Identification of *Brettanomyces*/*Dekkera* Species Based on Polymorphism in the rRNA Internal Transcribed Spacer Region

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Brettanomyces/*Dekkera* yeasts grow in wine mainly during barrel aging. Their presence is often associated with formation of off-flavors. This potential spoilage generates a strong demand for a sensitive, rapid, and reliable identification procedure. Ribosomal DNA restriction fragment length polymorphism and comparative sequence analysis of the two internal transcribed spacer (ITS) regions located between the ribosomal RNA genes was carried out using *Brettanomyces*/*Dekkera* yeast reference strains and wine isolates. ITS1 and ITS2 were found to contain distinct regions with sufficient sequence divergence to make them suitable as specific identification target sites. Specific oligonucleotides were designed for each *Brettanomyces*/*Dekkera* species and evaluated for specificity and reliability. No cross-reaction products were detected when the specific primers were assayed in a PCR reaction with *Brettanomyces*/*Dekkera* strains of different species or other wine-related non-*Brettanomyces*/*Dekkera* yeasts. Thus, PCR using a combination of all four specific primers gave a specific and reproducible detection assay for the genus *Brettanomyces*/*Dekkera*. Use of these specific primers allowed for species-specific discrimination. *Brettanomyces*/*Dekkera* yeast isolates from wine were shown to uniquely belong to the species *B. bruxellensis*.

Key words: Brettanomyces/Dekkera spp., yeast, ribosomal RNA, internal transcribed spacer, enology

Brettanomyces/*Dekkera* yeasts are reported to be involved in spoilage by off-flavor production in beer and wine [1, 10,11,13,22,32]. In wine, these yeasts typically grow in low cell numbers after completion of the alcoholic and malolactic fermentation during the aging of wine in barrels, tanks, and bottles. The aroma characteristics of their spoilage-causing metabolites were described as burnt plastic, smoky, barnyard, horse sweat, leather, wet wool, and mousy [7,10,14,17,18,20,23]. Because of the high spoilage potential by *Brettanomyces*/*Dekkera,* there is a demand for a fast and reproducible monitoring method. The earlier their detection in wine, the better the chances for winemakers to prevent further growth and spread of these yeast to other batches. Physiological tests have been shown to fail not only because of the extensive time required for identification but also because they often generate ambiguous or even incorrect results [26].

Brettanomyces, the anamorph form of the genus *Dekkera*, includes five species. *B. nanus* was added to the four recognized species *B. bruxellensis*, *B. anomalus*, *B. custersianus*, and *B. naardenensis* following the renaming of *Eeniella nana* on the basis of rDNA sequence homology [1,6,19,35]. Molecular studies of species within this genus were initiated based on the mitochondrial genome [19] and the rRNA genes [27]. Molina et al. [27] carried out restriction analysis of the 18S rRNA from various *Brettanomyces* yeasts and found good correlation with isoenzyme electrophoresis and DNA homology analysis. Further investigation of the ribosomal RNA genes on the partial sequence level of the 18S and 26S rRNA was done by Yamada et al. [34] and Cai et al. [9], who determined the sequence of various *Brettanomyces* and *Dekkera* yeast strains by PCR and direct sequencing. Whereas these studies used molecular techniques to determine the phylogenetic relationship among these *Brettanomyces* yeast strains, other studies have applied molecular techniques for identification purposes. Ibeas et al. (20] used nested PCR to track one single strain in sherry wine. Mitrakul et al. [26] used RAPD-PCR to discriminate yeasts within the species *B. bruxellensis* [26]. Although strain discrimination worked well by RAPD-PCR in the latter study, results can be difficult to reproduce with the RAPD-PCR method. In addition, RAPD data often cannot be interpreted in terms of relatedness among strains, and no sequence data from RAPDs of *Brettanomyces* spp. is available in databases. Alternatively, ribosomal genes have been used extensively to give information about the phylogenetic relationship of taxa. As in many eukaryotes, ribosomal genes in yeast are organized as 18S-5.8S-26S operons, which are tandemly repeated 50 to 200 times per haploid genome [30]. The two internal transcribed spacers (ITS1 and ITS2) separate the conserved 18S and the 26S from the 5.8S rRNA gene. ITS regions are less conserved due to less evolutionary constraints [28], and therefore can be used to discriminate species within some genera.

