Evaluation of ITS PCR and RFLP for Differentiation and Identification of Brewing Yeast and Brewery 'Wild' Yeast Contaminants

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ABSTRACT

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A reference library of ITS PCR/RFLP profiles was collated and augmented to evaluate its potential for routine identification of domestic brewing yeast and known 'wild' yeast contaminants associated with wort, beer and brewing processes. This library contains information on band sizes generated by restriction digestion of the ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer (ITS) region consisting of the 5.8 rRNA gene and two flanking regions (ITS1 and ITS2) with the endonucleases *Cfo*I, *Hae*III, *Hinf*I and includes strains from 39 non-*Saccharomyces* yeast species as well as for brewing and non-brewing strains of *Saccharomyces*. The efficacy of the technique was assessed by isolation of 59 wild yeasts from industrial fermentation vessels and conditioning tanks and by matching their ITS amplicon sizes and RFLP profiles with those of the constructed library. Five separate, non-introduced yeast taxa were putatively identified. These included *Pichia* species, which were associated with conditioning tanks and *Saccharomyces* species isolated from fermentation vessels. Strains of the lager yeast *S. pastorianus* could be reliably identified as belonging to either the Saaz or Frohberg hybrid group by restriction digestion of the ITS amplicon with the enzyme *Hae*III. Frohberg group strains could be further sub-grouped depending on restriction profiles generated with *Hinf*I.

Key words: brewery, contaminant, identification, ITS, rDNA, RFLP, yeast.

INTRODUCTION

The use of pure, uncontaminated cultures of specific strains of brewing yeast for fermentation has been a central tenet of the brewing industry for over a century[4]. Use of pure cultures minimizes the risk of microbial contamination and ensures consistent fermentation performance and product quality. The closed systems employed in modern breweries reduce the risk of contamination by 'wild' yeast, which may be considered to be any yeast occurring other than the specified production yeast. Wild yeasts can therefore be non-*Saccharomyces* yeast, *Saccharomyces* yeast species other than brewing production yeast, or even production yeast strains other than those intended for a specific fermentation. In other words, yeast not deliberately used and under full control[20]. Contamination, albeit at a low concentration and rarely influencing the fermentation process or product, is however normal[55] and is used a quality control indicator within modern breweries.

Beer spoilage due to the presence of wild yeast contaminants can take a number of forms and is influenced by the contaminant taxa[10,31]. Reported problems include the production of off-flavours, particularly phenolic off-flavours, which are formed by the carboxylation of ferulic and cinnamic acids[49], as well as the production of other off flavours such as acetic acid or esters. Competition for nutrients may also occur if wild yeast growth rates are sufficiently fast. An extreme case of competition involves the presence of so-called killer yeast, which can completely replace a brewing yeast through the production of killer factors or zymocins[61]. The presence of small or non-flocculating wild yeast cells can, in addition, result in unacceptably high levels of turbidity and difficulties with clarification[10].

The wild yeast isolated from raw materials, brewery facilities, malt extract (wort) in fermentation vessels or beer in collection vessels, kegs or bottles are rarely identified to species level and little published data is available regarding the dynamics of wild yeast contamination in the brewery, i.e. the relative abundance of different species at different stages in the production of beer. An improved understanding of wild yeast and their occurrence is necessary to inform decisions regarding the optimization of processes to eliminate unwanted yeast contaminants and thus prevent unnecessary beer spoilage.

Conventional methods used to detect and isolate wild yeast contaminants have mainly been based on morphological traits and especially their physiological abilities[5,30]. The most popular method for differentiation is cultivation of unknown yeasts on a wide range of selective media, e.g., lysine medium[57], actidione medium[23], crystal violet medium[1], MYGP+copper medium[2,46] and CLEN media[46]. Incubation at 37°C for detection of wild yeast in lager breweries has also been used[56]. Different organisms have different growth requirements with regard to nutrients,

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carbon sources and oxygen availability, thus selective media can aid in detection of contaminants. However, no single medium is capable of detecting or differentiating all wild yeast strains and in many cases, e.g. selection of non-brewing *Saccharomyces* yeast based on Cu-sensitivity, the mechanistic reasons for the differentiation are obscure. Breweries typically focus on detection and differentiation of brewing and non-brewing yeast rather than identification of contaminant yeast taxa. Traditional identification of wild yeasts to species level is a complex, laborious and time-consuming process which requires approximately 50–100 biochemical tests[5,30,34]. Final results are usually available several days or even weeks after beer packaging; therefore, these methods are often used as a retrospective view of quality rather than proactive process control[51].

Though not routinely used in breweries, molecular techniques for the identification of wild yeast contaminants have the advantage over traditional techniques in that they can often be used to accurately identify yeast isolates to species level. One such technique is ITS-PCR involving the amplification of the ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer (ITS) region consisting of the 5.8S rRNA gene and two variable flanking regions ITS1 and ITS2. Interspecific polymorphism in this region allows for differentiation of species[59]. Restriction fragment length polymorphism (RFLP) can be used in conjunction with ITS-PCR to differentiate closely-related species such as those of the *Saccharomyces* sensustricto complex[18].

PCR amplification of rDNA and restriction digestion of the amplicon has not been used extensively for the identification of yeasts isolated during brewing or from beer[27,53], but has seen wide application in other fermentation industries, particularly for the identification of yeasts occurring during oenological fermentations[13,14,17,18,21,22,36,43,44,48,62]. The technique has also been used to identify yeasts occurring as contaminants or natural, spontaneous fermenters of cider[15,39] and rice wine[28] as well as yeast associated with non-alcoholic citrus juice beverages[3,32]. Despite its accuracy and relative ease of use, this technique has not been widely adopted for routine identification of isolated wild yeast in breweries. One possible reason for this is that there is currently no comprehensive reference library of ITS-PCR fragment sizes and RFLP band sizes for yeast associated with brewery fermentation and beer.

The aims of this study were, firstly, the collation and augmentation of rDNA-PCR (ITS1, 5.8S and ITS2) data and gel-detectable RFLP profiles for known brewery wild yeast contaminants, as well as common production yeast, for use as a reference tool and, secondly, to critically evaluate the potential of this reference library for identification of wild yeast contaminants isolated from industrial fermentation vessels and beer conditioning tanks.

MATERIALS AND METHODS

Yeast strains

Fifty-nine wild yeast isolates were obtained from wort and beer sampled from five different fermentation vessels and three different conditioning tanks used for the production of two different lager beers. All samples were obtained from one brewery and at the same time. Known yeast strains, including several type strains were also obtained from the National Collection of Yeast cultures (NCYC; Norwich, UK), Dipartimento Biologia Vegetale Perugia, Yeasts Industrial Collection (DBVPG; Perugia, Italy), European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF; University of Frankfurt, Frankfurt, Germany) and the VTT Culture Collection (VTT; Technical Research Centre of Finland, Espoo, Finland). Also included was the production lager strain CB11. Yeast strains were maintained on YPD agar (1% yeast extract, 2% neutralized bactopeptone, 2% dextrose and 1.2% agar) at 4°C.

