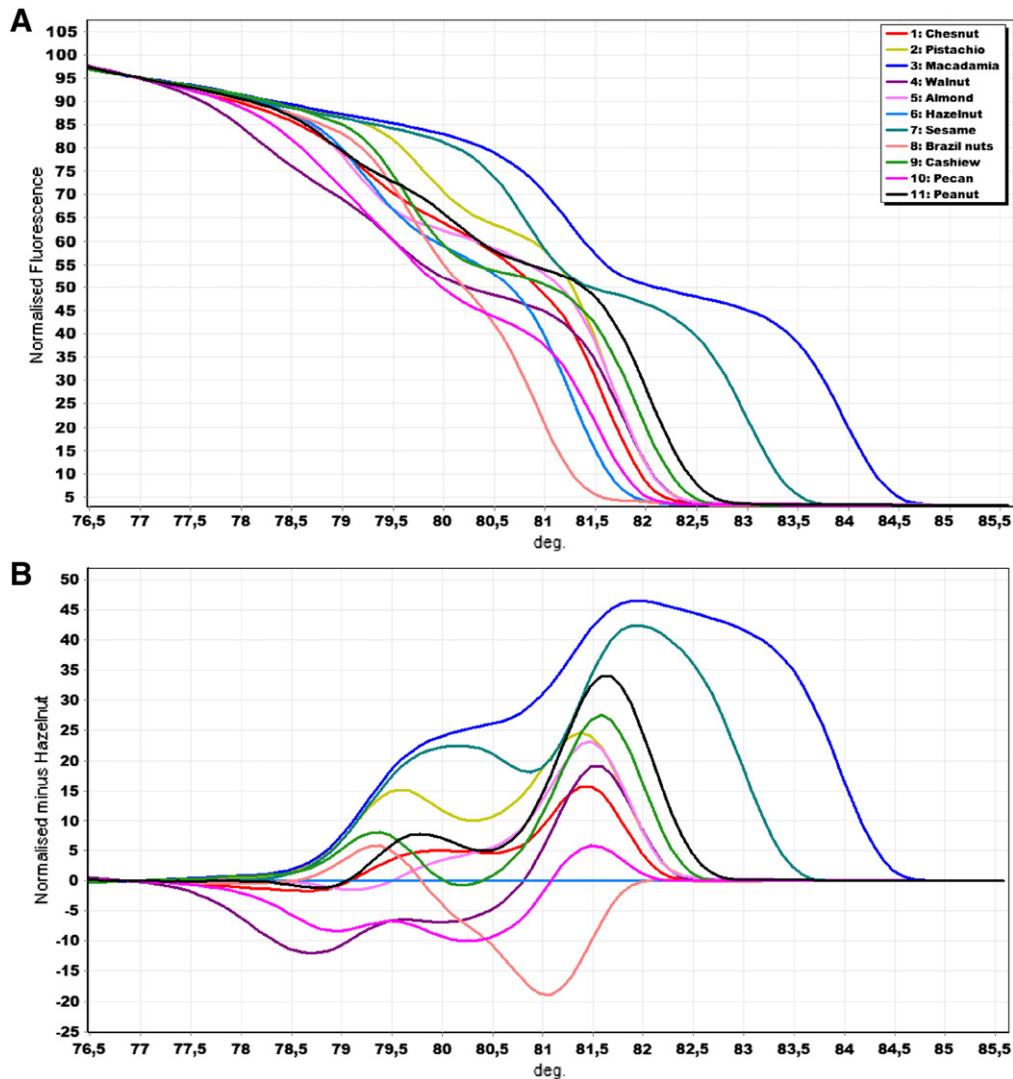


Fig. 2. A) Identification of nut species using HRM analysis with the universal *trnL* chloroplast marker. B) Difference graph of 11 species using hazelnut as reference genotype. Assigned genotypes using a cut-off confidence value of 90%. Used as hazelnut control for HRM assay. The HRM of all other species were compared to this control and were resulted as hazelnut at >90% confidence or as variation if <90% confidence. Color code table with the species used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3Average genotype confidence percentages (± 3.7) resulting from HRM analysis of the *trnL* region of eleven nut species at a ramp of 0.1 °C.

