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Molecular Detection and Identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalus* in Spoiled Wines

Luca Cocolin,* Kalliopi Rantsiou, Lucilla Iacumin, Roberto Zironi, and Giuseppe Comi

Dipartimento Scienze degli Alimenti, Università degli studi di Udine, 33100 Udine, Italy

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In this paper we describe the development of a PCR protocol to specifically detect *Brettanomyces bruxellensis* and *B. anomalus*. Primers DB90F and DB394R, targeting the D1-D2 loop of the 26S rRNA gene, were able to produce amplicons only when the DNA from these two species were used. No amplification product was obtained when DNA from other *Brettanomyces* spp. or wine yeasts were used as the templates. The 305-bp product was subjected to restriction enzyme analysis with *Dde*I to differentiate between *B. bruxellensis* and *B. anomalus*, and each species could be identified on the basis of the different restriction profiles. After optimization of the method by using strains from international collections, wine isolates were tested with the method proposed. Total agreement between traditional identification and molecular identification was observed. The protocol developed was also used for direct detection of *B. bruxellensis* and *B. anomalus* in wines suspected to be spoiled by *Brettanomyces* spp. Application of culture-based and molecular methods led us to the conclusion that 8 of 12 samples were spoiled by *B. bruxellensis*. Results based on the application of molecular methods suggested that two of the eight positive samples had been infected more recently, since specific signals were obtained at both the DNA and RNA levels.

A wide variety of yeast species have been implicated in wine spoilage. Of the spoilage yeasts, species of *Brettanomyces* (imperfect state, *Dekkera*) are probably the most serious (21) and controversial. These organisms are most frequently found in red wine 6 to 10 months after barrelling (4), but they have also been isolated from other fermented beverages, such as beer and cider. *Brettanomyces* species can survive, multiply, and contaminate wines from transfer piping or cooperage that has been insufficiently cleaned and disinfected after use. These potential spoilage yeasts have been identified in almost every wine-producing area of the world (12). Wines infected with *Brettanomyces/Dekkera* yeasts develop off-flavors, which are described as animal, stable, barnyard, horse blanket, and burnt plastic (5, 12, 13, 16, 18). *Brettanomyces* can produce a distinct haziness when the concentration of cells present in the wine is $<3 \times 10^3$ cells/ml (4) or higher (13). *Brettanomyces* species can synthesize volatile phenolic compounds, including phenol, syringol (16), and several ethylphenols (6). Control of these organisms is difficult due to their relative resistance to normally used concentrations of sulfur dioxide (15). The physiology and ecology of this spoilage genus are still unclear, and little is known about it (14).

Current methods for identification and enumeration of *Brettanomyces* contamination take 1 to 2 weeks and rely on growth on semiselective culture media or selective culture media, followed by final identification by biochemical and physiological analysis and morphology as determined by microscopic examination (19). Newer techniques for rapid detection and identification of *Brettanomyces*, such as a nested PCR, PCR-restriction fragment length polymorphism, and fluorescence in situ hybridization, have been described recently (10, 18, 23, 26).

Unfortunately, strain isolation, which is necessary prior to taxonomic characterization, is known to introduce potential biases. For example, this approach fails to characterize the microorganisms for which selective enrichment and culturing are problematic or impossible (2), thereby eliminating these populations from consideration. Recently, direct methods to characterize the yeast diversity within wine fermentations by using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified rRNA genes (rDNA) have been developed (7, 8, 22). This approach has the advantage of eliminating the requirement for strain isolation, thereby negating the potential biases inherent in microbial enrichment. Indeed, studies in which such direct analyses have been employed have repeatedly demonstrated that there is a tremendous variance between cultivated and naturally occurring species, which dramatically alters the perception of the true microbial diversity present in various habitats (17).

The application of molecular techniques to wine yeast identification has proved to be very powerful for elucidating recent and classical issues concerning taxonomy. The nomenclature of *Brettanomyces* used in the wine industry differs from that of the recently revised taxonomy of yeasts. Enologists refer to the spoilage organism as *Brettanomyces* (Brett) or by the species names *Dekkera intermedia*, *Brettanomyces intermedius*, *Brettanomyces lambicus*, *Brettanomyces custersii*, and *Dekkera bruxellensis*. Today, only *Brettanomyces bruxellensis* and *Brettanomyces anomalus* are accepted species names, and the other names are considered synonyms. The spoilage organism in wine belongs to the species *B. bruxellensis* (26).

In this paper we describe the development of a PCR-restriction enzyme (RE) analysis protocol to detect and identify, directly in wine samples, *B. bruxellensis* and *B. anomalus*.

* Corresponding author. Mailing address: Dipartimento di Scienze degli Alimenti, via Marangoni 97, 33100 Udine, Italy. Phone: 39/0432/590-759. Fax: 39/0432/590-719. E-mail: lscocolin@uniud.it.

TABLE 1. Yeast strains used in this study

Species	Strain	Source[a]
Brettanomyces (*Dekkera*) spp.		
B. bruxellensis	DBVPG6706^T	DBVPG
B. anomalus	NCAIM960^T	NCAIM
B. nanus	NCAIM637	NCAIM
B. naardenensis	NCAIM700	NCAIM
B. bruxellensis	NCAIM547	NCAIM
B. bruxellensis	NCAIM666	NCAIM
B. bruxellensis	ISA 1792	ISA
B. bruxellensis	ISA 1700	ISA
B. bruxellensis	DBA 1	DBA
B. bruxellensis	DBA 2	DBA
B. bruxellensis	DBA 3	DBA
B. bruxellensis	DBA 4	DBA
B. bruxellensis	DBA 5	DBA
B. bruxellensis	DBA 6	DBA
B. bruxellensis	DBA 7	DBA
B. bruxellensis	DBA 8	DBA
B. bruxellensis	DBA 9	DBA
B. bruxellensis	DBA 10	DBA
B. bruxellensis	ERSA B1	ERSA
B. anomalus	ERSA B2	ERSA
B. bruxellensis	ERSA 22A	ERSA
B. anomalus	ERSA MNT B	ERSA
B. bruxellensis	ERSA 7C	ERSA
B. bruxellensis	ERSA 13B	ERSA
B. bruxellensis	ERSA 22B	ERSA
B. bruxellensis	ERSA 7A	ERSA
Other yeast species		
Saccharomyces cerevisiase	DBVPG6173^T	DBVPG
Hanseniaspora uvarum	DBVPG6717^T	DBVPG
Pichia anomala	DBVPG6612^T	DBVPG
Candida stellata	DBVPG6714^T	DBVPG
Metschnikowia pulcherrima	UCD125	UCD
Zygosaccharomyces bailii	DBVPG6287^T	DBVPG
Kluyveromyces thermotolerans	DBVPG6480^T	DBVPG