Isolation of industrial wild yeast contaminants

Multiple wort samples (10 mL) and beer samples (100 mL) were filtered through Millipore® membrane (pore size of 0.45 μm) filters, which were transferred to MYGP+copper plates (0.3% malt extract, 0.3% yeast extract, 0.5% bactopeptone, 1% glucose, 1% agar technical and 100 ppm Cu added as copper sulphate). The high copper concentrations prevented growth of the production lager yeast strains and allowed single colony wild yeast strains to be isolated. These wild yeasts were taken from the original copper plates and subcultured on 100 and 200 ppm copper-supplemented Bacto™ agar (Becton, Dickinson and Company) to ensure that the production yeast strain was not present. All plates were incubated at 25°C for 48 h before subsequent subculturing on plates without additional copper.

DNA extraction, PCR and RFLP

Rapid DNA extraction was carried out by transferring a fresh yeast colony (approx. 2 days old) to a 50 μL yeast DNA extraction buffer (2×10^{-3} M NaOH and 0.001% sarcosine) and boiling for 10 min at 100°C. Sarcosine was included to permeabilise cell membranes. The supernatant containing DNA crude extract was collected by brief centrifugation and stored at –20°C. The PCR reactions were performed as described by White et al.[59] Briefly, 50 μL PCR reaction mixture contained 0.5 μM primer ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′), 0.5 μM primer ITS4 (5′ TCCTCCGCTTATTGATATGC 3′), 25 μL Master mix (Quick Load Taq 2× from New England Biolabs), 3 μL DNA crude extract and water. The PCR conditions were as follows: 95°C for 15 min (initial denaturing), 35 cycles of 95°C for 1 min (denaturing), 55°C for 2 min (annealing), 72°C for 2 min (elongation) followed by a final elongation step at 72°C for 10 min. A quantity of 6 μL of product from each reaction was separated on a 1.4% (w/v) agarose gel in TAE buffer and 8 μL of the PCR product was digested with *Cfo*I (Promega), *Hae*III or *Hinf*I endonucleases (New England Biolabs) and separated on a 4% agarose gel. Gels were stained with ethidium bromide, visualized under UV light and photographed. Band sizes were calculated with reference to a 100 bp ladder (New England Biolabs). Tentative identification of unknown brewery contaminant yeast was carried out by first comparing ITS amplicon sizes with those of known species, including type strains, to produce a short-list of candidate species and secondly by comparing restriction profiles produced using the enzymes *Cfo*I, *Hae*III and *Hinf*I.

Where available, ITS sequence data was obtained from databases such as MycoBank (http://www.mycobank.org/), Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and CBS-KNAW Fungal Biodiversity Centre database (http://www.cbs.knaw.nl/). Virtual restriction profiles of the full length sequences (ITS sequences containing sequences of both ITS1 and ITS4 oligonucleotides) were determined using DNAMAN v4.1 (Lynnon BioSoft) and Clone Manager 5 software (Scientific & Educational Software).

RESULTS

While numerous methods are available for the extraction of DNA from yeast cultures, the one used here was found to be particularly simple and rapid. Better results were obtained than with methods involving extraction with SDS or colony PCR (data not shown). However, any method that produces relatively pure DNA for PCR is sufficient.

Collation of the available data and grouping of yeasts according to ITS amplicon size in ascending order revealed that the many species and indeed genera can be separated according to amplicon size (Table I). Species of the genus *Candida*, with amplicon sizes ranging from 400–700 bp were found in all amplicon size categories. *Candida rugosa, C. pararugosa, C. intermedia, C. sake* and *C. stellata*, however, all had amplicon sizes of 500 bp or less. A characteristic of these species was the lack of restriction sites with the *Hae*III enzyme. Restriction with *Hinf*I resulted in production of double bands, with restriction sites in each case being close to the centre of the amplified region. The similar amplicon sizes and restriction profiles of species within the group prevent definitive identification of individual species based on ITS/RFLP, though smaller amplicon sizes within this group may suggest *C. rugosa* or *C. intermedia* rather than, for example, *C. stellata*. Predicted bands sizes, based on available sequence data, were similar to observed gel-detectable band sizes, with differences in restricted band sizes typically less than 20 bp (Table I) when different strains are compared. Other *Candida* species, including *C. tropicalis*, *C. norvegica* and *C. parapsilosis* had larger amplicon sizes (>500 bp) and while the data suggests that *C. tropicalis* and *C. parapsilosis* are not differentiable based on ITS amplicon size and restriction pattern, *C. norvegica* due to its larger size (580 bp) and unique banding pattern may be differentiated using this technique. Banding patterns for *C. tropicalis* and *parapsilosis* corresponded well to predicted band sizes (Table I). *Candida* species with amplicon sizes above 600 bp included *C. mesenterica*, *C. santamariae* (basionym *C. beechii)* and *C. tenuis*. The latter species were observed to have similar amplicon sizes and restriction profiles. The exceptions here were the profiles generated with *Hae*III and *Hinf*I for *C. tenuis* by Guillamon et al.[22], which deviated significantly from the predicted profile. *Candida mesenterica*, on the other hand could be distinguished due to its larger amplicon size (approx. 650 bp) and lack of restriction with *Cfo*I. Amplicon size and restriction profile with *Hae*III were similar to those of *Scheffersomyces stipitis* (basionym *Pichia stipitis*), emphasising the requirement for more than one restriction profile to be generated to reliably identify a species or genus. *Candida boidinii* can be distinguished from other members of the genus by its larger amplicon size (≥700 bp). Care should be taken however to avoid confusion of this strain with *Hanseniaspora uvarum* when basing identification on visible fragment sizes.

The *Dekkera* species, *D. bruxellensis* and *D. anomala*, while producing similar restriction profiles may be distinguished by the larger size of the *D. anomala* amplicon (>500 bp) compared to the *D. bruxellensis* amplicon (≤500 bp) and the tendency of *D. anomala* to generate a double band with the *Hinf*I enzyme. *Dekkera bruxellensis* can likewise be distinguished from *Pichia fermentans* based on the *Cfo*I restriction profile. Of note here is the discrepancy in the *D. anomala* results obtained by Granchi et al.[21] and the authors of this study who found amplicon sizes of 514 and 540 bp, respectively, compared with an amplicon size of 800 bp obtained by Esteve-Zarzoso et al.[17] and Morrissey et al.[39]

Of the *Pichia* species, those with the smallest ITS amplicon sizes were *P. fermentans, P. membranifaciens* and *P. kudriavzevii* (basionym *Issatchenkia orientalis*), with *P. fermentans* strains normally having a smaller size (~450 bp) than the other two species. Results suggest that *P. fermentans* and *P. membranifaciens* cannot be reliably differentiated based purely on observation of gel-detectable fragment sizes with the enzymes used here. However, it is possible to differentiate these species with *Xho*II and *Mae*II restriction enzymes according to *in silico* data: *P. fermentans* ITS amplicon is digested into 322 and 123 bp fragments with *Xho*II and undigested with *Mae*II, whereas *P. membranifaciens* ITS amplicon is digested into 175, 170, 105 and 29 bp fragments with *Xho*II and 260, 125 and 94 bp fragments with *Mae*II (data not shown). These two species could, in the majority of cases, be differentiated from *P. kudriavzevii* by the restriction profiles obtained with *Cfo*I and *Hae*III.

