UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Wine Authenticity – a genomic approach

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Genética Molecular Comparativa e Tecnológica

Maria Leonor Gonçalves Pereira

Orientador: Professor Doutor Henrique Guedes-Pinto

Co-Orientadora: Professora Doutora Paula Martins-Lopes

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Maria Leonor Gonçalves Pereira

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(Maria Leonor Gonçalves Pereira)

Wine Authenticity - a genomic approach

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RESUMO

A globalização do sector alimentar disponibiliza aos consumidores uma grande diversidade de produtos, aumentando a sua preocupação em relação à sua origem. As vantagens associadas às metodologias baseadas em DNA, fiabilidade e reprodutibilidade, têm potenciado a sua aplicação nesta temática. Na última década, as ferramentas bioinformáticas, surgiram como uma necessidade para analisar o crescente volume de dados obtidos em biologia molecular.

A vitivinicultura é uma actividade economicamente sustentável, com necessidade de mão-de-obra especializada e com relevância para a sociedade, não só em termos económicos, como também ambientais e sociais. O valor e qualidade dos vinhos devem-se, entre outros factores, às castas envolvidas na sua produção. As regiões vínicas com a Denominação de Origem (DO) contemplam apenas a utilização de um número restrito de castas. No entanto, é permitido por lei a introdução de outras castas, em percentagens legalmente definidas, constituindo uma oportunidade para práticas fraudulentas.

Com o presente estudo pretende-se desenvolver um sistema para autenticidade, de vinhos e mostos, eficiente no controle de prácticas fraudulentas e detecção de rotulagem incorrecta, visando a valorização dos vinhos DO. Numa primeira abordagem, DNA genómico extraído de *Vitis vinifera* L. (folhas e mostos monovarietais) de 10 castas portuguesas e uma referência (Cabernet Sauvignon) foi analisado utilizando marcadores moleculares microssatélites (SSR) e intermicrossatélites (ISSRs). Os perfis obtidos para as amostras de folhas e mostos, com marcadores ISSRs não foram coincidentes. Por outro lado, a utilização de seis marcadores SSRs (estabelecidos pela *International Organization of Vine and Wine* - OIV), revelou a presença de 42 alelos coincidentes, permitindo a discriminação das amostras de mosto baseado no perfil alélico da respectiva casta. Este estudo demonstrou a eficiência dos marcadores moleculares SSRs, com aplicação na autenticação varietal dos mostos.

Na autenticidade de vinhos, a extracção de DNA a partir de vinho é a principal limitação quando se pretende aplicar metodologias baseadas no DNA. Neste estudo foi desenvolvido um protocolo de extracção de DNA considerando as características específicas da matriz do vinho. O protocolo desenvolvido permitiu a extracção de grandes quantidades de DNA e de elevada qualidade. As concentrações de DNA variaram entre 260 e 465 ng/µL e as razões qualitativas, A_{260nm}/A_{280nm} e A_{260nm}/A_{230nm}, entre 1,71 - 1,81 e 1,66 - 1,98, respectivamente. O DNA obtido de amostras de vinho foi amplificado por PCR utilizando os marcadores SSRs, com total correspondência

entre o perfil do vinho monovarietal (tinto e branco) e a respectiva casta. O mesmo protocolo foi aplicado a vinhos de lotes comerciais e os resultados mostraram que o tamanho dos alelos obtidos era o esperado, considerando as castas referidas no rótulo. Assim, este método optimizado pode ser a base para a implementação de um sistema de autenticidade fidedigno.

Atualmente, os *Single Nucleotide Polymorphism* (SNPs) são os marcadores moleculares mais aplicados em plantas e referem-se à identificação de polimorfismos baseados na alteração de um único nucleótido. Devido às suas características inerentes apresentam um enorme potencial para fins de autenticidade. A técnica de *High Resolution Melting* (HRM) é presentemente utilizada para a deteção de SNPs e eventos de Inserções-Deleções (INDELs) no genoma de *Vitis*. Esta técnica baseia-se na variação da temperatura média de desnaturação do DNA na presença de um fluoróforo saturado que se intercala na cadeia dupla de DNA.

O perfil antociânico é específico de cada casta, sendo um factor potencialmente interessante para a identificação varietal e, portanto, para a detecção de SNPs. Com base neste pressuposto, foi realizado um estudo molecular com a finalidade de detetar SNPs em genes que codificam as enzimas da via biossintética das antocianinas (*i.e.*, Chalcona isomerase - CHI e UDP-glucose:flavonóide-3-O-glucosil-transferase - UFGT), a fim de diferenciar um conjunto específico de castas. A sequência do gene *CHI* apresentou apenas 5 SNPs. Por sua vez, o gene *UFGT* mostrou ser muito polimórfico, com um total de 58 SNPs e 1 INDEL para um grupo de 22 castas estudadas. Um ensaio de HRM foi estabelecido com base em um fragmento de 704 bp, pertencente ao gene *UFGT*, permitindo a discriminação de 18 haplótipos, tendo em conta a forma da curva de desnaturação.

Um outro estudo foi realizado para avaliar a futura aplicação da técnica de HRM, como método de análise na autenticidade de mostos e vinhos. Para validar os diferentes ensaios utilizaram-se três tipos de amostras: folha, mosto e vinho monovarietal, tendo em consideração o tamanho do fragmento amplificado (*Vv1*-704 bp, *Vv2*-375 bp e *Vv3*-119 bp). O ensaio *Vv1*-HRM, baseado no gene *UFGT* e considerando 32 variações nucleotídicas, foi bem-sucedido quando aplicado a amostras de mostos, permitindo a sua total discriminação. Os fragmentos *Vv2* (F3H) e *Vv3* (UFGT) foram amplificados com sucesso em todas as amostras testadas, permitindo a discriminação dos genótipos. A análise HRM baseou-se na temperatura média de desnaturação (T_m) para o fragmento *Vv2* e na forma da curva de desnaturação para os fragmentos *Vv1* e *Vv3*. O presente estudo descreve um ensaio HRM sensível, rápido, eficiente e promissor, aplicado pela primeira vez em amostras de vinhos, com excelentes perspectivas de aplicação na autenticidade de vinhos.

Palavras-chave: *Vitis vinifera* L.*, Vinho, Extracção de DNA, SNP, Antocianinas, HRM*

ABSTRACT

Food market globalization offers consumers a great diversity of food products, increasing their concern in relation to the products' origin. The development of food authenticity/traceability systems in the last decades have aimed to re-establish consumers' confidence. The use of DNA-based systems for this particular purpose has widely increased in the last years, as DNA technology has become more precise and robust, along with bioinformatics' tools and databases available.

Vine-growing and wine production is an essential economic and labour intensive activity and plays a major role to society in socioeconomic, environmental and societal terms. Wine quality and market value greatly depend on the grapevine varietal composition. The wine producing region, specified by the Denomination of Origin (DO), can only use a limited number of grapevine varieties. However, other varieties can be included in wine production under certain legally defined percentages which is by itself an attraction for fraudulent practices. With the present study we intend to contribute for the establishment of an efficient wine authenticity system targeting the valorization of DO wines, fraud and mislabeling detection, considering must and wine samples.

Primarily, DNA extracted from *Vitis vinifera* L. (leaf and monovarietal must) of ten different Portuguese grapevine varieties and one of reference (Cabernet Sauvignon) were screened using microsatellite (SSR) and inter-microsatellite (ISSRs) marker systems. The ISSR markers did not produce coincident results between reference leaf and must samples. Concerning the 6 SSRs markers used (established by the International Organization of Vine and Wine – OIV), a total of 42 alleles were detected, allowing the discrimination of the must samples according to the respective leaf sample. This study indicates that SSR markers may be successfully applied to must varietal authentication.

The DNA extraction from wine samples is the main bottleneck when aiming to apply DNA-based methodologies to wine authenticity. In this study, a DNA extraction protocol was established considering the particular characteristics of the wine matrices. The developed protocol allowed the extraction of large amounts of high-quality DNA from wines (ranges from 260 to 465 ng/µL) with optimal A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} ratios ranging from 1.71 to 1.81 and from 1.66 to 1.98, respectively. All DNA extracted from wine samples were amplifiable with a specific *Vitis vinifera* L. SSR markers, with a total correspondence between monovarietal wine (red and white) and its respective variety. The same protocol was applied to commercial blended wines producing amplicons

with the expected size range, taking into consideration the varieties referred on the label. This enhanced method can be the basis of a successful authenticity system.

Single Nucleotide Polymorphisms (SNPs) have become the most popular genetic marker system in plants and are interesting for authenticity purposes due to their ability to identify small genetic variations. High Resolution Melting (HRM) is a recent advance technique for the detection of SNPs and Insertion-Deletions (INDELs) in the *Vitis* genome and depends on DNA melting in the presence of saturating intercalating double-stranded DNA binding dyes.

The anthocyanin profile is specific of each variety, being potentially interesting for varietal identification and therefore for SNP detection. Based on these assumptions, a molecular study was conducted aiming to detect SNPs within the genes encoding the enzymes of the anthocyanin pathway (*e.g.*, chalcone isomerase - CHI and UDP-glucose:flavonoid 3-*O*-glucosyl-transferase - UFGT) in order to differentiate a selection of grapevine varieties. The *CHI* gene sequence presented a low polymorphism rate, 5 SNPs. Whereas the *UFGT* gene was highly polymorphic, with a total of 58 SNPs and 1 INDEL, allowing the discrimination of 18 different genotypes within the 22 grapevine varieties studied. A HRM assay was designed based on a large UFGT fragment, 704 bp, allowing the discrimination of 18 haplotypes based on the melting curve shape.

Another study was performed to evaluate the application of this HRM assay, as a screening method for must and wine authenticity purposes. Three sample types (leaf, monovarietal must and wine) were used to validate the different HRM assays according to the amplified fragment length (*Vv1*–704 bp, *Vv2*–375 bp and *Vv3*–119 bp). The *Vv1* HRM assay, based on *UFGT*, was only successful applied to monovarietal must samples, allowing the discrimination of the must varietal composition considering 32 nucleotide differences. The *Vv2* (*F3H*) and *Vv3* (*UFGT*) fragments were successfully amplified in all the sample types, allowing the discrimination of the genotypes. The HRM analysis was based on the melt temperature (T_m) (*Vv2* fragment) and in the melting curve shape (*Vv1* and *Vv3* fragments). This current study reports on a sensitive, rapid, efficient and promising HRM assay applied for the first time to wine samples with excellent prospects for wide application in wine authenticity.

Keywords**:** *Vitis vinifera* L.*, Wine, DNA extraction, SNP, Anthocyanins, HRM*

THESIS OUTLINE

Portugal has a long tradition in winemaking and is one of the most recognized wine producers in the world. There are vineyards throughout the country with different characteristics regarding *terroir*, complemented by several winemaking techniques allowing the existence of great diversity in terms of wine offer. However, wine price, quality and value also depend on the grapevine variety, region and winery reputation. Nowadays, wine consumers are demanding more detailed information regarding grapevine varietal composition. In this context, and considering both wine consumers and producer' interests, the main aim of this thesis was to increase the current knowledge on wine authenticity and developed an efficient authenticity system to trace through the entire wine chain, from the vineyards to the bottle.

The present thesis is organized in six main chapters: one revision chapter, four chapters related to the developed scientific research (three already published in International Scientific Journals and one in preparation), and a final chapter with the main conclusions of this study. The central objective of this work was to develop a potential wine authenticity system based on DNA fingerprinting. In order to achieve the main goal several others needed to be target:

1. the development of a successful DNA extraction protocol from must and wine samples;
2. the use of DNA molecular markers for grapevine varietal identification in must and wine matrices;
3. the development of HRM analysis system for SNPs genotyping, associated with anthocyanins genes pathway, suitable for grapevine varietal discrimination and;
4. the validation of developed methods applied to wine authenticity.

Chapter 1 focuses on the thematic directly involved in the food authenticity, particularly food products that are protected by Denomination of Origin (DO). Establishing a genomic approach for wine authentication involves many factors and requires several steps. The first step is one of the most important and crucial and refers to the DNA extraction from complex food/beverage matrices, in particular must and wine. The purpose of this chapter is to present the current state-of-the-art considering the DNA extraction constrains and possible solutions to overcome problems related to

different food matrices. Followed by a brief review of the existent DNA molecular markers available for grapevine varietal identification and the particularities of the development of molecular markers based technologies that maybe adequate for the purpose of the study, *e.g.*, authenticity of must and wine samples. It also points out the genes that were targeted in this particular study.

In Chapter 2 the work concerning the use of DNA molecular markers for assessing varietal authentication in must samples is presented. Wine quality and market value greatly depend on the grapevine varietal composition; thus, it is of great importance that grapevine varieties are correctly identified as soon as they are received in wine cellars. Herein, two DNA-based molecular markers were tested and results are compared in order to define the best strategy for grapevine varietal identification in must samples.

The first step concerning DNA-based wine authenticity approach is to develop a DNA extraction protocol capable of yielding good quality DNA. Thus, in Chapter 3 an enhanced DNA extraction protocol from different wine samples, experimental and commercial, is described. The strategy selected to overcome all the limitations presented in the DNA extraction from processed matrices are reported, as well as the advantages and the validation of the new developed protocol.

In Chapter 4 a rapid and high sensitive method - High Resolution Melting - for grapevine varietal identification based on the detection of SNPs, insertions-deletions (INDELs) in two key genes involved in the anthocyanin pathway are described. *Vitis vinifera* L. is a species with a large number of varieties, which differ in terms of their anthocyanin content. The genes involved in the anthocyanin biosynthesis pathway have a direct effect in the anthocyanin profile of each variety, being potentially interesting for varietal identification. Thus, the purpose of this chapter is the design of an assay suitable for the discrimination of the largest number of grapevine varieties based on the high number of polymorphisms found within the UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*) gene. Twenty-two grapevine varieties were sequenced and results showed a total of 58 SNPs and 1 INDEL found for the *UFGT* gene, allowing the discrimination of 18 different genotypes.

The implementation of an authenticity system implies the validation of the developed methods. Thus, Chapter 5 reports on the validation of the established HRM assays using DNA extracted from must and wine samples by the accurately identification of the grapevine varieties through SNPs determination. The possible application of such a system to authenticity procedures is discussed, along with the limitations and advantages of such a system.

In the last chapter, Chapter 6, the main outcomes of the described work are presented, bringing together chapters 2, 3, 4 and 5, and taking into consideration the main objective initially proposed. Furthermore, new approaches and research possibilities will be present based on the information gained through this research study.

CHAPTER 1

DENOMINATION OF ORIGIN AUTHENTICITY THROUGH DNA TECHNOLOGY

DENOMINATION OF ORIGIN AUTHENTICITY THROUGH DNA TECHNOLOGY

Based on:

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- Martins-Lopes P, Baleiras-Couto M, Fernandes JR, Pereira L, Brazão J, Eiras-Dias JE (2015) Origem varietal – castas de videira utilizadas. Química enológica - métodos analíticos. Avanços recentes no controlo da qualidade de vinhos e de outros produtos vitivinícolas. (Eds) Curvelo-Garcia A S & Barros P, Publindústria. Capítulo 13.2, pp539-563, ISBN: 9789897231186.
- Martins-Lopes P, Gomes S, Pereira L, Guedes-Pinto H (2013) Molecular markers for food traceability. Food Technology and Biotechnology 51(2), 198-207.

ABSTRACT

There is an increasing demand for the improvement of food quality control. The existence of traceability systems in the food chain helps, in some extent, to control it. However, these systems do not guarantee the complete authenticity of food products. DNA-based food authenticity assays are being developed and used for the identification of both raw and processed food products. The main constraint for wide application of DNA-based methodologies in food authenticity is linked to the nucleic acid extraction. The optimization of DNA extraction protocol from food products needs to take into consideration the food matrices complexity and the amount of starting material. In this chapter, an overview of the problems associated with nucleic acid extraction from food matrices concerning DNA degradation, extraction methods, and PCR inhibitors is provided and possible resolution of the existent constraints are explored. Different DNA-based molecular markers are also covered to solve authenticity issues, concentrating more in the wine particular case. The DNA molecular markers more adequate for authenticity purposes are short fragment length, such as SNPs. In *Vitis* even though some SNPs have been defined the search for more discriminative markers are pursued. Herein, the anthocyanin pathway is pointed as a possible source of discriminative SNP markers.

1.1 INTRODUCTION

Food quality, safety, and authenticity are major concerns that are seriously taken into consideration by producers, retailers, and consumers. Food safety concept has changed in the last years due to the several food chain outbreaks that have been reported, diminishing the consumers' trust on the food sector (Fajardo et al. 2010, Galimberti et al. 2013, Lockley and Bardsley 2000). This problematic is linked to food globalization that has extensively increased the occurrence of mislabelling and fraudulent practices.

In order to re-establish the consumers' confidence and to protect the brand of several food products, a number of strategies were undertaken by the European Union (EU). The EU established various regulations in order to protect food, feed and food production, by the definition of protected denominations: *Protected Designation of Origin* (PDO) and *Protected Geographical Indication* (PGI) under EU regulation 510/2006 (CR 2006), and *Traditional Specialities Guaranteed* (TSG), under EU regulation 1216/2007 (CR 2007). In 2012, the EU updated some of the regulation considering the previous denominations aiming to guarantee the quality of such products (Regulation 1115/2012; CIR 2012). The market has slowly started to associate these products with reliable ones, recognizing their high value and quality.

Nowadays, consumers are willing to pay more for these highly quoted products. Therefore, these food products are vulnerable to fraudulent practices at the producers' and retailers' level, requiring a tight control suitable for all food chain sectors. Several traceability systems have been regulated and implemented throughout the food chain, mainly consisting on the registration of the product from the geographical origin through the processing and finally ending at the retailer. This system is based on a digital control ensured by a code placed on the products' label to guarantee quality and to prevent outbreaks related to food adulterations (Mafra et al. 2008).

Authenticity requires a more deep knowledge of the food product, with the recognition of each food component (*e.g.*, species and cultivars) and its geographical origin. Several techniques have been applied for food authentication concerning species/variety identification, geographical origin, and manufacturing processes, namely spectroscopy, isotopic analysis, chromatography, electronic nose, immune biochemical assays, and thermal analysis (Asensio et al. 2008, Cajka and Hajslova 2011, Cavazza et al. 2013, Peris and Escuder-Gilabert 2009, Sanahuja et al. 2011, Voerkelius et al. 2010, Zhao et al. 2013). However, these analytical techniques are directly dependent on external conditions, such as: soil composition, weather, production year, among others, affecting the detection efficiency of species/variety identification. Additionally, despite the entire analytical

techniques available presently, there are still some adulterations that are difficult to detect. Therefore, alternative and environmental independent methods, suitable for species/variety identification, are required. DNA-based methods became available and a more attractive solution, since the DNA molecule presents a high stability even under high temperatures, pressures and chemical treatments used during food processing (Poms et al. 2004).

The use of DNA molecular markers has been vastly used for food authenticity purposes since their high stability allows them to be applied to transformed food products (Agrimonti et al. 2011, Baleiras-Couto and Eiras-Dias 2006, Doveri and Lee 2007, Faria et al. 2008, Intrieri et al. 2007, Jérôme et al. 2008, Martins-Lopes et al. 2008, Pafundo et al. 2007, Pereira et al. 2011, 2012, Vietina et al. 2011).

The aim of the present chapter is to update the state-of-the-art concerning the use of nucleic acids as a universal molecule for food authenticity purposes and the available molecular marker systems. Through this chapter a special focus will be given to must and wine emphasizing the problems associated with DNA extraction procedures and the availability of solutions to overcome some of them; the molecular markers and new PCR-based methodologies for grapevine varieties identification and consequently wine authenticity will be discussed. Additionally, a brief reference concerning the analysis of anthocyanins pathway genes and its implications as a new molecular marker for wine authenticity will be presented.

1.2 Problems Associated with Nucleic Acid Extraction from Food Matrices

DNA is one of the most reliable molecules for food authenticity. However, the success of DNA-based authenticity systems is highly dependent on the extracted DNAs' quality, quantity, and purity. These factors vary according to the samples' nature, the technology applied in the food processing stages and the DNA extraction method applied. DNA quality is a critical factor and is determined by the DNA degradation and the average fragment length of the nucleic acid obtained. The purity of DNA is essential for PCR-based methods and when it is not achieved it can compromise seriously the amplification process (Di Bernardo et al. 2007, Lockley and Bardsley 2000, Madesis et al. 2014, Turci et al. 2010). In this context, some of the main problems associated with DNA extraction from food matrices will be generally described, focusing on the DNA extraction from must and wine matrices.

1.2.1 DNA DEGRADATION

Food industry developed several chemical and biotechnological technologies to apply in processed food ensuring the quality and safety of the final product. Food processing technologies include drying, fermentation, salting, various forms of cooking (*e.g.*, roasting, frying, smoking, steaming, and oven baking), canning, freeze pasteurization, vacuum packing, osmotic dehydration, sugar crystallization, addition of food preservatives, high-temperature short time pasteurization, and high-pressure low-temperature food processing (Hellberg and Morrissey 2011, Meusnier et al. 2008, Pafundo et al. 2007).

Damage concerning the DNA fragmentation is mainly caused by nucleases enzymatic degradation, temperature, ionic strength, chemical agents, and pH values used throughout the industrial process (Gryson 2010). DNA fragmentation occurs when submitted to enzymatic hydrolysis as in fermentation processes found in wine, milk, and dairy products (Drábek et al. 2008, Klein et al. 1998, Mafra et al. 2008, Pereira et al. 2011), and when subjected to grinding and milling processes where raw materials are submitted to shear forces and mechanical stress such as, in some vegetable oil matrices (Adam and Zimm 1977, Busconi et al. 2003, Consolandi et al. 2008). Exposure to heat causes fragmentation of high molecular weight DNA whereas physical or chemical treatments cause random breaks in DNA strands reducing the average DNA fragment size (Bauer et al. 2004, Bergerová 2010, Vijayakumar et al. 2009). The mechanism of DNA destruction by heat is based on depurination or deamination. At temperatures above 100 °C, a significant strand scission and irreversible loss of secondary structure occurs (Bauer et al. 2003).

Matrices nature as well as food processing and storage conditions strongly affect the DNA degradation, due to pH influence (Bauer et al. 2003). Several food products, such as fruits and vegetables, are characterized by their acidity, thus accelerating the acid catalysed reactions in the course of thermal treatments. However, processing at alkaline pH is part of the production of several food products such as milk and dairy products and vegetable oils. DNA is very sensitive to acid and alkaline agents because of the mechanism of DNA hydrolytic degradation (Gryson 2010). The combination of high temperature, low pH, and pressure represents the technology generally used for food products preservation. Nevertheless, these treatments negatively influence the integrity of DNA in processed foods and therefore PCR detection of food components through DNA-based diagnostic techniques are compromised (Bauer et al. 2003, Bergerová et al. 2011, Jonas et al. 2001).

1.2.2 DNA EXTRACTION

DNA extraction is a critical step for further PCR based food analysis. Factors such as sampling methods, sample size, matrix type, and inhibitors can affect the quantity and quality of the extracted DNA (Demeke and Jenkins 2010). Therefore, the main objective of a DNA extraction procedure is to provide suitable DNA for posterior analyses. Several studies have been published to establish the most suitable DNA extraction method for each food matrix.

The most widely used DNA extraction methods from plant material and plant derived products are the detergent-based methods and the commercial kits that use silica-based columns or magnetic beads. The cetyltrimethylammonium bromide (CTAB) extraction method has been considered efficient for a large range of plant derived food products, especially due to its ability to separate polysaccharides from DNA. Doyle and Doyle (1987), originally described the CTAB method, and some variations of this method have been proposed to improve the DNA quality depending upon the sample matrix (Demeke and Jenkins 2010, Martins-Lopes et al. 2008, Pereira et al. 2011). A large number of commercial kits are available for DNA extraction from different matrices, but only a limited number can be used for the isolation of DNA from processed food products (Gryson et al. 2004). In general, DNA extraction protocols contemplate the following steps:

1. Breakage of cell wall by grinding the tissue in dry ice or liquid nitrogen;
2. Disruption of cell membranes using a detergent (*e.g.*, CTAB or SDS);
3. Inactivation of endogenous nucleases by the addition of detergents and/or EDTA, which bind to the cofactor of several enzymes (*e.g.*, Mg);
4. Inactivation and degradation of proteins by Proteinase K addition;
5. Separation of inhibitory polysaccharides through the differential solubility of polysaccharides and DNA in the presence of CTAB;
6. Separation of hydrophobic cell constituents, *e.g.*, lipids and polyphenols achieved with the extraction using an organic solvent, such as chloroform; and
7. Separation and concentration of DNA carried out through alcohol/salt precipitation.

The selection and optimization of the DNA extraction protocol, eliminating all the potential inhibitory components, is of crucial importance for the success of a given PCR-based methodology. A range of DNA extraction methods from plant tissue and food products can be found in the literature, enhanced and applied to different matrices, always taking into account the particularities of each sample type.

1.2.3 PCR INHIBITORS

PCR-inhibitors are one of the major limitations when using PCR-based technology for food authenticity purposes. Most of the PCR-inhibitors result from two main sources: food matrices nature and the DNA extraction protocol itself. Genomic DNA isolation from food matrices is difficult due to the natural presence of proteins, polysaccharides, lipids, and polyphenols. These components are found in different proportions in all food products and need to be taken into account when developing a protocol. The chemicals within the DNA extraction protocol, such as CTAB, phenol, and other compounds (*e.g.*, salts and carbohydrates) are *per se* strong inhibitors of *Taq* DNA polymerase (Di Pinto et al. 2007). The composition of the extraction buffer differs among protocols. However, the presence of CTAB is essential when using plant material, not only for the dissolution of the membranes as well as for the precipitation of DNA in the presence of high concentrations of sodium chloride (NaCl) or other salt and polysaccharides reduction. The addition of polyvinylpyrrolidone (PVP) and β-mercaptoethanol in the extraction buffer are also proposed and justified due to their antioxidant properties and their ability to eliminate polysaccharides, polyphenols, and proteins found in several food matrices, *e.g.*, wine, vegetable oils, and honey (Consolandi et al. 2008, Jobes et al. 1995, Pereira et al. 2011, Valentini et al. 2010). The PVP binds to polyphenolic compounds and can be separated by centrifugation in the chloroform–isoamyl alcohol extraction step. N-lauryl-sarcosine is also used as an antioxidant preventing the oxidation of polyphenolic compounds during cell lysis. The use of organic solvents (*e.g.*, phenol, chloroform, isoamyl alcohol, and hexane) is a problematic issue due to their high toxicity, misleading results in terms of DNA concentration and the fact that they are PCR-inhibitors. However, in most situations it is a necessary request in order to obtain DNA enable for PCR. The choice and optimization of the DNA extraction procedures which eliminate PCR inhibitors may be of major importance for the success of a given PCR method (nested PCR, Real-Time PCR, multiplex PCR) (Elsanhoty et al. 2011). The heterogeneity of various food products require the development of new DNA extraction protocols or the adaptation of existing ones in order to achieve a more suitable and adequate for each particular food matrices.

1.3 FOOD MATRICES

To ensure compliance with the food labelling, reliable techniques have been proposed for the detection and quantification of adulterants in several food matrices. Recently, DNA-based methods have provided a reliable tool for a wide range of food matrices screening (Madesis et al.

2014). Most of the commercially available and published DNA extraction methods from food and agricultural products include cereals, beans, vegetables, olive oils, fruit products, honey, nuts, wines and musts, and spices, among others. As previously mentioned, DNA extraction from food products possesses several problems and limitations and requires differential treatment according to the sample. The food matrices contain a large range of substances (*e.g.*, carbohydrates, fat and chemicals) which are often inhibitory of the PCR reaction leading to false and non-reproducible results. As an example, for vegetable oils (olive, sunflower, rapeseed, soybean, and palm oil) a high number of DNA extraction methods were developed to obtain DNA from these different oil matrices.

One of the main constraints in extracting DNA from vegetable oils is associated with the complex matrix enclosed by a lipidic layer. Consolandi et al. (2008) reported the disruption of neutral olive oil micelles by addition of the detergent Tween 20 after solubilization with hexane. Thus, the use of surfactant Tween 20, is crucial to emulsify the lipidic fraction of oil samples, and has been used instead of organic solvents to separate the oily from the aqueous and the pellet phases. Besides this constrain there are other factors that may affect the DNA extraction in these matrices such as the refining treatment, the presence of many interfering substances, including seed embryo DNA, and the storage period after milling (Costa et al. 2010, Doveri et al. 2006, Martins-Lopes et al. 2008, Pafundo et al. 2007, 2010).

Another interesting food product is honey as it is mainly composed of carbohydrates, which represent 95% of honey dry weight (White 1975). It also contains organic acids, proteins, amino acids, minerals, polyphenols, vitamins and aroma compounds which difficult even more the DNA extraction. Thus, a critical step of the DNA extraction from honey is to separate DNA from carbohydrates, namely fructose and glucose. The honey DNA is routinely extracted using both CTAB-based methods and commercial kits (DNeasy Blood and Tissue Kit—QIAGEN GmbH) (Jain et al. 2013). Prior to DNA extraction, honey samples need to be dissolved overnight in water and pre-warmed to 60–65 °C to permit easier handling. An important step in honey DNA extraction is to use phosphate buffered saline solution (PBS at pH 7.6) to separate the sugars through several centrifugation steps that produce a supernatant phase (where sugars are present) and a pellet that contains DNA and minerals. Other reagents such as, PVP40, β-mercaptoethanol, N-lauroyl-sarcosine and proteinase K are normally used to remove PCR-inhibitors and improve the amount and quality of DNA extracted not only for honey samples but also for other food products. The procedures followed in the must and wine DNA extraction method and their utility for authenticity purposes will be discussed in the following section.

