Genetic traceability of Asti Spumante and Moscato d'Asti musts and wines using nuclear and chloroplast microsatellite markers

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Abstract

The final characteristics of a wine are strongly influenced by must varietal composition. Further, wine quality and value can be heavily modified if grape varieties other than those expected/allowed are used, especially in the case of monovarietal wines. 'Moscato bianco', which is one of the main grape varieties grown in Piedmont (north-western Italy), is used for the production of two renowned monovarietal sparkling wines: Asti Spumante and Moscato d'Asti. Here, the genetic traceability of these wines was assessed using a simple sequence repeat (SSR or microsatellite) DNA-based method. Must and wine samples from two local wineries were collected at different winemaking steps: after grape crushing and pressing, without the skins (must sample 1, M1); after static clarification or flotation (M2); halfway through fermentation (M3); and finished wines. A DNA extraction protocol was developed, and samples were analysed using a set of 9 nuclear (nSSR) and 7 chloroplast (cpSSR) markers. The application of nSSR markers was successful for M1 and M2, but was inadequate for M3 and wines. CpSSR gave better results as amplifications were achieved using DNA extracted from M1, M2 and wines, despite the lack of amplification in M3. Furthermore, the amplified cpSSR loci showed high polymorphism, allowing the identification of 5 distinct chlorotypes among 7 muscat-flavoured and 2 non-aromatic grapevines. Altogether, these results suggest that this technique could be extended to wine quality and authenticity control, as well as origin protection.

Keywords

Grapevine Vitis vinifera L. Moscato bianco DNA extraction Simple sequence repeat (SSR)

Introduction

The Muscats are a large family of grapevines (Vitis vinifera L.) that share the characteristic 'muscat' aroma, which distinguishes them from other grapes. This pleasant flavour is one of the reasons for their widespread diffusion and cultivation all over the world, as table grapes or for wine production. 'Moscato bianco' (syn.

‘White muscat’, ‘Muscat blanc à petits grains’, ‘Moscatel de grano menudo’) is considered one of the most ancient cultivars and main progenitors of the Muscat family [1]. This variety has been cultivated in many temperate European areas since ancient times, like the equally popular descendent ‘Muscat of Alexandria’.

‘Moscato bianco’ is one of the main grape varieties grown in Piedmont (north-western Italy), where it accounts for one-fourth of the total cultivated area. The plants are cultivated in the Langhe and Monferrato hills, including part of the provinces of Alessandria, Asti, and Cuneo. The ‘Moscato bianco’ grapes are used for the production of two renowned sparkling wines: Asti Spumante and Moscato d’Asti (semi-sweet/sweet wines). These wines are obtained with a double fermentation in pressure-resistant closed tanks, employing the method developed in Italy by Federico Martinotti (1860–1924), but more widely known as the “Charmat” method. This technique is more suitable for aromatic musts than the traditional “champagne” method, because it allows a better preservation of the primary aromas. In Moscato d’Asti wine, in comparison with Asti Spumante, the fermentation is stopped sooner so that the residual sugar content is higher, the alcohol level lower, and the wine less fizzy. Both wines are certified DOCG (Denominazione di Origine Controllata e Garantita), the most prestigious Italian Appellation of Origin. DOCG wines are analysed by government-licensed personnel before bottling, and then, bottles are sealed with a numbered governmental seal across the cork to prevent later manipulations.

The vinification process and the geographical origin of the grapes are two important parameters of wine quality. Nevertheless, the final characteristics of the wine are strongly influenced by must varietal composition, especially in monovarietal wines, for which only one cultivar is used. Thus, wine quality and value can be heavily modified if grape varieties other than those allowed are employed. In that respect, the development of methods allowing the authentication of grape varieties in musts and wines would be of great value for quality and authenticity control, as well as origin protection.