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Our goal in this study was to develop a specific and reproducible identification method for the four yeast species, *B. bruxellensis*, *B. anomalus*, *B. custersianus*, and *B. naardenensis*, to provide an essential tool for the study of their role in winemaking. We focused on the ITS regions of 19 *Brettanomyces* strains from the four species by initially comparing their lengths and patterns based on rDNA restriction fragment length polymorphism (RFLP) after digestion with 16 endonucleases. The sequences of ITS1 and ITS2, including the 5.8S rRNA gene, were subsequently determined and compared. Oligonucleotides were designed for each species based on the polymorphic portions of the amplified fragments and found to be useful for identifying *Brettanomyces* strains to each of the four species. Control experiments did not show any cross-reaction with other yeasts often present in wine, making this method useful for identification of *Brettanomyces* species among wine isolates.

Material and Methods

Yeast strains. *Brettanomyces* yeast type strains from the four species *B. bruxellensis*, *B. anomalus*, *B. custersianus*, and *B. naardenensis* were used in this study (Table 1). The *Brettanomyces* type strain selection reflects the situation in the type culture collections with the most strains deposited for *B. bruxellensis* and the least for *B. custersianus* and *B. naardenensis* [1]. In addition to *Brettanomyces* yeast strains, other yeast species commonly occurring in wine were included in the experiments (Table 1).

PCR amplification and ITS RFLP. Cell lysates were generated as described by Schütz and Gafner [31]. An estimate of the DNA concentration was conducted using the spectrophotometric method described by Maniatis et al. [25]. The lysates were diluted to 10 ng/µl and stored at -20°C. DNA concentration in the reactions was 1 ng/µl and the conditions were 1xTaq Polymerase buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 µM dNTPs, and 1 µM of each primer. The following were the reaction parameters on the Robocycler 40 (Stratagene, La Jolla, CA): 1 cycle, 94°C for 1 min; 30 cycles, 94°C for 1 min, 50 to 65°C (depending on the primer) for 2 min, 72°C for 1 min; and 1 cycle, 72°C for 5 min. The oligonucleotides pITS1 and pITS4 [33] were used to amplify the internal transcribed spacer regions, including the 5.8S gene of the rDNA repeats. The primers derived from the sequences (Figure 1) were pB1 (5'-GTGGA TAAGCAAGGATAAAAATAC-3') and pB2 (5'-AGAGTG AGGGGATAATGATTTAAGG-3') specific for *B. bruxellensis*, pA1: TATAGGGAGAAATCCATATAAAAC for *B. anomalus*, pC1 (5'-CATTAGCATACAAACACAAC AAACC-3') for *B. custersianus*, and pN1 (5'-CGTTTC ATTTTTGTGACGTCCC-3') for *B. naardenensis*.

Separation of the PCR fragments was performed on a 3% (w/v) NuSieve® agarose 3:1 gel (FMC, Philadelphia, PA), using 1xTAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8) containing 200 ng/mL ethidium bromide. A 100-bp ladder (Promega, Madison, WI) was run alongside the samples as a molecular weight marker. DNA was visualized by UV transillumination and processed using the Gel Doc 1000 Video Gel Documentation System (Bio-Rad, Hercules, CA). PCR products were purified with the QiaQuick PCR Purification kit (Qiagen, Chatsworth, CA) and subsequently digested with the 16 endonucleases: *Alu* I, *Bfa* I, *Bst*N I, *Bst*U I, *Cfo* I, *Dde* I, *Hae* III, *Hin*f I, *Hpa* II, *Hsp*92 II, *Mbo* I, *Rsa* I, *Sau*3A I, *Scr*F I, *Taq* I, and *Tru*9 I. For the purpose of simplified presentation, a matrix of Jaccard similarity coefficients was computed using Genstat (Genstat 5 Release 3.2; NAG Inc., Downers Grove, IL). Clusters were derived from the similarity matrix using the average-linkage hierarchical method. Comparison with sequence data was performed manually from the printout.