The two *Kazachstania* species included here[31], *K. exigua* and *K. unispora* could be distinguished from each other by the profiles generated with *Hae*III and *Hinf*I and could furthermore be differentiated from other species due to their large amplicon size and restriction profile with *Hae*III. In the same amplicon size class were the *Kluyveromyce*s species, which could not be distinguished from one another based on amplicon size or restriction profile, but could be distinguished from non-*Kluyveromyces* species as long as more than one restriction profile was available. The profile generated with *Cfo*I was, for example, similar to that obtained from *Zygosaccharomyces rouxii*. *Torulaspora delbrueckii* was another species with a large ITS amplicon (800 bp) and which generated numerous bands with *Cfo*I. A distinctive characteristic of this species was the lack of restriction sites for *Hae*III. This unrestricted DNA could be distinguished from that of *H. uvarum* (also without restriction sites) by size. According to *in silico* data, it is possible to use restriction enzymes other than those employed here to differentiate *Kluyveromyces* species. Namely, *Ava*I, *Hind*III and *Msl*I restriction enzymes do not cleave the *K. lactis* ITS amplicon, whereas the *K. marxianus* ITS amplicon is cleaved into two fragments, based on a partial ITS amplicon sequence available, having sizes of 458 and over 240 bp (*Ava*I), 573 and over 125 bp (*Hind*III) and 494 and over 204 bp (*Msl*I).

Table I. Sizes of amplified products of the 5.8S-ITS regions and restriction fragments from non-*Saccharomyces* species identified as brewery contaminants. Italicised sizes represent virtual fragment sizes determined from published sequences and known restriction sites using DNAMAN v4.1 and Clone Manager 5 software.

Species	Code	PCR sizes	Restriction fragments (bp)			References for identification
			*Cfo*I	*Hae*III	*Hinf*I	
PCR amplicon ≤500 bp						
Candida rugosa	CBS 613	399	*208, 191*	*399*	*179, 144, 68, 8*	40
C. rugosa	CECT 1447	420	195, 185	410	180, 150	22
C. rugosa	VTT C-02465	380	210, 170	380	200, 180	This study
C. pararugosa	ATCC 38774^T (CBS 1010)	415	*245, 170*	*415*	*218, 197*	40
C. pararugosa	VTT C-02469	450	270, 180	450	210, 190	This study
C. intermedia	CBS 5159	386	*212, 174*	*386*	*202, 184*	11
C. intermedia	CECT 10118, 11054, 11154	425	220, 190	410	225, 200	17
C. intermedia	VTT C-98309	400	240, 160	400	220, 180	This study
C. sake	CBS 159	441	*236, 205*	*441*	*226, 207, 8*	40
C. sake	CECT 1044	470	250, 210	445	230, 210	22
C. sake	CECT 10034, 10276, 1044	450	250, 200	450	230, 220	17
C. sake	VTT C-94208	450	250, 200	450	230, 220	This study
C. stellata	CBS 157^T	468	220, 130	468	245, 230	21
C. stellata	CECT 11406, 11109, 11110	475	215, 110, 80, 60	475	235, 235	17
C. stellata	CECT 11108, 11110	500	220, 130	480	260, 240	22
Dekkera bruxellensis	CBS 74^T (CECT 1451^T)	468	*231, 123, 70, 44*	*372, 96*	*260, 208*	11
D. bruxellensis	CBS 74^T	459	226, 121, 82	350, 95	261, 197	21
D. bruxellensis	CECT 1009, 1010, 1451^T, 1452, 11045	485	255, 140, 90	375, 95	270, 215	17
D. bruxellensis	CECT 1009^T (CBS 72)	500	230, 130, 80	375, 105	265, 105	22
D. bruxellensis	NCYC 2818	485	250, 140, 90	375, 95	270, 215	39
D. bruxellensis	VTT C-00348	490	240, 120, 80, 50	390, 100	220, 180, 90	This study
D. bruxellensis	CECT 1452^T (CBS 4914)	500	230, 130, 80	375, 105	265, 215	22
Pichia fermentans	CBS 187^T	445	*154, 92, 87, 69, 23, 16, 2, 2*	*331, 79, 35*	*251, 194*	19
P. fermentans	CECT 1455^T (CBS 187), 10064, 10078, 10413, 10454	450	170, 100, 100, 80	340, 80, 30	250, 200	17
P. fermentans	CECT 1455^T (CBS 187)	470	170, 110, 80	340, 85	260, 210	22
P. fermentans	CBS 187^T	449	149, 139, 91, 74	325, 80	246, 193	21
P. fermentans	NCYC 562	450	170, 100, 100, 80	340, 80	250, 200	39
P. membranifaciens	CBS 5516	479	*162, 101, 83, 69, 64*	*326, 88, 48, 12, 5*	*274, 205*	19
P. membranifaciens	CECT 1115, 10570, 10568	500	175, 110, 90, 75	330, 90, 50	275, 200	17
P. membranifaciens	CECT 10037, 10113	500	260, 110, 75	330, 90, 50	275, 200	17
P. membranifaciens	CECT 1115^T (CBS 107^T)	500	180, 120	340, 100	280, 220	22
P. membranifaciens	CBS 107^T	475	160, 110, 90, 80	320, 80, 50	275, 200	21
P. membranifaciens	VTT C-05624, C-86170	500	160, 110, 90, 80, 60	350, 100, 50	280, 220	This study
Yarrowia lipolytica	CECT 1240	380	210, 270	380	190, 190	17,**52**
Y. lipolytica	VTT C-02463, C-0038^T	380	210, 170	380	190, 110, 70	This study,
PCR amplicon 500–600 bp						
Candida norvegica	CECT 10310	580	510	370, 190	290, 260	22, 17
C. parapsilosis	CBS 604	520	*295, 225*	*401, 105, 14*	*266, 246, 8*	40
C. parapsilosis	CECT 10437	520	290, 220	410, 110	270, 250	22
C. parapsilosis	CECT 10434, 1449, 10437	550	300, 240	400, 115	290, 260	17
C. parapsilosis	VTT C-82056	450	260, 190	450	230, 220	This study
C. tropicalis	ATCC 750	525	*284, 241*	*444, 81*	*262, 255, 8*	40
C. tropicalis	CECT 1440	550	280, 250	450, 90	270, 270	17,22

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Table I. (continued from previous page)

