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Novel Preparation Method of Template DNAs from Wine for PCR To Differentiate Grape (*Vitis vinifera* L.) Cultivar

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Template DNAs were extracted from wine and purified for use as samples for PCR to differentiate grape cultivars. It has been pointed out that the authentication of grape material by PCR using wine as a material is very difficult. The problems are (1) decomposition of DNAs during fermentation; (2) contamination of DNAs from microorganisms such as yeast; (3) interference of DNA extraction by polysaccharides and polypeptides in the beverages; and (4) coexistence of PCR inhibitors, such as polyphenols. For this study was developed a novel preparation method of template DNA from wine to differentiate grape cultivars using PCR by (1) lyophilizing and pulverizing the fermented beverage to concentrate the DNAs; (2) decomposition of polysaccharides and proteins so as not to inhibit DNA extraction using heat-resistant amylase and proteinase K without DNA damage by endogenous DNase; and (3) separation of the template DNAs for PCR from PCR inhibitors, such as polyphenols, by purification using 70% EtOH extraction and isopropyl alcohol precipitation. To prevent the amplification of microorganisms' DNAs during PCR, suitable PCR primers closely related to the specific plant DNAs, such as chloroplast DNA and mitochondrial DNA, were selected. The sequences of the amplified DNAs by PCR were ascertained to be the same as those of grape materials.

KEYWORDS: Cultivar identification; fermented beverages; PCR; plant-specific primers; wine

INTRODUCTION

Fermented alcoholic beverages, such as rice wine, beer, and wine, have a very long history and have been enjoyed by people all over the world. The kind of plant material, such as rice, barley, or grape, affects the quality of the fermented alcoholic beverages, such as rice wine, beer, or grape wine (*1*–*3*). As many wine brewers label the wines with the name of the cultivars, development of a scientific method to identify or differentiate grape cultivars using wine itself as a sample is a worthwhile pursuit; because the qualities of wine are greatly affected by the grapes used, authentication of the grape material is very important (*4*, *5*).

In the past decade, proteins or amino acids (*6*–*10*), phenolic substances (*11*), minerals (*12*–*15*), and aromatic substances (*16*–*18*) have been used for the differentiation or identification of cultivars or the geographical origin of grapes.

Although there are many reports on cultivar identification of grape material for wine breweries such as the random amplified polymorphic DNA (RAPD) method (*19*, *20*), SSR primer combination (STMS)s method (*21*–*23*), amplified fragment length polymorphism (AFLP) method (*24*, *25*), and mass spectroscopy (*26*), it has been pointed out that the differentiation of grape cultivars by Polymerase Chain Reaction (PCR) using wine as a sample is very difficult (*4*, *5*). The problems are (a) decomposition of plant DNA during the fermentation; (b) contamination of DNAs from microorganisms such as yeasts; (c) interference of DNA extraction by polysaccharides and polypeptides in the beverages; and (d) coexistence of pigment substances such as polyphenols, which inhibit DNA polymerase for PCR (*4*, *5*).

We reported cultivar differentiation of rice grains (*27*, *28*) and rice material of rice cakes (*29*) and rice crackers (*30*) by the PCR method. We developed various kinds of PCR primers related to plant-specific proteins, such as starch-branching and starch-debranching enzymes (*31*).

The aim of the present study is to improve the method to prepare template DNAs from wine to identify or differentiate grape cultivars by PCR using wine as a sample.

MATERIALS AND METHODS

Materials. Leaves of six kinds of grape, Chardonnay, Cabernet Sauvignon, Cabernet Franc, Riesling, Merlot, and Koshu, cultivated at the National Institute of Fruits, were used for the preparation of the template DNAs.

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Table 1. Sequences of STS Primers and PCR Condition

primer		sequence[a]	annealing temp (°C)	primer (25 pmol/μL)	location (gene)	accession no.
a	F	AGC CGT TCG CAG CG	46	F: 0.3 μL	*O. sativa* mitochondrial SSR (*32*)	AM463372
	R	ATG AGA CCT CGA ATT		R: 0.3 μL		
b	F	CCA AGA GAG GAC AAC CTG T	42	F: 0.3 μL	*O. sativa* mitochondrial SSR (*32*)	AM463372
	R	ATT CCT CAC CTA TCC TGT CA		R: 0.3 μL		
c	F	TTC CTA CGT GAA CCA ATT TT	42	F: 0.3 μL	*O. sativa* chloroplast SSR (*32*)	AP008211
	R	TTC AAA GGG TTA GGT TTT TCT		R: 0.3 μL		
d	F	CTT TGT TTA TGC TTC GGA TT	42	F: 0.3 μL	*O. sativa* mitochondrial SSR (*32*)	X15901
	R	GTT CGC CTA GAG AAT GAC AC		R: 0.3 μL		
e	F	ATG ACT ATA AGG AAC CAA CGA	36	F: 0.2 μL	*V. vinifera* cytochrome *b* gene (*33*)	AY727902
	R	TTG TAA TTA CTG TAG CTC CCC		R: 0.2 μL		

[a] F, forward primer; R, reverse primer. A, adenine; T, thymine; G, guanine; C, cytosine.

