

# A Review of the Authentication of Wine Origin by Molecular Markers

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## ABSTRACT

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Viniculture is one of the oldest agricultural activities of humans. The selection of grape varieties is of primary concern for factors such as wine quality and is dependent on the grapevine variety employed. Under today's conditions, and the sanctions imposed by world markets in the competitive wine sector, wine producers have been compelled to use different analysis methods in order to prove quality. However, some of these methodologies can be very time-consuming. Recently, highly polymorphic molecular markers have been applied to the characterisation and differentiation of grapes in must and wine. With DNA profiles, the determination of origin and the use of this information on the label can act as a powerful quality control tool in the wine sector. In this review, the advantages of molecular markers and the applications of the markers in determining the origin in must and wine, are discussed with the aim of bringing together the limited number of studies (studies conducted with DNA markers) related to the determination of the grape variety in must and wine.

**Key words:** DNA, grape, molecular marker, wine.

**Abbreviations:** amplified fragment length polymorphism (AFLP), capillary gel electrophoresis and laser-induced fluorescence (CGE-LIF), polymerase chain reaction (PCR), polyacrylamide gel electrophoresis (PAGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), sequence characterized amplified region (SCAR), sequence-tagged microsatellites (STMs), simple sequence repeats (SSR).

## INTRODUCTION

The grape has always had a key role in the life and trade of people, because grapes (*Vitis vinifera* L.) have been cultivated for table consumption, for raisins, for juice, liquor, vinegar or wine production.

Grapevines have been grown commercially for many thousands of years in various parts of the world, especially in the warm climates of Europe, Australia, New Zealand, the USA, South Africa and Turkey. Thousands of grape cultivars have existed in these regions and many of them have been distributed to other parts of the world, far

from their regions of origin. Grape varieties originate in the genus *Vitis*. There are more than 30,000 grape varieties in the world; however, only approximately 15,000 genotypes are currently grown and only a few hundred varieties are cultivated for commercial purposes.

A large diversity of *Vitis vinifera* L. varieties can be used in the production of wine and the wine sector is a major economic agricultural activity. Factors such as climatic and soil differences have an influence on the quality and taste of the wine. These factors cause wines of the same grape variety to have a different taste depending on the growing area and vintage. Wine quality and value are dependent on the grapevine varieties used, and only small numbers of cultivars are of commercial importance<sup>12</sup>. Several works concerning characterisation and differentiation of grapes in must and wine have been based primarily on the analysis of chemical and biochemical parameters, namely the protein and amino acid profiles, analysis of trace elements or phenolic compounds. However, these methodologies are very time-consuming and for this reason molecular markers have been adapted to the analysis<sup>1,15</sup>. These are powerful alternatives for varietal identification and have been developed over the last 20 years. Molecular markers provide significant advantages for grape cultivar identification. DNA typing has enhanced the potential for cultivar characterisation. Some recently developed DNA typing methods based on PCR technologies offer an objective method for the characterisation of grape varieties<sup>15–17</sup>.

DNA extraction from grapes, especially from plant tissues and young shoot tips, is a feasible technique, however there is little literature available regarding grape juice, grape must and commercial wine<sup>1,9,21</sup>. Efficient DNA extraction and amplification from musts and wines is difficult, owing to several factors such as the decomposition of plant DNA during maceration, the contamination of DNA due to microorganisms such as yeasts, and the interference from polysaccharides, tannins and polyphenols in the matrix, which inhibit DNA polymerase in the polymerase chain reaction (PCR)<sup>1,10,27</sup>.

## MOLECULAR MARKERS AND GRAPE

In the past, grape cultivars were identified by comparison of morphological characters, a method based on the visual evaluation of leaf, fruit, shoot tip and other organs. Traditionally, grapevine identification has relied on the skills of ampelography. Ampelography searches for differences among varieties and ensures classification. The expression of many of these traits is often affected by

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diseases or developmental and environmental factors. Morphological characteristics are used for cultivar identification; however, it is known that morphological characteristics can be affected by different environmental conditions. The morphological variability and the subjective evaluations made by man limit ampelographic identification<sup>1,2,11</sup>.

Molecular marker technology uses a DNA sequence that is easily detected and whose inheritance can be readily monitored. The use of molecular markers is based on innately occurring DNA polymorphisms. DNA is easily extracted from plant materials and analysis can be cost and labour effective. DNA-based molecular markers have been widely used in the last few years for genetic studies and cultivar characterisation and identification. Grapevine identification is carried out almost exclusively through DNA marker analysis by the polymerase chain reaction (PCR), using techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR) and simple sequence repeats (SSR).