Species	Chestnut	Pistachio Aigina	Macadamia	Walnut	Almond	Hazelnut	Sesame	Brazilnuts	Cashew	Pecan	Peanut
Chestnut	100										
Pistachio Aigina	9.91	100									
Macadamia	0	0	100								
Walnut	4.76	0.02	0	100							
Almond	53.91	10.82	0	5.05	100						
Hazelnut	9.92	0.04	0	0.81	1.03	100					
Sesame	0	0	0	0	0	0	100				
Brazilnuts	0.01	0	0	0	0	3.12	0	100			
Cashew	10.63	9.4	0	1.05	24.05	0.12	0	0	100		
Pecan	1.82	0	0	13.44	0.39	13.88	0	0.74	0.05	100	
Peanut	1.44	3.49	0	0.16	8.08	0	0	0	26.33	0	100

carousel using an initial denaturing step of 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, then a final extension step of 72 °C for 2 min. The fluorescent data were acquired at the end of each extension step during PCR cycles. Before HRM, the products were denatured at 95 °C for 5 s, and then annealed at 50 °C for 30 s to randomly form DNA duplexes. HRM was performed as follows: pre-melt at the first appropriate temperature for 90 s, and melt at a ramp of 10 °C in an appropriate temperature range at 0.1 °C increments every 2 s. The fluorescent data were acquired at the end of each incremental step. End point fluorescence level was acquired following the melting process by holding at 60 °C for 5 min. The Rotor-Gene 6000 proprietary software (version 2.0.2) was used to genotype the different varieties and the food products. The negative derivative of fluorescence (F) over temperature (T) (dF/dT) curve primarily displaying the T_m, the normalized raw curve depicting the decreasing fluorescence vs increasing temperature, and difference curves were mainly used. Furthermore, a 2-step procedure was followed to assess similarity of unknown HRM curves with a known one. Each species was set as a 'genotype' (reference species) and the average HRM genotype confidence percentages (GCPs) (value attributed to each species being compared to the genotype, with a value of 100 indicating an exact match) for the replicates (disregarding the most outlying replicate) were tabulated (Ganopoulos et al., 2012).

For the detection of contamination with hazelnuts a second duplex PCR assay was developed according to Mader, Ruzicka, Schmiderer, and Novak (2011), containing a universal primer pair for the *rbcl* gene F-CTTGATTTCACAAAGATGATGA, R-TTCTTCGCATGTACCCGACG, (Han et al., 2012) and a *C. avellana* specific primer pair HaizeltrnL F-CAAAATAAAACAAATTAAGGGTTCATAAAG, HaizeltrnL R-CGGAACGA AGTCAACTCCAATTG.

Here a ratio of universal to specific primer pair of 1.5:1 was used after optimization (data not shown). In both cases the T_m (melting temperature) value of the *C. avellana* amplicon was intentionally lower than that of the amplicon of the adulteration(s).

To verify the sensitivity and accuracy levels of the methodology in detection and quantification of admixture, standard samples of hazelnut were prepared by mixing with biscuit materials, at progressive ratios of 10, 5, 2, 1, 0.1 and 0.01%, to generate data at six score points. All experiments were repeated three times with three different independent samples. PCR and HRM analysis was performed as described before (Ganopoulos, Argiriou, & Tsaftaris, 2011a; Ganopoulos et al., 2011b).

2.3. Sequence analysis

PCR products were directly sequenced in two directions with the Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in an automated ABI 3730 sequencer (PE Applied Biosystems). The sequences were aligned with the CLUSTAL W program.

3. Results

Simple and rapid identification of species in market products is of great importance not only for traceability control in the food chain but also for the production and release of safe for the consumer processed products. In this sense, the HRM analysis facilitates and simplifies this task compared to conventional techniques where the final PCR amplification requires great handling and processing of samples. In real-time PCR, results are obtained automatically, without any post-PCR processing. It also allows monitoring of PCR performance at each time, since it records the process from cycle to cycle, as opposed to conventional PCR which records only at final time. Moreover, it is highly sensitive and specific.

3.1. Identification of nut species with HRM analysis of universal *trnL* barcoding region

Herein, we have used the shape of the melting curves in order to distinguish differences among the nut species examined. Fig. 1 presents the analysis by means of conventional derivative plots in the "genotyping" mode. This analysis showed that each genotype was represented by two peaks except for walnut, peanut and pecan which were represented by three peaks. The melting temperature peaks of nut species are presented in Table 2. The first peak ranged from 78.2 °C to 81.28 °C, the second peak ranged from 79.5 °C to 83.98 °C and the third peak from 81.53 °C to 82.05 °C. However, the different nut species tested did generate distinctive HRM profiles and normalized HRM profiles (Fig. 2), allowing the discrimination and the differentiation of each species. Analysis of the normalized HRM curves produced with the *trnL* primer set revealed that most of the nut species could easily be distinguished. Assigning the hazelnut as a genotype we were able by subtracting the area (difference graph) from the rest of the produced melting curves by the other species, to estimate the confidence value of similarity between hazelnut and the other nut species used in the study and show that *trnL* was a sufficient region to distinguish the tested species (Fig. 2A, B). The average genotype confidence percentages (GCPs) resulting from HRM analysis of the *trnL* region of eleven nut species are shown in Table 3. GCPs were calculated and a cut-off value of 90% was used to assign a genotype for each region. The highest GCP (53.91) was found between the almond and chestnut species.

