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Analytical Methods

Barcode High Resolution Melting (Bar-HRM) analysis for detection and quantification of PDO "Fava Santorinis" (*Lathyrus clymenum*) adulterants

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ABSTRACT

Legumes considered as one of the most important crops worldwide. Due to high price as a PDO product, commercial products of "Fava Santorinis" are often subjected to adulterations from other legume products coming from other *Lathyrus* or *Vicia* and *Pisum* species. Using plant DNA barcoding regions (*trnL* and *rpoC*) coupled with High Resolution Melting (Bar-HRM) we have developed a method allowing us to detect and authenticate PDO "Fava Santorinis". Bar-HRM proved to be a very sensitive tool able to genotype *Lathyrus* and its closed relative species and to detect admixtures, being sensitive enough to as low as 1:100 of non-"Fava Santorinis" in "Fava Santorinis" commercial products. In conclusion, Bar-HRM analysis can be a faster, with higher resolution and cost effectiveness alternative method to authenticate PDO "Fava Santorinis" and to quantitatively detect adulterations in "Fava Santorinis" with other relative commercial "Fava" food products.

1. Introduction

Lathyrus clymenum belongs to the genus *Lathyrus* in Fabaceae family. *Lathyrus* species are cultivated as human and animal food. *L. clymenum* ("Fava") is an important legume in the Mediterranean diet, not only because of its taste, but also for its nutrients that contribute to a healthy diet. As a human nutrient, its quality is governed by food safety legislation, with a particular focus on the exclusion of contaminants such as pesticides, metals, toxins, and allergens (http://www.fda.gov/Food/FoodSafety/default.htm). In recent years the European Union (EU) has supported efforts to protect producers and consumers from fraudulent activities involved in the production of highly prized food. In particular, it has introduced regulations aimed at ensuring traceability, allowing the possibility of tracking batches of product throughout its production, processing and distribution, to define the origin of a food product, and to govern its labelling. Moreover, raw materials used in food production must meet certain standards and appropriate legislative measures to which suppliers, manufacturers and retailers must comply need to be enforced (Ganopoulos, Argiriou, & Tsaftaris, 2011b, Ganopoulos, Argiriou, & Tsaftaris, 2011a). In order to protect those foods produced within the EU which have unique characteristics, thanks to their production process and/or nutritional value, the concept of the certification brands PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) has been introduced.

The PDO "Fava Santorinis" also called "Fava" and "arakas" offers high income to growers since it is 4 times more expensive than regular "Fava" coming from favabeans (*Vicia faba*) or from peas (*Pisum sativum*) locally called "arakas". For all the above reasons PDO "Fava Santorinis" is prone to adulterations. Furthermore, since cultivated Fabaceae (*V. faba*) subsp. *major* (cultivated for food) and subsp. *minor* (cultivated for feed) also called Fava, frequently could mislead the consumers. The same is true for peas whose seed products are locally called "arakas". Hence, *L. clymenum* is often mixed with other *Lathyrus* species such as *Lathyrus cicera*, *Lathyrus ochrus* and *Lathyrus sativus* but some of them contain high levels of the neurotoxin α,β-diamino propanoic acid (ODAP) in their seeds which may cause a kind of paralysis known as lathyrism (Jiao et al., 2011; Kumar, Bejiga, Ahmed, Nakkoul, & Sarker, 2011; Yan et al., 2006).

Chloroplast sequences have been used as a means for species identification (Hollingsworth et al., 2009; Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005). The sequences used are usually of short length known as DNA barcodes (Kress et al., 2005). Chloroplast DNA barcoding is mainly used in order to identify different plant

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species, but its applications could be extended to the food industry, evolution studies and forensic (Taberlet et al., 2007). Different regions of the plastid genome have been proposed to serve as DNA barcodes, including the *rbcL*, *matK*, *rpoB* and *C* genes and the non-coding spacers *atpF–atpH*, *trnH–psbA*, and *psbK–psbI trnL-F* the *trnL* (UAA) intron and the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal DNA, with different groups having their preferred plant barcodes, yet no consensus has emerged for the use of a standard region (Edwards, Horn, Taylor, Savolainen, & Hawkins, 2008; Hollingsworth et al., 2009; Kress et al., 2005; Taberlet et al., 2007; Yao et al., 2010).

Chloroplast DNA regions of the Fabaceae family have been used for barcoding certain genus or for phylogenetic studies, among the regions used are the *psbA–trnH* region *trnT–trnL* and the nuclear ribosomal *ITS2* region (Edwards et al., 2008). Wojciechowski, Lavin, and Sanderson (2004) preferred the *matk* region in order to construct the phylogeny of legumes. Asmussen and Liston (1998), used *rpoC* and *psbA*, *trnHGUG*, *ndhF*, and their intergenic regions in order to construct the phylogeny of *Lathyrus* genus. Later, Kenicer, Kajita, Pennington, and Murata (2005) used *trnL-F* and *trnS-G* and ITS in order to study the systematic and biogeography of *Lathyrus*.

