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PCR Differentiation of Commercial Yeast Strains Using Intron Splice Site Primers

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The increased use of pure starter cultures in the wine industry has made it necessary to develop a rapid and simple identification system for yeast strains. A method based upon the PCR using oligonucleotide primers that are complementary to intron splice sites has been developed. Since most introns are not essential for gene function, introns have evolved with minimal constraint. By targeting these highly variable sequences, the PCR has proved to be very effective in uncovering polymorphisms in commercial yeast strains. The speed of the method and the ability to analyze many samples in a single day permit the monitoring of specific yeast strains during fermentations. Furthermore, the simplicity of the technique, which does not require the isolation of DNA, makes it accessible to industrial laboratories that have limited molecular expertise and resources.

In contrast to traditional wine-making in which the indigenous yeasts ferment the grape must, most modern wine-makers inoculate with a pure culture of a selected yeast strain to ensure a rapid, reliable, and predictable fermentation (29). The availability of a range of commercial yeasts with different wine-making characteristics and applications means that a number of yeast strains may be used in a single winery. It is generally assumed that indigenous yeasts are suppressed by the competitive effect of addition of a high-density starter monoculture, but studies show that indigenous yeast can still participate in the fermentation (12, 26, 32). For these reasons, rapid and simple methods for the routine verification of yeast strain identity in starter cultures and fermentations would be useful.

Classical physiological methods are of limited use in the identification of commercial wine-making yeasts. First, the vast majority of the strains are of a single species, *Saccharomyces cerevisiae*. A second difficulty is that many strains of industrial importance are either diploid or aneuploid and therefore phenotypically wild type (3, 5). Furthermore, low sporulation frequency, poor spore viability, and homozygosity for the homothallism gene HO leading to immediate diploidization of meiotic segregants have made genetic analysis of these yeasts difficult (2, 37). For these reasons, molecular methods have been adopted for strain identification. Fatty acid composition (22) and yeast protein fingerprinting (38) have been utilized with some success, but these methods appear to be inadequate for differentiating strains of the same species (27). In recent years, the genetic diversity of commercial *S. cerevisiae* strains has become apparent and has lead to a number of nucleic acid-based identification techniques. The variability in the chromosomal constitution of commercial yeast strains is considerable, making chromosome karyotyping a useful method (14, 27). Restriction fragment length polymorphism, which has been an important development for mapping genes of agricultural and medical importance, has also been successfully applied to yeast strain differentiation. Both the nuclear (7) and mitochondrial (8) genomes have been analyzed by restriction fragment length polymorphism. The potentials of these methods have been compared and discussed elsewhere (27).

The development of the PCR (31) has opened up new avenues for yeast strain identification. The application of the PCR for discrimination between wine yeast strains by the amplification of random (28) or specific (16) target sequences has recently been reported. In the present study, oligonucleotides have been synthesized with sequences complementary to yeast intron splice sites to target mutable regions of the genome. With the use of these primers in the PCR, we are able to differentiate between commercial yeasts commonly used in the Australian wine industry. The method is rapid, reliable, and simple, making the technique available to laboratories with limited resources for verifying strain identity and monitoring yeast growth during fermentations.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this study are listed in Tables 1 and 2. All are strains of *S. cerevisiae* except AWRI 1034, which belongs to the species *Torulaspora delbrueckii*. The 15Du strains (6) were obtained from Steven Reed, Research Institute of Scripps Clinic, San Diego, Calif. The source of yeast B431 is Brigalow Brewing Company, Slacks Creek, Queensland, Australia. The source of K5088 is Cerebos Ltd., Seven Hills, New South Wales, Australia. The other yeasts listed in Table 1 were obtained from the culture collection at The Australian Wine Research Institute. In Table 2, AWRI 814 is the original AWRI 729 yeast that was obtained from a dry yeast labelled Epernay. Isolates of this yeast were sent to other culture collections and then returned to The Australian Wine Research Institute at a later date (30). Yeasts were grown on yeast extract-peptone-dextrose (YEPD) at 25°C by standard procedures (33).

Preparation of DNA template for PCR. To obtain a DNA preparation method suitable for the PCR, cells of strain AWRI 796 grown on YEPD plates and then stored at 4°C were resuspended in either sterile water, 0.1% Triton X-100 (Sigma), or 500 μg of Zymolyase 20T per ml (Seikagaku Corp., Tokyo, Japan). The cells resuspended in water were then treated in one of the following ways: (i) no treatment, (ii) boiled for 10 min, (iii) frozen in liquid nitrogen and then boiled for 10 min, (iv) boiled for 10 min followed by freezing in liquid nitrogen, or (v) same as method (iii) but followed by a second freeze-boil cycle. The cells resuspended in Triton X-100 were boiled for 10 min, and the cells resuspended in Zymolyase were incubated for 10 min at 37°C. A cell suspension of 2 μl (5×10^4 cells) was used for each PCR amplification with primer EI1. The results were compared with those obtained when purified DNA was used as a template.

