Differentiation and species identification of yeasts using PCR

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A PCR-based method has been developed that permits both intraspecies differentiation and species identification of yeast isolates. Oligonucleotide primers that are complementary to intron splice sites were used to produce PCR fingerprints that display polymorphisms between different species of indigenous wine yeasts. Although polymorphisms existed between isolates of the same species, the banding patterns shared several amplification products that allowed species identification. Importantly, the method was able to distinguish between species of the closely related *Saccharomyces sensu stricto* yeasts. In two cases where isolates could not be positively identified there was discrepancy between the phenetic and phylogenetic species concept. The method has applications in yeast ecological studies, enabling the rapid grouping of isolates with related genomes and the investigation of population dynamics of strains of the same species.

Keywords: PCR, identification, yeasts

INTRODUCTION

The yeasts are a phylogenetically diverse group of unicellular fungi. In a recent monograph, yeasts comprise 83 genera and 590 species (Barnett *et al.*, 1990). These numbers are constantly fluctuating as new species are discovered and the principles and methodologies of yeast taxonomy are modified and updated (reviewed by Boekhout & Kurtzman, 1996, and by van der Walt, 1987). Although morphological and biochemical phenotypes continue to be the main criteria used for the identification of an unknown isolate (Barnett *et al.*, 1990; Kreger-van Rij, 1984), these physiological characters may be determined by only a small fraction of the genome (Winge & Roberts, 1949). The resulting confusion is apparent in the changes that have occurred in the taxonomy of the industrially important yeast *Saccharomyces cerevisiae*, which had previously been divided into a number of distinct species due to variations in fermentation and assimilation of carbon sources (Barnett, 1992). It is now clear that many of the characters are unstable and due to a single mutation (Scheda & Yarrow, 1966).

The most widely accepted definition of a species is the biological species concept, which is based upon genetic relatedness or hybridization (Dobzhansky, 1937). Lack of fertility, however, does not preclude conspecificity and is not applicable to the imperfect species that constitute the majority of the yeasts. Hybridization studies are also difficult in yeasts that have low spore viability as is the case in many commercial strains.

The phenotypic characters used in yeast systematics have more recently been combined with molecular-based criteria. Determining DNA relatedness using reassociation studies has been of exceptional value in yeast systematics. For example, Vaughan Martini and colleagues have reclassified several of the yeasts in the genus *Saccharomyces*, that could not be differentiated from *S. cerevisiae* on the basis of phenotypic characters (Vaughan Martini, 1989; Vaughan Martini & Kurtzman, 1985). The results are in excellent agreement with the hybridization studies of Naumov (1987). More recently, phylogenetic relationships amongst yeasts have been studied by comparing the sequences of the rRNA genes. The principal advantage of this method is that, since ribosomes share a common evolutionary origin in all organisms, it permits the comparison of both closely and distantly related species (Kurtzman, 1992).

The GenBank accession numbers for the 26S rRNA sequences determined in this study are AF020437 (CBS 187^T), AF020438 (CBS 107^T), AF020435 (AWRI 1271) and AF020436 (AWRI 1272).

Species identification and strain differentiation continue to be important in yeast utilizing industries. In winemaking, several studies have analysed the diverse microbial flora of grapes and must during fermentation and it is apparent that a number of species are involved (see Fleet *et al.*, 1984). The identification methods described above are time-consuming, laborious and are generally of limited value for strain differentiation. A rapid PCR-based method has recently been developed in our laboratories for differentiating commercial wine making strains (de Barros Lopes *et al.*, 1996). The method uses primers with sequences complementary to intron splice sites of *S. cerevisiae* to target variable regions of the genome. As intron splice site sequences appear to be conserved in all fungi, the primers are useful for all yeasts (Jacob & Gallinaro, 1989). In this report, we extend the PCR method to study indigenous grape juice microflora. As the PCR fingerprint is species specific, this method also provides a rapid yeast identification system.

METHODS

Yeast strains and media. The yeast strains used in this study are listed in Tables 1 and 2. Reference strains (Table 1) are

species type strains obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Delft, The Netherlands. Winery isolates (Table 2) were obtained from: The Australian Wine Research Institute (AWRI) culture collection, grape must and winery equipment at Petaluma Ltd, Piccadilly, South Australia, and a grape juice fermentation at Sonoma-Cutrer Winery, Windsor, CA, USA. Yeasts were isolated aseptically from juice and must by sterile collection and dilution in 0.1% peptone followed by plating onto glucose-yeast extract-peptone agar (GYEPA, yeast extract 1%, w/v, peptone 0.5%, w/v, glucose 4%, w/v, agar 2%, w/v) and lysine medium (Oxoid). Yeasts were isolated from the surfaces of winery equipment by swabbing with sterile buffered swabs (Disposable Products). The swabs were then rolled onto the surface of GYEPA plates. Prior to PCR, yeasts were grown on YEPD plates (yeast extract 1%, w/v, peptone 2%, w/v, glucose 2%, w/v, agar 2%, w/v).

