Profiling the Yeast Communities of Wine Fermentations Using Terminal Restriction Fragment Length Polymorphism Analysis

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Abstract: Terminal restriction fragment length polymorphism (TRFLP), used most often to describe bacterial communities, presents a high-throughput, low-cost solution for analyzing mixed yeast communities in wine and other fermentations. In this study, a TRFLP approach was developed for the identification and discrimination of yeasts and used to construct a TRFLP database comprising 121 strains of yeast (representing 24 genera and 72 species) associated with wine and food fermentations. This database exhibits sensitive discrimination among species and robust intraspecific conservation of TRFLP profiles, enabling reliable characterization of mixed yeast communities. The yeast ecology of sweet, botrytized wine fermentations from two separate vintages was analyzed using this database, demonstrating the utility of this method for fast-paced, qualitative detection and identification of differences in yeast community structures over time in a complex, diverse fermentation system.

Key words: TRFLP, culture-independent methods, community profiling

Wine fermentations typically involve a complex, multispecies consortium of yeasts. The most important are *Saccharomyces* species, but yeasts of the genera *Hanseniaspora*, *Kluyveromyces*, *Metchnikowia*, *Candida*, *Zygosaccharomyces*, *Brettanomyces*, and many others are often present and active at different stages of the fermentation, introduced either from grape surfaces or from the winery environment (Boulton et al. 1996). Most of these yeasts are considered beneficial or benign, but several are among the most destructive contaminants in the wine industry, spoiling wines through the production of off-flavors, haze/films/sediment, or gas production (Boulton et al. 1996). Thus, characterizing these communities requires techniques that enable rapid, sensitive discrimination of yeast species in musts and wines.

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Acknowledgments: This work was supported in part by grants from the Oregon Wine Board and the California Competitive Grants Program for Research in Viticulture and Enology to DM. NB was supported in part by the D.E. Gallo educational enhancement fund, A.L. & R.C. Heck research fellowship, Wine Spectator scholarship, R.C. Briess scholarship, M.J. Lewis endowment, and Jastro & Shields fellowship. KBM was supported in part by NIH award 1U01TW008160 and USDA-AFRI 2008-35621-04750.

The authors thank Greg Allen (Dolce Winery, Oakville, CA) for the generous contribution of wine samples and critical review of the manuscript.

Manuscript submitted Aug 2011, revised Sept 2011, accepted Nov 2011. Publication costs of this article defrayed in part by page fees.

doi: 10.5344/ajev.2011.11077

Traditionally, culture-based enrichment methods are used for detection and identification of yeasts in wine, but these methods can be time-consuming and can misrepresent the community present by biasing for the growth of less fastidious organisms (Heard and Fleet 1986), and the occurrence of viable-but-not-culturable (VBNC) cells in the low-pH, high-ethanol environment of wine can limit the sensitivity of these methods (Millet and Lonvaud-Funel 2000). To surmount these issues, culture-independent methods for comprehensively profiling wine microbiota have received more attention, with denaturing gradient gel electrophoresis (DGGE) the most popular (Cocolin et al. 2000, 2001, Mills et al. 2002). However, community profiling using DGGE is complicated by a number of technical issues related to gel-based separation (Nubel et al. 1996, Sekiguchi et al. 2001, Kisand and Wikner 2003), but above all DGGE is a technically challenging method that requires a high level of skill and ultimately only separates different strands, necessitating a gel extraction/sequencing step to complete identification. These issues limit the application of this technique in situations that require high-throughput processing of large and ecologically diverse sample sets, such as multivintage, multitreatment studies of wine fermentations.

