Real-Time PCR Assay for Detection and Enumeration of *Hanseniaspora* Species from Wine and Juice

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Abstract: *Hanseniaspora* species (anamorph *Kloeckera* sp.) are common yeast constituents on grapes and often dominate in the early stages of wine fermentations. Growth of *Hanseniaspora* sp. in must has been linked to changes in sensory attributes of wine and is proposed as a factor in stuck and sluggish fermentations. A real-time quantitative polymerase chain reaction (PCR) assay was developed to rapidly profile *Hanseniaspora* sp. populations. The assay allows direct enumeration of *Hanseniaspora* sp. populations in either must or wine and is not impacted by high concentrations of *Saccharomyces cerevisiae* DNA. The development of this assay will enable high throughput surveys of must samples to better explore the relationship between early *Hanseniaspora* sp. populations and the ensuing wine fermentation or sensory changes.

Key words: fermentation, real-time PCR, *Hanseniaspora*, *Kloeckera*

The initial stages of wine fermentations contain a diversity of organisms including bacteria and yeasts. The dominant yeast on ripe grapes and throughout the initial stages of the fermentation are from the genus *Hanseniaspora/Kloeckera* (Fugelsang 1997, Sabate et al. 2002). Depending on the nature of the grapes, *Hanseniaspora* sp. populations may reach high levels (10^6 to 10^7 cells/mL) during the first few days of the fermentation; however, they die off as a *Saccharomyces cerevisiae* population dominates and completes the fermentation (Fleet and Heard 1993). In some cases, *Hanseniaspora* populations have been found to persist throughout wine fermentations at a lower level than *S. cerevisiae* (Heard and Fleet 1986, 1985). Growth of these apiculate yeasts may contribute to the final complexity of the wine through production of esters, glycerol, and acetoin (Gil et al. 1996), and the use of apiculate wine yeasts as adjunct starters has been suggested (Heard 1999, Romano, et al. 1997). However, growth of *Hanseniaspora* sp. may also negatively impact wine fermentations. *Hanseniaspora* sp. have been associated with ester taints and high levels of acetic acid (du Toit and Pretorius 2000). Strains of *H. uvarum* have also been reported to produce killer toxins that may affect certain *S. cerevisiae* strains (Fleet 2003), and the growth of *Hanseniaspora* sp. during the initial fermentation may deplete the juice of nutrients, particularly thiamin, needed by *S. cerevisiae* and thus cause stuck or sluggish fermentations (Bisson 1999).

While traditional methods to identify yeasts in wine rely on culturing (Boulton et al. 1996), recent advances in molecular typing have dramatically enhanced the ability to identify yeasts colonies once isolated from wine. Over 20 different molecular biology techniques have been used to identify yeasts isolated from wine or pertinent environments (Loureiro and Malfeito-Ferreira 2003). The majority of these studies used some type of polymerase chain reaction (PCR) to identify organisms that had been previously isolated from wine by plating.

Relatively few researchers have employed methods to directly identify yeasts from wine, without any enrichment steps. Different *S. cerevisiae* strains were followed through fermentation with a direct multiplex PCR approach (Lopez et al. 2003). A two-step PCR was developed that could detect as few as 10 intact *Dekkera* cells in contaminated sherry (Ibeas et al. 1996). Other researchers have used PCR-denaturing gradient gel electrophoresis (DGGE) approaches to directly profile yeast communities on grapes (Prakitchaiwattana et al. 2004) and in wine fermentations (Mills et al. 2002). There are two main advantages of direct characterization of wine microbial DNA as opposed to yeast enrichment and plating. The first is that many microbial populations might not respond to standard enrichment plating because of injury, lack of appropriate nutrients, or persistence in a metabolically active but nonculturable state. For example, PCR-DGGE approaches have identified nonculturable yeast populations in commercial wine fermentations (Mills et al. 2002). A second advantage, in comparison to plating methods, is that direct molecular analyses take less time. Since DNA samples can be stored for later analysis, molecular approaches permit screening of higher numbers of samples than would be reasonably

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operable for plating studies, allowing more comprehensive ecological surveys to define regional or varietal-based influences in the microbial populations associated with wine.