For identification using physiological methods, the isolates were sent to CBS.

Preparation of DNA template for PCR. For all species except the *Dekkera/Brettanomyces* yeasts, DNA was prepared using a simple freeze/boil method as described previously (de Barros Lopes *et al.*, 1996). Yeast colonies grown on YEPD plates and stored at 4 °C overnight were resuspended in 100 µl sterile water, frozen in liquid nitrogen and then

Table 1. Yeast strains of known identity used in study

Species Synonym	CBS no.	AWRI no.
<i>Saccharomyces cerevisiae</i> Meyen ex E. C. Hansen	1171 ^{NT}	1219
<i>Candida robusta</i> Diddens et Lodder	1907	1144
<i>Saccharomyces paradoxus</i> Bachinskaya	432 ^{NT}	1172
<i>Saccharomyces bayanus</i> Saccardo	380 ^T	1146
<i>Saccharomyces uvarum</i> Beijerinck	395	1145
<i>Saccharomyces pastorianus</i> Reess ex E. C. Hansen	1538 ^{NT}	1173
<i>Saccharomyces unisporus</i> Jörgensen	398 ^T	1218
<i>Saccharomyces exiguus</i> Reess	379 ^T	1216
<i>Saccharomyces kluyveri</i> Phaff <i>et al.</i>	3082 ^T	1217
<i>Dekkera bruxellensis</i> van der Walt	74 ^T	1205
<i>Brettanomyces bruxellensis</i> Kufferath <i>et van Laer</i>	72	1102
<i>Dekkera intermedia</i> van der Walt	4914	1207
<i>Brettanomyces intermedius</i> Krumbholz <i>et Tauschanoff</i>	73	1104
<i>Brettanomyces lambicus</i> Custers	75	1127
<i>Brettanomyces custersii</i> Florenzano	5512	1103
<i>Brettanomyces abstinentis</i> Yarrow <i>et Ahearn</i>	6055	1130
<i>Dekkera anomala</i> M. T. Smith <i>et van Grinsven</i>	8139 ^T	953
<i>Brettanomyces claussenii</i> Custers	76	1128
<i>Brettanomyces anomalus</i> Custers	77	1168
<i>Brettanomyces naardenensis</i> Kolfshoten <i>et Yarrow</i>	6042 ^T	951
<i>Brettanomyces custersianus</i> van der Walt	4805 ^T	950
<i>Brettanomyces nana</i> M. T. Smith <i>et al.</i> (formerly <i>Eniella</i>)	1945 ^T	1201
<i>Torulaspora delbrueckii</i> Lindner	1146 ^T	1152
<i>Issatchenkia orientalis</i> Kudryavtsev	5147 ^T	1220
<i>Hanseniaspora uvarum</i> (Niehaus) Shehata <i>et al.</i>	314 ^T	1158
<i>Hanseniaspora guilliermondii</i> Pijper	465 ^T	1200
<i>Metschnikowia pulcherrima</i> Pitt <i>et Miller</i>	5833 ^T	1149
<i>Pichia fermentans</i> Lodder	187 ^T	1199
<i>Pichia membranifaciens</i> E. C. Hansen	107 ^T	1095

Table 2. Winery isolates studied

AWRI strain no.	Source*	Species
870	NSW	<i>Saccharomyces cerevisiae</i> †
871	NSW	<i>Saccharomyces cerevisiae</i>
1265‡	SA	<i>Saccharomyces cerevisiae</i>
1266	SA	<i>Saccharomyces bayanus</i> †
948	SA	<i>Saccharomyces bayanus</i>
872	NSW	<i>Torulaspora delbrueckii</i> †
873	NSW	<i>Issatchenkia orientalis</i> †
868	NSW	<i>Hanseniaspora uvarum</i> †
1274	SA	<i>Hanseniaspora uvarum</i>
1275	CA	<i>Hanseniaspora uvarum</i>
1276	CA	<i>Hanseniaspora uvarum</i>
1277	CA	<i>Hanseniaspora guilliermondii</i> †
1267‡	SA	<i>Metschnikowia pulcherrima</i> †
1268‡	SA	<i>Metschnikowia pulcherrima</i>
1269‡	SA	<i>Metschnikowia pulcherrima</i>
1270‡	SA	<i>Metschnikowia pulcherrima</i>
1271	SA	<i>Pichia fermentans</i> §
1272	SA	<i>Pichia membranifaciens</i> §

* SA strains isolated in South Australia and CA strains isolated in California, USA (this study). NSW strains isolated in New South Wales, Australia (Heard, 1988; Petering *et al.*, 1990). Karyotypes of the NSW isolates can be compared (Petering *et al.*, 1990).

