Yeasts isolated from New Zealand vineyards and wineries

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Abstract

Background and Aims: The yeast flora from a range of New Zealand commercial wineries was surveyed to estimate the incidence of yeast species in grape juice.

Methods and Results: Molecular analysis of the internal transcribed spacer region was performed for 1279 yeast colonies isolated from 17 different fresh grape juices sampled in eight New Zealand wineries between 2003 and 2009. The 17 juices contained at least 25 different species of yeast from nine genera. Microsatellite fingerprinting of *Saccharomyces cerevisiae* showed that some strains were identical to known commercial yeast varieties, but we also found evidence for local populations of *S. cerevisiae* common to individual wineries or regions. Five genotypes from Central Otago, New Zealand, were very closely related to a single sequenced strain derived from Chile, which in turn is related to European wine isolates.

Conclusions: The yeast flora found in New Zealand grape juices is broadly similar to that found in wineries elsewhere around the world. Genotyping of *S. cerevisiae* suggests recent dispersal of both commercial and non-commercial yeast strains from Europe to New Zealand.

Significance of the Study: These data are consistent with two human-mediated modes for the international dispersal of *S. cerevisiae*: one via the escape of strains traded commercially, and another via long distance dispersal of non-commercial strains.

Keywords: *internal transcribed spacer (ITS)*, *microsatellite*, Saccharomyces cerevisiae, *winery*, *yeast*

Introduction

Wine quality is strongly influenced by the yeast species or strains involved in the fermentation process. Grape juice initially contains a range of yeast species, but *Saccharomyces cerevisiae* usually predominates towards the end of wine fermentations because it is more tolerant to stresses associated with these conditions, including alcohol, anaerobiosis and the presence of sulphur dioxide (reviewed by Pretorius 2000, Fleet 2008).

Molecular analysis of yeast strains has identified over 15 yeast genera associated with winemaking (Pretorius 2000, Fleet 2008). To date, there has been limited work undertaken on the yeast flora present in New Zealand vineyards and wineries. Standard yeast taxonomy and genetics have been used to identify several yeast species (Parle and Di Menna 1965, Thornton 1991). More recently, molecular analysis has identified several yeasts present in a single winery (Anfang et al. 2009).

Here, we present results of a larger survey of wild yeasts from grape juice samples from New Zealand vineyards and wineries. Species are identified using a combination of restriction fragment length polymorphism (RFLP) and DNA sequence analysis of the internal transcribed spacer (ITS) region. In addition, we utilise microsatellite genotyping to characterise strains of *S. cerevisiae* obtained, and analyse their relationships and likely origins.

Methods

Sampling

The majority of juice samples were taken in the winery after commercial pressing and cold settling. However, two samples were taken from grapes sampled in the vineyard and pressed by hand to give juice; and a Chardonnay harvest was sampled from juice at various stages of commercial processing. All samples were plated directly, with dilution as appropriate, on rich media yeast peptone dextrose (YPD) agar (Sherman 2002), containing chloramphenicol at 100 μg/mL to inhibit bacterial growth) and incubated at 28°C until colonies appeared.

RFLP analysis

DNA extraction from randomly chosen colonies was from Ling et al. (1995). ITS polymerase chain reaction (PCR) mixtures were set up with the following components: 2.5 μL 10 × PCR buffer (including Mg^{2+}), 1 μL each of 10 μM primers (ITS1 and ITS4 for ITS (see Bradbury

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et al. 2006), NL1 and NL4 for 26S (Kurtzman and Robnett 1997)), 0.5 μL of 10 mM of each dNTP, 0.2 μL of 5 U/μL Taq DNA polymerase, 2 μL of template DNA and dH_2O up to 25 μL final volume. The programme used for PCR was: 5 min at 95°C; followed by 35 cycles of 30 s at 95°C, 30 s at 55°C annealing and 60 s at 72°C, followed by 7 min at 72°C. Yields of PCR products were assayed by agarose gel electrophoresis. Fragments were separated on 1% agarose gels made in Tris-borate-Ethylenediaminetetraacetic acid buffer. Gels were stained with ethidium bromide, visualized and photographed under ultraviolet light. Fragment sizes were estimated by comparison against a DNA standard (1 Kb plus ladder; Invitrogen, Carlsbad, California, USA). ITS PCR products were digested without further purification with the restriction endonuclease *Hae*III (and sometimes *Hin*fI), as recommended by the manufacturer, and analysed by gel electrophoresis as above.