High Resolution Melting analysis (HRM) is an automated analytical molecular technique that measures the rate of double stranded DNA dissociation to single stranded DNA with increasing temperature. A fluorescent dye, which is homogenously intercalated into DNA, is included in the PCR reaction in order to follow the dissociation of the double stranded DNA. The thermodynamic properties like the sequence length GC content complementarity and nearest neighbour of the particular DNA product cause a specific change in fluorescence and the observed melting curve during HRM DNA dissociation (Reed & Wittwer, 2004). Increased resolution and precision of the instruments and the development of saturating DNA dyes facilitated the use of HRM for genotyping (SNP, SSR markers) (Ganopoulos et al., 2011b; Mader, Lukas, & Novak, 2008; Wu, Wirthensohn, Hunt, Gibson, & Sedgley, 2008), for methylation analysis, as an alternative to gel electrophoresis, and for quantitative detection of adulterants in "Basmati" rice and *Helleborus niger* (Ganopoulos et al., 2011b, 2011a; Mader, Ruzicka, Schmiderer, & Novak, 2010). HRM could be used as an alternative technique to investigate microsatellites, especially for those laboratories that do not have immediate access to capillary sequencers (Ganopoulos et al., 2011b; Mackay, Wright, & Bonfiglioli, 2008). The sensitivity of the method has already been widely demonstrated (Martino, Mancuso, & Rossi, 2010; Reed et al., 2004). Results are obtained, without additional post-PCR processing, in less than 2 h approximately.

Herein, we describe a new application of HRM which is coupled with the DNA barcoding (Bar-HRM) using universal regions for the rapid detection, quantification and adulteration measurement of "Fava Santorinis" species and their commercial food products. Bar-HRM was proven capable not only to distinguish among different *L. clymenum* legume relatives but in addition to detect a ratio of 1:100 of non-Fava Santorinis in Faba Santorinis commercial products.

2. Materials and methods

2.1. Plant and food material

The plant materials used in this study are shown in Table 1 together with their source origin. The species have been selected either because their name is confused with either the common name Fava (*V. faba*) or the scientific name *Lathyrous* (*Lathyrous cicera, L. ochrus* and *L. sativum*) or because they are cheap substitutes of the expensive "Fava Santorinis" like *Pisum*. In addition 10 commercial products were used; one was original "Fava Santorinis" product originating from the local producers, three were putative "Fava Santorinis" and five were "Fava" products (Table 1).

Isolation of DNA was performed with 0.1 g starting material of fine powder of seeds or leaves with the Qiagen DNeasy plant mini kit, according to the manufacturer's instructions. Different percentage mixes of *L. clymenum* "Fava Santorinis" and *L. cicera* "non-Fava Santorinis" species were obtained by mixing, in the appropriate quantities, seed fine powder from *L. clymenum* and *L. cicera*. The DNA concentration was estimated by standard spectrophotometric methods at 260 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/uL work concentration.

2.2. PCR amplification

PCR amplification, DNA melting, and end point fluorescence level acquiring PCR amplifications were performed in a total volume of 15 μL on a Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia). The reaction mixture contained 20 ng genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 300 nM forward and reverse primers (Table S1), 1.5 mM Syto® 9 green fluorescent nucleic acid stain, and 0.5 U Kapa Taq DNA polymerase (Kapa Biosystems, USA). A third generation DNA intercalating dye, Syto® 9, that at high concentrations can saturate all available sites within double stranded DNA was used. Syto® 9s' fluorescence provides a more accurate assessment of DNA melt status compared to SYBR Green I (Monis, Giglio, & Saint, 2005) and can be used to monitor both the accumulation of the amplified product during PCR and also the subsequent product melting on the RotorGene 6000 (software version 1.7.87 Corbett Life Science, Cambridge, UK).

A rapid PCR protocol was conducted in a 36-well carousel using an initial denaturing step of 94 °C for 3 min followed by 30 cycles of 94 °C for 20 s, 50–54 °C for 40 s, and 72 °C for 20–40 s, then a final extension step of 72 °C for 2 min. The fluorescent data was acquired at the end of each extension step during PCR cycles. Before HRM, the products were denatured at 94 °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 increment step. End point fluorescence level was acquired following the melting process by holding at 60 °C for 5 min. In order to further increase the reproducibility and reliability of the HRM curve analysis (by obtaining similar amplified quantities of final PCR products before melting), finer adjustments, by diluting, were made to the genomic DNA templates obtained from the species leaves, where no dilution was made to the DNA extracted from the seed commercial products. A Ct parameter of 19 ± 4 cycles at a threshold of 0.01 of the normalised fluorescence was established. All samples were examined in duplicate.