Ten kinds of commercial wines, for which the grape cultivars were labeled, were used as samples. These were eight kinds of Chardonnay [France and Chile, 2002; France (2) and Chile, 2004; France (2) and Chile, 2005], Cabernet Sauvignon (Chile, 2002), Sauvignon Blanc (Chile, 2002), Riesling (Germany, 2004), Merlot (France, 2004), Pinot Noir (France, 2004 and 2005), and Koshu (Japan, 2004 and 2005). As an example of fermentation microorganisms, yeast, *Saccharomyces cerevisiae* (NFRI 3069), stored in the National Food Research Institute, Japan, was used as the DNA source.

Preparation of Materials. Thirty milliliters of each wine was put into a plastic centrifugation tube (50 mL) and stored in the freezer at −80 °C. Thereafter, each sample was lyophilized using a freeze-dryer (FD-1, Eyela, Tokyo, Japan) and pulverized with a coffee mill (IF-201, Iwatani, Tokyo, Japan).

Preparation of Template DNA for PCR from Wine. Lyophilized wine powder (100 mg) was put into a sterilized microcentrifugation tube (SMT), and 100 μL (white wine) or 500 μL (red wine) of Tris-HCl buffer (0.1 M, pH 8.0, 0.1 M NaCl) was added. Heat-stable α-amylase (100 μL, *Bacillus licheniformis*, 790 units/mg of solid, 50 mg/mL, Sigma, St. Louis, MO) was added to the above-mentioned sample solution and stored for 1 h at 80 °C using a heat block (Cool Thermo Unit CTU-N, TAITEC, Tokyo, Japan), followed by protein digestion by the addition of 100 μL of proteinase K (Worthington Biochemical Corp., *Tritirachium album*, 27.7 units/mg, 20 mg/mL, Lakewood, OH) with 0.2% sodium dodecyl sulfate (SDS) and 1 h of standing at 55 °C.

After the enzyme reaction, the hydrolysate was centrifuged for 15 min at 4 °C (8000*g*, himac CR 21F, Hitachi Co., Hitachi, Tokyo, Japan), the supernatant was moved to a different SMT, and 2 volumes of cold isopropyl alcohol was added; the mixture was left standing for 15 min. Crude DNA was collected as a precipitate of centrifugation (8000*g*, 15 min). Thereafter, DNA was redissolved in 300 μL of Tris-EDTA buffer (TE, 0.1 M Tris-HCl, pH 8.0, 0.1 M ethylenediamine tetraacetate). Cold 70% EtOH was added to the crude DNA solution, and the mixture was left to stand for 10 min at 0 °C. The DNA in the transparent supernatant was moved at the different 1.5 mL of SMT, and purified DNA was precipitated by the addition of 3 M sodium acetate (10 μL, pH 5.2) and 2 volumes of cold isopropyl alcohol. Thereafter, the DNA was collected by centrifugation (8000*g*, 15 min, 4 °C) and dissolved in 300 μL of TE.

RNA in the crude DNA in TE was decomposed with RNase (RNase A, 10 mg/mL, Nippongene Co., Tokyo, Japan) for 30 min at 55 °C.

Thereafter, neutral phenol solution was added, and the upper layer was transferred to another SMT after centrifugation (8000*g*, 15 min, 4 °C). The same volume of the solution of phenol/chloroform (1:1, v/v) was added to the solution followed by centrifugation (8000*g*, 15 min, 4 °C), and the upper layer was transferred to another tube. The solution was amended with 0.2 M NaCl and 2 volumes of cold ethanol to precipitate the DNAs. The DNAs were washed with 50 μL of 70% ethanol and dissolved in 30 μL of 0.1 TE and then subjected to PCR.