Yeast DNA purification. Yeast DNA was isolated by standard procedures (1). A cell suspension (10 ml) from an overnight culture grown in YEPD was resuspended in 200 μl of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris [pH 8], 1 mM EDTA [pH 8]). The yeast cells were homogenized by vortexing for 3 min with glass beads (0.3 g) in the presence of 200 μl of phenol-chloroform-isoamyl alcohol. Tris-EDTA buffer (200 μl) was

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TABLE 1. Laboratory and commercial yeast strains used in this study

Strain[a]	Application
15Dua	Laboratory strain
15Dua/α	Laboratory strain
15Duα	Laboratory strain
B431	Brewer's yeast
K5088	Baker's yeast
AWRI 939	Sake yeast
AWRI 350	Wine yeast
AWRI 729	Wine yeast
AWRI 834	Wine yeast (secondary fermentation)
AWRI 81	Wine yeast (flor yeast)
AWRI 1017	Wine yeast
AWRI 1034	Wine yeast (sweet wines)
AWRI 838	Wine yeast
AWRI 796	Wine yeast

[a] All yeasts are *S. cerevisiae* strains except AWRI 1034, which is a strain of the osmotolerant species *T. delbrueckii*.

added, and the aqueous layer was collected after centrifugation. The DNA was precipitated with ethanol. DNA concentrations were determined by measuring the A_{260} after incubation with RNase. For each PCR, 0.5 μg of DNA was used.

Optimization of amplification. For all subsequent experiments, the DNA template was prepared for PCR by freezing cells in liquid nitrogen followed by 10 min of boiling in a water bath.

The reproducibility of the method was tested with *S. cerevisiae* AWRI 796 and the 15Du strains. Three isogenic 15Du strains that differ only at the mating locus were analyzed. These strains were plated to single-colony density, and each amplification reaction was performed with an individual colony.

For determining the effect of cell concentration on PCR, AWRI 796 cells were resuspended in water at concentrations of 5×10^2 to 2.5×10^5 cells per μl before the freeze-boil treatment.

For comparing results of stationary-phase and dividing cells, yeast strain AWRI 796 was grown for 24 h at 25°C on YEPD plates. These plates were then stored at 4°C for either 0, 2, 4, or 8 h prior to preparing DNA template and PCR.

Intron splice site primers and PCR conditions. The following primers were used in this study: EI1, CTGGCTTGGTGTATGT; EI2, CTGGCTTGCTACATAC; LA1, GCGACGGTGTACTAAC; LA2, CGTGCAGGTGTTAGTA. The nucleotides that are conserved with the intron splice sites are underlined (Fig. 1). Primers were used either singularly or in pairs. PCRs were performed in 50 μl with 50 pmol of primer, 2 μl of DNA template, 32 μM of each deoxynucleoside triphosphate, 2.5 mM MgCl, and 0.2 U of *Taq* polymerase (Advanced Biotech). The reactions were run for 33 cycles: denaturation at 94°C for 1 min, annealing at 45°C for 2 min, and extension at 74°C for 1.5 min. An initial denaturation for 3 min at 94°C and a final 5-min extension at 74°C were used. Products of each amplification reaction were resolved on a 2% agarose gel (Agarose NA; Pharmacia), stained with ethidium bromide, and visualized under UV light. The gels were photographed with the Gel Cam Documentation System (Sony), and the photographs were scanned to produce a computer image (Hewlett-Packard Scan Jet 11CXIT).

Cloning and sequencing of amplified products. Amplified products from PCRs were either gel isolated with a silica matrix (Geneclean II Kit; Bio 101) or isolated by spin column chromatography (PCR Spinclean; Progen). The purified fragments were then cloned into the pGEM-T vector (Promega), and the ligated products were transformed into *Escherichia coli* DH5α (1). Inserts were sequenced with M13 forward and reverse primers with *Taq* polymerase (Prism Ready Reaction Dye Primer Cycle Sequencing Kit; Applied Biosystems). The sequences obtained were analyzed on SeqEd version 1.0.3 software (Applied Biosystems) and compared with that of the yeast genome by use of a BLAST search (Basic Local Alignment Search Tool) with the *Saccharomyces* Genome Database (internet address, http://genome-www2.stanford.edu:5555/cgi-bin/blastsgd). Predicted open reading frames were detected with DNA Strider 1.0 msoftware (Commissariat a l'Energie Atomique, Gif-sur-Yvette, France).

TABLE 2. Possible isolates of AWRI 729

Strain	Source
AWRI 729	University of California, Davis
AWRI 814	Australian Wine Research Institute
AWRI 825	Department of Agriculture, Western Australia
AWRI 835	Department of Agriculture, Western Australia
AWRI 925	University of California, Davis
AWRI 947	Australian Wine Research Institute
AWRI 1116	Epernay, France
AWRI 1117	Epernay, France
AWRI 1118	Epernay, France

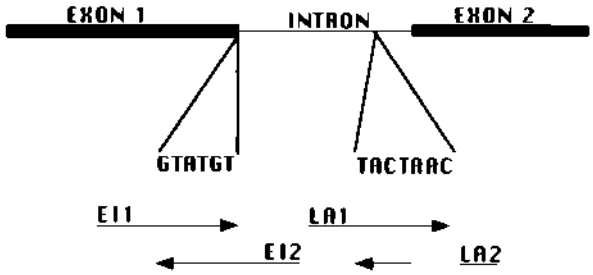

FIG. 1. Design of intron primers. The sequences of primers EI1 and EI2 were based on the consensus sequence GTATGT at the 5′ exon-intron splice site. The sequences of primers LA1 and LA2 were based on the lariat branch point consensus sequence TACTAAC.