Species	Code	PCR sizes	Restriction fragments (bp)			References for identification
			*Cfo*I	*Hae*III	*Hinf*I	
Dekkera anomala	VTT C-91183	540	270, 150, 70, 50	400, 140	270, 270	This study
D. anomala	Unknown	514	270, 145, 72	400, 110	225, 225, 81	21
Pichia kudriavzevii	CBS 5147^{T}	509	*206, 169, 52, 6*	*381, 90, 38*	*221, 151, 137*	11
P. kudriavzevii	CBS 5147^{T}	494	185, 170, 69, 56	370, 90	225, 160, 145	21
P. kudriavzevii	VTT C-89178	500	200, 180, 70, 50	360, 90, 50	220, 150, 130	This study
PCR amplicon 600–700 bp						
Candida mesenterica	CECT 1025	640	580	490, 140	305, 220	22
C. mesenterica	CECT 1025	650	600	500, 150	320, 220, 100	17
C. santamariae	CBS 4261	623	*290, 276, 47, 10*	*412, 132, 79*	*309, 306, 8*	40
C. santamariae	CECT 11165^{T}	650	300, 300	400, 140, 90	330, 330	17
C. tenuis	CBS 7047	629	*288, 279, 52, 10*	*412, 126, 78, 13*	*312, 309, 8*	40
C. tenuis	CECT 10188	680	310, 275	500, 150	305, 180, 140	22
Debaryomyces hansenii	CBS767^{T}	639	*295, 284, 50, 10*	*421, 138, 80*	*316, 315, 8*	40
D. hansenii	CBS767^{T}	656	295, 280	400, 120, 75	315, 315	21
D. hansenii	CECT 10286	650	300, 300, 50	420, 150, 90	325, 325	17
Filobasidium capsuligenum	CBS 4736	620	*324, 296*	*499, 67, 54*	*345, 267, 8*	11
F. capsuligenum	CECT 11190 (CBS 4736)	650	340, 300	550, 100	360, 280	17
Millerozyma farinosa	ATCC MYA-4447	669	*296, 295, 68, 10*	*416, 174, 79*	*344, 166, 151, 8*	19
M. farinosa	CECT 1447 (CBS 185), 10348	700	300, 300, 80	440, 175, 80	350, 185, 160	17
Meyerozyma guilliermondii	UAF-214	607	*293, 255, 49, 10*	*390, 116, 79, 17, 5*	*314, 285, 8*	19
M. guilliermondii	CECT 1019, 1021, 1438, 1456^{T} (CBS 2030^{T})	625	300, 265, 60	400, 110, 90	320, 300	17
M. guilliermondii	NCYC 443 (CBS 2030^{T})	625	300, 265, 60	400, 115, 90	320, 300	39
M. guilliermondii	CBS 2030^{T}	605	320, 270	380, 120, 80	340, 300	21
Rhodotorula mucilaginosa	CBS 316	616	*298, 222, 96*	*401, 215*	*339, 210, 59, 8*	11
R. mucilaginosa	CECT 11016 (CBS 316)	640	320, 240	425, 215	340, 225	22
R. mucilaginosa	VTT C-89179	600	300, 210	400, 200	360, 220	This study
R. mucilaginosa	CECT 11010	640	320, 240, 80	425, 215	340, 225, 75	22, 17
Scheffersomyces stipitis	CBS 6054	628	*297, 273, 48, 10*	*493, 135*	*313, 307, 8*	
S. stipitis	CECT 1922^{T} (CBS 5773)	650	300, 285	490, 140	310, 310	17
S. stipitis	NCYC 1540, 1542	650	300, 280	500, 140	325, 325	This study
Schwanniomyces occidentalis	VTT C-02471	600	280, 280, 40	400, 170, 130	320, 130, 100	This study
Wickerhamomyces anomalus	MUCL 51252	618	*559, 49, 10*	*618*	*310, 300, 8*	19
W. anomalus	CECT 1110,1114,10320, 10410,10590,10591,10667	650	575	600, 50	310, 310	17, 39
W. anomalus	NCYC 750	650	575	600, 50	310, 310	39
W. anomalus	VTT C-74201^{T}, C94191	650	570	650	310, 310	This study
W. anomalus	CBS 5759^{T}	615	615	615	310, 290	21
W. subpelliculosus	VTT C-82050	600	560, 40	600	300, 300	This study

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Table I. (continued from previous page)

Species	Code	PCR sizes	Restriction fragments (bp)			References for identification
			*Cfo*I	*Hae*III	*Hinf*I	
PCR amplicon ≥700 bp						
Candida boidinii	CBS 2428	704	*321, 290, 73, 20*	*704*	*352, 136, 33, 8*	11
C. boidinii	CECT 1014^{T}	700	330, 330	700	370, 180, 145	22
C. boidinii	CECT 1029	700	330, 295	700	370, 180, 145	22
C. boidinii	CECT 1014^{T}, 11153	750	350, 310, 90	700	390, 190, 160	17
Hanseniaspora uvarum	KW 2662	747	*319, 313, 105, 10*	*747*	*342, 188, 155, 62*	19
H. uvarum	CBS 314^{T}	745	324, 315, 105	745	330, 180, 150, 70	21
H. uvarum	CECT 1444, 11105, 11106, 11107, CECT 1120^{T}	760	320, 315, 105	760	360, 200, 180	22
H. uvarum	NCYC 2739	750	320, 310, 105	750	350, 200, 180	39
H. uvarum	CECT 1444, 10389, 11105, 11106, 11107, 11156, 11172	750	320, 310, 105	750	350, 200, 180	17
H. uvarum	CBS 104^{T}	745	324, 315, 105	745	330, 180, 150, 70	21
H. valbyensis	CBS 479^{T}	740	640, 110	739	235, 210, 155, 105	21
H. valbyensis	CECT 1445^{T}, 10122	750	630, 120	750	240, 220, 170, 105	17
Kazachstania exigua	IFO 1128	720	*349, 286, 75, 10*	*483, 237*	*342, 238, 132, 8*	19
K. exigua	CECT 11192^{T}	675	320, 200, 90	400, 200, 75	400, 275	18
K. exigua	CECT 11192^{NT}	750	375, 300	500, 250	350, 250, 150	18
K. unispora	MUCL 51234	740	*337, 309, 84, 10*	*508, 117, 115*	*374, 358, 8*	19
K. unispora	CECT 10682^{T} (CBS 398)	775	350, 310, 115	500, 110	400, 375	18
Kluyveromyces lactis	UOA/HCPF BUL74	719	*283, 187, 159, 80, 10*	*645, 74*	*285, 181, 112, 77, 59, 5*	19
K. lactis	CECT 1121, 1961^{NT}, 10356, 10669	740	285, 190, 165, 90	655, 80	290, 180, 120, 80, 65	17
K. marxianus	CECT 1123,10368,10585, 10668	740	285, 185, 140, 100	655, 80	240, 185, 120, 80, 65, 50	17
K. marxianus	VTT C-75007^{T} (CBS 834)	720	280, 200, 160, 80	340, 380	280, 190, 120	This study
K. marxianus var. *drosophilarum*	CECT 10390,11337	740	285, 190, 165, 90	655, 80	240, 185, 120, 80, 65, 50	17
K. marxianus var. *drosophilarum*	CECT 11340	740	285, 190, 165, 90	655, 80	240, 185, 185, 65, 50, 15	17
Dekkera anomala	CECT 11162^{T}, NCYC 749	800	340, 340, 120	800	360, 190, 160, 80	17, 39
Torulaspora delbreuckii	MUCL 51211	798	*327, 220, 139, 102, 10*	*798*	*410, 380, 8*	19
T. delbreuckii	VTT C-05716^{T} (CBS 1146^{T})	800	330, 220, 150, 100	800	410, 390	This study
T. delbrueckii	CBS 1146^{T}	803	330, 215, 140, 100	803	420, 380	21
T. delbrueckii	CECT 1880, 10558, 10589, 10651, 10676, 10683, 10693, 10694, 11146, 11199 (CBS 1146^{T})	800	330, 220, 150, 100	800	410, 380	17
Zygosaccharomyces bisporus	VTT C-94195^{T} (CBS 702)	790	300, 280, 100, 90	750	280, 240, 140, 90	This study
Z. bisporus	CECT 11055^{T} (CBS 702)	790	300, 275, 110, 90	690, 100	390, 225, 150	17
Z. bailii	ATCC MYA-4549	785	*322, 272, 95, 84, 10, 2*	*696, 89*	*347, 221, 158, 51, 8*	19
Z. bailii	VTT C-05662	790	320, 280, 100, 90	500	360, 220, 160	This study
Z. bailii	CECT 10674^{T}, 11042, 11043	790	320, 270, 95, 95	690, 90	340, 225, 160, 55	17
Z. bailii	CECT 10674^{T}	775	330, 295	700	340, 230, 175	22
Z. bailii	CBS 680^{T} (CECT 10674)	785	313, 273, 98	700, 75	356, 226, 160	21
Z. rouxii	CBS 732^{T}	725	*271, 191, 165, 88, 10*	*395, 213, 84, 33*	*345, 244, 128, 8*	19
Z. rouxii	CBS 732^{T}	735	272, 186, 159	400, 220	356, 250, 130	21
Z. rouxii	CECT 1232^{T} (CBS 732^{T})	725	295, 205, 175	400, 220	335, 240, 150	22
Z. rouxii	CECT 1230, 1232, 10137, 11136, 11189	750	290, 200, 170, 90	400, 210, 90	350, 260, 140	17