Terminal restriction fragment length polymorphism (TRFLP) is another culture-independent profiling method that, like DGGE, has become popular for characterizing complex environmental communities. This method (Figure 1) enables sensitive discrimination of mixed microbiota based on restriction site heterogeneity of hypervariable DNA sequences (most typically rRNA genes) amplified using fluorescently labeled PCR primers. As only the terminal fragments retain the fluorescent tag, each organism present should be represented by one fluorescent peak. However, fragment size parity among taxonomically similar organisms for a given restriction enzyme can complicate identification (Liu et al. 1997),

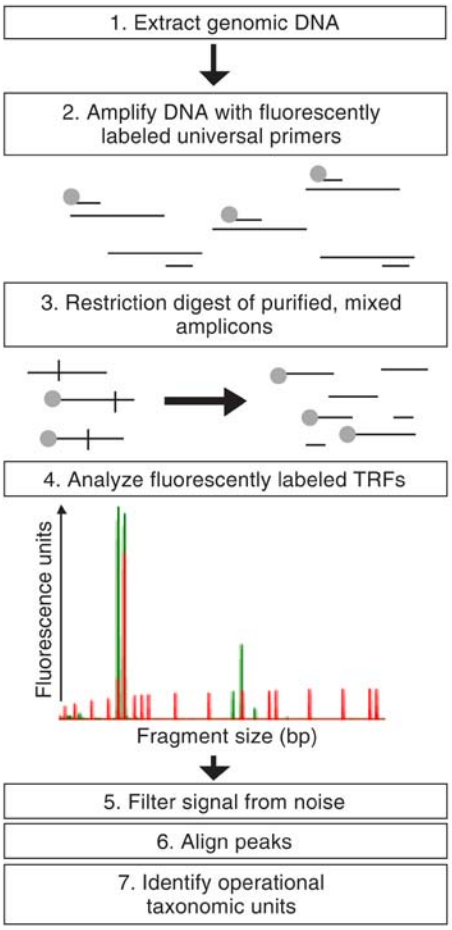

Figure 1 Process flow schematic for terminal restriction fragment length polymorphism.

and in practice these are grouped as operational taxonomic units (OTU) matching the terminal restriction fragment (TRF) profile. TRFs are compared to a database of known TRF profiles to unravel community structure. For identifying bacteria, this database is most commonly derived by *in silico* digests of 16S rRNA sequences deposited in public databases. However, studies that use TRFLP to describe complex fungal communities in soils and other environments (e.g., Adams Krumins et al. 2009, Edwards and Zak 2010, Macdonald et al. 2008) have instead relied on TRFLP as a tool to describe large-scale shifts in fungal diversity as observed by changes in TRF profiles, but not for identification of individual OTUs against a reference database, providing specific community structure information. That is partly due to the paucity of complete fungal sequence data deposited in public databases, making putative identification of most OTUs impossible. Nor does a comprehensive fungal TRFLP database exist, which would provide more reproducible identification of OTUs, because of the variability of restriction sites among members of the same genus/species (Anderson and Cairney 2004). Thus, the creation of a robust TRFLP database is essential for the adoption of this technique to study fungal communities.

TRFLP presents a promising solution for community profiling in wine with the following advantages over other profiling techniques: (1) it is adapted for high-throughput sample processing and thus can describe variation in large time-based and multivariate-treatment-based studies; (2) it is a robust, sensitive method that does not require additional sequencing steps following OTU separation (as with DGGE); and (3) it is a low-cost, relatively low-tech method providing fairly sensitive data when characterizing communities with low-to-moderate diversity, such as wine and food fermentations. TRFLP is not a perfect method, and as with any PCR-based method there are potential limitations of primer binding bias (Leuders and Friedrich 2003), operon copy number heterogeneity (Crosby and Criddle 2003), and polymerase error (Osborn et al. 2000), although many of these issues can be ameliorated by procedural modification (Polz and Cavanaugh 1998, Osborn et al. 2000). When conducted with appropriate methodological and statistical treatment, TRFLP has been shown to yield reliable, accurate, pseudo-quantitative representations of complex microbial community structure (Blackwood et al. 2003, Leuders and Friedrich 2003, Liu et al. 1997, Osborn et al. 2000, Schutte et al. 2008, Trotha et al. 2002).