Real time PCR and barcoding with HRM were carried out on standard samples prepared by mixing fine powder from seeds of *L. clymenum* with *L. cicera* in different proportions of 1%, 5%, 7%, 10%, 15%, 30%, and 50%. PCR amplification was performed as described earlier. The difference plot curves of genotypes were generated in 3 steps: (1) normalisation, (2) temperature shift, and (3) difference plot. A base curve was chosen arbitrarily to create a more distinguishable plot, especially for those with similar melting curves (Li et al., 2010).

2.3. Barcoding of PCR products by high-resolution melting analysis

The Rotor-Gene 6000 proprietary software (version 1.7.87) was used to genotype the different varieties and the food products. The

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Table 1
Commercial "Fava" products, *Lathyrus* and related species used in this study.

No. genotype	Species/type	Abbreviation	Source
1	Commercial 1 ("Fava")	Com1	Market
2	Commercial 2 ("Fava")	Com2	Market
3	Commercial 3 ("Fava")	Com3	Market
4	Commercial 4 ("Fava")	Com4	Market
5	Commercial 5 ("Fava")	Com5	Market
6	Commercial 6 ("Fava")	Com6	Market
7	Commercial 7 ("Fava Santorinis")	Com7	Local producers
8	Commercial 8 (putative "Fava Santorinis")	Com8	Market
9	Commercial 9 (putative "Fava Santorinis")	Com9	Market
10	Commercial 10 (putative "Fava Santorinis")	Com10	Market
11	*Lathyrus sativus*	Lsa	NAGREF
12	*Lathyrus clymenum*	Lcl	AUTH, Seed Bank
13	*Pisum sativum* subsp. *eliatus*	Psel	NAGREF
14	*Vicia faba* subsp. *minor*	Vfmin	NAGREF
15	*Vicia faba* subsp. *major*	Vfmaj	NAGREF
16	*Lathyrus cicera*	Lci	NAGREF
17	*Pisum sativum*	Psa	NAGREF
18	*Lathyrus ochrus*	Loc	NAGREF

negative derivative of fluorescence (F) over temperature (T) (dF/dt) curve primarily displaying the Tm, the normalised raw curve depicting the decreasing fluorescence vs increasing temperature, and difference curves (Wittwer, Reed, Gundry, Vandersteen, & Pryor, 2003) were mainly used. Furthermore, a 2-step procedure was followed according to Ganopoulos et al. (2011a, 2011b) 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 (Hewson, Noormohammadi, Devlin, Mardani, & Ignjatovic, 2009). GCPs were re-coded from a 1–100 to a 1–20 range of values to decrease the number of different genotypes causing small differences in the shape of the melting curves giving slightly different GCPs (Ganopoulos et al., 2011b). The means of the confidence percentage of the species replicates assigned to a representative genotype, together with the standard deviation, were generated using Microsoft Office Excel.

2.4. Sequence analysis

PCR products were directly sequenced in two directions for each product with 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

3.1. Lathyrus and relative legumes barcoding using Bar-HRM analysis

The use of HRM to differentiate species and by this "Fava Santorinis" PDO products belonging to *L. clymenum* was tested using two chloroplast regions as markers to amplify polymorphic products, in eight species and 10 commercial "Fava" products (Table 1). The potential resolving power of this approach is much greater than conventional melting curve analysis. In HRM, melting curves from different amplicons can be differentiated on the basis of shape, even when they define the same Tm values because of the composite melting curves of heterozygotes. Heterozygotes include 2 homoduplexes and 2 heteroduplexes contributing to the Tm and thus Tm is ambiguous and less useful as a metric than is the curves' shape. In this study we used the shape of the more informative melting curves assessing differences in the species under investigation (Fig. 1A and B) and to apply this information in food traceability.

Analysis of the normalised HRM curves with the barcode marker *trnL* (Fig. 1A and B) revealed that most of the species could easily be distinguished, for example *L. clymenum* and *P. sativum*, the curve profiles of some species were similar and could therefore not be visually differentiated. Assigning species *L. clymenum* 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 *L. clymenum* (Fig. 1B). GCPs were calculated and a cut off value of 90% was used to assign a genotype for each barcode region (Table 2). Furthermore, closer examination of the *L. clymenum* HRM difference curve, with the mean *L. ochrus* curve as the baseline, revealed part of the curve sitting outside the 90% CI curve, suggesting that the *L. clymenum* and *L. ochrus* HRM curves are indeed different (Fig. 1C).