RFLP is utilized in genetic fingerprinting and paternity testing. RFLP can be applied in diversity and phylogenetic studies ranging from individuals within populations or species, to closely related species. The DNA is first extracted from fresh tissue and purified. The purified DNA can be amplified by PCR. The DNA is then cut into restriction fragments using suitable endonucleases, which only cut the DNA molecule where there are specific DNA sequences and these recognition sequences or restriction sites are recognized by specific enzymes. The restriction fragments are then separated according to length by agarose gel electrophoresis. The resulting gel can be enhanced by Southern blotting. Alternatively, fragments may be visualized by pre-treatment or post-treatment of the agarose gel, using methods such as ethidium bromide staining or silver staining.

With RAPD, the markers are DNA fragments from PCR amplification of random segments of genomic DNA with a single primer of arbitrary nucleotide sequence and they are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. The method is based on enzymatic amplification of target or random DNA segments with arbitrary primers. There are several advantages to the RAPD method relative to RFLP and isoenzymes. The main advantage of the RAPD method is that it is a rapid and simple assay. RAPD does not require labor intensive and costly procedures such as Southern transfers, filter hybridizations or autoradiography. It is not necessary to construct or maintain a genomic library. RAPD requires far smaller quantities of genomic DNA than what is needed for RFLP analysis<sup>5</sup>.

AFLP is a PCR technique based on genetic fingerprinting and was developed in the early 1990's by KeyGene N.V. The AFLP technique can be applied in studies involving genetic identity, parentage and identification of clones and cultivars and, phylogenetic studies of closely related species. AFLP has been preferred to the other markers by scientists in recent years for cultivar characterisation and identification since AFLP-PCR is a highly

sensitive method for detecting polymorphisms in DNA. The technique was originally described by Vos et al.<sup>29</sup>

SSR, microsatellite sequences, also called simple sequence repeats, are regions of tandem repeats of two to five nucleotides that are ubiquitous in eukaryotic genomes. Since they are usually less than 100 bp long and are embedded in DNA with a unique sequence, they can be amplified *in vitro* using PCR. Microsatellites are simple to clone, characterize and display due to variations in the number of repeat units. The polymorphism is sufficiently stable to be used in genetic analyses<sup>6</sup>. Microsatellite markers are highly polymorphic, show a codominant mode of inheritance, and allow simple data interpretation. They have been proven to be superior markers for grapevine DNA typing due to their capacity to detect small amounts of DNA.

Since they are highly polymorphic and codominant, these molecular markers have been proven to be useful in characterizing grape cultivars. The detection is performed using several microsatellite markers accepted internationally as a reference<sup>7,13,14,24</sup>.

## AUTHENTICATION OF WINE ORIGIN BY MOLECULAR MARKERS AND WINE QUALITY TECHNIQUES

Today, it is more important than ever to identify varieties correctly. Not only is accurate identification important to nurseries, growers and winemakers, but recent international trade regulations and wine labeling laws require that varietally labeled wines be correctly identified. Grape varieties have a direct effect on the quality of the finished product. The selection of grape varieties is a primary concern for wine quality. Determining the varietal composition of a wine is useful for wine producers, for consumers and for governmental control and consumer-protection bodies. For the wine producer or wholesaler, such a method can certify the authenticity of the grape variety or varieties used<sup>25</sup>.

During the last decade, the wine sector has implemented programs to encourage the development of biotechnology based testing, as these tests are not affected by different environmental conditions and are thus more reliable. Monovarietal wines are made primarily from a single variety of grape, which is put on the label. However, the inclusion of other varieties in their production is permitted under legally defined percentages, which differ from country to country.

Several works regarding characterisation of grapes in musts and wines have been based on the analysis of chemical and biochemical parameters. Identification of grapevine cultivars or analysis of the geographical origin of the grape from the must or the wine has been attempted using different techniques such as polyacrylamide gel electrophoresis (PAGE) analysis of the native must proteins, thermal ionization mass spectrometry, or <sup>2</sup>H NMR spectroscopy. One of the most successful methods is the native electrophoretic analysis of total grape must proteins using polyacrylamide gels (native-PAGE). Other methods are based on the analysis of phenolic profiles, amino acid profiles, trace elements and isotopes, or terpenes and other aroma compounds<sup>26</sup>.