RESULTS

Design and use of intron splice site primers. Since introns are not essential for gene function, they have evolved with minimal constraint. There are conserved sequence motifs within all introns, however, that are necessary for their removal during the synthesis of mRNA. In the yeast *S. cerevisiae*, the lariat branch point sequence TACTAAC is strictly conserved, with mutations in this site preventing spliceosome assembly and cleavage of the 5′ intron junction. The sequence GTATGT is also almost exclusively utilized at the 5′ splice site. A third sequence, (C/T)AG, defines the 3′ end of the intron (40). To detect polymorphisms in yeast strains by PCR, primers were designed which contain sequences at their 3′ end complementary to either the 5′ splice site or lariat branch consensus sequences. The primers were extended at their 5′ end to produce 16-mer oligonucleotides by using random sequence. The primer sequences were checked on the program Oligo4 (National Biosciences) to test for possible secondary structure and to obtain oligonucleotides with similar annealing temperatures. Primers were used singularly or in pairs. When used alone, the amplification is between two different intron splice sequences.

To develop a rapid PCR technique, several different methods of preparing DNA template were tested (Fig. 2). Cells from the wine yeast AWRI 796, grown to the stationary phase on YEPD plates, were resuspended in 100 μl of either water, 0.1% Triton X-100, or Zymolyase. The cells resuspended in water were either used for PCR directly or subjected to different freezing and boiling treatments. Cells resuspended in Triton X-100 or Zymolyase were incubated at 100 or 37°C for 10 min, respectively. The cell suspension (2 μl) was then used in a 50-μl PCR with primer EI1 as described in Materials and Methods. The different cell treatments were compared with a reaction using purified DNA. The amplification products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. The results in Fig. 2 demonstrate that each of the different cell preparations generated a more complex amplification pattern than the cells that were resuspended in water without any further treatment. Although there are several differences in the amplification products with each of the DNA preparations, all treatments generated patterns comparable to that obtained with purified DNA.

The most important criterion for the usefulness of the technique was the reproducibility of the results. Five colonies of the wine strain AWRI 796 and the laboratory yeast strains 15Du (both haploid mating types and a diploid strain) were picked to

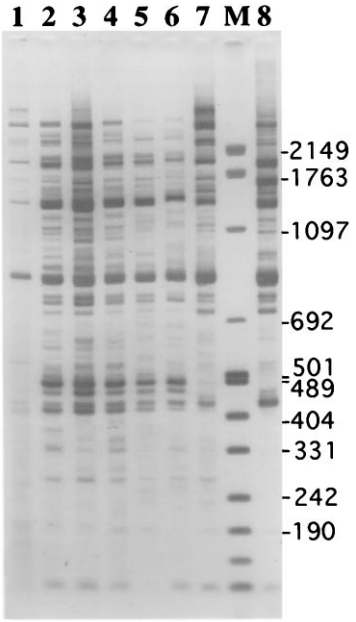

FIG. 2. Comparing methods of DNA template preparation. Lanes: 1 to 5, cells resuspended in sterile water (1, no treatment; 2, boiled; 3, freeze-boiled; lane 4, boil-frozen; 5, two cycles of freeze-boiling); 6, cells resuspended in Triton X-100 and boiled; 7, cells resuspended in Zymolyase and incubated at 37°C; 8, purified DNA; M, molecular size standards (sizes are indicated on the right in base pairs). All reactions were with primer EI1 and strain AWRI 796.

determine whether reliable results could be obtained when cells were prepared for PCR by simply freezing in liquid nitrogen and then boiling for 10 min. Figure 3A demonstrates that although there are polymorphisms generated between strains 15Du and AWRI 796, the amplification fingerprints within each strain are very similar. Although cells prepared by only boiling exhibited an amplification pattern comparable to that of the freeze-boil samples (Fig. 2), this treatment generated less consistent results (data not shown). Since the freeze-boil treatment of cells was simple and rapid, this method was used for all subsequent experiments.

To determine the sensitivity of the reaction, a serial dilution of cells was made before the freeze-boil treatment. The results in Fig. 3B illustrate that when 5×10^3 to 5×10^5 cells were used in the reaction, the amplification pattern remained consistent, although some minor bands were variable. Further dilution led to a significant change in the pattern, with only the major fragments being amplified. These results demonstrate that the amplification fingerprint was relatively insensitive to the number of yeast cells used in the reaction, making it unnecessary to accurately quantify the cell number.