Table 1 Yeast strains used in this study. (FAW, Forschungsanstalt für Obst-, Wein- und Gartenbau, Wädenswil, Switzerland; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; UCD, University of California, Davis, USA; CA, Cornell University, NYSAES, Geneva, NY.)

Strain or species name	Synonym	Strain number
Saccharomyces cerevisiae		FAW HK 8
Cryptococcus curvatus		FAW 1
Rhodotorula glutinis		FAW 9
Torulaspora delbrueckii		FAW 6
Hanseniaspora uvarum		FAW HS 94
Candida guillermondii		FAW2
Pichia kluyveri		FAW 6
Pichia anomala		FAW 10
Dekkera bruxellensis		CBS 72[b]
	Brettanomyces abstinens	CBS 6055[b]
	Dekkera bruxellensis	CBS 74[b]
	Dekkera bruxellensis	FAW 13[a]
	Brettanomyces custersii	CBS 5512[b]
	Brettanomyces intermedius	CBS 73[b]
	Dekkera intermedia	CBS 4914[b]
	Dekkera intermedia	UCD 605[a]
	Brettanomyces lambicus	CBS 75[b]
	Brettanomyces sp.	UCD 615[c]
	Brettanomyces sp.	CA 89[a]
	Brettanomyces sp.	CA 92[a]
	Brettanomyces sp.	CA 94[a]
	Brettanomyces sp.	CA 95[a]
Dekkera anomala		CBS 8139[b]
	Dekkera claussenii	CBS 76[b]
	Brettanomyces anomalus	CBS 77[b]
Dekkera naardenensis		CBS 6042[b]
Dekkera custersiana		CBS 4805[b]

[a]Wine isolates, identified as *Dekkera bruxellensis* in this study. *Brettanomyces*/*Dekkera* yeast strains from wine samples were isolated by wine-filtration and yeast plating on *Brettanomyces*/*Dekkera* selective medium (36). The medium was modified by decreasing the cycloheximide concentration from 50 to 10 mg/L for the purpose of being less selective for less resistant strains.

[b]Type strains.

Sequencing and alignment of sequence data. DNA sequencing was performed using the ABI Prism 373A Stretch automated DNA sequencer (Applied Biosystems, Foster City, CA), which uses cycle sequencing, dye terminator chemistry, and AmpliTaq-FS DNA polymerase. Editing and comparison of both DNA strands were carried out in the program SeqEd v 1.0.3 by PE Applied Biosystems. DNA alignment based on the sequence as shown in Figure 1 was performed by the program