† The identity of the yeasts in Fig. 3 are in agreement with results obtained by CBS using standard physiological methods.

‡ All strains were isolated from grape juice or fermenting must except for the *M. pulcherrima* strains and one *S. cerevisiae* yeast which were isolated from winery equipment.

§ Species description using standard physiological methods. Inconsistency with molecular methods.

boiled for 10 min. The suspensions were stored at -20°C . For the *Dekkera/Brettanomyces* yeasts DNA was purified using standard procedures (Ausubel *et al.*, 1994). A cell suspension from a 10 ml overnight culture grown in YEPD media was resuspended in 200 μl breaking buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8), 1 mM EDTA (pH 8)]. The yeast cells were homogenized by vortexing for 3 min with 0.3 g glass beads in the presence of 200 μl phenol/chloroform/isoamylalcohol. Tris/EDTA buffer (200 μl) was added and, after centrifugation, the aqueous layer collected. The DNA was precipitated with ethanol and resuspended in 100 μl water.

PCR fingerprinting. PCR experiments were done as previously described (de Barros Lopes *et al.*, 1996). PCRs were performed in 50 μl with 50 pmol intron primer EI1 (5'-CTGGCTTGGTGTATG), 2 μl DNA template, 32 μM each dNTP, 2.5 mM MgCl₂, and 0.2 U *Taq* polymerase (Advanced Biotechnology). The reactions were run for 33 cycles: denaturation was at 94°C for 1 min, annealing at 45°C for 2 min, and extension at 74°C for 1.5 min. An initial 3 min denaturation 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 (Pharmacia Agarose NA) containing 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) with Adobe Photoshop (Adobe Systems).

rDNA isolation and sequencing. Partial sequences approximately equivalent to position 83–610 of the *S. cerevisiae* 26S rDNA were obtained using a strategy based on Boekhout *et al.* (1994). A divergent 5' domain of the gene was amplified using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5'-GGTCCGTGTTTCAAGACGG). PCRs were run for 25 cycles: denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 74°C for 1.5 min. An initial 3 min denaturation at 94°C and a final 5 min extension at 74°C were used. Amplified products were isolated with a silica matrix (GeneClean II Kit; Bio101). The purified rDNA genes were sequenced in both directions using the external primers NL1 and NL4 and two internal primers NL2 (5'-CTCTCTTTTCAAAGTTCTT-TTCATCT) and NL3 (5'-AGATGAAAAGAACTTTGA-AAAGAGAG). Sequencing was performed using dye-deoxy sequencing chemistry (Prism Ready Reaction DyeDeoxy Termination Cycle Sequencing Kit; Perkin Elmer). Phylogenetic relationships were determined using the PAUP program (version 3.1) (Swofford, 1993).

RESULTS

Differentiation of indigenous wine yeasts

In a previous study it was demonstrated that commercial strains of *S. cerevisiae* can be differentiated using PCR with primers that target intron splice site sequences (de Barros Lopes *et al.*, 1996). Fig. 1 demonstrates that primer EI1, complementary to the yeast intron 5' splice site, is also effective in differentiating yeasts isolated from grape juice and winery equipment. Amplification fingerprints of several of the isolates are readily distinguishable. Other yeasts produce similar amplification patterns. Lanes 1–3, 4–5, 8–11 and 13–16 all share amplified fragments with each other, but in most cases all the strains can be differentiated by at least a single polymorphism. The exceptions are yeasts AWRI 1266 and 948 in lanes 4 and 5, and AWRI 1267 and 1268 in lanes 13 and 14. The use of other intron primers uncovered polymorphisms between AWRI 1267 and 1268 (data not shown). These yeasts, which are both *Metschnikowia pulcherrima*, were isolated from different locations in the same winery. The *Saccharomyces bayanus* strains AWRI 1266 and 948 were isolated from cold-stored juices (-2°C) from the same winery in different years. Interestingly, the two yeasts could not be differentiated using any of the intron primers indicating they may be identical. The use of other methods are required to test this further.

Species possess characteristic amplification fingerprints

Fig. 1 shows that several yeasts produced related amplification fingerprints. In a previous study it was also observed that, although polymorphisms exist between *S. cerevisiae* strains, the strains also shared a

Fig. 1. Differentiation of indigenous wine yeasts (AWRI strain nos given) using PCR with intron primer EI1. Lanes: 1, 870; 2, 871; 3, 1265; 4, 1266; 5, 948; 6, 872; 7, 873; 8, 868; 9, 1274; 10, 1275; 11, 1276; 12, 1277; 13, 1267; 14, 1268; 15, 1269; 16, 1270; 17, 1271; 18, 1272. The isolates are arranged into groups with similar amplification fingerprints. This is indicated above the lane numbers. The DNA fragments, 650, 420, 360, 220 and 200 bp, discussed in the text in reference to lanes 13–16 are marked. The photographs were scanned to produce a computer image using Adobe Photoshop.

number of common amplified fragments (de Barros Lopes *et al.*, 1996). This finding suggested that individual species produce a characteristic fingerprint. To test this further, a number of type strains for different species obtained from the CBS culture collection were analysed using the PCR method. Results from two genera are shown in Fig. 2.