In the initial stages of the study, there were problems in obtaining uniform amplification fingerprints. Results indicated that this was due to cells being at different stages of growth prior to PCR. The reason for this is unclear. One explanation is that the interaction of proteins with the genome in dividing cells is either blocking the annealing of the primers to the splice site sequences or inhibiting the *Taq* polymerase extension reaction. Since strains grow at different rates, this could be a drawback for the technique, particularly when rapid identification is necessary. To avoid this problem, we have tested whether transferring the yeast to 4°C to stop cell division was sufficient to obtain reliable results (Fig. 3C). Cells that were not incubated at 4°C gave poor and inconsistent amplification (Fig. 3C, lane 4). Incubating cells at 4°C for 2 h or more, however, ensured a dependable amplification. Although the physiological state of the cells at the different time points was uncertain, the result is important in that it permits PCR analysis of strains soon after the formation of visible single colonies.

Differentiation of *S. cerevisiae* strains. Figure 4 shows the amplification patterns obtained with the four intron primers used separately for five *S. cerevisiae* strains, namely, a laboratory yeast and four commercial yeasts used in either the wine, sake, baking, or brewing industry (Table 1). As well as amplifying fragments shared by all of these yeasts, the method generated distinctive polymorphisms that permit unmistakable differentiation of these strains. The brewing, baking, and sake yeasts are most readily distinguished from each other and from the two other strains by using primer LA2. In the baker's yeast, the LA2 primer amplified products of 280 and 320 bp. Only the 280-bp fragment was amplified with the brewing yeast, and only the 320-bp fragment was amplified by the sake yeast. The laboratory and wine strains did not produce either of these fragments but can be distinguished by other changes in the LA2 amplification pattern. Primer LA1 shows similar differences, but the amplification fingerprints of the baker's and brewer's yeasts are very similar. The laboratory yeast 15Du is most easily identified by the use of primer EI1, which gave an amplification product of 1.55 kbp, which is not seen in any other yeast analyzed to date. The wine yeast AWRI 796 is differentiated by a product of 800 bp with primer EI1 that has not been observed in other wine yeasts plus a 300-bp fragment with primer EI2.

Differentiation of wine-making strains. All of the *S. cerevisiae* strains shown in Fig. 4 have very different physiological characteristics that make them suitable for their specific application. Figure 5 demonstrates that the primers are also useful in differentiating commercial wine-making strains from each other. The yeast strains analyzed are those commonly used in the Australian wine industry as starter cultures for grape juice fermentation and have been previously karyotyped by pulsed-

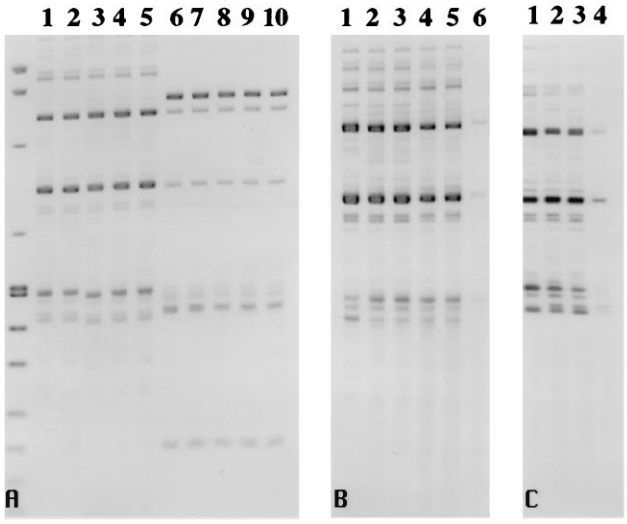

FIG. 3. Optimization of PCR method. (A) Reproducibility of PCR method. Lanes: 1 to 5, AWRI 796; 6, 15Du**a**; 7, 15Du**α**; 8 to 10, 15Du**a**/**α**. All DNA templates were prepared by the freeze-boil method. All reactions were with primer EI1. (B) Effect of DNA template concentration. Lanes: 1, 5×10^5 cells; 2, 1×10^5 cells; 3, 5×10^4 cells; 4, 1×10^4 cells; 5, 5×10^3 cells; 6, 1×10^3 cells. All reactions were with primer EI1 and strain AWRI 796. (C) Stationary and dividing cells. Strain AWRI 796 was grown on plates for 24 h at 25°C and then stored at 4°C for 8 (lane 1), 4 (lane 2), 2 (lane 3), and 0 (lane 4) h. All reactions were with primer EI1.

