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High Resolution Melting (HRM) applied to wine authenticity

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ABSTRACT

Wine authenticity methods are in increasing demand mainly in Denomination of Origin designations. The DNA-based methodologies are a reliable means of tracking food/wine varietal composition. The main aim of this work was the study of High Resolution Melting (HRM) application as a screening method for must and wine authenticity. Three sample types (leaf, must and wine) were used to validate the three developed HRM assays (*Vv1*–705 bp; *Vv2*–375 bp; and *Vv3*–119 bp). The *Vv1* HRM assay was only successful when applied to leaf and must samples. The *Vv2* HRM assay successfully amplified all sample types, allowing genotype discrimination based on melting temperature values. The smallest amplicon, *Vv3*, produced a coincident melting curve shape in all sample types (leaf and wine) with corresponding genotypes. This study presents sensitive, rapid and efficient HRM assays applied for the first time to wine samples suitable for wine authenticity purposes.

1. Introduction

It is estimated that about 10,000 different *Vitis vinifera* L. varieties exist worldwide (Gallet, 2000). However, only a few are used in wine production. When considering high valuable wines and the wines belonging to Denomination of Origin (DO), the number of varieties with interest is even lower.

Portugal is characterized by having a huge *V. vinifera* germplasm collection, with hundreds of varieties (Cunha, Teixeira-Santos, Brazão, Fevereiro, & Eiras-Dias, 2013). In 1756, the regulation defining the Port Wine's region was established, being the first Demarcated region in the World (*Região Demarcada do Douro*). In 2001, the Douro region was classified by UNESCO as a world heritage site. In this region premier wines are produced mainly due to the grapevine varieties used and its terroir. In the Douro region there are old vineyards with a wide number of grapevine varieties, which are contrasting with the modern vineyards that have defined grapevine varieties used to produce monovarietal wines with high commercial value.

Governmental bodies have an obligation to control, promote and defend the DO appellations. In monovarietal wines the occasional addition of other grapevine varieties, above the percentage permitted by law, can occur and are considered illegal, unless stated, under labelling legislation (European Union Regulation n° 607/2009). Thus, wine authenticity has become a subject of great concern since the incorrect labelling represents a commercial fraud. Therefore, the precise identification of the grapevine varietal composition is a key point to combat fraudulent practices and to assure commercial fairness.

Traditional methods used for must and wine grapevine varietal identification and authentication rely on protein and metabolites analysis and on the isotope ratios of certain bio-elements (Arbulu, Sampedro, Gómez-Caballero, Goicolea, & Barrio, 2015; Camin et al., 2013; Sen & Tokatli, 2014; Versari, Laurie, Ricci, Laghi, & Parpinello, 2014). However, these analytical techniques are influenced by winemaking processes, environment and storage conditions (Arbulu et al., 2015), therefore leading to inconsistencies related to the accurate and reliable identification of grapevine varieties (Fang et al., 2008). DNA based methods are considered to be more reliable based on the fact that DNA is a stable molecule. Furthermore, DNA has been applied to several food matrices with remarkable success considering authenticity purposes (Faria, Magalhães, Nunes, & Oliveira, 2013; Madesis, Ganopoulos,

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Sakaridis, Argiriou, & Tsaftaris, 2014; Martins-Lopes, Gomes, Pereira, & Guedes-Pinto, 2013).

Grapevine varietal identification is easily guaranteed with the use of nuclear molecular markers, namely, Simple Sequence Repeat (SSR) approved and supported by the International Organization of Vine and Wine (OIV; OIV, 2007). Although SSR markers have been used for food authenticity purposes several problems have arisen related to the DNA quality, a result of the extraction procedures (reviewed by Pereira, Gomes, & Martins-Lopes, 2016). When considering must/wine matrices the presence of large quantities of polyphenols, polysaccharides and proteins sometimes inhibit PCR reactions (Işçi, Yildirim, & Altindisli, 2014). Another drawback in the application of SSR markers, in such sample type, is related with DNA degradation, result of alcoholic fermentation process. However, several DNA extraction protocols have been improved and have managed to increase both the yield and the quality of the extracted DNA (*e.g.*, Baleiras-Couto & Eiras-Dias, 2006; Bigliazzi, Scali, Paolucci, Cresti, & Vignani, 2012; Boccacci, Akkak, Marinoni, Gerbi, & Schneider, 2012; Nakamura, Haraguchi, Mitani, & Ohtsubo, 2007; Pereira, Guedes-Pinto, & Martins-Lopes, 2011; Savazzini & Martinelli, 2006). The use of small molecular markers, such as, Single Nucleotide Polymorphisms (SNPs) could be a way to overcome the natural DNA degradation found in such samples.