The sequence of each amplicon and the alignment between nut species are depicted in Fig. 3. The sequences of the dry nut species used in this study possess variation capable to produce differences in the HRM analysis profile. Specifically we observed deletions, insertions and a number of single nucleotide polymorphisms (SNPs) responsible for the differences in the observed melting curves between the different nut species amplicons. Sequences for *trnL* UAA intron for the eleven nut species have been submitted to the NCBI GenBank and the accession numbers are given below, *P. dulcis* (Almond) JQ769094, *A. occidentale*

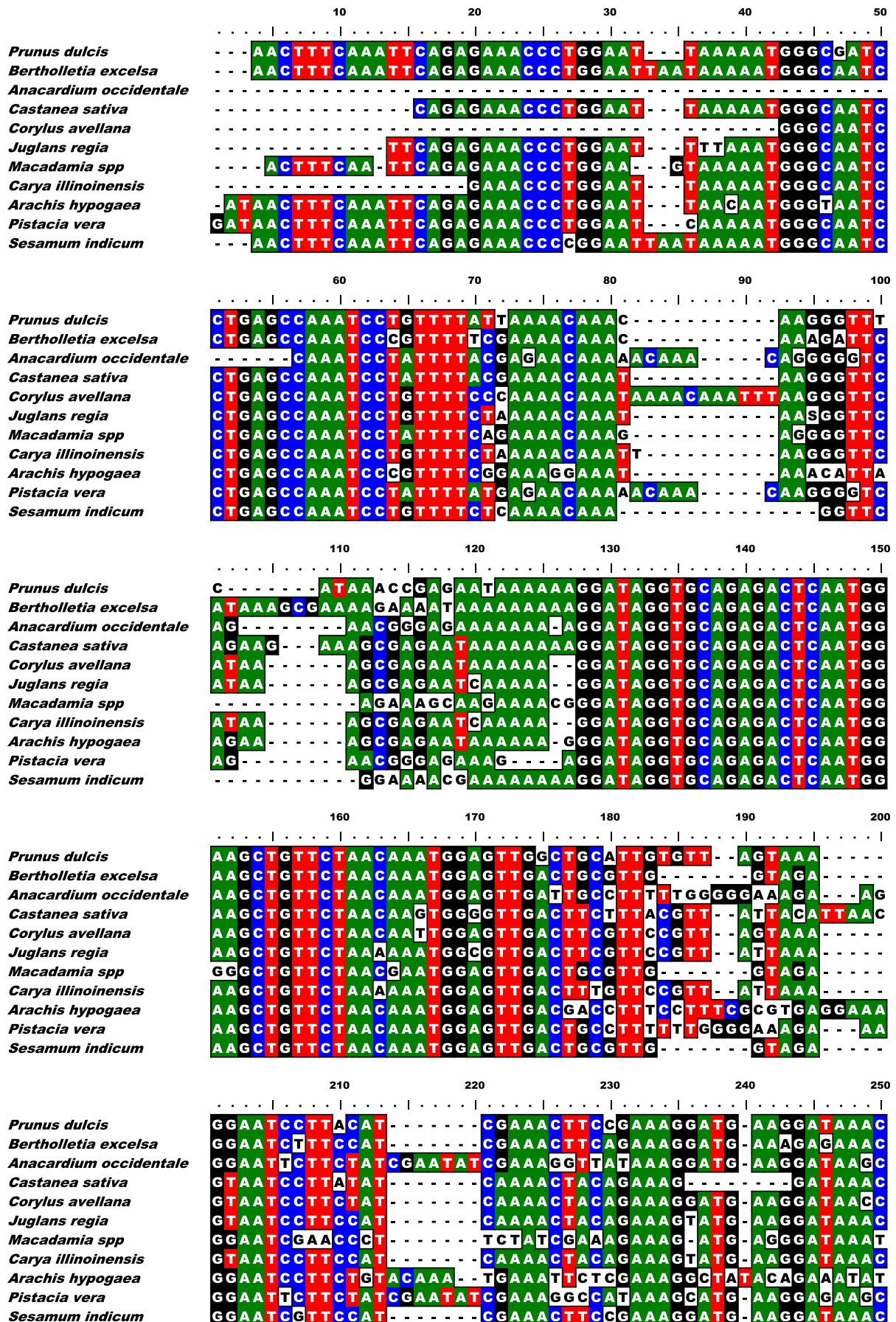


Fig. 3. DNA sequence alignment analysis of dry nut species shows differences in the DNA level alignment thus confirming the Bar-HRM results.