[a] Abbreviations: DBVPG, Dipartimento di Biologia Vegetale, Università di Perugia, Perugia, Italy; NCAIM, National Collection of Agriculture and Industrial Microorganisms, Szent Istvan University, Budapest, Hungary; ISA, Instituto Superior de Agronomia, Lisbon, Portugal; DBA, Dipartimento di Biotecnologie Agrarie, Università di Firenze, Florence, Italy; ERSA, Ente Regionale Sviluppo Agricolo, Gorizia, Italy; UCD, Department of Viticulture and Enology, University of California, Davis.

Moreover, other molecular techniques, such as PCR-DGGE, reverse transcription (RT)-PCR-DGGE, and hybridization with a *B. bruxellensis*-specific probe, were used as confirmatory tests for detection of spoilage yeasts in wine samples.

MATERIALS AND METHODS

Yeast strains. Yeast strains used in this study are listed in Table 1. They were routinely propagated in YPD agar (1% [wt/vol] yeast extract, 2% [wt/vol] bacteriological peptone, 2% [wt/vol] glucose, 1.5% [wt/vol] agar; all components obtained from Oxoid, Milan, Italy) at 25 to 28°C for 36 to 48 h.

Extraction of nucleic acids from yeast cultures. Strains were grown for 36 to 48 h in YPD broth at 25 to 28°C, and 2 ml for DNA extraction and 2 ml for RNA extraction were centrifuged in tubes containing 0.3-g glass beads. The nucleic acids were extracted by using the protocol suggested by Cocolin et al. (7). For DNA phenol-chloroform-isoamyl alcohol (25:24:1; pH 6.7; Sigma, Milan, Italy) was used, whereas for RNA, phenol-chloroform (5:1; pH 4.7; Sigma) was employed. The DNA and RNA were collected by centrifugation at 14,000 × *g* and 4°C for 10 min, and the pellets were dried under a vacuum at room temperature. Fifty microliters of sterile water was added, and 30 min of incubation at 45°C was used to facilitate nucleic acid solubilization. One microliter of DNase-free RNase (Roche Diagnostics, Milan, Italy) and 1 μl of RNase-free DNase (Roche Diagnostics) were added to digest the RNA and DNA, respectively, by incubation at 37°C for 1 h. The RNA solution was checked for the presence of residual amounts of DNA by performing PCR amplification. When positive signals were detected, the DNase treatment was repeated to eliminate the DNA.

Design of primers. Primers were selected by using the CLUSTAL W multiple-sequence alignment program (version 1.82; available at www.ebi.ac.uk/clustalW). The D1-D2 loops of the 26S rDNA of the yeasts belonging to the genus *Brettanomyces* and of yeast species normally present in wine fermentations and/or the wine environment were aligned, and homologous regions in the *B. bruxellensis* and *B. anomalus* molecules were selected to design specific primers (Fig. 1).

Specific amplification of *B. bruxellensis* and *B. anomalus*. Primers DB90F (5′-GAY ACT AGA GAG AGR RGG ARG GC-3′, where Y = C or T and R = A or G) and DB394R (5′-ACG AGG AAC GGG CCG CT-3′) were used for specific amplification of *B. bruxellensis* and *B. anomalus*. PCR amplification was performed in a 50-μl (final volume) mixture containing 10 mM Tris-HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, each primer at a concentration of 0.2 μM, 1.25 U of *Taq* polymerase (Applied Biosystems, Milan, Italy), and 2 μl (about 100 ng) of extracted DNA. The thermal cycler parameters were as follows: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1.5 min; and final extension at 72°C for 7 min. Amplification was carried out with a Minicycler (Genenco, Florence, Italy).

RT-PCRs were performed with the *Tth* DNA polymerase (Roche Diagnostics). One hundred nanograms of RNA was mixed with each primer at a concentration of 450 nM, and the volume was brought to 25 μl with DNase-RNase-free sterile water. After denaturation at 70°C for 5 min, the tubes were placed immediately in ice, and 25 μl of a mixture containing 25 mM bicine-KOH (pH 8.2), 57.5 mM potassium acetate, 4% (vol/vol) glycerol, 2.5 mM manganese acetate, and 5 U of *Tth* DNA polymerase was added. The reaction mixtures were incubated for 30 min at 68°C, and after denaturation at 95°C for 2 min, the amplification reaction described above was carried out. PCR products were electrophoresed in a 2% Tris-borate-EDTA (TBE) agarose gel and observed under UV light.

RE analysis. Five microliters of *Brettanomyces*-specific PCR products was subjected to RE analysis. The enzymes used were selected by using the DNA Strider molecular biology software, which also determined the molecular weights of the restriction fragments resulting from the digestion. In particular, *Bst*UI, *Dde*I, *Mse*I, and *Taq*I (Roche Diagnostics) were tested for the ability to distinguish strains belonging to *B. bruxellensis* and *B. anomalus*. After digestion at 37°C for at least 2 h, performed by using the instructions of the manufacturer, restriction fragments were separated on a 3% TBE agarose gel and stained for 30 min in TBE buffer containing 0.5 μg of ethidium bromide (Sigma) per ml. Gels were examined under UV light by using the GeneGenius BioImaging system (SynGene, Cambridge, United Kingdom).

Universal amplification of 26S rDNA. Primers NL1 (5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′) (20) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) (7) were used for amplification of the 26S rDNA of the yeasts present in the wine samples analyzed. Each PCR was performed in a 50-μl (final volume) reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1.25 U of *Taq* polymerase (Applied Biosystems), and each primer at a concentration of 0.2 μM. Two microliters (100 ng) of template DNA was added to the mixture. The reactions were performed for 30 cycles; denaturation was at 95°C for 60 s, annealing was at 52°C for 45 s, and extension was at 72°C for 60 s. An initial denaturation step at 95°C and a final 7-min extension step at 72°C were used. Five microliters of each PCR product was analyzed by electrophoresis in a 0.5× TBE agarose gel. RT-PCRs were carried out by using the *Tth* enzyme (Roche Diagnostics) as described above for specific amplification. A GC clamp (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G) was added to primer NL1 when it was used for DGGE analysis (25).