3.2. Adulterations of "Fava" products detected by Bar-HRM analysis

After the confirmation that single species can be identified by HRM analysis we applied the same approach for the identification of the species used in "Fava" food products. Eight legume species four of which are *Lathyrus* species were used as controls (Table 1). The normalised HRM curves for the amplicons, from the eight species and 10 commercial "Fava" food products, based on HRM analysis with barcode marker *rpoC* are shown in Fig. 2A. Each genotype produced a unique melting plot that was easily distinguishable from other species. Thus all samples have been successfully distinguished and assigned to species origin genotypes. In 3 commercial (Com8, Com9, and Com10) "Fava Santorinis" products adulterations were revealed by applying our genotype method (Fig. 2A). Only *L. ochrus* shared a plot similar to *L. clymenum*. To avoid mistyping these 2 genotypes, we generated a separate subset involving only the two species using their HRM curves, thus in (Fig. 2B) we show that the two species are clearly distinguished. Results obtained from the 10 different commercial "Fava" food products indicate that HRM can be effectively used to assess if a single known species or more than one has been used during the process of the commercial product production.

In Table 2 we present the average genotype confidence percentages (±3.15) resulting from HRM analysis of the barcode region *rpoC* of eight species and 10 commercial "Fava" products at a ramp of 0.1 °C. To further improve our analysis we elaborated a statistical analysis where the GCPs values were re-coded in a range of 1–20 from 1 to 100 to decrease the number of different genotypes.

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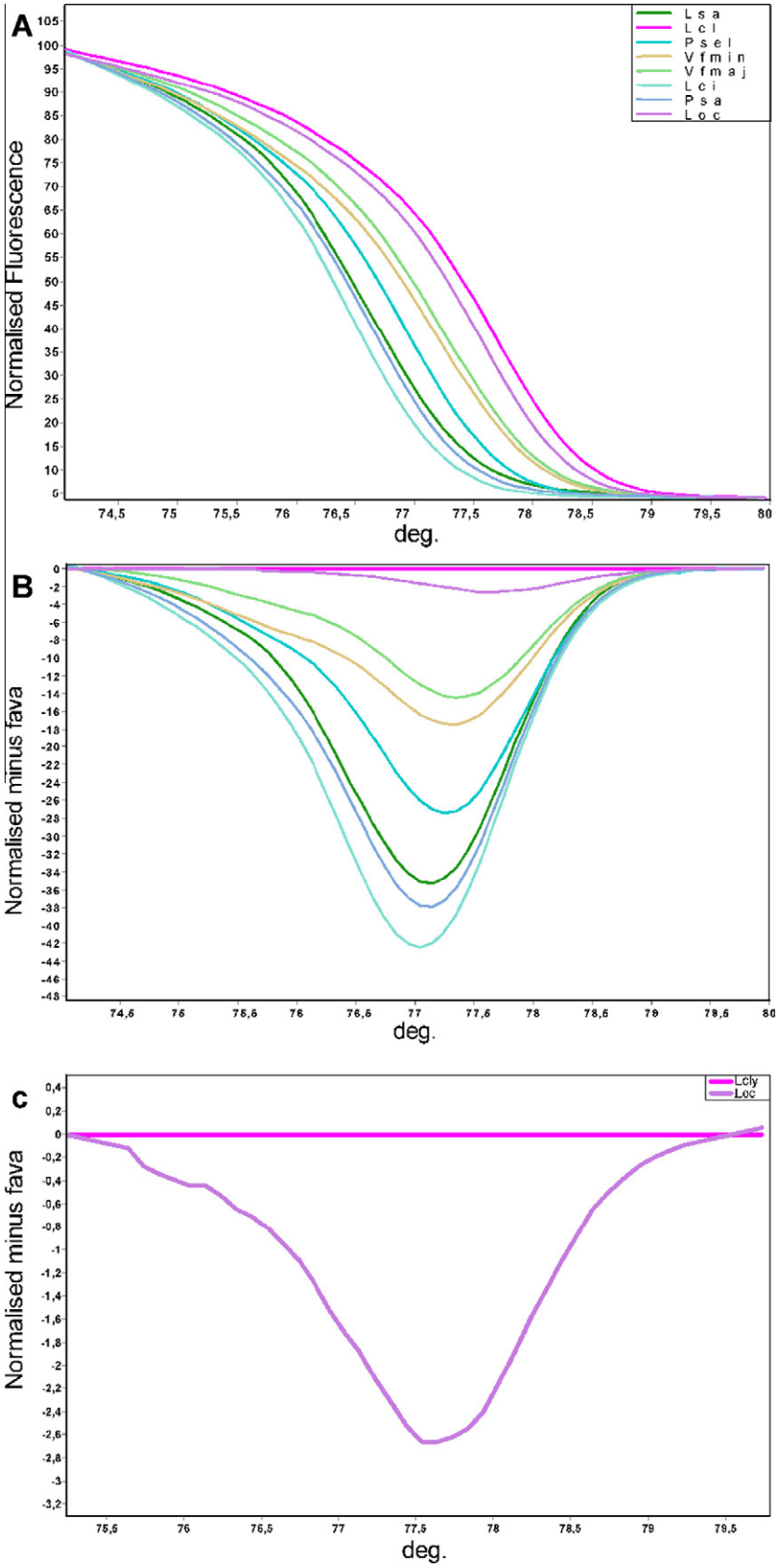