One molecular method for enumeration of microbial populations in wine is real-time or quantitative PCR (QPCR). QPCR assays have been developed for the detection of various wine-related microorganisms, including *Oenococcus oeni* (Pinzani et al. 2004), lactic acid bacteria (Furet et al. 2004, Neeley et al. 2005, Stevenson et al. 2006), acetic acid bacteria (Gonzalez et al. 2006), *Dekkera* (*Brettanomyces*) *bruxellensis* (Delaherche et al. 2004, Phister and Mills 2003), *S. cerevisiae* (Martorell et al. 2005), and *Zygosaccharomyces* species (Casey and Dobson 2004). QPCR offers significant advantages over other molecular methods by accurately quantifying the target populations as opposed to simply identifying a population above a specific threshold. Moreover, the method can be performed in several hours and, depending on the thermocycler used, can examine numerous samples (up to 384 samples per QPCR plate). In this study, we developed a QPCR method for the detection and quantification of *Hanseniaspora* sp. in must in order to facilitate ecological surveys of this yeast in the winery environment.

Materials and Methods

Microbial strains and propagation. Yeast and bacteria used in this study were obtained from the Agricultural Research Service Culture Collection (USDA-ARS, Peoria, IL), the Herman J. Phaff Yeast Culture Collection, University of California, Davis (UCDFST), and the UCD Department of Viticulture and Enology Culture Collection (UCDVEN) (Table 1). All yeasts were grown in YM broth (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g dextrose, and 1.0 L H_2O) (Becton Dickinson, Sparks, MD) at 25°C.

Primer design. Sequence analysis was performed using the Seqweb GCG sequence analysis program (version 2; Accelrys, San Diego, CA). The D1/D2 domain of the large subunit ribosomal RNA gene from *H. osmophila* (GenBank sequence accession number U84228), *H. uvarum* (U84229), *H. valbyensis* (U73596), *H. guilliermondii* (U84230), and *S. cerevisiae* (U44806) were aligned, and primers HanF 5'-GGCGAGGATACCTTTTCTCTG-3' and HanR 5'-ACCACCCACTTTGAGCTG-3' were selected to produce a 77 bp fragment specifically from *Hanseniaspora*. All accession numbers are for type strains and were obtained from a published source (Kurtzman and Robnett 1998).

Specificity of PCR assays. DNA from all yeast was isolated as described previously (Mills et al. 2002). PCR reactions were performed at a final volume of 50 µL. All PCR reagents were obtained from Applied Biosystems (Foster City, CA). Each reaction contained 5 µL AmpliTaq gold buffer; 2.0 mM $MgCl_2$; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 0.2 mM primers; 1.25 U AmpliTaq Gold and 2 µL (~20 ng) of extracted DNA. The reactions were run for 40 cycles on a GeneAmp 2700 thermalcycler (Applied Biosystems), denaturation was 95°C for 60 sec, annealing was 58°C for 45 sec, and extension was 72°C for 7 sec. An initial 5 min denaturing step at 95°C and a final 7 min extension at 72°C were used. The products were analyzed by agarose gel electrophoresis on a 3% gel and stained with 0.5 mg/mL ethidium bromide (Ausubel et al. 1995). The gels were visualized under UV transillumination

Table 1 HanF–HanR PCR amplification from wine-related yeast and bacteria.

Microorganism	Strain number[a]	PCR result
***Hanseniaspora* sp.**		
H. osmophilia	UCDFST40-417	–
H. uvarum	UCDFST54-192	+
H. valbyensis	UCDFST68-28	+
H. guilliermondii	UCDFST57-20	+
Other yeast		
Brettanomyces custersianus	NRRLY-6653	–
B. intermedius	UCDFST82-30	–
B. lambicus	UCDFST89-4	–
B. nanus	NRRLY-17527	–
B. naardenensis	NRRLY-17526	–
Dekkera abstinens	UCDFST82-24	–
D. anomala	NRRLY-17522	–
D. bruxellensis	UCDVEN2050	–
Candida kefyer	UCDFST76-22	–
C. krusei	UCDFST94-157.1	–
C. lambica	UCDFST72-232	–
C. mesenterica	UCDFST67-35	–
C. sake	UCDFST51-18	–
C. sorboxylosa	UCDFST91-491.3	–
C. stellata	UCDFST72-1034	–
C. veronae	UCDFST86-32	–
C. vini	UCDFST40-36	–
Debaryomyces hansenii	UCDFST74-86	–
D. carsonii	UCDFST66-20	–
Issatchenkia terricola	UCDFST91-489.3	–
I. orientalis	UCDFST75-62	–
Kloeckera japonica	UCDFST94-153.2	–
Kluyveromyces marxianus	UCDFST55-82	–
Metschnikowia pulcherrima	UCDFST40-214	–
Pichia. anomala	UCDFST76-71T	–
P. membranaefaciens	UCDFST57-22	–
S. baynanus	UCDFST01-159	–
Saccharomyces cerevisiae	NRRLY-12632	–
Schizosaccharomyces japonicus	UCDFST71-26	–
Zygosaccharomyces bailii	UCDFST68-113	–
Bacteria		
Acetobacter aceti	UCDVEN114	–
Lactobacillus plantarum	UCDVEN1	–
Oenococcus oeni	UCDVEN154	–