DGGE analysis. The Dcode universal mutation detection system (Bio-Rad, Hercules, Calif.) was used for DGGE analysis. Electrophoresis was performed in 0.8-mm polyacrylamide gels (ratio of 8% [wt/vol] acrylamide to bisacrylamide, 37.5:1) by using a 30 to 60% denaturant gradient (100% denaturant was 7 M urea and 40% [wt/vol] formamide) that increased in the direction of electrophoresis. The gels were subjected to a constant voltage of 120 V for 4 h at 60°C, and after electrophoresis, they were stained for 20 min in 1.25× TAE containing 1× (final concentration) SYBR Green (Molecular Probes, Eugene, Oreg.). The gels were visualized under UV light and were analyzed by using the GeneGenius BioImaging system (SynGene) for recognition of the bands present.

RNA hybridization. A probe specific for the D1-D2 26S rDNA sequence of *B. bruxellensis*, BRE26S14 (5′-CGG TCT CCA GCG ATT-3′) (26), was used for RNA hybridization analysis. The probe used in the assays was synthesized with a 3′-digoxigenin-UTP modification. One-microgram portions of RNA purified from wine samples were applied to Zeta-probe GT membranes (Bio-Rad) by

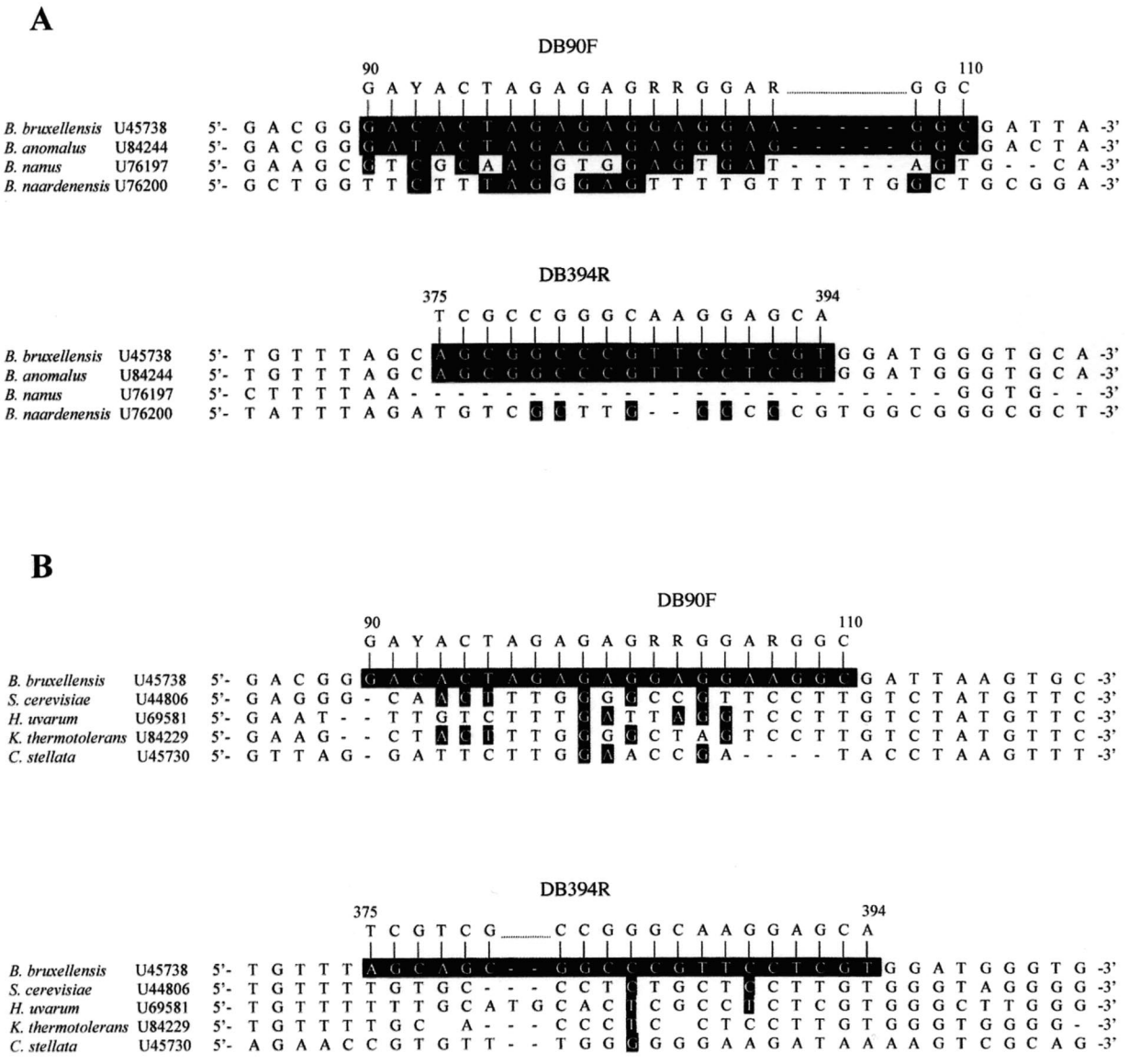

FIG. 1. Alignments of partial yeast D1-D2 26S rDNA sequences (5′-3′) for selection of the specific *B. bruxellensis* and *B. anomalus* primers. (A) Sequences of *Brettanomyces* spp.; (B) sequences of yeast species involved in wine fermentation. The sense (5′-3′) sequence of primer DB90F and the antisense (3′-5′) sequence of primer DB394R are shown above the alignments. Similarities between the primers and the target sequence are highlighted. The GenBank accession numbers of the sequences are indicated on the left. The primer positions were calculated based on the *B. bruxellensis* sequence (accession number U45738).

using a BioDot blotting apparatus (Bio-Rad) as recommended by the manufacturer. Hybridization was performed overnight in 20 mM sodium phosphate (pH 7.4)–7% (wt/vol) sodium dodecyl sulfate (SDS) at 37°C. The membranes were washed twice in 20 mM sodium phosphate (pH 7.4)–5% (wt/vol) SDS for 15 min each time and twice in 20 mM sodium phosphate (pH 7.4)–1% (wt/vol) SDS for 15 min each time. The washes were carried out at 37°C. Detection was performed with a digoxigenin chemiluminescence kit (Roche Diagnostics), used as recommended by the manufacturer. Control RNA samples were purified from the reference strains used in the study as described above.