These methodologies are very time-consuming and therefore molecular markers have been adapted, as a wide range of molecular markers are already in use to characterize and classify grape germplasm<sup>4</sup>. It is important for these analyses to obtain suitable DNA from the must and wine. DNA extraction from must and wine is difficult due to the presence of contaminants such as polyphenols and polysaccharides. Polysaccharides and polyphenolic compounds can form a complex which can become irreversibly bound to nucleic acids<sup>8,19,22,25</sup>. Therefore, an effective DNA extraction technique for must and wines was recently developed. There is only a small amount of published research on the authentication of varietal wines by the analysis of grape (*Vitis vinifera* L.) residual DNA in must and wine using molecular markers. There are a lot of parameters important for the choice of molecular markers for the identification of the grapes from the must and the wine. It is necessary to select a molecular technique which is highly polymorphic and independent of intravarietal variability. Microsatellites were chosen as the basic technique to study the characteristics of wine grape varieties. SSR (microsatellite markers) are true genetic markers, locus-specific, and codominant<sup>2,3,4,18</sup>. The first grape markers were reported by Thomas and Scott<sup>28</sup> and many more have been developed<sup>2,3,4</sup>. For all of the reasons above, microsatellite DNA analysis is a powerful objective and reliable technique for grapevine characterization in musts and wines<sup>8</sup>.

The French doctoral thesis "Study of the genetic polymorphism of the cultivated grapevine (*Vitis vinifera* L.) by means of microsatellite markers: application to the characterisation of grape varieties in wines" by Siret<sup>25</sup> is one of the first published research works. In this work, a new method was developed where DNA extraction could be used to identify and characterize the varietal composition of wines. DNA was analyzed during the fermentation process for six cultivars (Chardonnay, Clairette Blanche, Grenache Noir, Merlot, Muscat Blanc à Petits Grains and Syrah) and the extractions and analyses of DNA from must and experimental wines were conducted using molecular techniques<sup>25</sup>.

In this thesis work, the extractions and PCR analyses of the DNA from the must were successful (day 1). At the beginning of the fermentation, the quantity of DNA extracted from the pellet rose, whereas the pellet weight decreased slowly, which was probably due to structural changes in tissue integrity. DNA was not visible on the agarose gel after staining with ethidium bromide at the end of the fermentation and thus the method was not regarded as very sensitive. PCR amplification was possible with all pellets, except after day 13 with the Muscat Blanc à Petits Grains fermentation. Nevertheless, the whole process was designed to maintain the same conditions for all of the fermentations. In conclusion, the DNA extraction procedure was adequate for the purification of the DNA from the possible inhibitors that could be found in the must or wine. The researcher demonstrated that DNA was present in the wines at the end of the fermentation, and in some cases, as solid particles as well as in solution. This DNA could be analyzed using the microsatellite technique, but improvements in the detection technique still required development to characterize commercial wines.

A second study that employed a microsatellite DNA-based method for *Vitis vinifera* grape must authentication was presented by Faria et al.<sup>8</sup> Five of the most significant port wine producing grape cultivars (Tinta Roriz, Tinto Cão, Touriga Francesa, Touriga Nacional, and Tinta Barroca) were typed at four microsatellite loci as described by Bowers et al.<sup>3</sup> and Thomas and Scott<sup>28</sup>. The corresponding five varietal musts and 26 must mixtures, that resulted from the combination of the five varieties, were also typed at the four loci. There were no differences between the corresponding leaf and varietal must profiles. All must combinations showed the expected band profiles corresponding to the sum of the varietal band profile components. Among the 26 must mixtures, eight could be discriminated using the four loci VVMD5, VVMD6, VVMD7 and VVS2<sup>4,8,28</sup>.

Siret et al.<sup>27</sup> studied the usefulness of SSR markers for the analysis of must and wine mixtures. It is known that wine quality depends not only on the vinification process and the geographical origin of the grapes, but also relies highly on varietal composition. Cultivars of standardized quality are used, but grapevine identification can sometimes pose a problem for wine-growers. Wine aromatic compounds, particularly monoterpenes, pigments, or other organic trace compounds, have been tested as indicators of the grape variety used to produce a given wine. Must and juice varietal differentiation has also been possible by means of isoenzyme patterns or by the use of electrospray mass spectrometry for the determination of grape juice proteins. However, until now, no tests have been available for wines. In this research, methods based on DNA typing were developed using the PCR technology of recent years. Microsatellite markers were applied to the analysis of grape juices<sup>25</sup>.

