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Authentication of carnaroli rice by HRM analysis targeting nucleotide polymorphisms in the *Alk* and *Waxy* genes

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ABSTRACT

Carnaroli is a high quality and priced variety, being considered as one of the finest Italian rice varieties due to its sensorial and rheological properties and, thus being a potential adulteration target. The present work aimed at exploiting polymorphisms in the *Alk* (A/G and GC/TT in exon 8) and *Waxy* ((CT)n and G/T in intron 1) genes by HRM analysis to differentiate Carnaroli rice from closely related varieties. The HRM method targeting the *Alk* gene did not allow gathering the Carnaroli subgroup genotypes in the same cluster. The HRM approach targeting *Waxy* gene successfully discriminated the varieties sold as Carnaroli from all the others with high level of confidence (>98%), which corroborated sequencing data. Its applicability to commercial rice samples was successful. Therefore, the proposed new HRM method can be considered a simple, specific, high-throughput and cost-effective tool for the authentication of Carnaroli rice, contributing to valorise such premium variety.

1. Introduction

Rice (*Oryza sativa* L., Poaceae family) is one of the most important cereals for human consumption, being a staple food for nearly half the world population. Its global consumption has seen a slight increase over the past years, from about 437 million tonnes in the 2008/2009 crop year to 487 million tonnes in the 2018/2019 (Statista, 2021). The world production of paddy rice has also increased in the past 10 years, from 680 million tonnes in 2009 to 755 million tonnes in 2019 (FAOSTAT, 2021). In Europe, Italy leads the production of rice with about 1.49 million tonnes in 2019 (FAOSTAT, 2021). Most of the rice fields are located in the regions of Piedmont and Lombardy, in the north-western part of the Po valley (Faivre-Rampant et al., 2011), where about 230 different varieties registered in the Plant variety database of the European Commission (European Commission, 2021) are cultivated, the majority of which belonging to the japonica ecotype (Cai et al., 2013; Faivre-Rampant et al., 2011). Among them, Carnaroli is a high quality and valued variety, being considered as one of the finest Italian rice genotypes. Carnaroli is a long-grain rice type A, characterised by an excellent cooking resistance due to its low tendency to lose starch and good ability to absorb liquid while creaming, making it ideal for the preparation of traditional risotto (https://risodeltapoigp.it/en/carnaroli-rice/). Owing to its high commercial value, this variety is a potential target for adulteration. Therefore, the development of methods to differentiate and identify rice varieties, in particular Carnaroli, is of utmost importance to avoid fraudulent practices. Since morphological characteristics might be minimal among other rice varieties of the same group, disabling their differentiation, exploiting DNA polymorphisms associated to the distinct quality traits could provide useful authentication markers.

Starch, the most relevant compound to determine rice quality and end-use, is composed of two polysaccharides, namely amylose and amylopectin, whose ratio is determinant for the rice cooking properties. After cooking, varieties with high amylose content have dry, firm and separate grains, while low amylose ones usually have tender, cohesive and glossy texture (Biselli et al., 2014; Dobo et al., 2010). Amylose synthesis is catalysed by the granule bound starch synthase (GBSS) that is encoded by the *Waxy* (*Wx*) gene, being located on chromosome 6. Various nucleotide polymorphisms have been associated with the *Wx* gene, namely a $(CT)_n$ microsatellite or simple sequence repeats (SSR), located in the 5' untranslated region of the gene and closely related to apparent amylose content, and several single nucleotide polymorphisms

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(SNP), such as the G/T and the A/C, located in the first intron and sixth exon, respectively (Biselli et al., 2014; Bligh et al., 1995; Dobo et al., 2010; Larkin & Park, 2003). Although these polymorphisms might be useful, their individual correlation with a specific quality trait is not absolute, thus, the combination of different polymorphisms has been used to distinguish rice varieties with different amylose contents (Biselli et al., 2014; Chen et al., 2008; Dobo et al., 2010; Larkin & Park, 2003).

The amylopectin content is related to the gelatinization temperature, a critical point at which the starch granules start to swell and undertake an irreversible process of crystallinity melting, loss of birefringence and solubilisation (Gao et al., 2003; Zhou et al., 2016). Its synthesis involves the activity of starch synthase IIa (SSIIa), encoded by the *Alk* gene, also located on chromosome 6 (Gao et al., 2003; Umemoto & Aoki, 2005; Zhou et al., 2016). As for the *Wx* gene, several SNP can also be identified in the *Alk* gene, namely G/A and GC/TT, located on exon 8. The G/T polymorphism from the first of the two contiguous nucleotides is a silent SNP because it does not cause amino acid change, whereas the polymorphisms G/A and C/T (second of the two contiguous SNP) lead to the replacements of valine (encoded by the common allele <u>G</u>TG) with methionine (encoded by <u>A</u>TG) and leucine (encoded by <u>C</u>TC) with phenylalanine (encoded by <u>T</u>TC), respectively. These amino acid changes affect the SSIIa activity and, consequently, starch gelatinization properties (Nakamura et al., 2005; Umemoto & Aoki, 2005; Zhou et al., 2016).