Preparation of Template DNA from Grape Leaves. DNAs of grape leaves were extracted according to the CTAB method (*28*). Each fragment of grape leaves (0.1 g each) was placed in a microcentrifuge tube (2 mL), and DNAs were extracted into 0.6 mL of 2 × CTAB [2% CTAB, 20 mM EDTA (ethylene diamine-*N*,*N*,*N*′,*N*′-tetraacetic acid), 1.4 M NaCl, 0.1 M trishydroxyaminomethane-HCl buffer, pH 8.0] solution and 0.2 mL of distilled water for 30 min at 65 °C. The solution of chloroform and isoamyl alcohol (24:1, v/v) (0.8 mL) was added and stirred gently for 15 min using a rotator. Thereafter, the solution was centrifuged (8000*g*, 15 min) in a refrigerated centrifuge (hi-mac CR21F, Hitachi Co. Ltd.), and the upper layer was transferred to another microtube. CTAB solution (10%, 0.08 mL) and chloroform/isoamyl alcohol (24:1, v/v) were added to the solution, and it was stirred gently for 15 min followed by centrifugation (8000*g*, 15 min). The upper layer was transferred to another tube and was left standing for 5 min in the freezer (−80 °C) after the addition of 2.5 volumes of the precipitation buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% CTAB). The precipitate was collected by centrifugation (6000*g*, 15 min) and dissolved in 0.5 mL of Tris-EDTA buffer (TE) and was amended with the same volume of isopropyl alcohol. After gentle stirring with a rotator for 15 min, the precipitate was collected by centrifugation (6000*g*, 15 min). The precipitate was dissolved in 0.2 mL of TE followed by the decomposition of RNAs by the addition of 1 μL of RNase (RNase A, bovine pancreas, 10 mg/mL, Nippon-Gene Co., Ltd., Tokyo, Japan) and incubation for 1 h at 55 °C. Thereafter, neutral phenol solution was added, and the upper layer was transferred to another tube after the centrifugation (8000*g*, 15 min). The same volume of the solution of phenol/chloroform (1:1, v/v) was added to the solution followed by centrifugation (8000*g*, 15 min), and the upper layer was transferred to another tube. The solution was supplemented with 0.2 M NaCl and 2 volumes of isopropyl alcohol to generate the precipitate of DNAs, and the precipitate was dissolved in 30 μL of 0.1 TE and was subjected to PCR.

Preparation of Template DNA for PCR from Fermentation Microorganisms. *S. cerevisiae* (NFRI 3069) was cultured for 40 h at 30 °C in 10 mL of the medium of yeast extract (5 g/L, Bacto Yeast Extract, Becton Dickinson Microbiology Systems, Sparks, NV), tryptone (10 g/L, Tryptone Peptone, Becton Dickinson Microbiology Systems), and 5 g/L NaCl, pH 8.0. Nine milliliters of the culture suspension was centrifuged (4300*g*, 4 °C) for 10 min. The collected yeast was subjected to DNA preparation using a commercial kit (ISOPLANT II, Nippon-Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's manual.

Primers and Conditions for PCR. The primers for PCR to differentiate grape cultivars used for wine were SSR primers reported as rice mitochondrial SSR (*32*), rice chloroplast SSR (*32*), and grape SSR (*33*–*35*).

Nucleotide sequences of DNAs amplified by PCR using the template DNAs prepared from wines were determined to ascertain that the proliferated DNAs were derived from grape, and not from *S. cerevisiae*. Nucleotide sequences of the most suitable STS primers used for PCR