1.3.1 MUST AND WINE

Economically, the wine sector is one of the most important agricultural activities worldwide. The protection of local and regional wines with Denomination of Origin (DO) is essential for authenticity purposes, protecting consumers against fraud. Wine is, among other products, one of the most susceptible to food adulterations, even though there is specific legislation protecting its authenticity. The main adulterations found in wine are:

1. Sugaring (chaptalization);
2. Addition of foreign sugars (*e.g.*, beets, sugarcane, and corn);
3. Addition of water; and
4. Falsification of its varietal composition and geographical origin.

These adulterations are difficult to detect and thus require the use of specific methodologies. Chemical analyses *per se* are not sufficient to verify must/wine authenticity, therefore DNA has been applied to solve varietal composition issues. Several studies have reported the ability to extract and genotype DNA from different grapevine products, including grape juice (Faria et al. 2000), grape must (Baleiras-Couto and Eiras-Dias 2006, Faria et al. 2008, Pereira et al. 2012, Rodríguez-Plaza et al. 2006), experimental wines collected immediately after the end of fermentation (Baleiras-Couto and Eiras-Dias 2006, Garcia-Beneytez et al. 2002, Siret et al. 2002), and aged wine samples (Drábek et al. 2008, Nakamura et al. 2007, Pereira et. al. 2011, Savazzini and Martinelli 2006). Must and wine matrices present high DNA degradation either due to the nature of the sample or to the winemaking process (decanting and filtration), stabilization (clarification and racking), and aging (Garcia-Beneytez et al. 2002). The DNA extraction protocols developed for wine samples had these factors into consideration and included several steps in order to increase the yield of DNA extraction (Fig. 1.1):

1. Precipitation of wine sample in 2-propanol;
2. Treatment with proteinase K;
3. Extraction with phenol, chloroform, and isoamyl alcohol;
4. Additional precipitations; and
5. Several washes.

The volume of wine needed for DNA extraction varies according to the protocols and in some cases is considered even limitative (Baleiras-Couto and Eiras-Dias 2006, Drábek et al. 2008, Pereira et al. 2011, Savazzini and Martinelli 2006). With the continuous optimization of protocols a decrease in the samples volume was possible achieving a volume of 10 mL of wine (Pereira et al. 2011). Prior

precipitation of the sample in 2-propanol was considered a crucial step in order to optimize the DNA extraction from wine (Pereira et al. 2011, Savazzini and Martinelli 2006).

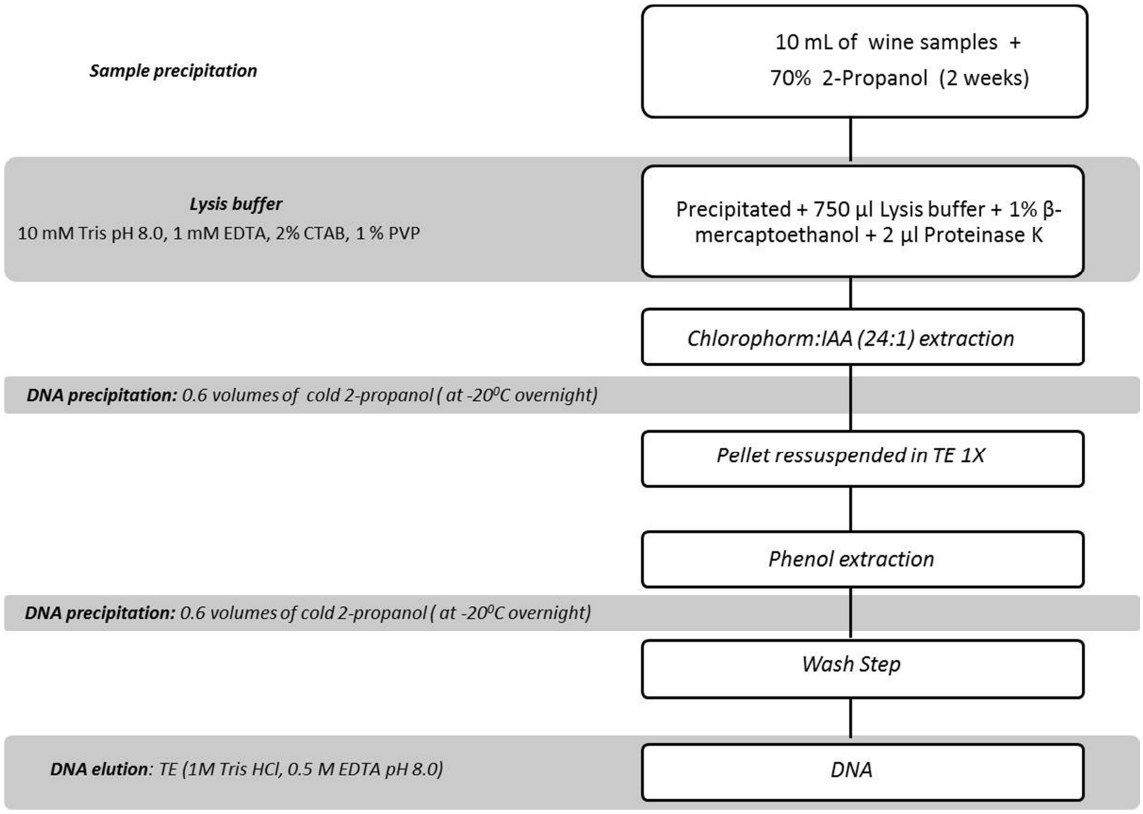

Fig. 1.1 Schematic representation of DNA extraction method from wine (*adapted from* Pereira et al. 2011).

The precipitation time has also been tested in different types of samples (red and white), and a period of at least 2 weeks at - 20 °C is the most effective procedure (Boccacci et al. 2012, Pereira et al. 2011). In some extraction protocols proteinase K is added at different concentrations and at different extraction stages (Bigliazzi et al. 2012, Nakamura et al. 2007, Pereira et al. 2011, Siret et al. 2000). All DNA extraction protocols from wine described include one or more extractions with chloroform-isoamyl alcohol (24:1) and/or phenol (Bigliazzi et al. 2012, Nakamura et al. 2007, Pereira et al. 2011, Savazzini and Martinelli 2006). Additionally, several 2-propanol precipitations are required (Baleiras-Couto and Eiras-Dias 2006, Bigliazzi et al. 2012, Drábek et al. 2008, Nakamura et al. 2007, Pereira et al. 2011, Siret et al. 2000). These steps are considered essential to improve the DNA extraction (Fig. 1.1).

Despite the factors that affect the DNA extraction including the heterogeneity of the food matrices and their specific characteristics, the DNA extraction has been successfully achieved in

different matrices such as wine, olive oil, cheese, fish and meat, among others. This major achievement has allowed the developed of PCR-based methods suitable for authenticity of food products and to some extent they will contribute to restore consumer's confidence.

1.4 MOLECULAR MARKERS

Molecular markers present numerous advantages over conventional phenotyping. The selection of the most suitable marker systems enters into consideration with several aspects, such as: level of polymorphism detected by its means; inheritance co-dominant; evenly distributed throughout the genome; stable over generations, simple, quick and inexpensive; DNA amount request (Agarwal et al. 2008, Hatzopoulos et al. 2002). Over the years, several molecular markers have been developed for grapevine varieties accurate identification and characterization.

The *V. vinifera* L. (Euroasian group) is one the oldest cultivated plants worldwide being mainly used in wine production. The total grapevine variety number is estimated to be approximately 11 000 (Maul et al. 2003). Even though most of them are in ampelographic collections, a large number has not been characterized and/or has been incorrectly classified (misnamed, synonyms and homonyms) (Gismondi et al. 2014). The Portuguese ampelographic collection contains approximately 720 registered grapevine varieties (250 putative autochthonous varieties are included in the collection), with 343 being legally authorized for wine production. However, only a restricted group of 51 native grapevine varieties are commonly used in viticulture (Almadanim et al. 2007). Grapevine varieties have traditionally been characterized and identified by standard amplelographic descriptors recognized by the *International Ampelography Committee*. The Organization *Internationale de la Vigne et du Vin* (OIV, International Organization of Vine and Wine) has established a methodology for the grapevine variety amplelographic description. However, standard ampelography methods can sometimes result in wrong classifications since they result on morphological characters which depend on the plant's development phase, health and nutritional status, *terroir*, and environmental conditions, as well as on the ampelographist expertise. Furthermore, the vast number of established grapevine varieties difficult even more the differentiation morphologically based. In order to overcome some of the drawbacks given by ampelography, grapevines have been additionally characterized considering chemical and biochemical features. The biochemical approaches included the isoenzymatic, phenolic and aromatic compounds, aminoacids, minerals, stable isotopes and organic compounds profiles (Tomić et al. 2013). Nevertheless, these methodologies also presented some limitations since they are highly dependent on the environmental conditions and the sample

development stage, making these methods unreliable approaches for botanical grapevine classification.

With the advent of high-throughput sequencing technologies, the grapevine reference genome sequence, using a near homozygous Pinot Noir line (PN40024), was published by the French-Italian Public Consortium (Jaillon et al. 2007) being an excellent platform for genome genetic analysis based on sequence comparisons between the reference genome and available varieties variants (Jaillon et al. 2007, Velasco et al. 2007). Thus, grapevine variety characterization is currently performed using DNA-based molecular markers, providing a more accurate identification and characterization, since the profiles obtained are independent of environmental conditions. In grapevine, various DNA-based techniques, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Single Strand Conformation Polymorphism (SSCP), Amplified Fragment Length Polymorphism (AFLP), Inter-Simple Sequence Repeats (ISSRs), Simple Sequence Repeat or Microsatellites (SSR) and Single Nucleotide Polymorphism (SNP) have been proposed as useful means for identifying grapevine varieties (Benjak et al. 2005, Cabezas et al. 2011, Cunha et al. 2009, Fanizza et al. 2003, Herrera et al. 2002, Moreno et al. 1998). However, SSR and SNP markers are nowadays the markers of choice, mainly due to their interesting characteristics: co-dominance and high specificity and reproducibility (Vezzulli et al. 2008).

1.4.1 MICROSATELLITE

The microsatellites (SSR) are specific and co-dominant *loci*, being therefore considered as excellent molecular markers. The SSRs consist on a small sequence repeat, 2 to 6 bp, spread throughout the genome, which present a high variation level between individuals in what the number of single sequence repeats is concerned. The sequences that limit the repeat motif is highly conserved within a particular species. The SSR molecular markers have demonstrated to be very useful for grapevine varietal characterization, and a list of SSRs for *V. vinifera* are in the database (http://www.eu-*Vitis*.de/index.php). From the initial list of SSR markers, six were selected within an European project GENRES 081 (European network for grapevine genetic resources conservation and characterization, www.genres.de/eccdb/*Vitis*) as being the most adequate for grapevine varietal characterization and have been considered by the OIV as a descriptor for *Vitis* species and varieties (OIV, 2009; Table 1.1). Nowadays, these six microsatellites are used to characterize the majority of the grapevine varieties. In Portugal, this set has been used to characterize the 341 grapevine varieties (Veloso et al. 2010) used in wine production (Portaria nº 428/2000 of 17th of July).

Table 1.1 SSR list considered by the OIV as descriptors.

Locus	Repeat Motif	Code Number OIV	Reference
VvS2	(AG)n	801	Thomas and Scott 1993
VvMD5	(CT)nTA(CT)nATAG(AT)n	802	Bowers et al. 1996
VvMD7	(CT)n	803	Bowers et al. 1996
VvMD27	(CT)n	804	Bowers et al. 1999
VrZAG62	(GA)n	805	Sefc et al. 1999
VrZAG79	(GA)n	806	Sefc et al. 1999

Recently, three more SSRs *loci* (VVMD25, VVMD28 and VVMD32) were added to the list for grapevine variety identification (GrapeGen06, http://www1.montpellier.inra.fr/grapegen06). These nuclear SSRs have been widely used in grapevine for several purposes: varietal identification, synonyms and homonyms clarification, genetic origin and germplasm collection diversity assessment, marker assisted selection and genetic mapping. The authenticity of wine involves the varietal identification through PCR-based methodologies (Table 1.2). Different research groups have used SSRs for must and wine authenticity purposes (Table 1.2). Some of these studies were successful when experimental wines were used (Baleiras-Couto and Eiras-Dias 2006, Faria et al. 2000, García-Beneytez et al. 2002, Nakamura et al. 2007, Pereira et al. 2011, Rodríguez-Plaza et al. 2006, Siret et al. 2000, 2002b). Baleiras-Couto and Eiras-Dias (2006) using multivarietal musts, reported a relationship between the proportion of each variety in the mixture and the signal intensity of the alleles obtained using an automatic sequencer, suggesting the possibility of quantifying the presence of each variety in the mixture. A similar result was obtained by Işçi et al. (2009) in experimental wine and must samples. Even though SSR requires a small amount of DNA (approximately 10-25 ng per PCR reaction), the major constrain associated to its wide application for varietal identification in wine is associated with low DNA amount available in wine and the fact that the existent DNA is highly degraded due to the wine fermentation and aging steps (García-Beneytez et al. 2002). Faria et al. (2000) and Siret et al. (2002b) evaluated the SSR limit of detection when analyzing mixed varietal wines during fermentation. The authors were able to detect about 10 % of a varietal presence in the beginning of the fermentation process. Whereas in the end of the fermentation process a variety needed to be present on a percentage around 30 % in order to be amplifiable by SSR markers (Siret et al. 2002b). Although, several constrains have occurred when using SSR markers in aged wines, because of the DNA low recovery. Pereira et al. (2011) have developed a DNA extraction method that

allowed the recovery of DNA from commercial aged wines and have successfully achieved DNA amplification by PCR-SSR markers.

Table 1.2 DNA-based methods applied in must/wine authentication.

Samples	Target	Technique	Reference
Monovarietal and blended musts experimental wines	nSSR	PCR *acrylamide gel*	Faria et al. 2008 Siret et al. 2002a Siret et al. 2000 Faria et al. 2000
Monovarietal and blended musts Experimental and commercial wines	cpSSR nSSR	CE	Bigliazzi et al. 2012 Pereira et al. 2012 Pereira et al. 2011 Nakamura et al. 2007 Baleiras-Couto and Eiras-Dias 2006 García-Beneytez et al. 2002
Monovarietal musts and wines (6, 12, 24 months old)	nSSR *VvNCED*	CE Real-time PCR	Scali et al. 2014 Drábek et al. 2008 Savazzini and Martinelli 2006
Monovarietal musts Blended musts	nSSR Genomic DNA	CGE- LIF (CAPS)-based assay	Rodríguez-Plaza et al. 2006 Spaniolas et al. 2008
Monovarietal musts and wines from sparkling wines	nSSR cpSSR	CE	Boccacci et al. 2012

CE- capillary electrophoresis; CGE- LIF - Capillary gel electrophoresis with laser-induced fluorescence; CAPS - Cleaved Amplified Polymorphic Sequence.

The SSR profiles obtained in the wine samples corresponded to the leaf profiles. The use of cloroplastidial SSRs (cpSSR) have been used for varietal composition analysis in experimental wines (Baleiras-Couto and Eiras-Dias 2006, Boccaci et al. 2012). Even though they were successful applied in experimental wine samples, they present some limitations for wide application due to their low polymorphism level. Although SSRs have been used to trace must and wines samples, they frequently present amplification problems, due to their motif length. Therefore, a search for alternative markers, producing smaller amplicons has been pursued.

1.4.2 Single Nucleotide Polymorphism (SNP)

The single nucleotide polymorphism is the smallest genetic variation unit, representing the most frequent polymorphism among plant and animal genomes. The SNPs are characterized as being bi-allelic, in diploids, revealing the exact allele information, which is an advantage in relation to other

molecular marker systems. Another advantage is that they are distributed throughout the genome, not being limited to a certain region. Their allele binning is based on nucleotide sequence instead of amplicon length, which makes these markers particularly suitable for comparing the data obtained by different laboratories. In 2007, with the release of the genome grapevine sequence, opening a new window of opportunities was opened for the development of new markers, especially SNP markers that could be targeted at the genome level (Velasco et al. 2007). Previously, This et al. (2004) have reported that SNP frequency in grapevine genes was high.

In grapevine, SNPs have been widely applied in different fields: parental search (Hunt et al. 2010), linkage maps (Troggio et al. 2008, Vezzulli et al. 2008), associative mapping and QTLs (Fournier-Level et al. 2009), evolution studies and population genetics (Emanuelli et al. 2013, Fournier-Level et al. 2010, Riahi et al. 2013) and varietal identification (Cabezas et al. 2011). Several studies have reported a number of standard sets of SNPs suitable for grapevine genotyping (Cabezas et al. 2011, Di Génova et al. 2014, Lijavetzky et al. 2007). Concerning food authenticity, SNPs have been already applied to trace olive oil (Consolandi et al. 2008, Doveri et al. 2006) and meat samples (Negrini et al. 2008). Until now, in the wine sector, there are no reports, of the PCR-based SNP markers for wine authenticity purposes. However, it was previewed that it could be applied in the near future, since the grapevine varietal identification is being implemented (Cabezas et al. 2011, Di Génova et al. 2014).

1.4.3 Real-Time PCR

The Real-Time PCR methodology is a highly sensitive allowing the detection and quantification of a given sequence. The chemistry used in the sequence detection is variable according to final application purpose, being the most widely applied: SYBR® Green, TaqMan®, and Molecular Beacon. Between the chemistries used several particularities need to be considered, such as the necessity to design specific assays for the TaqMan® and Molecular Beacon. Such particularities make such assays more expensive in comparison to the SYBR® Green based assays.

In grapevine their application is still very limited, because when Real-time PCR is used for diagnostic purposes there is a serious of normatives, which is required to be previously established. The implementation of such a system imposes that primers, probes, PCR condition, and protocol are validated for endogenous and exogenous genes and that reference material is developed. These procedures have been established for transgenic detection, however in grapevine this type of normative does not exist for varietal detection (Savazzini and Martinelli 2006). In grapevine, some

studies have been developed in order to select the endogenous genes that can be used for such purpose. Savazzini and Martinelli (2006) selected a serious of genes and have defined that the locus *VvNCED2* presented the best features since it is a single copy gene (Savazzini et al. 2005). The authors were able to develop a Real-Time PCR assay, TaqMan-based, suitable to quantify the DNA amount available in grape, must and wine samples. SNP-based markers used in association with Real-Time PCR will be the most reliable molecular approach for the varietal composition determination in wine samples.

1.4.4 High Resolution Melting (HRM)

High Resolution Melting (HRM) analysis emerged as highly specific technique that allows the quick, high-throughput and cost-effective analysis of specific DNA amplicons. Recent advances in the development of saturating DNA dyes made the use of HRM easier for bar coding, genotyping and for adulterants quantification (Ganopoulos et al. 2011, 2012, 2013, Sakaridis et al. 2013). HRM is a simple, PCR-based method for detecting DNA sequence variation by measuring DNA duplex melting temperature changes result of sequence differences. The PCR fragments are amplified using unlabeled primer pairs, and a melting analysis is performed at the end of the PCR reaction in the presence of a high fidelity dye that binds to double-stranded DNA (dsDNA) (Fig. 1.2). Prior to the high-resolution melting analysis, the PCR products are denatured and then quickly reannealed. This quick melting/reannealing step is the core of the technique, as it influences the subsequent HRM analysis. During this critical step, complementary strands for the unique allele of a homozygous sample reanneal perfectly to form a perfect complementary dsDNA product (homoduplex). However, in the case of heterozygous, samples that have more than one allele present, in the PCR product half the alleles reanneal to the complementary strand of the same allele and the other half reanneal to the complementary strand of the other allele (heteroduplexes). The last step of the HRM analysis involves a slow melting of PCR products while a high frequency, high accuracy fluorescence capture is performed. The duplex melting temperature (Tm) is always different among the homozygous sequence, the homozygous sequence with variant and the heterozygous sequence, resulting in different melting temperatures and melting curve shapes (Reed et al. 2007). Several software programs are available for HRM data analysis, adequate to each HRM platform. Following samples normalization, the software automatically groups melting curves based on their similarity. For HRM assay, to be highly accurate, some factors must be controlled, such as sample source and

preparation, amplicon length, GC content, DNA dyes, and the precision of the equipment (Wittwer, 2009).

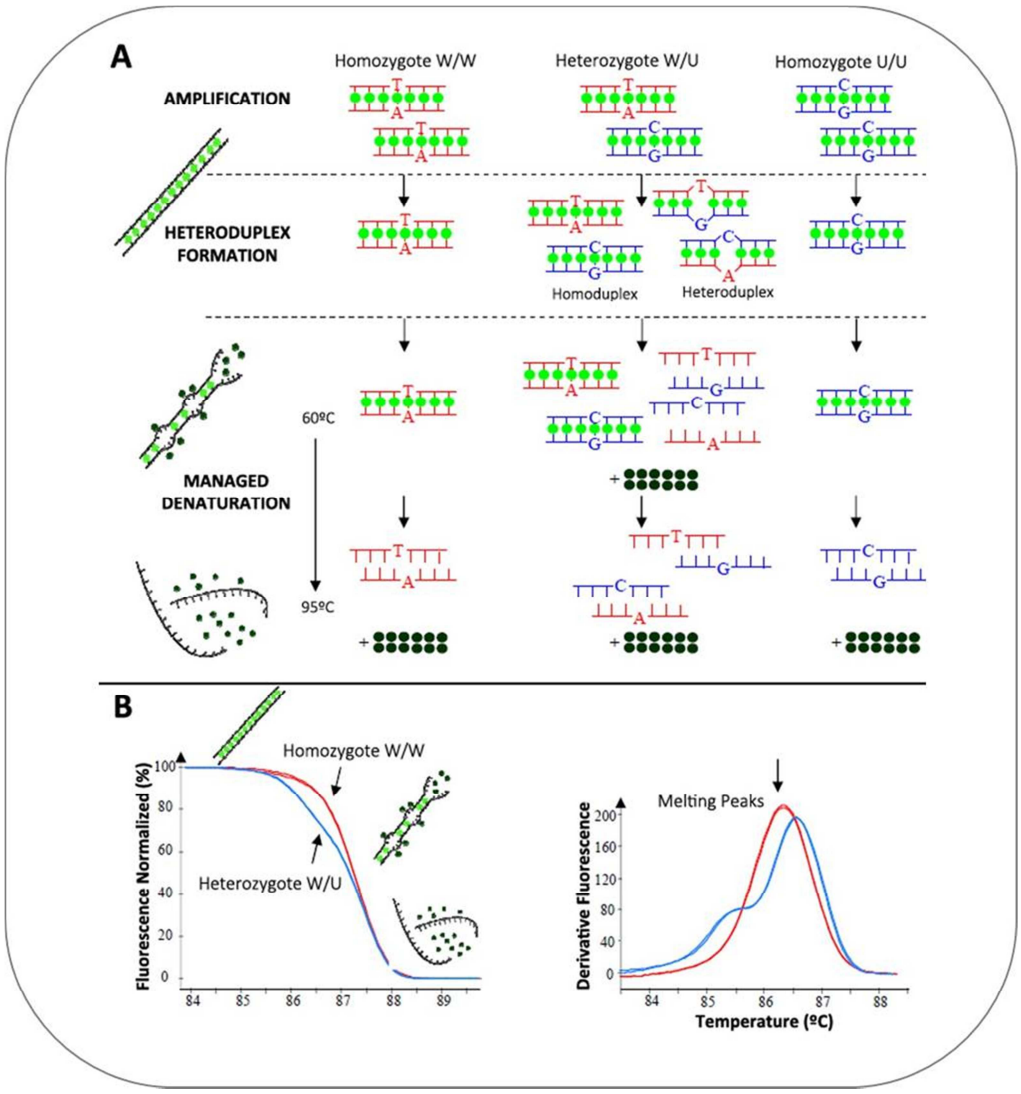

Fig. 1.2 Schematic principle of High Resolution Melting (HRM) analysis. **A.** DNA fragments are amplified using fluorescently intercalating DNA dye, heat-denatured and cooled. Heterozygote (W/U) variant formed after denaturation and rehybridization, two homoduplexes (W/W and U/U) and two heteroduplexes (W/U). **B.** The results are illustrated as denaturation melting curves (fluorescence normalized or derivative fluorescence in function of temperature). HRM detects mutations in DNA fragments due to temperature shift of the melting curve caused by variation of the amplicon T_{ms} or variation of the curve shapes in heteroduplexes presence (*Adapted from* Meistertzheim et al. 2012).

Nowadays, HRM analysis is used for food authenticity purposes since it is a sensitive, stable, and reliable technique that allows the rapid verification of specific genotype amplicons (Ganopoulos et al. 2011, 2012, 2013). In *Vitis*, HRM was applied for grapevine varietal identification using as amplicons several microsatellites *loci* (Mackay et al. 2008). HRM application for the development of

single nucleotide polymorphism, for grapevine varietal identification and for further application in wine authenticity will be presented in Chapter 5.

1.5 ANTHOCYANINS

The oenological potential of grapevine varieties are directly associated to their chemical composition. Several groups of compounds have been proposed as having good features to be used in wine authentication purposes, among these the anthocyanins have been considered to have such potential (Muccillo et al. 2014). Anthocyanins are phenolic compounds synthesized via the flavonoid pathway. These compounds confer colour to the tissues where they accumulate, ranging from magenta and red to blue, violet and purple. The levels of anthocyanins in wine are linked to grape skins' colour, wines' colour stability, and organoleptic characteristics result of their interaction with phenolic compounds, proteins and polysaccharides (Mazza and Miniati 1993, Ribéreau-Gayon 1982). Anthocyanins are mainly accumulated in the berries' skin, being transferred to wine during the vinification process (He et al. 2010). The proportion and amount of each anthocyanin in wine is determined by the grapevine variety and the viticulture conditions. However, Dimitrovska et al. (2013) and He et al. (2010) refer that the grapevine varietal anthocyanins pattern or fingerprint is relatively constant and independent of the environmental conditions; therefore they suggest that such profiles can be considered as varietal markers.

The biosynthesis of anthocyanins begins in *véraison* (onset of ripening) and reaches maximum expression around harvest time (Ribéreau-Gayon 1982). Within the flavonoids group, anthocyanins are characterized by a C_6-C_3-C_6 carbon backbone. In *V. vinifera* the most common anthocyanins are: delphinidin, cyanidin, petunidin, peonidin, and malvidin. Malvidin derivatives are the main anthocyanins in most of the *V. vinifera* varieties (Liang et al. 2008). The anthocyanins biosynthetic pathway has been well characterized (Boss et al. 1996) (Fig. 1.3). The flavonoid synthesis is regulated by the action of two gene classes: (1) structural genes, encoding enzymes for anthocyanins and other flavonoids synthesis; (2) and the regulatory genes involved in structural genes spatial and temporal regulation (Deluc et al. 2008). Thus, plant pigmentation patterns are mainly controlled by the regulatory genes expression profiles (Grotewold 2006, Holton and Cornish 1995, Velasco et al. 2007). The anthocyanin biosynthetic pathway includes genes encoding the following enzymes: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3'-hydroxylase (F3H), dihydroflavonol reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) and UDP-glucose:flavonoid 3-O-glucosyltransferase(UFGT), that were partially cloned in grapevine by Sparvoli et al. (1994). These

genes are located in different positions on the biosynthetic pathway playing diverse roles in plant development (Fig.1.3).

Fig. 1.3 Scheme of the anthocyanin biosynthetic pathway in plants. Enzymes for each step are shown in bold. Flavonoid intermediates are boxed and principle flavonoid end products are in gray boxes. PAL, Phe ammonia lyase; CHS, Chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3*β*-hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP-glycose:flavonoid-3-*O*-glycosyltransferase; FLS, flavonol synthase; GT, glycosyl transferase; LAR, leucoanthocyanidin reductase; ANR, anthocyanin reductase (*Adapted from* Jeong et al. 2008).

Genes encoding for the enzymes present in the early steps of the biosynthetic pathway belong to multicopy families, having different temporal and spatial partitioned expression profiles, sometimes coincident with the particular flavonoid biosynthesis (Harris et al. 2013, Jeong et al. 2008). The CHI and UFGT are considered key enzymes of the pathway (Kuhn et al. 2014). Enzyme CHI leads to the formation of flavanones in a central branching point of the flavonoid pathway; F3H influence the levels of anthocyanins and flavonol precursors (Yoder et al. 1994); and UFGT is located in a final position of the anthocyanin pathway and is associated with the accumulation of anthocyanins in the berries' skin. The genes *CHI, PAL*, *CHS*, *F3H*, *DFR*, *LDOX* are expressed in both white and red grapevine varieties whereas, *UFGT* gene is only expressed in red grapevine varieties,

being crucial for the anthocyanin biosynthesis (Boss et al. 1996, Zheng et al. 2013). The search of SNPs in these genes sequence can provide knowledge concerning different grapevine genotypes and be used for further traceability/authenticity purposes.

1.6 GENERAL OVERVIEW

The development of food traceability/authenticity systems is becoming almost an imposition worldwide since consumers and producers are seeking for high quality food products that can also be guaranteed in terms of safety. As a consequence, food traceability/authenticity mechanisms have been required by consumers and government organizations. Consumers are interested in choosing what they are eating/drinking based on nutritional value and product origin, where labelling information plays a central role.