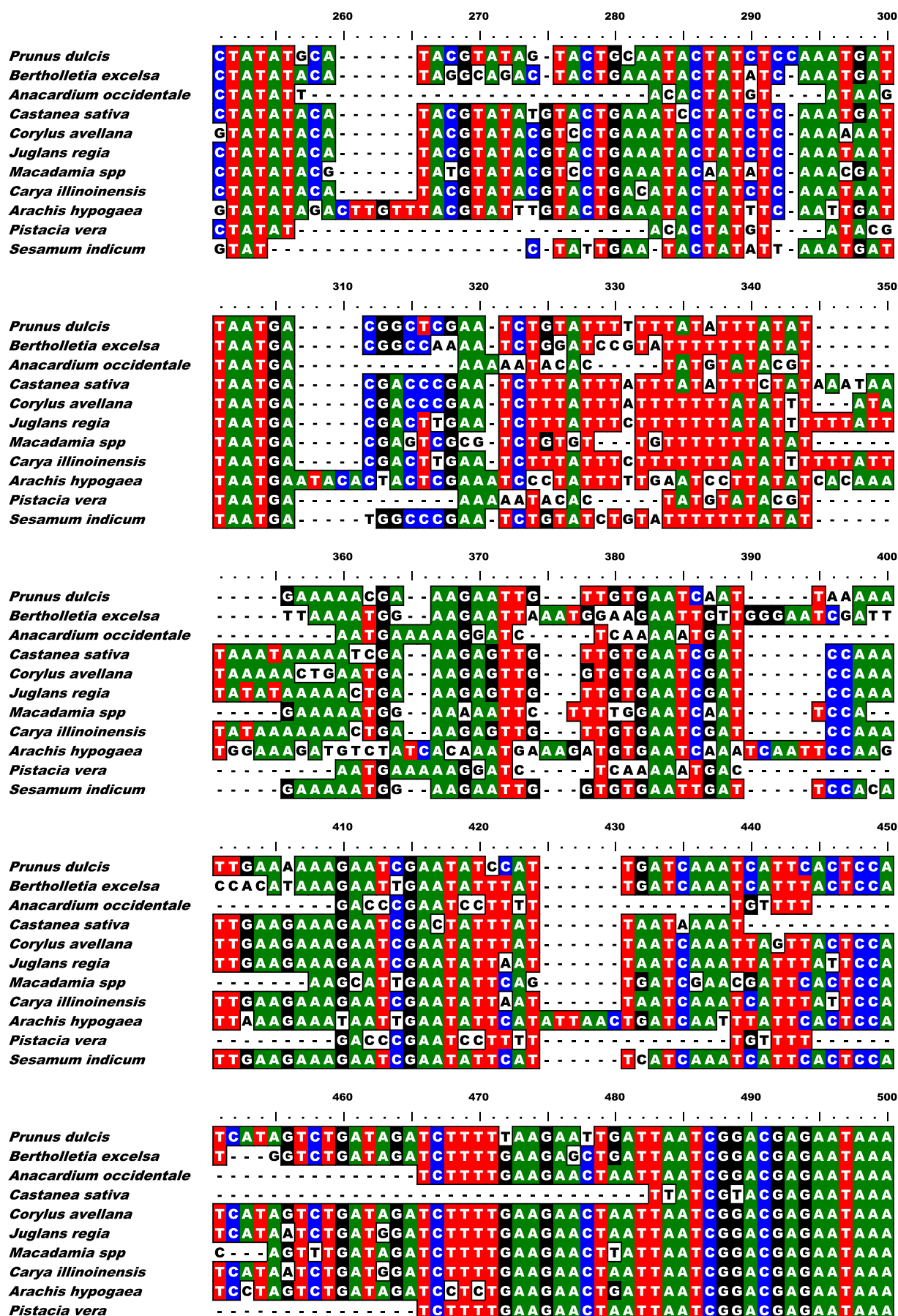


Fig. 3 (continued).