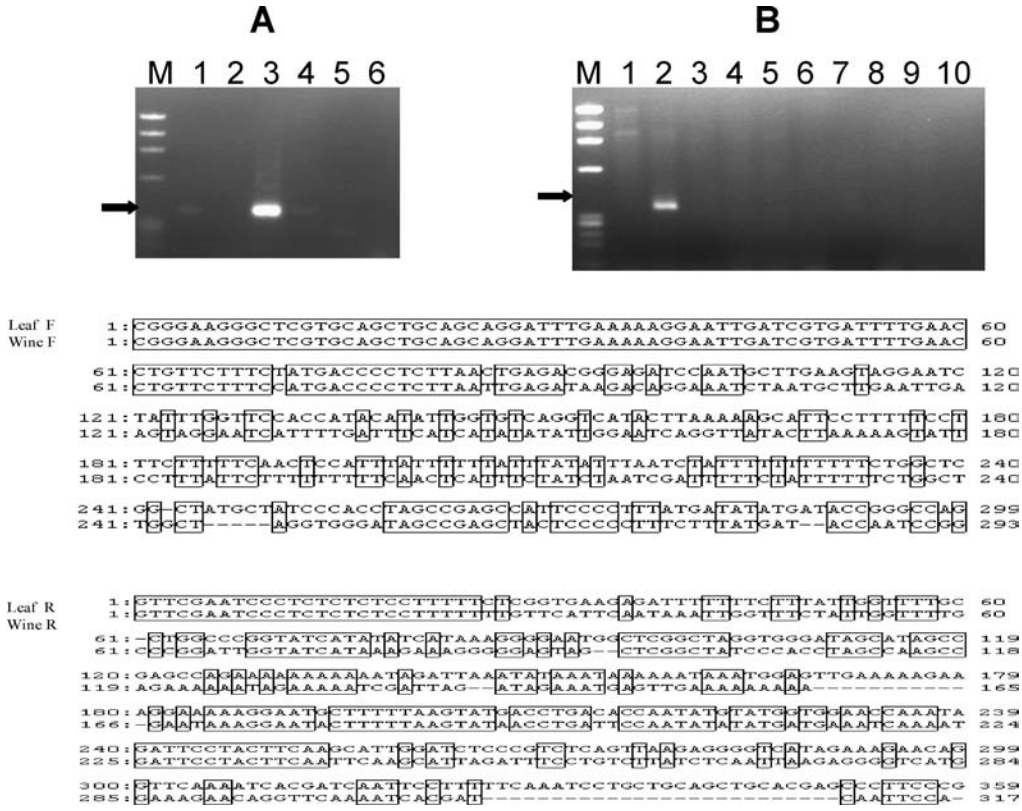

Figure 1. Results of PCR using template DNAs prepared from primer f with grape leaves or various wines as shown in **Table 1**: (**A**) template DNAs prepared from grape leaves (1, Cabernet Sauvignon; 2, Cabernet Franc; 3, Chardonnay; 4, Merlot; 5, Riesling; 6, Koshu); (**B**) template DNAs prepared from wine (1, Cabernet Sauvignon; 2, Sauvignon Blanc; 3, Pinot Noir; 4, Pinot Noir; 5, Chardonnay; 6, Chardonnay; 7, Koshu; 8, Koshu; 9, Riesling; 10, Merlot); (**C**) nucleotide sequences of amplified DNA (forward) (upper line, amplified DNA from grape leaf; lower line, amplified DNA from wine); (**D**) nucleotide sequences of the amplified DNA (reverse) (upper line, amplified DNA from grape leaf; lower line, amplified DNA from wine).

of the grape material DNA or wine DNA are shown in **Table 1**. The solution for PCR consisted of 30 ng/μL of template DNA, 0.2 μL of DNA polymerase (Taq DNA polymerase, Takara-Bio Inc., 5 units/μL, Ohtsu, Japan), 2.0 μL of reaction buffer (12 mM Tris-HCl buffer, 60 mM KCl, pH 8.3), 2.0 μL of dNTPs (100 μM), and 2.0 μL of $MgCl_2$ (25 mM), which was added with 0.2–0.8 μL of STS primer solution (5 pmol/μL), and the total amount of reaction mixture was adjusted to 20 μL by sterilized water. Although annealing temperature differs for each primer, PCR was carried out under denaturing conditions for 1 min at 96 °C, annealing for 1 min at 36–46 °C, and extension for 2 min at 72 °C, which was repeated for 35 cycles. A Thermal Cycler 9700 (Applied Biosystems Japan, Tokyo, Japan) and a Thermal Cycler Dice (Takara-Bio Inc., Otsu, Japan) were used for the PCR. Electrophoresis was carried out using 2% agarose gel and a Mupid electrophoresis system (Cosmo-Bio Co., Ltd., Tokyo, Japan) according to the method of previous papers (*27–31*). The molecular weight standard used was Marker 4 (φX174/*Hae*III digest, Wako Pure Chemicals Industries, Ltd., Osaka, Japan), which ranged from 194 to 1353 bp of DNA.

Development of STS Primer a. In the present study, STS primer a shown in **Table 1** was developed on the basis of the nucleotide sequence of the DNA amplified by PCR using the primer b shown in **Table 1**, as reported in our previous papers (*27–29*). DNA was extracted from the agarose gel after electrophoresis of the PCR product using EASYTRAP (Takara-Bio, Otsu, Japan). DNA cloning was carried out using a TOPO XL PCR Cloning Kit (Invitrogen Corp., Carlsbad, CA). The DNA sequence was determined using a commercial DNA preparation kit (QIAprep Spin Miniprep kit, Qiagen, K.K., Tokyo, Japan), a DNA proliferation kit (Big Dye Terminator Cycle Sequencing kit, V1-1, Applied Biosystems Japan), and automatic DNA sequencing system (DNA Sequencer, ABI PRISM Genetic Analyzer 310, Applied Biosystems Japan). STS primer a was designed on the basis of the sequence of amplified DNA by PCR using the primer b with 14-mer for the forward primer and 15-mer for the reverse primer so that the amplified DNA would be specific to Chardonnay.

Homology Search between DNA Sequences of Lactic Bacteria and PCR Products. All five DNAs amplified by PCR using the selected primers (a−e in **Table 1**) were sequenced and subjected to homology search against the registered whole genome of *Oenococcus oeni* (CP000411) using DDBJ BLASTN (http://www.ddbj.nig.ac.jp/) (*34*).

PCR Using Grape SSR Primer. PCR was carried out using the primers reported for the cultivar identification of grapevines (*33*, *35*). The primer is related to mitochondrial cytochrome *c* (*33*), and another primer pair was VrZAG21F and VrZAG21R (*35*). The conditions of the PCR were the same as above.

RESULTS

Purity of Template DNAs from Wine. Separation of DNA from the pigment substances was investigated. As a result of the extraction and purification procedure as described under Materials and Methods, the template DNA was separated from pigment substances such as polyphenols. The UV spectrum of the above-mentioned template DNA showed a peak at 260 nm but no shoulder peak at 280 nm, indicating the combination of enzyme hydrolysis followed by purification with isopropyl alcohol and 70% EtOH was effective for the purification of template DNA even though the amount of DNA decreased markedly.