DNA-based methods have provided useful tools for plant and animal species identification, being widely applied in food authentication (Amaral et al., 2016; Böhme et al., 2019; Druml & Cichna-Markl, 2014; Garino et al., 2014; Grazina et al., 2020; Mafra et al., 2008; Soares et al., 2017). Among them, high resolution melting (HRM) analysis is considered a promising, cost-effective and high-throughput tool to differentiate closely related species or even varieties in several food matrices (Ballin et al., 2019; Costa et al., 2012, 2016; Druml & Cichna-Markl, 2014; Mackay et al., 2008; Soares et al., 2018; Villa et al., 2016; Wu et al., 2008). HRM is a post real-time PCR analysis that relies on monitoring the gradual denaturation of double stranded DNA (dsDNA) amplicons, allowing the detection of small nucleotide variations, such as deletions, insertions, SNP and microsatellites, without requiring further sequencing (Druml & Cichna-Markl, 2014; Grazina et al., 2021; Simko, 2016). HRM analysis targeting such polymorphisms has been applied to differentiate varieties of rice (Ganopoulos et al., 2011), grapevine (Pereira et al., 2017), olive oil (Gomes et al., 2018), common bean (Ganopoulos et al., 2012) and lentils (Bosmali et al., 2012). However, to our knowledge, its application to differentiate rice varieties or any other DNA fingerprint approaches to authenticate Carnaroli rice have not been described.

Therefore, the present work intends to exploit polymorphisms in the *Alk* and *Wx* genes, namely SNP and microsatellites, by the HRM analysis aiming at differentiating Carnaroli rice from closely related varieties. For this purpose, 35 rice varieties, including several under the commercial name of Carnaroli, others closely related, also classified as long-grain rice type A, and some belonging to long B and round types were acquired and sequenced to identify polymorphisms. The sequences were deposited in the Genbank and the identified polymorphisms were targeted by HRM analysis. Additionally, the proposed HRM method was applied to authenticate commercial rice samples, aiming at detecting possible adulterations of Carnaroli rice.

2. Materials and methods

2.1. Rice samples

Thirty five rice specimens of pure varieties, namely Carnaroli, S. Andrea, Carnise, Karnak, Gladio, Volano, Barone, Ronaldo, Gloria, Sole Cl, Carnaval, Caravaggio, Keope, Poseidone, L202, L252, Roma, Baldo, Cammeo, Galileo, Casanova, Fedra, Proteo, Telemaco and Generale (Table S1, supplementary material) were provided by Italian producers. According to their morphological characteristics, Italian rice varieties have distinct classifications and groups (Table S1, supplementary material) (Gazzetta Ufficiale Della Repubblica Italiana, 2017). Additionally, different varieties are gathered in subgroups and go under the same commercial name, which is usually the same designation of the most representative variety.

Fourteen commercial rice samples were acquired from Italian (8 samples) and Portuguese (6 samples) markets (Table S2, supplementary material). Additionally, to assess assay specificity, a total of 35 species commonly used as food were acquired in local markets, including 30 plant (peanut, pine nut, chestnut, almond, hazelnut, walnut, broad bean, rapeseed, sunflower, oat, rye, barley, wheat, tomato, maize, soybean, potato, cassava, pumpkin, lupine, onion, garlic, parsley, white pepper, laurel, paprika, chilli, oregano, basil, coriander, turmeric) and 4 animal (pork, cow, chicken, codfish) species (Table S3, supplementary material).

All rice samples were ground separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different materials and different blender containers, previously treated with DNA decontamination solution. The prepared samples were immediately extracted or stored at −20 °C until further analysis.

2.2. DNA extraction

The NucleoSpin Food kit (Macherey-Nagel, Düren, Germany) was chosen to perform the DNA extraction from ground rice samples (100 mg), according to the manufacturer instructions with slight modifications, as described by Costa et al. (2012), and with the addition of 2 μL of RNase (2 mg/mL) after the lysis step. All extracts were immediately kept at −20 °C until further analysis.

2.3. DNA quality and purity

Yield and purity of DNA extracts were assessed by UV spectrophotometric using a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA) with a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for dsDNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA). The purity of the extracted DNA was determined by the ratio of the absorbance at 260 and 280 nm (A_{260}/A_{280}).

The integrity of DNA extracts was also evaluated by electrophoresis in 1% agarose gel stained with 1 × Gel Red (Biotium, CA, USA) and ran in 1 × SGTB buffer (GRISP, Porto, Portugal) for 20–25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Oligonucleotide primers

In silico analysis of the *Alk* and *Wx* genes located on chromosome 6 of *Oryza sativa* was performed. For HRM analysis, new primers were designed to amplify a 178-bp fragment targeting the nucleotide polymorphisms A/G and GC/TT in the exon 8 of the *Alk* gene (Table 1). For sequencing analysis of the *Alk* gene, a set of primers available from the literature were used to amplify a fragment of 922 bp (Table 1, Kadaru et al., 2006). For the *Wx* gene, the polymorphisms of the (CT)n microsatellite and the G/T SNP in the first intron were targeted by two newly designed sets of primers to amplify fragments of 183 bp and 341 bp for HRM and sequencing analysis, respectively (Table 1). To assess the amplification capacity of the DNA extracts, universal eukaryotic primers targeting the conserved nuclear 18S rRNA gene (Table 1) were used. The absence of hairpins and self-hybridization was assessed using the software OligoCalc (http://www.basic.northwestern.edu./biotools/oligocalc.html). The specificity of the oligonucleotides primers was

Table 1
Oligonucleotide primers targeting *Alk* and *Waxy* genes of rice and a universal eukaryotic gene.