Table II. Sizes of amplified products of the 5.8S-ITS regions and restriction fragments from *Saccharomyces* species including type (T) and new type (NT) strains. Italicised sizes represent virtual fragment sizes determined from published sequences and known restriction sites using DNAMAN v4.1 and Clone Manager 5 software.

Species	Origin	Code	PCR sizes	*Cfo*I	*Hae*III	*Hin*fI	References for identification
Restriction group 1							
Saccharomyces bayanus var. *uvarum*	*Mesophylax adopersus*	MCYC 623 (CBS 7001)	*838*	*363, 336, 132, 10*	*311, 230, 172, 128*	*362, 352, 119, 8*	19
S. cerevisiae	Infected blood	UOA/HCPF EM10049	*842*	*363, 335, 134, 10*	*311, 231, 172, 128*	*362, 352, 116, 8*	19
S. cerevisiae	Fig fruit	S288c	*841*	*363, 336, 132, 10*	*311, 230, 172, 128*	*362, 352, 119, 8*	SGD
S. cerevisiae	Fig fruit	BY4741	880	340, 320, 120	320, 240, 180, 140	380, 380	This study
S. cerevisiae	Ale	CECT 1485 (CBS 4309), 1883, 1942^{NT} (CBS 1171)	850	375, 325, 150	325, 230, 170, 125	375, 365, 110	18
S. cerevisiae	Ale	NCYC 2593, 1046	880	340, 320, 120	320, 240, 180, 140	390, 390, 100	This study
S. cerevisiae	Ale	NCYC 76	880	385, 365	325, 230, 170, 125	360, 350, 160	39
S. cerevisiae	Ale yeast	CBS 1171^{T}	850	380, 340	320, 225, 180, 145	360, 350, 120	21
S. cerevisiae	Ale yeast	CECT 1942^{T} (CBS 1171^{T})	880	385, 365	320, 220, 180, 145	365, 155	22
S. cerevisiae	Ale	CECT 1942^{T}, 1971	880	385, 365	320, 230, 180, 150	365, 155	17
S. paradoxus	Tree exudate	NRRL Y-17217 (CBS 432, CECT 1939)	*841*	*364, 335, 132, 10*	*312, 229, 172, 128*	*363, 351, 119, 8*	19
S. paradoxus	Tree exudate, soil	CECT 1939^{NT} (CBS 432, 11143 (CBS 5829)	850	375, 325, 150	325, 230, 170, 125	375, 365, 110	18
S. paradoxus	Tree exudate	CBS 432^{T}	850	380, 340	320, 225, 180, 145	360, 350, 120	21
S. paradoxus	Tree exudate, soil	CECT 1939^{T} (CBS 432), 11143 (CBS 5829)	880	385, 365	320, 230, 180, 150	440, 440	17
S. pastorianus (Hybrid group 2; Frohberg)	Lager	DBVPG 6285, 6282, 6257 (CECT 11188, CBS 1260)	880	385, 365	320, 220, 180, 140	365, 365, 130	This study
S. pastorianus	Lager	W34, CB11	880	340, 320, 120	320, 220, 180, 140	365, 365, 130	This study
S. pastorianus (Hybrid group 2; Frohberg)	Lager	DBVPG 6560, 6283	880	385, 365	320, 220, 180, 140	365, 220, 180, 115	This study
S. pastorianus	Lager	NCYC 1056	880	385, 365	320, 220, 180, 140	365, 220, 180, 115	This study
Restriction group 2							
Saccharomyces bayanus var. *bayanus*	Turbid beer	CBS 380^{T} (CECT1941)	850	380, 340	490, 225, 145	360, 350, 120	21
S. bayanus	wine	CECT 1369, 1969^{T} (CBS 395)	850	375, 325, 150	495, 230, 125	375, 365, 110	17, 22, 18
S. bayanus	Turbid beer blackcurrant juice	CECT 1941^{T} (CBS 380), 1969 (CBS 395)	880	385, 365	500, 220, 145	365, 155	22, 17, 18
S. pastorianus	Lager	IFO 1167 (CBS 1513)	*838*	*361, 333, 134, 10*	*481, 229, 128*	*360, 354, 116, 8*	19
S. pastorianus (Hybrid group 1; Saaz)	Lager	DBVPG 6047 (CECT 1940; CBS 1538), 6033 (CBS 1513; CECT 11037), 6258 (CBS 1486, CECT 11201), 6261 (CBS 1503, CECT 1970), 6284	880	385, 365	500, 220, 140	365, 130	This study
S. pastorianus	Lager	CBS 1538^{T}	850	380, 340	490, 225, 145	360, 350, 120	21
S. pastorianus	Lager	CECT 1940 (CBS1538)	880	385, 365	500, 220, 145	365, 155	22
S. pastorianus	Lager	CECT 1940^{NT} (CBS1538) 1970 (CBS 1503)	850	375, 325, 150	495, 230, 125	375, 365, 110	18
S. pastorianus	Lager	CECT 1940^{T} (CBS1538), 1320 (CBS1538)	880	385, 365	500, 220, 145	365, 155	17
S. kudriavzevii	Decayed leaf	IFO 1802	*841*	*364, 335, 132, 10*	*484, 229, 123, 5*	*363, 351, 119, 8*	7, 19
S. mikatae	Soil	CBS 8839	*841*	*364, 334, 130, 10*	*484, 228, 126*	*363, 350, 117, 8*	19,31