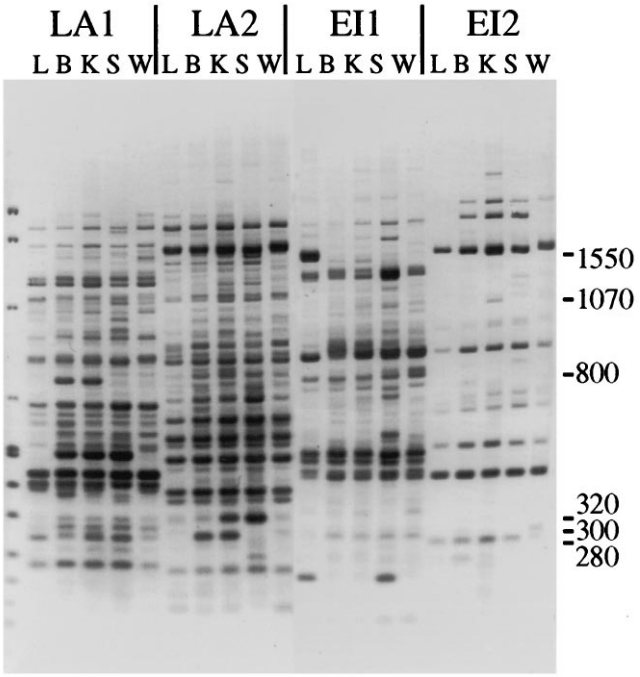

FIG. 4. Differentiation of *S. cerevisiae* strains with intron primers. Lanes: 1 to 5, primer LA1; 6 to 10, primer LA2; 11 to 15, primer EI1; 16 to 20, primer EI2. L, laboratory strain 15Du; B, brewing strain B431; K, baking strain T5088; S, sake strain AWRI 939; W, wine strain AWRI 796. The numbers indicate the sizes, in base pairs, of the polymorphic fragments discussed in the text.

field gel electrophoresis (25) (strains listed in Table 1). By using primer EI1, seven of the eight wine yeasts can be differentiated from each other and from the *S. cerevisiae* strains tested in Fig. 4. Yeast AWRI 1034, shown in lane 7 of Fig. 5A, produced an amplification pattern distinct from that of the other yeasts. This yeast is *T. delbrueckii* and is the only non-*Saccharomyces* yeast used in this study. An amplified product at 1.33 kb separates the *S. cerevisiae* strains into two groups. The 800-bp fragment is unique to AWRI 796 (Fig. 5A, lane 9). The 200-bp fragment, which is detected in the laboratory yeast 15Du (lane 1) and was amplified in the sake yeast (Fig. 4), permits the differentiation of wine yeast strain AWRI 1017 (lane 6).

Although the differences between the strains are consistently amplified with primer EI1, the polymorphisms can be minor. The increased intensity of the 330-bp fragment distinguishes AWRI 350 (Fig. 5B, lane 2) from AWRI 838 (lane 8). These two strains, however, are more readily differentiated by use of primers LA1 and LA2 (Fig. 5B). Primer LA1 amplifies 1,000- and 1,100-bp fragments in yeast AWRI 838 but not in AWRI 350 (lanes 1 and 2). Primer LA2 (lanes 3 and 4) amplifies the 280-bp fragment in AWRI 838, which was also amplified in the brewer's and baker's yeasts in Fig. 4 but not in AWRI 350.

Strain AWRI 81 (Fig. 5B, lane 7) is most easily differentiated from strains AWRI 729 and AWRI 834 (lanes 6 and 7) by using primer EI2. With this primer, the 1,070-bp fragment present in the AWRI 729 and AWRI 834 fingerprints is not amplified for AWRI 81. The use of each of the intron primers separately did not allow the differentiation of AWRI 729 and AWRI 834. To distinguish between these two yeasts, it was necessary to use a primer pair. The use of primers EI1 and EI2 or LA1 and LA2 together gives poor amplification because of the complementarity of the primer sequences. However, when primers LA1 and EI1 (lanes 8 and 9) or LA1 and EI2 (lanes 10 and 11) are used, the two strains could be differentiated. The first primer combination produced a difference in the amplification of a 700-bp fragment in strains AWRI 834 and AWRI 729. When primers LA1 and EI2 were used simultaneously, several polymorphisms were detected, including a 1,680-bp amplification product for strain AWRI 834.