In terms of the wine industry, the European Union has developed legislation that includes several features from origin and geographical indications, to traditional terms, and labeling specifications. This definition has led to the discrimination of Denomination of Origin appellations that besides limiting the geographical areas also defines grapevine varieties in vineyards, with a limited inclusion of other varieties under demarcated percentages. The wine labels contain information regarding grapevine variety, geographical origin, and production year. Even though grapevine variety information is not mandatory by European law, consumers appreciate its definition in labels especially in high quoted wines. Thus, accurate grapevine variety identification suitable for the entire vitiviniculture production chain would be a distinguishing factor benefiting consumers, producers and traders.

In order to develop a solid wine traceability/authenticity system the vineyards should be planted with certified grapevine material. In fact, genetic assessment with SSRs markers has already become a recommended protocol by OIV to certify new plant material at the propagation phase. Identifying DNA variations associated with important oenological traits as well as for authenticity purposes is a major focus of producers and researchers. Nowadays, the DNA-based methods are considered more reliable based on the stability of the DNA, and have been applied for wine authenticity and traceability.

Identifying DNA variations associated with important oenological traits as well as for authenticity purposes is a major focus of producers and researchers. Molecular markers are used widely as tools for traceability purposes, through the identification of grapevine varieties more rapidly and efficiently may be used in wine authenticity.

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CHAPTER 2

MOLECULAR MARKERS FOR ASSESSING MUST VARIETAL ORIGIN

MOLECULAR MARKERS FOR ASSESSING MUST VARIETAL ORIGIN

Adapted from:
Pereira L, Martins-Lopes P, Batista C, Zanol GC, Clímaco P, Brazão J, Eiras-Dias JE, Guedes-Pinto H (2012) Molecular Markers for Assessing Must Varietal Origin. Food Analytical Methods 5, 1252–1259.

ABSTRACT

Wine quality and market value greatly depend on the grapevine varietal composition, which may be characteristic of specific regions. In order to defend the distinct regions, Denominations of Origin were defined to protect against fraudulent practices. In this study, we evaluated the efficiency of two microsatellite-based systems: microsatellite (SSR) and inter-microsatellite (ISSR) for must varietal composition determination and their potential role in certification purposes. Eleven *Vitis vinifera* L. varieties from leaf and monovarietal must DNA samples were screened with six SSR and 14 ISSR primers to discriminate polymorphisms. Principal coordinates analysis was performed with DCENTER on the resultant data using unweighted pair group mathematical average and revealed that ISSRs markers were not suitable for certification procedures, whereas nuclear SSR markers presented a complete correspondence between leaf and must samples, demonstrating that they were adequate for traceability purposes.

2.1 INTRODUCTION

European grapevine (*Vitis vinifera* L.) is an economically important crop, grown for both table grapes and for winemaking. The Portuguese National Ampelographic Collection contains about 760 accessions, mainly Portuguese specific. However, the number of distinct varieties may be significantly lower, since there are probably duplicates related to regional denomination (Lopes et al. 2006). A large number of *V. vinifera* varieties can be used in wine production, although only a small number are commercially important. Portuguese legislation allows 341 varieties for wine production (Baleiras-Couto and Eiras-Dias 2006). Grapevine varieties deeply influence the wine quality and therefore have a direct impact on wine's market price, particularly in referenced market segments such as Denomination of Origin (DO) wines. For that reason, these highly quoted wines are the preferential target for fraudulent practices. Thus, wine authenticity is important in protecting the reputation of DO wines and ensuring consumers' confidence in quality control. The control process by grapevine varietal identification should comprise all stages of the vinification process starting with the cellar reception and ending with bottled wine. This control may be more accurate and efficient when DNA-based methodologies are used, since they are independent from environmental conditions. Currently, DNA-based techniques are being widely used for food traceability purposes (Agrimonti et al. 2011, Baleiras-Couto and Eiras-Dias 2006, Doveri and Lee 2007, Faria et al. 2008, Intrieri et al. 2007, Jérôme et al. 2008, Martins-Lopes et al. 2008, Pafundo et al. 2007, Vietina et al. 2011). DNA extraction from grapevine vegetative material is well established (Lodhi et al. 1994). However, efficient DNA extraction and amplification from other matrices such as grape must and wine remain difficult, mainly due to:

1. Plant DNA decomposition during the maceration process;
2. Presence of microorganisms' DNA, namely yeasts; and
3. DNA polymerase inhibitors such as polysaccharides, tannins, and polyphenols, present in matrices especially further down the processing chain.

Nevertheless, several reports have been successful using must and experimental wine samples (Baleiras-Couto and Eiras-Dias 2006, Faria et al. 2000, Garcia-Beneytez et al. 2002, Nakamura et al. 2007, Rodríguez-Plaza et al. 2006, Siret et al. 2000, 2002). Grapevine collections were formerly described using ampelographic descriptions including morphological and phenological aspects (Alleweldt and Dettweiler 1986, Dettweiler 1993, Ortiz et al. 2004, Santiago et al. 2005). With the development of DNA-based markers, grapevine collections have been characterized at the DNA level. Microsatellites markers have been the molecular tool selected for the identification and

documentation of *Vitis* gene banks (Almadanim et al. 2007, Botta et al. 1995, Bowers et al. 1996, Cipriani et al. 1994, Le Cunff et al. 2008, Lopes et al. 1999, 2006, Sefc et al. 1998, This et al. 2004, Veloso et al. 2010). A European project (GENRES#081, http://www.genres.de/*Vitis*/*Vitis*.htm) selected a set of six microsatellite primer pairs (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79) as the most adequate for grapevine varietal characterization due to their high polymorphism level. These six SSRs are also included in the "OIV descriptor list for grapevine cultivars and *Vitis* species" (OIV 2009). Recently, the GrapeGen06 Project has suggested the use of three more SSRs markers (VVMD25, VVMD28, and VVMD32) for additional genetic data (http://www1.montpellier.inra.fr/grapegen06).

Other molecular markers have been applied to characterize grapevine varieties such as inter-simple sequence repeat (ISSR), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and chloroplastidial microsatellites (cpSSR), among others (Benjak et al. 2005, Cunha et al. 2009, Fanizza et al. 2003, Herrera et al. 2002, Moreno et al. 1998). ISSR markers are frequently used on genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology studies (Godwin et al. 1997, Gupta et al. 1994, Reddy et al. 2002, Zietkiewicz et al. 1994). However, they have been limitedly applied for food certification purposes (Martins-Lopes et al. 2008). The need of certifying methods to determine grapevine varieties used in the winemaking process leads to the main goal of the present study which focuses on the possibility of using ISSR and SSR molecular markers for must varietal authentication, using, as reference, leaf DNA samples, and their possible role for certification purposes.

2.2 MATERIALS AND METHODS

2.2.1 GRAPEVINE MATERIAL

Eleven *V. vinifera* L. varieties, used in Portuguese wine production, were selected. Young leaf samples from six white grapevine varieties (Alvarinho, Loureiro, Fernão Pires, Moscatel Galego, Malvasia Fina, and Viosinho) and five red grapevine varieties (Touriga Nacional, Touriga Franca, Tinto Cão, Aragonez, and Cabernet Sauvignon) were collected (field collection of the University of Trás-os-Montes and Alto Douro, in Vila Real, Portugal) and immediately stored at −80 °C. Monovarietal musts were prepared at National Institute of Biological Resources in Dois Portos, using freshly harvested grapes from the same grapevine varieties. All must samples were collected after maceration and immediately frozen at −20 °C.

2.2.2 DNA Extraction

Genomic DNA was extracted from leaves using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987). Must DNA extractions were performed using an adapted version of Doyle and Doyle (1987), due to the samples' nature. Two milliliters of homogenized monovarietal must samples were centrifuged at 10,000 rpm (Eppendorf Centrifuge 5430 R) for 5 min. Pellet was recovered, and 1 mL of CTAB extraction buffer, containing 2% of polyvinylpyrrolidone (PVP) and 1% of β-mercaptoethanol, was added. Two extractions with chloroform/isoamyl alcohol were performed. The final pellet was washed twice with washing buffer (76% EtOH, 10 mM ammonium acetate). DNA was resuspended in 50 µL of distilled water. DNA concentration was determined using NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and DNA quality was verified on a 0.8% (w/v) agarose gel stained in 7 µg/mL ethidium bromide solution.

2.2.3 SIMPLE SEQUENCE REPEAT

Grapevine varieties and correspondent must samples were genotyped at six SSR *loci*: VVS2 (Thomas and Scott 1993), VVMD5 and VVMD7 (Bowers et al. 1996), VVMD27 (Bowers et al. 1999), VrZAG62, and VrZAG79 (Sefc et al. 1999). Forward primers were purchased from Sigma Aldrich (Sigma, St. Louis, MO, USA) and labeled with specific fluorochrome for the automatic sequencer (Beckman Coulter Sequencer, Beckman Coulter, Inc, Fullerton, USA). Polymerase chain reactions (PCR) and conditions were performed as described by Baleiras-Couto and Eiras-Dias (2006). Amplicon separation was carried out through capillary electrophoresis on an automated sequencer, and fragment size was established with the help of internal size standards (CEQ™ DNA Size Standard Kit-400) using the software package CEQ™ 8000 Fragment Analysis System.

2.2.4 INTER-SIMPLE SEQUENCE REPEAT

ISSR amplifications were tested using 14 University of British Columbia (UBC) primers (Table 2.2). Each reaction consisted of 30 and 50 ng of genomic leaf and must DNA sample, respectively. PCR reactions were performed at the conditions described by Martins-Lopes et al. (2008). Amplicons were separated by electrophoresis onto 1.5% (w/v) agarose gels (Seakem® LE Agarose) in 1×

Tris/borate/EDTA buffer at 80 V for 150 min, after which the gels were stained in 7 µg/mL ethidium bromide solution and digital image was obtained directly under UV light. Each DNA sample was independently amplified at least twice with each primer for each DNA extraction, and only reproducible amplified products were scored.

2.2.5 Data Analyses

The PCR fragments were scored for the presence (1) or absence (0) of equally sized bands, and two matrices of the different ISSR and SSR markers were assembled and used in the statistical analysis. These matrices were used to perform a Principal Coordinate Analysis (PCA) based on the ISSR data carried out by using the SIMQUAL (Jaccard's Coefficient), DCENTER (double-centering analysis) and EIGEN modules. All computations employed the appropriate procedures within NTSYS.pc v2.02 (Rohlf 1998).

2.3 Results and Discussion

2.3.1 DNA Extraction and Quantification

The CTAB method was found to be suitable for must samples. The DNA extracted from monovarietal must samples was of high quality (A_{260nm}/A_{280nm}, 1.7 to 2.0), which ran as high-molecular-weight band, comparable to the leaf DNA samples in agarose gel (Fig. 2.1). The must DNA sample concentrations ranged from 211 to 401 ng/µL. The must DNA yields were higher than those reported by other authors using similar samples (Faria et al. 2000, 2008, Garcia-Beneytez et al. 2002, Siret et al. 2002), ranging from 10 to 20 µg/mL of starting material. One of the reasons that may explain the higher yields obtained may be the fact that we have used immediately frozen fresh must samples, which preserved high-quality DNA. Another reason could rely on the DNA extraction protocol, which was slightly different from the previous described. The inclusion of PVP in the extraction buffer helped to eliminate possible PCR inhibitors, such as polysaccharides, polyphenols, and tannins that are present in high concentrations in these types of samples. The fact that the DNA extracted from these samples presented a high quality can anticipate PCR amplification success. A similar approach was shown to be efficient when dealing with wine samples (Pereira et al. 2011a).

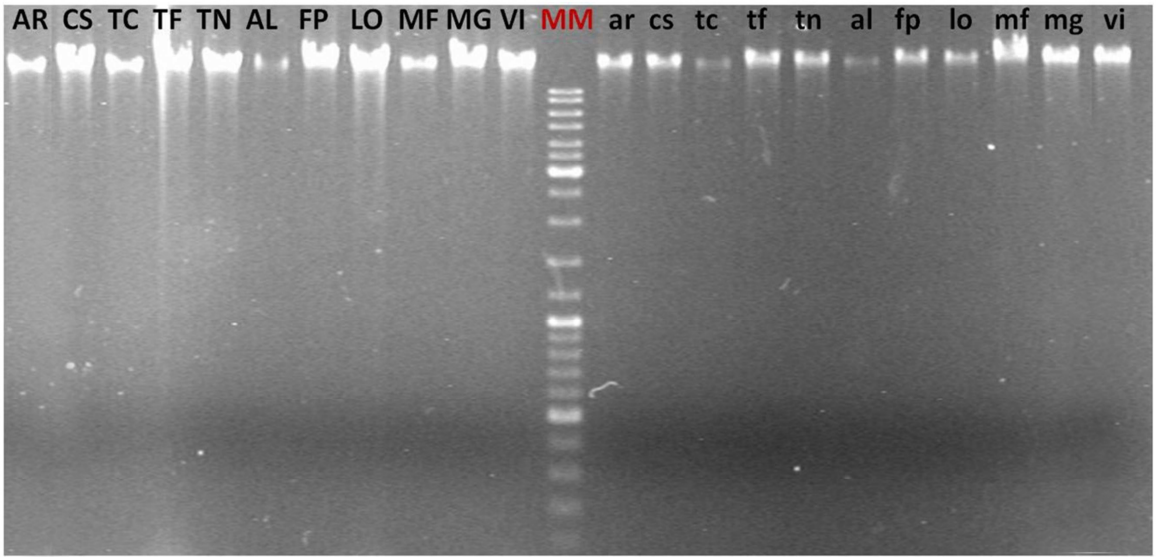

Fig. 2.1 DNA extracted from leaves and monovarietal musts. Uppercase corresponds to leaf, and lowercase corresponds to monovarietal must samples: lanes: AR/ar Aragonez; CS/cs Cabernet Sauvignon; TC/tc Tinto Cão; TF/tf Touriga Franca; TN/tn Touriga Nacional; AL/al Alvarinho; LO/lo Loureiro; FP/fp Fernão Pires; MF/mf Malvasia Fina; MG/mg Moscatel Galego; and VI/vi Viosinho and MM molecular marker GeneRuler^{TM} DNA ladder Mix 10 kbp (MBI Fermentas, Burlington, ON, Canada).

2.3.2 NUCLEAR SSR

The six nuclear markers selected were suitable and sufficient for differentiating the varieties studied. All monovarietal must samples were amplified by the six microsatellite primers, opposite to what was observed by Baleiras-Couto and Eiras-Dias (2006) and Garcia-Beneytez et al. (2002) where some must samples failed to amplify. Applying 45 cycles in the PCR reaction instead of 35 cycles used for leaf DNA samples increased the signal intensity in must DNA samples. The microsatellite alleles' size obtained in monovarietal must DNA samples was in accordance with the leaf DNA samples (Fig. 2.2) and with those referred to in the literature for the grapevine varieties under study (Almadanim et al. 2007, Veloso et al. 2010). The six nuclear SSR markers revealed a high level of polymorphism, with a total of 42 alleles, an average of seven alleles per primer (Table 2.1). The number of alleles per locus ranged from five (VrZAG79) to nine (VrZAG62), which is in accordance with the average number of alleles expected *per locus* (Cipriani et al. 2010). The *Ho* varied between 0.636 (VrZAG79; VVMD7) and 0.909 (VrZAG62; VVS2) while the He varied between 0.684 (VVMD7) and 0.861 (VrZAG62; VVMD27). Polymorphic information content (PIC) values ranged between 0.622 (VVMD7) and 0.800 (VrZAG62), VrZAG62 being the highest discriminative marker. All genetic parameters obtained by SSR data analysis were in accordance to literature (Almadanim et al. 2007, Cipriani et al. 2010, Ibañez et al. 2003, Lopes et al. 1999, 2006, Sefc et al. 2000).

Table 2.1 Genetic parameters calculated on data of 6 SSR *loci* in 11 samples considering grapevine leaf, and monovarietal must.

Locus	Samples	*No*	*PIC*	*He*	*Ho*
VVS2	11	7	0.759	0.827	0.909
VVMD5	11	7	0.770	0.835	0.727
VVMD7	11	7	0.622	0.684	0.636
VVMD27	11	7	0.799	0.861	0.818
VrZAG62	11	9	0.800	0.861	0.909
VrZAG79	11	5	0.640	0.727	0.636

No-number of alleles, *Ho*-observed heterozygosity, *He*-expected heterozygosity, *PIC*-polymorphic information content

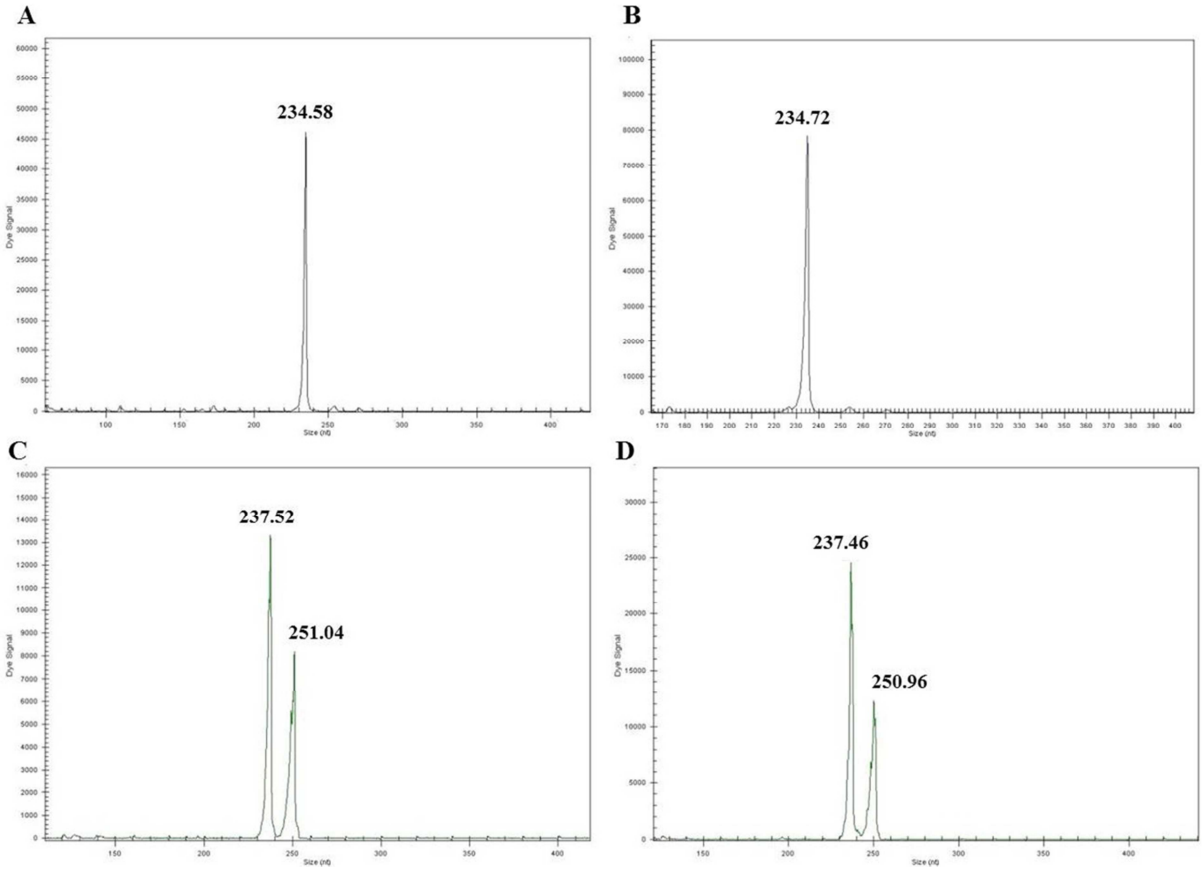

Fig. 2.2 Plots of the dye signal traces provided by CEQ 8000 Fragment Analysis Software using Tinta Roriz leaf (A, C), and monovarietal must (B, D) samples amplified with VVMD5 (A, B), and VVMD7 (C, D) microssatelite *loci*.

In this study, profiles generated by all microsatellite *loci* matched between samples from the same genotypes, reinforcing the view that nuclear SSR markers are suitable for certification purposes, as suggested by Baleiras-Couto and Eiras-Dias (2006), Savazzini and Martinelli (2006), and Siret et al. (2000, 2002).

2.3.3 INTER-SSR

The selection of the UBC primers was based on literature that referred to these primers as the most suitable for grapevine amplification (Herrera et al. 2002, Moreno et al. 1998). For ISSR analyses, all reproducible amplicons from leaf and monovarietal must DNA samples were considered. All selected ISSR primers contained dinucleotide repeats. Thus, considering only the bands present in the leaf samples, the average percentage of polymorphism obtained with (AC)n (UBC827, 841, 856, 857 and 889) (TG)n (UBC891), and (GA)n (UBC811) repeats was approximately 70%, whereas (CA)n (UBC817, 846, 888) and (GT)n (UBC849, 890) repeats presented an inferior percentage of polymorphism of 58% and 57%, respectively (Table 2.2).

Table 2.2 Total number and coincident bands found for each ISSR primer among grapevine leaf (L), and monovarietal must (M) samples. The number of polymorphic bands and percentage of polymorphism obtained per each ISSR primer was calculated considering only grapevine leaf samples (L).

Primer	Sequence 5'- 3'	No. of bands			No. of coincident bands	No. of polymorphic bands	% Polymorphism
		L	M	Total		(leaf)	(leaf)
807	$(AG)_8T$	17	12	18	11	10	59
809	$(AG)_8G$	19	17	19	17	14	74
811	$(GA)_8C$	10	12	12	10	7	70
817	$(CA)_8A$	8	9	9	8	3	38
827	$(AC)_8G$	16	18	19	15	12	75
841	$(AC)_8G$	14	12	15	11	8	57
846	$(CA)_8RT$	16	17	22	11	15	94
849	$(GT)_8YA$	12	11	12	11	7	58
856	$(AC)_8YA$	13	15	16	12	10	77
857	$(AC)_8YG$	16	18	21	12	12	75
888	$BDB(CA)_7$	21	21	21	21	9	43
889	$DBD(AC)_7$	23	21	23	21	15	65
890	$VHV(GT)_7$	22	22	22	22	12	55
891	$HVH(TG)_7$	23	19	23	19	16	70
Total		229	224	252	201	150	65

B=C/G/T;D=A/G/T;H=A/C/T;R=A/G;V=A/C/G;Y=C/T; UBC primers from University of British Columbia

A total of 252 reproducible ISSR fragments were scored; however, we only considered the leaf samples to calculate the percentage of polymorphism. The bands size ranged from 300 bp to 2.5 kbp in the leaf DNA samples and 120 bp to 1.5 kbp in the monovarietal must DNA samples. UBC889 and UBC891 primers presented the maximum number of bands (23), whereas primer UBC817 presented the lowest band number (eight). The number of polymorphic bands ranged from three

(UBC817) to 16 (UBC891). UBC846 primer presented the highest percentage of polymorphism (94%), whereas the lowest percentage was found for UBC817 primer (38%). The different profiles obtained through this molecular marker system can result in the DNA contamination from microorganisms, resulting in different bands amplified. Another constraint may be linked to the presence of PCR inhibitors that may favor certain fragments over others. Based on the data, a principal component analysis was performed (Fig. 2.3).

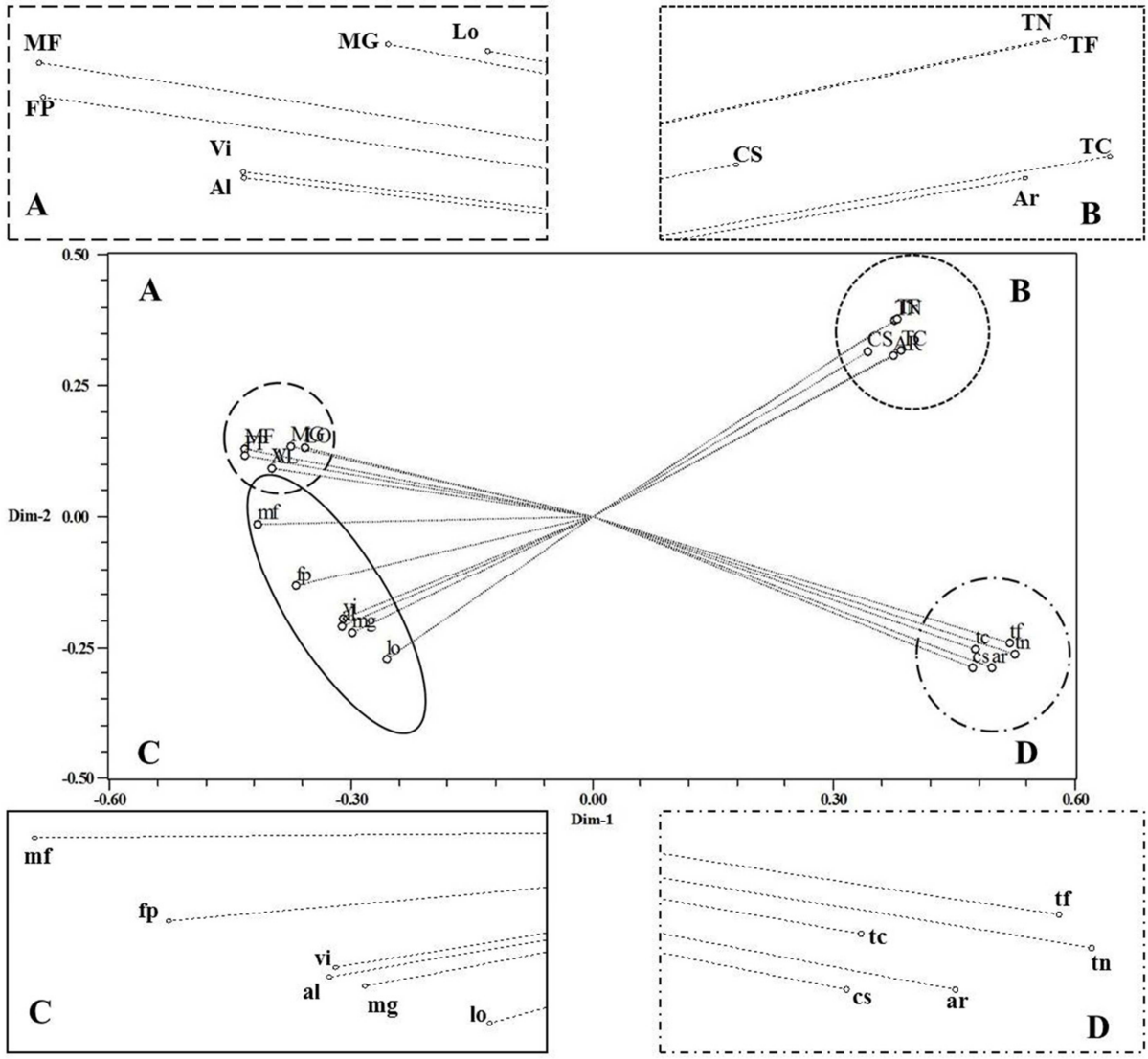

Fig 2.3 PCA performed with DCENTER among the grapevine leaf and correspondent monovarietal must samples. When an ISSR matrix is factored using the EIGEN program, the elements of the eigenvectors corresponding to positive eigenvalues are interpreted as the coordinates of each point in a Cartesian space (dotted lines represent Eigen-vectors). Uppercase corresponds to leaf, and lowercase corresponds to must samples: AR/ar Aragonez; CS/cs Cabernet Sauvignon; TC/tc Tinto Cão; TF/tf Touriga Franca; TN/tn Touriga Nacional; AL/al Alvarinho; FP/fp Fernão Pires; LO/lo Loureiro; MF/mf Malvasia Fina; MG/mg Moscatel Galego; and VI/vi Viosinho. Groups: a White grapevine leaf varietal samples; b red grapevine leaf varietal samples; c monovarietal white must samples; and d monovarietal red must samples.

It was not possible to find a total correspondence between leaf and monovarietal must samples due to the presence of high-molecular-weight bands amplified in leaf DNA samples but absent in must DNA samples. Nevertheless, the presence of low-molecular-weight bands in monovarietal must samples was also observed. The different behavior of the samples according to their origin lead to a distribution in the PCA graphic in four main groups: (a) leaf white grapevine variety samples, (b) leaf red grapevine variety samples, (c) white monovarietal must samples, and (d) red monovarietal must samples (Fig. 2.3). However, regarding white grapevine varieties, some of the high molecular weight bands were present in both leaf and monovarietal must samples, which justifies the higher proximity found between groups a and c (Fig. 2.3). The fact that the white must samples produce high-molecular-weight bands may be linked to the fact that they do not present as many PCR inhibitors as do red must samples. When A_{260nm}/A_{230nm} ratio is considered, a difference between white and red must samples is evident; white must samples present generally higher ratio values (1.51 to 2.26) than red must samples (1.40 to 2.13). Another interesting observation is that the A_{260nm}/A_{230nm} ratio values obtained in different DNA extraction from the same variety present similar values. This could be explained by the unique composition of each variety (Dimitrovska et al. 2011). Similar results were observed recently in wines (Pereira et al. 2011b). ISSRs have been widely applied to grapevine mainly to detect intravarietal differences and to assess genetic diversity and relationships among grapevine varieties (Dhanorkar et al. 2005, Herrera et al. 2002, Moreno et al. 1998, Sabir et al. 2008, Wu et al. 2009, Zietkiewicz et al. 1994). To our knowledge, for certification purposes, this marker system has only been applied in olive oil (Martins-Lopes et al. 2008), being this study the first approach to applying ISSR markers in monovarietal musts for this specific goal.