Purification of PCR products and sequencing

PCR products for sequencing were purified using High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions and analysed on an Applied Biosystems (Foster City, California, USA) DNA Sequencing 3130XL machine. All accession number sequences were sequenced completely on both strands. Sequences were edited using Vector NTI, and compared to the GENBANK database using BLAST. For taxonomy of *Pichia* and *Issatchenkia* species, we followed Kurtzman et al. (2008).

Microsatellite analysis

Primers for the microsatellite multiplex (Richards et al. 2009) are given on our website (http://www.sbs.auckland.ac.nz/uoa/science/about/departments/sbs/research/wine-science/wine-science_home.cfm). Amplifications were performed using the Qiagen (Valencia, California, USA) multiplex PCR kit (#206143) with standard reaction conditions (54°C annealing). PCR products were assayed for yield on agarose gels and analysed on an Applied Biosystems DNA Sequencing 3130XL machine using Genemapper software (Applied Biosystems, Foster City, California, USA).

Individual microsatellite alleles (190 in total) were scored as 1 (presence) or 0 (absence), based on their sizes. A dendrogram was generated with the unweighted pair-group method with arithmetic average algorithm (Sneath and Sokal 1973) using Nei's distance coefficient (Nei 1973). The procedures above were performed using Numerical Taxonomy SYStem-pc, Version 2.1.

Results and discussion

RFLP and sequencing of the yeast flora in grape juices

Grapes and grape juice were sampled from vineyards spanning the geographic range of the wine-growing regions of New Zealand, as listed in Table 1. The titre of yeasts in the juices was typically 10^4–10^6 colony-forming units per millilitre, with the exception of one low-titre juice from the Villa Maria winery in Marlborough (10^2 cfu/mL). In total, 1279 yeast colonies were analysed from 22 samples of 17 different juice harvests in eight New Zealand wineries between 2003 and 2009.

The ITS region was amplified by PCR from 1279 individual colonies and digested with *Hae*III. For each juice, 1–2 individual strains of each RFLP type were identified by direct sequencing of ITS PCR products. Table 2 lists sequences submitted to GENBANK, along with their suggested identity based on BLAST hits to identical or nearly identical sequences. The species names of the closest matches will be used to describe these colonies (Table 1 and Table 2).

Table 1 shows that the predominant species in each juice varied, with different juices having as their most numerous components *Metschnikowia pulcherrima* or *chrysoperlae* (five juices), *Hanseniaspora uvarum* (4), *S. cerevisiae* (4), *Candida zemplinina* (3), *S. bayanus* (1), or *C. oleophila* (1). Collectively, these seven yeast species represented over 94% of colonies plated.

There were examples of intra-specific sequence variation in ITS, with minor differences between strains of *Torulaspora delbrueckii*, *S. cerevisiae* and *H. uvarum*. For *C. albicans*, there was a *Hae*III polymorphism within otherwise closely related sequences, and conversely the *Metschnikowia* species could not be reliably separated by their RFLP patterns. Several distinct sequences were obtained from within the genus *Metschnikowia*, whose species are not well defined (Lopandic et al. 1996, Giménez-Jurado et al. 2004). One sequence from Central Otago (GU931320) was 1-bp different from the *M. chrysoperlae*-type species, while another (GU931319) differed at four bases (all in ITS1) from *M. pulcherrima* BIO126. An additional variant of *M. pulcherrima* with 5 bp different in ITS2 was obtained, as well as two single-stranded sequences that appeared to be recombinants between these other three sequences (data not shown). Finally, we noted colonies with mixed sequences, which both were from within the genus (data not shown), indicative of either hybrids or polyploids. These results suggest that there may be a hybrid complex from the genus *Metschnikowia* in New Zealand wineries.

Because the juice samples were primarily from commercial juices taken in the winery, we could generally not distinguish whether the yeast were derived from the vineyard or the winery. However, a series of 2003 samples from a single juice harvest (Table 1 upper section) included samples taken from the vineyard, as well as later samples as the juice progressed through the winery. In this series, there were no major changes to the vineyard flora distribution during the early winery handling, up until the pressing stage, when there was an increase in the abundance of three species (*S. cerevisiae*, *P. fermentans* and *T. delbrueckii*). A subsequent pasteurisation step (involving an approximately 15-second treatment at 80°C) reduced total plated cell counts 10-fold and again changed the distribution of yeasts in the juice; the relative enrichment of *S. cerevisiae* suggests that it preferentially survives this heat treatment.

In addition to fresh juices, colonies were analysed from seven juices frozen at –20°C. The frequency data

Table 1. Number of individual yeast colonies identified for 16 yeast species in 17 grape juices.