Target gene	Primer	Sequence (5′→ 3′)	Amplicon size (bp)	Reference/NCBI accession No.
***Alk* (Exon 8)**	Alk-F	TCGGCGGGCTGAGGGACAC	178	AP003509.3
	Alk-R1	TCCTGCGACATGCCGCGCA		
	Alk-F1	GTGGGGTTCTCGGTGAAGAT	922	Kadaru et al. (2006)
	Alk-Rn	AAGCAAGAGGCAAACAGCTC		
***Waxy* (5′UTR and Intron 1)**	M6I1–F	TCTATCTCAAGACACAAATAACTG	183	AP014962.1
	M6I1-R	GAAAGATGCATGTGATCGATCTG		
	M6I1–F2	CGTGCCCCGCATGTCATC	341	AP014962.1
	M6I1-R	GAAAGATGCATGTGATCGATCTG		
18S rRNA	EG-F	TCGATGGTAGGATAGTGGCCTACT	109	Villa et al. (2017)
	EG-R	TGCTGCCTTCCTTGGATGTGGT		

assessed using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), allowing to reveal homologies between all sequences available in the GenBank database. The primers were synthesised by STABVIDA (Lisbon, Portugal).

2.5. Qualitative PCR

PCR assays were carried out in 25 μL of total reaction volume, containing 20 ng of DNA extract, buffer (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 3 mM of $MgCl_2$, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany) and 200 nM of each primer targeting the *Alk* and *Wx* genes (Table 1). All reactions were performed with 200 μM dNTP (GRISP, Porto, Portugal), except for Alk-F1/Alk-Rn primers, for which 400 μM were used. The reactions were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The temperature programs for the primers targeting the *Alk* and *Wx* genes were as follows: initial denaturation at 95 °C for 5 min; 40 cycles of amplification at 95 °C for 30 s, 58 °C, 62 °C or 66 °C (for primers M6I1–F/M6I1-R, M6I1–F2/M6I1-R or Alk-F/Alk-R1, respectively) for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min. For primers Alk-F1/Alk-Rn the temperature program was: initial denaturation at 95 °C for 5 min; 40 cycles of amplification at 95 °C for 45 s, 60 °C for 45 s and 72 °C for 1.5 min; and a final extension at 72 °C for 10 min.

The amplified fragments were further analysed by electrophoresis in a 1.5% agarose gel containing 1 × Gel Red (Biotium, Hayward, CA, USA) for staining and carried out in 1 × SGTB buffer (GRISP, Porto, Portugal) for about 20–25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each extract was amplified at least in two independent assays.

2.6. Real-time PCR and HRM analysis

The real-time PCR assays were carried out in 20 μL of total reaction volume, containing 2 μL of DNA (20 ng), 1 × of SsoFast® Evagreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 320 nM of each primer (Alk-F/Alk-R1 or M6I1–F/M6I1-R) (Table 1). The assays were performed in a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), using the following temperature conditions: 95 °C for 5 min; 50 cycles at 95 °C for 20 s, 60 °C (for primers M6I1–F/M6I1-R) or 66 °C (for primers Alk-F/Alk-R1) for 50 s, with collection of fluorescence signal at the end of each cycle. Data were processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA).

For HRM analysis, PCR products were denatured at 95 °C for 1 min and then annealed at 65 °C and 70 °C, with primers M6I1–F/M6I1-R and Alk-F/Alk-R1, respectively, for 3 min, in order to promote the correct formation of the DNA duplexes. These steps were followed by the melting curves from 65 °C (M6I1–F/M6I1-R) or 70 °C (Alk-F/Alk-R1) up to 95 °C, with temperature increments of 0.2 °C every 10 s. Fluorescence data were acquired at the end of each melting phase and processed using the Precision Melt Analysis, Software 1.2 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves, as a function of temperature, and respective difference curves for easy visual identification of clusters. Melting curve shape sensitivity determines the stringency used to classify into different clusters, and melting temperature (Tm) difference threshold determines the lowest Tm difference between samples. Cluster detection parameters were set to high sensitivity and threshold yields, providing more heterozygote clusters. Therefore, melting curve shape sensitivity parameter was adjusted to percentage value > 50% and Tm difference threshold was set to 0.19 ± 0.02.

2.7. Sequencing of PCR products

The fragments obtained with the primers M6I1–F2/M6I1-R and Alk-F1/Alk-Rn (Table 1) were purified using the GRS PCR & Gel Band Purification Kit (GRISP, Porto, Portugal) to remove any possible interfering components. Then, the purified products were sent to a specialised facility (Eurofins Genomics, Ebersberg, Germany) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions to allow the production of two complementary sequences of high quality. Sequencing data were aligned using the available software BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and the electropherograms were analysed with FinchTV (Geospiza, Seattle, WA, USA). The obtained sequences were deposited in the GenBank database.

3. Results and discussion

3.1. DNA quality and primer specificity

In general, the quality assessment of DNA extracts showed adequate yields (12.7–560.9 ng/μL) and purities ($A_{260}/A_{280} = 2.19 \pm 0.06$). Prior to the specific amplification of the selected sequences of *Alk* and *Wx* genes, DNA extracts were tested with universal eukaryotic primers targeting the 18S rRNA gene, as described by Villa et al. (2017), confirming their amplification capacity (Table S3, Supplementary material). For the specific amplification of the selected regions, two sets of primers were used for each gene, one for HRM analysis and a second for sequencing, encompassing the target fragment (Table 1). The primers used in HRM analysis were assayed for cross-reactivity using several plant and animal species commonly used as food ingredients, confirming the absence of any unspecific amplification (Table S3, Supplementary material).