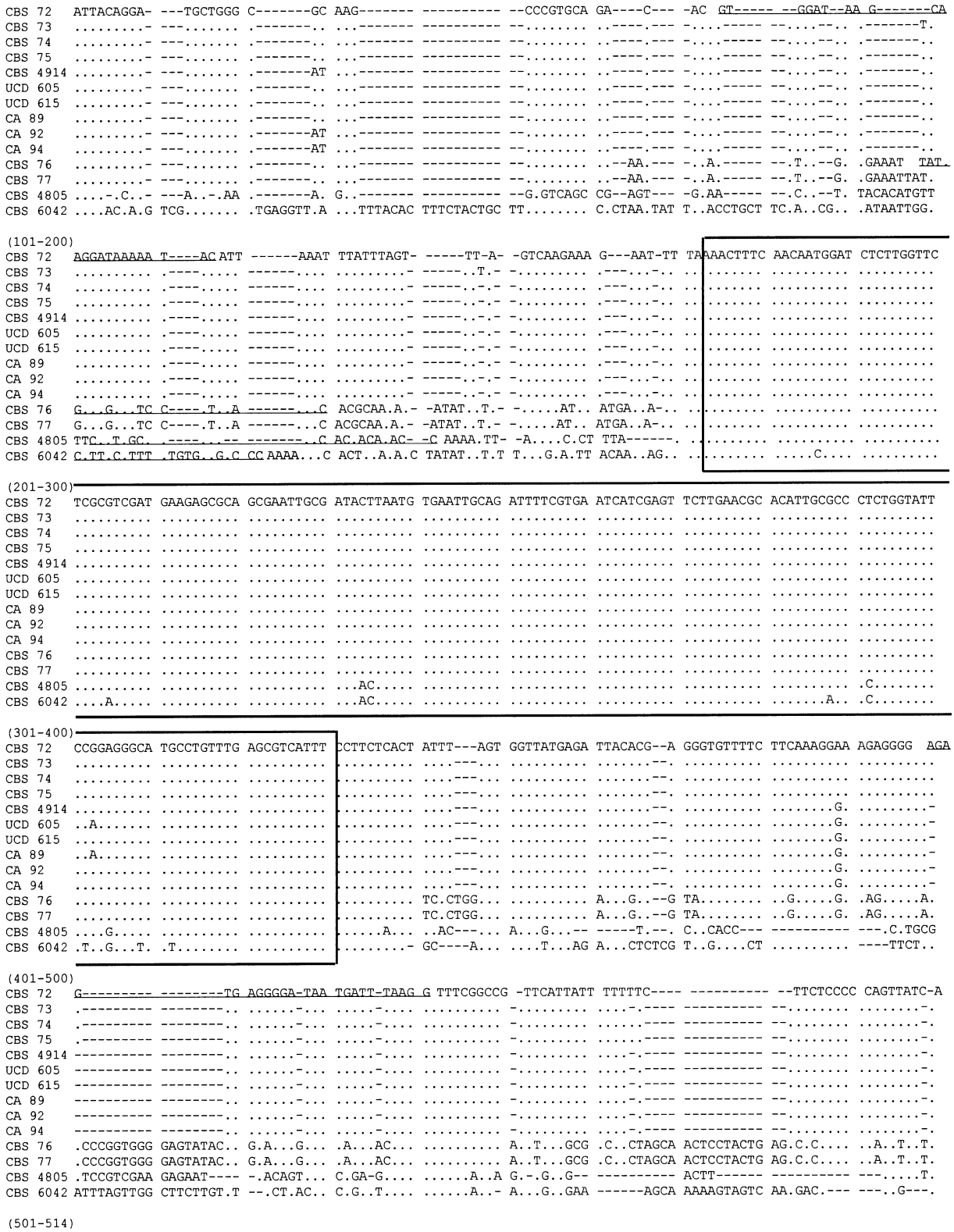

Figure 1 Comparison of the sequence of the ITS regions including the 5.8S rDNA and partial sequences of 18S and 26S rDNA from *Brettanomyces/Dekkera* strains. Sequences were aligned using the program MEGALIGN from the DNASTAR software package. The sequence of *B. bruxellensis* CBS 72 was used as reference. Dots indicate identical nucleotides as in the reference sequence; dashes indicate deletions. The 5.8S rDNA stretches from position 172 to 330, 18S rDNA ends at position 4, and 26S rDNA starts at position 504. Numbers in parentheses indicate nucleotide positions in alignment and fragment lengths. Primer binding sites are underlined; pB1 (from position 74 to 117) and pB2 (from position 398 to 441) in the line CBS 72, pA1 (from position 97 to 130) in the line CBS 76, pC1 (from position 103 to 142) in the line CBS 4805, and pN1 (from position 101 to 122) in the line CBS 6042.

MEGALIGN from the DNASTAR package (www.DNASTAR.com) and formatted with the fseq utility [4].

Nucleotide sequence accession numbers. The 5.8S rRNA gene sequences, including partial sequences from ITS1 and ITS2, which were determined from the various *Brettanomyces* yeast strains, have been deposited in the National Center for Biotechnology Information (NCBI) GenBank data library under the accession numbers AF043499 to AF043512.