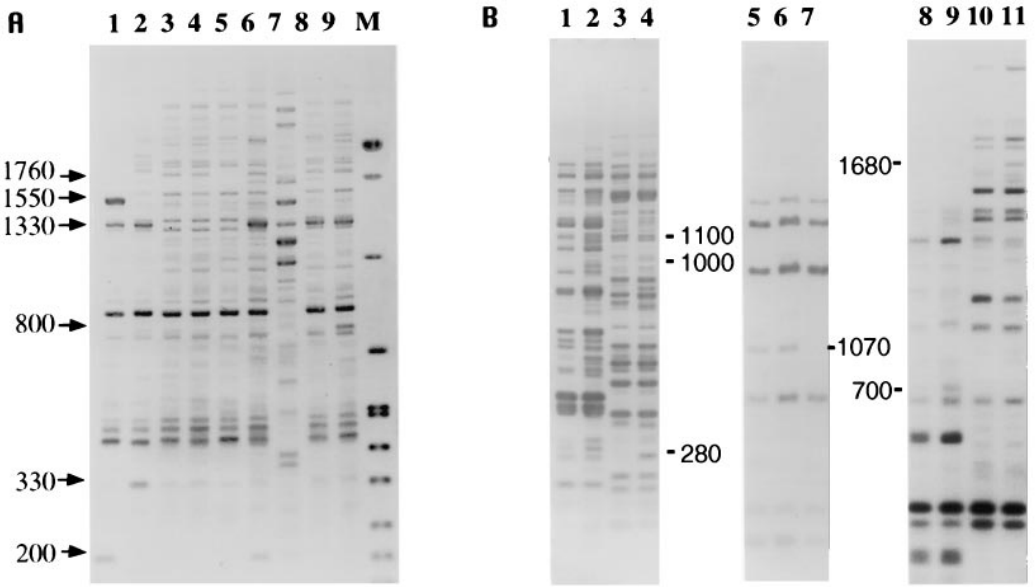

FIG. 5. Differentiation of commercial wine-making strains. (A) PCR using primer EI1. 15Du is a laboratory yeast. The AWRI yeasts are commercial wine-making strains. Lanes 1, 15Du; 2, AWRI 350; 3, AWRI 729; 4, AWRI 834; 5, AWRI 81; 6, AWRI 1017; 7, AWRI 1034; 8, AWRI 838; 9, AWRI 796. (B) PCR using other intron primers. Lanes: 1, primer LA1, strain AWRI 350; 2, primer LA1, strain AWRI 838; 3, primer LA2, strain 350; 4, primer LA2, strain AWRI 838; 5, primer EI2, strain AWRI 729; 6, primer EI2, strain AWRI 834; 7, primer EI2, strain AWRI 81; 8, primers LA1 and EI1, strain AWRI 729; 9, primers LA1 and EI1, strain AWRI 834; 10, primers LA2 and EI1, strain AWRI 729; 11, primers LA2 and EI1, strain AWRI 834. The numbers indicate the sizes, in base pairs, of the polymorphic fragments discussed in the text.

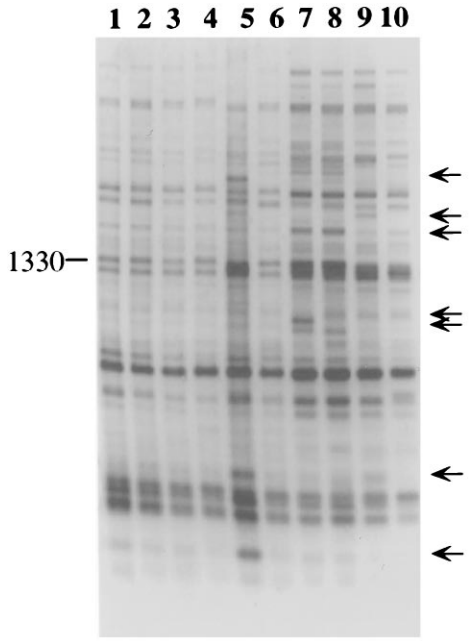

FIG. 6. Verification of *S. cerevisiae* strain identity. Lanes: 1, AWRI 729; 2, AWRI 814; 3, AWRI 825; 4, AWRI 835; 5, AWRI 925; 6, AWRI 947; 7, AWRI 1116; 8, AWRI 1117; 9, AWRI 1118; 10, AWRI 796. All reactions were with primer EI1. The unlabelled arrows indicate polymorphisms between the strains with fingerprints distinct from that of AWRI 729.

Verification of wine yeast identity. A number of yeasts believed to be isolates of strain AWRI 729 exist (13, 27, 30) (Table 2). To assess whether this method is effective for verifying the identity of *S. cerevisiae* strains, the putative 729 strains were analyzed. The results with primer EI1 are shown in Fig. 6 and clearly indicate that several of the strains are unrelated to AWRI 729. The original yeast strain 729 (now named AWRI 814) was isolated in 1964 from a sample of dried yeast labelled Epernay. The current AWRI 729 yeast was obtained from The University of California, Davis, and shows an amplification fingerprint identical to that of the original isolate (Fig. 6, lanes 1 and 2). A second isolate of strain 729 was retrieved from The University of California, Davis, at a later date (and named AWRI 925), and interestingly, this yeast has an amplification fingerprint completely different from that of AWRI 729 (lane 5). Two isolates of AWRI 729 acquired from The University of Western Australia (AWRI 825 and AWRI 835) are indistinguishable from the original strain (lanes 3 and 4). However, three other yeasts from Epernay (AWRI 1116, AWRI 1117, and AWRI 1118) are distinct from strain AWRI 729 (lanes 7 to 9). The results were confirmed by using the other intron primers and demonstrate the usefulness of the technique for yeast strain authentication in culture collections.

Sequence analysis of PCR products. The specificity of the PCR has been determined by sequencing several of the amplified fragments (Table 3). Generally, the analysis was performed with products of primers EI1 and LA2 since PCR with these primers amplifies towards the other conserved splicing motif, assisting in the recognition of intron sequences. These primers were used singularly in PCRs with strains 15Du and AWRI 796. The results demonstrate that the conditions used in the experiments are effective in annealing to the conserved sequence motifs of introns. Of the 11 primer binding sites that could be established by a comparison with the *Saccharomyces* genome database, seven are conserved in the intron splice site motifs of the primer (Table 3). In all sequences, at least one of the primers has annealed to an exact match of the conserved motif. As expected, the homology with the target DNA is less specific at the 5′ end of the primers.