Fig. 2(a) shows the results for several *Saccharomyces* yeasts. The type strain for *S. cerevisiae* (CBS 1171^{NT}) and its asexual anamorph (CBS 1907, previously *Candida robusta*) are compared to other yeasts in the genus. The two *S. cerevisiae* yeasts have similar amplification fingerprints, which are different from distantly related yeasts in the genus as well as from closely related species of the *Saccharomyces sensu stricto* group. The PCR fingerprints of the two *S. bayanus* yeasts, the type strain (CBS 380^T) and a second strain (CBS 395, formerly the type strain of *Saccharomyces uvarum*), are less conserved than those obtained for *S. cerevisiae*, possibly indicating that this species is less clearly defined. The shared amplification fragments, for example at 860, 550 and 490 bp, still clearly demonstrate a kinship with each other. Furthermore, the *S. bayanus* PCR fingerprint is notably different from that of the closely related species *Saccharomyces pastorianus* (CBS 1538^{NT}) and shows no similarity to the other yeasts in the genus.

Fig. 2. Intron primer PCR fingerprints of *Saccharomyces* and *Dekkera/Brettanomyces* yeasts (CBS strain nos given). (a) Genus *Saccharomyces*. Lanes: 1, 1171^{NT}; 2, 1907; 3, 432^{NT}; 4, 380^T; 5, 395; 6, 1538^{NT}; 7, 398^T; 8, 379^T; 9, 3082^T. Sc, *S. cerevisiae*; Sr, *S. paradoxus*; Sb, *S. bayanus*; Ss, *S. pastorianus*; Su, *S. unisporus*; Se, *S. exiguus*; Sk, *S. kluyveri*. The 860, 550 and 490 bp DNA fragments discussed in the text in reference to *S. bayanus* are marked. (b) Genus *Dekkera/Brettanomyces*. Lanes: 1, 74^T; 2, 72; 3, 4914; 4, 73; 5, 75; 6, 5512; 7, 6055; 8, 8139^T; 9, 76; 10, 77; 11, 6042^T; 12, 4805^T; 13, 1945^T. Db, *D. bruxellensis*; Da, *D. anomala*; Br, *B. naardenensis*; Bc, *B. custersianus*; Bn, *B. nana*. The photographs were scanned to produce a computer image using Adobe Photoshop.

Banding patterns for *Dekkera* and *Brettanomyces* yeasts are shown in Fig. 2(b). The *Dekkera bruxellensis* strains (lanes 1–7) all have related amplification patterns. The *Dekkera anomala* strains (lanes 8–10) also share similar amplification patterns to each other but are unrelated to the other yeasts in the genus. Each of the fingerprints of *Brettanomyces naardenensis*

Fig. 3. Species identification of indigenous wine yeasts using PCR with primer EI1. Lanes: 1, CBS 1171^{NT}; 2, AWRI 870; 3, CBS 380^T; 4, AWRI 1266; 5, CBS 1146^T; 6, AWRI 872; 7, CBS 5147^T; 8, AWRI 873; 9, CBS 314^T; 10, AWRI 868; 11, CBS 465^T; 12, AWRI 1277; 13, CBS 5833^T; 14, AWRI 1267. Sc, *S. cerevisiae*; Sb, *S. bayanus*; Td, *T. delbrueckii*; Io, *I. orientalis*; Hu, *H. uvarum*; Hg, *H. guilliermondii*; Mp, *M. pulcherrima*. The photographs were scanned to produce a computer image using Adobe Photoshop.

(CBS 6042^T), *Brettanomyces custersianus* (CBS 4805^T) and *Brettanomyces nana* (CBS 1945^T, formerly *Eeniella nana*) are unique (lanes 11–13) (Boekhout *et al.*, 1994). These results indicate that, at least for some genera, the PCR fingerprints are not only shared by strains of a species, but also unique to that particular species.

Species identification of indigenous wine yeasts

By comparing the amplification fingerprints of unknown isolates to those produced by type strains of yeasts associated with the wine industry, an effective identification system has been developed. Several of the yeasts isolated from grape juice have been shown to be strains of *S. cerevisiae*. Lanes 1 and 2 of Fig. 3 are the PCR fingerprints of the *S. cerevisiae* type strain (CBS 1171^{NT}) and an isolated yeast AWRI 870. The similarity of the patterns is evident and indicates that AWRI 870, 871 and 1265 (Fig. 1, lanes 1–3) are strains of *S. cerevisiae*. AWRI 1265 produced identical PCR fingerprints to the commercial strain used in the winery suggesting that it is the commercial yeast and not an indigenous yeast from the grapes (results not shown). Strains AWRI 1266, 948 and several other yeasts isolated from cold stored juice were also initially identified as *S. cerevisiae* using physiological tests. The results in Fig. 3 (lanes 3 and 4) demonstrate that the

Fig. 4. Comparison of PCR fingerprints of *Pichia* type strains and wine isolates. Lanes: 1, CBS 187^T (*P. fermentans*); 2, AWRI 1271; 3, CBS 107^T (*P. membranifaciens*); 4, AWRI 1272. The photographs were scanned to produce a computer image using Adobe Photoshop.