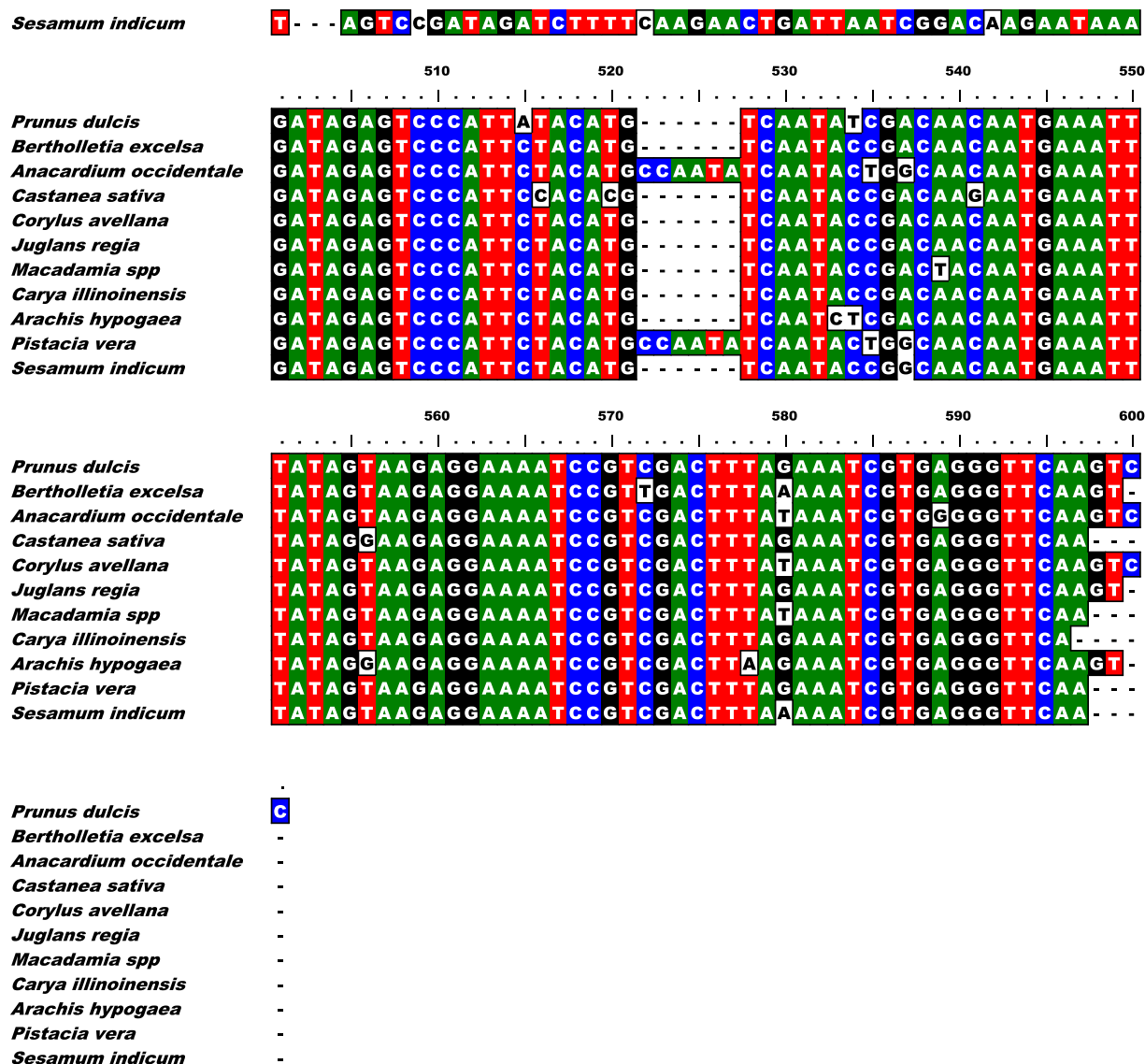


Fig. 3 (continued).

(Cashew) JQ769096, *C. avellana* (Hazelnut) JQ769098, *A. hypogaea* (Peanut) JQ769102, *J. regia* (Walnut) JQ769099, *B. excelsa* (Brazilnut) JQ769095, *C. illinoensis* (Pecan) JQ769101, *C. sativa* (Chestnut) JQ769097, *P. vera* (Pistachio Aigina) JQ769103, *M. spp.* (Macadamia) JQ769100, and *S. indicum* (Sesame), JQ769104.

3.2. Quantitative detection of hazelnut traces in biscuits with HRM analysis

To specifically detect hazelnut, the chloroplast non coding *trnL* UAA intron was chosen. Using the obtained sequences we have designed specific primers (HazeltrnL-F and HazeltrnL-R) for hazelnut identification, which were blasted against DNA/mRNA sequences in the Genbank database of NCBI. The choice was based on the absence of homolog sequences in other plant sources and especially to closely related species. By using the primer-BLAST tool when designing the primers, it was possible to verify their 100% specificity towards the *trnL* intron and the lack of sequence similarity with other food species (Costa, Mafra, & Oliveira, 2012). Moreover, the hazelnut pair of primers was proven to be specific for hazelnuts as it did not detect any common food ingredient such as nuts, seeds, oils, dairy products

and grains including the 14 most common allergens in a preliminary PCR experiment (data not shown) (Schöringhumer & Cichna-Markl, 2007).

The method was further validated with biscuits spiked with hazelnut in different proportions (10%, 5%, 2%, 1%, 0.05% and 0.01%). DNA was extracted in duplicate from all the validation samples and HRM analysis was performed using the *rbcl* and specific hazelnut *trnL* primers (HazeltrnL-F and HazeltrnL-R). Different proportions of hazelnut traces in biscuits were analyzed and detected via HRM analysis (Fig. 5). The process of the hazelnut amplicon dissociation reveals the actual degree of contamination resulting from admixture on a more precise level, as it is suggested (Mader et al., 2011). To validate polymorphisms detected by HRM analysis, amplicon sequences of the representative species for each melting curve type were analyzed. A relative limit of detection and quantification of 0.01% with high correlation ($R^2=0.959$) was obtained with real-time PCR (Fig. 4A). The respective melting curve analysis revealed the amplification of similar products since they exhibited the same melting temperature of 78.51 ± 0.2 °C (Fig. 4B).