Wine samples. Twelve samples of red wine suspected to be spoiled by *Brettanomyces* spp. were collected from wineries in northern Italy. They were samples of Lambrusco, Cabernet Sauvignon, Merlot, Barbera, Langhe rosso (a vintage of Barbera and Cabernet), and Docetto. These samples were subjected to culture-dependent and culture-independent methods of detection and identification of *Brettanomyces* spp. Wine samples were plated on Wallerstein laboratory nutrient (WLN) agar (Oxoid) and incubated at 28°C for 10 days. When present, at least five suspected colonies, based on their morphotype characteristics (3), were isolated on YPD agar and after DNA extraction were identified by specific PCR and RE analyses. Nucleic acids were extracted directly from wine samples by using the protocols described by Mills et al. (22). For extraction of DNA, a DNeasy Plant minikit (Qiagen, Milan, Italy) was used; the amount of wine used in each analysis was 50 ml. RNA was extracted with the Concert Plant RNA reagent (Invitrogen, Milan, Italy) from 1 ml of wine. DNase-free RNase (Roche Diagnostics) and RNase-free DNase (Roche Diagnostics) were added to digest coextracted RNA and DNA, respectively, during incubation at 37°C for 1 h. Nucleic acids were subjected to PCR and RT-PCR by using specific primers for *B. bruxellensis* and *B. anomalus*, and when a positive signal was obtained, RE analysis was performed for molecular identification. Moreover, amplification and RT were performed with universal primers NL1 and LS2, and this was followed by DGGE analysis to distinguish yeast populations present in the wine samples.

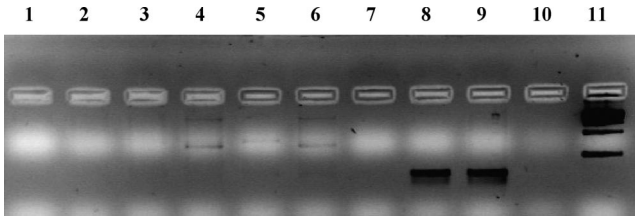

FIG. 2. PCR amplification results obtained with the specific primers developed in this study. Lane 1, *Candida stellata* DBVPG6714; lane 2, *Hanseniaspora uvarum* DBVPG6717; lane 3, *Saccharomyces cerevisiae* DBVPG6173; lane 4, *Metschnikowia pulcherrima* UCD125; lane 5, *Zygosaccharomyces bailii* DBVPG6287; lane 6, *Kluyveromyces thermotolerans* DBVPG6480; lane 7, *Pichia anomala* DBVPG6612; lane 8, *Brettanomyces anomalus* NCAIM960; lane 9, *Brettanomyces bruxellensis* DBVPG6706; lane 10, negative control; lane 11, molecular weight marker (1-kb ladder; Sigma).

For both RT-PCRs, 1 μg of RNA extracted directly from a sample was used in the reaction mixture. Finally, a specific probe for *B. bruxellensis* was used for RNA dot blot hybridization analysis.

RESULTS

Design of specific primers for *B. bruxellensis* and *B. anomalus*. Partial alignments of the D1-D2 loop of the 26S rDNA from *Brettanomyces* spp. and other yeasts involved in wine fermentations, corresponding to the annealing sites of primers DB90F and DB394R, are shown in Fig. 1. In Fig. 1A, the homology of the specific primers with sequences belonging to *Brettanomyces* species is shown, whereas in Fig. 1B the identity with wine yeast 26S rDNA is shown. Primers DB90F and DB394R were located within the 26S rDNA of *B. bruxellensis* (accession number U45738) at positions 90 to 110 and 375 to 394, respectively, which resulted in amplification of a 305-bp PCR product. As shown in Fig. 1, primer DB90F had a high level of homology only with the sequences of *B. bruxellensis* and *B. anomalus*. In particular, a deletion present in the 3′ region of the primer for these two species (Fig. 1A) was exploited to allow amplification only when *B. bruxellensis* and *B. anomalus* DNA were used as the PCR templates. When reverse primer DB394R was used, low levels of homology were observed for other *Brettanomyces* spp., as well as for other wine yeasts (Fig. 1B).

Specific amplification. The results obtained from specific amplification of *B. bruxellensis* and *B. anomalus* are shown in Fig. 2. The specific 305-bp PCR product was obtained only when DNA from *B. anomalus* (Fig. 2, lane 8) and *B. bruxellensis* (Fig. 2, lane 9) were used as templates. No signal was observed when DNA from *Brettanomyces nanus*, *Brettanomyces naardiensis*, and non-*Brettanomyces* spp. were subjected to PCR amplification with primers DB90F and DB394R (data not shown). All the strains belonging to *B. bruxellensis* and *B. anomalus* used in this study and shown in Table 1 gave the specific PCR product that was the expected size. The detection limit of the PCR protocol developed here was determined to be 10^4 to 10^5 cells of *B. bruxellensis* DBVPG6706 (data not shown).

Identification of *B. bruxellensis* and *B. anomalus* by RE analysis. *Bst*UI, *Dde*I, *Mse*I, and *Taq*I restriction endonucleases were used to digest the specific PCR product obtained with primers DB90F and DB394R. Of the REs tested, only the *Dde*I endonuclease was able to differentiate *B. bruxellensis* and *B. anomalus* (Fig. 3). The restriction patterns obtained had a band at about 154 bp for both species and a second band at 129 bp for *B. bruxellensis* and at 106 bp for *B. anomalus*. The molecular weights of restriction fragments were calculated by using the DNA Strider software. Two additional bands at 23 and 22 bp for *B. anomalus* and one band at 22 bp for *B. bruxellensis* were also determined by computer analysis, but these bands were not observed on the gel. This was probably due to the lack of resolution of small DNA fragments by agarose gel electrophoresis. All *Brettanomyces* strains used in this study were cut based on the control strain used for optimization of the method.