3.2. Alk gene

3.2.1. HRM analysis

A real-time PCR assay using EvaGreen dye was successfully developed and further combined with HRM analysis targeting the *Alk* gene to amplify a 178-bp fragment of rice (Fig. 1). This approach intends to take advantage of HRM analysis as a high-throughput tool capable of discriminating fragments with small nucleotide differences, such as the

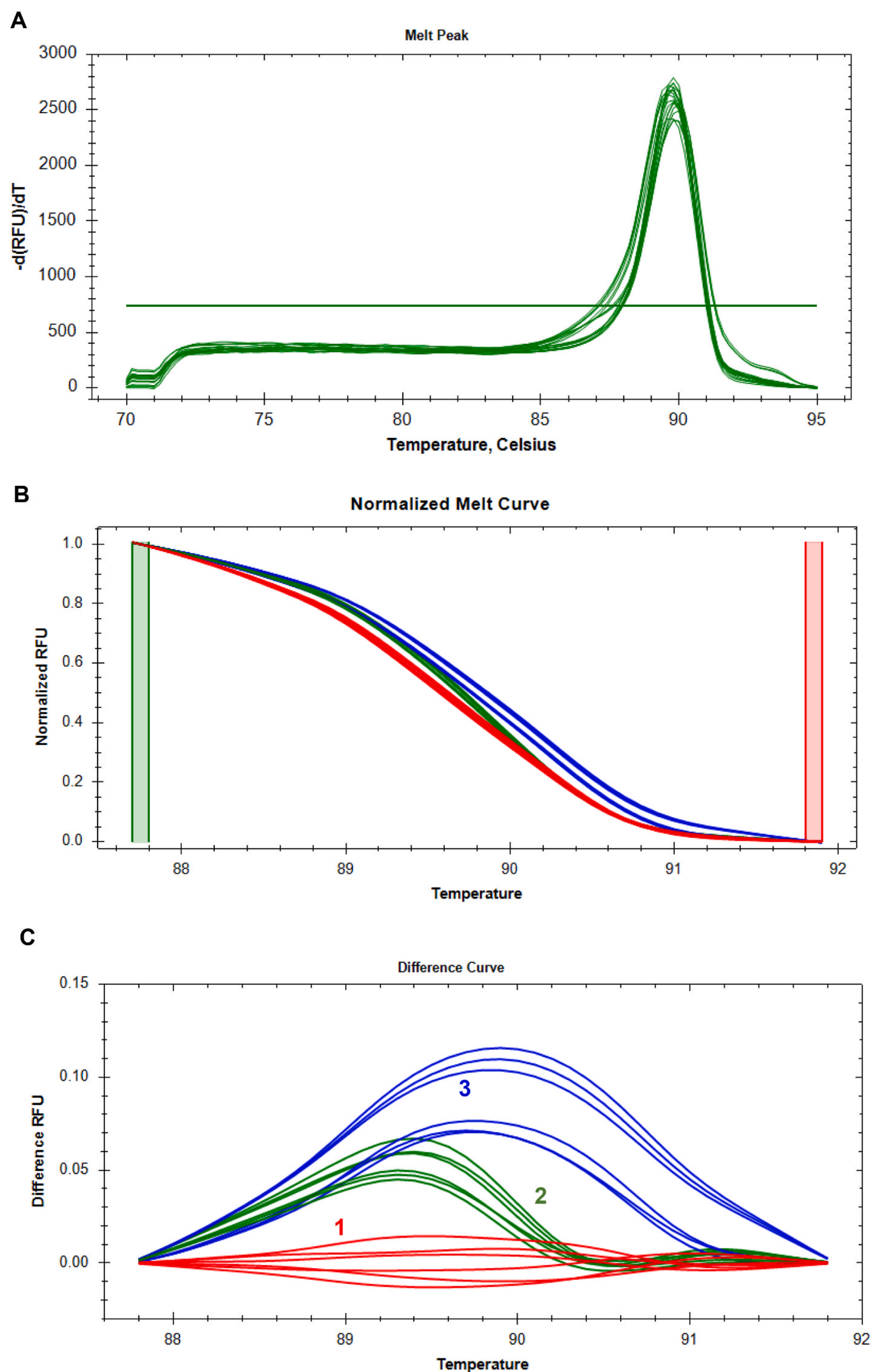

Fig. 1. Conventional melting (A), normalised melting (B) and temperature shift difference (C) curves obtained by real-time PCR with EvaGreen dye and HRM analysis, targeting the *Alk* gene with primers Alk-F/Alk-R1, applied to different rice varieties. Legend: Cluster 1 (red lines), Carnaroli; Cluster 2 (green lines), Carnise and Sole Cl; Cluster 3 (blue lines), Gladio and Ronaldo.

nucleotide polymorphisms A/G and GC/TT in the exon 8 of the selected amplicon. Fig. 1A presents the conventional melting curve analysis of selected rice varieties representative of all subgroups (Table S1, supplementary material), including Carnaroli and Carnise, as example varieties under the commercial name of Carnaroli, and others, namely Sole Cl, Gladio and Ronaldo. Data provide close melt peaks around 88.8 °C, suggesting very similar amplicons for all tested varieties. The application of HRM analysis discriminated the selected varieties in 3 clusters, namely Carnaroli (cluster 1), Carnise and Sole Cl (cluster 2), and Gladio and Ronaldo (cluster 3), which can be noted in the normalised (Fig. 1B) and difference (Fig. 1C) curves. These results showed that the method enabled differentiating Carnaroli from all the other tested Italian rice varieties with high level of confidence (>98%). However, Carnaroli was also distinguished from Carnise, a variety from the same subgroup also sold as Carnaroli, which is not an intended result. Therefore, the polymorphisms of the *Wx* gene were further investigated.

3.2.2. Sequencing

To further correlate HRM data with the varietal polymorphisms and confirm the obtained results, fragments of the *Alk* gene (922 bp) using the primers Alk-F1/Alk-Rn were sequenced in opposite directions, providing electropherograms with high resolution. The sequences were deposited in the GenBank database with the accession numbers OK334524-OK334536. The alignment results of selected rice varieties clearly highlight (Fig. 3) the polymorphisms on the exon 8 of *Alk* gene, namely A/G and GC/TT located at positions 145 and 246–247, respectively, which are summarised in Table 2. As it can be inferred, Carnise and Sole Cl have both polymorphisms, which justifies their clustering in a distinct group from Carnaroli. The GC/TT polymorphism identified in Gladio and Ronaldo corroborates their differentiation in cluster 3.