Results

Polymorphism within the ITS elements of the rRNA genes. A region spanning the internal transcribed spacers ITS1 and ITS2 and including the 5.8S rDNA was amplified from the selected *Brettanomyces* and non-*Brettanomyces* yeasts (Table 1) using the primers pITS1 and pITS4]33]. Size of the generated fragments was between 450 and 550 bp for all *Brettanomyces* yeast strains (Figure 2A), whereas size of the seven common wine yeasts *Saccharomyces cerevisiae*, *Cryptococcus curvatus*, *Rhodotorula glutinis*, *Torulaspora delbrueckii*, *Hanseniaspora uvarum*, *Candida guillermondii*, and *Pichia anomala* varied between 600 and 900 bp (Figure 2B; lanes 1-6, 8). An exception was the fragment of *Pichia kluyveri*, which was in the same size range as those of the tested *Brettanomyces* yeasts. Sizes were the same for each of the seven *B. bruxellensis* strains and the three *B. anomalus* strains, but differed among the four species (Figure 2A, lanes 1-12). *B. custersianus* yielded a fragment size close to 500 bp, as did *B. bruxellensis*. The fragment length of *B. naardenensis* was approximately 100 bp larger and resembled *B. anomalus*. Fragments from all *Brettanomyces* yeast strains isolated from wines originating from different regions worldwide were similar in size compared to the species *D. bruxellensis* and *D. custersiana.*

In order to further investigate the polymorphic value of these fragments and to specify unequivocally the species of the *Brettanomyces* wine isolates, RFLP was conducted based on the ITS elements including the 5.8S rDNA gene. Fragments from *B. bruxellensis* strains were digested with 16 different restriction enzymes. The nine enzymes *Alu* I, *Taq* I, *Tru*9 I, *Bst*N I, *Scr*F I, *Dde* I, *Hin*f I, *Hsp*92 II, and *Sau*3A I were noninformative, yielding identical patterns for all strains. The seven enzymes *Cfo* I, *Bfa* I, *Mbo* I, *Hpa* II, *Hae* III, *Rsa* I, and *Bst*U I provided distinctive restriction profiles. These enzymes were then screened across the whole selection of *Brettanomyces* yeasts. The restriction digest using *Cfo* I is shown in Figure 3A. Polymorphic variation allowed for classing strains within one of the four species (Figure 3B). Whereas differentiation of four out of the seven different CBS *B. bruxellensis* strains was possible, the three tested *B. anomalus* strains shared the same unique pattern and therefore could not be discriminated (Figure 3A). The fragments were sequenced by direct PCR sequencing and aligned (Figure 1). With the exception of a few nucleotide positions, the 5.8S rRNA genes were identical for all tested yeast strains. ITS regions, however, were polymorphic among the four different species. Among strains within a species, the two *B. anomalus* strains CBS 76 and CBS 77 were identical at all 452 nucleotides. The ten *B. bruxellensis* strains differed by less than six of the 392 and 395 bp (Figure 1). Five of 19 strains—the two *B. bruxellensis* strains CBS 5512 and CBS 6055, the *B. anomala* strain CBS 8139, and the *Brettanomyces* yeast isolates FAW 13 and CA 95 from wine—could not be sequenced directly, which will be discussed.

Specific primers for species-specific discrimination of *Brettanomyces* yeasts. Specific primers were designed for each of the four *Brettanomyces* species based on the sequence data and tested by PCR using pITS 4 as reverse primer. The four specific primers, pB1, pA1, pC1, and pN1, were tested with all the *Brettanomyces* type strains and wine isolates. PCR products were only generated with the strains from the corresponding *Brettanomyces* species. The two exceptions were the *Brettanomyces abstinens* type strain CBS 5512 and the *Dekkera bruxellensis* wine isolate FAW 13 [15]. Both did not yield a PCR product when primer pB1 was used. Primer pB2 was therefore designed on the basis of the polymorphic sequence within ITS2 and tested with all the listed *Brettanomyces* yeasts. A PCR product was generated for every strain of *B. bruxellensis*, but for none of the other strains (data not shown), indicating that primer pB2 was suitable for *B. bruxellensis* identification. To reduce the number of test tubes and to facilitate the identification procedure with an unknown *Brettanomyces* strain, all four species-specific primers were mixed in equal amounts and used in combination with pITS4 in a PCR reaction. Figure 4A shows the reaction products of PCR reaction with these primers for each species ("B" lanes). As positive control, reactions were performed using pITS1 and pITS4 ("A" lanes). To discriminate the genus *Brettanomyces*