Fig. 1. Bar_HRM on *Lathyrus* and related species using HRM analysis with the *trnL* chloroplast marker. (A) Normalised melting profiles of *Lathyrus* species with the *trnL* type indicated. (B) Difference graph of eight species using *Lathyrus clymenum* as reference genotype. Colour code table with the species used. (C) Difference plot of two closely related species (*Lathyrus clymenum* (fuchsia) and *Lathyrus ochrus* (lilac). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 2
Average genotype confidence percentages (±3.15) resulting from HRM analysis of the barcode chloroplast region *rpoC* 10 commercial "Fava" products and eight Fabaceae species at a ramp of 0.1 °C. Values over the 90% confidence percentage threshold are presented in bold.

	Com1	Com2	Com3	Com4	Com5	Com6	Com7	Com8	Com9	Com10	Lsa	Lcl	Psel	Vfmin	Vfmaj	Lci	Psa	Loc
Com1	100																	
Com2	46.16	100																
Com3	3.02	25.85	100															
Com4	30.74	**94.45**	39.60	100														
Com5	50.02	12.69	0.33	0.00	100													
Com6	0	0.00	0.03	57.40	0.00	100												
Com7	88.68	47.00	3.99	34.15	65.05	0.00	100											
Com8	6.47	38.99	**92.26**	55.26	30.63	1.08	9.05	100										
Com9	15.44	1.04	0.01	0.45	0.00	0.00	13.82	0.03	100									
Com10	51.72	10.26	0.21	7.75	26.95	0.00	34.25	0.51	45.13	100								
Lsa	0	0.00	0.00	34.19	0.00	0.66	0.00	0.00	0.00	0.00	100							
Lcl	82.5	46.03	4.13	0.00	66.79	0.00	**98.80**	9.55	11.87	27.84	0.00	100						
Psel	4.31	41.43	69.36	6.73	0.47	0.08	4.79	65.44	0.01	0.46	0.00	4.72	100					
Vfmir	46.82	11.08	0.29	0.25	**99.36**	0.00	62.12	0.96	30.74	24.92	0.00	64.27	0.38	100				
Vfmaj	10.48	0.60	0.00	66.82	42.38	0.00	11.38	0.01	66.12	18.90	0.00	10.81	0.00	88.04	100			
Lci	21.5	56.74	44.58	5.33	8.54	0.00	33.13	68.17	0.35	2.33	0.00	36.20	32.76	8.00	0.24	100		
Psa	**91.93**	68.17	5.85	49.86	39.72	0.00	**90.82**	11.44	8.83	40.64	0.00	78.61	9.69	36.16	5.68	30.40	100	
Loc	65.28	13.15	0.37	7.59	81.95	0.00	68.58	1.08	51.42	56.04	0.00	64.69	0.50	80.70	44.06	7.12	48.20	100