In addition we tested five commercial biscuits, the 3 samples labeled as containing hazelnut tested positively for specific amplicon,

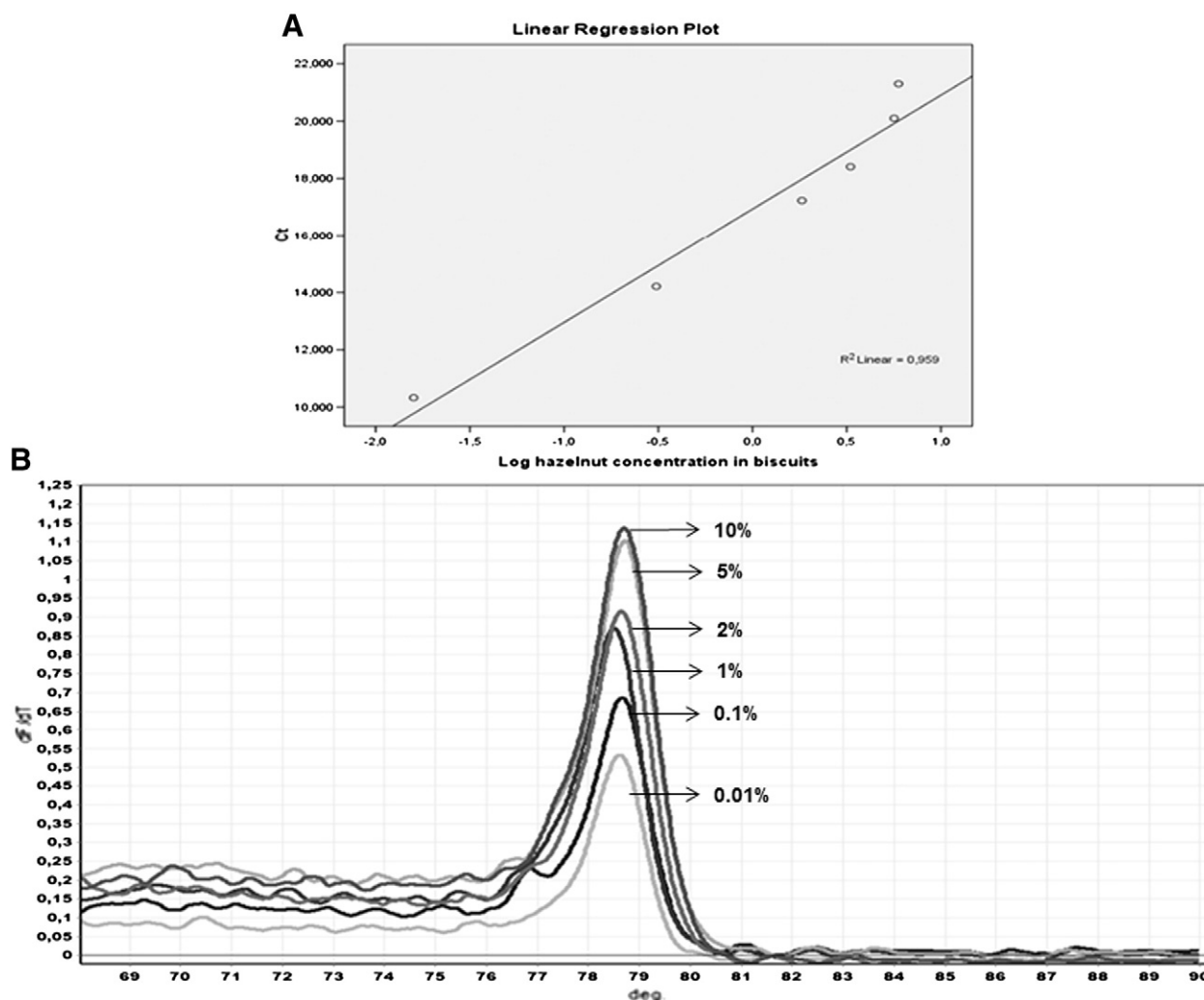


Fig. 4. Standard curve (A) and melting curve analysis (B) obtained by real time PCR with Syto9® dye amplification targeting the *trnL* UAA intron in the chloroplast of hazelnut and applied to reference mixtures containing 10, 5, 2, 1, 0.1 and 0.01% of hazelnut.

suggesting the effectiveness of the technique in processed foods (Costa et al., 2012). From the two food samples with no allegation for hazelnut, a sample of biscuit (Commercial5) gave the expected melting profile, suggesting the presence of unlabeled hazelnut in this product (Table 1).

The fluorescence level at a predefined temperature between the melting domains of the hazelnut amplicon and the amplicons of the universal primer was used as a standard measurement for the level of contamination for calibration ($R^2 = 0.929$). The high R suggests high correlation of the fluorescence mean with the genotypes used especially in the case of mixtures (Fig. 6).