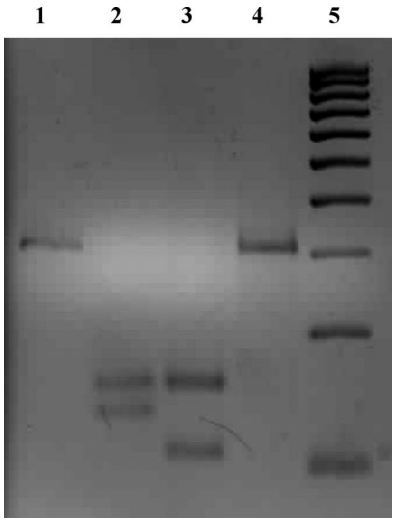

FIG. 3. *Dde*I RE analysis of the PCR product obtained by using specific primers DB90F and DB394R. Lanes 1 and 4, uncut *B. bruxellensis* DBVPG6706 and *B. anomalus* NCAIM960 controls, respectively; lanes 2 and 3, restriction patterns of *B. bruxellensis* DBVPG6706 and *B. anomalus* NCAIM960, respectively; lane 5, molecular weight marker (100-bp ladder; Sigma).

Detection and identification of *B. bruxellensis* and *B. anomalus* in wine samples. Twelve samples that were suspected to be spoiled by *Brettanomyces* spp. were collected and analyzed. A polyphasic approach involving culture-dependent and culture-independent methods was used. Samples were plated on WLN medium, and suspected *Brettanomyces* colonies were isolated and identified by PCR-RE analysis. Nucleic acids were also extracted directly from wine and used subsequently in molecular analyses. In particular, DNA and RNA were subjected to specific amplification for detection of *B. bruxellensis* and *B. anomalus*, and to confirm the results obtained, DNA and RNA were subjected to DNA and RNA-DGGE analysis with universal primers for amplification. Moreover, RNA was blotted onto membranes and hybridized with a *B. bruxellensis*-specific probe. The results obtained are summarized in Table 2.

WLN medium plates showed that there was growth in the case of samples 2, 3, 4, 10, 11, and 12, while for the rest of the sample yeasts <10 CFU/ml was observed. Only in the case of sample 12 was there a mixed yeast population on the plate, which was characterized by colonies of at least two morphotypes as described by Cavazza et al. (3). On all the plates colonies with the characteristic *Brettanomyces* appearance were observed. Counting revealed that in samples 2, 3, 4, and 10 the size of the population was about 10^3

TABLE 2. Results obtained from the wine samples tested by traditional and direct molecular methods[a]

Sample	*Brettanomyces* counts on WLN medium (CFU/ml)	PCR with specific primers	RT-PCR with specific primers	PCR-DGGE with universal primers	RT-PCR-DGGE with universal primers	RNA dot blot
1	<10	−	−	FB	+	−
2	1.9×10^3	+	−	+	−	−
3	2×10^3	+	−	+	−	−
4	3.2×10^3	+	−	+	−	−
5	<10	+	−	+	−	−
6	<10	+	−	+	+	−
7	<10	−	−	FB	+	−
8	<10	−	−	FB	+	−
9	<10	−	−	−	−	−
10	1.5×10^3	+	−	+	−	−
11	1.5×10^4	+	−	+	+	+
12	1×10^4	+	−	+	+	+

[a] +, *Brettanomyces*-specific signal present; −, *Brettanomyces*-specific signal absent; FB, faint band.

CFU/ml, and higher counts (10^4 CFU/ml) were obtained only for samples 11 and 12 (Table 2). Five colonies suspected to be *Brettanomyces* spp. were isolated, and after DNA extraction they were subjected to PCR with specific primers and RE analysis with *Dde*I. All the isolates gave a PCR product of the expected size, and when samples were subjected to RE analysis, the restriction profiles obtained were identical to that of *B. bruxellensis*. Some restriction profiles for representative isolated strains are shown in Fig. 4.

When specific primers were used for amplification of the DNA isolated directly from wine, a positive signal was obtained for some samples. As shown in Table 2 and Fig. 5, when DNA extracted from samples 2, 3, 4, 5, 6, 10, 11, and 12 were used for PCR amplification, the expected *Brettanomyces* band was observed. When an RE analysis with *Dde*I was performed, all the samples produced the profile specific for *B. bruxellensis* (Fig. 6). A residual uncut PCR product was also present, highlighting the need for an increase in the enzyme concentration or in the digestion time, which are modifications of the conditions suggested by the manufacturer. The second band in the gel was very faint only for sample 12 (Fig. 6, lane 11). No positive results were obtained when the RNA extracted directly from wine were subjected to RT-PCR with the specific primers developed in this study (Table 2).

To confirm the detection and identification of *B. bruxellensis* from all the wines analyzed, PCR and RT-PCR with primers NL1 and LS2, followed by DGGE analysis, and RNA dot blot analysis with a specific *B. bruxellensis* probe were carried out.

When universal primers NL1 and LS2 were used, almost all the wine samples produced a PCR product of the expected size (Table 2). Sample 9 did not produce any amplicon, and samples 1, 7, and 8 produced faint bands in the agarose gel; thus, these samples could not be used for DGGE analysis. The results obtained by DNA DGGE analysis for the other wine samples are shown in Fig. 7, in which control wine yeasts were used as migration markers in lanes 1 to 7. This figure shows that all the samples that produced a good PCR product during amplification produced a band that migrated at the same position in the gel as a band that *B. bruxellensis* produced. Moreover, for samples 5, 6, and 12 (Fig. 7, lanes 10, 11, and 14, respectively) a second band, comigrating with the *Saccharomyces cerevisiae* control strain, was observed. RT-PCRs carried out with the universal primers resulted in amplicons in the case of samples 1, 6, 7, 8, 11, and 12 (Table 2). When analyzed by DGGE (Fig. 8), only samples 11 and 12 produced a *B. bruxellensis* band. In sample 11 no other bands were visible, whereas in sample 12 an *S. cerevisiae* signal was detected. Also, in samples 1 and 8 (Fig. 8, lanes 7 and 10, respectively) very faint

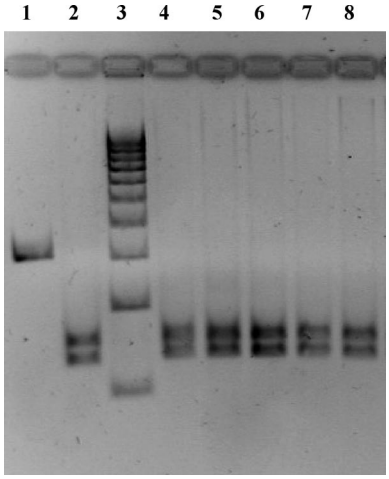

FIG. 4. *Dde*I RE analysis of PCR products obtained from strains isolated from wine samples. Lanes 1 and 2, uncut and cut amplicon of *B. bruxellensis* DBVPG6706; lane 3, molecular weight marker (100-bp ladder; Sigma); lanes 4 to 8, wine isolates.