[a]NRRLY: Agricultural Research Service Culture Collection, Peoria, IL; UCDVEN: University of California, Davis, Department of Viticulture and Enology culture collection; UCDFST: The Herman J. Phaff Yeast Culture Collection.

using a multimage light cabinet (Alpha Innotech, San Leonardo, CA).

QPCR reactions. QPCR reactions were performed on a Prism 7700 sequence detection system, with SybrGreen master mix used according to manufacturer's instructions (Applied Biosystems). Optimized reactions were performed in 0.5 mL MicroAmp optical tubes or plates, and each 25 µL reaction contained 1 x SybrGreen master mix, 300 nM HanF, 300 nM HanR, and 2 µL purified DNA. Each reaction was performed in triplicate. Reactions were run for 40 cycles, denaturation was 95°C for 60 sec, annealing was 58°C for 45 sec, and extension was 72°C for 7 sec. An initial 10 min denaturing step at 95°C was used. The cycle threshold (Ct), or PCR cycle where fluorescence first occurred, was determined automatically using sequence detection software (version 1.7; Applied Biosystems).

Artificial contamination of juice and wine. *Hanseniaspora uvarum* 54-192 (5.8 x 10^7 cfu/mL) was serially diluted in YM media (RM: rich medium) to 10^{-7}, plated on YM agar, and incubated for 24 hr at 30°C. This culture was also serially diluted in filter-sterilized juice (Chardonnay, Brix 22.5, pH 3.36, titratable acidity 7.4 g/L) and juice containing ~10^7 *S. cerevisiae* cells. DNA was isolated from 700 µL of each dilution using a MasterPure yeast DNA purification kit according to manufacturer's instructions (Epicentre Technologies, Madison, WI). This DNA was then used in QPCR reactions described above. Standard curves for quantification of unknown samples and determination of amplification efficiency were generated by plotting the Ct values of QPCR reactions performed on DNA from these dilution series in YM media against log input cells.

Reproducibility of QPCR assay. A fresh culture of *H. uvarum*, grown in YM, was serially diluted in Chardonnay juice (previously filtered through a 0.2-µm filter) and plated on YM medium to obtain colony forming units per milliliter at each dilution. DNA was also isolated from the juice sample dilutions and a QPCR assay was run, as described above, to determine cell number. Three trials were performed on three separate cultures.

Determination of *Hanseniaspora* population from juice. Fifty mL samples of juice were collected from a local winery; 10 mL were harvested by centrifugation and resuspended in 1 mL dH_2O. Concentrated cells were then serially diluted and plated on WL media to establish the number of *Hanseniaspora* sp. cells (Pallmann et al. 2001). DNA was also extracted from the juice samples and used in a QPCR assay, as described above. Ct values from the assay were compared to those from a standard curve to determine the cfu/mL of the *Hanseniaspora* sp. in the juice.

Results

Primer design and specificity. 26S rDNA gene sequences for the five common *Hanseniaspora* sp. wine isolates and *S. cerevisiae* were aligned and regions specific to *Hanseniaspora* sp. used to create primers HanF and HanR (Figure 1). These primer sequences were then checked against both GenBank and EMBL databases. Primer HanF exhibited specific homology to *Hanseniaspora* sp., while primer HanR exhibited general homology to yeasts. The primers were then empirically tested by PCR against various yeast and bacteria known to have been isolated from wine. Only *H. uvarum*, *H. guilliermondii*, and *H. valbyensis* produced the expected 79 bp PCR product and no amplicons were produced from the other yeasts or bacteria (Table 1).

QPCR detection limits. The QPCR assay was carried out on *H. uvarum* cells contained within rich media, Chardonnay juice, and Chardonnay juice supplemented with *S. cerevisiae*. The supplemented juice was used to gauge the impact of a large amount of nontarget DNA on the QPCR assay. *Hanseniaspora uvarum* cells were serially diluted in RM, and DNA isolated from each dilution was used to construct a standard curve. The same culture was also serially diluted in juice or supplemented juice to determine the effects of this matrix on the QPCR assay (Figure 2). In all cases, the detection limit was ~10 cfu/mL, and the assay was linear over four orders of magnitude (Figure 2). These results suggest that samples obtained from wine or wine containing nontarget yeasts do not significantly impact the assay. However, the accuracy of quantification at levels less than 100 cfu/mL of *Hanseniaspora* may be impacted by the presence of *Saccharomyces*.