4. Discussion

The development of allergen detection methods is heavily depended on the specificity of the method. In particular the method should be able to specifically detect the allergenic food and not any other species that could give false positives and thus will result in unnecessary food avoidance (Watanabe et al., 2012).

The use of chloroplast DNA for the identification of plants is well established method (Hollingsworth et al., 2009). Chloroplast DNA barcoding is a powerful technique which has been used in food industry, in forensic science, in diet analyses based on feces and in ancient DNA studies. In plants the *trnL* region has been identified as a suitable region (Taberlet et al., 2007). This region has been used extensively

for phylogenetic studies between closely related species (McDade, Daniel, Kiel, Vollesen, & Lavin, 2005; Scharaschkin, Doyle, & Zomlefer, 2005) or for identifying plant species.

A significant aspect of our results is that we were able to transfer and adapt the universal *trnL* primers and standard PCR conditions previously designed to discriminate species by DNA sequencing to more rapid, closed-tube, melting curve assays like the HRM analysis; as it is shown here by the successful genotyping of the eleven nut species tested. The use of HRM to differentiate nut species was performed using a universal chloroplast *trnL* region, which amplifies polymorphic products in eleven nut species. The potential resolving power of this approach is much greater than conventional melting curve analysis because, in HRM, melting curves from different amplicons can be differentiated on the basis of shape even when they define the same T_m values (Ganopoulos et al., 2011a, 2011b).

Platteau et al., 2011 developed PCR assays to detect hazelnut traces in cookies and the detection limits of these lies somewhere between 10 and 100 ppm of hazelnut, moreover in the same study the authors could not identify if ELISA or real time PCR is best suited for the detection of hazelnuts. Another study regarding the detection of a different almond allergen (Pru du 5) was proposed by Costa et al. (2012) by means of HRM analysis in a real-time PCR system with EvaGreen DNA binding dye. The authors reported the detection of the gene encoding for Pru du 5 allergen with a relative LOD of 50 mg/kg of almond in walnut material and an absolute LOD of