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Chapter 3

An Enhanced Method for *Vitis vinifera* L. DNA Extraction From Wines

An Enhanced Method for *Vitis vinifera* L. DNA Extraction From Wines

Adapted from:
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ABSTRACT

Wine market value largely depends on grape variety, which is of primary importance in wine identification. The aim of the present work was to enhance a wine DNA extraction protocol and, subsequently, grapevine variety identification. This enhanced method is an outcome from several previously developed extraction methods and effectively allows obtaining large amounts of high-quality DNA exhibiting an optimal $260_{nm}/280_{nm}$ ratio. Grapevine variety DNA extracted from wine was amplifiable with a specific SSR primer. This procedure was applicable for monovarietal and older commercial red and white wines. The potential of this enhanced method relies on its use for traceability as part of protecting both consumer and producer interests.

3.1 INTRODUCTION

Wine character and quality depend on grapevine variety, together with other factors such as *terroir* and winemaking technologies. Wine is usually made from one or more varieties of the European species *Vitis vinifera* L. Depending on the wine production region, especially if it is a Denomination of Origin (DO) wine, only a limited number of varieties are allowed. The inclusion of other varieties is only permitted under legally defined percentages. The fact that different grape varieties may be used in wine production is in itself an attraction for fraudulent practices. Scientific techniques and legislative guidelines have been developed for grape, must, and wine traceability to guarantee product origin and detect fraud and mislabeling.

Methods used for grapevine varietal identification or grape geographical origin determination comprise several features, such as must protein profiles (Gonzalez-Lara et al. 1989, Moreno-Arribas et al. 1999, Pueyo et al. 1993), anthocyanins (Garcia-Beneytez et al. 2003, Revilla et al. 2001), amino acids (Vasconcelos and Chaves das Neves 1989), aromatic compounds (Muñoz-Organero and Ortiz 1987), and chemical elements (Almeida and Vasconcelos 2003, Coetzee and Vanhaecke 2005, Monaci et al. 2003). These methods are time-consuming and influenced by various parameters such as soil composition, weather conditions, vinification methodologies, and wine aging. Grapevine DNA can be extracted from any part of the plant, although the preferred material is young leaves (Lodhi et al. 1994). Several studies have reported the ability to extract and genotype DNA from different grapevine products, including grape juice (Faria et al. 2000), grape must (Baleiras-Couto and Eiras-Dias 2006, Faria et al. 2008, Rodríguez-Plaza et al. 2006), experimental wines collected immediately after the end of fermentation (Baleiras-Couto and Eiras-Dias 2006, Garcia-Beneytez et al. 2002, Siret et al. 2002), and aged wine samples (Drábek et al. 2008, Nakamura et al. 2007, Savazzini and Martinelli 2006). Nevertheless, efficient DNA extraction and amplification from must and wine samples remains difficult. Previous studies hypothesized that these difficulties could be due to various processes involved in winemaking, such as decanting, clarification, and filtration, which completely remove grapevine DNA (Garcia-Beneytez et al. 2002).

Developing DNA extraction protocols using wine as a sample for grapevine varietal identification and/or differentiation is a worthwhile pursuit. The detection of *V. vinifera* variety by polymerase chain reaction (PCR) using wine DNA is hampered by the insufficient quantity and quality of template DNA obtained after extraction, given the degradation that plant DNA suffers during the fermentation process (Faria et al. 2000, Savazzini and Martinelli 2006). There are other constraint, including the interference of polysaccharides and polypeptides in beverages and the coexistence of pigment substances such as polyphenols, all of which interfere with or even inhibit DNA polymerase

during PCR (Garcia-Beneytez et al. 2002, Siret et al. 2000). Nevertheless, several studies have attempted to overcome these difficulties (Drábek et al. 2008, Nakamura et al. 2007, Savazzini and Martinelli 2006). The strategy selected for this study included gathering reported protocols and choosing the most suitable approach to define a DNA extraction protocol from wine that would be suitable for PCR amplification.

3.2 MATERIALS AND METHODS

3.2.1 DNA EXTRACTION

Leaves from *V. vinifera* varieties Tinta Roriz and Fernão Pires were obtained from the vineyards of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal, and used as a standard. An established CTAB method (Doyle and Doyle 1987) was used for leaf DNA extraction. Two monovarietal wines produced from Tinta Roriz (red) and Fernão Pires (white) varieties were collected at the end of the fermentation process. Three commercial blended red wines (2006 Torre de Ferro, 2005 Vinha do Côro Reserva, and 2004 Estremadouro Reserva) and three commercial blended white wines (2007 Porta da Ravessa, 2006 Caves Santa Marta, and 2005 Monte Novo) were purchased in a local market. The labels of these wines identified that Fernão Pires and Tinta Roriz varieties, among others (Tinta Barroca, Tinta Amarela, Touriga Franca, Roupeiro, Arinto, Malvasia Fina, Gouveio and Cerceal), were used in their production, although no information was given about the percentage of each variety.

The wine samples were precipitated in a plastic centrifugation tubes (35 mL), using 10 mL of sample and 0.7 vol of 2-propanol (Merck, Darmstadt, Germany) and maintained at -20 °C for 2 weeks, after which crude DNA was collected as a precipitate by 30 min of centrifugation at 4,000 *g* at room temperature (Hettich Universal Zentrifugen D-7200, Tuttlingen, Germany). The pellet was dissolved in 750 µL preheated extraction buffer [20 mM ethylenediaminetetraacetic acid (EDTA), 10 mM tris-(hydroxymethyl) aminomethane–hydrochloride (Tris-HCl) pH 8.0, 1.4 M sodium chloride (NaCl) and 2% (w/v) hexadecyltrimethylammonium bromide (CTAB; Sigma-Aldrich, St. Louis, MO), included just prior to use, 1% (v/v) 2-mercaptoethanol (Sigma-Aldrich), 2% (w/v) polyvinylpyrrolidone (PVP; Sigma-Aldrich), and Proteinase K (20 mg/mL; Sigma-Aldrich)] by briefly vortexing. The samples were incubated at 65 °C for 60 min. An equal volume of chloroform:isoamyl alcohol (24:1) (v/v) was added to the sample, followed by centrifugation at 13,000 *g* for 15 min at 4 °C (Biofuge Fresco Heraeus, Kendro Laboratory Products, Hanau, Germany). The upper layer was transferred to another tube and the samples were treated with RNase (10 mg/mL; MBI Fermentas, Burlington, Canada) at 37 °C for 30

min. DNA was precipitated with 0.6 vol of cold 2-propanol and incubated at -20 °C overnight. After precipitation, DNA was collected by centrifugation at 10,000 *g* for 15 min at 4 °C. DNA was dissolved in 300 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). An equal volume of neutral phenol (Sigma-Aldrich) was added and homogenized. The upper layer was transferred to another centrifuge tube after centrifugation at 13,000 *g* for 15 min at 4 °C. DNA was precipitated with 0.6 vol of cold 2-propanol and incubated at -20 °C overnight. After precipitation, DNA was collected by centrifugation at 10,000 *g* for 15 min at 4 °C. The supernatant was discarded and the DNA pellet was washed with buffer (76% ethanol, 10 Mm ammonium acetate) for 5 min. The DNA pellet was dried at room temperature, eluted in 50 µL TE, and maintained at -20 °C until use.

One commercial DNA extraction method, DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), five academic methods (Baleiras-Couto and Eiras-Dias 2006, Drábek et al. 2008, Nakamura et al. 2007, Rodríguez-Plaza et al. 2006, Savazzini and Martinelli 2006), and the protocol described in this study were used to test each of the commercial wine samples. These methods were compared in terms of wine sample starting volume, average DNA concentration, and total DNA extraction efficiency.

3.2.2 DNA QUANTIFICATION AND QUALITY

Nucleic acid concentration and extract quality was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). All measurements were repeated three times, presenting the average value.

3.2.3 GRAPE MICROSATELLITE ANALYSIS

Analyses of DNA extracted from grape leaf, monovarietal wine, and commercial wine samples were performed using VrZAG79 microsatellite *loci* (Sefc et al. 1999). Forward primer was labeled at the 5'-end with a specific fluorochrome (5–carboxy-fluorescein; 5-FAM) compatible with the Beckman analysis system (Beckman Coulter, Fullerton, CA), synthesized by Sigma-Genosys (Woodlands, TX).

3.2.4 PCR CONDITIONS

PCR reactions were performed in a 20 µL vol containing 10 × PCR buffer containing NH_4SO_2, 2.5 mM $MgCl_2$, 10 µM each of dATP, dCTP, dGTP, and dTTP, 0.5 U Taq polymerase (MBI Fermentas), 0.3 µM of each VrZAG79 primer, and 40 ng DNA extracted from leaves and monovarietal and

commercial wines. PCR was performed in a T Gradient 96 cycler (Whatman-Biometra, Göttingen, Germany). PCR conditions for DNA from leaves were as follows: initial denaturation at 95 °C/5 min, followed by 35 cycles with a temperature profile of 95 °C/20 s, 61 °C/30 s, and 72 °C/30 s, and a final extension step at 72 °C/5 min. For DNA analysis from monovarietal and commercial wines, 45 cycles were applied.

3.2.5 ALLELIC SIZE DETERMINATION

Separation of the amplified products was carried out through capillary electrophoresis on a Beckman Coulter automated sequencer with the help of internal size standards (CEQ DNA Size Standard Kit-400; Beckman Coulter) using the software package CEQ 8000 fragment analysis system (Beckman Coulter).

3.3 RESULTS and DISCUSSION

3.3.1 DNA EXTRACTION

A consistent wine DNA extraction method is the basis for any marker-based assessment of wine varietal composition. To overcome the difficulties found throughout the extraction process, the development or improvement of a DNA extraction protocol from wine is essential. As this is an improved protocol, it is necessary to justify some options. Initially, wine samples (commercial) were precipitated over several time scales: 24 and 48 hours and one and two weeks. The present protocol was applied to all precipitation times and results showed that for commercial white wines there was a pronounced decrease in DNA extraction efficiency (31%), while for commercial red wines the decrease was only 7% when comparing 25 hours and two weeks (Table 3.1). If in red wine samples no significant differences were found in terms of total DNA yields, regarding the time-scale used for precipitation, in what concerns PCR amplification there were clear differences found among white and red wine samples; all white wine samples were amplifiable by PCR, whereas red wine samples were only amplifiable when precipitated for two weeks. Thus, precipitations for two weeks were preferred to all other time-scales. The constitution and the concentration of the extraction buffer components varied widely among the protocols reviewed. Only one cationic detergent (CTAB) was chosen, for its capacity to dissolve the membranes and to increase DNA precipitation with high NaCl concentrations; CTAB also reduces sample polysaccharide contamination. DNase performance was inhibited by the addition of Tris-HCl and EDTA. The β-Mercaptoethanol was chosen to promote

protein denaturation and to eliminate polyphenols. PVP was used because of its antioxidant effect and ability to eliminate polysaccharides.

Table 3.1 Wine precipitation in 2-propanol over 2 weeks, 1 week, 48 hours, and 24 hours in white and red commercial wines. The results refer to genomic DNA concentration and extraction efficiency, considering average values.

Samples	[DNAg] ng/µL	Extraction Efficiency (ng)
2 weeks		
White Commercial wine	229	11450
Red Commercial wine	481	24050
1 week		
White Commercial wine	200	10000
Red Commercial wine	483	24150
48 hours		
White Commercial wine	189	9450
Red Commercial wine	480	24100
24 hours		
White Commercial wine	157	7850
Red Commercial wine	447	22350

The concentrations used in the present protocol were adapted to sample characteristics. The protocol involves the addition of chloroform:isoamyl alcohol and includes phenol and proteinase K to facilitate the separation of chromatin proteins. The present protocol was compared to six other DNA extraction methods (Table 3.2). The Qiagen DNeasy Plant Kit was not adequate for the wine matrix, and although the results are shown, they are not referred to. The protocol described in this study presented the highest yield and DNA concentrations, even though it began with the smallest volume sample. The present protocol needs only a 10 mL wine sample for DNA extraction, a great advantage when compared with other methods that require a sample volume ranging from 30 to 400 mL (Baleiras-Couto and Eiras-Dias 2006, Drábek et al. 2008, Nakamura et al. 2007, Savazzini and Martinelli 2006). Additional information regarding A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} ratios are supplied (Table 3.2). Once again the present protocol presents the optimal values for DNA quality (Table 3.2), which may explain why the samples are amplifiable, as none of the other samples with low A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} ratios were amplifiable by PCR. In terms of total extraction time, this method describes entirely the steps required. In all the protocols available in the literature, some specifications are not given (*e.g.*, incubation, precipitation, centrifuge, and washing times), and for

that reason the extraction time is variable and cannot be accurately determined. Therefore, comparison between methods is not possible.

Table 3.2 Comparison of seven different DNA extraction methods, evaluating initial quantity of wine, DNA concentration average, 260/280 and 260/230 ratios, and total DNA yield.

Method	Starting volume (mL)	[DNA]g (ng/µL)	260/280 ratio	260/230 ratio	Extraction Efficiency (ng)
(Baleiras-Couto and Eiras-Dias 2006)	400	25.7	1.34	0.36	2570
(Rodríguez-Plaza et al. 2006)	15	13.2	1.23	0.29	264
(Drábek et al. 2008)	40	11.5	1.19	0.18	575
(Nakamura et al. 2007)	30	148.4	1.37	0.32	4452
(Savazzini and Martinelli 2006)	45	98.3	1.43	0.38	4915
Qiagen – DNeasy Kit plant	2	1.6	0.98	0.34	80
Present protocol	10	268.2	1.74	1.79	13410

3.3.2 QUANTIFICATION AND PURITY OF DNA TEMPLATES

The measurements performed with the NanoDrop spectrophotometer revealed high genomic DNA concentrations and purity of all samples extracted from grapevine leaf and monovarietal and commercial blended wines (Table 3.3).

Table 3.3 Nucleic acids quantification and extract purity evaluation from grapevine leaf and monovarietal and commercial wine samples.

Samples	[DNA] ng/µL	260/280 ratio	260/230 ratio
Leaves			
Tinta Roriz	716	1.93	2.10
Fernão Pires	981	1.94	2.12
Monovarietal wines			
Tinta Roriz	465	1.71	1.94
Fernão Pires	343	1.79	1.98
Red Commercial wines			
Torre de Ferro-2006	452	1.81	1.79
Vinha do Côro Reserva-2005	330	1.71	1.81
Estremadouro Reserva-2004	260	1.73	1.77
White Commercial wines			
Porta da Ravessa-2007	206	1.74	1.66
Caves Santa Marta-2006	197	1.72	1.97
Monte Novo-2005	164	1.73	1.82

This study therefore demonstrates that DNA from *V. vinifera* remains available in wine samples even after fermentation and other winemaking processes, given that wine samples were either collected at the end of fermentation process or two to six years after bottling, contrary to previous reports (Baleiras-Couto and Eiras-Dias 2006, Drábek et al. 2008, Garcia-Beneytez et al. 2002, Nakamura et al. 2007, Savazzini and Martinelli 2006, Siret et al. 2002).

Phenol is frequently used in DNA extraction methods and increases contamination risk. Nevertheless, this reagent has a maximum absorbance of A_{270} to A_{275nm}, which is close to that of DNA, and phenol contamination at times mimics both higher yields and purity because of an upward shift in the A_{260nm} value, giving misleading results. Other contaminants such as polysaccharides, proteins, solvents, and salts are also present and absorbed at A_{280}, A_{270}, and A_{230nm}. Considering the toxicity and contamination properties of phenol, it was eliminated from the DNA extraction protocol in a first approach, but the results revealed both poor DNA yield and quality. Thus, phenol extraction was shown to be crucial for good DNA extraction from wine samples. The UV spectra performed with the NanoDrop spectrophotometer on two samples of genomic DNA extracted from wine, one presenting phenol contamination and other using the method reported here without contamination, demonstrated that the enhanced protocol overcame the phenol contamination problem (Fig. 3.1).

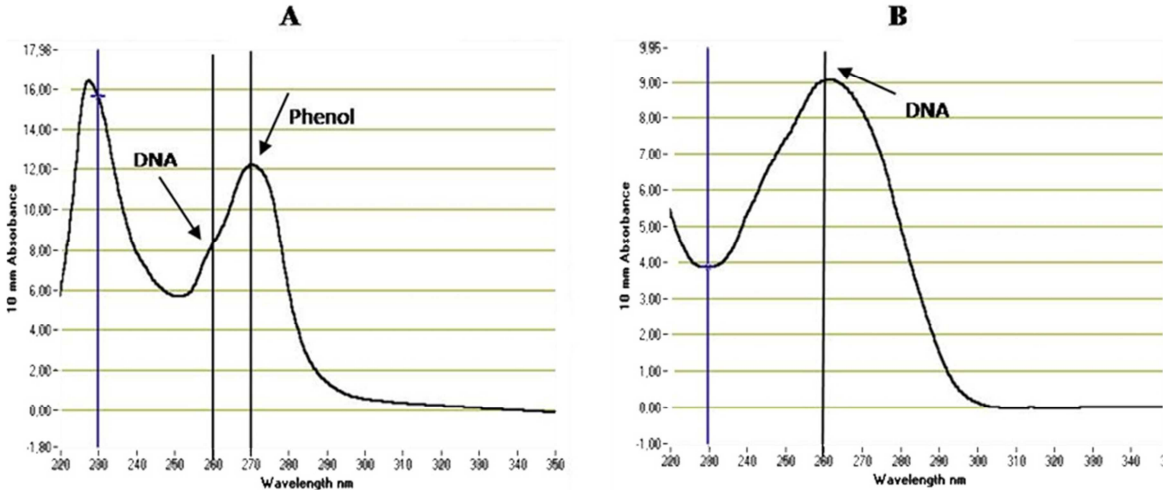

Fig. 3.1 UV spectrum performed by NanoDrop ND-1000 spectrophotometer showing (A) phenol contamination and (B) the absence of phenol and/or other contaminations in DNA wine samples.

The UV spectrum also showed that other contaminations usually present when DNA is extracted from these matrices were removed efficiently from the DNA template, supported by the DNA purity from monovarietal and commercial wine samples, which presented values between 1.71 and 1.81 for red wines and between 1.72 and 1.79 for white wines (Table 3.3).

3.3.3 Amplification of genomic DNA from wine

PCR reaction can be inhibited by several factors. In wine samples, the presence of phenolic compounds and polysaccharides are the main cause of PCR impasse (Garcia-Beneytez et al. 2002, Siret et al. 2000, 2002). Other difficulties related to PCR failure are the small DNA quantities and its degradation status (Drábek et al. 2008, Savazzini and Martinelli 2006). Several strategies have been adopted to overcome these problems, such as improving DNA extraction methods and the use of chloroplast microsatellite markers in wine (Baleiras-Couto and Eiras-Dias 2006) and real-time PCR (Drábek et al. 2008, Savazzini and Martinelli 2006). In this protocol, both concentration and purity were high. The combination of 2-propanol precipitation, enzyme treatment, phenol and chloroform extraction, and several washes was positive and favorable for PCR reaction, eliminating all possible contaminants found in both white and red wine samples.

In order to confirm the presence of *V. vinifera* DNA, a suitable primer combination for the PCR reaction was selected. The first condition for primer selection was that it would not amplify the DNA of microorganisms responsible for alcoholic and malolactic fermentation, such as yeasts and lactic acid and/or acetic acid bacteria, and be specific for grapevine varietal identification. Although six SSR nuclear primers have been accepted as universal markers for grapevine genotyping (GENRES 081 research project; www.genres.de/*Vitis*/*Vitis*.htm), only one primer was chosen (VrZAG79), given that the main purpose of this study was to prove that the improved DNA extraction method was efficient and the extracted DNA was suitable for PCR amplification of specific *V. vinifera* DNA segments. All samples amplified successfully (Fig. 3.2).

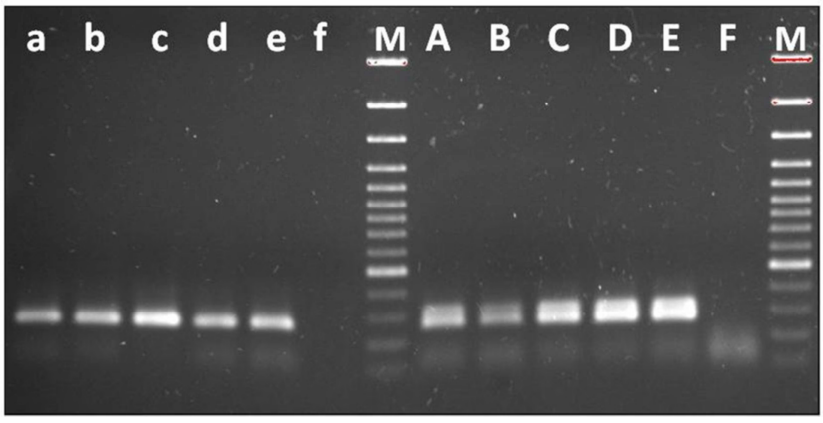

Fig. 3.2 Amplification profile obtained with VrZAG79 nuclear primer. Lanes a, b, c: commercial white wines; d: monovarietal white wine; e: Fernão Pires variety (leaf), f: negative control. Lanes A, B, C: commercial red wines; D: monovarietal red wine; E: Tinta Roriz (leaf); F: negative control; M: molecular marker GeneRuler 100 bp DNA Ladder (MBI Fermentas).

The microsatellite VrZAG79 allele sizes were obtained on an automatic sequencer and are expressed in base pairs (Table 3.4). The microsatellite allelic sizes found in the monovarietal wine samples (red and white) correspond to those detected in the leaf samples.

Table 3.4 Microsatellite genotype using VrZAG79 nuclear locus, expressed as the alleles size and size range, in base pairs obtained with DNA from grape leaves, monovarietal wines and commercial wines.

Samples	Microssatelite VrZAG79	
	Allele sizes (bp)	Size range (bp)
Leaf		
Tinta Roriz	245:249	---
Fernão Pires	245:245	---
Monovarietal wine		
Tinta Roriz	245:249	---
Fernão Pires	245:245	---
Red Commercial wines		
Torre de Ferro- 2006	---	243-251
Vinha do Côro Reserva-2005	---	243-251
Estremadouro Reserva-2004	---	243-249
White Commercial wines		
Porta da Ravessa-2007	---	245-251
Caves Santa Marta-2006	---	245-251
Monte Novo-2005	---	243-247

For commercial blended wines, a size range was found instead of a specific allelic size, which is acceptable for commercial wines, given that several grapevine varieties were used in their production. Furthermore, VrZAG79 profiles provided by the automatic sequencer for monovarietal and commercial blended red (Fig. 3.3) and white (Fig. 3.4) wine samples are presented. Apart from the expected alleles (Tinta Roriz in red wines and Fernão Pires in white wines), the commercial blended wines presented other peaks, possibly belonging to other varieties present in these blends. It was not possible to associate the intensity of the peaks (alleles) with the relative proportion of each variety in the wine, since they are unknown. Several authors have reported PCR amplification from experimental wine samples collected immediately after fermentation (Garcia-Beneytez et al. 2002, Siret et al. 2002), from stabilized wines 8 months after fermentation and ready for bottling (Baleiras-Couto and Eiras-Dias 2006), and from 24-month-old monovarietal wines (Savazzini and Martinelli 2006). Nevertheless, difficulties were found in all of the reports when amplifying genomic DNA due to low DNA quantity and integrity. To our knowledge, this is the first report on PCR amplification using DNA extracted from 6-year-old bottled wines.

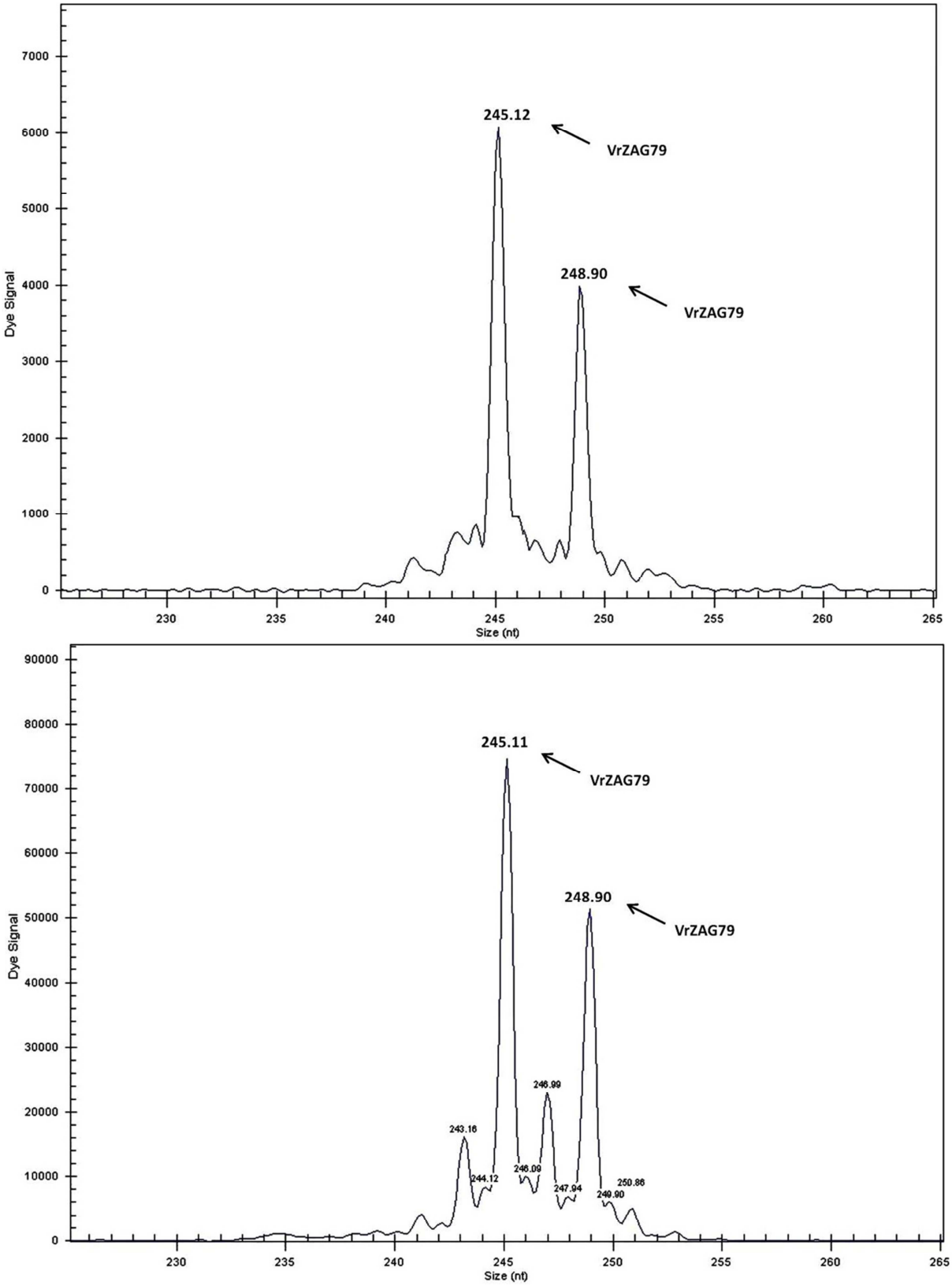

Fig. 3.3 Plots of the dye signal traces provided by CEQ 8000 Fragment Analysis Software for microsatellite amplification of DNA at VrZAG79 *loci* for monovarietal (up) and commercial (bottom) red wine using Tinta Roriz variety.