Musts and wines are characterized and distinguished mostly through the analysis of chemical and biochemical parameters, such as protein and amino acid profiles [2–4], trace elements and isotopes [5], terpenes and other aroma compounds [6, 7]. However, such methods are time-consuming and although they can be successful in determining the grape cultivars used in musts, they generally do not give definitive and reliable results in wines. In the last two decades, DNA-typing has proved to be a valuable technique for accurately identifying grape cultivars due to its high discriminating power at a relatively low cost. Among the available DNA markers, nuclear simple sequence repeats (nSSR or microsatellites) are the markers of choice for grapevine fingerprinting [8]. They are polymerase chain reaction (PCR)-based, highly polymorphic and highly reproducible, and they permit data exchanges among laboratories as well as the construction of integrated databases. SSR markers have been used to distinguish between grape cultivars using residual grape DNA extracted from monovarietal, multivarietal, or fermenting musts [9–16], and from experimental [12–16] or commercial wines [16, 17]. Moreover, a simple single nucleotide polymorphism

(SNP)-based method using a cleaved amplified polymorphic sequence (CAPS) has been applied to must mixtures during alcoholic fermentation [18]. In general, all research groups obtained positive results with respect to must analysis and concluded that the main limiting factors with respect to DNA authentication in wines were the small amount and the degradation of residual DNA extracted. The presence of potential PCR inhibitors was also considered a limiting factor for marker amplification.

The use of markers amplifying mitochondrial or chloroplast sequences may be useful due to their relative abundance and stability in comparison with nuclear sequences. Chloroplast SSR (cpSSR) markers have been developed in the last years [19–21]. Their ability to identify grape cultivars has been assessed, but findings from these studies show a low level of polymorphism [22–25]. Nevertheless, cpSSR have also been used to demonstrate their usefulness for DNA authentication of wines [16, 26].

In the present work, we assessed the usefulness of a microsatellite DNA-based method for the genetic traceability of the monovarietal wines Asti Spumante and Moscato d'Asti. Unlike in the previous works, based on the analysis of wines obtained from experimental microvinifications, in our work, musts and wines were directly sampled during the winemaking process in two important wineries of the Asti area (Piedmont). A new DNA extraction protocol was developed, and samples were analysed using a set of 9 nSSR and 7 cpSSR in order to identify cultivars in musts and wines.

Materials and methods

Plant material

Young grapevine leaves were collected from 'Cortese', 'Italia', 'Malvasia moscata', 'Moscatello selvatico', two 'Moscato bianco' clones ('CVT4' and 'Muscat blanc à petits grains'), 'Moscato di Alessandria' (syn. 'Muscat of Alexandria', 'Zibibbo'), 'Moscato giallo', 'Moscato rosa', 'Muscat Ottonel', and 'Orange muscat' true-to-type accessions grown at Bosca Industries in Canelli (Asti, Italy) and CNR-IVV in Grinzane Cavour (Cuneo, Italy). Leaves were stored at −80 °C until DNA extraction.

Must and wine samples

Monovarietal musts and wines of two sparkling wines made from 'Moscato bianco', Asti Spumante and Moscato d'Asti were sampled during the main winemaking steps in the two wineries (Vallebelbo and Terrenostre, respectively) located in the Cuneo province (Piedmont, north-western Italy). In both cases, 5 l of must was collected at three winemaking steps and stored at −30 °C: (1) must after grape crushing and pressing, without the skins; (2) must after static clarification (Terrenostre) or flotation (Vallebelbo); and (3) half-fermented must. Wines were sampled from 750-ml bottles ready for marketing and stored at 4 °C. Since the winemaking process of Moscato wines does not provide any contact with the pomace, two red musts were also analysed in order to test the performance of the developed DNA extraction protocol. These musts

were obtained from ‘Croatina’ and ‘Nebbiolo’ cultivars by skin and seed maceration for 24 h followed by pressing.

The commercial winemaking process of Asti Spumante and Moscato d’Asti is as follows: after sedimentation, the grape must is centrifuged and/or filtered and stored in tanks at near-freezing temperatures to avoid natural fermentation. Filtration and/or centrifugation is repeated, if necessary, to prevent further fermentation. One to several months after crushing and stabilization (to prevent protein haze), the must is heated (from 0 to 18 °C) and inoculated with yeast to induce fermentation (18–20 °C). This step is conducted in special sealed tanks (called autoclave) to allow the solubilisation of the CO2 into the wine. The winemakers make batches according to demand so that the resulting wines can be as “fresh” (fresh flavours and sparkles) as possible. Once the desired alcohol (6–9 % in Asti Spumante, 3.5–6.5 % in Moscato d’Asti) and residual sugar (about 80 g/l in Asti Spumante, ≥100 g/l in Moscato d’Asti) levels are reached, the wine is rapidly chilled to stop fermentation, filtered (microfiltration), bottled, and corked. Asti Spumante is usually packaged like ‘champagne’ with a wired-down cork, while Moscato d’Asti typically has the traditional cork used for most still wines.