Other studies have also enabled the analyses of DNA during the fermentation process, in experimental monovarietal wines. Analysis of must and wine mixtures (Chardonnay B/Clairette B and Syrah N/Grenache N) were performed on experimental fermentations<sup>27</sup>. In this research, the usefulness of the microsatellite technology for the analysis of must mixtures and for the analysis of wine mixtures during the fermentation process was assessed. As a preliminary test, researchers analyzed leaf DNA mixtures with microsatellite markers, in order to estimate the detection limit of a foreign DNA. Then, grape mixtures were made, and experimental fermentations were carried out. DNA was extracted during the fermentation process and analyzed using five microsatellite loci. Wine mixtures could be identified with this technique when up to 30% of a second cultivar was added. In this study, leaf DNA extraction methodology was successfully applied to musts with some modifications, and the microsatellite DNA-based method was successfully applied to the authentication of mono and multivarietal musts. However, this method relies on a *Vitis vinifera* microsatellite genotype database for the determination of not only the presence or absence of a certain variety, but also the positive identification of others that may also exist in the must. This type of analysis becomes easier when one is working with musts that originate from demarcated regions, where only a few varieties are authorized for the production of quality wines. The presented methodology is particularly

important when one needs to know if a must sample contains only one variety as this information has particular importance for the production of monovarietal wines. DNA was extracted during the fermentation process and analyzed using five microsatellite loci. The 70:30 (v/v) mixtures were successfully analyzed until the end of the fermentation<sup>27</sup>.

García-Beneytez et al.<sup>11</sup> identified several musts and wines using microsatellite markers. DNA was extracted from the solid phase of 16 monovarietal and 5 multivarietal musts (mixtures of two musts down to a 4:1 proportion) and these 21 samples were genotyped at seven microsatellites through a multiplex PCR reaction and automated fluorescence detection. PCR multiplexing was successful in the monovarietal musts, but should be used with caution with at least some markers in multivarietal musts. The same extraction and detection methods were unsuccessfully applied to the solid and liquid phases of five monovarietal commercial wines in this study. This was the case even after using different concentration procedures. The presence of nucleic acids was then studied in a recent must, during the fermentation process, and during the subsequent steps of winemaking. Genotyping was possible in the resulting experimental wine until decanting, when the particles in suspension were removed. These results suggested that wine authentication through DNA analysis was not possible in commercial wines under the tested conditions.

Siret et al.<sup>27</sup> provided some suggestions to improve their method such as increasing the volume, concentrating the samples, using other microsatellite loci, and employing more sensitive technology such as automatic sequencers. This analysis for the varietal identification of musts and wines was evaluated during their investigation. Seven microsatellite markers were applied to the analysis of 16 monovarietal and 5 multivarietal musts (mixtures of 2 musts in different proportions), 5 monovarietal wines, and 1 must during the fermentation process and subsequent steps of winemaking (decanting process) until the complete transformation into a wine. In this work, all the previous suggestions were followed and applied to commercial wines. This can be considered as an important step towards wine authentication, because, up to this time, no reports about authentication of experimental or commercial wines were available<sup>25,27</sup>.

In this work, DNA analysis was also possible in wines a few days after the end of fermentation. Several steps followed the fermentation process to obtain a final commercial wine: decanting, clarification and filtration. During these steps, particles in suspension were removed, eliminating the main source of DNA present. This removal and the DNA degradation that occurred during the winemaking process, when the berries were crushed, prevented the use of sequence-tagged microsatellites (STMs) analysis for wine authentication under the tested conditions. However, STMs analysis is considered a useful tool for cultivar identification of both monovarietal and multivarietal musts.

Another study on wines and must was carried out by Baleiras-Couto and Eiras-Dias<sup>1</sup>. Molecular methods based on residual DNA analysis of *Vitis vinifera* L. cultivars were applied to musts and wines produced from five

different cultivars, namely Touriga Franca, Fernão Pires, Tinta Barroca, Tinto Cão and Marselan. Initially, three DNA extraction methods were compared and optimised for the isolation of DNA from the must and wine. Afterwards, six nuclear and two chloroplast microsatellite markers were used to identify single-varietal must and blends of two varieties of musts prepared in the laboratory and single-varietal wines produced in microvinifications. Preliminary results on the multivarietal musts indicated a possible relationship between the proportion of each variety in the mixture and the signal intensity of the alleles obtained in an automatic sequencer, suggesting that it could be possible to quantify the presence of each variety in the mixture<sup>1</sup>.