PCR fingerprint of strain AWRI 1266 is very similar to that of the type strain of the closely related species *S. bayanus*. The majority of the yeasts isolated in grape juice were identified as *Hanseniaspora uvarum* (lanes 9 and 10) with more than 11 polymorphic patterns observed using the single primer EI1 (four of which are shown in Fig. 1, lanes 8–11). The PCR fingerprints obtained from these yeasts are more heterogeneous than those of the other isolates, but all amplify fragments of 650, 420, 360, 220 and 200 bp. Several strains isolated from winery equipment (Fig. 1, lanes 13–16) produced amplification patterns similar to the type strain of *Metschnikowia pulcherrima* (Fig. 3, lanes 13 and 14). Using this method yeasts belonging to the species *Torulaspora delbrueckii* (Fig. 1, lane 6, and Fig. 3, lanes 5 and 6), *Issatchenkia orientalis* (Fig. 1, lane 7 and Fig. 3, lanes 7 and 8) and *Hanseniaspora guilliermondii* (Fig. 1, lane 12, and Fig. 3, lanes 11 and 12) were also identified.

The identity of the yeasts in Fig. 3 are in agreement with results obtained by CBS using standard physiological methods.

Discrepancy between the phenetic and phylogenetic species concept

Of the yeasts studied in grape juice from three different wine regions, all but two were positively identified using PCR. Strains AWRI 1271 and 1272 (Fig. 1, lanes 17 and 18) were identified as *Pichia fermentans* and *Pichia membranifaciens* using physiological methods. Fig. 4 displays the PCR fingerprints of these two yeasts compared to the *P. fermentans* (CBS 187^T) and *P. membranifaciens* (CBS 107^T) type strains. The results

Fig. 5. Phylogenetic tree for the type strains of *S. cerevisiae* (CBS 1171^N), *P. fermentans* (CBS 187^T) and *P. membranifaciens* (CBS 107^T), and the grape juice isolates AWRI 1271 and 1272 based on partial 26S rDNA sequence. The phylogenetic relationships were determined by using the branch-and-bound search option on the PAUP program. Numbers on the branches indicate bootstrap values (100 replicates). Branch lengths are proportional to the number of steps.

show that there is no similarity between the pattern of the isolated yeasts and the type strains. The difficulty in using solely phenotypic methods for yeast systematics could explain this disparity. To further test the relationship between the yeasts at the genomic level, partial sequences of the 26S ribosomal genes were obtained. The data are expressed as a phylogram (Fig. 5). Comparison of the AWRI 1272 and CBS 107^T rDNAs suggests relatedness with *P. membranifaciens*, but the 5% sequence divergence indicates that the yeasts should be considered a different species. The 26S rDNA of AWRI 1271 is very different to that of the *P. fermentans* type strain.

DISCUSSION

A PCR-based method is described that is effective in the differentiation of yeast strains of different species. In a previous study, oligonucleotide primers that anneal to *S. cerevisiae* intron splice site motifs were used in PCR for uncovering polymorphisms in commercial yeast strains (de Barros Lopes *et al.*, 1996). In this report the method is extended to include indigenous yeasts of grape must and wine (Fig. 1). Although introns are highly mutable, the splice site sequences are conserved in all yeasts studied to date. In the yeast *S. cerevisiae*, three sequence motifs are essential; GTATGT at the 5' splice site, complementary to primer E11, TACTAACA at the lariat branch point, and C/TAG at the 3' splice site (Woolford, 1989). In the distantly related fission yeast *Schizosaccharomyces pombe*, approximately 22% of the introns contain the same 5' consensus sequence, GTATGT, and another 60% vary from this sequence at only a single nucleotide (Zhang & Marr, 1994). The same consensus sequence is also commonly observed in other yeasts and higher fungi (Jacob & Gallinaro, 1989).

Analysis of commercial yeasts showed that *S. cerevisiae* strains generate similar PCR fingerprints (de Barros Lopes *et al.*, 1996) suggesting that each yeast species produces a characteristic amplification pattern.