To test reproducibility of the QPCR assay, *H. uvarum* was serially diluted in juice to create samples with known levels of contamination. The *H. uvarum* level in these samples was then determined by QPCR and correlated to plating analysis from the same dilution (Figure 3). Three separate trials were performed, and in each case the relationship between colony forming units determined by plating and that determined by QPCR produced high R^2 values of 0.979, 0.988, and 0.947.

Quantification of *Hanseniaspora* sp. from juice. Actual juice samples were obtained from a local winery in order to establish the accuracy of the *Hanseniaspora* sp. QPCR assay. Each juice sample was concentrated, serially diluted,

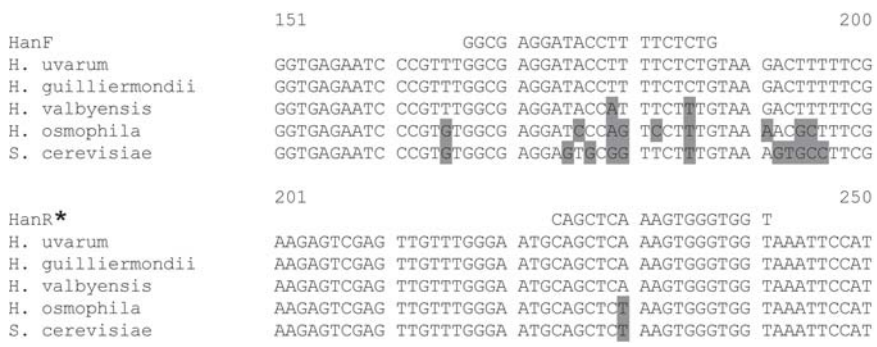

Figure 1 Alignment of D1/D2 26S rDNA partial sequences and HanF and HanR primers. Shaded sequences are regions of nonidentity to the *H. uvarum* 26S rDNA sequence. *Reverse complement of HanR is presented in order to view homology.

and plated onto WL medium, which allows for the differentiation of yeast strains due to their colony morphology (Pallmann et al. 2001). *Hanseniaspora* sp. were identified as green flat colonies. DNA was also extracted from the juice samples and quantified by QPCR (Table 2). Overall an excellent correlation was seen between the QPCR estimated *Hanseniaspora* sp. population and the actual population as determined by plating.

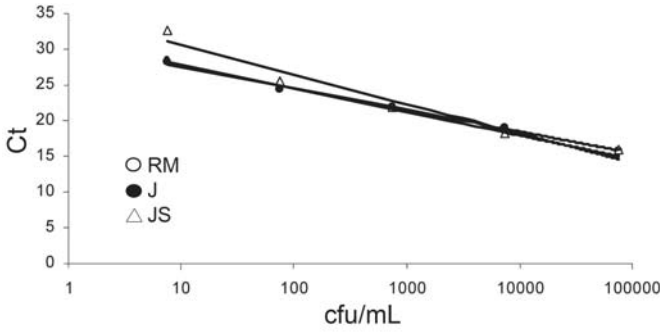

Figure 2 Determination of amplification efficiency and detection limits of *H. uvarum* diluted in rich media (RM), juice (J), and juice supplemented with 10^7 *S. cerevisiae* cells (JS). DNA was then extracted from each dilution and used in a QPCR reaction. Ct values were plotted against log 10 dilution. Lines represent the regression of log cell numbers in each matrix. R^2 values: RM, 0.996; J, 0.997; SJ, 0.959. (Note that RM and J lines overlap.)

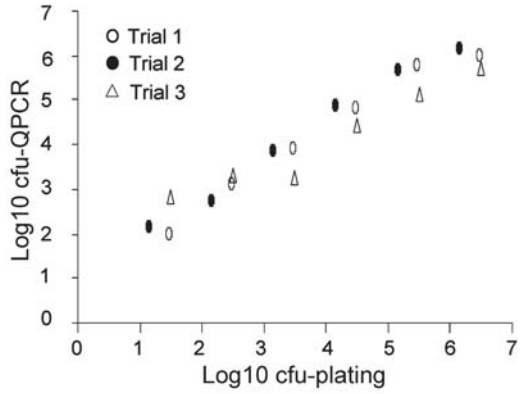

Figure 3 Sensitivity and accuracy of the QPCR assay compared to plating for determination of *H. uvarum* in juice. *H. uvarum* was serially diluted and plated on YM medium. The same dilution was also performed in sterile-filtered Chardonnay juice providing samples with a known cfu/mL. DNA was isolated from these samples and a QPCR assay run to determine cell number. Three trials were performed. Estimated cell numbers were compared to those established by plating. (R^2 values: trial 1, 0.979; trial 2, 0.988; trial 3, 0.947.)