Example of Different Amplified DNAs by PCR Using Template DNAs from Grape Leaves and Wine. In the case of PCR using primer f (*34*), shown in **Table 1**, PCR using the template DNA from grape or wine generated amplified DNAs of which the nucleotide sequences were different, even though their molecular weights were similar as shown in **Figure 1**. Except for the amplified DNA by primer f shown in **Figure 1**,

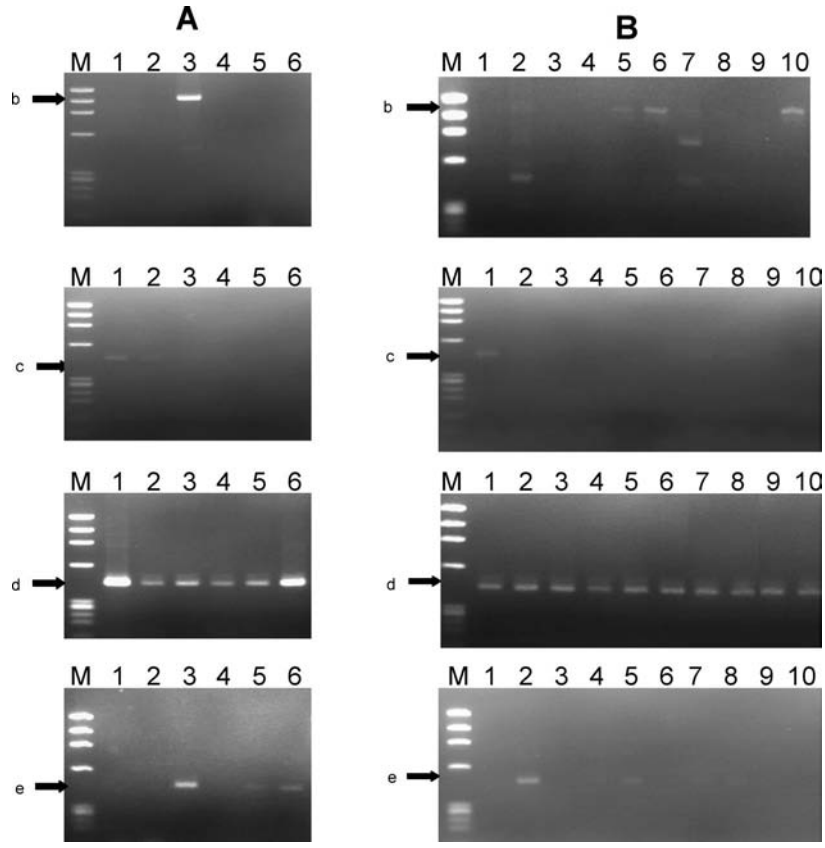

Figure 2. Results of PCR using template DNAs prepared from primers b−e with grape leaves or various wines as shown in **Table 1**: (**A**) template DNAs prepared from grape leaves (1, Cabernet Sauvignon; 2, Cabernet Franc; 3, Chardonnay; 4, Merlot; 5, Riesling; 6, Koshu); (**B**) template DNAs prepared from wine (1, Cabernet Sauvignon; 2, Sauvignon Blanc; 3, Pinot Noir; 4, Pinot Noir; 5, Chardonnay; 6, Chardonnay; 7, Koshu; 8, Koshu; 9, Riesling; 10, Merlot; b, c, d, and e indicate amplified DNA by PCR using primers b, c, d, and e shown in **Table 1**). M indicates molecular weight marker for DNA. The molecular weight standard used was Marker 4 (φ X174/*Hae*III digest, Wako Pure-Chemicals Industries, Ltd.).

all of the amplified DNAs were sequenced and subjected to a DDBJ homology search against the reported accessions as shown in **Table 1**. The search showed almost perfect agreement between the sequences of the amplified DNAs and those of the corresponding accessions.

PCR Using Template DNA from Grape or Wine as a Positive Control. In the case of PCR using primer d, shown in **Table 1**, all of the template DNAs generated the amplified DNAs of which the molecular weights were about 500 bp, as shown in **Figure 2**. The nucleotide sequences of the amplified DNAs were matched from base number 1 to 317 for the forward primer and from base number 1 to 299 for the reverse primer except for a single nucleotide difference at number 225 (C and T).

PCR for Differentiation of Cabernet Sauvignon. In the case of PCR using template DNAs from grape leaves and primer c, shown in **Table 1**, only the grape leaves of Cabernet Sauvignon and Merlot showed amplified DNA, of which the molecular weights were about 500 bp, after PCR, and only Cabernet Sauvignon revealed amplified DNA bands among the 10 various wines, as shown in **Figure 2**. The nucleotide sequences of the amplified DNAs of which the molecular weights were about 500 bp were the same from base number 1 to 300 for the forward primer and from base number 1 to 300 except for 8 bases for the reverse primer.