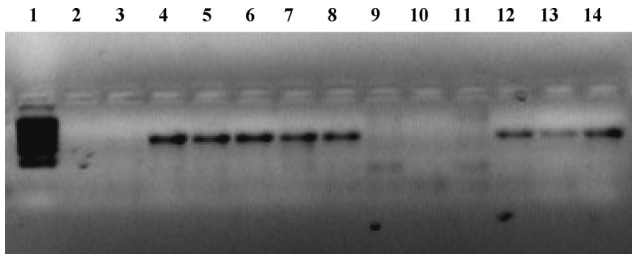

FIG. 5. Amplification of DNA extracted directly from wine samples with specific primers DB90F and DB394R. Lane 1, molecular weight marker (1-kb ladder; Sigma); lane 2, negative control; lanes 3 to 14, wine samples 1 to 12, respectively.

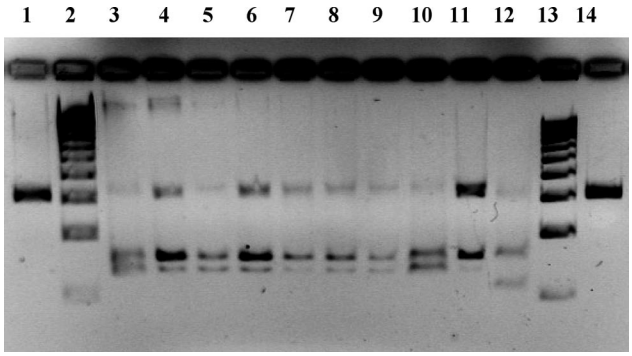

FIG. 6. RE analysis of the wine samples that were positive as determined by specific amplification. Lanes 1 and 14, uncut *B. bruxellensis* DBVPG6706 and *B. anomalus* NCAIM960 controls, respectively; lanes 2 and 13, molecular weight marker (100-bp ladder; Sigma); lanes 3 and 12, *B. bruxellensis* DBVPG6706 and *B. anomalus* NCAIM960 restriction patterns, respectively; lanes 4 to 11, wine samples 2, 3, 4, 5, 6, 10, 11, and 12, respectively.

bands of *B. bruxellensis* were detected. Samples 1 and 6 (Fig. 8, lanes 7 and 8) were mainly characterized by an *S. cerevisiae* population, while samples 7 and 8 (Fig. 8, lanes 9 and 10) produced a *Candida stellata* band. Identification of yeast species was based on comigration with control strains and by sequencing the bands marked in Fig. 7 and 8.

The results obtained by RNA hybridization with probe BRE26S14 are shown in Fig. 9. The results for RNA extracted from several dilutions of *B. bruxellensis* (Fig. 9A), decimal dilutions of *B. bruxellensis* RNA (Fig. 9B), control strains used in this study, and samples 11 and 12 (Fig. 9C) are shown. The protocol was determined to have a detection limit of 10^4 cells of *B. bruxellensis* (Fig. 9A) and 20 ng of RNA extracted from *B. bruxellensis* (Fig. 9B). The probe used in the study was confirmed to be highly specific because it did not hybridize to any of the yeast strains used as controls except *B. bruxellensis* strains (Fig. 9C). When the protocol was used to detect *B. bruxellensis* in wine, only samples 11 and 12 produced a positive

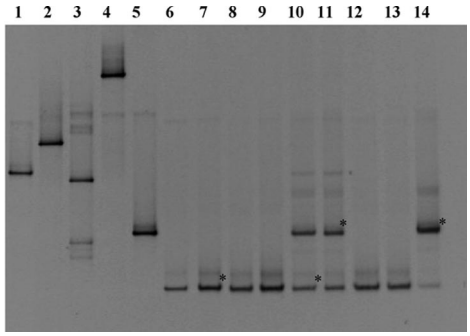

FIG. 7. DGGE analysis of the PCR products obtained by amplification of DNA extracted directly from wine samples with universal primers NL1 and LS2. Lane 1, *Candida stellata* DBVPG6714; lane 2, *Pichia anomala* DBVPG6612; lane 3, *Kluyveromyces thermotolerans* DBVPG6480; lane 4, *Hanseniaspora uvarum* DBVPG6717; lane 5, *Saccharomyces cerevisiae* DBVPG6173; lane 6, *Brettanomyces bruxellensis* DBVPG6706; lanes 7 to 14, wine samples 2, 3, 4, 5, 6, 10, 11, and 12, respectively. Bands marked with an asterisk were excised, reamplified, sequenced, and identified by sequence analysis.