Year	Region	Variety, vineyard, sample		Number of colonies per sample by species															
				Huv	*Sce*	*Cze*	*Sba*	*Mpu + Mch*	*Col*	*Cal*	*Pkl*	*Tde*	*Pte*	*Csa*	*Pan*	*Pku*	*Cdi*	*Hoc*	Sample Total
2003	Gisborne	Samples of single Chardonnay harvest (grapes/juice), Putatahi Vineyard, Montana Winery	Vineyard	40	1					1									42
			Truck	34		1					1		1						37
			Post-crushed	73									1						74
			Post press	52	3						3	8	1						67
			Pasteurisation	10	20						4	3							37
2004	Auckland	Three different Chardonnay grapes/juice, Kumeu River Winery	MVCH Vineyard	19		71													90
			MVCH Juice		14	3							4						21
			KCH juice		90											1			91
			SCH juice		25														25
2007	Marlborough	Sauv. Blanc juice	Coopers Creek	1	2		70												73
2008	Marlborough	Seven different Sauvignon Blanc juices	Pernod Ricard 1	6	76		3						1						86
			Pernod Ricard 2	87	2	6													95
			Saint Clair 1	12		81										1	1		95
			Saint Clair 2	61	12			1		16		1			1				92
			Saint Clair 3	62		1		8											71
			Villa Maria			84		1			1								86
			Delegats	1	1	1	28		38	11	7			5	1			1	94
2009	Central Otago	Five different Pinot Noir juices	Wanaka Rd	4				16											20
			Cromwell		3			9											12
			Bendigo Reach	12	2			13											27
			Cromwell (Rose)	1	9			18											28
			Bannockburn					16											16
			Species totals	475	260	248	101	82	38	28	16	12	8	5	2	2	1	1	1279

Three-letter species designations refer to the first 16 species in Table 2. Colony numbers were combined for the two *Metschnikowia* species (which had indistinguishable RFLP pattern) and for *C. albicans* (which has two different RFLP patterns). Colonies that failed to amplify or gave results suggestive of mixtures are not included. Grey shading indicates the numerically predominant species in each sample; for the 2003 Chardonnay series, only the sample most similar to the other juices was used.

from these platings are not included in Table 1, since the frozen juices had very low titres, presumably as a result of cell death during freezing or storage. However, the frozen juice sampling provided sequences of an additional 11 yeast species (lower part of Table 2). In total, the molecular analyses identified 22 different yeast species in New Zealand grape juices, with five others that could not be completely characterized. These data greatly expand previous assessments of both the number of genera and species of yeast present in New Zealand vineyards and wineries (Parle and Di Menna 1965, Thornton 1991, Anfang et al. 2009).

These results demonstrate that a similar range of yeast genera and species is present in New Zealand grape juices as those found overseas. *H. uvarum* was the predominant yeast of grape juice found previously in New Zealand (Parle and Di Menna 1965), and it again predominated in our survey. Of the other main non-Saccharomyces yeasts found, both *C. zemplinina* (e.g. Li et al. 2010) and *Metschnikowia* (e.g. Peter et al. 2005, Lopandic et al. 2008) have been widely found in overseas vineyards and wineries. The levels of both *S. cerevisiae* and *S. bayanus* were reasonably high in some of our samplings, but are not outside previous observations (e.g. Naumov et al. 2000, Nisiotou and Nychas 2007). Different juices had different dominant flora, but the basis of these differences is unknown. We sampled widely in terms of geography and from three different grape cultivars but from a relatively small number of juices in each growing region. Consequently, few significant conclusions can be drawn about regional differences in New Zealand's yeast flora. However, there were two preliminary indications of regional differences in the data: *S. bayanus* was present only in Marlborough, while *Metschnikowia* species predominated (55–95%) in all five different Central Otago juices but were rare elsewhere. These trends need to be examined in larger surveys.

Genotyping of S. cerevisiae

Microsatellite fingerprinting using a multiplex of 10 variable loci was used to determine the genotypes of a subset of the 260 *S. cerevisiae* colonies identified in the juices above, as well as more than 250 others found in fresh juices or uninoculated ferments. Most genotypes contained 1–2 alleles at each microsatellite locus and had bands corresponding to both MATa and MATα, consistent with their being diploid. However, a few strains had more than two alleles at some loci and may be polyploid (see Bradbury et al. 2006, Legras et al. 2007).

Table 2. ITS RFLP patterns and species identities of New Zealand wine yeasts.