This is supported by the analysis of type strains in Fig. 2 demonstrating that, at least for some species, the amplification pattern is unique to the species. By comparing the PCR fingerprints of unknown isolates to those produced by type strains, it has been possible to identify yeasts of *T. delbrueckii*, *I. orientalis*, *H. uvarum*, *H. guilliermondii*, *M. pulcherrima*, *S. cerevisiae* and *S. bayanus* (Fig. 3). Differentiation of the *Saccharomyces* species was of particular interest. Phenotypic characters led to the identification of AWRI 1266 and 948 as *S. cerevisiae* but PCR suggested these isolates, and several others collected from juice, to be *S. bayanus*. This is an important finding since traditional taxonomic methods do not allow the differentiation of all *Saccharomyces sensu stricto* species, although molecular methods have been described recently that allow their discrimination (Cardinali & Martini, 1994; Guillaumon *et al.*, 1994; Lewicka *et al.*, 1995; Naumov *et al.*, 1992).

The amplification fingerprints of *Saccharomyces* yeasts shown in Fig. 2 suggest that the PCR method will not lead to false positives. The *Saccharomyces sensu stricto* yeasts were previously considered to be a single species, *S. cerevisiae* (Barnett, 1992; Kreger-van Rij, 1984). DNA reassociation and hybridization analyses have led to the separation of these yeasts into four distinct species. The sequence similarity between *S. bayanus* and *S. pastorianus* is 72% using reassociation analysis (Vaughan Martini & Kurtzman, 1985), yet the amplification fingerprints of the two species are clearly distinct. As this is the highest DNA similarity known for two different yeast species, it is unlikely that other species will have sufficient homology to produce related amplification patterns. The divergence observed using PCR is presumably related to the primer targeting regions with high rates of sequence change.

As for all molecular techniques, the PCR method does not provide a means of differentiating the teleomorph and anamorph states of a species. This is shown for *S. cerevisiae* and its asexual synonym *C. robusta*, and the *Dekkera/Brettanomyces* yeasts (Fig. 2). Therefore, although identified isolates are labelled as the teleomorphic species, nothing is known of their sexual reproduction. The results obtained with the *Dekkera/Brettanomyces* yeasts agree with recent systematic changes to the genera. Molecular analysis of these yeasts (Hoeben & Clark-Walker, 1986; Boekhout *et al.*, 1994) has led to the grouping of the original twelve species into four: *D. anomala*, *D. bruxellensis*, *B. custersianus* and *B. naardenensis*. The PCR amplification fingerprints also led to the grouping of these yeasts into the four species (Fig. 2b). Mitochondrial DNA analysis (Hoeben *et al.*, 1993) and 26S ribosomal sequence (Boekhout *et al.*, 1994) analysis have also indicated that *Eeniella nana* is derived within the genus *Brettanomyces*. The PCR amplification fingerprint of this yeast is also compared in Fig. 2(b) but the method does not seem useful for demonstrating relationships between species.

Reassociation analysis of *Saccharomyces sensu stricto* yeasts and of other genera (Price *et al.*, 1978) have led to the conclusion that if two yeasts have more than 80% DNA homology they are conspecific. Determining a lower limit of DNA reassociation for hybridization has been more difficult. Kurtzman *et al.* (1980) showed that *Issatchenkia scutulata* var. *scutulata* and *I. scutulata* var. *exigua* were able to mate and produce 3–6% viable ascospores, and sibling matings between the progeny gave increased ascospore viability. Yet these two varieties have only 24% DNA homology. This means, that when using the PCR method, strains producing distinct amplification patterns cannot be assumed to be different species as some species will be represented by more than a single fingerprint.

Of the yeasts collected from grape must, the PCR technique was unable to identify two isolates. Using the phenetic system of classification, these two yeasts were identified as *P. fermentans* and *P. membranifaciens*. Comparison of the PCR fingerprints with the type strains of these species showed no kinship. Further molecular analysis of these yeasts emphasized the inconsistency. The sequence of the 26S ribosomal gene of the isolate identified as *P. membranifaciens* indicates that the yeast is related to the *P. membranifaciens* type strain but is not conspecific. This agrees with fatty acid analysis and DNA reassociation experiments that demonstrate heterogeneity within the *P. membranifaciens* species (Noronha-da-Costa *et al.*, 1996). Furthermore, PAUP analysis of the 26S rDNA sequences demonstrates that the AWRI 1271 isolate is distant from the type strain of *P. fermentans*. The *Pichia* genus is known to contain a diverse group of yeasts (Yamada *et al.*, 1996) and more detailed analysis is necessary before the systematics of these species is resolved.

Discrepancies between molecular methods and physiological methods of classification were also observed with other yeasts. Several strains isolated in the winery were phenotypically identified as *Rhodotorula mucilaginosa*. The intron primer produced several different PCR fingerprints with these isolates, none of which resembled the fingerprint of the type *R. mucilaginosa* strain (results not shown). Similarly, three *Candida stellata* strains obtained from the CBS culture collection produced similar PCR fingerprints to each other but unrelated to that of the type *C. stellata*. Sequence comparison of the 26S rDNA showed the type strain sequence to be markedly different from the other three yeasts (results not shown).