The conserved splicing motifs are obviously present in both intron and nonintron sequences, and it is expected that both regions are being amplified. Analysis of the sequences supports this expectation. Sequence LA2-25 is a 592-bp fragment. A search of the *Saccharomyces* genome database indicates that it is amplified from a region of chromosome V (cosmids 9747, 8198, and 9781 and lambda clones 3612 and 6052; nucleotides 38192 to 38784). This has not previously been identified as an open reading frame (ORF) since there is a stop codon in all six reading frames. The region, however, meets all the criteria of a spliced gene (15, 40). The TACTAACA lariat branch point motif is at position 38208. A potential 5′ splice site, GTATGT, exists 44 nucleotides upstream from the lariat branch point. This distance agrees with that found in other yeast introns. There are three possible ATG start codons within 127 bp of the 5′ splice site. This is consistent with introns being located at the 5′ end of genes, always in the first 150 bp, downstream of the ATG. The region also contains a 3′ splice site motif 15 bp from the lariat branch point. This would confer an intron of 75 nucleotides, which is well within the common maximum size of 550 bp. Importantly, splicing of this sequence would lead to the excision of a stop codon near the lariat branch point, producing an ORF of 132, 146, or 161 amino acids, depending upon the ATG utilized in translation. The predicted protein sequences have no significant homologs in other organisms.

Sequences obtained from other amplified products also met the criteria of spliced genes, but in these cases, the significance is unclear. Two of these sequences were in coding regions of previously identified genes (sequences EI1-15 and LA2-24). A third sequence (LA2-34) was in a region of the yeast genome that is not yet in the genome database; therefore, there is not

TABLE 3. Sequence analysis of PCR fragments

Sequence name	Target DNA[a]	Matching sequence in database[b]
EI1-13	**TTCAGCTGGTGCATGT** **TTGGCTTGTTGTATGT**	Chromosome XVI sequence SC9367
EI1-15	**CTGGCTTGGGGGGTGT** AAATA**T**ACTG**GTATGT**	*SMX3* SCSMX3
EI1-17	**TTGGCTTGTTGTATGT** Second primer not in yeast genome database	Chromosome XVI and unidentified region SC9367
LA1-1	CGGTTGA**TG**TACTAAC **CTACCGGTGTTCTAAC**	Encoding hypothetical Ser-Thr-rich protein—chromosome IX SC9402
LA2-24	TCGTCCT**GTGTTTGTA** AATTTCA**GTGTTAGTA**	*MDM10/CCR4* gene SCMSM10
LA2-25	CTGG**GGAGCTG-TAGTA** **GTAGACGGTGTTAGTA**	Predicted intron containing ORF SCE9747
LA2-34	Not in genome database	Unidentified sequence

[a] The nucleotides which are identical to the primer are shown in bold. The sequences that correspond to the intron splice site motif are underlined. Sequences for first and second primers are given.

[b] Designations with the prefix SC are *Saccharomyces* Genome Database locus numbers.

sufficient information to establish whether an ORF would be created. Interestingly, an amplified fragment from AWRI 796 (EI1-17) is conserved at one end with a region of chromosome XVI but at the other end has no homology to sequences in the yeast genome database. This suggests that the primer has exposed a change in chromosome structure of the wine yeast. Whether this is due to the presence of an intron or to chromosome translocation cannot be determined with the sequence data obtained.

DISCUSSION

Many precursor mRNAs in eukaryotes contain intervening sequences or introns. The introns are precisely spliced from the pre-mRNA to form functional mature mRNAs in a process that requires the spliceosome, a large ribonucleoprotein complex (40). The reason for the existence and distribution of introns is debated continuously, and whether these intervening sequences have a function remains unclear (9, 19). It is known, however, that many introns are very close to selectively neutral, and except for the sites that have been conserved for recognition by the spliceosome, their sequences are highly variable. These changes in intron structure include nucleotide substitutions, deletions, or insertions or the presence or absence of introns in a gene (21, 23, 36).

Since introns have potentially high rates of sequence evolution, their analysis has become an important tool in studies of genome relatedness. Evolutionary relationships between species have been analyzed by determining the divergence of intron sequences (35). Similar analyses have been used to study the population genetics of a species (23). Furthermore, by comparing the intron sequences of X-linked and Y-linked genes, the extent of male-driven evolution has been analyzed in a number of mammals (34). Primers that anneal to intron splice sequences have been used to identify and map genetic polymorphisms in cereals with the PCR (39). Splice site primers have also been employed for identifying genes in complex genomes by using both PCR and hybridization methods (20).