However, there are no studies to date that have considered TRFLP for analysis of wine/food fermentation microbial ecology or that have developed/optimized a method for characterization of yeast ecology of wine/food microbial communities using TRFLP. For the purposes of this study, a yeast TRFLP profile database was generated from 121 different strains of yeast (representing 24 genera and 72 species) common in wines and fermented foods. This database was created targeting the internal transcribed spacer (ITS) region of the fungal rRNA gene. This noncoding spacer region is one of the most frequently chosen molecular targets for identifying fungi because of its polymorphism, allowing for subtle differentiation of species (Gardes and Bruns 1993). Method robustness was assessed by testing TRFLP profile consistency among six strains of *Saccharomyces cerevisiae* and nine of *Candida zemplinina* and the detection threshold for minority species in mixed culture titrations. The yeast ecology of sweet, botrytized wine fermentations was then analyzed using this database and results for specific populations validated by quantitative PCR (qPCR). Results demonstrate the utility of TRFLP for characterizing the yeast communities present in wine fermentations with a high degree of sensitivity.

Materials and Methods

DNA extraction. Must/wine samples collected from Dolce Winery (Oakville, CA) were immediately frozen on site, transported on ice, and stored at -80°C until processing. Two separate vintages were sampled: 2008 (inoculated with *S. cerevisiae*), sampled directly from the press pan (day 0) and after 1, 6, 19, and 51 days of fermentation, and 2009 (native fermentation), sampled directly from the press pan (day 0), and after 6, 9, 10, 17, 21, 26, 32, 38, 49, 59, and 66 days of fermentation. Frozen samples were thawed and centrifuged at 8,000 × *g* for 15 min and decanted, retaining the cell pellet, which was processed immediately. All wine samples were extracted and analyzed in duplicate.

Pure yeast cultures used for construction of the ITS TRFLP database were obtained from the Phaff Yeast Culture

Collection, University of California (UC) Davis, and the Viticulture and Enology Culture Collection, UC Davis, and grown in YEPD (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, and 2% [wt/vol] glucose) at room temperature. Whole cultures were centrifuged at 8,000 × *g* for 15 min and decanted, retaining the cell pellets, which were stored at -80°C until processing.

From the resulting cell pellet, 100 µL were washed three times by suspension in 1 mL ice-cold PBS, centrifugation at 8,000 × *g* (5 min), and the supernatant discarded. The pellet was then suspended in 200 µL DNeasy lysis buffer (20 mM Tris-Cl [pH 8.0], 2 mM sodium EDTA, 1.2% Triton X-100) supplemented with 40 mg/mL lysozyme and incubated at 37°C for 30 min. From this point, the extraction proceeded following the protocol of the Qiagen Fecal DNA Extraction Kit protocol (Qiagen, Valencia, CA), with the addition of a bead-beater cell lysis step of 2 min at maximum speed following addition of "buffer ASL" using a FastPrep-24 bead-beater (MP Biomedicals, Solon, OH). DNA extracts were stored at -20°C until further analysis.

TRFLP. PCR amplification was performed in 50 µL reactions containing 5 to 100 ng/µL of DNA template, 25 µL 2X Promega GoTaq Green Master Mix (Promega, Madison, WI), 1 mM $MgCl_2$, and 2 pmol of each primer. Each PCR was performed in triplicate and the products combined prior to purification to limit the potential for PCR amplification bias.

For amplification of the ITS1/ITS4 domain, the forward primer used was ITS1HEX (5'-[5HEX] TCCGTAGGTGAACCTGCGG-3') and the reverse primer was ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCR conditions were an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 7 min. All amplifications were performed in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA). PCR products were analyzed by electrophoresis on a 1% (wt/vol) agarose gel in 1X TAE stained with Gel Red (Biotium, Hayward, CA) and visualized under UV light. A 100-bp DNA ladder (New England BioLabs, Ipswich, MA) was used as a size standard. All PCR products were purified using QIAquick PCR Purification Kit (Qiagen), following the manufacturer's instructions.