Of the three *Zygosaccharomyces species* included in this study, all were in the largest amplicon size class with sizes ranging from 725–790 bp. *Zygosaccharomyces rouxii* could be distinguished from *Z. bailii* and *Z. bisporus* based on its smaller amplicon size, but as indicated previously care must be taken to avoid misidentification of this species as *Kluyveromyces*. The available data suggests that *Z. bailii* and *Z. bisporus* are not readily distinguishable using this technique and, furthermore, that there is the possibility of this species being confused with *Hanseniaspora uvarum* based on amplicon sizes and restriction profiles.

For the remaining non-*Saccharomyces* species within this library, amplicon sizes ranged from 600–700 bp and these species included *Millerozyma farinosa* (synonym *P. farinosa*), *Meyerozyma guilliermondii* (basionym *P. guilliermondii*), *S. stipitis, Wickerhamomyces anomalus* (synonym *P. anomala)*, and *Wickerhamomyces subpelliculosus* (basionym *P. subpelliculosa*). (Table I). Of these, the most difficult to differentiate are the *Wickerhamomyces* species.

Yeasts belonging to the *Saccharomyces sensu stricto* group have ITS sizes of 840–880 bp (Table II), a feature which distinguishes them immediately from the known non-*Saccharomyces* contaminants. In many cases, restriction profiles were identical in different species. Restriction with *Hae*III however reveals that these species fall into one of two groups. The first group yields 4 distinct bands (approx. 310, 230, 170, 130 bp) which correspond in size to the predicted ITS fragments that would be generated with *Hae*III digestion of the *S. cerevisiae* S288c ITS region (Table II). All *S. cerevisiae* strains, including the investigated ale strains, belong to this group. Other species that fall into this group (based on observed and predicted restriction profiles) include *S. bayanus* var. *uvarum* and *S. paradoxus*. The second *Hae*III restriction group generates three fragments of approx. 480, 230 and 130 bp and includes *S. kudriavzevii* and *S. mikatae*[7].

Interestingly, *Hae*III restriction can separate *S. pastorianus* strains into either restriction group 1 (with 4 bands) or restriction group 2 (with 3 bands); this differentiation is consistent with the study of hybrid groups of *S. pastorianus*[16]. The majority of *S. pastorianus* restriction group 1 strains were indistinguishable from *S. cerevisiae* ale strains as well as the sister laboratory strains BY4741 and S288c. Those belonging to restriction group 2 had profiles similar to those observed with several strains of *S. bayanus* including the *S. bayanus* var. *bayanus* type strain. Previously categorized hybrid group 1 (Saaz) strains included in this study showed the same restriction profiles with individual enzymes. Restriction group 1 lager strains could however be further sub-grouped depending on profiles generated with *Hinf*I restriction of amplicons. This profile was found with three strains belonging to restriction group 1 lager yeasts. Otherwise, *Hinf*I could not differentiate different *Saccharomyces* species or strains investigated (fragment sizes were approx 360, 350 and 120, with the two larger bands frequently observed as a single band on gels). Strain W34 and the production strain (CB11) used in this brewery had RFLP patterns consistent with Frohberg strains. Some differences were observed when comparing restriction profiles with *Cfo*I, though these differences were as often observed when comparing strains of one species as comparing different species and are therefore not applicable for differentiation of species within the *Saccharomyces sensu stricto* group.

Wild yeast contaminants, 59 in total, were isolated from samples collected from conditioning tanks and fermentation vessels. Results showed that ITS-PCR/RFLP can be used, to some extent, to differentiate a number of wild yeast taxa present in the brewing process. Wild yeast species putatively identified mainly belonged to the genera *Candida, Pichia, Dekkera, Rhodotorula* and *Saccharomyces.* Putative identifications are listed in Table III according to the constructed 5.8S ITS library. All isolates with PCR products of 880 bp belonged to *Saccharomyces* restriction group 1 (Table III), which includes *S. cerevisiae, S. paradoxus, S. pastorianus* hybrid group 2 (Frohberg) and *S. bayanus* var. *uvarum.* It is therefore not possible to differentiate the isolated *Saccharomyces* species. *Pichia* species were the most commonly isolated yeasts from conditioning tanks but were not detected in fermentation vessel samples. *Saccharomyces* spp. made up the largest proportion of yeast found in fermentation tanks. Strains belonging to *Saccharomyces* restriction group 1 were the most abundant contaminants found in fermentation vessels (Table III). They made up around 82% of the wild yeasts found in these vessels and 10% in conditioning tanks. Approximately 8–10% of *Saccharomyces* isolates found in both conditioning tanks and fermentation vessels were putatively identified as *S. paradoxus* based on a distinctive double band generated with *HinfI* digestion. *Dekkera bruxellensis* made up 16% of wild yeast isolates in conditioning tanks. *Candida* species occurred at a low frequency of 3% in conditioning tanks and fermentation vessels. Composition of the wild yeast population varied depending on vessel and beer type; two fermentation vessels were only contaminated by *Saccharomyces* restriction group 1 species while the others had additional *S. paradoxus, C. intermedia/C. pararugosa/ C. rugosa, D. bruxellensis* and *R. mucilaginosa* as contaminants. One lager beer type was contaminated with *Pichia* spp. only while the other had a mixture of *Saccharomyces*, *Pichia*, *Candida* and *Rhodotorula* (data not shown).