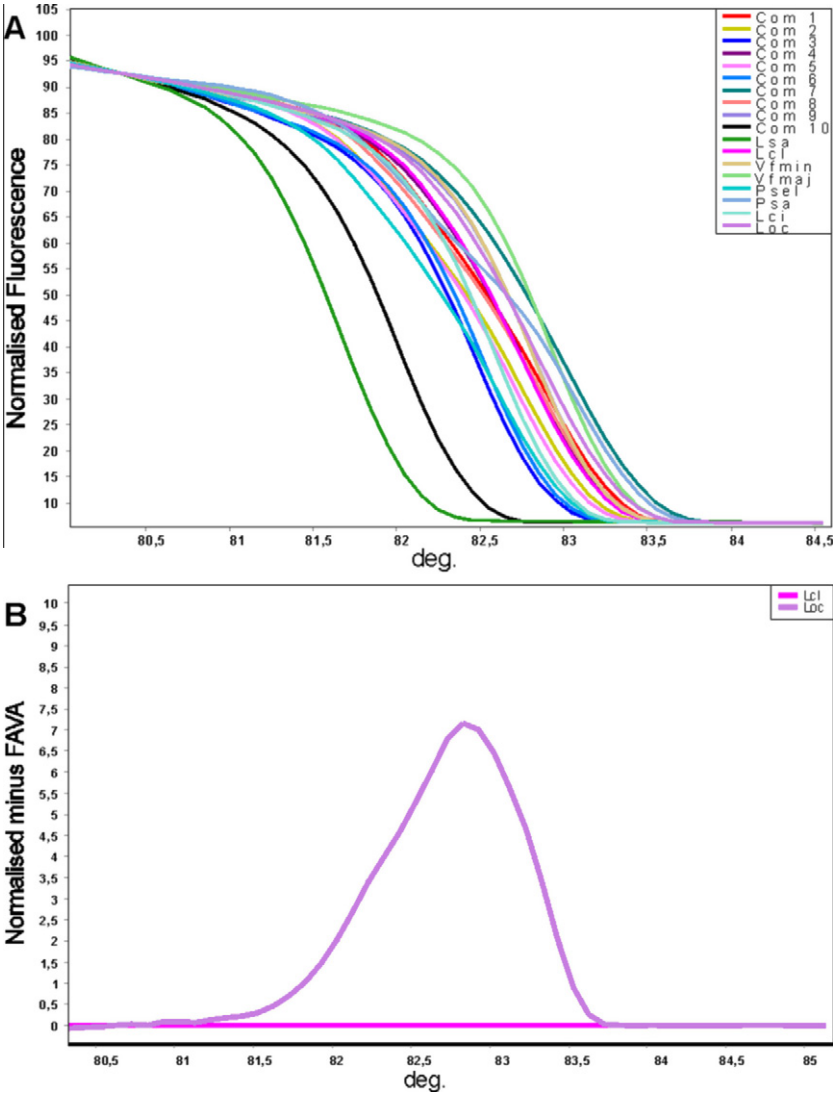

Fig. 2. Bar-HRM on eight *Lathyrus* and related Fabaceae species and 10 commercial "Fava" food products using HRM analysis with *rpoC* chloroplast marker. (A) Normalised melting profiles of *Lathyrus* species with the *rpoC* type indicated. (B) Difference graph of eight species using *Lathyrus clymenum* as reference genotype. Colour code table with the species used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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The method was validated with "Fava Santorinis" (*L. clymenum*) spiked with *L. cicera* in different proportions and also with commercial market samples. DNA was extracted in duplicate from all the validation samples and HRM analysis was performed using the *trnL* region. Mere fluorescence normalisation of temperature-shifted melting curves suffices to distinguish the genotypes with melting curve shapes after applying the Corbett software (Fig. 3A). When our data were analysed by means of conventional derivative plots, in the "genotyping" mode, each genotype was represented by a single peak (Fig. 3B). Heterozygous samples had only one shoulder instead of forming double peaks (Fig. 3B). The process of the "Fava Santorinis" amplicon dissociation reveals the actual degree of contamination resulting from adulteration on a more precise level, as it is suggested by Mader et al. (2010). These differences depicted in Fig. 3B, between the two closely related species *L.* clymenum and *L.* cicera were confirmed via sequencing *L. clymenum* and *L. cicera* (Fig. 3C). *L. clymenum trnL* region comprises 94 bp while *L. cicera* 89 bp. *L. cicera* has a deletion of 1 G and another deletion of 4 bp (CAAA) and three SNPs G (instead of T) and two A (instead of C).

Based on the approaches above and using the mean fluorescence in Fig. 3A we were able to produce a standard curve (Fig. 4) that shows significant correlation between the level of fluorescence and the percentage of contamination (R^2 = 0.926) further validating the results obtained via the Bar-HRM method.