Another study in the field employed a method that combined the use of microsatellite markers (VVMD5 and ZAG79), capillary gel electrophoresis and laser-induced fluorescence (CGE-LIF), and was developed and applied to the identification of Albariño and Moscatel Grano Menudo musts by Rodríguez-Plaza et al.<sup>21</sup> It was shown that the microsatellite markers (VVMD5 and ZAG79) provided DNA amplification patterns specific for Albariño and Moscatel Grano Menudo grapes that could be adequately differentiated using CGE-LIF. Furthermore, the DNA sizes determined by the CGE-LIF method were corroborated using a more standard procedure (i.e., an automatic genetic analyzer with a commercial kit), which demonstrated the usefulness of this new methodology. The GCE-LIF method utilized commercially available products including polymers, DNA-intercalating dyes and bare fused silica capillaries, to provide reproducible and sensitive separations of the DNA fragments for grapevine characterization. Extraction of DNA from must and wine was achieved yielding SSR amplification for nuclear and chloroplast loci. This research suggests that nuclear SSR markers can distinguish all varieties used in the authentication of wine origin.

Researchers assert that preliminary results on multivarietal musts indicate a possible relationship between the proportion of each variety in the mixture and the signal intensity of the alleles obtained in an automatic sequencer, suggesting the possibility of quantifying the presence of each variety in the mixture. Results showed that amplifications of the chloroplast SSR loci were achieved using DNA extracted from leaves, must and wine, but a low level of polymorphism was observed. Results demonstrated that markers targeting short DNA fragments in chloroplast genomes could be a useful tool to detect grapevine DNA in wines and suggested the possible application of these techniques in controlling origin certification and in detecting wine falsification. This method could be applicable for forensic applications.

In the light of all the above-mentioned research, further investigations are required in order to develop new chloroplast markers with a higher level of polymorphism and to demonstrate their usefulness in wine authenticity.

## CONCLUSIONS

Grapewine is an ancient fruit species whose domestication goes back nearly 5000 years. The wine sector is one of the economically important agricultural activities in the

world. A large diversity of *Vitis vinifera* L. varieties (cultivars) can be used in the production of wine, although only small numbers are of commercial importance. Production yield, alcohol level, acidity and anthocyanin levels are among the important characteristics of grape varieties. Since these characteristics are highly correlated with the final wine quality, it is important to be able to detect and correctly identify the grape varieties (cultivars) present in musts and wines. This is particularly relevant in controlling the quality and authenticity of monovarietal wines. The cultivars used strongly influence the quality and specific characteristics of the wine. Several studies have shown a relationship between grape varieties and the presence of certain compounds in must and wine such as flavour compounds, polyphenols, acids and sugars. Wine which specifies its respective cultivar name has both a commercial and marketing advantage and fulfils consumer demand for wine value. This is particularly relevant in monovarietal wines. For this reason, wine authenticity is of prime importance, especially in relation to quality control and consumer information. Since wine quality and value are dependent on the grapevine cultivars used, it is of great importance to be able to detect and identify the grape varieties present in musts and wines. The characterisation and differentiation of musts have been based mostly on the analysis of chemical and biochemical parameters, namely protein and amino acid profiles, analysis of trace elements or phenolic compounds. However, these methods do not always give definitive results<sup>21</sup>.

Researchers have demonstrated that DNA is present in wines at the end of the fermentation, in solid particles in some cases, as well as in solution. There are some problems with DNA analysis in wine. Limiting factors for the analysis of wine include the low quantity of DNA present in solution and/or the possible degradation of DNA during the fermentation and aging process. Another limiting factor is the complexity of the wine, as it contains potential inhibitors of the PCR reaction, such as tannins and polyphenols or polysaccharides<sup>10,20-24</sup>.

DNA can be analyzed using the microsatellite techniques, but improvements in the detection technique must still be developed to characterize commercial wines. The DNA extraction procedure is adequate for the purification of the DNA from possible inhibitors that may be present in the must or wine. A method for DNA extraction from wine was developed and was shown to be adequate for microsatellite amplification.

Results have shown for the first time that markers targeting short DNA fragments in the chloroplast genome are useful tools to detect grapevine DNA in wines. This suggests that it may be possible to apply these techniques in controlling wine quality and origin certification, as well as in detecting wine falsification.

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