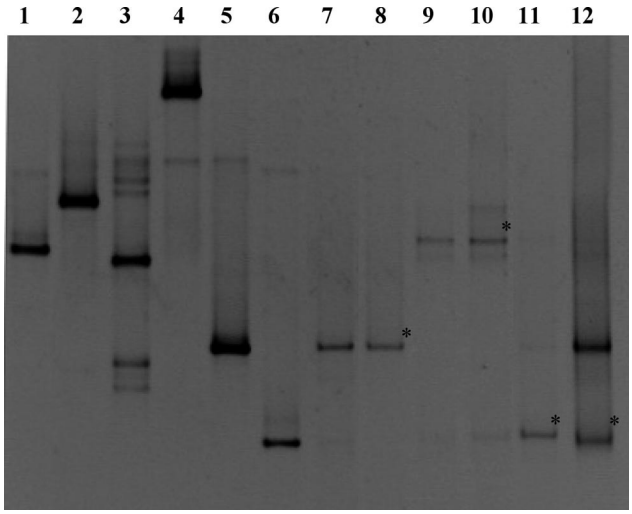

FIG. 8. DGGE analysis of RT-PCR products obtained from RNA extracted directly from wine samples with universal primers NL1 and LS2. Lane 1, *Candida stellata* DBVPG6714; lane 2, *Pichia anomala* DBVPG6612; lane 3, *Kluyveromyces thermotolerans* DBVPG6480; lane 4, *Hanseniaspora uvarum* DBVPG6717; lane 5, *Saccharomyces cerevisiae* DBVPG6173; lane 6, *Brettanomyces bruxellensis* DBVPG6706; lanes 7 to 12, wine samples 1, 6, 7, 8, 11, and 12, respectively. Bands marked with an asterisk were excised, reamplified, sequenced, and identified by sequence analysis.

spot comparable to the signal obtained when the RNA was extracted from 10^4 cells of *B. bruxellensis.*

DISCUSSION

Brettanomyces is one of the most complex and controversial yeast issues that a winemaker encounters when making wine.

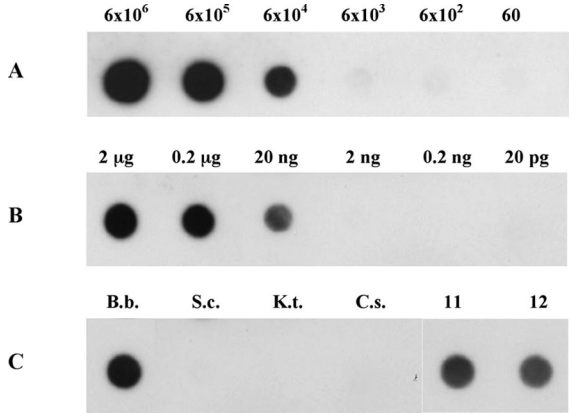

FIG. 9. RNA dot blot with the *B. bruxellensis* BRE26S14-specific probe. (A) Total RNA purified from serial dilutions of *B. bruxellensis* DBVPG6706 cells. The values at the top indicate the total number of cells from which RNA was extracted, as determined by plate counting on WLN medium. (B) Serial dilutions of *B. bruxellensis* DBVPG6706 RNA. The values at the top indicate the amount of total RNA blotted onto the membrane. (C) RNA samples extracted from yeast cultures and directly from wine samples. The abbreviations and numbers at the top indicate the yeast species used or the wine sample analyzed. One microgram of total RNA was blotted onto the membrane. Abbreviations: B.b., *Brettanomyces bruxellensis* DBVPG670; S.c., *Saccharomyces cerevisiae* DBVPG6173; K.t., *Kluyveromyces thermotolerans* DBVPG6480; C.s., *Candida stellata* DBVPG6714.

This genus is usually not included in the genera of yeasts found on the grape surface (11); however, it is quite common to find *Brettanomyces* in a winery (1, 4, 12).The use of different selective and/or differential media (1, 5, 9, 12, 24) is time-consuming and/or very expensive. Rapid and reliable detection, quantification, and characterization methods are required for a successful wine microbiological analysis. A novel approach involves application of molecular techniques. Among these techniques, the PCR-based methods are attractive because of their rapidity.

Previous studies have described detection and discrimination of *Brettanomyces/Dekkera* species by different PCR methods. Mitrakul et al. (23) used PCR-randomly amplified polymorphic DNA analysis for discriminating different strains of *B. bruxellensis*. A new fluorescence in situ hybridization method, in which peptide nucleic acid probes are used for identification of *Brettanomyces*, was proposed by Stender et al. (26). This method is easily adapted to microscopic techniques currently used in wine laboratories, but a fluorescence microscope is required. Identification of *Brettanomyces/Dekkera* species based on polymorphism in the rRNA internal transcribed spacer region has been reported (10). In this study the authors used four primers to identify the species of the genus *Brettanomyces/Dekkera*. They used the discriminatory potential of the internal transcribed spacer regions located between the rRNA genes. However, for the analyses described in the papers mentioned above the workers used isolated strains and classical techniques and thereby included biases inherent in traditional plating or enrichment. The use of culture-independent methods has repeatedly demonstrated that there is tremendous variance between cultivated and naturally occurring species. These approaches have been used recently to study the ecology of different ecosystems, including wine fermentations (8, 22). Only Ibeas et al. (18) described a nested PCR method for identification of *Brettanomyces/Dekkera* strains directly from sherry wine. The protocol described in this paper was found to be specific for *D. intermedia*, *B. bruxellensis*, and *B. lambicus*, and no PCR product was obtained for *B. anomalus* and *Brettanomyces claussiensis*, thus eliminating the possibility that the technique could be used to detect one species considered to be a wine spoilage organism, *B. anomalus*.

The goal of this study was to optimize a culture-independent, molecular technique-based protocol that would allow detection and differentiation of *B. bruxellensis* and *B. anomalus*, the main agents of wine spoilage. The protocol which we developed is based on PCR amplification and RE analysis and can be used for suspected colonies isolated from spoiled wine for identification purposes, but more significantly, it can be used directly with wine samples. DNA and/or RNA may be extracted from spoiled wine, and by using PCR-RE analysis the presence of *B. bruxellensis* and/or *B. anomalus* can be determined without prior traditional, culture-dependent isolation. Moreover, since the method could be also applied to directly extracted RNA, studies of the activity and viability of *B. bruxellensis* and *B. anomalus* can be performed.