DNA extraction

Leaves

Total genomic DNA was extracted from 0.25 g of leaves using the Tris/EDTA-Sarcosyl protocol described by Thomas et al. [27]

Musts and wines

DNA extraction from each must type was initially performed from 0.25 g of must pellet following the method described by Thomas et al. [27] and from 2 ml of homogenized must as described by Faria et al. [9]. DNA was also extracted from 0.2, 0.5 and 1.0 g of must pellet using the Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI, USA) following the manufacturer’s instructions.

Later, a new DNA extraction protocol was developed adding the two red ‘Croatina’ and ‘Nebbiolo’ musts as control. In must samples (50 ml), the solid fraction (pellet from which DNA was extracted) was obtained by centrifugation at 4,000 rpm for 40 min at 4 °C. Then, 0.25 g of the pellet was placed in a 2.0-ml reaction tube containing 800 µl of extraction buffer preheated to 65 °C [20 mM EDTA pH 8.0, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0, 2 % cetyltrimethylammonium bromide (CTAB), 1 % polyvinylpyrrolidone (PVP, MW 40,000), and 2 % β-mercaptoethanol]. In wine samples, the solid fraction was precipitated with 2-propanol prior to extraction in order to concentrate the nucleic acids. Thus, 2-propanol (0.7 v/v) was added to 30 ml of wine samples in a 50-ml tube, homogenized, and kept at −30 °C for 2 weeks. After precipitation, wine samples were centrifuged at 4,000 rpm for 40 min at 4 °C. The pellet was dissolved by vortexing in 800 µl of extraction buffer preheated to 65 °C and transferred to a 2-ml microtube. All must and wine samples were

vortexed and incubated in a water bath at 65 °C for 60 min, with occasional mixing (every 10–15 min). Then, 1 volume of chloroform:isoamyl alcohol (24:1) was added and homogenized by vortexing. After centrifugation at 10,000 rpm for 10 min at 4 °C, the supernatant was transferred to a clean 1.5-ml reaction tube and the extraction with chloroform:isoamyl alcohol repeated. Then, the DNA-containing upper phase was transferred to a clean 1.5-ml microtube and thoroughly mixed with 0.7 v/v of ice-cold 2-propanol and 0.1 v/v of sodium acetate (3 M, pH 5.2). After 2 h (must) to overnight (wine) incubation at −30 °C, the precipitated DNA was collected by centrifugation at 13,500 rpm for 30 min at 4 °C, washed once with 700 µl of 70 % ethanol, and centrifuged again at 13,500 rpm for 10 min at 4 °C. Residual ethanol was removed by evaporation at room temperature, and the pellet was resuspended in 50 µl of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0).

DNA quality and quantity was estimated on 0.8 % agarose gels by staining with ethidium bromide and by visual comparison with known quantities of E-Gel Low Range Quantitative DNA ladder (Invitrogen, Carlsbad, CA, USA), using a Gel Doc EQ System (Bio-Rad Laboratories, Hercules, CA, USA).

PCR amplification and microsatellite analysis

The DNA extracted from the leaves, the two red musts (‘Croatina’ and ‘Nebbiolo’), and each monovarietal ‘Moscato bianco’ must type and wine was genotyped using two sets of microsatellite markers. A set of 9 nSSR loci were chosen based on our experience in grapevine fingerprinting: the VVS2 [28], VVMD5, VVMD7 [29], VVMD27 [30], VrZAG62, and VrZAG79 [31] loci, which have recently been proposed by the Organisation Internationale de la Vigne et du Vin (OIV) as molecular markers for the varietal identification in grape [32], and the VMC2F10 (AgroGene S.A., France, unpublished data), VVIQ52 and VVIB66 [33] loci, which were chosen for their very little allele size (≤100 base pairs, bp). A set of 7 cpSSR loci developed for tobacco were also analysed: ccmp3, ccmp5, ccmp10 [19], ccSSR5, ccSSR14 [20], NTCP-8, and NTCP-12 [21] were chosen as they were identified as the most polymorphic loci in 1201 samples of Vitis sylvestris and Vitis vinifera genotypes [24].