The gelatinization temperature is a key parameter for rice cooking quality that is mainly regulated by the *Alk* gene, encoding SSIIa, which is involved in the synthesis of amylopectin. Therefore, the nucleotide diversity of *Alk* gene and its correlation with rice traits has been the subject of several studies. Particularly, the nucleotide polymorphisms of G/A and GC/TT on exon 8 have been associated with gelatinization temperature of rice. Rice varieties with high starch gelatinization temperature have the G/GC haplotype, while those with low values have the A/GC or G/TT haplotypes (Waters et al., 2006; Gao et al., 2011; Zhou et al., 2016). In the present study, three haplotypes were identified in the Italian tested varieties, namely G/TT, A/GC and G/GC (Table 2). These findings suggest that the tested varieties have mostly low gelatinization temperature, which is in good agreement with results of Caffagni et al.

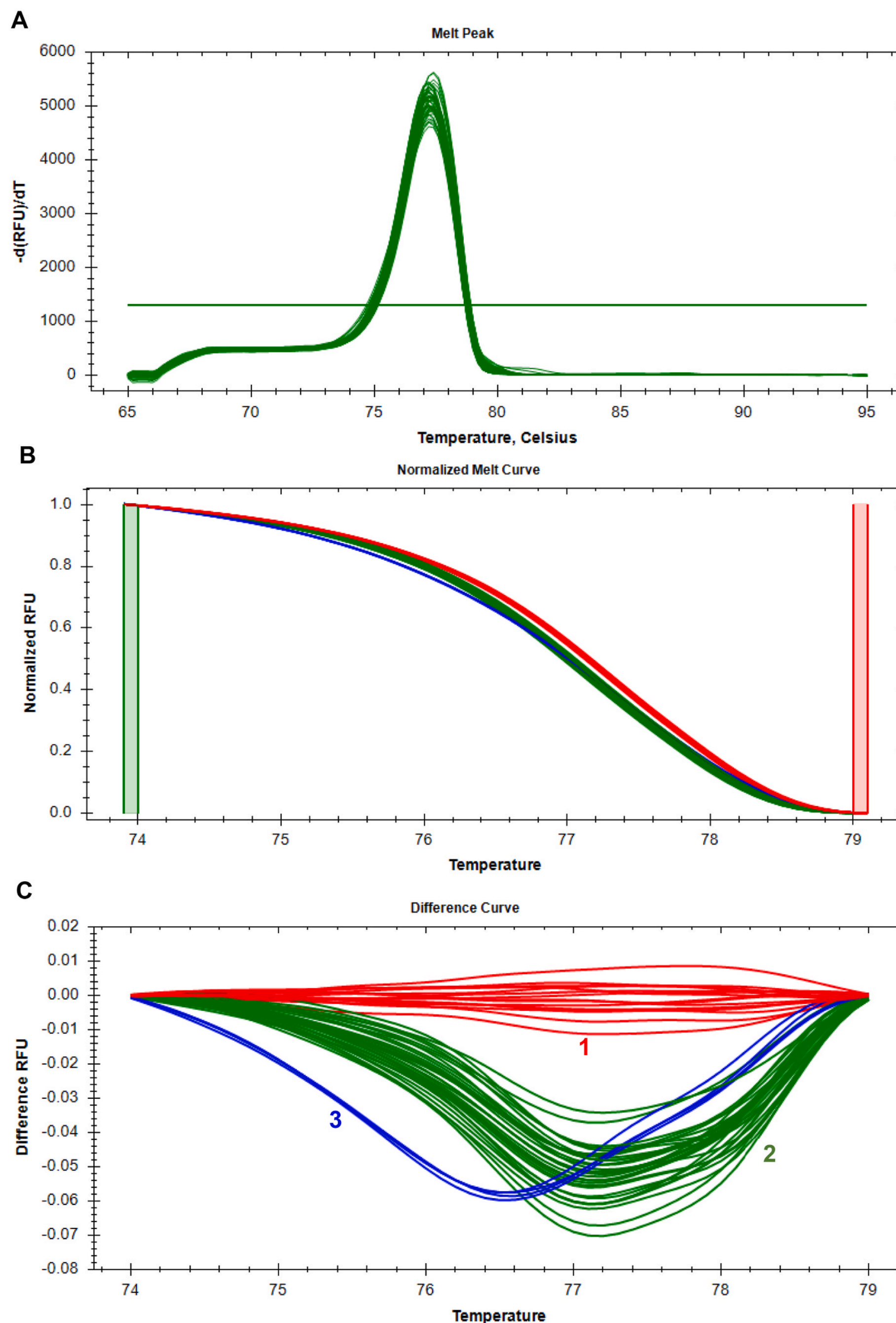

Fig. 2. Conventional melting (A), normalised melting (B) and temperature shift difference (C) curves obtained by real-time PCR with EvaGreen dye and HRM analysis, targeting the *Waxy* gene with primers M6I1–F/M6I1-R, applied to different rice varieties. Legend: Cluster 1 (red lines), Carnaroli, Carnise and Karnak; Cluster 2 (green lines), S. Andrea, Volano, Ronaldo, Gloria, Sole Cl, Roma, Baldo and Generale; Cluster 3 (blue lines), Gladio.