PCR for Differentiation of Other Grape Cultivars. In the case of PCR using template DNAs from the grape leaves and primer e, shown in **Table 1**, only Chardonnay, Riesling, and Koshu showed positive DNA bands, of which the molecular weights were about 500 bp. Cabernet Sauvignon, Sauvignon Blanc, and Merlot did not reveal amplified DNA bands, as shown in **Figure 2**. On the contrary, Sauvignon Blanc, Chardonnay, and Koshu revealed amplified DNAs, of which the molecular weights were about 500 bp. Cabernet Sauvignon, Pinot Noir, Riesling, and Merlot did not show amplified DNA bands, as shown in **Figure 2**. The nucleotide sequences of the amplified DNAs were the same from base number 1 to 387 for the forward primer except for 11 SNPs and from base number 1 to 361 except for 8 bases for the reverse primer.

Chardonnay-Specific PCR Primer b. In the case of PCR using template DNAs from the grape leaves and primer b, shown in **Table 1**, only Chardonnay, among the six grape varieties tested, generated amplified DNAs of which the molecular weights were about 1 kbp, as shown in **Figure 2**. On the contrary, Chardonnay and Merlot revealed amplified DNAs of which the molecular weights were about 1 kbp. Sauvignon Blanc revealed amplified DNA of 300 bp as shown in **Figure 2**. The nucleotide sequences of the amplified DNAs, of which the molecular weights were about 1 kbp, were perfectly matched from base number 1 to 319 for the forward primer and from base number 1 to 331 for the reverse primer.

Improvement of PCR Primer. As a result of the PCR using mitochondrial SSR primer b according to the study by Nishikawa et al. (*32*), template DNAs prepared from wine of Chardonnay and Merlot were amplified to show DNA bands of 1 kbp, as shown in **Figure 2**.

We carried out cloning of the amplified DNA and determined its nucleotide sequence, followed by designing the novel STS primer pair using the downstream nucleotide sequence to

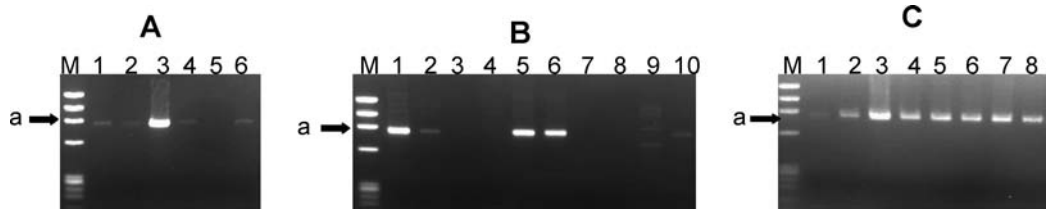

Figure 3. Results of PCR using template DNAs prepared from grape leaves or various wines. Primer a was used as shown in **Table 1**: (**A**) template DNAs prepared from grape leaves (1, Cabernet Sauvignon; 2, Cabernet Franc; 3, Chardonnay; 4, Merlot; 5, Riesling; 6, Koshu); (**B**) template DNAs prepared from wine (1, Cabernet Sauvignon; 2, Sauvignon Blanc; 3, Pinot Noir; 4, Pinot Noir; 5, Chardonnay; 6, Chardonnay; 7, Koshu; 8, Koshu; 9, Riesling; 10, Merlot); (**C**) template DNAs prepared from wine labeled as Chardonnay (1, France, 2002; 2, Chile, 2002; 3, France, 2004; 4, France, 2004; 5, Chile, 2004; 6, France, 2005; 7, France, 2005; 8, Chile, 2005).

generate a clearer low molecular weight DNA band by PCR using template DNA prepared from wine.

As a result of cloning and sequencing of the amplified DNAs by PCR (**Figure 3 A**,**B**) using the template DNAs from grape leaf and wine (Chardonnay), the nucleotide sequences were the same.

In the case of PCR using the above-mentioned template DNAs prepared from eight kinds of Chardonnay wine and primer a, all of the template DNAs were amplified and their molecular weights were about 800 bp as shown in **Figure 3C**.

Homology Search between DNA Sequences of the Lactic Bacteria and the PCR Products. During the wine fermentation, lactic acid bacteria, such as *O. oeni*, sometimes increase in addition to the yeasts. Therefore, all five DNAs amplified by PCR using the selected primers (a−e in **Table 1**) were sequenced and subjected to homology search against the registered whole genome of *O. oeni* (CP000411). As a result of the homology search, only two homologous sequences were found; the first was from primer a with an *e* value of 8.7; and the second one was from primer b with an *e* value of 27. In addition to the homology as low as 8.7 and 27, both of the common sequences were proved to be located in different regions with the regions of primers a and b. Therefore, DNA would never be amplified or would be amplified with different molecular weights during PCR even if the DNA derived from *O. oeni* were contaminated in the wine. Thus, it was shown that contamination of DNA derived from lactic acid bacteria would not interfere in the identification of grape cultivars.