Purified PCR products were digested using the restriction enzymes HaeIII, DdeI, and HinfI. The digested DNA was submitted to the UC Davis College of Biological Sciences Sequencing Facility. Traces were visualized using Peak Scanner Software v1.0 (Applied Biosystems) using a baseline detection value of 10 fluorescence units. Peak filtration and clustering was performed with R software (http://www.R-project.org/) using the program scripts and statistical analysis protocols designed by Abdo et al. (2006).

The TRF profiles of the pure yeast cultures were used to create the ITS TRFLP database (Table 1). Putative species assignments of the unknown fragments in the wine must samples were then made by referencing this ITS database. TRF profile consistency was tested by analyzing six strains of *S. cerevisiae* and nine of *C. zemplinina.* TRFLP detection sensitivity was tested by analyzing mixtures containing decreasing concentrations of *Pichia membranifaciens* titrated against a known, constant concentration of *S. cerevisiae.*

Quantitative PCR. Quantitative PCR was performed in 20-µL final volume reactions containing 2 µL DNA template, 0.2 µM each respective primer, and 10 µL Takara SYBR 2X Perfect Real Time Master Mix (Takara Bio Inc., Otsu, Japan). The primers YEASTF (5'-GAGTCGAGTTGTTTGGGAATGC-3') and YEASTR (5'-TCTCTTTCCAAAGTTCTTTTCATCTT-3'), producing a 124-bp fragment, were used to enumerate total yeast (Hierro et al. 2006). Reaction conditions involved an initial step at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C, and 30 sec at 72°C, followed by a melt curve to assess amplicon specificity. Cell concentration was calculated by comparing sample threshold values (C_T) to a standard curve of serially diluted genomic DNA extracted from a known concentration of *S. cerevisiae*. The primers SC1 (5'-GAAAACTCCACAGTGTGTTG-3') and SC2 (5'-GCTTAAGTGCGCGGTCTTG-3') were used to enumerate total *Saccharomyces* present, and the primers CZ2 (5'-CTTGGGTGTCGAAAGGCG-3') and CAST (5'-CAATATGCGTTCAAAAATTCAAT-3') for *Candida zemplinina* (Zott et al. 2010). Reaction conditions involved an initial step at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 1 min at 63°C (*S. cerevisiae*) or 62°C (*C. zemplinina*), and 30 sec at 72°C, followed by a melt curve to assess amplicon specificity. Cell concentration was calculated by comparing sample threshold values (C_T) to a standard curve of serially diluted genomic DNA extracted from a known cell concentration of *S. cerevisiae* or *C. zemplinina*.

All reactions were performed in triplicate in optical-grade 96-well plates on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems). The instrument automatically calculated cycle threshold (C_T), efficiency (E), and confidence intervals.

Results

One of the main goals of this work was to develop a TRFLP database specifically targeted for wine and food yeast discrimination. The TRF profiles for 121 yeast strains were compiled to develop the yeast TRFLP database (Table 1). This database presents the TRF sizes resulting from each restriction digest for each yeast species. Where multiple TRF sizes exist for a single species (e.g., due to heterogeneity among multiple ITS copies within the yeast genome), values given in boldface type indicate the major (i.e., most abundant) TRF size for a given cut. To use the database, sample TRF sizes from each restriction digest are compared to the appropriate column. A species is considered "present" if all the corresponding major TRFs are found in a sample. Minor TRFs appearing for each yeast are provided in the database to aid filtration of these supplemental peaks from true, unknown TRFs and to limit the overestimation of sample diversity. The presence of these minor TRFs should not hinder data processing, as in most cases they do not match the major TRF sizes of other yeasts in the database, so remain unique. Additionally, these minor TRFs

Table 1 Wine yeast internal transcribed spacer terminal restriction fragment database.