All nSSR and cpSSR amplifications were performed in a volume of 20 µl containing 2 µl of 10× NH4 buffer [160 mM (NH4)2SO4, 670 mM Tris–HCl (pH 8.8 at 25 °C), 0.1 % Tween-20], 2.25 mM MgCl2, 250 µM dNTP, 0.55 µM of each primer, 0.75 U BioTaq DNA polymerase (Bioline, London, UK), 1 µl of 10 % bovine serum albumin (BSA) (Panvera, Madison, WI, USA), and 50 ng of DNA extracted from leaves. For DNA extracted from musts and wines, a volume of 3 and 5 µl of DNA solution was used, respectively. PCRs were performed using a thermal iCycler (Bio-Rad Laboratories) under the following conditions for leaf DNA: an initial denaturation step at 95 °C for 3 min; 28 cycles of 30 s of denaturation at 95 °C, 50 s at the annealing temperature (50 °C for nSSR and 54 °C for cpSSR), and 2 min of extension at 72 °C; and a final 10 min extension step at 72 °C. For DNA amplification from musts and wines, 40 and 45 cycles were applied, respectively.

The forward primers were then labelled with a fluorochrome (6-FAM, HEX, NED or PET). One microlitre of a mix containing amplification products of differently labelled loci was added to a mix containing 10:1 parts of Hi-Di formamide and GeneScan-500 LIZ size standard, respectively (Applied Biosystems, Foster City, CA, USA). Samples were denatured at 95 °C for 4 min and analysed on a capillary ABI-PRISM 3130 Genetic Analyzer (Applied Biosystems). Results of the run were then analysed with GeneMapper software (Applied Biosystems), and alleles were designated by their size in bp.

Results and discussion

DNA extraction methods

The DNA extraction method currently used in our laboratory for grape leaf or bud tissue is the Tris/EDTA-Sarkosyl protocol described by Thomas et al. [27], which has also been successfully applied to musts and wines by Baleiras-Couto and Eiras-Dias [16]. Other DNA extraction protocols have been developed for grape musts and wines, most of which are CTAB-based, such as the ones described by Faria et al. [9] and Siret et al. [13]. The first method has been widely used by several authors [10, 14, 15] in comparison with the second one, which is more laborious.

As a first step, DNA extraction from each 'Moscato bianco' must type was performed following the Thomas et al. [27] and Faria et al. [9] protocols. In both cases, DNA was not visible on the ethidium bromide stained gel, while RNA was only observed using the Faria et al. [9] protocol (data not shown). The presence of a low-molecular-weight band or smear, consisting essentially of degraded RNA, was useful to evaluate the efficiency of the extraction procedure, as a rough direct relationship between DNA and RNA band intensities could be established in these musts. The extracted DNA was examined further by PCR analysis, which is a much more sensitive approach than ethidium bromide staining and UV-visualization, but GeneMapper analysis confirmed the absence of nSSR amplification in all samples. In addition, no DNA or RNA bands were found using Wizard Magnetic DNA Purification System for Food, a commercial kit for the separation of nucleic acids based on a magnetic bead technology.

As a second step, DNA was extracted using our CTAB-based method. The quantity of extracted DNA, estimated by visual comparison with known quantities of standard DNA, varied between the 'Moscato bianco' must types tested and was unrelated to the industry (Terrenostre vs. Vallebelbo) (Fig. 1). The DNA quantity obtained from musts after grape crushing without the skins (M1) ranged from 20 to 100 ng/µl, while for musts after static clarification or flotation (M2), DNA concentration was lower, ranging from 5 to 10 ng/µl. The amount of DNA extracted from musts sampled at mid-fermentation (M3) could not be estimated because of the very low quantities obtained. In contrast, a high DNA concentration (≥100 ng/µl) was observed for the two control red musts with skins. RNA bands were detected in all samples with variable intensity as PCRs were generally performed without RNAse digestion; however, since RNA is considered an

amplification suppressor at high concentrations [34], it was digested in recalcitrant samples where no amplification occurred. Unlike the protocol proposed by Faria et al. [9], based on a constant volume of must sample (2 ml), our method relies on a constant quantity of solid must parts (0.25 g). In fact, since the pellet recovered from a constant volume varied according to must type (white or red) and winemaking step (M1, M2, and M3), using a constant weight in our DNA extraction protocol was successful. Nevertheless, the amount of extracted DNA decreased dramatically from M1 to M3, a result that contrasts with those obtained by Siret et al. [13]. The authors, working on samples obtained from microvinifications, observed an increase of the DNA quantity up to the beginning of fermentation, whereas the pellet weight decreased slowly, probably due to structural changes in tissue integrity. Consequently, more DNA was recovered when the cells were degraded. Here the amount of DNA in the pellet decreased dramatically from M1 to M3 along with its weight. The main explanation is the strong processes of purification applied to 'Moscato' musts during the industrial winemaking. In fact, although M1 is still a grape juice, in M2 the solid parts are partially eliminated, and in M3 the settling, fining, filtration, and centrifugation steps are repeated many times before the beginning of fermentation.