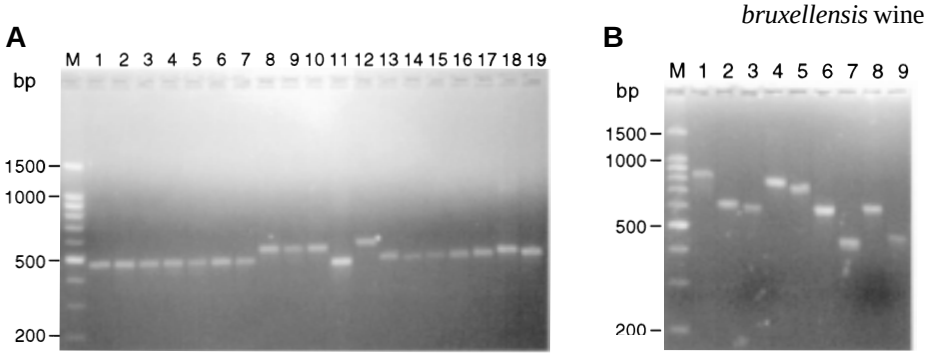

Figure 2 Size differences of the ITS regions including the 5.8S DNA of *Brettanomyces* yeasts. PCR was performed with primers pITS1 and pITS4. Fragments were separated by electrophoresis on a 3% NuSieve agarose 3:1 gel in 1 X TAE buffer. (**A**) *Brettanomyces*/*Dekkera* yeasts (by lane number): 1, CBS 72 (*Dekkera bruxellensis*); 2: CBS 73 (*Brettanomyces intermedius)*; 3, CBS 74 (*Dekkera bruxellensis)*; 4, CBS 75 (*Brettanomyces lambicus*); 5, CBS 4914 (*Dekkera intermedia*); 6, CBS 5512 (*Brettanomyces custersii*); 7, CBS 6055 (*Brettanomyces abstinens*); 8, CBS 76 (*Dekkera claussenii*); 9, CBS 77 (*Brettanomyces anomalus*); 10, CBS 8139 (*Dekkera anomala*); 11, CBS 4805 (*Dekkera custersiana*); 12, CBS 6042 (*Dekkera naardenensis*); and 13 to 19, *Brettanomyces*/*Dekkera* isolates from wines, referred to as FAW 13, UCD 605, UCD 615, CA 89, CA 92, CA 94, and CA 95. (**B**) Other wine yeast isolates (by lane number): 1, *Saccharomyces cerevisiae*; 2, *Cryptococcus curvatus*; 3, *Rhodotorula glutinis*; 4, *Torulaspora delbrueckii*; 5, *Hanseniaspora uvarum*; 6, *Candida guillermondii*; 7, *Pichia kluyveri*; 8, *Pichia anomala*; 9, *Dekkera bruxellensis.* Lane M in both **A** and **B**, 100-bp ladder molecular weight marker (Promega).