The main advantages of the intron-based PCR method described here is that it is rapid, simple and relatively cheap to perform. In all yeasts studied to date, except for the *D. bruxellensis* group, suitable PCR fingerprints could be obtained without first isolating the DNA. The method suffers from the same disadvantage as DNA reassociation experiments in that similarities can only be observed between closely related yeasts. The DNA reassociation method, however, is of little use for routine yeast identification as a number of experiments

must be performed for each strain to be identified until the analogous type strain is determined. The information obtained using PCR is clearly not as precise as sequencing and is not useful in determining phylogenetic relationships between new species. One advantage of the PCR method is that several areas of the genome are compared. Analysis of a single genetic locus can lead to erroneous conclusions (see Palumbi & Baker, 1994). This is especially relevant with yeast hybrids, and the limitation is observed with *S. bayanus* and *S. pastorianus* which have identical 26S rDNA sequences (Peterson & Kurtzman, 1991).

In conclusion, a method based on PCR with intron primers has been developed that permits both strain differentiation and species identification. The PCR method was not developed to replace existing techniques in yeast taxonomy and systematics, but as a preliminary tool in these disciplines. The method can assist taxonomists in rapidly detecting unrelated yeasts in a heterogeneous species, as was observed with the *Pichia* and *Candida* yeasts. Similarly, PCR fingerprinting is capable of exposing the teleomorph of asexual species. As shown in this study, the technique is useful to ecologists for grouping yeasts with related genomes. By comparing the PCR amplification pattern of isolates to a database of type strain fingerprints it is possible to identify the species of a completely uncharacterized yeast.

ACKNOWLEDGEMENTS

We thank Jeff Cohn and staff at Vinquiry, Healdsburg, CA, USA for isolation of yeasts at Sonoma Cutrer Winery. We thank Neil Shirley and Ata-Ur-Rehman for DNA sequencing, Paul Grbin for initial observations, Sonia Dayan and Sandra Rainieri for critical reading of the manuscript. Anna Martens acknowledges Brian Croser, Andrew Markides and Andrew Yap for helpful discussions. Alison Soden acknowledges the receipt of an Australian Postgraduate Research Award (Industry) in conjunction with Lallemand Australia, Lallemand Inc, Australia. This work was funded by the Grape and Wine Research and Development Corporation and the Cooperative Research Centre for Viti-culture.

REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994). *Current Protocols in Molecular Biology*. New York: Wiley.
- Barnett, J. A. (1992). The taxonomy of the genus *Saccharomyces* Meyen ex Reess: a short review for non-taxonomists. *Yeast* **8**, 1–23.
- Barnett, J. A., Payne, R. W. & Yarrow, D. (1990). *Yeasts: Characteristics and Identification*, 2nd edn. Cambridge: Cambridge University Press.
- Boekhout, T. & Kurtzman, C. P. (1996). Principles and methods used in yeast classification, and an overview of currently accepted yeast genera. In *Nonconventional Yeasts in Biotechnology*, pp. 1–81. Edited by K. Wolf. Berlin Heidelberg: Springer.