DISCUSSION

Restriction analysis of the rDNA region spanning the 5.8S rRNA gene and flanking internal transcribed spacers (ITS1 and ITS2) has previously been shown to be an effective, rapid and simple method to identify a variety of yeasts isolated from alcoholic fermentations. Compared to traditional methods based on selective media and incubation conditions, this method has potential advantages in terms of speed, efficiency and reduced work load[6,25,55]. Although the method has been applied for detection of wild yeast contaminants of food and wine, there are few reports of its application for differentiation of wild yeast contaminants in breweries and their occurrence at different stages of brewing process[26,27,47,55,60]. Yeast contaminants in the brewing industry are rarely identified to species level, possibly due to a lack of appropriate reference strains, the continued use of traditional, non-specific

Table III. Identification of the predominant yeast species isolated from an industrial brewery and their frequency at different stages of the brewing process

Amplicon size	Genera within size class	Restriction fragment			Putative identification	%	Origins	References for identification
		*Cfo*I	*Hae*III	*Hin*fI				
400	*Candida/Dekerra/Pichia/ Yarrowia*	240, 160	380	220, 180	*C. intermedia, C. pararugosa, C. rugosa*	3	Fermentation	This study
600	*Candida/Dekkera/ Meyerozyma/Rhodotorula/ Wickerhamomyces*	300, 210, 80	400, 200	360, 320	*R. mucilaginosa*	7	Fermentation	This study
880	*Saccharomyces*	385, 365	320, 230, 180, 150	440, 440	*S. paradoxus*	8	Fermentation	18
880	*Saccharomyces*	340, 320, 120	320, 230, 180, 150	360, 350, 110	Restriction group 1	16	Fermentation	This study
880	*Saccharomyces*	385, 365	320, 240, 170, 120	360, 350, 160	Restriction group 1	41	Fermentation	39
880	*Saccharomyces*	385, 365	320, 230, 180, 150	365, 155	Restriction group 1	25	Fermentation	18,This study
400	*Candida/Dekerra/Pichia/ Yarrowia*	240, 160	380	220, 180	*C. intermedia, C. pararugosa, C. rugosa*	3	Conditioning	This study
450	*Candida/Dekerra/Pichia/ Yarrowia*	180, 100, 90, 80	340, 85, 25	260, 200	*P. fermentans, P. membranifaciens*	11	Conditioning	This study, 18
450	*Candida/Dekerra/Pichia/ Yarrowia*	180, 100, 90, 80	310, 90, 40	250, 200	*P. fermentans, P. membranifaciens*	18	Conditioning	This study
500	*Candida/Dekerra/Pichia/ Yarrowia*	160, 110, 90, 80, 60	350, 100, 50	280, 220	*P. fermentans, P. membranifaciens*	14	Conditioning	This study
500	*Candida/Dekerra/Pichia/ Yarrowia*	260, 110, 75	330, 90	275, 200	*D. bruxellensis*	16	Conditioning	18
450	*Candida/Dekerra/Pichia/ Yarrowia*	170, 100, 85, 80	340, 80, 30	250, 200	*P. fermentans, P. membranifaciens*	18	Conditioning	This study, 18
880	*Saccharomyces*	385, 365	320, 230, 180, 150	440, 440	*S. paradoxus*	10	Conditioning	18
880	*Saccharomyces*	340, 320, 120	320, 230, 180, 150	380, 380	Restriction group 1	3	Conditioning	This study
880	*Saccharomyces*	385, 365	320, 240, 170, 120	360, 150	Restriction group 1	7	Conditioning	This study, 18

means of identification and the occurrence of misleading information in brewing literature, for example, the name *Candida mycoderma* has been used to describe a mixture of different yeasts, moulds and bacteria, which contribute to surface films[24,25] rather than the actual species. Additionally, nomenclature for asexual/sexual stages of yeasts cause confusion, e.g., *Pichia kudriavzevii* (basionym *Issatchenkia orientalis*) the teleomorph of *Candida acidothermophilum* (synonym *Candida krusei*)[54]. For these reasons, we have constructed a reference library for known wild yeast contaminants found in breweries and tested this library using unknown yeast contaminants isolated from industrial fermentation vessels and conditioning tanks. A number of production and non-production yeast strains were included to determine if these can be differentiated using the PCR-RFLP technique. Strains included laboratory strains, ale strains and lager strains from both hybrid groups identified by Dunn and Sherlock[16].

A comparison of PCR fragment sizes and restriction profiles indicated that the data compiled in Table I could be used to identify a number of non-*Saccharomyces* wild yeast contaminants. Species with unique restriction profiles that have been observed in two or more independent investigations and with multiple strains include *D. bruxellensis, H. valbyensis, K. exigua, K. unispora, M. farinosa, M. guilliermondii, P. kudriavzevii, R. mucilaginosa, S. stipitis, T. delbrueckii* and *Z. rouxii*. Other species may potentially provide reliable restriction profiles for identification but these first require verification, in most cases because only one strain has been included in one or more studies (*C. mesenterica, C. norvegica, Filobasidium capsuligenum, Schwanniomyces occidentalis, W. subpelliculosus, Z. bisporus*). In other cases, species within a genus have displayed similar restriction profiles (*C. santamariae* and *C. tenuis* or *C. intermedia, C. rugosa, C. sake* and *C. stellata* or *C. tropicalis* and *C. parapsilosis* or *P. fermentans and P. membranifaciens* as well as *Z. bisporus and Z. bailii*), meaning that the profiles generated cannot be considered to be species-specific. In other cases similar profiles are generated with species of different genera (*Kluyveromyces lactis* and *W. anomalus* or *C. boidinii* and *H. uvarum*) and such profiles can therefore only be considered indicative of a species and not reliable for definitive identification.

In some cases, there are discrepancies in restriction profiles among strains of a given species and, while the majority of these differences are observed with only one of the three restriction enzymes, there are cases in which profiles bear no similarity to each other. One notable example occurs with *Dekkera anomala,* in which a distinct profile was observed in two independent studies, while a different profile was obtained by two other independent studies. The strongly conflicting results in this case may suggest that mis-identification or mis-labelling of strains may have occurred, mostly likely as a consequence of the rapidly changing taxonomic status of yeasts. Otherwise, small differences in fragment sizes

may be related to sequence differences between strains of a given species or due to the way in which the DNA was amplified and restricted or the bands sizes determined. It may be expected that differences in recorded fragment sizes as great as 20 bp are possible simply due to the manner in which band size was calculated and this would account for many of the small size variations reported by different researchers for the same strain of a species.