In the present study, introns have been targeted to detect polymorphisms in commercial yeast strains. A PCR-based method that does not require the purification of DNA, making the technique simple and rapid, has been developed. Sequencing of the amplified fragments shows that the conditions used for PCR are effective in targeting the splice site motifs, and fragment LA2-25 reveals that intron-containing ORFs are targeted. These conserved splice site motifs are also present in nonintron DNA, however, and sequencing demonstrates that these regions of the genome are similarly amplified. The results from the use of the intron primers have been compared with that of random primers. Random primers have either given no amplification or else have provided few polymorphisms between the wine-making strains studied (results not shown). This has also been observed by Quesada and Cenis (28), who used randomly amplified polymorphic DNA analysis for yeast strain differentiation. The ability of the intron primers to produce such high polymorphisms may reflect the yeast genome structure. Recent models argue that introns in multicellular organisms have evolved to have a function in providing an extra level of gene regulation for differentiation (19). The most apparent example is alternative splicing of genes in higher eukaryotes. It has also been found that for several introns in higher eukaryotes, the intron sequences are very large and well conserved across species boundaries, indicating a regulatory function. Apart from an unusually large yeast intron of 1,001 nucleotides in the *DBP2* gene of *S. cerevisiae* that has recently been shown to possess a regulatory function (4), the smaller yeast introns (10) are less likely to be evolutionarily constrained. This potential for intron sequence evolution, and the sexual isolation of wine yeast as a result of their inability to produce viable meiotic products, might explain the high degree of polymorphism detected by using the intron primers.

The use of specific primers that target the delta elements of transposons as a method for *S. cerevisiae* strain identification has been reported (16). The authors declare that these primers permit the differentiation of more than 95% of their commercial strains. Furthermore, the few examples shown indicate striking polymorphisms, although previous use of the transposon primers has shown a lesser degree of difference (18). The intron primers provide an alternative strategy to transposon and randomly amplified polymorphic DNA (28) analysis for strain differentiation, and targeting both transposons and introns may lead to an efficient *S. cerevisiae* identification system, making the need for chromosome karyotyping unnecessary. The intron primers have a significant advantage, however, for characterization of non-*Saccharomyces* yeasts. The transposon delta sequences are not present in non-*Saccharomyces* yeasts, and therefore the transposon primers are limited to *S. cerevisiae* and very closely related species (11, 24). Intron splice sites are conserved in all yeasts that have been studied to date. For example, in the unrelated fission yeast *Schizosaccharomyces pombe*, the 5′ splice site is either GTAAGT or the *S. cerevisiae* consensus sequence GTATGT. The branch point sequence is also less stringent than for *S. cerevisiae*, but many contain the TACTAAC site (41). The usefulness of this can be seen with yeast AWRI 1034, which is a strain of *T. delbrueckii* (Fig. 5A). *T. delbrueckii* is a highly osmotolerant fermenting yeast, capable of growth in 50% glucose. For this reason, it is occasionally used in the production of some sweet wine styles. The amplification pattern of this yeast is unique when compared with that of the *S. cerevisiae* yeasts used in the study, clearly identifying it as an unrelated yeast.

The intron splice site primers are effective in differentiating between the main commercial wine yeasts used by the Australian wine industry. These wine yeasts (excluding AWRI 796) have been previously subjected to chromosome karyotyping using transverse alternating field electrophoresis (25). Transverse alternating field electrophoresis, although sensitive in detecting polymorphisms in yeast strains, is time-consuming and technically difficult, and the results can be difficult to interpret. For example, AWRI 350 and AWRI 838 are easily separated by use of a single primer with the PCR technique (Fig. 5), but the chromosome karyotypes are difficult to distinguish. The PCR technique is much quicker and less labored than chromosome karyotyping. Apart from strains AWRI 729 and AWRI 834, the commercial strains could all be differentiated by performing a single amplification reaction. For these two strains, polymorphisms were detected only when two primers were used simultaneously. It is curious to note, however, that these two yeasts have very different fermentation characteristics and exhibit considerable chromosome polymorphism when analyzed by pulsed-field gel electrophoresis (25).

An interesting result was also observed with strains AWRI 729 and AWRI 814 (Fig. 6). These are putative isolates of the same yeast that have been stored in different culture collections. The intron primers were unable to detect polymorphisms between these two strains. The strains display similar growth characteristics, amino acid uptake properties, and fermentation characteristics in the laboratory but have been reported to produce different levels of volatile acidity by the wine industry. Whether the reason for this difference is environmental or genetic is not clear. Preliminary pulsed-field gel electrophore-

sis studies were unable to discriminate between the strains (25), but more recently, a minor polymorphism has been noticed by increasing the resolution of the chromosomal fingerprint method (13). The explanation for this modification remains unknown. The genetic composition of some yeasts has been demonstrated to be quite unstable (17), and combined with the freeze-drying method used for the long-term storage of these strains, closely related yeasts could give differences in karyotype. It is unlikely, however, that the PCR method will detect these changes except in rare cases where the amplified fragment spans the chromosome break. A more detailed analysis will be required to resolve the evolutionary divergence of these strains, so as to determine the sensitivity of the intron primers.

In summary, a PCR-based method using intron splice site primers has been developed for identification of commercial yeast strains. The main advantage of this technique is that it is simple, rapid, and accessible to industrial laboratories with limited molecular expertise and resources. The speed of the technique and the ability to analyze many samples in a single day permit its use in monitoring the growth of yeasts during propagation and fermentation for routine quality control. The method has been used for confirmation of the identity of active dried strains used for wine-making (6a) and has been effective in differentiating commercial winery strains from both the indigenous *S. cerevisiae* and non-*Saccharomyces* yeast flora present in the juice and on winery equipment in one winery studied (17a).

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