Yeast	Strain[a]	HaeIII	HinfI	DdeI
Brettanomyces anomalus	VEN2066	**405**[b], 375	**218**, 437	**435**
Brettanomyces bruxellensis	04-212	368	203	462
Brettanomyces custersianus	VEN2027	**442**, 408	**208**, 193, 442	**443**
Brettanomyces/Dekkera anomalus/anomala	VEN2826	407	220	437
Brettanomyces/Dekkera bruxellensis	VEN2079	**367**, 341, 462	**202**, 461	**462**, 427
Brettanomyces/Dekkera bruxellensis	VEN2807	**367**, 341, 461	**205**, 190, 461	**462**, 427
Candida apicola	69-111	400	**221**, 230, 341, 454	**454**, 417
Candida boidinii	72-163	**690**, 636	383	**691**, 637
Candida bombi	54-194	369, **396**	202, **218**	**452**, 418
Candida carphphila	74-101	112	281	390
Candida ethanolica	81-102	276, **297**	195	251
Candida guilliermondii	65-4	112, 103	281	**389**, 214, 44
Candida krusei	40-371	372	213	499
Candida krusei	40-274	370	213	498
Candida lactis-condensi	68-139-	405	232	59
Candida lipolytica	79-100.4	357	170	357
Candida maritima	68-692.2	**568**, 401, 523, 649	**316**, 193, 225, 293	**197**, 181, 495
Candida oleophila	62-4	129	307	112
Candida oleophila	68-35	120, **129**	307	**112**, 613
Candida sake	51-18	402, **435**	204	90
Candida salmanticensis	71-181	420	420	421
Candida solani	51-36	**619**, 547	276	129
Candida stellata	59-13	**401**, 371, 455	**220**, 204	**455**, 419
Candida tropicalis	54-195	**441**, 406	250	405
Candida versatilis	51-32	417, **454**	232	456
Candida zemplinina	72-1034	457	231	59
Candida zemplinina	VEN2097	**456**, 422	**234**, 214, 454	**60**, 457
Candida zeylanoides	40-019	**129**, 119, 553	307	**112**, 415
Candida zeylanoides	62-1033	**129**, 119, 98, 554	**300**, 224, 316	**106**, 94, 416
Clavispora lusitaniae	80-84	377	177	377
Cryptococcus kuetzingii	68-196	456, **498**	267	97
Cryptococcus laurentii	76-106	531	50	371
Cyberlindnera veronae	68-1000.1	**125**, 115, 530	296, **235**, 274, 305, 608	**32**, 83, 610
Debaryomyces hansenii var. *hansenii*	40-56.	124, **134**	313	420
Debaryomyces hansenii var. *hansenii*	40-59	123, **134**, 569	313	420
Debaryomyces pseudopolymorphus	57-3	**136**, 126, 566	**301**, 227, 316	**417**, 241, 392
Dekkera bruxellensis	82-30	365	202	461
Dekkera bruxellensis	77-105	110, 341, **366**	204	461
Hanseniaspora guilliermondii	40-023	809	154	158
Hanseniaspora guilliermondii	05-782	**807**, 274	**153**, 143, 215, 566	**157**, 521
Hanseniaspora uvarum	54-192	802	153	**157**, 444
Hanseniaspora uvarum	55-310	**805**, 271	**153**, 140, 214, 402	**157**, 88, 444
Hanseniaspora valbyensis	68-28	500, **793**	131, **160**, 401	125, **135**
Issatchenkia occidentalis	75-63	274, **299**	**87**, 197, 443	409, **443**
Issatchenkia occidentalis	52-171	273, **295**	178, **192**	249
Issatchenkia orientalis	05-784	**373**, 348	**215**, 197, 343, 350	**499**, 462
Issatchenkia terricola	99-57.1	262, **282**	88	381, **411**
Issatchenkia terricola	56-108	282	87	412
Kloeckera apiculata	94-155	**271**, 315	**153**, 401	**157**, 443
Kluyveromyces dobzhanskii	50-45	70	99, 344	555
Kluyveromyces dobzhanskii	51-200	70, 65	76, 344	556
Kluyveromyces lactis	72-12	70	56	**554**, 454, 500, 274
Kluyveromyces lactis var. *lactis*	70-4	70	52, 56, 342	554
Kluyveromyces marxianus	40-351	70	55	554
Kluyveromyces marxianus	50-84	70	56	553
Kluyveromyces thermotolerans	55-41	**78**, 171	336	442, **488**
Kluyveromyces thermotolerans	40-185	79	336	488
Kluyveromyces waltii	72-13	79	336	488
Kluyveromyces waltii	72-42	79	**336**, 95	488
Lachancea thermotolerans	51-208	**79**, 171	336	488
Lanchanea thermotolerans	40-193	79	337	488