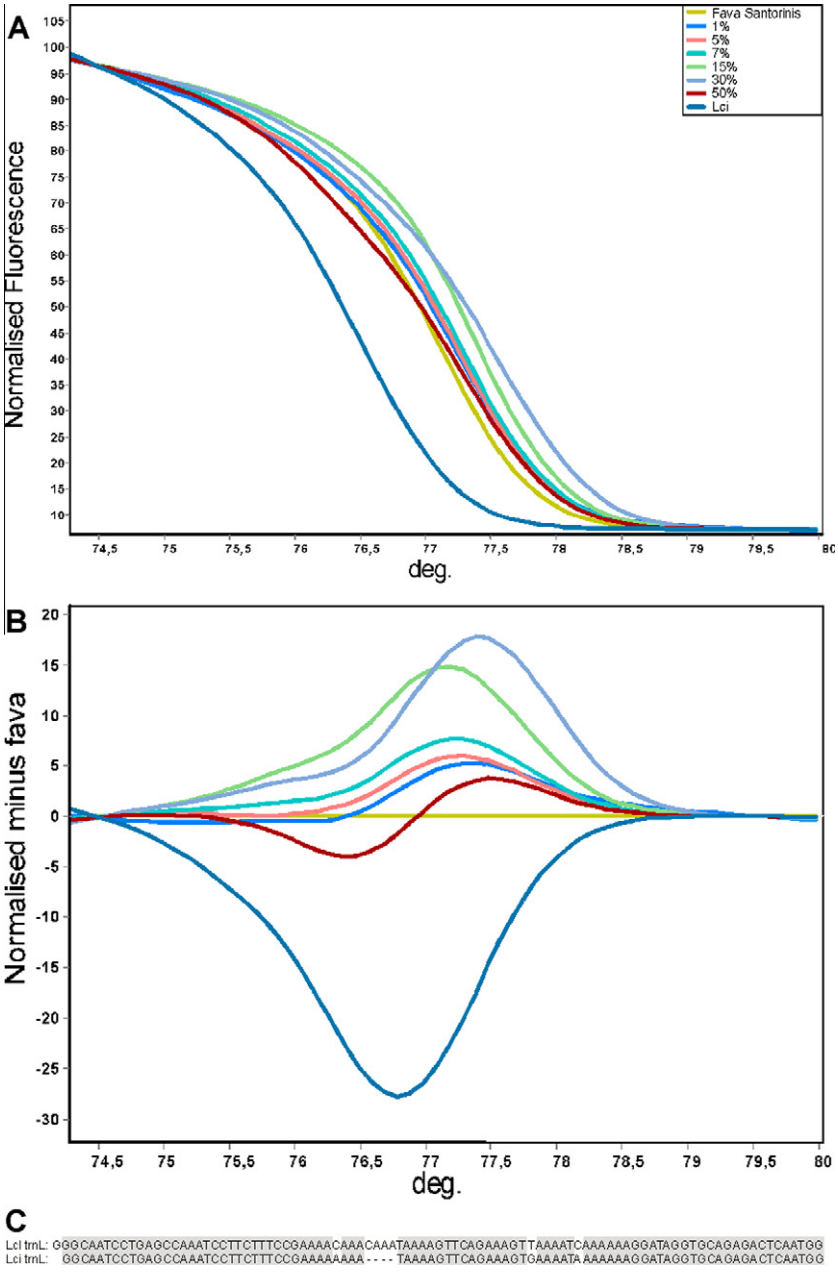

Fig. 3. Adulterations of "Fava Santorinis" detected by HRM analysis. (A) High-resolution melting curves of the *trnL* region primer assay. The HRM data are presented as graphs displaying the difference in fluorescence (ΔF) of the two different amplicons and *Lathyrus cicera* amplicon mixtures in relation to the "Fava Santorinis" (*Lathyrus clymenum*). Each variant amplicon was mixed with a "Fava Santorinis" amplicon at a ratio of 1:99, 5:95, 7:93, 15:85, 30:70 or 50:50, respectively. (B) Diagrams of normalised fluorescence of mixed "Fava Santorinis"/no "Fava Santorinis". DNA dilutions normalised against "Fava Santorinis" control fluorescence. (C) Sequence analysis of *Lathyrus clymenum* and *Lathyrus cicera* shows differences in the DNA level alignment thus confirming the Bar-HRM results.

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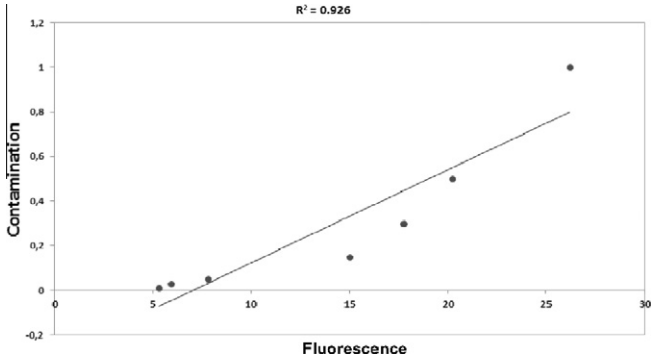

Fig. 4. Pure "Fava Santorinis" was mixed with *Lathyrus cicera* in progressive proportions (1%, 3%, 7%, 15%, 30%, and 50%). Values of fluorescence were plotted against the percentage of adulteration of each dilution to generate a typical standard curve. All the experiments were performed in duplicate.