- Boekhout, T., Kurtzman, C. P., O'Donnell, K. & Smith, M. T. (1994). Phylogeny of the yeast genera *Hanseniaspora* (anamorph *Kloeckera*), *Dekkera* (anamorph *Brettanomyces*), and *Eeniella* as inferred from partial 26S ribosomal DNA nucleotide sequences. *Int J Syst Bacteriol* **44**, 781–786.
- Cardinali, G. & Martini, A. (1994). Electrophoretic karyotypes of authentic strains of the sensu stricto group of the genus *Saccharomyces*. *Int J Syst Bacteriol* **44**, 791–797.
- de Barros Lopes, M. A., Soden, A., Henschke, P. A. & Langridge, P. (1996). PCR differentiation of commercial yeast strains using intron splice site primers. *Appl Environ Microbiol* **62**, 4514–4520.
- Dobzhansky, T. (1937). Genetics and the origin of species. In *Columbia Biological Series*, pp. 364. Edited by L. C. Dunn. New York: Columbia University Press.
- Fleet, G. H., Lafon-Lafourcade, S. & Ribéreau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl Environ Microbiol* **48**, 1034–1038.
- Guillamón, J. M., Barrio, E., Huerta, T. & Querol, A. (1994). Rapid characterization of four species of the *Saccharomyces* sensu stricto complex according to mitochondrial DNA patterns. *Int J Syst Bacteriol* **44**, 708–714.
- Heard, G. M. (1988). *Occurrence and growth of yeasts during the fermentation of Australian wines*. PhD thesis, The University of New South Wales.
- Hoeben, P. & Clark-Walker, G. D. (1986). An approach to yeast classification by mapping mitochondrial DNA from *Dekkera*/*Brettanomyces* and *Eeniella* genera. *Curr Genet* **10**, 371–379.
- Hoeben, P., Weiller, G. & Clark-Walker, G. D. (1993). Larger rearranged mitochondrial genomes in *Dekkera*/*Brettanomyces* yeasts are more closely related than smaller genomes with a conserved gene order. *J Mol Evol* **36**, 263–269.
- Jacob, M. & Gallinaro, H. (1989). The 5' splice site: phylogenetic evolution and variable geometry of association with U1RNA. *Nucleic Acids Res* **17**, 2159–2180.
- Kreger-van Rij, N. (1984). *The Yeasts, a Taxonomic Study*, 3rd edn. Amsterdam: Elsevier Biomedical Press.
- Kurtzman, C. P. (1992). rRNA sequence comparisons for assessing phylogenetic relationships among yeasts. *Int J Syst Bacteriol* **42**, 1–6.
- Kurtzman, C. P., Smiley, M. J. & Johnson, C. J. (1980). Emendation of the genus *Issatchenkia* Kudriavzev and comparison of species by deoxyribonucleic acid reassociation, mating reaction, and ascospore ultrastructure. *Int J Syst Bacteriol* **30**, 503–513.
- Lewicka, K., Mallie, M. & Bastide, J.-M. (1995). Genetic variability in the *Saccharomyces* sensu stricto complex revealed by multi-locus enzyme electrophoresis. *Int J Syst Bacteriol* **45**, 538–543.
- Naumov, G. I. (1987). Genetic basis for classification and identification of the ascomycetous yeasts. *Stud Mycol* **30**, 469–475.
- Naumov, G. I., Naumova, E. S., Lantto, R. A., Louis, E. J. & Korhola, M. (1992). Genetic homology between *Saccharomyces cerevisiae* and its sibling species *S. paradoxus* and *S. bayanus*: electrophoretic karyotypes. *Yeast* **8**, 599–612.
- Noronha-da-Costa, P., Rodrigues, C., Spencer-Martins, I. & Loureiro, V. (1996). Fatty acid patterns of film-forming yeasts and new evidence for the heterogeneity of *Pichia membrani-faciens*. *Lett Appl Microbiol* **23**, 79–84.
- Palumbi, S. R. & Baker, C. S. (1994). Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Mol Biol Evol* **11**, 426–435.
- Petering, J., Langridge, P. & Henschke, P. (1990). Identification of yeast strains by molecular biology techniques. In *Ninth International Oenological Symposium, Cascais, Portugal*, pp. 178–199. Breisach, Germany: International Association for Modern Winery Technology and Management.
- Peterson, S. W. & Kurtzman, C. P. (1991). Ribosomal RNA sequence divergence among sibling species of yeasts. *Syst Appl Microbiol* **14**, 124–129.
- Price, C. W., Fuson, G. B. & Phaff, H. J. (1978). Genome comparison in yeast systematics: delimitation of species within the genera *Schwanniomyces*, *Saccharomyces*, *Debaryomyces*, and *Pichia*. *Microbiol Rev* **42**, 161–193.
- Scheda, R. & Yarrow, D. (1966). The instability of physiological properties used as criteria in the taxonomy of yeasts. *Archiv Mikrobiol* **55**, 209–225.
- Swofford, D. L. (1993). PAUP: phylogenetic analysis using parsimony, version 3.1. In *Illinois Natural History Survey*. Champaign: Illinois Natural History Survey.
- Vaughan Martini, A. (1989). *Saccharomyces paradoxus* comb. nov., a newly separated species of the *Saccharomyces* sensu stricto complex based upon nDNA/nDNA homologies. *Syst Appl Microbiol* **12**, 179–182.
- Vaughan Martini, A. & Kurtzman, C. P. (1985). Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces* sensu stricto. *Int J Syst Bacteriol* **35**, 508–511.
- van der Walt, J. P. (1987). The typological yeast species, and its delimitation. In *Biology of Yeasts*, 2nd edn, pp. 95–121. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.
- Winge, Ö. & Roberts, C. (1949). Inheritance of enzymatic characters in yeasts, and the phenomenon of long-term adaptation. *C R Trav Lab Carlsberg Ser Physiol* **24**, 263–315.
- Woolford, J. L. J. (1989). Nuclear pre-mRNA splicing in yeast. *Yeast* **5**, 439–457.
- Yamada, Y., Higashi, T. & Mikata, K. (1996). Should *Petasospora* Boidin et Abadie (Saccharomycetaceae) be retained? – the phylogeny based on the partial sequences of 18S and 26S ribosomal RNAs. *Biosci Biotechnol Biochem* **60**, 818–822.
- Zhang, M. Q. & Marr, T. G. (1994). Fission yeast gene structure and recognition. *Nucleic Acids Res* **22**, 1750–1759.