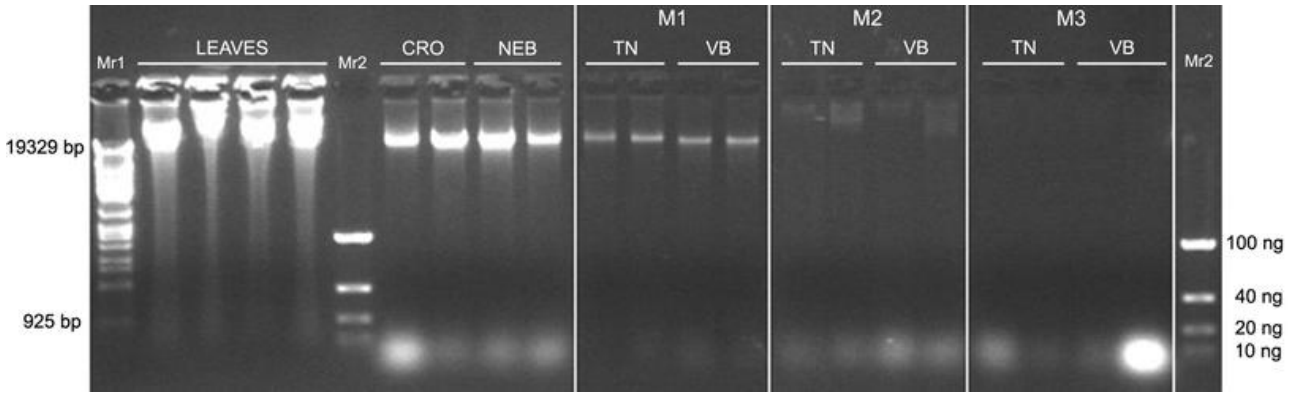

Fig. 1

DNA extracted from 'Moscato bianco' leaves, 'Croatina' (CRO) and 'Nebbiolo' (NEB) red musts, and each 'Moscato bianco' must type collected from Terrenostre (TN) and Vallebelbo (VB) wineries: M1 must after grape crushing, without the skins; M2 must after static clarification (Moscato d'Asti, TN) or flotation (Asti Spumante, VB); M3 half-fermented must. Mr1 molecular weight marker (λ DNA/Eco130I(StyI)/MluI,); Mr2 quantitative DNA ladder (E-Gel® Low Range Quantitative DNA ladder)

As reported by other authors [12–17], the nucleic acids recovered from wine extraction were not visible on agarose gels, despite the additional precipitation step with 2-propanol. DNA extracted from wine is expected to be scarce and of low quality/integrity because of degradations due to enzyme activity and wine processing. Nevertheless, the extracted wine samples were submitted to PCRs because traces of DNA could be present and therefore amplified.

Nuclear SSR analysis

The genetic traceability of residual grapevine DNA in Moscato d'Asti and Asti Spumante monovarietal musts and wines was tested with nuclear microsatellite analysis, using leaf DNA as control.

PCR amplification at the 9 nSSR loci was successful in M1 and M2 and in the two red 'Croatina' and 'Nebbiolo' musts. The nSSR allele sizes were identical in must and leaf samples of the same cultivar (Table 1). With respect to the VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79 loci, accepted by the OIV as universal markers for grapevine genotyping [32], DNA amplification was successful in all must samples. Conversely, no amplifications were reported by Savazzini and Martinelli for the same set of loci in some varietal wines [15] and by others for VVMD5 and VVMD7 [14] or VrZAG79 and VVMD7 [16], due to possible DNA degradation in the primer-annealing regions. Other limiting factors can inhibit Taq polymerase activity during DNA amplification. Phenols and polysaccharides, among others, occur in high quantities in must and wine tannins. As a consequence, an extensive purification is often required to generate PCR-compatible material. However, because purification increases the time and cost of sample preparation as well as the loss of target nucleic acids, a more satisfying approach to the problem of PCR inhibition would be to relieve interference rather than attempt to remove all of the inhibitory substances. BSA has been widely used for relieving PCR and other enzymatic reaction interference [34]. Therefore, BSA was included in our PCR protocol, and amplifications were successful for all nSSR tested.