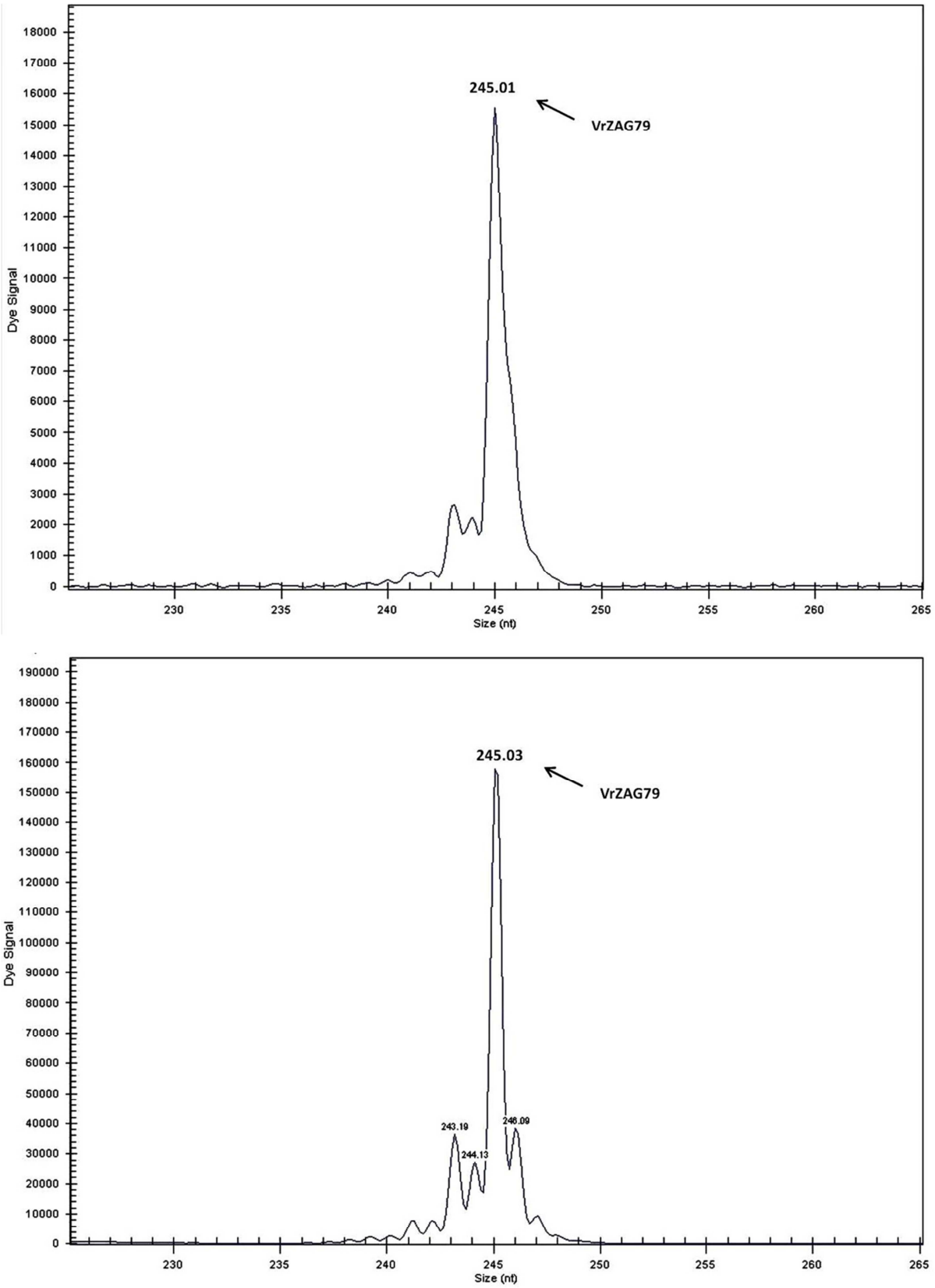

Fig. 3.4 Plots of the dye signal traces provided by CEQ 8000 Fragment Analysis Software for microsatellite amplification of DNA at VrZAG79 *loci* for monovarietal (up) and commercial (bottom) white wine using Fernão Pires variety.

3.4 CONCLUSIONS

This study reports on an efficient *V. vinifera* DNA extraction method from wine samples suitable for amplification. The main steps for a successful DNA extraction protocol from wine were 2-propanol precipitation, enzyme treatment, phenol and chloroform extraction, and several washes. Although these steps were performed equally on red and white wine samples, they should be strictly followed for red wine samples. With white wine samples the precipitation time can be reduced significantly without affecting PCR efficiency.

In regard to previously published data in the literature, the method described here presents several advantages: type of sample—allows for efficient extraction and consequent amplification of genomic DNA extracted from wines after several years of bottling; initial volume of sample allows for grapevine DNA extraction from a wine sample volume of only 10 mL, which is advantageous compared to the volumes presented in the current literature; DNA quantity and purity quantification of genomic DNA extracted from wine samples revealed a high concentration and purity level, thereby showing that *V. vinifera* DNA remains after the entire vinification process; PCR reaction inhibition removes all possible contaminants, providing an unambiguous amplification profile; and all DNA samples were amplifiable with a specific marker, thereby demonstrating that the DNA corresponded to a grapevine variety and excluding the hypothesis of belonging to a microorganism.

Wine quality, value, and price depend on several factors, but one of the main characteristics is grapevine variety. Thus, it is important that consumers trust wine labeling. An efficient traceability system is imperative. In the wine market the correspondence between the final product and grapevine varieties may be established through molecular marker technology, which requires a reliable and reproducible DNA extraction method. This protocol can provide the basis for a successful traceability methodology to guarantee product origin and to detect fraud and mislabeling. DNA extraction is the most relevant step for further analysis, such as *V. vinifera* detection in wine.

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Chapter 4

Vitis vinifera L. SNP Detection With High Resolution Melting Analysis Based on the *UFGT* Gene

Vitis vinifera L. SNP Detection With High Resolution Melting Analysis Based on the *UFGT* Gene

Adapted from:
Pereira L, Martins-Lopes P (2015) *Vitis vinifera* L. Single-Nucleotide Polymorphism Detection with High Resolution Melting Analysis Based on the UDP-Glucose:Flavonoid 3-O-Glucosyltransferase Gene. Journal of Agricultural and Food Chemistry (*published in the web:* DOI:10.1021/acs.jafc.5b03463)

ABSTRACT

Vitis vinifera L. is a species with a large number of varieties, which differ in terms of anthocyanin content. The genes involved in the anthocyanin biosynthesis pathway have a direct effect in the anthocyanin profile of each variety, being potentially interesting for varietal identification. The current study aimed at the design of an assay suitable for the discrimination of the largest number of grapevine varieties. Two genes of the anthocyanin pathway, chalcone isomerase (*CHI*) and UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*), were sequenced in 22 grapevine varieties. The *CHI* gene presented 5 SNPs within the sequence. A total of 58 SNPs and 1 INDEL were found among the *UFGT* gene, allowing the discrimination of 18 different genotypes within the 22 grapevine varieties. A HRM assay designed for *UFGT*, containing 704 bp, produced differentiated melting curves for each of the 18 haplotypes. The developed HRM assay is efficient in grapevine varietal discrimination.

4.1 INTRODUCTION

In the Vitaceae family, the *Vitis* genus is agronomically very important. Within this genus, the only European species, *Vitis vinifera* L., represents one of the oldest domesticated plants and is extremely relevant in the wine industry. The high adaptability of the *V. vinifera* species to different environmental conditions (Dal Santo et al. 2013) makes it difficult to unequivocally identify the grapevine varieties. The traditional methods used in the identification and differentiation of grapevine varieties, based on ampelography and ampelometry, are dependent on the plants' phenology, which is influenced by environmental, phytosanitary, and nutritional conditions. Nowadays there are several molecular marker based methods developed to guarantee grapevine variety identification, and they have been extended to must and wine samples, where morphological characterization is not applicable (Pereira et al. 2011). Among the main molecular marker systems, simple sequence repeat (SSR) markers represent one of the most suitable genetic tools currently adopted by the international scientific community to define a grapevine variety (OIV 2009). Recently, on the basis of the whole genome sequencing of the 12X *V. vinifera* PN40024/reference genome (Jaillon et al. 2007), sequence-based molecular markers, as single-nucleotide polymorphism (SNP), have been generated. According to Cabezas and collaborators (2011), SNP markers present several advantages concerning varietal identification, namely: (1) mostly biallelic; (2) abundant throughout the genome; (3) relatively stable during evolution; and (4) low mutation rate. Furthermore, SNPs can be easily reproduced between laboratories and when using different detection methodologies, because the different alleles are not distinguished on the basis of size but on the nucleotide presence at a given position. These features, in combination with their high availability, make SNPs the most popular marker system for several genetic analyses. High resolution melting (HRM) represents a method that enables the genotyping of SNPs in a large number of samples. The principle of HRM analysis is based on the generation of different melting curve profiles due to the sequence variation present in the double-stranded DNA. Single-nucleotide changes represent the smallest genetic variation and are divided into four classes, distinguished by the different melting temperature (Tm) shifts they produce. SNP class 1 involves C/T and G/A, and SNP class 2 involves C/A and G/T, base exchanges that are easily genotyped by HRM due to Tm differences >0.5 °C (Liew et al. 2004). In contrast, in SNP class 3, only C/G base exchange occurs, and SNP class 4 is described by A/T base exchange, producing very small Tm differences (<0.4 °C for SNP class 3 and <0.2 °C for SNP class 4) (Pietzka et al. 2009). The two major applications of HRM are targeted genotyping (Han et al. 2012) and gene scanning (Erali and Wittwer 2010). In *Vitis*, HRM was applied in grapevine variety

identification using various microsatellites (Mackay et al. 2008). The SNP identification in functional genes can not only constitute an advantage for varietal identification but also can be further used in functional studies. The berry color phenotype has been thoroughly studied, in recent years (Cardoso et al. 2012, Castellarin and Di Gaspero 2007). The anthocyanins are responsible for grapevine berry color, varying largely in concentration and composition depending on the grapevine variety (Mattivi et al 2006). Grapevine varieties with white-colored berries do not synthesize anthocyanins (Boss et al. 1996). The anthocyanin biosynthetic pathway has been well characterized and is genetically determined by structural and regulatory genes. The structural genes are regulated at the transcriptional level by regulatory genes, and thus plant pigmentation patterns are mainly controlled by the expression profiles of regulatory genes (Grotewold 2006, Holton and Cornish 1995, Velasco et al. 2007). The anthocyanin biosynthetic pathway includes genes encoding the enzymes chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3'-hydroxylase (F3H), dihydroflavonol reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), and UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT), which were partially cloned in grapevine by Sparvoli et al. (1994). The *CHI* (X75963) and *UFGT* (AF000372) genes have been identified in *V. vinifera* and are located in chromosomes 13 and 16, respectively. Despite their localization in the anthocyanin pathway, UFGT and CHI are considered key enzymes (Kuhn et al. 2014). CHI leads to the formation of flavanones in a central branching point of the flavonoid pathway (Grotewold 2006); on the other hand, UFGT is located in a final position of the anthocyanin pathway and is associated with the accumulation of anthocyanins in the berries' skin (Cutanda-Perez et al. 2009, Kobayashi et al. 2001, Zheng et al. 2013). The *CHI* gene and the remaining genes (*PAL*, *CHS*, *F3H*, *DFR*, *LDOX*) are expressed in both white and red grapevine varieties. UFGT is expressed only in red grapevine varieties, and its expression is crucial for anthocyanin biosynthesis (Boss et al. 1996, Zheng et al. 2013). The aim of this study was to develop a new HRM assay for grapevine varietal identification based on the detection of SNPs, insertions, and/or deletions (IN/DELs) in two genes involved in the anthocyanin pathway.

4.2 MATERIAL AND METHODS

4.2.1 VITIS VINIFERA L. SAMPLES

Twenty-two *V. vinifera* grapevine varieties (red and white) considered as the most ancestral genotypes used in wine production in Portugal, including both national and international accessions, were used. Young leaves of each grapevine variety (Table 4.1) were collected from the Sogrape Vinhos S.A. and Real Companhia Velha vineyards located in the Douro region and immediately frozen

in liquid nitrogen. In order to validate varietal genotype young leaves belonging to the clones of each grapevine variety were collected and immediately frozen in liquid nitrogen. The plants were sampled in established clonal field belonging to the Sogrape enterprise, to the Sociedade Borges S.A., the Direcção Regional de Agricultura e Pescas do Norte, and to the National Ampelographic Collection (CAN), located at Dois Portos in the National Vitivinicultural Research Station (EVN-INIAV) including samples of different regions of the country.

Table 4.1 List of 22 grapevine varieties used for SNP identification, corresponding code and berry color.

Grapevine variety name	Code	Berry color
Alicante Bouschet	AB	Red
Cabernet Sauvignon	CS	Red
Chardonnay	Ch	White
Côdega do Larinho	CL	White
Donzelinho Tinto	DT	Red
Fernão Pires	FP	White
Gouveio	Gou	White
Merlot	M	Red
Malvasia Fina	MF	White
Moscatel Galego	MG	White
Pinot Noir	PN	Red
Rufete	Ruf	Red
Sousão	Sou	Red
Tinta Amarela	TA	Red
Tinta Barroca	TB	Red
Touriga Brasileira	TBr	Red
Tinto Cão	TC	Red
Touriga Franca	TF	Red
Tinta Francisca	TFi	Red
Touriga Nacional	TN	Red
Tinta Roriz	TR	Red
Viosinho	Vio	White

4.2.2 GENOMIC DNA EXTRACTION

Total genomic DNA was extracted from frozen young leaves using the CTAB method (Doyle and Doyle 1990). Each DNA sample was eluted in 100 µL of 0.1X TE buffer (100 mM Tris-HCl, 0.1 mM EDTA, pH 8), and the purity, integrity, and quantity of all DNA samples were estimated on Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) measurements and by electrophoresis on 0.8% agarose in 1X Tris−acetate−EDTA (TAE).

4.2.3 SNP Detection: Primer Design, PCR Assays, and PCR Product Sequencing

For SNP detection, single-copy genes are preferred over multicopy to avoid problems associated with paralog genes. In *Vitis*, both *UFGT* and *CHI* genes are single-copy genes; however, the number of *CHI* genes is dependent on the species (Przysiecka et al. 2015, Shimada et al. 2003, Velasco et al. 2007).

The National Center for Biotechnology Information database (NCBI) was used to find sequences related to these genes using *V. vinifera* genome database (VvGDB). The *UFGT* and *CHI* genes were amplified using primers fwdUFGT (5'-ATGTCTCAAACCACCACCAAC-3') and revUFGT (5'-CCGGGAAACCTTTATTTTCA-3') (Fig. 4.1) and fwdCHI (5'-TATCCCCGAAGATGTCTCCA-3') and revCHI (5'-AAAGTGTCCCGGATGTTAC-3') generating 1500 and 1725 bp amplicons, respectively. Primers were designed in Primer3 software, considering the following parameters: size (between 18 and 22 bases in length), melting temperature (T_m) in the range of 50–60 °C; a % GC between 40 and 60, and absence of dimerization capability and of significant hairpin formation (>3 bp). The PCR reactions were performed in a 20 µL volume, containing 20 ng of genomic DNA, 1X PCR buffer, 25 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, and 0.3 U of *Taq* DNA polymerase (Roche). The reactions were incubated at 94 °C for 3 min, followed by 30 cycles of 94 °C/1 min, 58 °C/1 min, 72 °C/2 min, and a final step of 72 °C for 10 min.

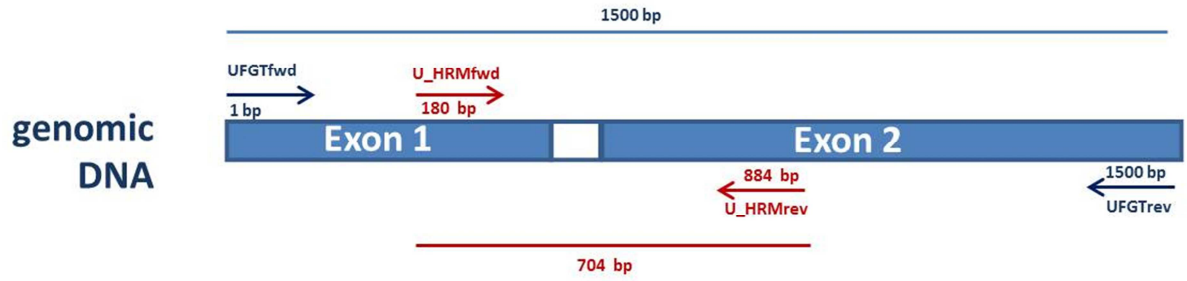

Fig. 4.1 Schematic representation of the primer set used in the *UFGT* gene. The empty space corresponds to the intron.

PCR amplicons resulting from the amplification of *UFGT* and *CHI* genes were directly sequenced (STAB VIDA; http://www.stabvida.com), and the genotypes of the 22 grapevine varieties were obtained. Sequence alignment was performed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The alignments of reverse and forward sequences were applied to produce consensus sequences. The sequences of each individual DNA fragments were aligned with the original sequence to identify the SNP presence (see Additional file 1).

4.2.4 High Resolution Melting Assay Design

HRM analysis was performed in a specific 704 bp fragment of the UFGT gene using primers U_HRMfwd (5'-GCAATGTAATATCAAGTCC-3') (starting at 180 bp) and U_HRMrev (5'-TTTCTTTCTTTGAGCCATT-3') (ending at 884 bp) (Fig. 4.1). PCR and HRM analyses were performed in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 µL containing the respective primer pair (5 pmol of each primer), 20 ng of gDNA, and the MeltDoctor HRM Master Mix (Applied Biosystems). PCR/HRM included an initial step of 10 min at 95 °C and 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. The melting curve was obtained continuously, performed as follows: 30 s at 95 °C, 1 min at 65 °C, 15 s at 65 °C, rising 0.3 °C/s, and 15 s at 95 °C. All reactions were performed in triplicate. High Resolution Melt Software v3.0.1 (Applied Biosystems) was used to analyze the data. After normalization and determination of the temperature shift, the different melting curves of the several plots were generated. To validate the reference HRM profile for each grapevine variety, DNA from the clones was analyzed using the developed HRM assay, using the previously described conditions.

4.2.5 Phylogenetic Analysis

Predicted amino acid sequences were used for phylogenetic analysis using COBALT software. COBALT is a constraint-based multiple protein alignment tool that finds a collection of pairwise constraints derived from conserved domain database, protein motif database, and sequence similarity, using RPS-BLAST, BLASTP, and PHI-BLAST. Pairwise constraints are then incorporated into a progressive multiple alignment. (http://www.stva.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi; Papadopoulos et al. 2007).

4.3 RESULTS AND DISCUSSION

4.3.1 SNP Identification

The *CHI* gene is located in a central position in the anthocyanin pathway. This gene was sequenced in 22 grapevine varieties (14 Portuguese and 8 international), revealing the existence of only 5 SNPs among all of these varieties (data not shown). The low number of SNPs detected within this gene is in accordance with previous studies, making it a noninteresting marker for varietal

identification purposes (Cardoso et al. 2012, Salmaso et al. 2005). On the contrary, the *UFGT* gene revealed to be highly polymorphic, presenting a high number of SNPs among the grapevine varieties studied. The *UFGT* gene length covered 1500 bp, and within this region a total of 58 SNPs and an insertion were detected (Table 4.2). Eighteen SNPs and the insertion were found within the exon 1 region (493 bp) with an average frequency of 1 SNP/25.9 nucleotides. Four SNPs were found in the intron (74 bp) with an average frequency of 1 SNP/18.5 nucleotides. The remaining 36 SNPs were positioned within the exon 2 region (881 bp) with an average frequency of 1 SNP/24.5 nucleotides. These results are interesting because the number of SNPs detected within the coding region is high, contrary to previous studies (sunflower (Kolkman et al. 2007), cotton (An et al. 2008) and grapevine (Riahi et al. 2013) where the majority of the SNPs were located in the noncoding region of the genes. The high SNP frequency found within this gene is particularly interesting because *UFGT* has been previously associated with several different types of anthocyanins (Cardoso et al. 2012) which are directly involved in berry skin color and grape organoleptic characteristics.

4.3.2 High Resolution Melting Curve PCR Analysis

The *CHI* gene was not considered for the HRM approach because the number of SNPs found did not allow the discrimination of the varieties under study. The HRM assay was designed for *UFGT* gene considering a fragment size of 704 bp. The *UFGT* HRM fragment included 32 of the 58 SNPs present and the detected insertion, considering the 22 grapevine varieties under study, with an average frequency of 1 SNP/22 nucleotides. Considering the same fragment length, Nicolè et al. (2013) reported a frequency of 1 SNP/31.69 nucleotides, revealing that this gene is highly polymorphic and, therefore, interesting for varietal identification. Fragment size influences the sensitivity of subsequent HRM analysis, and it is usually advisable that the size does not exceed 300 bp length (Druml and Cichna-Markl 2014). In addition, long amplicons may contain several melting domains, resulting in complex melting profiles, meaning that longer amplicons represent small differences in the melting curve caused by small sequence variation. In plants, the studies using HRM report a PCR amplicon range of 50−260 bp (De Koeyer et al. 2010, Han et al. 2012, Ó Lochlainn et al. 2011, Xanthopoulou et al. 2014). To our knowledge this is the first paper considering such a large fragment size (704 bp). Therefore, this method is quite promising as it broadens the genotyping potential using HRM analysis. The UFGT targeted sequence generated 18 different melting curve profiles (Fig. 4.2). The T_m values found within all of the samples were similar (T_{m1} = 82.7−83.0 °C; T_{m2} = 86.6 − 86.9 °C), which did not distinguish the different haplotypes. However, the shapes of the

melting curves were very informative, and even when the grapevine variety sequence differed by only a single SNP, this variation was detectable for (a) CS, Sou, and Vio and (b) FP, TR, and MF grapevine varieties (Table 4.2).

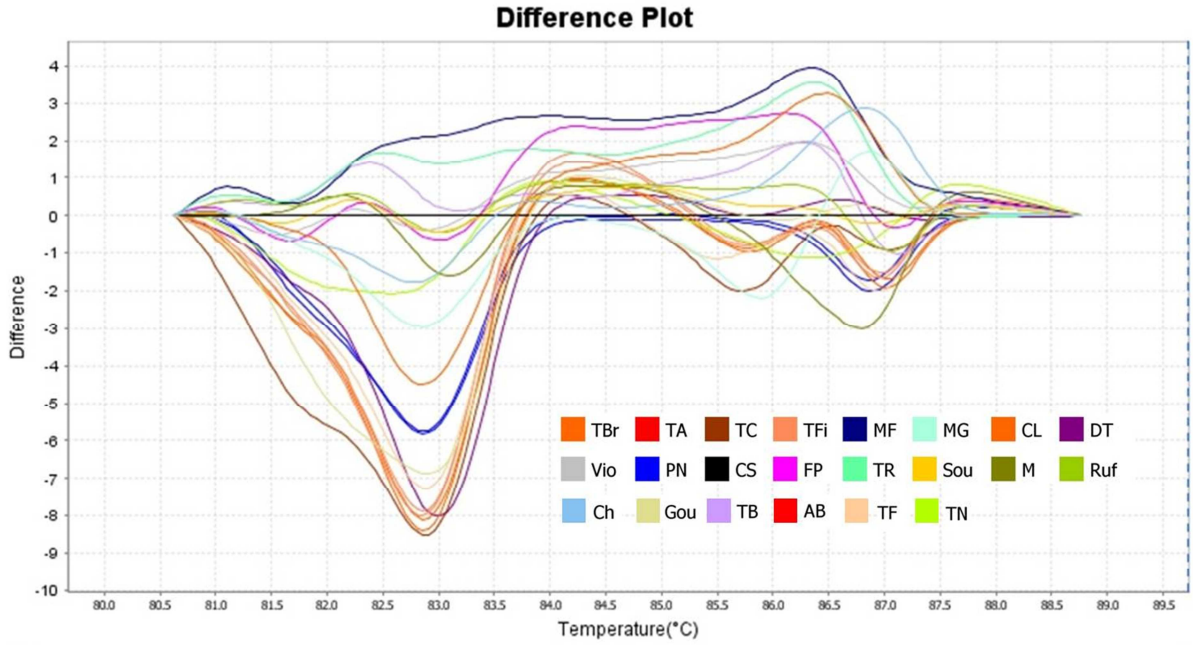

Fig. 4.2 HRM difference plot of 22 grapevine varieties for UFGT fragment based on the shape of the melting curves. 18 different genotypes were found since AB, TFi, TA and CL grapevine varieties present the same profile.

Additionally, all of these SNPs belong to class 4, A/T, which is the most challenging genotype variation mainly due to the difficulty associated with the difference in T_m (Pietzka et al. 2009). These results demonstrate the power, sensitivity, and specificity of this particular HRM assay that allows the identification of several complex genotypes and subsequently the detection of different melting curves (Fig. 4.2). The assay was successful in the definition of the several haplotypes since it was based on the combination of a high number of SNPs within the amplified fragment. Even though the assay is based on a long fragment, this HRM assay proved to be highly sensitive and was able to distinguish 18 haplotypes based on a combination of 33 nucleotide differences in a unique assay. Previous studies reported the detection of several SNPs and INDELs but always considering simple events (De Koeyer et al. 2010, Emanuelli et al. 2014, Han et al. 2012, Li et al. 2010, Yan et al. 2012) and not a combination of nucleotide differences. Never, until now, has it been reported that by using a unique assay was it possible to detect such a high number of events in one reaction. The designed HRM assay proved to be powerful and specific. The shape of the HRM curve for each grapevine variety needed to be further validated in relation to their varietal specification, since there are a high number of clones available within each grapevine variety (Ocana et al. 2013). Therefore, DNA samples from different clones of each grapevine variety were used and tested for reproducibility.

The plants were sampled in established clonal field collections, maintained jointly in private and public institutions, including samples of different regions of the country. The melting curve profiles obtained for each variety were coincident among clones of the same haplotype, indicating that such an assay can be used in the genotype identification of these particular grapevine varieties (Fig. 4.3).

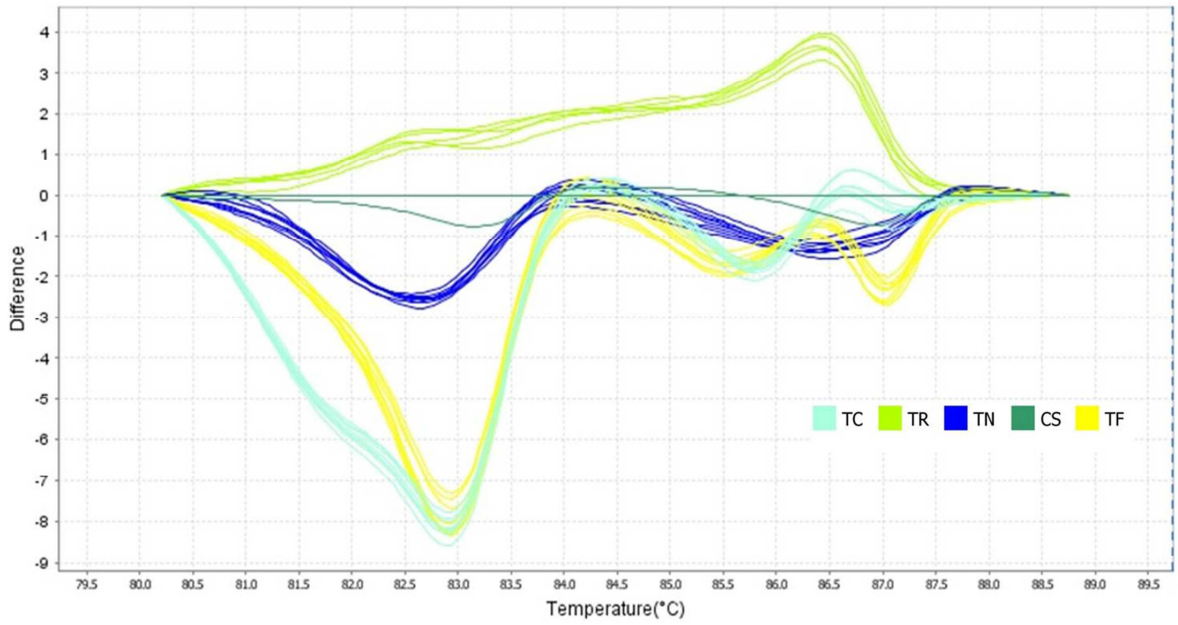

Fig. 4.3 HRM different melting curves of the most representative grapevine varieties used in wine production in Portugal and corresponding clones for UFGT fragment.

The shapes of the melting curve profiles of five red grapevine varieties used in the Douro region are presented as an example in Fig. 4.3. Among them, Cabernet Sauvignon (CS) is used as an international grapevine reference variety. The clones of each grapevine variety present the same genetic profile, reinforcing the assay's robustness. Four of the 22 grapevine varieties studied (Tinta Francisca, Alicante Bouschet, Côdega de Larinho, and Tinta Amarela) could not be distinguished using this particular HRM assay (Fig. 4.4), since they presented the same sequence for this particular region (Table 4.2). The sequences of these grapevine varieties differ only from Touriga Franca (TF) in a unique SNP at the 425 bp position (Table 4.2), which is discriminated using this particular assay. The robustness of the HRM assay could also be confirmed by the profiles obtained in the clones of the undistinguishable grapevine varieties herein tested (Fig. 4.4), since all of the samples tested presented the same shape HRM melting curve, as expected. With all of the nucleotide sequences taken into consideration, in particular, the SNPs found in the 22 grapevine varieties, the amino acid sequences were deduced and aligned. A total of 31 amino acid residue variations were found within the grapevine varieties studied (Table 4.3).