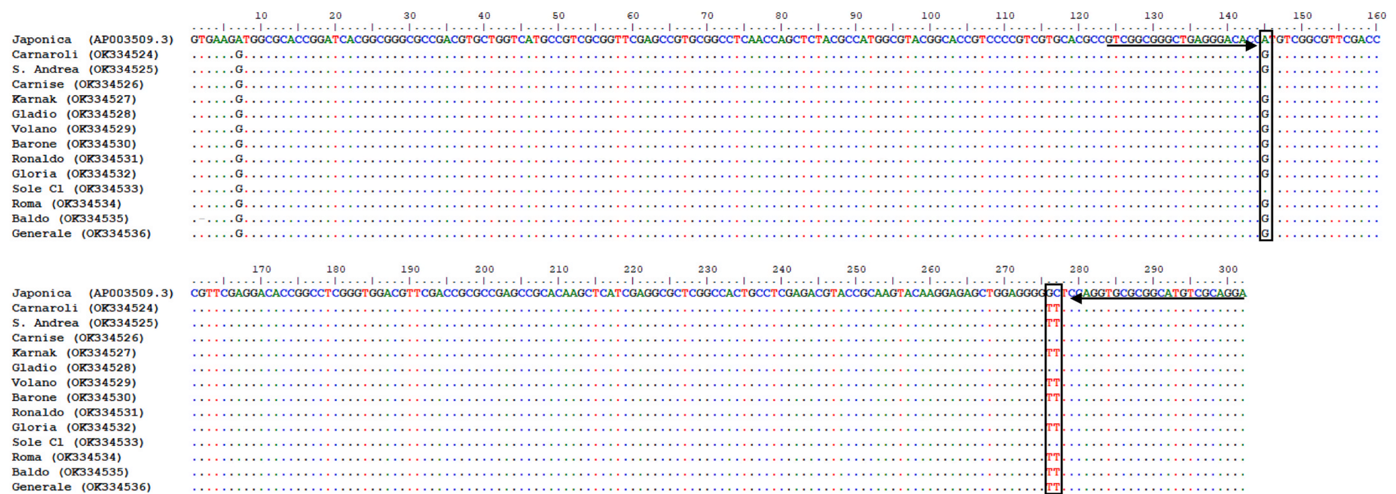

Fig. 3. Alignment of sequencing products of *Alk* gene of different *Oriza sativa* varieties. The arrows identify the region of primer annealing (Alk-F/Alk-R1), while the boxes identify the nucleotide polymorphisms (A/G and GC/TT at positions 145 and 246–247, respectively). The GenBank accession numbers are in brackets, being the obtained sequences deposited as OK334524-OK334536, while AP003509.3 corresponds to *Oryza sativa* Japonica Group genomic DNA, chromosome 6, PAC clone: P0525F01.

Table 2
Resumed sequencing results of identified polymorphisms in the *Alk* and *Waxy* genes of selected rice varieties.

Rice variety	*Alk* gene		*Waxy* gene	
	Exon 8 SNP (A/G)	Exon 8 polymorphism (GC/TT)	Microsatellite (CT)n	Intron 1 SNP (G/T)
Carnaroli	G	TT	17	G
Carnise	A	GC	17	G
Karnak	G	TT	17	G
Roma	G	TT	18	T
Baldo	G	TT	18	T
Generale	G	TT	18	T
S. Andrea	G	TT	18	T
Volano	G	TT	18	T
Barone	G	TT	17	T
Ronaldo	G	GC	18	T
Gloria	G	TT	18	T
Sole Cl	A	GC	18	T
Gladio	G	GC	20	G

(2013), who verified that Italian *japonica* genotypes most frequently carry either G/TT or A/GC haplotypes. According to Zhou et al. (2016), after testing several wild accessions of rice, the G/GC haplotype was considered wild-type, while both the G/TT and A/GC haplotypes were mutant. Therefore, most of the cultivated Italian tested varieties diverged from wild-type and arise most likely from mutants as a result of the selection of rice traits for cooking properties during domestication (Zhou et al., 2016).

3.3. Waxy gene

3.3.1. HRM analysis

To further improve the discrimination of Carnaroli rice, a new EvaGreen real-time PCR assay coupled to HRM analysis was developed targeting the *Wx* gene to amplify fragments of 183 bp, exploiting the nucleotide differences associated with the polymorphisms of the (CT)n microsatellite and the G/T SNP in the first intron. As expected, the conventional melting curve profiles of representative subgroup varieties of rice were very similar, exhibiting melt peaks around 77.2 °C (Fig. 2A). The subsequent application of HRM analysis, as displayed in the normalised (Fig. 2B) and difference (Fig. 2C) curves, grouped the varieties in 3 clusters: Carnaroli, Carnise and Karnak (cluster 1); S. Andrea,

Table 3
Results of HRM analysis targeting the *Waxy* gene of rice varieties.