Table 1 Allele size (in base pairs, bp) at 9 nSSR loci in leaves, musts, and wines

Cultivar	VVS2	VVMD5	VVMD7	VVMD27	VrZAG62	VrZAG79	VMC2F10	VVIQ52	VVIB66
Leave									
Croatina	139–151	235–235	247–249	188–194	186–196	244–244	90–94	79–79	90–108
Nebbiolo	155–155	231–235	247–249	184–188	194–200	242–250	94–104	79–79	96–106
Moscato bianco	133–133	227–235	233–249	178–194	186–196	250–254	94–100	79–79	90–90
Must									
Croatina	139–151	235–235	247–249	188–194	186–196	244–244	90–94	79–79	90–108
Nebbiolo	155–155	231–235	247–249	184–188	194–200	242–250	94–104	79–79	96–106
Moscato bianco M1	133–133	227–235	233–249	178–194	186–196	250–254	94–100	79–79	90–90
Moscato bianco M2	133–133	227–235	233–249	178–194	186–196	250–254	94–100	79–79	90–90
Moscato bianco M3	–	–	–	–	–	–	–	–	–
Wine									
Moscato d'Asti	–	–	–	–	–	–	–	–	–
Asti Spumante	–	–	–	–	–	–	–	–	–

M1 must after grape crushing, without the skins, M2 must after static clarification (Moscato d'Asti) or flotation (Asti Spumante), and M3 half-fermented must.

The identification of the grape variety in fermenting musts (M3) and wines (Moscato d'Asti and Asti Spumante) was not possible using nSSR markers. The amplifications were not consistent, and the few successful ones were not repeatable (data not shown). Such results, together with the absence of visible DNA bands on agarose gels, indicated that no DNA or low traces of highly degraded DNA were extracted. nSSR amplifications have been observed in samples collected during or at the end of fermentation in experimental microvinifications [12–16] but not in

commercial wines [16]. All winemaking steps aimed at "cleaning" (i.e., clarifying) the wine are more intensively applied in wine industries than in experimental wines. The solid parts, basically composed by traces of grape, seed and skin tissues, are gradually removed during the post-fermentation steps (decanting, clarification, and filtration), thus eliminating the main source of DNA. This DNA depletion and degradation is even more severe in Moscato d'Asti and Asti Spumante than in other wines as different clarification steps, including the use of adjuvants such as bentonite, are applied many times before and after the fermentation. Savazzini and Martinelli [15] have obtained a nSSR amplification of approximately 240 bp in DNA samples extracted from 18- to 24-month-old wines, but the analysis was not successful in all of the 20 tested wines nor for the 6 tested loci. Only an endogenous target of 80 bp in length was systematically amplified in the 20 wine samples. Therefore, the VMC2F10, VVIQ52, and VVIB66 nSSR, characterized by low allele sizes, were tested in the present work, but no amplification was obtained in M3 musts and bottled Moscato wines.

Chloroplast SSR analysis

The use of markers amplifying mitochondrial or chloroplast genomes may be useful in the genetic traceability of wines due to their relative abundance when compared to nuclear genome. In particular, it has been suggested that their circular form increases stability and resistance against heat disintegration [35]. Arroyo-García et al. [24] have analysed chloroplast DNA polymorphism in Vitis. Only 9 out of 54 primer pairs tested revealed length polymorphism within 1201 genotypes of two Vitis subspecies (Vitis sylvestris and Vitis vinifera), identifying 8 different chlorotypes. Among these cpSSR loci, two (ccmp3 and ccmp5) were also tested in must and wine by Baleiras-Couto and Eiras-Dias [16].