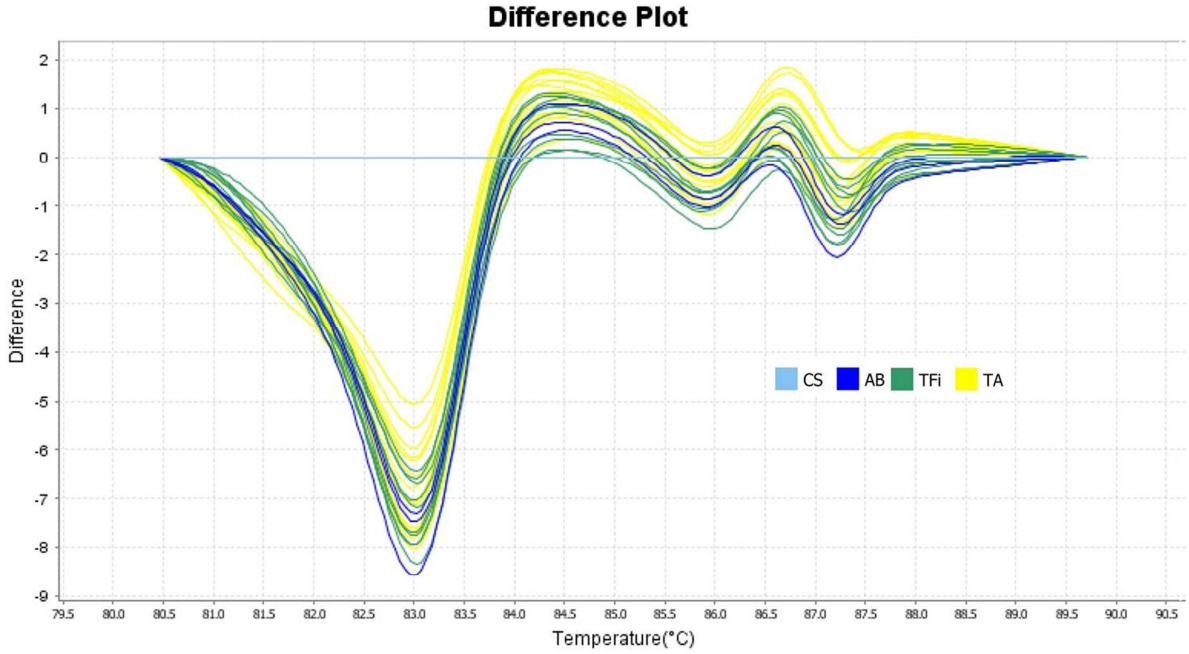

Fig. 4.4 HRM difference plot of AB, TFi and TA grapevine varieties and corresponding clones presenting the same shape of the melting curve and consequently the same genotype for UFGT fragment.

Table 4.3 Predicted amino acid sequence variations in UFGT, using the reference genome, based on the SNPs found across the twenty-two grapevine varieties (*Vitis vinifera* L.).

Amino acid position	33	47	69	74	80	86	88	89	91	142	153	161	174	181	182	204	256	259	290	293	312	327	357	362	364	373	386	400	424	444	445
Sample																															
UFGT	A	Q	V	A	A	D	E	L	M	A	T	G	N	S	K	M	I	T	L	A	R	Y	L	A	G	Y	A	E	G	F	K
CS	.	.	I	P	.	.	.	.	T	.	I	A	.	.	.	.	V	.	V	S	.	H	.	.	.	F	V	K	R	.	I
Sou	.	.	I	P	.	.	.	.	T	.	I	A	.	.	.	.	V	.	V	S	.	H	.	.	.	F	V	K	R	.	I
Vio	.	.	I	P	.	.	.	.	T	.	I	A	.	.	.	.	V	.	V	S	.	H	.	.	.	F	V	K	R	.	I
FP	.	.	I	P	.	.	.	M	T	.	I	.	Y	.	.	.	V	.	V	S	.	H	.	.	.	F	V	K	R	.	I
MF	.	.	I	P	.	.	.	M	T	.	I	.	Y	.	.	.	V	.	V	S	.	H	.	.	.	F	V	K	R	.	I
TR	.	.	.	P	.	.	.	M	T	.	I	.	Y	.	.	.	V	.	.	S	.	H	.	.	.	F	V	K	R	I	I
Ruf	.	.	.	P	T	.	.	M	T	.	I	.	Y	.	.	.	V	.	.	.	.	H	.	.	W	F	V	K	R	.	.
Ch	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	V	.	.	.	.
MG	.	.	.	P	.	.	.	.	T	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TBr	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GOU	.	.	.	P	.	.	.	.	.	.	.	.	.	Y	E	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.
M	.	.	.	.	.	.	.	.	.	S	.	A	.	.	.	.	V	.	.	.	.	H	.	.	.	F	.	K	R	.	.
TB	.	.	.	.	.	.	.	.	.	S	.	A	.	.	.	.	V	.	.	.	.	H	.	.	.	F	.	K	R	.	.
TN	.	.	.	.	.	.	.	.	.	S	.	A	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.
PN	V	E	.	P	P	G	D	.	.	.	I	.	.	.	.	L	L	.	.	.	.	H	.	.	.	.	V	.	.	.	.
TFi	V	E	.	P	T	G	D	.	.	.	.	A	.	Y	E	.	V	S	.	.	.	H	.	G	.	F	V	K	R	.	.
AB	V	E	.	P	T	G	D	.	.	.	.	A	.	Y	E	.	V	S	.	.	.	H	.	G	.	F	V	K	R	.	.
CL	V	E	.	P	T	G	D	.	.	.	.	A	.	Y	E	.	V	S	.	.	.	H	.	G	.	F	V	K	R	.	.
TA	V	E	.	P	T	G	D	.	.	.	.	A	.	Y	E	.	V	S	.	.	.	H	.	G	.	F	V	K	R	.	.
TF	V	E	.	P	T	G	D	.	.	S	.	A	.	Y	E	.	V	S	.	.	.	H	.	G	.	F	V	K	R	.	.
DT	V	E	.	P	T	G	D	.	.	.	.	.	.	Y	E	.	V	S	.	.	.	.	V	G	.	F	V	.	R	.	.
TC	V	E	I	P	T	G	D	.	T	.	I.	.	.	Y	E	.	V	S	V	S	.	.	.	G	.	F	V	K	R	.	I

Amino acid code: A (Alanine); D (Aspartic Acid); E (Glutamic Acid); F (Phenylalanine); G (Glycine); H (Histidine); I (Isoleucine); K (Lysine); L (Leucine); M (Methionine); N (Asparagine); P (Proline); Q (Glutamine); R (Arginine); S (Serine); T (Threonine); V (Valine); W (Tryptophan); Y (Tyrosine).

The *UFGT* gene expression is correlated with the grapevine phenotype, and its transcription is needed for berry pigmentation (no color/red color) (Lijavetzky et al. 2006, Pelsy et al. 2015). The anthocyanin profile and content can deeply influence the final wine quality (Bindon et al. 2014). With

the variation of the red grapevine variety amino acid sequences studied taken into consideration, a Cobalt tree was constructed, giving rise to three major groups (Fig. 4.5). Group A included CS, Sou, TR, and Ruf; group B incorporated M, TB, TBr, and TN; and group C comprised TFi, AB, TA, TF, DT, and TC. The Pinot Noir clones analyzed were completely distinguished from all of the grapevine varieties, and therefore they generated an independent branch.

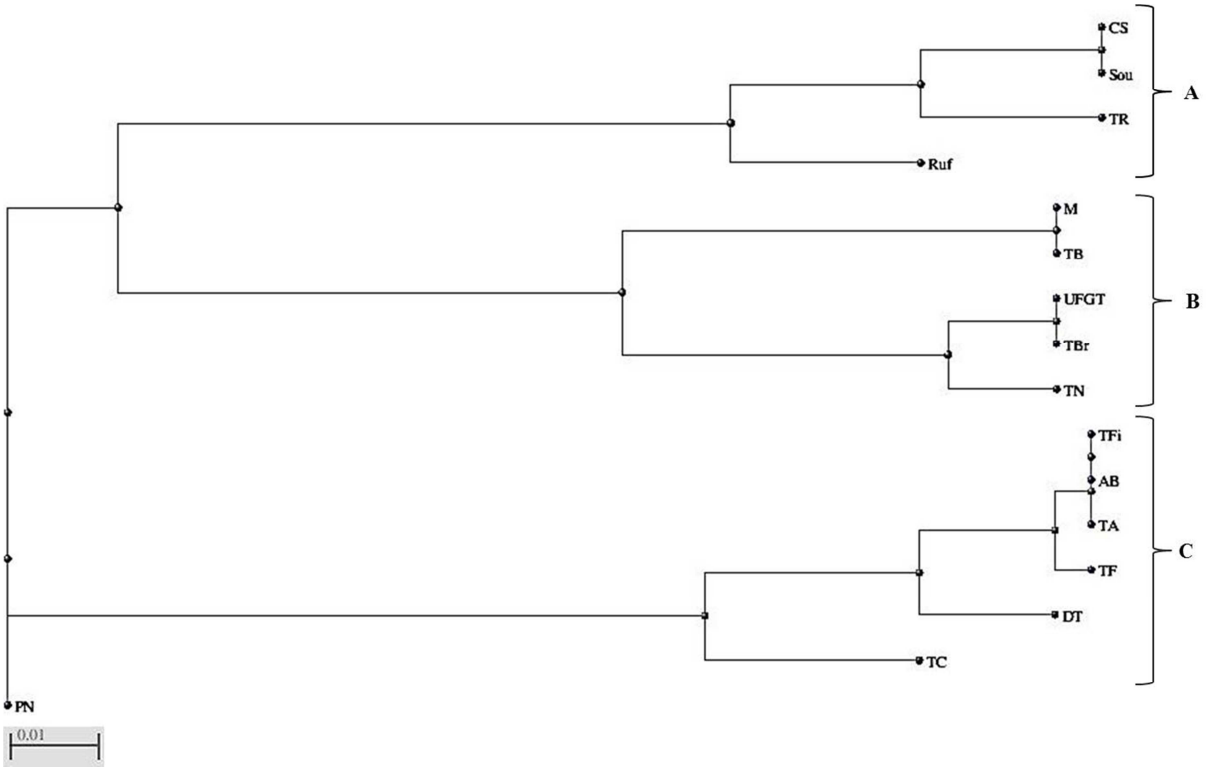

Fig. 4.5 Phylogenetic tree based on deduced amino acid sequence variations of UFGT from the red grapevine varieties using the Cobalt multiple sequence alignment tool.

The differences found between the amino acid sequence of Pinot Noir and the sequence of the NCBI database were expected, because the NCBI reference genome is a hybrid of Pinot Noir (Jaillon et al. 2007). Touriga Brasileira presented the same amino acid sequence as reported in the NCBI database relative to the *UFGT locus*. Although the *UFGT* gene expression is related to the anthocyanin profile of the grapes (Lijavetzky et al. 2006), there is a lack of information on the specific profile of each grapevine variety. Some of the most widely used grapevine varieties have been characterized, among them Pinot Noir, Merlot, and Cabernet Sauvignon. Pinot Noir grapes have a differentiated anthocyanin profile, with only monoglucoside anthocyanins, in contrast with other grapevine varieties (Merlot, Cabernet Sauvignon, Touriga Franca, Tinta Roriz, Touriga Nacional, Sousão, Rufete, Tinta Amarela, Tinta Barroca and Tinto Cão) that present all three types of anthocyanin (monoglucosides, acetates, and coumarates) (Costa et al. 2014, Dimitrovska et al. 2015,

Jordão et al. 1998, 2012). This may justify the outstanding position of Pinot Noir in relation to the other grapevine varieties studied. However, the amino acid variation present within the UFGT sequence has a direct implication on the protein composition, which may or not influence the protein function. The results obtained through this work represent a good landmark in this particular area and should be considered as a suitable platform for further studies. The deduced amino acid sequences set the bases for a better understanding of the correlation between anthocyanin content and the amino acid profiles.

4.4 CONCLUSIONS

The present work confirmed the low level of polymorphism of the *CHI* gene and revealed a high level of polymorphism present within the *UFGT* gene of grapevine. The *UFGT* gene allowed the discrimination of 18 different grapevine genotypes among 22 grapevine varieties. From the 58 SNPs and 1 INDEL were predicted a total of 31 amino acid residual changes, enhancing the potential effect on the anthocyanin profile of each variety, which opens doors for further omic studies considering this particular gene. This study provides the first report on the use of a large fragment in HRM assays, thereby allowing the detection of multiple events (SNP and INDEL) in a unique assay, which can be adapted for large-scale genotyping and mapping in *Vitis*. This assay can be extended for grapevine varietal certification procedures, which are imperative throughout the entire wine chain (plant nursery to wine), because it allows varietal identification.

ASSOCIATED CONTENT

ACCESSION CODES

Sequence data from this study can be found in the GenBank database under accession numbers (see Additional file 2).

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Table 4.2 Single Nucleotide Polymorphisms identified in *UFGT* gene with information on the genotypes composition found across the 22 grapevine varieties (*Vitis vinifera* L.).

Nucleotide position / Sample	90	98	139	205	207	220	238	240	257	264	265	272	309	366	375	424	425	442	459	483	525	555	560	562	598	600	617	619	636	663	685	762	789
UFGT	C	C	C	G	G	G	G	C	A	G	C	T	G	T	T	-	G	C	C	G	A	A	A	G	A	T	C	A	C	C	A	C	C
TBr	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ch	S	.	.	R	S	S	.	.	.	.	.	.	.	.	Y	-	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.
MG	S	.	.	R	S	S	.	.	.	.	.	Y	R	.	Y	-	.	.	Y	.	W	M	.	.	.	.	.	.	.	.	.	.	.
GOU	.	.	.	.	.	S	.	M	.	.	.	.	.	.	Y	-	.	.	.	.	.	M	R	.	.	Y	M	R	Y	Y	.	.	.
CS	S	.	.	R	S	S	.	.	.	.	.	Y	A	.	C	T	.	.	Y	S	W	C	.	.	.	.	.	.	.	.	.	.	.
Sou	S	.	.	R	S	S	.	.	.	.	.	Y	A	.	C	T	.	.	T	S	W	C	.	.	.	.	.	.	.	.	.	.	.
Vio	S	.	.	R	S	S	.	.	.	.	.	Y	A	.	C	-	.	.	Y	S	W	C	.	.	.	.	.	.	.	.	.	.	.
FP	G	.	.	A	C	C	.	.	.	.	M	C	A	.	C	-	.	.	T	.	T	C	.	.	T	.	.	.	.	.	.	.	.
TR	G	.	.	A	C	C	.	.	.	.	M	C	A	.	C	-	.	.	T	.	T	C	.	.	W	.	.	.	.	.	.	.	.
MF	G	.	.	A	C	C	.	.	.	.	A	C	A	.	C	-	.	.	T	.	T	C	.	.	W	.	.	.	.	.	.	.	.
Ruf	G	.	.	R	S	C	R	.	.	.	M	C	A	.	C	-	.	M	Y	.	W	C	.	.	W	.	.	.	.	.	.	.	.
M	.	.	.	.	.	.	.	.	.	.	.	.	W	W	C	-	K	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.
TB	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	-	K	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.
TN	.	.	.	.	.	.	.	.	.	.	.	.	R	.	Y	-	K	.	.	S	.	M	.	.	.	.	.	.	.	.	.	.	.
TF	.	Y	S	.	.	S	R	.	R	K	.	.	R	.	Y	-	K	.	.	S	.	C	R	R	.	.	M	R	Y	Y	.	Y	M
TFi	.	Y	S	.	.	S	R	.	R	K	.	.	R	.	Y	-	.	.	.	S	.	C	R	R	.	.	M	R	Y	Y	.	Y	M
AB	.	Y	S	.	.	S	R	.	R	K	.	.	R	.	Y	-	.	.	.	S	.	C	R	R	.	.	M	R	Y	Y	.	Y	M
TA	.	Y	S	.	.	S	R	.	R	K	.	.	R	.	Y	-	.	.	.	S	.	C	R	R	.	.	M	R	Y	Y	.	Y	M
CL	.	Y	S	.	.	S	R	.	R	K	.	.	R	.	Y	-	.	.	.	S	.	C	R	R	.	.	M	R	Y	Y	.	Y	M
TC	S	Y	S	R	S	C	R	.	R	K	.	Y	R	.	Y	-	.	.	Y	.	W	C	R	R	.	.	M	R	Y	Y	.	Y	M
DT	.	Y	S	.	.	C	R	M	R	K	.	.	.	.	Y	-	.	.	.	.	.	C	G	R	.	Y	A	G	T	T	.	Y	M
PN	.	Y	S	.	.	S	S	.	R	K	.	.	.	.	.	-	.	.	Y	.	W	M	.	.	.	.	.	.	.	.	W	.	.

Numbering starts with A of the start codon. Nucleotide Code: A (Adenine); C (Cytosine); T (Thymine); G (Guanine); M (A or C); R (A or G); W (A or T); S (C or G); Y (C or T) and K (G or T).

Table 4.2 (Continued)

Nucleotide position / Sample	816	841	843	850	943	955	1014	1054	1083	1122	1131	1134	1144	1146	1160	1165	1193	1232	1245	1273	1345	1404	1405	1409	1459	1481
UFGT	T	A	A	A	C	G	G	T	G	A	T	A	T	G	C	G	A	C	A	G	G	T	T	A	T	A
TBr	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ch	.	R	.	.	.	.	K	.	.	.	.	.	.	.	.	.	W	Y	.	R	R	.	.	.	.	.
MG	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	R	.	.	.	.	.
GOU	.	R	.	.	.	.	.	.	.	.	.	.	K	.	.	.	W	.	.	.	R	.	.	.	.	.
CS	Y	G	W	.	S	K	.	Y	.	M	Y	T	.	.	.	.	T	Y	M	A	A	.	.	W	.	.
Sou	Y	G	W	.	S	K	.	Y	.	M	Y	T	.	.	.	.	T	Y	M	A	A	.	.	W	.	.
Vio	Y	G	W	.	S	K	.	Y	.	M	Y	T	.	.	.	.	T	Y	M	A	A	.	.	W	.	.
FP	Y	G	.	.	S	K	.	Y	.	M	Y	T	.	.	.	.	T	T	M	A	A	.	.	W	Y	.
TR	Y	G	.	.	S	K	.	Y	.	M	Y	T	.	.	.	.	T	T	M	A	A	.	W	W	Y	R
MF	Y	G	.	.	S	K	.	Y	.	M	Y	T	.	.	.	.	T	T	.	A	A	.	.	W	.	.
Ruf	.	G	.	.	.	.	.	Y	.	.	.	T	.	.	.	K	T	T	M	A	A	.	.	.	Y	R
M	.	G	T	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	A	A	.	.	.	.	.
TB	.	G	T	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	A	A	.	.	.	.	.
TN	.	R	W	.	.	.	.	Y	.	.	.	W	.	.	.	.	W	.	.	R	R	.	.	.	.	.
TF	Y	G	W	W	.	.	.	Y	.	.	.	T	.	R	S	.	T	Y	.	A	A	.	.	.	.	.
TFi	Y	G	W	W	.	.	.	Y	.	.	.	T	.	R	S	.	T	Y	.	A	A	.	.	.	.	.
AB	Y	G	W	W	.	.	.	Y	.	.	.	T	.	R	S	.	T	Y	.	A	A	.	.	.	.	.
TA	Y	G	W	W	.	.	.	Y	.	.	.	T	.	R	S	.	T	Y	.	A	A	.	.	.	.	.
CL	Y	G	W	W	.	.	.	Y	.	.	.	T	.	R	S	.	T	Y	.	A	A	.	.	.	.	.
TC	C	G	.	W	S	K	.	.	.	M	Y	T	.	R	S	.	T	T	M	A	A	.	.	W	.	.
DT	Y	G	.	W	.	.	.	.	.	.	.	W	K	R	S	.	T	Y	.	R	A	.	.	.	.	.
PN	.	S	W	.	.	.	.	Y	R	.	.	W	.	.	.	.	W	Y	.	R	R	Y	.	.	.	.

Numbering starts with A of the start codon. Nucleotide Code: A (Adenine); C (Cytosine); T (Thymine); G (Guanine); M (A or C); R (A or G); W (A or T); S (C or G); Y (C or T) and K (G or T

Chapter 5

High Resolution Melting (HRM) Applied to Wine Authenticity

High Resolution Melting (HRM) Applied to Wine Authenticity

Adapted from:
Pereira L, Gomes S, Castro C, Eiras-Dias JE, Brazão J, Fernandes JRA, Martins-Lopes P (2015) High Resolution Melting (HRM) Applied to Wine Traceability (*in preparation*).

ABSTRACT

The need for accurate and reliable methods for grapevine varietal identification has increased with market demands, especially in Denomination of Origin designation and highly quoted wines. The DNA-based methodologies are fast and reliable means of tracking varietal composition in food products. The main aim of this work was to study the application of High Resolution Melting (HRM), as a screening method for must and wine authenticity analysis. Three sample types (leaf, must and wine) were used to validate the three tested HRM assays, which were designed considering the amplified fragment length (*Vv1* – 704 bp; *Vv2* – 375 bp; and *Vv3* – 119 bp). The *Vv1* HRM assay was only successful when applied to leaf and must samples, allowing the discrimination of the must varietal composition. The *Vv2* HRM assay amplified successfully all the sample types and allowed the discrimination of the genotypes based on the melting temperature values (T_m). The smallest amplicon, *Vv3,* produced a coincident melting curve shape in all sample types of the same genotype. This current study reports on a sensitive, rapid and efficient HRM assay applied for the first time to wine samples that can be applied for wine authenticity purposes.

5.1 INTRODUCTION

It is estimated that about 11 000 different *Vitis vinifera* L. varieties exist worldwide (Galet 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 for having a huge *V. vinifera* germplasm collection, with hundreds of varieties (Cunha et al. 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 high quality 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 ancestral grapevine varieties in opposite to the modern vineyards that have vineyards with defined grapevine varieties used to produce monovarietal wines with high commercial value.

Governmental organisms have the 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 nº 607/2009). Thus, wine authenticity has become a subject of great concern since the incorrect labeling 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 et al. 2015, Camin et al. 2013, Sen and Tokatli 2014, Versari et al. 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 et al. 2013, Madesis et al. 2014, Martins-Lopes et al. 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 2009). Although SSR markers have been used for food authenticity purposes several problems have arisen related to the DNA quality, result of the extraction procedures (reviewed by Pereira et al. 2016). When considering must/wine matrices the

presence of large quantities of polyphenols, polysaccharides and proteins sometimes inhibit PCR reactions (Işçi et al. 2014). Another drawback in the application of SSR markers in such sample type is related with DNA degradation, a 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 and Eiras-Dias 2006, Bigliazzi et al. 2012, Boccacci et al. 2012, Nakamura et al. 2007, Pereira et al. 2011, Savazzini and Martinelli 2006). The use of small molecular markers, such as, Single Nucleotide Polymorphisms (SNPs) maybe 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 versatility it can be considered as an alternative approach for food authenticity purposes (Ganopoulos et al. 2011a, 2011b, 2013, 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 et al. 2008). Recently, HRM has been applied by our group in the grapevine varieties identification based on the SNPs changes detected within genes belonging to the anthocyanins pathway (Pereira and Martins-Lopes 2015, Castro et al. *in preparation*).

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.

5.2 MATERIAL AND METHODS

5.2.1 LEAF, MUST AND WINE SAMPLES

Thirteen *V. vinifera* grapevine 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 5.1). 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 5.1 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

5.2.2 VINIFICATION OF WHITE GRAPE VARIETIES

White variety 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 hours for defecation. After defecation, 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 liters 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.

5.2.3. *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 remontage 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.

5.2.4 *GENOMIC DNA EXTRACTION*

Total genomic DNA was extracted from frozen young leaf samples using the described CTAB method (Doyle and 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 was 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).

5.2.5 *HIGH-RESOLUTION MELTING ASSAY DESIGN*

The primer pairs tested for the HRM analysis are summarized in Table 5.2. The HRM primer pairs selected for the study were based on previous sequencing data described in Pereira and Martins-Lopes (2015) and Castro et al. (*in preparation*). Three length fragments were tested in leaf, must and wine DNA samples (Fig. 5.1, Table 5.2). All PCR reactions were conducted in a 48-well plate using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a 20 µL/well total volume according to manufacture instructions. The final reaction mixture contained 20 ng total DNA, 0.2 µM forward and reverse primers, and 1X MeltDoctor HRM Master Mix (Applied Biosystems). The PCR amplification was followed by the HRM and included an initial denaturating

step of 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 58 -60 °C for 30 s and 72 °C for 30 s, then a final extension step of 72 °C for 2 min. The melting curve was obtained in continuous, performed as follow: 95 °C for 30 s, 65 °C for 1 min rising 0.3 °C/s, 95 °C for 15 s. During the incremental melting step, fluorescence data were continuously acquired. All reactions were performed in triplicate. High Resolution Melt Software v3.0.1 (Applied Biosystems, Foster City, CA, USA) was used to analyse the data. This software uses an improved clustering algorithm that helps to accurately distinguish control and variant genotypes. After normalization and the temperature shift determination, the different melting curves of the several plots were generated.

Table 5.2 List of primers used in the HRM assays and the details of the amplified fragments.

Fragment/Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	T_m (°C)	Nº and type of polymorphisms within Fragment
Vv1 – *UFGT*	Fwd: GCAATGTAATATCAAGTCC Rev: TTTCTTTCTTTGAGCCATT	704	82.7–83.0 86.6–86.9	32 SNPs 1 INDEL
Vv2 – *F3H*	Fwd: AGAGAAAGAAGGCGACGT Rev: GATGGCTGGAAACGATGA	375	84.6-84.9	5 SNPs
Vv3 – *UFGT*	Fwd: AGCAGAGATGGGGGTGGCTT Rev: AGCAGGTAAAACCACCTGAA	119	78.6-78.8 81.7-81.9	4 SNPs 1 INDEL

Note: (Ta) Annealing temperature; (T_m) Melting temperature.

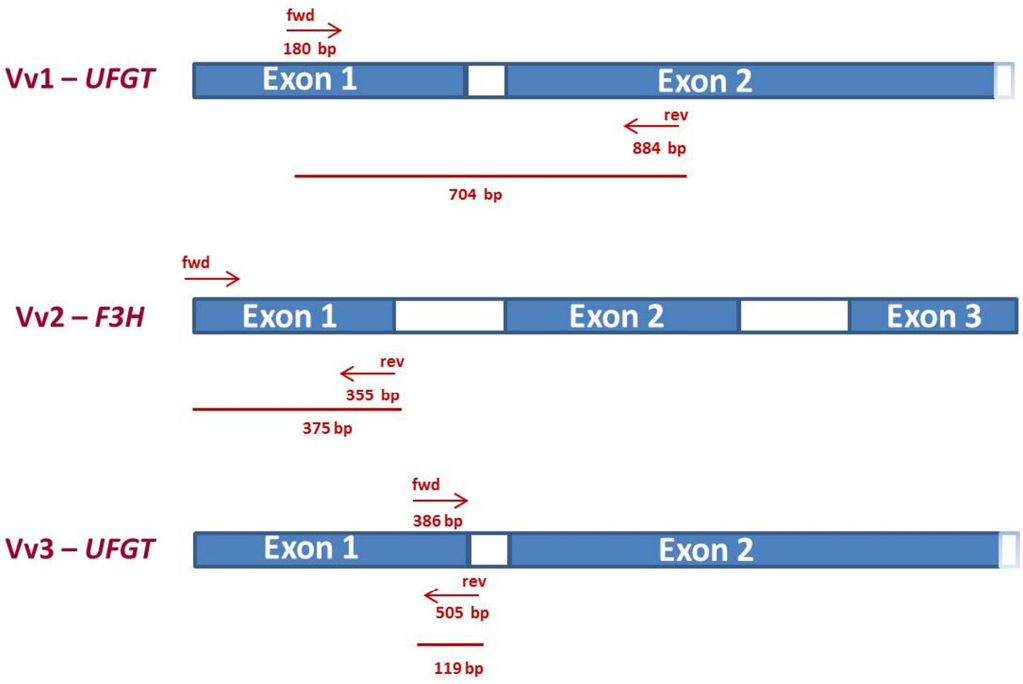

Fig. 5.1 Schematic representation of the different HRM specific fragments of *UFGT* (704 bp; 119 bp) and *F3H* genes. Empty spaces refer to intron regions.

Several HRM assays were used. Assays Vv1 and Vv2 fragments were previously optimized using leaf samples (Pereira and Martins-Lopes 2015, Castro et al. *in preparation*). The *Vv3* fragment was developed based on the sequencing information of the *UFGT* gene (Pereira and Martins-Lopes 2015).

5.3 Results and Discussion

5.3.1 HRM analysis applied to must using Vv1 – 704 bp fragment (UFGT gene)

HRM analysis of a specific fragment belonging to the *UFGT* gene which allowed the identification of several grapevine varieties (Pereira and Martins-Lopes 2015) was used. In the current study the HRM assay was tested in must samples and compared to the leaf DNA reference material (Fig. 5.2). The HRM profile obtained between the two differentiated samples, leaf *versus* must, completely corresponded in all the grapevine varieties used (4 genotypes).

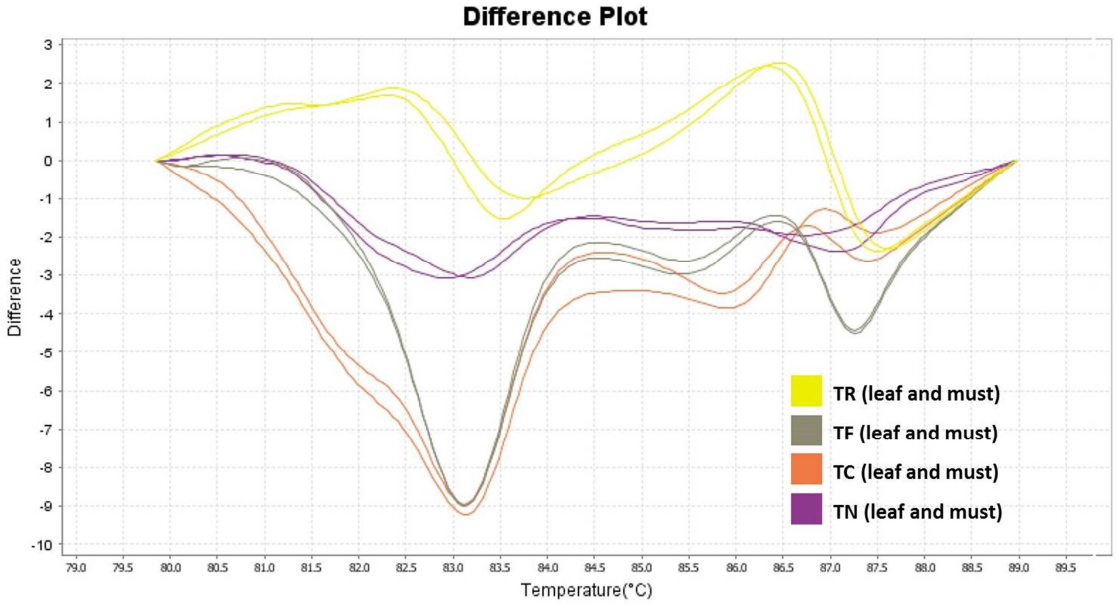

Fig. 5.2 HRM different profiles obtained for a specific fragment of *UFGT* gene (704 bp) for the grapevine varieties, considering leaf and corresponding must (TN – Touriga Nacional; TF – Touriga Franca; TR – Tinta Roriz; TC – Tinto Cão).