SNPs are considered the newest type of molecular markers that offer several advantages since they are abundant in the genome, genetically stable and can be used to overcome the degradation limitations allowing DNA amplification and the use of more sensitive techniques (Cabezas et al., 2011). High Resolution Melting (HRM) analysis has been widely used for mutation detection and genotyping. Due to its nature, it can be considered as an alternative approach for food authenticity purposes (Ganopoulos, Argiriou, & Tsaftaris, 2011a; Ganopoulos, Argiriou, & Tsaftaris, 2011b; Ganopoulos, Bazakos, Madesis, Kalaitzis, & Tsaftaris, 2013a; Ganopoulos, Sakaridis, Argiriou, Madesis, & Tsaftaris, 2013b; Madesis et al., 2014; Wittwer, 2009).

HRM analysis is a sensitive, stable, and reliable screening method that allows the rapid analysis of specific amplicons, characteristic of a particular genotype, previously amplified by PCR. In *Vitis*, HRM was applied in grapevine variety identification using various microsatellites (Mackay, Wright, & Bonfiglioli, 2008). Recently, HRM has been applied by our group to the identification of grapevine varieties based on the SNPs changes detected within genes belonging to the anthocyanins pathway (Castro et al., submitted; Pereira & Martins-Lopes, 2015).

The aim of this study was to evaluate the capacity of HRM to access varietal identification in must and wine samples, in order to establish a future alternative authenticity procedure.

2. Material and methods

2.1. Leaf, must and wine samples

Thirteen *V. vinifera* varieties were selected based on their importance to the Portuguese wine sector, in particular to the Douro region. The sampling comprised national and international varieties (Table 1). Young leaf samples from each grapevine variety were harvested from certified vineyards (Sogrape Vinhos S.A. and Real Companhia Velha) and immediately frozen in liquid nitrogen until DNA extraction. Grape samples were harvested from the certified vineyards in two consecutive production years, 2012 and 2013. Monovarietal must and wine samples were produced at the National Institute for Agricultural and Veterinary Research (INIAV) in Dois Portos, Portugal, using freshly harvested grapes. All must samples were collected immediately after wine maceration and immediately frozen at −20 °C. The wines were vinified using two procedures according to grape color.

Table 1
List of 13 grapevine varieties used, corresponding code and berry color.

Grapevine variety name	Code	Berry color
Alicante Bouschet	AB	Red
Cabernet Sauvignon	CS	Red
Donzelinho Tinto	DT	Red
Merlot	M	Red
Malvasia Fina	MF	White
Pinot Noir	PN	Red
Rufete	Ruf	Red
Tinto Cão	TC	Red
Touriga Franca	TF	Red
Tinta Francisca	TFi	Red
Touriga Nacional	TN	Red
Tinta Roriz	TR	Red
Viosinho	Vio	White

2.1.1. Vinification of white grape varieties

White varieties grapes were weighed, crushed, destemmed and pressed separately. Immediately, 80 mg/L of sulfur dioxide (SO_2) was added to each must and then placed in a cold room at 4 °C for about 48 h for cold settling. After cold setting, the musts were transferred to two glass containers, in two equal parts for each variety, and the alcoholic fermentation was conducted by adding an active dry yeast (QA23) in the ratio of 30 g/hL of must and 1 g of diammonium phosphate per 10 L of must. The must fermentation took place in a controlled temperature chamber (16–18 °C). At the end of alcoholic fermentation (reducing sugars <3 g/L) the wines were racked and 40 mg/L of the SO_2 was added. Approximately, 2 months after fermentation (December), the wines were transferred and the free SO_2 levels were corrected up to 20 mg/L. In January, all wines were sampled for further laboratory analysis, after which the free SO_2 was fixed up to 40 mg/L. Finally, the wines were bottled in 0.375 L glass bottles.

2.1.2. Vinification of red grape varieties

The red grape varieties were weighed, crushed and destemmed separately. Shortly after, 80 mg/L of SO_2 and an active dry yeast (D254) was added in the ratio of 20 g/hL of must. The musts were fermented in a controlled temperature chamber (24–26 °C). During this phase, the punch down of wine grapes was made twice a day. When the must density was lower than 1.0 g/cm^3, wine grapes pressing was made. The must/wine resulting was transferred into glass bottles to almost full capacity. When the fermentation ended, the operations performed were the same as described in white wines. Wine sampling was performed one year after bottling. When the bottle was opened, samples were taken and immediately frozen at −20 °C until the DNA extraction procedure was pursued.

2.2. Genomic DNA extraction

Total genomic DNA was extracted from frozen young leaf samples using the described CTAB method (Doyle & Doyle, 1987). Must DNA extractions were performed using a modified CTAB protocol (Pereira et al., 2012). Wine genomic DNA extractions were performed according to the method described by Pereira et al. (2011). The DNA samples were diluted in 100 μL of 0.1X TE buffer (Tris-HCl 100 mM, EDTA 0.1 mM pH = 8) to 10 ng/μL of working concentration. The determination of the samples' purity, integrity and quantity were based on measurements performed using a Nanodrop™ 1000 Spectrophotometer and by electrophoresis on a 0.8% agarose gel in 1X TAE buffer (Tris-acetate-EDTA).

2.3. High-Resolution Melting assay design

2.3.1. HRM primer design

The primer pairs tested for the HRM analysis are summarized in Table 2. The HRM primer pairs selected for the study were based on