Primers DB90F and DB394R were designed on the basis of the differences between 26S rDNA sequences belonging to *Brettanomyces* spp. and yeast species involved in wine fermentations (Fig. 1), and this was possible because of the extensive database for the D1-D2 loop created in the last few years (20). The primers selected in this study had a high specificity for *B. bruxellensis* and *B. anomalus*. This specificity made it possible to amplify DNA extracted directly from wine for detection of *B. bruxellensis* and *B. anomalus*. The protocol described here had a high detection limit. When the DNA and RNA were extracted from wine, a visible signal was not obtained for concentrations less than 10^4 to 10^5 cells/ml. This finding could be explained by considering different factors. The first factor is the possible presence of residual inhibitory substances that are present in the nucleic acid preparations that are not completely removed with the specific extraction kit and interfere with the amplification step. As a matter of fact, other authors have reported a substantial difference in the detection limit for *Dekkera* cells when the PCR was performed with DNA extracted from pure cultures or directly with wine (18). Moreover, the use of degenerate primers for simultaneous amplification of *B. bruxellensis* and *B. anomalus* may affect the stability of the primer match (defined as the measure of how tightly the primer and target are bound), as well as the primability of the primer match (which indicates how easily the DNA polymerase is able to extend the sequence). Detection of *B. bruxellensis* and *B. anomalus* in wines containing lower numbers of cells could be partially overcome by using a volume of wine larger than 1 ml, as suggested by other authors (22), in a way that more cells could be collected and processed. On the other hand, overloading of the kit used for extraction of the nucleic acids should be avoided, to prevent false-negative results due to inhibition of DNA polymerase by wine compounds, such as polysaccharides and polyphenols.

The sequence divergence in the fragment amplified was subsequently exploited to identify the two species considered by means of RE analysis. The *Dde*I restriction reaction could be used to differentiate the two species. As shown in Fig. 3, distinct restriction patterns were obtained, which allowed straightforward identification.

Twelve samples of wine that were suspected to be spoiled by *Brettanomyces* spp. because of their odor characteristics were analyzed by culture-dependent and culture-independent methods. When plating on WLN medium (a culture-dependent method) was used, six samples were positive for the presence of *Brettanomyces* spp. The counts (Table 2) refer to the *Brettanomyces* population, based on the specific characteristics of morphology and the color on WLN medium. The concentrations were about 10^4 CFU/ml only for samples 11 and 12, whereas for the other positive samples concentrations of about 10^3 CFU/ml were obtained. The remaining six samples exhibited no growth on the plates, underlining the conclusion that the concentration was less than 10 CFU/ml. Sample 12 was the only sample that produced a mixed population on the WLN plates. Another yeast population was observed along with *Brettanomyces* spp. All the other positive samples had a single colony morphology and color characteristic of *Brettanomyces* spp. At least five suspected colonies from the positive samples were isolated and subjected to molecular identification with the protocol developed in this study. After PCR amplification and RE analysis, all the isolates produced a profile identical to that of *B. bruxellensis* (Fig. 4).

Interesting results were obtained when culture-independent methods were used to detect and identify the two species considered in the wine samples. Because of the high detection

limit of the method, DNA was extracted from 50 ml of wine. When the specific PCR was used, a larger number of positive samples were detected than when the traditional plating method was used. All the samples that produced colonies on the plates also gave the specific PCR product when the concentration was not greater than 10^3 CFU/ml, but samples 5 and 6, containing a concentration of <10 CFU/ml, also produced the 305-bp amplicon (Fig. 5). This evidence could be explained by the presence of nonculturable or dead cells of *Brettanomyces* spp. This issue was immediately addressed when the RNA extracted from wine was subjected to specific RT-PCR. One important aspect is that RNA was extracted from 1 ml of wine. When higher volumes were used for RNA preparation, the pellets always were pink to dark brown, indicating that the purity of the nucleic acid obtained was poor. No positive results were obtained by specific RT-PCR (Table 2), even for the samples that gave positive PCR results. A possible explanation for this inconsistency is that different volumes of wine samples were processed. On the basis of these results, samples 5 and 6 were considered to contain only dead cells of *Brettanomyces* spp. The specific PCR products obtained from the wine samples were then subjected to RE analysis with the *Dde*I restriction endonuclease. All the amplicons were cut, and the patterns obtained were identical to the *B. bruxellensis* pattern. These results were in agreement with the identification of the isolates from the samples that exhibited growth on WLN medium.

The results of *Brettanomyces* detection by specific PCR were confirmed by DGGE analysis of PCR and RT-PCR products generated with universal primers and RNA hybridization with a *B. bruxellensis*-specific probe. In the DNA DGGE gels, all positive samples, as determined by specific PCR, produced a band that comigrated with the *B. bruxellensis* band (Fig. 7). Samples 5, 6, and 12 also produced a second band that was referable to *S. cerevisiae*. The specific *B. bruxellensis* band in sample 12 was faint, probably because of a masking effect due to the presence of a high concentration of *S. cerevisiae*, as demonstrated by plate counting (data not shown). After DGGE RNA analysis, samples 11 and 12 produced the cognate *B. bruxellensis* band, while the other samples contained different yeast populations (Fig. 8). Finally, RNA hybridization with probe BRE26S14 specific for *B. bruxellensis* was carried out. The detection limit of the assay was determined to be 10^4 cells of *B. bruxellensis* (Fig. 9), and it was 10-fold lower than the level of specific PCR amplification. A positive spot was observed only for samples 11 and 12, which were characterized by high counts of *Brettanomyces* spp. on the plates.

The results obtained by the multiphasic approach used allowed us to study the microbial ecology of the samples considered, with the specific aim of detecting *Brettanomyces* spp. Only samples 11 and 12 contained an active *B. bruxellensis* population consisting of at least 10^4 cells/ml. For the rest of the positive samples the size of the active population was 10^3 CFU/ml, as determined by plate counting. For samples 5 and 6, characterized by positive signals at the DNA level but not from the RNA, we speculate that there was a dead population of *B. bruxellensis*. Moreover, if the results obtained by PCR and RT-PCR with both specific and universal primers were combined, it could be possible to determine that spoilage by *B. bruxellensis* in samples 11 and 12 occurred more recently than spoilage in sample 5 or 6, in which only dead cells were detected by molecular methods.

Since the time required for extraction of DNA from cells and for differentiation of *Brettanomyces* strains by RE analysis is only approximately 8 h, this method could also be used for fast identification of *B. bruxellensis* and *B. anomalus* strains isolated from wine. Thus, this method is faster than traditional methods, which take 1 to 2 weeks. The availability of a rapid technique permits easy identification of *Brettanomyces* species during wine maturation. The disadvantage of the protocol described here is the high detection limit. However, this deficiency is less important if it is considered that the typical unpleasant odor appears when the *Brettanomyces* concentration reaches 10^5 CFU/ml or higher (5).

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