These genotypes were selected based on the previous work since they produced different melting curve shapes (Pereira and Martins-Lopes 2015). The melting curve profiles were coincident with the previous obtained in leaf samples, demonstrating the robustness of the assay.

Even though it is well known that wine fermentation yields DNA highly degraded (García-Beneytez et al. 2002), it is also true that must samples still contain high fragment genomic DNA, which enables it

to be successfully used in marker systems that target large fragments, such as SSR and ISSR markers (Baleiras-Couto and Eiras-Dias 2006, Bigliazzi et al. 2012, Pereira et al. 2012, Rodríguez-Plaza et al. 2006). The fragment size used in this assay is the biggest specific fragment reported so far in must samples, and it is interesting because it allows the discrimination of multiple events in one unique reaction, overcoming the potential obtained through SSR analysis which detects repeat variation. When HRM assay was applied, as a screening method, to DNA extracted from wine samples no amplification was detected. This result was expected since the assay comprises a large fragment size that is not available after the wine fermentation procedures (Baleiras-Couto and Eiras-Dias 2006, Işçi et al. 2009). The developed HRM assay demonstrated that it is possible to apply HRM to: (i) large DNA fragments; (ii) fragments with a high number of SNPs; and (iii) must genomic DNA samples. In order to overcome constrains found in the wine DNA samples, two HRM assays were further tested using different fragment length.

5.3.2 HRM ANALYSIS APPLIED TO MUST AND WINE USING Vv2 – FRAGMENT 375 bp (F3H GENE)

The main drawback associated to wine authenticity is the nature of DNA. The low quantity and the lack of integrity in wine DNA is associated with fermentation process. Baleiras-Couto et al. (2006) reported that the identification of the grapevine varieties, in monovarietal wines, was not possible with nuclear markers. Several efforts have been conducted to create short fragment length markers targeting varietal identification (Lijavetzky et al. 2007, Myles et al. 2010).

The HRM analysis is preferably applied to small fragment sizes (up to 300 bp) and with a low number of SNPs (Druml and Cichna-Markl 2014). Aiming the identification of the grapevine varieties in monovarietal wines, an HRM assay with a smaller fragment *Vv2* (375 bp) was used (Fig. 5.3). The amplified fragment comprehended a total of five SNPs and was designed within the *F3H* gene sequence (Castro et al. in preparation). The same group of grapevine varieties used in the first experiment was used and the international red grape variety Cabernet Sauvignon was added (Fig. 5.3). A total correspondence was found between leaf and the correspondent must (Fig. 5.3). These five varieties presented all the same sequence (Castro et al. in preparation), and through the HRM assay it is evident that the exactly same profile is obtained in leaf and must samples (based on the melting temperature - T_m). When this HRM assay was applied to wine samples the exact same profile was found (Fig. 5.4), demonstrating that although the fragment is still considerable big it is possible to obtain amplification within wine samples when appropriated extraction methods are used. In order to validate this HRM assay for wine authenticity purposes, a bigger number of grapevine

varieties were included to discriminate the different genotypes, previously obtained through the sequencing analysis (Castro et al. *in preparation*).

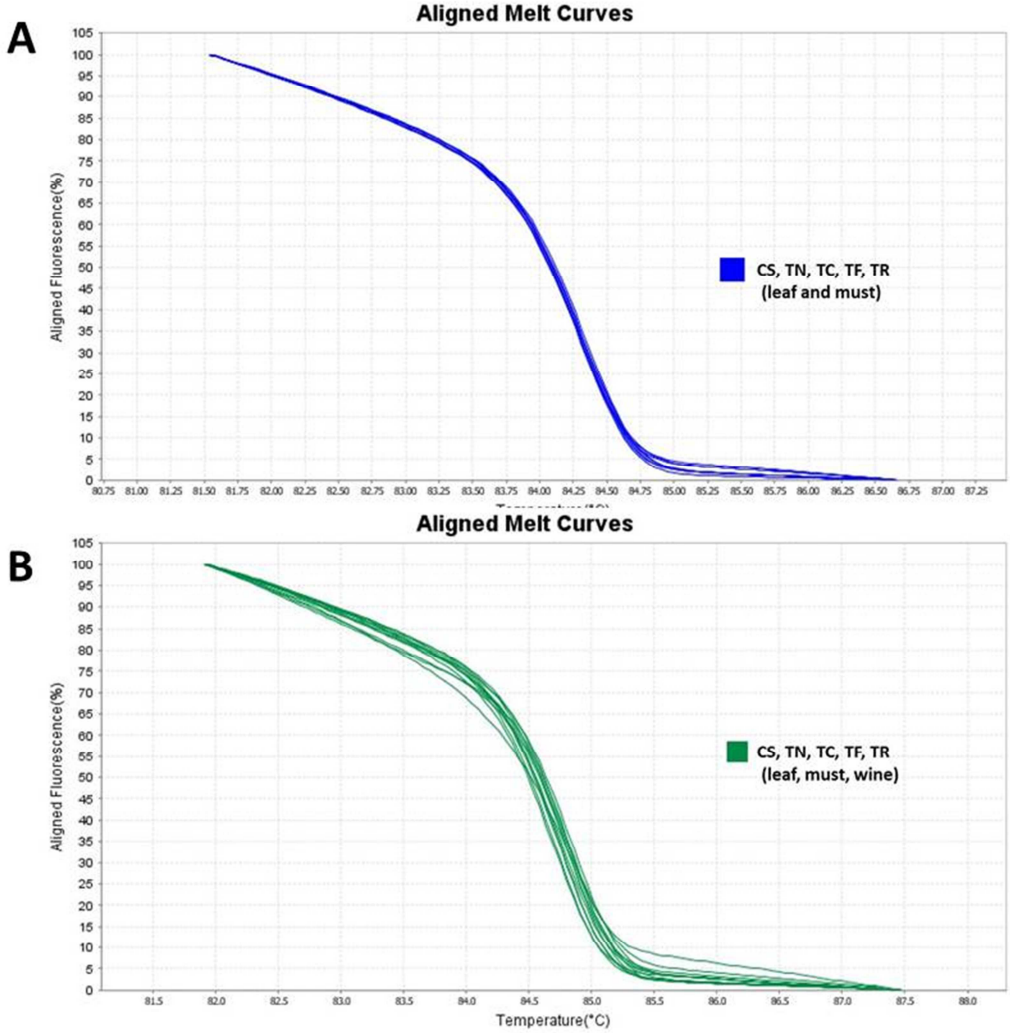

Fig. 5.3 HRM melt curve, based on Tm, obtained for a specific fragment of F3H gene for the grapevine varieties, considering leaf and corresponding must (A) and for the grapevine varieties, considering leaf and corresponding must and wine (B) (TN – Touriga Nacional; TF – Touriga Franca; TR – Tinta Roriz; TC – Tinto Cão).

In Fig. 5.4, four different genotypes, variants, were separated according to their T_m. The grapevine varieties were grouped according to their sequence, independent of their grape skin color, and the vinification process. Malvasia Fina is a white variety that was vinified with a different process and it did not interfere with amplification procedure. Not only the genomic DNA extracted from must and wine samples presented the required size for this particular assay but the DNA extraction procedures was efficient in the elimination of PCR-inhibitors (Pereira et al. 2011). HRM has been applied to fruit juice authenticity using the DNA barcode *trnL*, allowing the discrimination of five species (Faria et al. 2013).The fragment used was around 500 bp long, however it is a chloroplastidial sequence,

therefore it is less subjected to DNA fragmentation. The authors also refer in this study that DNA extractions needs to be optimized so PCR-inhibitors are not present.

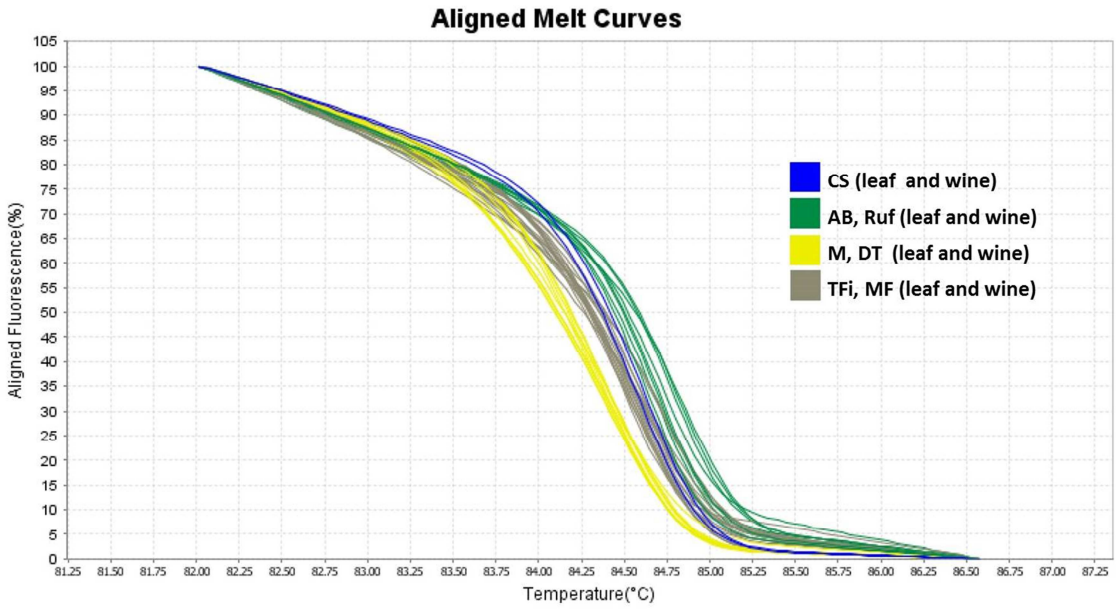

Fig. 5.4 HRM different profiles, based on Tm, obtained for a specific fragment of *F3H* gene for the grapevine varieties, considering leaf and corresponding wine(CS – Cabernet Sauvignon; AB – Alicante Bouschet; Ruf – Rufete; M – Merlot; DT – Donzelinho Tinto; TFi – Tinta Francisca; MF – Malvasia Fina).

5.3.3 HRM ANALYSIS APPLIED TO WINES USING Vv3 – FRAGMENT 119 bp (UFGT GENE)

In wine samples, the DNA amplicons length is an inherent disadvantage that influences the HRM sensitivity. With ageing the length of the DNA fragments, recovered from wine samples are smaller, and therefore it is imposed that shorter amplicons sequences are used for grapevine varietal identification (Baleiras-Couto and Eiras-Dias 2006). Thus, taking into consideration the known problematic a new approach was developed based on a redesigning of the UFGT primer pair, using a smaller fragment, *Vv3*. This fragment is 119 bp long, targeting short DNA fragments recovered in wine samples. The developed HRM assay generated 8 different HRM curve profiles, according to the grapevine varieties and wine samples genotypes (Fig. 5.5). A total of 8 genotypes were expected based on the sequencing information (Pereira and Martins-Lopes 2015).

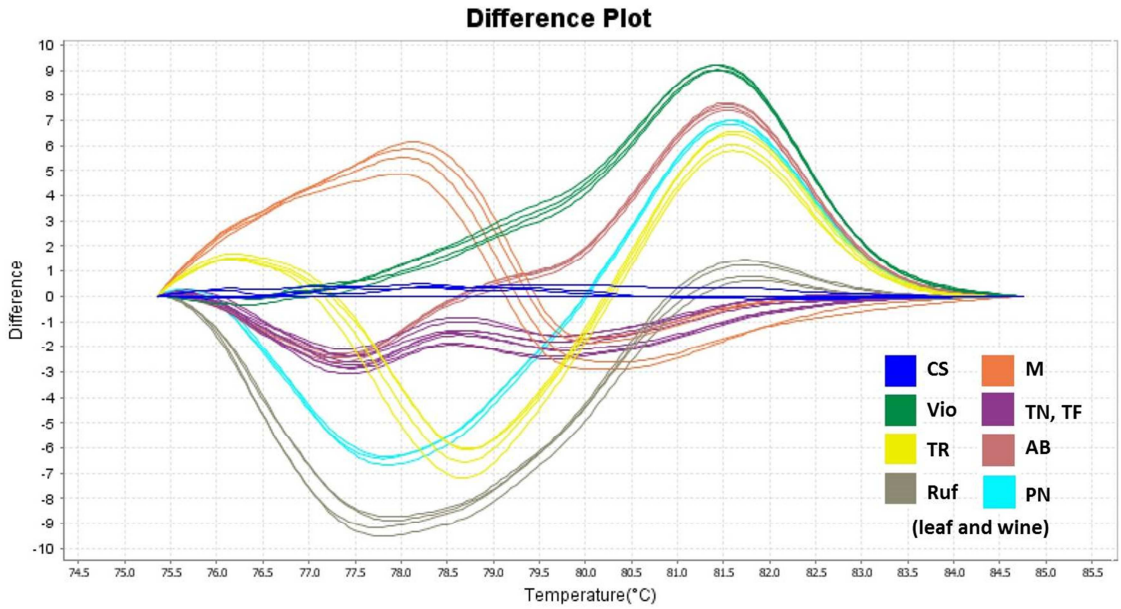

Fig. 5.5 HRM different profiles obtained by targeting *Vv3 UFGT* (119 bp) for the grapevine varieties, considering leaf and corresponding wine (CS – Cabernet Sauvignon; VIO – Viosinho; TR – Tinta Roriz; Ruf – Rufete; M - Merlot; TN – Touriga Nacional; TF- Touriga Franca; AB - Alicante Bouschet; and PN – Pinot Noir). Assigned genotypes using a cut off confidence value of 95%. Cabernet Sauvignon used as reference for HRM assay.

As depicted in Fig. 5.5, 7 grapevine varieties (CS– Cabernet Sauvignon; VIO– Viosinho; TR– Tinta Roriz; Ruf– Rufete; M– Merlot; AB– Alicante Bouschet; and PN– Pinot Noir) were discriminated from the nine varieties studied. Two grapevine varieties presented the same melting curve shape, Touriga Nacional and Touriga Franca, as expected since they present the same sequence in the fragment under consideration. A coincident profile was obtained between the leaf, and wine DNA samples belonging to the same genotypes. In this case the discrimination of the haplotypes was based on the shape of the melting curves since the melting temperature (T_m) values are similar between all samples. This analysis is more informative and allows a quick and visible accession of the genotypic differences found among grapevine varieties under study (Fig. 5.5) than the one based only on T_m results (Fig. 5.4). These results confirm that low-molecular weight markers, based on small sequence differences, are useful to detect grapevine DNA in wine samples, suggesting the possible application of HRM assay in grapevine varietal identification in DO wines and in undeclared admixture in wine.

Similar approaches were considered when applying HRM to highly processed food samples. Vietina et al. (2013) reported on the use of a small amplicon, 71 bp, to detect alien oil in olive oil samples, using the *Rbc1* chloroplast sequence. HRM assay can be applied to complex food/wine matrices as long as several conditions are attended:

1. an efficient DNA extraction protocol is used taking into consideration the specific matrices characteristics;

2. smaller DNA fragment are targeted and;
3. a combined number of single polymorphisms (SNPs) capable of differentiating several genotypes in one unique assay is used.

Within the developed HRM assays, based on MeltDoctor™, it was possible to have consistent and reproducible results independent of sample type (leaf, must, and wine). This is interesting since the assay can be designed based on a set of primers, and it can be used to identify multiple events within a fragment, without the need of designing multiple probes to detect each genotype, as it is required when using TaqMan or Molecular Beacon technology (Madesis et al. 2014), decreasing the cost required per analysis.

5.4 CONCLUSIONS

In the present work the use of HRM has proven to be successfully applied to must and wine samples, opening new opportunities for the use of DNA-based methods for must and wine authenticity purposes. The use of smaller fragments, such as the described for *Vv3*, guarantees a more robust and reliable method for wine authenticity, whereas for must samples higher fragments can be considered, increasing the discrimination power of the assay, since they are able to contemplate a higher number of SNP and INDEL events.

The application of HRM based assays in wine authenticity constitutes a consistent technology that can be cost effective, and therefore can revolutionize the wine sector, as long as a melt curve database is designed for such a purpose.

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Chapter 6

Concluding Remarks and Outlook

CONCLUDING REMARKS AND OUTLOOK

6.1 CONCLUSIONS

A wine with grapevine varietal composition specification and its origin has both a commercial and marketing advantage. The studies described in this doctoral thesis aimed to contribute to the establishment of a reliable and reproducible DNA-based molecular procedure suitable of identifying grapevine varietal composition in must and wine samples, considering the entire vinification process. The ideal system requires the development of accurate and reliable DNA isolation methods and the enlargement of grapevine markers suitable for varietal identification/discrimination in must and wine samples. The work undertaken is of great importance, especially to protect high quoted wines. A common adulteration in DO wines is the addition of other grapevine varieties, the detection of varietal composition is therefore a crucial step in the certification process. Through the application of specific DNA grapevine varietal molecular markers it would be possible to correctly characterize the varietal composition in wine.

The present study follows a wine authenticity research area. This thematic was constructed in a systematic form attempting to gradually solve the difficulties/constrains encountered, through the application of different experimental approaches, targeting the entire wine chain production, developing methodologies that will allow to identify the varietal composition of wine in every step of its production, back to its origin. The grape reception in wine cellars, are most of the times the first control point of grape varietal composition. Some grape producers are not interested in producing their own wine and sell their grapes to cellars that will transform these grapes. The grape quality and value is accordance to the variety and the geographical region where they are produced. For instance, in the Champagne region a kilogram of grape is payed between 5.5 and 6.5 Euros, whereas in California a kilogram of Cabernet Sauvignon grapes can reach 13 Euros (Falcão, 2014).

Molecular authenticity based on DNA molecular markers implies the extraction of high quality DNA from must samples. In Chapter 2, the work concerning the authenticity of must varietal composition is evaluated through two microsatellite-based systems: the microsatellite (SSR) and inter-microsatellite (ISSR). In this study, the DNA extraction method applied to must samples yielded high quality genomic DNA which was then used to study a group of *Vitis vinifera* L. varieties that deferred in grape skin color. The markers selected to screen the varietal composition were the OIV recommended, the SSR primer set and a group of ISSR primers. The results obtained revealed that

ISSRs markers were not suitable for certification procedures, whereas nuclear SSR markers presented a complete correspondence between leaf and must samples, demonstrating that they were adequate and could be properly applied for verifying the presence of protected designation of origin grapevine varieties.

The non-applicability of ISSR markers is due to the absence of high-molecular-weight bands which are present in the leaf DNA samples, demonstrating that DNA fragmentation occurs, conditioning the molecular marker choice. Must samples were collected immediately after the grape maceration process and DNA degradation was already detected, the vinification will increase the degradation even more limiting the selection of molecular marker system. To successfully achieve DNA amplification by PCR, the use of an efficient DNA extraction protocols is critical, which is affected by the matrices nature. The elimination of PCR inhibitors is a fundamental step when dealing with must/wine authenticity.

For the development of an authenticity system DNA-based, it is essential that a reliable and a reproducible DNA extraction method is available for the concerned matrices. Concerning wine, it is a very challenging matrix since it is variable and an evolving product, in the sense that its composition varies with the respective variety and with wine aging. In Chapter 3, a method for *Vitis vinifera* L. DNA extraction from wine was established. The protocol developed comprises several steps applied in a particular order with specific reagents in defined concentrations. The proposed protocol was effective when applied to different grapevine varieties and grape skin color, vinification procedures (controlled and non-controlled- commercial), and ages (new wines and 5 year old wines). Moreover, the extracted DNA samples, considering all the variables, were successfully amplified using SSR markers. Thus, this protocol can provide the basis for a successful authenticity system, guaranteeing the varietal identification in wines and therefore suitable for denomination of origin verification, wine fraud and mislabeling detection.

Even though the DNA extraction step has been exceeded, the choice of a reliable and wide applied molecular marker system still remained to be defined. The International Organization of Vine and Wine (OIV) recommended SSR set (OIV, 2009) is precise in the varietal definition, however it is not always successfully applied to DNA wine samples, mainly because of the high-molecular-weight fragments required, which in some cases are not present in the analysed wine samples due to DNA degradation (Siret et al., 2002). The use of molecular markers, such as Single Nucleotide Polymorphism (SNP), capable of overcoming the DNA degradation and targeting short DNA fragments is preferable.

In Chapter 4, new SNP markers, suitable for grapevine varietal identification, were targeted within the functional genes of the anthocyanin pathway (chalcone isomerase-*CHI* and UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*). SNPs represent a new marker system generation applied nowadays to grapevine characterization in parallel with microsatellites. The *UFGT* gene revealed to be highly polymorphic, presenting a high number of SNPs suitable to identify the grapevine varieties studied. In contrast to the *CHI* gene, that presented a low number of SNPs, therefore presenting low discriminatory power among grapevine varieties.

The novelty of this work was to evaluate the application of High-Resolution Melting (HRM) analysis on the grapevine SNP markers developed for molecular genotyping and detection as a way to guarantee the grapevine, must and wine authenticity. Another novelty associated to the developed HRM assay was the combination of a high number of nucleotide differences (33) within a long amplified fragment (704 bp), allowing the discrimination of 18 genotypes in a single assay. The improved HRM assay was optimized using leaf grapevine samples, from certified grapevines. All the genotyping was validated using clonal material. The DNA extraction procedure starting from leaf material is well established, allowing the recovery of pure DNA, which is a request to apply HRM technique. Although the DNA extraction procedures were optimized from must and wine samples, it was not certain that they would be sufficient for the requisites of the HRM chemistry.

In Chapter 5, the previously developed HRM assays (Chapter 4) flavanone 3'-hydroxylase (*F3H*)-based (Castro et al., *in preparation*) and a new assay based on the *UFGT* sequence data was tested using must and wine DNA samples, in order to establish a future authenticity procedure. The three developed HRM assays varied in terms of target fragment (704 bp, 375 bp and 119 bp), limiting their amplification to the sample type, in accordance to what was expected. The HRM assay with the longest amplified fragment (704 bp – *UFGT*-based) was successfully applied to must DNA samples, producing the exact same profile as obtained in leaf samples, allowing the discrimination of various genotypes using a unique assay. As previewed wine DNA samples were not amplified with this particular assay. The application of HRM assay producing smaller amplicons, 375 bp and 119 bp, revealed to be effective when using must and wine DNA samples, with reproducible results within all the sample types. However, the HRM assay targeting the smallest fragment allowed to apply the melting curve shape in the genotype differentiation, being easier to identify the different genotypes detected within this small fragment. This can also be due to the fact that the number of SNPs/fragment length found within *UFGT* is higher than in *F3H*, facilitating the multiple detection in a small fragment. However, the differences found between the HRM assays demonstrate clearly that every authenticity system needs to consider DNA fragmentation, and therefore the molecular marker system and technology to be applied.

Overall, the results obtained throughout this study prove that it is possible to establish a wine authenticity system DNA-based, that will guarantee the DO designation protection and will certainly contribute for a more even and fair trade. The HRM analysis is a rapid screening method that can analyze a large number of must and wine samples. In the future, this strategy can be performed as an authenticity tool for the correct label exposure in high value wines.

6.2 OUTLOOK

The outcomes of this study suggest that DNA molecular markers are by far the preferred choice for wine authentication. With the optimization of the DNA extraction protocol a wide range of molecular techniques can be applied for DNA polymorphism detection, and therefore grapevine varietal identification purposes.

The increasing amounts of sequence data generated using Next-Generation Sequencing (NGS) technologies have increased enormously the polymorphism discovery. The NGS technology has revolutionized plant genomics and combined with new software tools enables the discovery, validation, and assessment of genetic markers on a large scale. SSRs continue to be the markers of choice for large-scale germplasm collections characterization, genetic map construction, and quantitative trait locus (QTL) identification. Nevertheless, SNPs are the most abundant genetic variation and with higher frequencies throughout the plant species genome. The increase number of polymorphic marker, possible due to the efficient use of the sequence data generated, coupled with the SNPs' usefulness, has enabled the development of high-throughput genotyping assays. These strategies are being applied in *Vitis vinifera* L. species, with the genome-wide characterization performed by Myles et al. (2010) in several hundred grapevine varieties and its wild relative *Vitis sylvestris* using the grape 9000 SNP Infinium™ array. This study has allowed to understand not only the genetic profile of the grapevine varieties, but also to understand the *Vitis*' genetic structure and the domestication process (Myles et al. 2011).

The DNA barcoding is a molecular based system, which allows the identification of a particular species/variety within a species, using specific DNA regions. In order for it to be validated the DNA profiles need to be compared with reference sequences. Therefore, for the implementation of a DNA barcoding system certain conditions are mandatory, such as molecular variability between species/varieties and the availability of high quality reference sequences repository. DNA barcoding can be applied to several fields including food authenticity (Galimberti et al. 2013), and it is a reliable alternative to DNA fingerprinting approaches in plants identification, with a higher effectiveness/cost

ratio. In fact, DNA barcoding does not require an extensive knowledge of the genome of each organism, being based on the use of one or few universal markers (Hollingsworth et al. 2011).

Wine authenticity maybe based on such a DNA barcode, through the sequencing data accumulation and SNP identification and validation allowing the development of PCR based methodologies, such as the ones described in this thesis, allowing the simultaneous identification of different grapevine varieties in a unique reaction. The sequence data generated nowadays will certainly accelerate the process. Further request is the quantification of the varietal composition of wines, which is still today limited, because there is a lack of controls that could possibly allow to some extent the varietal quantification. However, at the moment this is still difficult and further investigation needs to be pursued for this issue to be attended.

Although the wine authenticity problematic approached in this thesis was linked to varietal identification, it's our opinion that a consistent, reliable and solid authenticity system should involve also the geographical origin. This type of approach imposes that an integrated analysis is undertaken, with subsequent multivariate data analysis. Thus, different scientific fields such as biology, genetics, oenology, geology, chemistry and physics, among others should contribute to the establishment of reliable and efficient traceability systems for food authentication, and in particular wine. Several examples exist in the literature applied to the wine sector (Catarinucci et al. 2011, Dutra et al. 2011). However, until now none of the systems guarantees the parameters required for a certified wine authenticity system. The searches for new forms of contributing are pursued.

Recently, our research group developed a label-free, simple, specific and reproducible biosensor for DNA detection and quantification, using optical fiber long-period grating (LPG) as physical support for the DNA immobilization, recognition and hybridization. This method potentially enables a fast, reagent free detection and quantification of the DNA present in a given sample without the need of probe labeling or the use of PCR. This methodology can be applied in certification and security of food products including wine, as long there is a suitable DNA extraction protocol to support such a device (Gonçalves et al. 2015).

All these new techniques and concepts provide new opportunities for enhancing the efficiency of wine authenticity systems in the wine market aiming the certification of high-value wines, with the accurate identification of valuable grapevine varieties. The findings directly help the wine sector in what authenticity is concerned, but it has a broader outcome, related to the preservation of endangered grapevine varieties that have potential oenological characteristics. The use of such differentiated varieties can be in the future validated through such a system.