4. Discussion

It is a challenge to develop accurate and reliable methods for the rapid identification of species which could have practical interest and application, like the food products coming from the plant species and their traceability. The HRM PCR method is a close tube post PCR method which permits the rapid analysis of genetic variation in PCR products. HRM involves accurate and precise monitoring of fluorescence changes caused by the release of an intercalating DNA dye from double stranded DNA during its denaturation caused by increased temperatures. Differentiation down to genus or even species level is possible based on melting temperature (Tm) of specific PCR products (Cheng et al., 2006). HRM analysis has already been used for the identification of fungi (Maeta et al., 2008) fish (Dalmasso et al., 2007) and plants (Ganopoulos et al., 2011b; Jaakola, Suokas, & Haggman, 2010).

Here we developed a method using HRM coupled with universal chloroplast DNA barcoding regions (Bar-HRM) in order to distinguish legume species (four *Lathyrus*, two *Vicia* and two *Pisum* species which potentially could be used as adulterants in "Fava Santorinis" commercial products) and moreover to authenticate "Fava Santorinis" commercial PDO products. In order to achieve this goal we have used two universal DNA barcoding regions, the *trnL* and *rpoC* chloroplast regions. The *trnL* region when used on *Lathyrus* and related species was able to adequately discriminate the eight different species used in this study. Only the two closely related species *L. clymenum* and *L. ochrus* show little difference yet they could be distinguished when we plot a difference graph of the two species. In addition, Bar-HRM proved to be a powerful method which allowed the quantification of "Fava Santorinis" adulterants. Using the *trnL* region we could quantify the adulteration of "Fava Santorinis" spiked with *L. cicera* as low as 1% in the commercial product. These results were also confirmed by sequence analysis of the *trnL* region which revealed that the two species differ in three nucleotides and moreover *L. cicera* has two deletions of 1 and 4 nucleotides. Thus, the sequence analysis of the two species used (*L. clymenum* and *L. cicera*) confirmed the power of the Bar-HRM method to distinguish the legume species and authenticate the PDO "Fava Santorinis" and reveal the adulterations in these PDO products. Moreover, the power of the method was further proved and validated via a standard curve of a very high correlation coefficient ($R^2 = 0.926$) suggesting that the use of Bar-HRM is a suitable cost effective and easy to use method to distinguish plant species and authenticate PDO products like "Fava Santorinis".

The use of chloroplast DNA for the identification of plants is well established (Hollingsworth et al., 2009). Chloroplast DNA barcoding is a powerful technique which could be used in food industry, in forensic science, in diet analyses based on feces and in ancient DNA studies. The animal predominant barcoding region is the CO1 gene, whereas in plants the *trnL* region has been identified as a suitable region (Taberlet et al., 2007) but it has been recognised that in plants it will be rather difficult to identify one region which will serve as a universal barcoding region (Hollingsworth et al., 2009; Kress et al., 2005). In addition, Taberlet et al. (2007) suggested the use of *trnL* (UAA) intron. 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 (Ronning, Rudi, Berdal, & Holst-Jensen, 2005; Ward, Peakall, Gilmore, & Robertson, 2005), although it is not the most variable region in the chloroplast genome (Shaw et al., 2005).

Moreover, we have employed the *rpoC* chloroplast region in order to discover "Fava Santorinis" adulterants. Using the *rpoC* region we discriminated the four *Lathyrous*, two *Vicia* and two *Pisum* species and furthermore we discovered that in 75% of the commercial products analysed, the name of the species displayed on the label was not in agreement with the species contained, as determined by genetic analysis using the methodology developed herein (Table 2).

The *rpoC* is suggested to have little discriminate power, the CBOL plant working group suggested than the discrimination power of single regions rank is *rpoC* < *rpoB* < *atpF–atpH* < *rbcL* < *matK* < *psbK–psbI* < *trnH–psbA*. (Chase, Cowan, Hollingsworth, & van den Berg, 2007) and proposed the use of combinations of three chloroplast regions for the universal DNA plant barcoding protocol *rpoC1*, *rpoB* and *matK* or *rpoC1*, *matK* and the plastid intergenic region *trnH-psbA* which is the most variable non coding chloroplast DNA region yet the *rpoC* region was adequate to discriminate the legume species and the commercial products used here.

It is common practice in plant barcoding the use of a combination of regions in order to identify plants. Kress et al. (2005) early on suggested the *trnH–psbA* spacer region as a suitable barcoding region. The *matK* region has also been proposed as a suitable barcoding region (Hollingsworth et al., 2009) but in our case *matK* was not appropriate because two bands were amplified (data not shown).

In conclusion we present, to our knowledge, the first adaptation of novel HRM assay using universal primers as a sensitive and reliable diagnostic tool for simultaneous detection and quantification of adulterants in PDO "Fava Santorinis" commercial food products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2012.01.015.

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