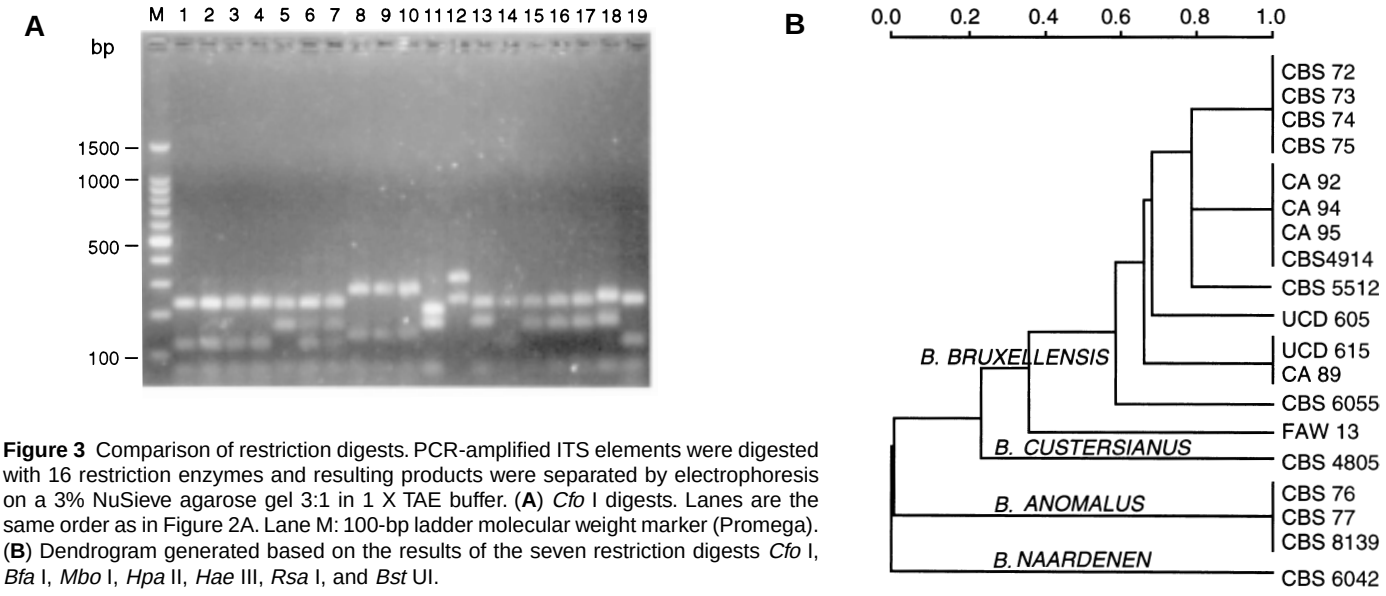

Figure 3 Comparison of restriction digests. PCR-amplified ITS elements were digested with 16 restriction enzymes and resulting products were separated by electrophoresis on a 3% NuSieve agarose gel 3:1 in 1 X TAE buffer. (**A**) *Cfo* I digests. Lanes are the same order as in Figure 2A. Lane M: 100-bp ladder molecular weight marker (Promega). (**B**) Dendrogram generated based on the results of the seven restriction digests *Cfo* I, *Bfa* I, *Mbo* I, *Hpa* II, *Hae* III, *Rsa* I, and *Bst* UI.

from other yeasts present in wine, the *Brettanomyces*-specific primer mix was assayed with the non-*Brettanomyces* wine yeasts (Figure 4B). Specific fragments were generated using pITS1 and pITS4 (Figure 4B, "A" lanes); however, no reaction products were observed when pITS1 was replaced with the mix of the four species-specific primers ("B" lanes).

Discussion and Conclusions

Two studies have described detection and discrimination of *Brettanomyces* yeasts. Ibeas et al. [20] identified one strain in sherry wine by nested PCR. RAPD PCR was adapted and applied to *Brettanomyces* species and *B. bruxellensis* strain identification by Mitrakul et al. [26]. The inherent disadvantage of RAPD PCR is the reproducibility of PCR product generation. Therefore, the discriminative potential of the ITS regions located between the rRNA genes was used in this study. The tandemly repeated arrays of rRNA genes are present in fungal nuclear genomes up to 200 times [3]. The nuclear small and large subunit rRNA genes have been successfully used for yeast identification purposes [21]. Whereas conserved portions of rRNA genes have been used for comparison of different fungal genera, sequence variation within ITS has been used to investigate species boundaries [8,16,24]. As expected, near identity was found in the 5.8S rRNA genes. The degree in ITS sequence variation enabled interspecific discrimination between the four *Brettanomyces* species. The fact that we did not find any reaction product with non-*Brettanomyces* yeasts using the specific primers confirms earlier data that ITS are highly variable among yeast genera [8,16,24].