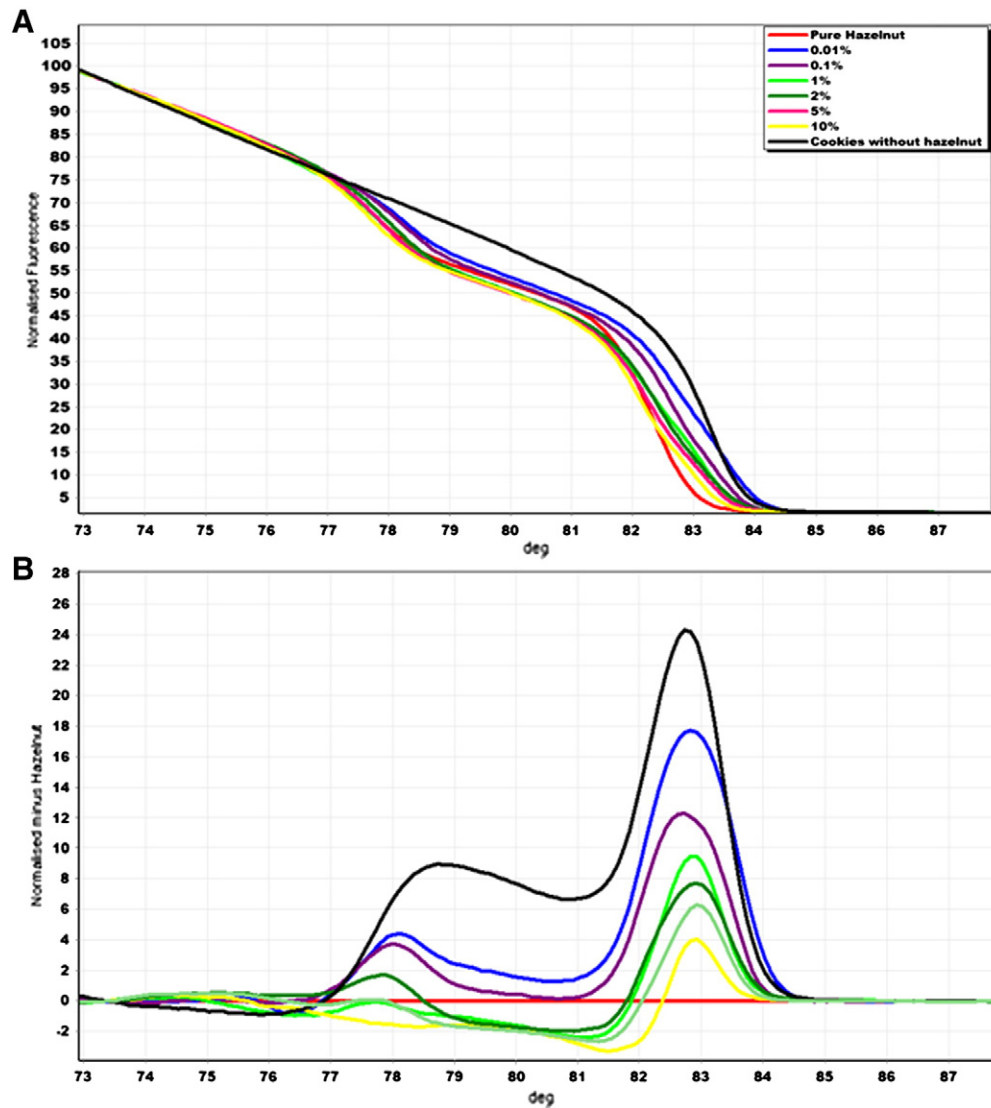


Fig. 5. Melting curves obtained by HRM analysis of the *rbcl* universal chloroplast gene and Hazelnut *trnL* specific amplicon when applied to reference mixtures containing 10, 5, 2, 1, 0.1 and 0.01% of hazelnut.

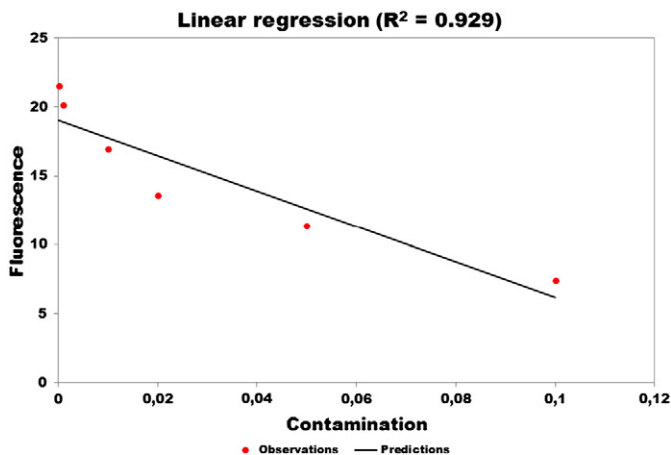


Fig. 6. Pure biscuits were mixed with hazelnut in progressive proportions (10, 5, 2, 1, 0.1 and 0.01%). Values of fluorescence were plotted against the percentage of adulteration of each dilution to generate a typical standard curve. All the experiments were performed in triplicate.

10 pg of almond DNA. The application of HRM analysis for almond detection allowed the distinction of almond from other fruits from the Rosaceae family such as peach, apricot and nectarine (Costa et al., 2012).

The Bar-HRM method allows the fast analysis of genetic variation in commercial food products using universal barcoding regions (chloroplast or nuclear) while at the same time is a close tube post PCR method reducing the risk of contamination. Moreover, HRM analysis embroil the accurate and precise monitoring of fluorescence changes due to the release of the intercalating DNA dye from double stranded DNA when it is denatured by increased temperatures. Differentiation down to genus or even species level is possible based on T_m of specific PCR products (Cheng et al., 2006). HRM analysis has already been used for the identification of plants (Ganopoulos et al., 2011b; Jaakola, Suokas, & Haggman, 2010). Universal barcoding marker *trnL* was able to differentiate among the eleven dry nut species, following HRM curve analysis. While the consumer awareness on food safety and authenticity increases, new techniques are developing to meet their demands. Food authentication is also of concern to food processors who do not wish to be subjected to unfair competition from processors who would gain an economic advantage from the misrepresentation of the food that they are selling (Galimberti et al., 2013; Reid, O'Donnell, & Downey, 2006).

The HRM analysis shows several advantages compared to the traditional SNP and quantitative probe methods (Bai, Wang, Yin, Tian, & Li, 2012): (a) the HRM analysis method sensitivity can reach 1%–0.1%; (b) it can analyze simultaneously a large number of samples; (c) it is highly specific (the specificity of PCR products approaches 100%); (d) it is a closed tube technique which avoids cross-contamination of the samples, thus there is no need for further processing; (e) the sample genotype can be evaluated directly by HRM curve analysis; (f) there is no need for specific probes and sequencing. Overall, HRM analysis is a low cost method which can significantly simplify the procedure and reduce the time of analysis (Sakaridis, Ganopoulos, Argiriou, & Tsaftaris, 2013).

In conclusion we present, to our knowledge, the first adaptation of novel HRM assay using highly specific primers as a sensitive and reliable diagnostic tool for simultaneous detection and quantification of allergenic nut in commercial processed food products using hazelnut as a case study. The applicability of the real-time PCR and Bar-HRM method for the detection of hazelnut DNA in biscuits was shown. The limit of detection of 0.01% was confirmed. Owing to the high sensitivity of the method, amplification is observed even in samples containing only very low amounts of hazelnut traces. In addition, we have barcoded all major nuts using *trnL* UAA intron universal primers.

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