ITS size (bp)	Hae III fragment sizes (bp)	Species	Genbank accession numbers
750	750	*Hanseniaspora uvarum*	AY796120, AY796201
850	325 + 230 + 170 + 125	*Saccharomyces cerevisiae*	AY796126, AY796191, AY796193, AY842480, GU931321
400	400	*C. zemplinina*	AY796117, AY796198, AY796195
850	495 + 320 + 125	*Saccharomyces bayanus*	AY942697
385	275 + 100	*Metschnikowia pulcherrima*	GU931319, GU931322
385	275 + 100	*M. chrysoperlae*	GU931320
625	425 + 125 + 75	*C. oleophila* (EU541359)†	–
450	410 + 40 (or 450)	*C. albicans*	AY796116, GU931325
495	440 + 55	*Pichia kluyveri*	AY796123, AY796197, AY796192, GU931324
730	730	*Torulaspora delbrueckii*	AY796127, AY796128, AY942695
350	250 + 100	*P. terricola*	AY796121, AY79619
700	450 + 150 + 100	*C. santamaria* (AY542869)†	–
550	530 + 20	*P. anomala*	AY796125
450	350 + 60 + 40	*P. kudriavzevii*	AY796199, GU931323
380	380	*C. diversa* (EU343824)†	–
685	605 + 80	*H. occidentalis*	AY796200
610	300 + 170 + 90 + 50	*Lachancea thermotolerans*	AY796122, AY942698
360	360	*C. salmanticensis*	AY942700
385	275 + 55 + 45 + 10	*C. inconspicua*	AY796202
570	420 + 110 + 45	*Debaromyces hansenii*	AY796119
720	660 + 60	*Zygosaccharomyces bailii*	AY796194
570	520 + 50	*C. cantarelli*	EF121771
390	290 + 100	(88% to *P. occidentalis*)‡	AY942699 (26S = GU985276)‡
730	660 + 70	(97% to *Z. microellipsoides*)‡	AY942701 (26S = GU985277)‡
730	730	(96% to *T. delbrueckii*)§	AY942696
470	420 + 50	(93% to *C. tropicalis*)§	AY796118
530	510 + 20	(91% to *Wickerhamomyces edaphicus*)§	AY796124

†For these three species (without an accession number), we obtained full-length but single-stranded ITS sequence that was identical to the GenBank entry and species indicated. ‡For these two species, identification is based on 100% match of the 26S ribosomal sequence (accession numbers in parentheses in right column) to the species indicated; the % nucleotide identity to the ITS sequence for this species is indicated in parentheses. §Species corresponding to the accession numbers in the lower three rows have not been clearly identified; the % identity of the closest database match is indicated.

The allele sizes were compared to strains from our existing database (Richards et al. 2009). In some cases (none from Table 1), strains in uninoculated juices or ferments corresponded to commercial wine yeasts – either all alleles matched exactly or there were minor differences at 1–2 loci out of 10 (similar to date reported by Valero et al. 2005, Schuller et al. 2007). These results provided clear evidence for the presence of escaped commercial strains within New Zealand wineries. The commercial strains represented in different wineries included Pris de Mousse (EC1118), Zymaflore VL2, Lalvin CY3079, Zymaflore X5, Lalvin D254 and Siha7; in all cases, they corresponded to commercial strains that had recently been used in the winery. Similar findings have been drawn for other winemaking regions where there is intensive use of commercial wine yeasts (e.g. Valero et al. 2005, Schuller et al. 2007). Based on these results, it is likely that at least some New Zealand wineries and vineyards have within them commercially imported strains of yeast.

In total, we identified 52 distinct *S. cerevisiae* genotypes (data are provided on our website, given in Methods) that did not resemble any of the 80 commercial yeast strains in our current database (Richards et al. 2009). The pair-wise similarities among all these *S. cerevisiae* strains ranged from 82 to 99%, and 88.4% of them were not more than 90%, which indicated a high level of genetic variation in this New Zealand population. Figure 1 shows the relationships among these *S. cerevisiae* strains. This analysis identified eight clusters of closely related genotypes, where all pair-wise similarities among members were larger than 93%. Three clusters (5, 6 and 8) contained closely related genotypes derived from a single winery, reminiscent of recent findings from New Zealand