Table 1 *(cont.)* Wine yeast internal transcribed spacer terminal restriction fragment database.

Yeast	Strain[a]	HaeIII	HinfI	DdeI
Metschnikowia pulcherrima	40-118	277	174	375
Metschnikowia pulcherrima	60-317	253, **273**	172	372
Pichia angophorae	65-106	668	396, 168?	186
Pichia angophorae	65-106.3	668, 185	**395**, 368, 404	**185**, 173
Pichia anomala	78-6	658	296	**432**, 394
Pichia anomala	40-128	655	297	433
Pichia burtonii	76-51	440	191	304
Pichia fermentans	76-39	**326**, 299	**190**, 175	246
Pichia guilliermondii	05-580	112	281	390
Pichia guilliermondii	05-746	112	281	389
Pichia kluyveri	53-50	333, **357**	**196**, 182, 435	**90**, 255, 382, 435
Pichia kluyveri	65-15	356	**196**, 182, 435	**91**, 255
Pichia kluyveri var. *kluyveri*	40-117	357	196	91, 252, 435
Pichia kluyveri var. *kluyveri*	40-324	333, **357**	182, **196**	91
Pichia membranifaciens	77-100	**320**, 326	200	255
Pichia membranifaciens	78-5	**320**, 295	**200**, 184	**256**, 236
Pichia opuntiae var. *thermotolerans*	77-448	460, 470	460, 470	460, 470
Pichia pinus	52-84	220	420	**665**, 167, 353
Pichia populi	68-628.3	**60**, 455	311	385
Pichia populi	68-603	60	**307**, 283, 314	**138**, 127
Pichia thermotolerans	80-203	**477**, 320	**478**, 320	**477**, 320
Rhodotorula glutinis var. *glutinis*	40-30	**561**, 640	261	34, **354**
Rhodotorula mucilaginosa	40-141	398	55	385
Rhodotorula mucilaginosa	40-188	398	**55**, 264	385
Saccharomyces bayanus	74-42	115, **125**	113	709
Saccharomyces bayanus	75-43	126	114	710
Saccharomyces cerevisiae	50-149	113, **124**	111	709
Saccharomyces cerevisiae	40-148	**124**, 298	**112**, 474	710
Saccharomyces cerevisiae	40-119	114, **124**	112	708
Saccharomyces exiguus	53-63	444, **484**	340	450
Saccharomyces exiguus	40-043	444, **484**	340	449
Schizosaccharomyces japonicus var. *versatilis*	71-26.	149	301	**701**, 233, 380
Schizosaccharomyces japonicus var. *japonicus*	60-255.1	136, **149**	300	233, 380, 611, **645**
Schizosaccharomyces pombe	65-116	411	409, **499**	411
Schizosaccharomyces pombe	70-49	410	495	409
Sporobolomyces roseus	67-302	531	290	**119**, 110, 646
Starmarella bombicola	62-133	323	223	**270**, 325, 464
Torulaspora delbrueckii	40-130	705	406	705
Torulaspora globosa	68-37	Not detected	426	101
Torulaspora pretoriensis	61-6	706	**120**, 407	706
Torulaspora pretoriensis	66-16	705	**120**, 407	705
Trichosporon pullulans	60-83	109	40	410
Zygoascus hellenicus	81-681	574, **650**	157	644
Zygosaccharomyces bailii	68-113	83	45	231
Zygosaccharomyces bailii	72-1032	**83**, 158, 192	45	236
Zygosaccharomyces cidri	75-9	Not detected	326	409
Zygosaccharomyces rouxii	40-293	**476**, 74, 80	**340**, 315, 347	**874**, 270, 371, 728
Zygotorulaspora florentina	66-12	600	246	437
Zygotorulaspora florentina	67-559	581	580	440