PCR Using Grape SSR Primer. PCR was carried out using the primers reported for the cultivar identification of grapevines (*33*–*35*). The primer is related to mitochondrial cytochrome *c* (*33*) and chloroplast SSR (*34*), with another primer pair being VrZAG21F and VrZAG21R (*35*).

In the case of mitochondrial cytochrome *c*, the result in **Figure 2** is described for primer e.

In the case of VrZAG21, the result is shown in **Figure 4**. Grape leaf DNA generated amplified DNA for Cabernet Sauvignon and Chardonnay, and wine DNA revealed amplified DNA for only Chardonnay. The molecular weight of the amplified DNA from the wine DNA was much lower than that from the grape leaf DNA.

DISCUSSION

Problems with the preparation of template DNAs from alcoholic beverages for PCR are (a) decomposition of plant DNA during the fermentation; (b) contamination of DNAs from the yeast microorganisms; (c) interference of DNA extraction by polysaccharides and polypeptides in the beverages; and (d) coexistence of pigment substances, such as polyphenols, which inhibits DNA polymerase during PCR (*4*, *5*).

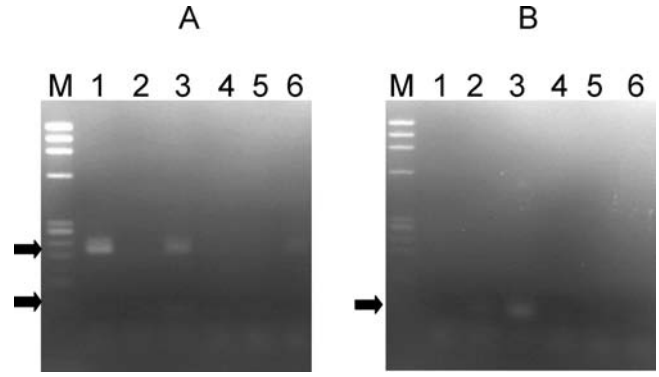

Figure 4. Results of PCR using SSR primer VrZAG21 and template DNAs prepared from grape leaves or various wines: (**A**) template DNAs prepared from grape leaves (1, Cabernet Sauvignon; 2, Cabernet Franc; 3, Chardonnay; 4, Merlot; 5, Riesling; 6, Koshu); (**B**) template DNAs prepared from various wines (1, Cabernet Sauvignon; 2, Sauvignon Blanc; 3, Chardonnay; 4, Merlot; 5, Riesling; 6, Koshu). Primer: VrZAG21 (forward), 5′-tca ttc act cac tgc att cat cgg c-3′; VrZAG21 (reverse), 5′-ggg gct act cca aag tca gtt ctt g-3′.

To concentrate DNA, lyophilization was adopted because it is effective without causing heat damage. As the DNAs are decomposed by the DNase of *S. cerevisiae* during fermentation, PCR using suitable primers to amplify small-size DNA fragments of <2 kbp was adopted as shown in **Table 1**.

It was reported that a commercial kit can be used for the preparation of template DNA for PCR from must or commercial wine; however, the preparation of template DNAs from five commercial wines was unsuccessful (*5*). It was reported that in the case of combination of the CTAB method with several kinds of commercial DNA extraction kit, the time course of change in the DNA pattern could be traced during experimental fermentation (*4*). However, this takes time and requires complicated procedures for the DNA preparation.

The previous papers are very valuable because they proved that the DNA remains even after fermentation and that it would be possible to analyze DNA patterns using must or wines as samples. Nevertheless, DNA patterns after PCR are not clear enough, requiring a precise electrophoresis system. Moreover, it was reported that application to commercial wines was rather difficult (*4*, *5*).

We investigated the preparation of template DNAs for PCR from wine and improved the method developed for Japanese rice wine (*36*).

As reported previously, polyphenols in wine inhibit PCR (*4*, *5*). Although the amount is less than in red wines, even white wines, such as Chardonnay, also contain polyphenols. Therefore, a purification procedure to remove most of the polyphenols was added.

It was reported that several factors, such as the kinds of extracting solutions, temperature, freezing, heating, juice runoff, pectolytic enzymes, and maceration time, affect the extraction of phenolic compounds from grape material or wine (*37*). Polyphenols have been reported to inhibit PCR (*4*, *5*, *38*). Although 70% EtOH is commonly used for washing DNA, it was proved to be useful for the separation of DNA from polyphenols in the present study. It became possible to prepare the template DNA for PCR using 70% EtOH for dissolution of DNA because polyphenols only slightly dissolve in 70% EtOH over a short time at low temperature. Although the amount of template DNA becomes less, this procedure led to high performance in PCR by the removal of the polyphenols. If the PCR proceeds without inhibition, the template DNA can be amplified from a million to a billion times.

To remove polysaccharides and polypeptides present in alcoholic beverages, enzymatic digestion by α-amylase and proteinase was used. A high temperature of 80 °C for amylase and SDS for proteinase K was effective for inhibiting DNase during enzymatic digestion. The UV spectrum showed that the polysaccharides and proteins were removed sufficiently and the template DNA was purified (ratio of A_{260} to $\underline{A}_{280}$ was 1.79).