Code	Variety	Subgroup	Cluster	Level of confidence (%, mean ± SD)
1	Carnaroli	Carnaroli	1	99.8 ± 0.2
2	Carnaroli	Carnaroli	1	99.9 ± 0.1
3	Carnaroli	Carnaroli	1	99.5 ± 0.6
4	Carnaroli	Carnaroli	1	99.9 ± 0.1
5	Carnaroli	Carnaroli	1	99.4 ± 0.6
6	Carnaroli	Carnaroli	1	100.0 ± 0.0
7	Carnaroli	Carnaroli	1	100.0 ± 0.0
8	Carnaroli	Carnaroli	1	99.9 ± 0.1
9	Carnaroli	Carnaroli	1	99.2 ± 0.8
10	S. Andrea	S. Andrea	2	99.2 ± 0.6
11	S. Andrea	S. Andrea	2	99.2 ± 0.2
12	Carnise	Carnaroli	1	99.7 ± 0.3
13	Karnak	Carnaroli	1	99.9 ± 0.1
14	Gladio	Thaibonnet	3	99.8 ± 0.3
15	Volano	Arborio	2	99.1 ± 0.6
16	Barone	Roma-Baldo	2	99.2 ± 0.6
17	Ronaldo	Ribe	2	99.2 ± 0.3
18	Gloria	Gloria	2	99.8 ± 0.1
19	Sole CL	Originário	2	99.7 ± 0.1
20	Carnaval	Carnaroli	1	99.4 ± 0.1
21	Caravaggio	Carnaroli	1	98.2 ± 0.6
22	Keope	Carnaroli	1	98.9 ± 0.2
23	Poseidone	Carnaroli	1	98.1 ± 0.3
24	L202	Carnaroli	1	99.7 ± 0.1
25	L252	Carnaroli	1	98.3 ± 0.4
26	Karnak	Carnaroli	1	98.7 ± 0.6
27	Roma	Roma-Baldo	2	99.1 ± 0.8
28	Baldo	Roma-Baldo	2	99.4 ± 0.6
29	Cameo	Roma-Baldo	2	99.0 ± 0.2
30	Galileo	Roma-Baldo	2	99.7 ± 0.1
31	Casanova	Roma-Baldo	2	91.4 ± 0.5
32	Fedra	Roma-Baldo	2	99.7 ± 0.1
33	Proteo	Roma-Baldo	2	99.6 ± 0.1
34	Telemaco	Arborio	2	99.1 ± 0.8
35	Generale	Arborio	2	99.5 ± 0.4

Volano, Ronaldo, Gloria, Sole Cl, Roma, Baldo and Generale (cluster 2); Gladio (Thaibonnet) (cluster 3). Table 3 presents the resumed results of HRM analysis using all the producer varieties, which shows that those belonging to the subgroup Carnaroli were discriminated from all the others with high level of confidence (>98%). The other varieties were grouped in cluster 2, also with generally high level of confidence (>99%), except Gladio that formed cluster 3 (99.8%). Therefore, the HRM method targeting the *Wx* gene allowed the successful discrimination of Carnaroli rice from all the other tested varieties, thus being a potential tool for its authentication.

3.3.2. Sequencing

Sequencing data regarding the *Wx* gene amplicons used in HRM analysis was obtained using the M6I1–F2/M6I1-R to amplify 341-bp fragments in opposite directions. Fig. 4 presents the sequence alignments of selected rice varieties with the highlighted polymorphisms of the (CT)n microsatellite between the positions 186–226 and the G/T SNP in the first intron located at the position 282. The sequences with sizes above 200 bp were deposited in the GenBank database with the accession numbers of OK3345217-OK334523, while others did not amply with M6I1–F2/M6I1-R primers, being their sequencing data obtained with M6I1–F/M6I1-R primers (Fig. 4). Table 2 summarises the obtained polymorphisms for the analysed rice varieties. Data show that Carnaroli, together with Carnise and Karnak, also sold as Carnaroli, possess 17 sequence repeats of CT and G as the SNP, which distinguishes them from all the other varieties and justifies their inclusion in cluster 1. All the other varieties, except Barone and Gladio, have a $(CT)_{18}$ microsatellite and T as the SNP, explaining their grouping in cluster 2. Barone was also grouped in cluster 2 despite holding a $(CT)_{17}$ microsatellite, which in fact corresponds to a single nucleotide difference (position 191, Fig. 4), comparing with the other varieties of cluster 2, not being sufficient to form an independent cluster. Finally, the longest microsatellite – $(CT)_{20}$ – and G as the SNP identified in Gladio variety justify its discrimination from all the others in cluster 3.

The *Wx* gene, located on chromosome 6, is a key gene that regulates amylose synthesis. Polymorphisms of the $(CT)_n$ microsatellite and G/T SNP in the *Wx* gene and their correlation with amylose content have been explored in several rice genotypes (Biselli et al., 2014; Caffagni et al., 2013; Jayamani et al., 2007). The haplotype CT_{18}/T has been the most frequently identified in numerous analysed rice genotypes from a wide range of origins, including Italy (Biselli et al., 2014; Caffagni et al., 2013; Jayamani et al., 2007), which corroborates the present results (Table 2). Besides, the identified $(CT)_n$-G/T polymorphisms were in good agreement with data obtained by Biselli et al. (2014) concerning the varieties of Carnaroli (Karnak, L202), Arborio (Volano), S. Andrea, Thaibonnet (Gladio), Ronaldo (Ribe), while data for other varieties (Gloria, Sole Cl, Barone, Volano, Generale, Carnise and Roma) were, as far as we know, herein presented for the first time.

Fig. 4. Alignment of sequencing products of *Waxy* gene of different *Oriza sativa* varieties. The arrows identify the region of primer annealing M2I1-f2/M6I1-R, while the boxes identify the nucleotide polymorphisms ((CT)n at positions 187–226 and T/G at position 282). The GenBank accession numbers are in brackets, being the obtained sequences deposited as OK3345217-OK334523, while AP014962.1 corresponds to *Oryza sativa* Japonica Group DNA, chromosome 6, cultivar: Nipponbare, complete sequence.