[a]For strain number: VEN denotes UC Davis Viticulture and Enology Culture Collection as source. All others obtained from UC Davis Phaff Yeast Culture Collection.
[b]**Bold** type indicates major peak for given restriction digest.

appear only when the associated yeast is highly abundant, as they typically appear at a very low abundance relative to the major TRF, so will not appear under most circumstances.

Most yeasts in the database can be distinguished by the HaeIII restriction digest alone (although all three digests should be performed to confirm findings). In only a few cases are genera/species not distinguished by all three digests, as with some closely related species, most notably *S. cerevisiae* and *S. bayanus*, which cannot be resolved by this technique, and teleomorphs from their anamorphs, such as *Candida krusei* and *Issatchenkia orientalis*. However, strains could not be distinguished within species, as demonstrated by analyzing multiple geographically disparate strains of *S. cerevisiae* and *C. zemplinina* (Table 2), although TRF base

pair lengths occasionally fluctuate slightly due to run-to-run variation in peak size calling. Within the database, two strains were analyzed for most yeast species, demonstrating the same result in most cases. The only exception was *Zygotorulaspora florentina*, which displayed high intraspecies heterogeneity, so while the strains tested do have unique TRF profiles, they had divergent TRF profiles for one or more restriction digests, and thus greater diversity may be expected for other strains of this species. However, this isolated example aside, the intraspecies consistency and interspecies separation of other yeasts in the database demonstrate that TRFLP is a robust means of distinguishing genera/species, as strain variation will not result in the detection of unknown peaks.

Although species could be robustly differentiated, a concern with this method was that an overabundance of *Saccharomyces* in a wine environment would quench the detection of minor yeast populations, thus limiting the sensitivity of this method for profiling complex fermentations. Mixtures of a constant concentration of *S. cerevisiae* (1×10^7 cells/mL) with serial dilutions of *P. membranifaciens* ($1 \times 10^6 - 1 \times 10^2$) were analyzed using TRFLP to determine whether the presence of a majority organism would suppress detection of a minority yeast and to determine the accuracy of the relative quantification provided by this method. The minority yeast, *P. membranifaciens*, could be detected down to 1×10^2 cells/mL (the detection threshold limit) in the presence of a high concentration of *Saccharomyces* (Figure 2A, B, C). This limit was separately determined by analyzing serial dilutions of a 1:1 mixture of known cell concentrations of *S. cerevisiae* and *P. membranifaciens*. Electropherograms of solutions of 1×10^4, 1×10^3, and 1×10^2 cells/mL were constructed (Figure 2D, E, F), and the latter was determined as the limit of detection, as a 1×10^1 cells/mL mixture had no detectable peaks.