Combination of enzyme treatment and purification by isopropyl alcohol/70% EtOH revealed favorable PCR and electrophoresis results using various wines as samples, as shown in **Figures 1**−**4**.

Although the template DNA was amplified by PCR, it cannot be used for authentication of grape cultivars if the amplified DNA is not derived from grape as in the case of primer f (see PCR shown in **Figure 1**).

Thus, it was necessary to select suitable PCR primers that do not amplify DNAs of microorganisms, such as yeasts, and which would be useful for the differentiation of grape cultivars. Among the various STS primers and SSR primers, useful for grape cultivar differentiation, primers a−e, as shown in **Table 1**, proved to be suitable because they were effective for cultivar differentiation. Moreover, the nucleotide sequences of the amplified DNAs from template DNAs prepared from wine were the same as those of amplified DNAs from those prepared from grape leaves as shown in **Figures 2** and **3**.

Therefore, as proposed here, it is recommended to ascertain that the nucleotide sequences of the amplified DNA markers are the same as those of the amplified DNA markers from the grape berries or leaves.

As shown in **Figure 2**, primer d can be used for positive control for PCR because it can amplify all template DNAs prepared from various kinds of grape cultivars by PCR. Positive control is indispensable to ascertain whether template DNA is purified sufficiently or not and whether the PCR has performed well or not.

As shown in **Figure 2**, the present method of preparation of template DNAs for PCR is useful because high molecular weight DNA can be amplified even from template DNA prepared from wine (primer b). It was reported that DNA in wine decomposes to a low molecular weight fragment during fermentation (*4*, *5*). This is true. However, PCR could amplify even DNA of molecular weight of >1 kbp when purified template DNA was prepared for PCR by an improved preparation method. Even though the amount of the template DNA was less, it could be amplified if the PCR inhibitors, such as polyphenols, polysaccharides, and proteins, are removed sufficiently.

Although identification of grape cultivars using grape berries or leaves as the sample has been reported (*20*–*25*), it is very difficult to differentiate grape cultivars using wine itself as a sample (*4*, *5*). As shown in **Figures 2** and **3**, using the novel method developed in this study, it would be possible to identify or differentiate these principal grape cultivars using wine as a sample.

As shown in **Figure 3**, this PCR method seems to be useful to ascertain the grape material of Chardonnay using commercial wine as a sample. In the case of grape leaf DNA, Chardonnay showed a clear DNA band, as shown in **Figure 3A**. In the case of comparison of wine DNAs, only two kinds of Chardonnay and Cabernet Sauvignon showed clear DNA bands, as shown in **Figure 3B**. However, there remains some possibility that the Chardonnay was blended as the sample was a commercial wine. By PCR using the eight different kinds of Chardonnay wines, all of the samples showed amplified DNA bands as shown in **Figure 3C**. Therefore, primer a could be used for authentication of Chardonnay using wine as a sample.

As shown in **Figure 4**, by PCR using ordinary grape SSR primer (VrZAG21) (*35*), amplified DNA could be detected in the case of Chardonnay wine, but its molecular weight was much lower than that of grape DNA. Although these primers could be used for identification or differentiation, it is more difficult compared with those primers as shown in **Figures 2** and **3**. As the DNA is decomposed during the fermentation as reported previously (*4*, *5*), it is recommended to select or develop suitable PCR primers such as mitochondrial SSR primers or chloroplast primers, as shown in the present paper.

Wine is the most popular alcoholic beverage in the world. The qualities, value, and price of wine vary depending on the grape cultivars, climate conditions, agronomic conditions of the cultivating area, brewing technology, and storage. Thus, it is important that consumers trust the labeling of the grapevine cultivars by the establishment of an efficient traceability system. As a future perspective, traceability studies are really interesting for customer defense; differentiation of material cultivars of wine is even more useful for autochthonous cultivars with a limited area of cultivation for which variety identification can be more related to area of origin of the grapes and thus of the wine.

DNA tests for traceability purposes need to attain even greater throughput. Fragment sequencing of the amplicon obtained from wine DNA is not cheap and immediate. To overcome this problem in industrial scale application of the method, we must make efforts to develop a time-saving, labor-saving multiplex primer set, such as a "PCR KIT for Chardonnay" in the future. The development of an easier and more rapid DNA preparation method is another objective in the next stage.

It would be useful for producers, dealers, and consumers of grape wines to increase the kinds of authenticated grape cultivars of wines using the novel preparation method of template DNA for PCR as proposed in the present paper.

ABBREVATIONS USED

BLAST, Basic Local Alignment Search Tool; CTAB, cetyltrimethylammonium bromide; DDBJ, DNA Data Bank of Japan; EtOH, ethyl alcohol; PCR, Polymerase Chain Reaction; STS, sequence tagged site.

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