In this work, 7 cpSSR primer pairs were used for the detection of grape cultivars in 'Moscato bianco' musts and wines. In a first step, PCR amplifications were carried out using DNA from young leaves in order to get the cpSSR profiles of two 'Moscato bianco' clones ('CVT4' and 'Muscat blanc à petits grains') and 9 other grape cultivars selected from those that may be fraudulently used to produce Moscato d'Asti or Asti Spumante wines. Among these, 7 were muscat-flavoured and 2 were non-aromatic ('Italia' and 'Cortese'). cpSSR analysis showed a high level of polymorphism among the genotypes. Considering the allele variants, 5 different chlorotypes were detected (Table 2). A similar pattern was observed between the two 'Moscato bianco' clones and 'Malvasia moscata', an ancient cultivar seldom mixed with 'Moscato bianco' in vineyards of the

Asti area. Yet, the ‘Moscato bianco’ chlorotype was different from all others, suggesting that this set of cpSSR is suitable for its genetic traceability in must and wine.

Table 2 Allele size (in base pairs, bp) and chlorotype at 7 cpSSR loci in 11 grape cultivars and monovarietal musts and wines

Cultivar	ccmp3	ccmp5	ccmp10	NTCP-8	NTCP-12	ccSSR5	ccSSR14	chlorotype
Leave								
Cortese	104	102	110	245	114	253	203	A
Italia	104	102	112	245	114	253	205	B
Malvasia moscata	105	101	111	246	113	252	204	C
Moscatello selvatico	105	101	111	246	113	252	205	D
Moscato bianco								
CVT4	105	101	111	246	113	252	204	C
Muscat blanc a` petits grains	105	101	111	246	113	252	204	C
Moscato di Alessandria (syn. Zibibbo)	104	102	111	245	114	253	204	E
Moscato giallo	104	102	110	245	114	253	203	A
Moscato rosa	104	102	112	245	114	253	205	B
Muscat Ottonel	104	102	112	245	114	253	205	B
Orange muscat	105	101	111	246	113	252	205	D
Must								
Moscato bianco M1	105	101	111	246	113	252	204	C
Moscato bianco M2	105	101	111	246	113	252	204	C
Moscato bianco M3	105	–	–	–	–	–	–	–
Wine								
Moscato d’Asti	105	101	111	246	113	252	204	C
Asti Spumante	105	101	111	246	113	252	204	C

M1 must after grape crushing, without the skins, M2 must after static clarification (Moscato d’Asti) or flotation (Asti Spumante); and M3 half-fermented must

The PCR amplifications at 7 cpSSR loci in M1 and M2 ‘Moscato bianco’ musts and two red musts (‘Croatina’ and ‘Nebbiolo’) were successful, and the results confirmed the allelic size obtained from ‘Moscato bianco’ leaf DNA (Table 2). In the M3 must (sampled at mid-fermentation), only ccmp3 was properly amplified, showing the same allele size (105 bp) as that obtained in leaf. Therefore, the fermentation process indeed prevents the identification of the grape variety in must. In contrast, cpSSR amplification was successful in wines, and the allele size was the same as that obtained for the reference leaf DNA (Table 2). These results are comparable to those obtained in monovarietal musts and wines by Baleiras-Couto and Eiras-Dias [16], although the number of markers used here allows defining a proper chlorotype that distinguishes most of the tested grape varieties.

Conclusion

In this work, we assessed the usefulness of a microsatellite DNA-based method for the genetic traceability of the monovarietal wines Asti Spumante and Moscato d’Asti during their main winemaking steps. In particular, this methodology was tested for the first time on musts and wines obtained from industrial processes, unlike most authors working on samples obtained from microvinifications. Extraction of DNA was achieved yielding SSR amplification for both nuclear

and chloroplast loci, although with different PCR amplification levels. The application of nSSR loci to the genetic traceability of 'Moscato bianco' wines during winemaking was successful only in the first two steps (M1 and M2 musts), but was inadequate in fermenting musts (M3) and finished wines. On the contrary, the amplification of cpSSR loci was achieved using DNA extracted from musts and wines, although difficulties were observed in fermenting musts. These results are encouraging, given that (1) several winemaking steps aimed at "clarifying" the wine are more intensively used in industrial winemaking than in experimental vinifications, and (2) DNA depletion and degradation are more important in Moscato d'Asti and Asti Spumante than in other wines, because settling, clarification with adjuvants, filtration, and centrifugation are performed many times before and after fermentation. Furthermore, although the level of polymorphism in cpSSR is generally low, in our cultivar set, it was high, suggesting the possible application of this technique in controlling origin certification to detect the authenticity of these wines.

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Conflict of interest

The authors declare that they have no conflict of interest.

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