Direct sequencing was successful for most *Brettanomyces* yeast strains but failed for the strains CBS 5512, CBS 6055, CBS 8139, CA95, and FAW 13. These results raise the issue whether all ITS sequences within *Brettanomyces* yeasts are completely uniform possibly due to nucleotide deletion, insertion, or major modifications within these regions. This counters the hypothesis that rDNA repeat units behave like single-copy genes [2,3,12]. The finding that primer pB1 was unable to act as a

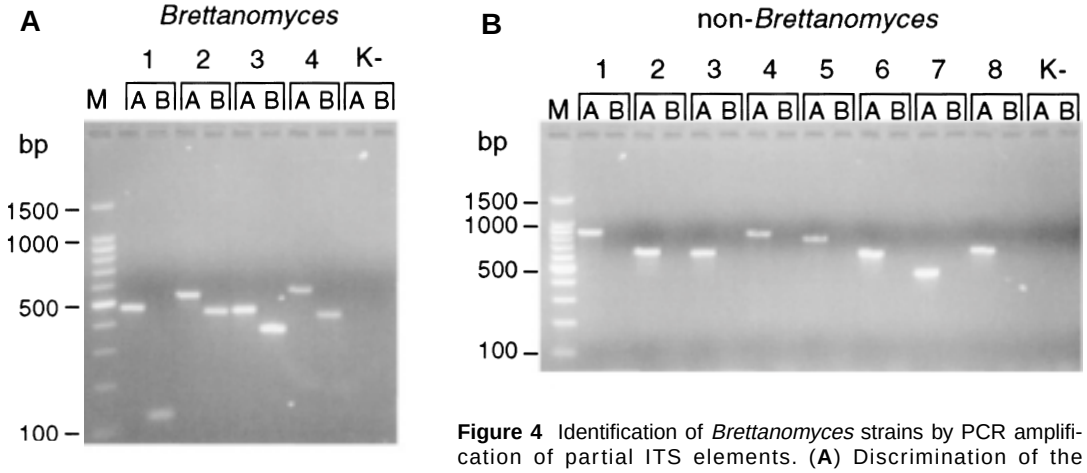

Figure 4 Identification of *Brettanomyces* strains by PCR amplification of partial ITS elements. (**A**) Discrimination of the *Brettanomyces* species (by lane number): 1, CBS 72 (*Dekkera bruxellensis*); 2, CBS76 (*Dekkera anomala*); 3, CBS 4805 (*Dekkera custersiana*); 4, CBS 6042 (*Dekkera naardenensis*). PCR reactions in lanes marked A contained the primer pair pITS1/pITS4, reactions in lanes marked B included the primer mix pB2, pA1, pC1, pN1)/pITS4 in equimolar amounts. (**B**) Proof of specificity for the *Brettanomyces*-primers came from testing them on the most abundant non-*Brettanomyces* yeasts in wine in the same reactions as described in (A): 1, *S. cerevisiae*; 2, *Cryptococcus curvatus*; 3, *Rhodotorula glutinis*; 4, *Torulaspora delbrueckii*; 5, *Hanseniaspora uvarum*; 6, *Candida guillermondii*; 7, *Pichia kluyveri*; and 8, *Pichia anomala*. No DNA template was added in the negative control reactions (K-). Lane M: 100 bp-ladder molecular weight marker (Promega).

specific primer for the two *B. bruxellensis* strains CBS 5512 and FAW 13 contrasts with the conserved ITS sequences. Cloning of the fragments prior to sequencing is necessary to investigate this further. For our purpose, primer pB2 showed specific binding to all *B. bruxellensis* strains indicating that this priming region within the ITS2 is conserved for *Brettanomyces* yeasts.

All tested *Brettanomyces* wine isolates belong to *B. bruxellensis,* as was earlier found by Mitrakul et al. for wine isolates [26]. The fact that different *Brettanomyces* spp. produce different off-flavors in wine [23] makes specific detection and identification assays necessary. Since the ITS-specific primers do not appear to be useful for strain identification, the RAPD PCR method described by Mitrakul et al. [26] represents the only method so far for this purpose. For many wineries, a reliable *Brettanomyces* identification assay is sufficient in order to detect the presence of these yeasts. In this respect, the *Brettanomyces* identification method presented here provides a fast and reliable way to identify *Brettanomyces* yeasts by assembling the four specific primers. Consequently, a promising approach would be a modified colony hybridization assay as described by Peterkin et al. [29] for *Listeria* spp., in which the primers described above would serve as specific probes and in which any volume of wine could be filtered.

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