Discussion

A rapid QPCR-based method for detection and enumeration of *Hanseniaspora* sp. in juice and wine was developed. Primers were designed to the D1/D2 loop of the 26S rRNA gene, as that is one region previously used to distinguish between yeast strains (Kurtzman and Robnett 1998). Primers were tested empirically in a series of PCR reactions with various wine-related yeast and bacteria. Only *Hanseniaspora* sp. produced a PCR product with the HanF and HanR primers (Table 1), indicating these primers could be used for generation of a QPCR-based assay for detection of *Hanseniaspora* sp. While *H. osmophila* was not detected using this primer set, we consider this to be a minor concern as *H. uvarum* is the most commonly isolated of the *Hanseniaspora* sp. (Capece et al. 2005).

We then needed to establish the detection limits of the QPCR assay. A culture of *H. uvarum* was serially diluted in rich media, juice, and juice with the addition of *S. cerevisiae* (Figure 2), the latter being an expected situation in inoculated must samples. With each of the trials, as few as 10 *H. uvarum* cells per milliliter could be detected. This limit of detection is in agreement with other QPCR methods for the detection of yeasts (Brinkman et al. 2003, Martorell et al. 2005, Phister and Mills 2003). The presence of juice-borne phenolic compounds, known inhibitors of PCR reactions (Wilson 1997), or high levels of nontarget *S. cerevisiae* did not impact the efficiency of the assay. The assay was found to be linear over four logs of detection for *Hanseniaspora* levels over 100 cell/mL.

The assay was tested on actual juice samples. *Hanseniaspora* sp. populations determined by both plating and QPCR assay were comparable, indicating the applicability of this assay. High levels of *Hanseniaspora* sp. are known to be associated with damaged grapes and have been implicated as a cause of stuck fermentations (Bisson 1999). This assay could be used as a rapid method to assess grape/must quality and potential risk for eventual fermentation problems. We have used the assay in prefermentation juice surveys to assess more fully the linkage between high levels of apiculate yeasts in prefermentation juice and eventual fermentation performance (Nierman et al. 2005). Such approaches will help identify the juice and fermentation conditions in which high levels of *Hanseniaspora* sp. populations might negatively impact fermentation performance.

Table 2 Comparison of *Hanseniaspora* sp. QPCR and plating results from juice samples.

Juice varietal (date sampled)	Brix (g/100 mL)	pH (g/100 mL)	TA (g/100 mL)	Plate (cfu/mL)	QPCR (cfu/mL)
Cabernet Sauvignon (10/28/05)	24.2	3.78	0.45	$4.8 \times 10^6 \pm 8.6 \times 10^5$	$3.33 \times 10^6 \pm 4.5 \times 10^5$
Cabernet Sauvignon (11/3/05)	23.5	3.69	0.51	$2.1 \times 10^6 \pm 3.2 \times 10^5$	$1.72 \times 10^6 \pm 4.1 \times 10^5$
Syrah (10/28/05)	27.5	3.87	0.45	$1.5 \times 10^4 \pm 2.3 \times 10^3$	$1.47 \times 10^4 \pm 8.8 \times 10^3$
Cabernet franc (11/1/05)	24.8	3.45	0.54	$2.9 \times 10^5 \pm 8.5 \times 10^4$	$2.6 \times 10^5 \pm 4.1 \times 10^4$
Tempranillo (10/26/05)	22.9	3.85	0.52	$7.5 \times 10^4 \pm 2.1 \times 10^4$	$5.6 \times 10^4 \pm 7.6 \times 10^3$

Conclusion

A QPCR assay for enumeration of *Hanseniaspora* sp. in must and wine has been developed that can detect as few as 10 cfu/mL, is linear over four orders of magnitude, and is not influenced by high concentrations of contaminating *S. cerevisiae* DNA. This assay will enable high throughput surveys of must samples in order to examine the impact of early growth of *Hanseniaspora* sp. populations on the ensuing wine fermentation or sensory attributes.

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