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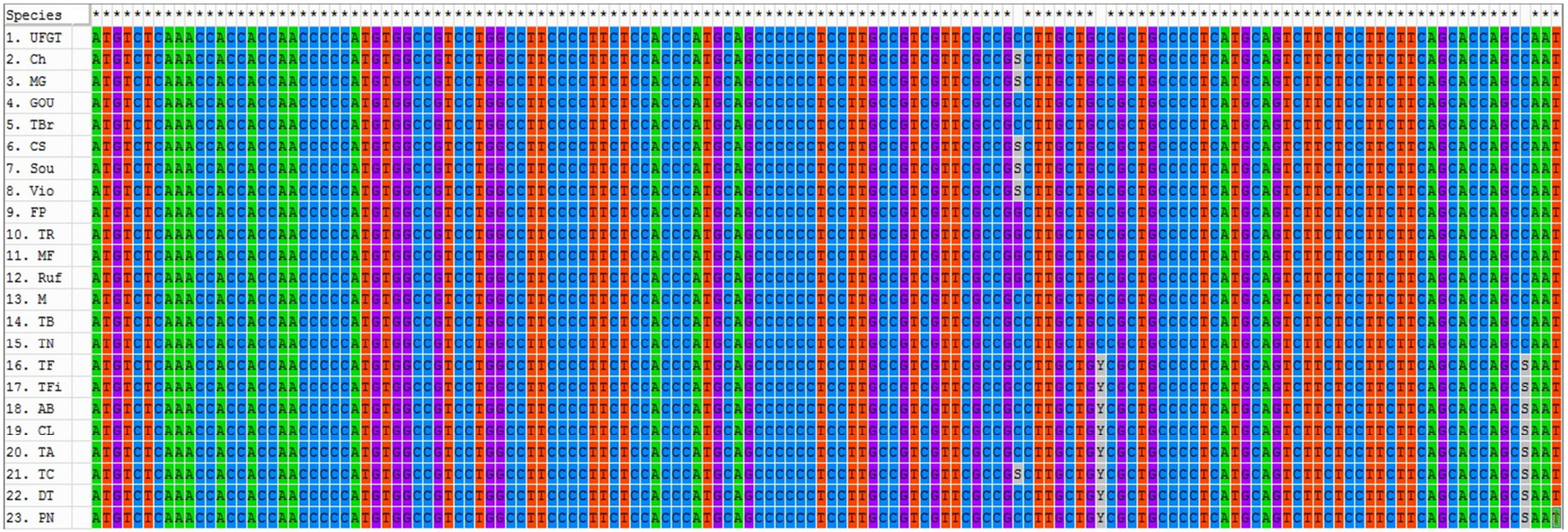
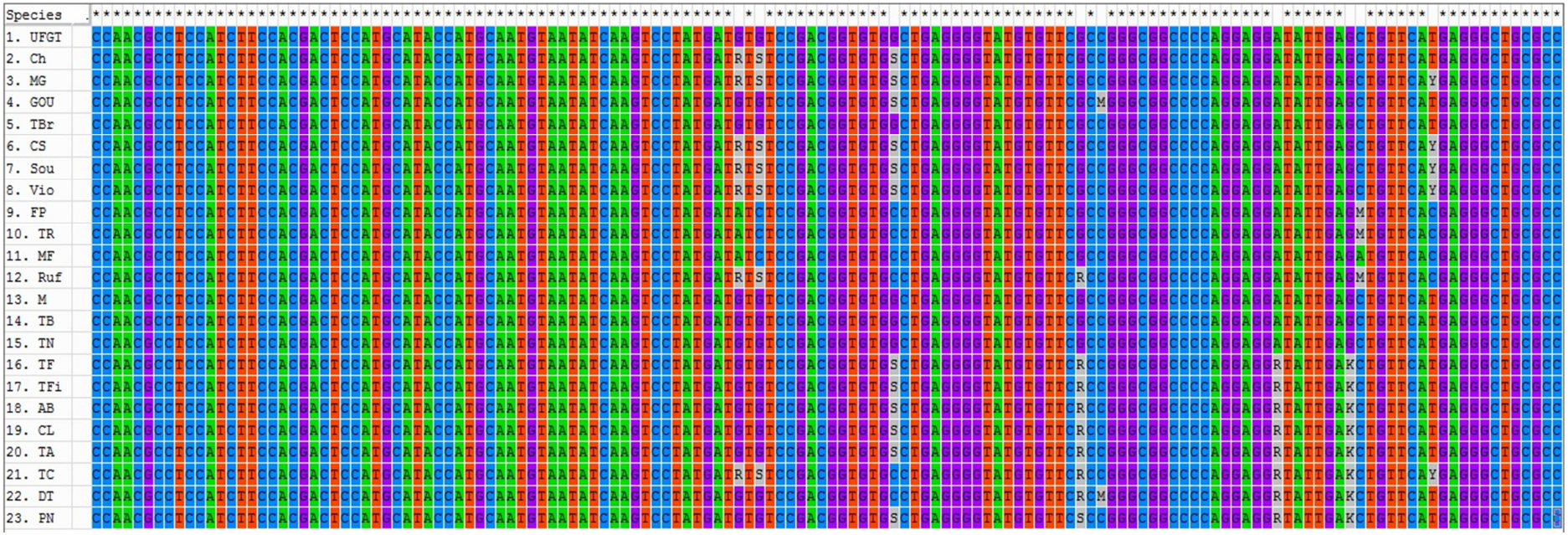
Additional Files

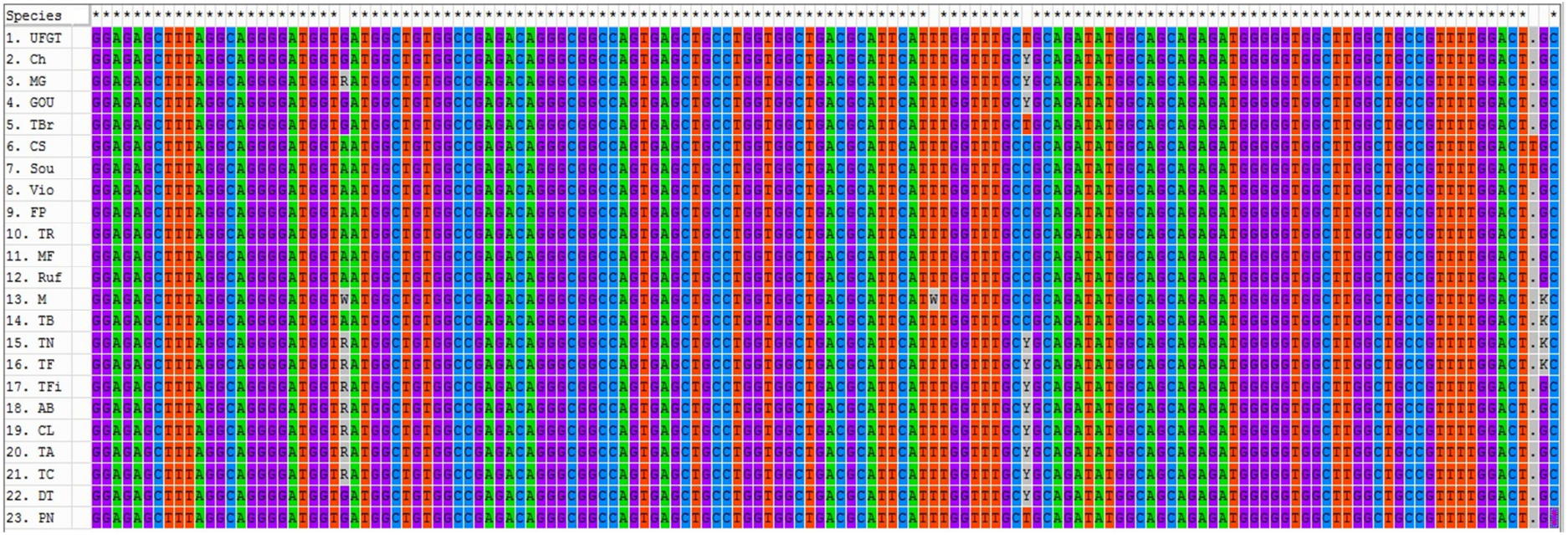
Additional file 1. *UFGT* gene alignment (0-142 bp) performed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).



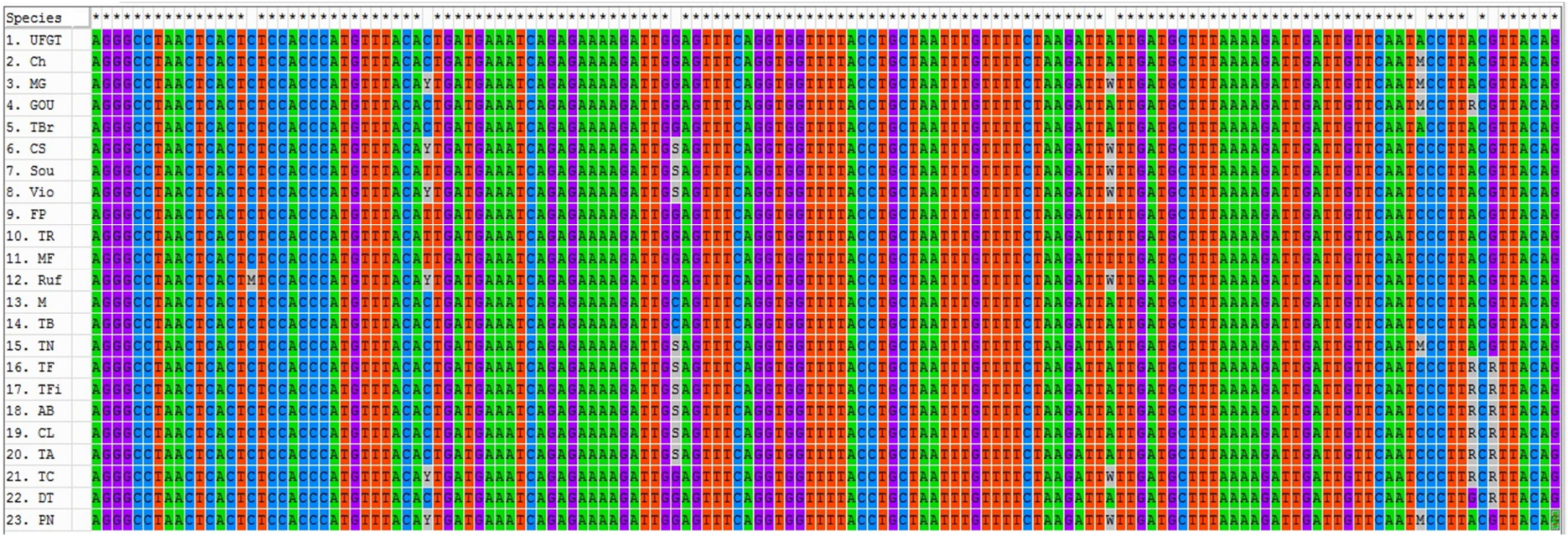
Additional file 1. *UFGT* gene alignment (143 bp-284 bp) (continued).



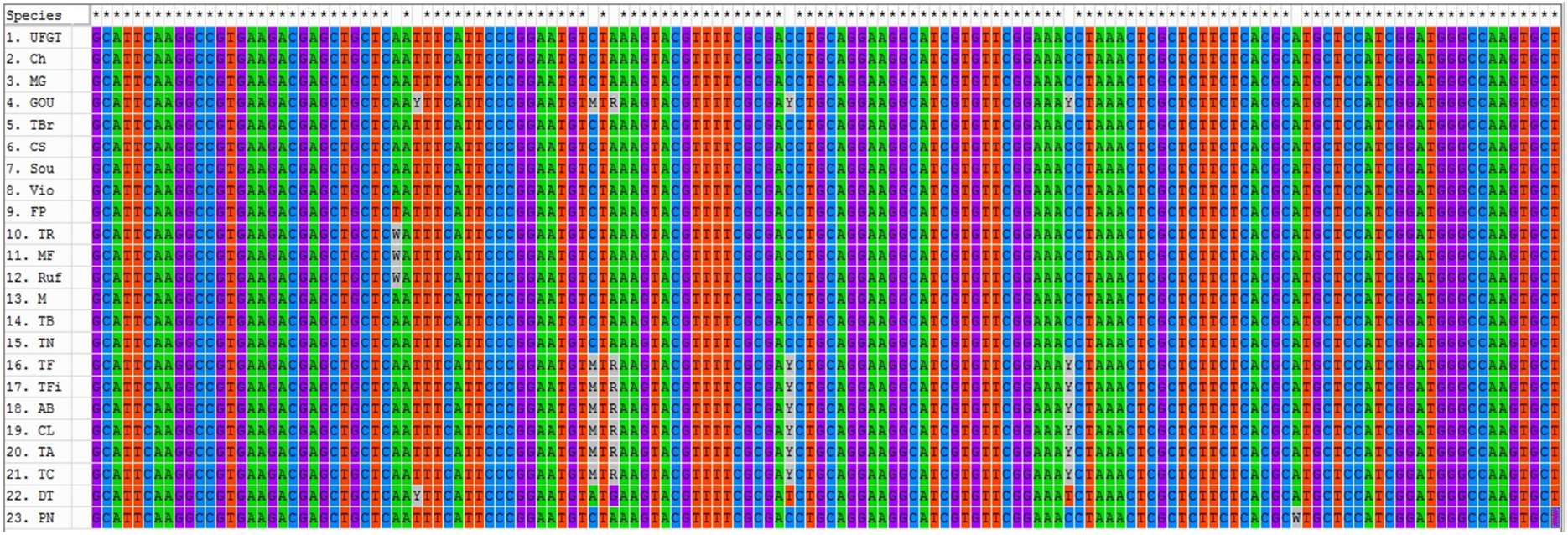
Additional file 1. *UFGT* gene alignment (285 bp-426 bp) (continued).



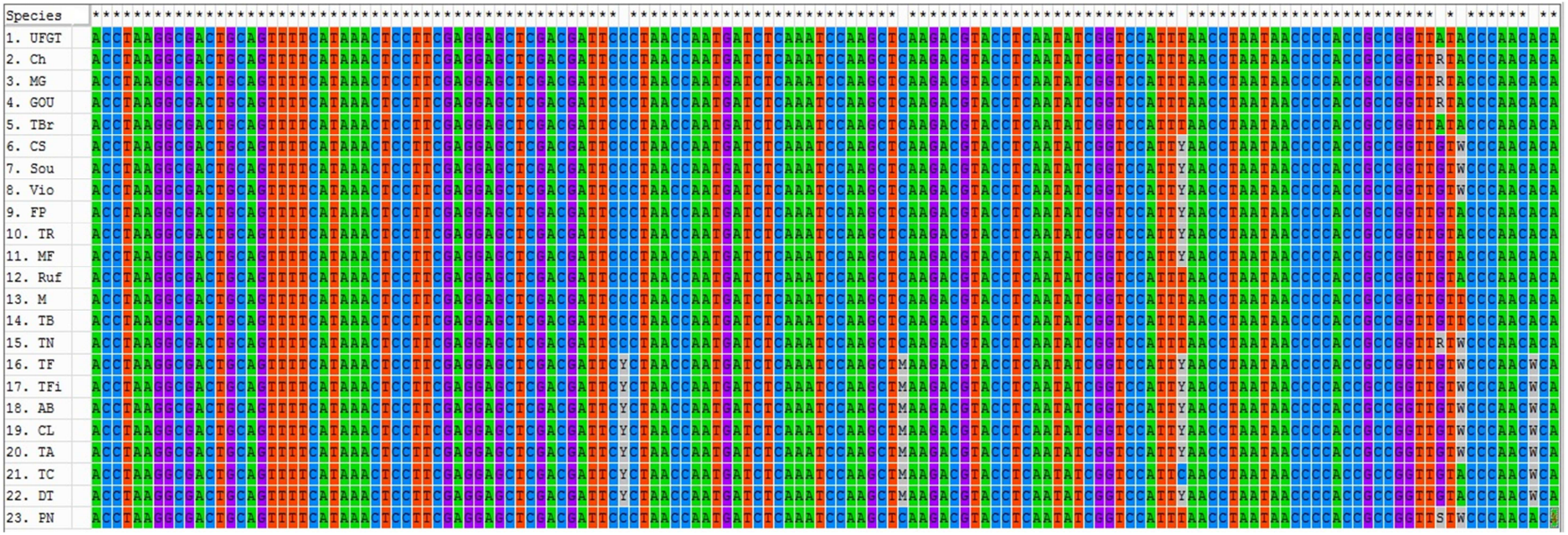
Additional file 1. *UFGT* gene alignment (427 bp-568 bp) (continued).



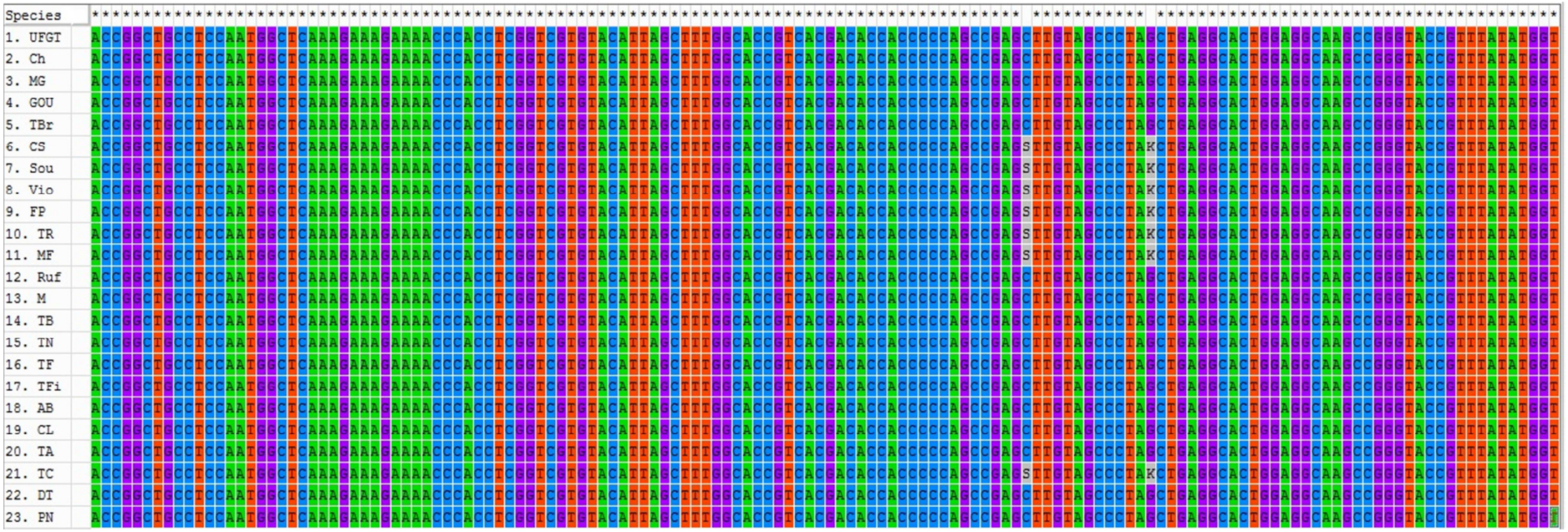
Additional file 1. *UFGT* gene alignment (569 bp-710 bp) (continued).



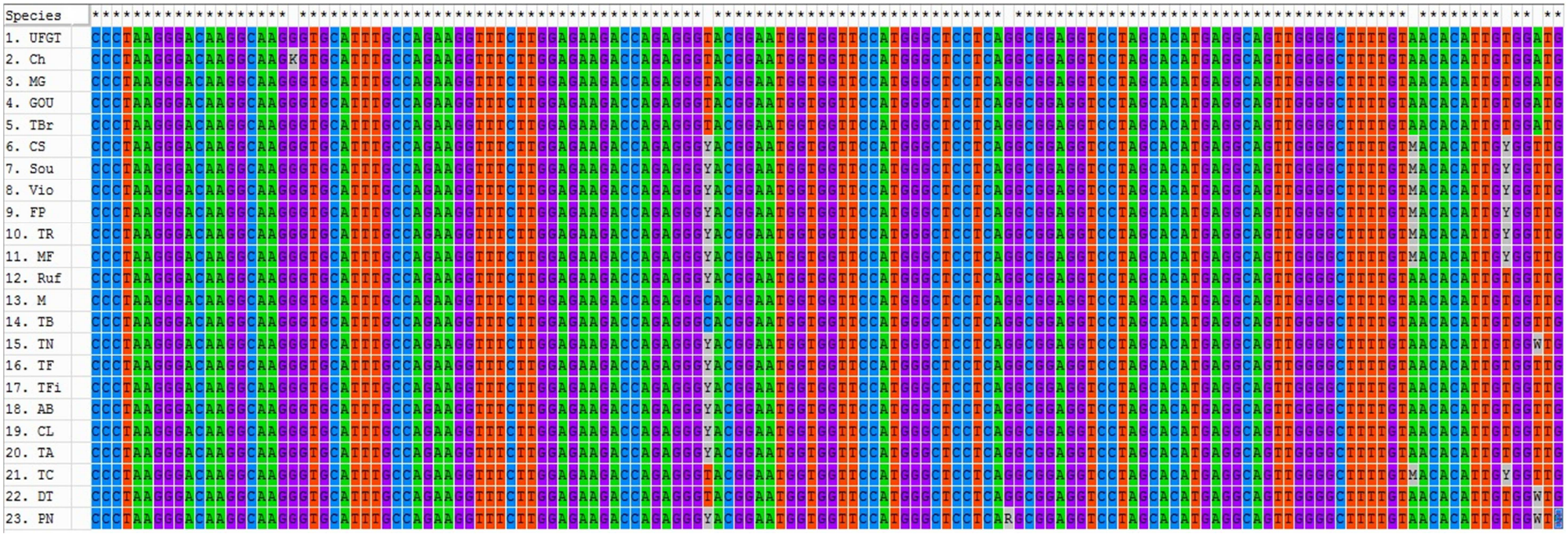
Additional file 1. *UFGT* gene alignment (711 bp-852 bp) (continued).



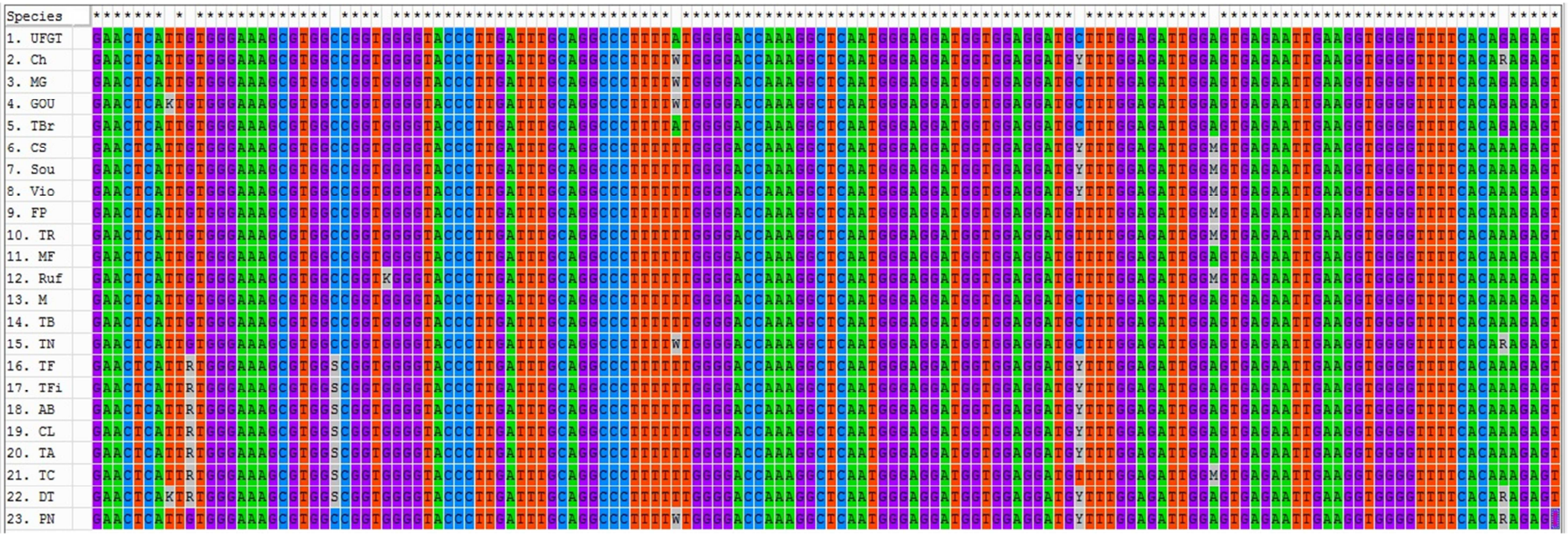
Additional file 1. *UFGT* gene alignment (853 bp-994 bp) (continued).



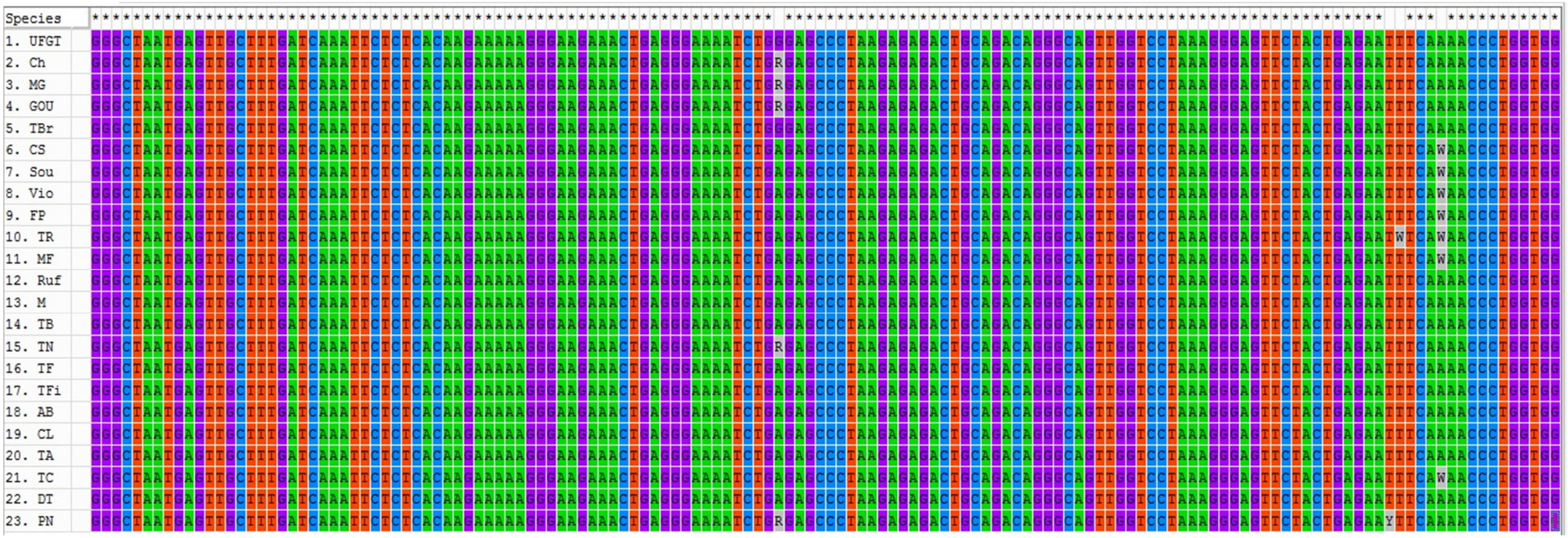
Additional file 1. *UFGT* gene alignment (995 bp-1136 bp) (continued).



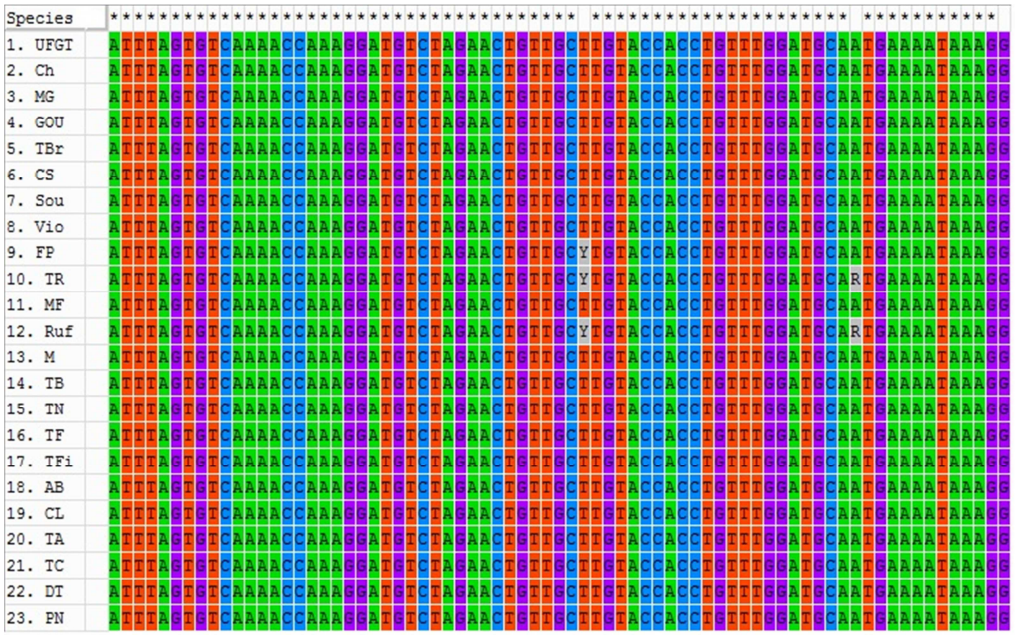
Additional file 1. *UFGT* gene alignment (1137 bp-1278 bp) (continued).



Additional file 1. *UFGT* gene alignment (1279 bp-1420 bp) (continued).



Additional file 1. *UFGT* gene alignment (1421 bp-1492 bp) (continued).



Additional file 2. Submitted SNPs (ss) accession numbers.

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TR	NC_012022.3:g.2333276A>T	1868281817
PN	NC_012022.3:g.2333277A>G	1868281828
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Ruf	NC_012022.3:g.2333516C>A	1868281845
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Additional file 2 (Continued).

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Alicante Bouschet (AB), Chardonnay (Ch), Cabernet Sauvignon (CS), Côdega do Larinho (CL), Donzelinho Tinto (DT), Fernão Pires (FP), Gouveio (Gou), Merlot (M), Malvasia Fina (MF), Moscatel Galego (MG), Pinot Noir (PN), Rufete (Ruf), Sousão (Sou), Tinta Amarela (TA), Tinta Barroca (TB), Touriga Brasileira (TBr), Tinto Cão (TC), Touriga Franca (TF), Tinta Francisca (TFi), Touriga Nacional (TN), Tinta Roriz (TR), Viosinho (Vio).