Previous studies suggested that the restriction enzyme *Hae*III could divide four siblings of the *Saccharomyces sensu stricto* group into two categories: *S. bayanus/S. pastorianus* which has a three-band pattern and *S. cerevisiae/S. paradoxus* which has a four-band pattern[18,27,38]. Interestingly, we found that *Hae*III digestion of *S. pastorianus* amplicons could also yield either a three or four band pattern, depending on the strain involved. Studies in recent years have shown that the *S. pastorianus* lager strains belong to at least two discrete groups[16,35]. The hybridization event[41,58] which led to the creation of *S. pastorianus,* believed now to be a cross between an *S. cerevisiae* ale strain and the recently discovered *S. eubayanus*[33], and the subsequent isolation of the hybrid, is believed to have occurred on at least two separate occasions. These hybridizations led to the formation of the Saaz type (group 1) strains used originally in Denmark and Bohemia[16] and the Frohberg type (group 2) used mainly in the Netherlands and Germany. It is probable that the success and proliferation of these strains in the brewing industry was brought about by improved cryotolerance due to the presence of the *S. (eu)bayanus* subgenome, as *S. bayanus* is known to be considerably more cryotolerant than *S. cerevisiae*[50] and *S. eubayanus* is found naturally in cold environments[33]. Increased tolerance to stress is typical of hybrid strains and appears to bestow an advantage to strains, particularly those used in the brewing and winemaking industries[47]. The increase in genome stability associated with the polyploid hybrid condition[47] may in fact encourage adaptive evolution of yeasts to their environment. Despite both lager hybrid groups sharing phenotypic characteristics allowing them to ferment wort efficiently at colder temperatures, they are genomically distinct and representatives of both groups were included in this study to determine the potential for ITS-PCR/RFLP to differentiate the two groups. This is the first study to directly compare ITS regions of strains from both of the known hybrids. Results were consistent with those reported by Dunn and Sherlock[16] who used cross genome hybridization to differentiate the two lager strain types. In this study, the *Hae*III enzyme was found also to distinguish the two hybrid groups, with one generating profiles typical of *S. cerevisiae* (hybrid group 2) and one generating profiles typical of *S. bayanus* var. *bayanus* (hybrid group 1). Dunn and Sherlock[16] and Nakao et al.[41] have established that, of the two hybrid groups, hybrid group 2 has retained more of the *S. cerevisiae* genome than the other, with 16 *S. cerevisiae*-type chromosomes, 12 *S. bayanus*-type chromosomes and eight chimeric chromosomes[16,41]. Hybrid group 2 strains appear to have also inherited their ITS region from *S. cerevisiae*.

Results of an investigation by Fernández-Espinar et al.[18] showed that *S. bayanus* and *S. pastorianus* strains could not be distinguished by ITS-PCR/RFLP using oligonucleotide primers and restriction enzymes identical to those used in this study[18]. Similarly, Manzano et al.[37] were unable to differentiate those yeasts using PCR-DGGE[37]. It was supposed that this result was due to the similarity between the ITS regions of both *S. bayanus* and *S. pastorianus*, leading to the difficulty in designing specific primers to distinguish them[29]. Those studies, however, only included *S. pastorianus* strains belonging to hybrid group 1 (Saaz type) and results are therefore in accordance with those of the current study. Barszczewski and Robak[6] found that RFLP patterns generated by a strain of *S. pastorianus* could not be distinguished from those of several *S. cerevisiae* strains. This result may have been influenced by the oligonucleotide primers and restriction enzymes chosen in that study or may have been due to the *S. pastorianus* strain belonging to hybrid group 2 and therefore having an ITS region similar to that of *S. cerevisiae*. Likewise, Tornai-Lehoczki and Dlauchy[53] found that four lager yeast strains could not be differentiated from three type strains of *S. cerevisiae* (and 12 ale strains) but were different to three type strains of *S. pastorianus.* Again, this result may be influenced by the hybrid group to which the strains belonged. The reference strains of *S. pastorianus* used in that study all belonged to hybrid group 1, as determined by Dunn and Sherlock[16]. The four lager strains used in the study may conceivably belong to hybrid group 2 (three of the strains originated in Germany and could be closely related).

Restriction endonucleases used in this study, as well as distinguishing the *S. pastorianus strains* as either Saaz or Frohberg type, also placed the Frohberg strains into one of two groups depending on the RFLP profile generated with *Hinf*I. The reason for this separation is not known but suggests a different post-hybridization history for these lager strains.

We could not distinguish brewing and non-brewing *Saccharomyces cerevisiae* strains from each other based on the technology described probably due to a low level of phylogenetic separation of industrial and non-industrial strains of the species. Based on 5.8S rDNA restriction patterns, they all independently belong to the *S. cerevisiae* species. However, one study by Yamagishi et al.[60] employed a combination of specific PCR of flocculation gene *FLO1* and amplification of rDNA followed by RFLP to distinguish brewing and non-brewing strains suggesting that differentiation of these may be possible with the right combination of amplicon and restriction enzyme. Similarly, Barszczewski and Robak[6] (2004) applied ITS-PCR RFLP and RAPD (randomly amplified polymorphic DNA) to discriminate between brewing and wild yeast isolates.

We have attempted to identify unknown contaminants found in conditioning tanks and fermentation vessels using the constructed ITS library. With reference to current taxonomy, 90% of isolates in fermentation vessels were found to be *Saccharomyces spp.* while *Pichia* species appeared to be dominant in conditioning tanks with 61% of the isolates further identified either as *P. fermentans* or *P. membranifaciens*. The genus *Pichia* is considered the most common of the non-fermentative spoilage yeasts which can cause turbidity and estery off-flavour of beer[8,45]. The predominance of *Pichia* in conditioning

tanks, but not in fermentation vessels, highlighted the possible effect of oxygen in beer post-fermentation. Air, if inadvertently introduced, can be the source of yeast or bacterial contamination, but even if not, can allow the growth of aerobic yeasts such as those from the *Debaryomyces, Dekkera,* or *Candida* genera[9]. A more detailed study with frequent sampling of all stages in the brewing process is, however, necessary to fully appreciate the wild yeast population changes that occur in response to changing environmental conditions.

CONCLUSIONS

The ITS-PCR RFLP technique has a sufficient level of resolution to identify a number of yeasts associated with industrial breweries. Its advantages in terms of reproducibility and ease of use may overcome limitations of classic identification methods. It should however be noted that certain profiles were only genus specific, while others profiles were not specific to either species or genus. Of note was the fact that all profiles generated from the 59 unknown brewery-isolated yeasts corresponded to a profile in the constructed library. The library therefore may not, in all instances, be reliably used to identify an isolate to species level, but may still be used to at least produce a list of possible candidate species which can be verified by other means.

Identification of wild yeasts, even if not to species level, may help brewers to detect if specific problems are occurring at specific steps of the brewing process. Because different species and genera occur under different conditions, the presence of particular yeast types may be a good indication of which process parameters need to be reviewed. For example, *Candida, Kluyveromyces, Pichia* and *Torulaspora* are opportunistic contaminants and usually occur during the aerobic phase of fermentation. Although the majority of these yeasts are obligate aerobes, some *Candida* and *Torulaspora* species show some growth in anaerobic conditions. *Dekkera* species can ferment sugar to ethanol when oxygen is available and their presence is associated with accumulation of high concentrations of acetic acid[12].

Individual *Saccharomyces sensu stricto* species cannot be identified using this technique. *Saccharomyces* species can, however, be separated into two different groups based on the restriction profiles generated with *Hae*III. This technique appears to be a simple and effective way to identify known lager yeast strains as belonging to either the traditional Saaz or Frohberg hybrid groups.

In this study, we generated and tested a 5.8S ITS RFLP library for brewery wild yeast contaminants as well as ale and lager brewing yeast strains based on findings in the literature. However, there are some yeasts reported as being brewery contaminants which have not been included here. Further studies should be conducted in order to strengthen and complete the database. ITS/RFLP profiles have yet to be generated for *Candida solani*, *C. humilis, C. oleophila*, *C. versatilis, Debaryomyces marama, Kodamaea ohmeri* (synonym *Pichia ohmeri*) and *Wickerhamomyces onychis* (basionym *P. onychis*)[5,25].

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