3.4. Application of HRM method targeting the Wx gene to authenticate commercial rice samples

Table 4 presents the summarised results of the application of the HRM method targeting the *Wx* gene to commercial rice samples. As it can be verified, from the five samples labelled as Carnaroli (C9–C14), four were grouped accordingly in cluster 1 (Carnaroli) with high levels of confidence (>97%), while one (C12) joined cluster 2, suggesting its incompliance with the labelled variety. Samples C6 and C8, labelled as Vialone Nano and Padano, were also grouped in cluster 1. Sequencing results confirmed that they have the same targeted polymorphisms as Carnaroli (CT_{17}/G) (data not shown). Regarding Vialone Nano, this finding should be due to its genetic proximity to Carnaroli since they share a parental line: Vialone Nano was created by cross breeding Nano × Vialone; and Carnaroli was formed from crossing Vialone × Lencino (Spada et al., 2004). However, this outcome should not interfere with the applicability of the method since both Vialone Nano and Padano are morphologically very distinct from Carnaroli, being classified as medium and semifino grains (Faivre-Rampant et al., 2011; Gazzetta Ufficiale Della Repubblica Italiana, 2017), thus unlikely to be used to adulterate Carnaroli – classified as Long A and superfine rice. As expected, samples of the other variety subgroups (Arborio, Roma, Ribe, Originario and Rosa Marchetti) were joined in cluster 2. Sample C3, labelled as Thaibonnet, was expected to be part of cluster 3, but it formed an independent cluster (4) with low and unreproducible level of confidence (87.9 ± 5.9). This finding suggests that this sample might contain a mixture of rice varieties possessing different polymorphisms, which disables its accurate clustering. In fact, this is a limitation of HRM analysis that, similarly to DNA barcoding, its application to mixtures might conduct to unreliable results (Grazina et al., 2020).

The molecular characterization of some Italian rice varieties has been attempted by several authors (Brandolini et al., 2006; Caffagni et al., 2013; Cirillo et al., 2009; Mantegazza et al., 2008). For this purpose, random amplified polymorphisms (RAPD), amplified fragment length polymorphism (AFLP), cleaved amplified polymorphic sequences (CAPS), SSR or microsatellites and SNP have been explored as molecular markers. RAPD profiles showed that Italian rice varieties are closely related (Ribe, Roma, S. Andrea, Selenio, Thaibonnet, Originario, Saturno, Baldo, Vialone Nano, Carnaroli, Arborio), but more genetically distant from Egyptian and Indian (Amber and Basmati, respectively), enabling to discriminate all tested varieties (Cirillo et al., 2009). SSR and SNP have been the most used markers, particularly associating their variability with cooking properties (Biselli et al., 2014; Caffagni et al., 2013; Gao et al., 2011; Jayamani et al., 2007; Zhou et al., 2016). However, their application to authenticate rice varieties is rather limited. Ganopoulos et al. (2011) have successfully a combined a SSR marker targeting the 8-bp deletion in the exon 7 of *badh2* gene of rice with HRM analysis to authenticate Basmati rice and differentiate it from non-Basmati rice varieties. In the present study, both SNP and SSR markers were combined for the first time with HRM analysis to differentiate a premium rice variety, Carnaroli, from other closely related Italian varieties.

4. Conclusions

In the present work, two methods based on HRM analysis targeting nucleotide polymorphisms of the *Alk* and *Wx* genes were successfully developed to differentiate Carnaroli rice among other Italian rice varieties. The HRM method targeting the A/G and GC/TT polymorphisms in the exon 8 of *Alk* gene allowed differentiating Carnaroli from other tested subgroup varieties, but it also discriminated it from Carnise that belongs to the same subgroup Carnaroli. The second HRM approach, targeting the (CT)n microsatellite and the G/T SNP in the first intron of *Wx* gene, successfully discriminated the genotypes of the subgroup Carnaroli from all the other tested varieties with high level of confidence (>98%). The varieties of the Carnaroli subgroup formed cluster 1, while the others were gathered in cluster 2 (S. Andrea, Arborio, Roma-Baldo, Ribe, Gloria and Originario) and cluster 3 (Thaibonnet). The clustering results corroborated sequencing data, which relied on the identified polymorphisms. The applicability of the HMR method targeting the *Wx* gene was successful using 14 commercial rice samples, from which one out of 5 samples labelled as Carnaroli suggests labelling incompliance/adulteration. Other eight samples were clustered according to their labelled variety, while one suggests mixed varieties instead of the labelled variety (Thaibonnet), being a possible fraudulent admixture.

Hence, the proposed new HRM method can be considered a simple, specific, high-throughput and cost-effective tool for the authentication of Carnaroli rice among other Italian rice varieties, being potentially useful for control laboratories of such products, contributing to valorise such premium variety.

Table 4
Results of HRM analysis targeting the *Waxy* gene of commercial rice samples.

Code	Label	Cluster[a]	Level of confidence (%, mean ± SD)
C1	Arborio	2	99.6 ± 0.5
C2	Roma	2	99.8 ± 0.1
C3	Thaibonnet	4	87.9 ± 5.9
C4	Ribe	2	99.8 ± 0.1
C5	Originario	2	99.9 ± 0.0
C6	Vialone Nano	1	99.3 ± 0.1
C7	Rosa Marchetti	2	99.8 ± 0.1
C8	Padano	1	99.7 ± 0.1
C9	Carnaroli	1	97.2 ± 0.9
C10	Carnaroli	1	98.5 ± 0.4
C11	Carnaroli	1	97.4 ± 0.6
C12	Carnaroli	2	99.0 ± 0.3
C13	Carnaroli	1	98.4 ± 0.2
C14	Arborio	2	94.9 ± 1.3

[a] Cluster 1 – subgroup Carnaroli, cluster 2 – subgroups S. Andrea, Arborio, Roma-Baldo, Ribe, Gloria and Originário; cluster 3 – subgroup Thaibonnet; cluster 4 – undefined.

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CRediT authorship contribution statement

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

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.108829.

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