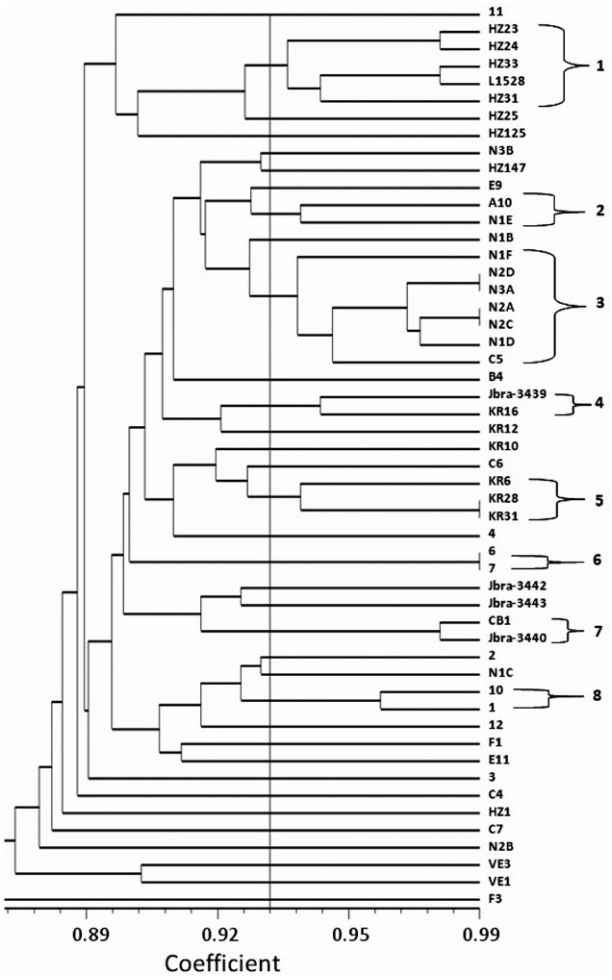

Figure 1. Dendrogram of *Saccharomyces cerevisiae* colonies based on pair-wise comparison of microsatellite profiles. The vertical line represents an overall similarity of alleles of 93% (for 190 alleles distributed among 10 loci); brackets with numbers 1–8 indicate closely related clusters of strains. The origin of the colonies according to their prefix is as follows: KR, Kumeu River, Auckland 2004; N, Neudorf, Nelson 2004 or 2006; HZ, Vinpro, Central Otago 2009; CB, Corbans winery, Hawkes Bay 2004; VE, Vidals Estate, Hawkes Bay 2004; Jbra, Montana, Gisborne 2003; B–F, Pernod Ricard winery, Marlborough 2005–6; numerals only, Coopers Creek 2007. L1528 refers to the sequenced stain derived from a winery in Chile (Liti et al. 2009).

(Goddard et al. 2010). However, five clusters were derived from more than one winery. For example, cluster 3 consists of four strains from the Neudorf winery in Nelson, plus a single genotype obtained from Marlborough (100 km away). Cluster 7 contained genotypes from Gisborne and Hawkes Bay, separated by 120 km, while cluster 4 consisted of genotypes from Auckland and Gisborne, vineyards that are 350 km apart. These findings considerably extend the geographic range of individual local New Zealand populations compared to our previous studies (Goddard et al. 2010).

The 52 New Zealand genotypes were compared to all others in our database, and revealed a startlingly close relationship between a group of five related strains from Central Otago and a single winery-derived strain from Chile, L1528 (Figure 1, cluster 1). The five New Zealand colonies were present as minor components of two different Central Otago juices. All were slightly different from each other, and all differed from the Chilean strain, so were not the result of mislabelling. The closest Otago genotype, HZ33, contained all 10 of the alleles present in L1528 (a homosoporic isolate), but was heterozygous at two of these loci and so had an additional two alleles. This degree of allele sharing indicates a very close genetic relationship (see Richards et al. 2009) and indicates a common origin for these geographically separated strains. The whole genome sequence of the Chilean strain has been determined and shows that it groups very closely with a cluster of wine-derived yeast isolates found primarily in Europe (Liti et al. 2009). The same cluster also includes vineyard- or winery-derived strains isolated in California and Australia. Our microsatellite data suggest that the five Central Otago strains also belong to this cluster. Although the direction of dispersal cannot be inferred from such data, the simplest hypothesis is that both the Chilean and New Zealand strains were originally derived from Europe, and that both were moved independently to either Chile or New Zealand. Human-mediated migration of yeast strains between wine-growing areas has been suggested previously (see Legras et al. 2007, Liti et al. 2009). Recently, one possible mechanism for long-distance spread of yeast strains has been established: commercial oak barrels imported into New Zealand from France were shown to contain isolates of *S. cerevisiae* (Goddard et al. 2010). Human-mediated migration associated with the wine industry would readily explain the dispersal of these European-derived yeast strains to both Southern hemisphere countries. This hypothesis could readily be tested by whole genome sequencing of the New Zealand isolate(s).

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