To validate this technique and database for describing community structure in actual wine fermentations, TRFLP was used to study the microbial ecology of a sweet, botrytized wine (Dolce, 2009 vintage, native fermentation) from 0 (press pan sample) through 66 days of fermentation (Figure 3A). Results show that TRFLP could sensitively detect shifts in the populations and could successfully identify most of the organisms present. At crush (time 0), the community was dominated by *Hanseniaspora* spp. (35% of the relative population) and a TRF profile putatively identified as *Botrytis cinerea* (20%), as well as two other OTUs putatively identified as *Penicillium* spp. (18%) and *Cladosporium* spp. (3%). These three mold OTUs declined rapidly after fermentation began, as expected. *Candida zemplinina*, detected only as a trace (0.3%) at crush, steadily grew concurrent with the decreasing population of *Hanseniaspora*. By day 32, *C. zemplinina* surpassed *Hanseniaspora* as the dominant yeast present and constituted 42% of the population at day 66. *Saccharomyces* only appeared at day 26 (1% relative population) and quickly grew from there, constituting 37% of the population at day 49, existing briefly as the most dominant yeast, but only 21% of the population at day 66. *Metchnikowia pulcherrima*, *Candida lactis-condensi*, and *Pichia angophorae* were also detected at low levels (<20% combined) fluctuating throughout the monitoring period.

Table 2 Yeast strain consistency trials.

	Restriction enzyme[a]	
Strain number/species	**HaeIII**	**HhaI**
148 *S. cerevisiae*	**123**, 298	**112**, 474
684 *S. cerevisiae*	**123**, 298	**112**, 474
40-148 *S. cerevisiae*	123	112
40-119 *S. cerevisiae*	123	112
687 *S. cerevisiae*	123	112
1332 *S. cerevisiae*	**123**, 298	**112**, 474
04-103 *C. zemplinina*	456	234
04-112 *C. zemplinina*	456	234
05-786 *C. zemplinina*	456	234
06-105 *C. zemplinina*	456	234
06-143 *C. zemplinina*	456	234
06-144 *C. zemplinina*	456	234
06-149 *C. zemplinina*	456	234
06-150 *C. zemplinina*	456	234
06-151 *C. zemplinina*	456	234

[a]**Bold** values indicate most prominent TRF where multiple values are given.

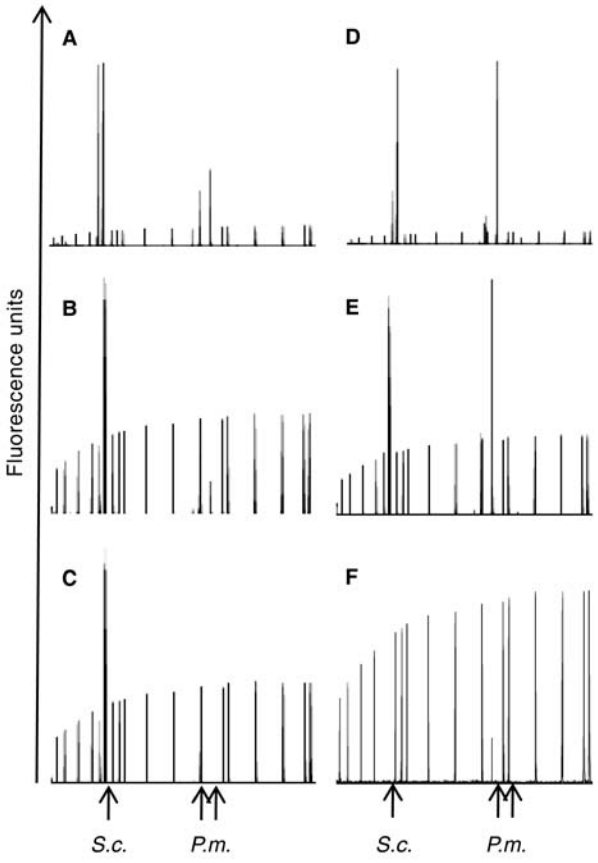

Figure 2 Electropherograms of decreasing cell concentrations of *P. membranifaciens* titrated against a known concentration of *S. cerevisiae*: (**A**) $10^7 : 10^6$ cells/mL (*S. cerevisiae* : *P. membranifaciens*); (**B**) $10^7 : 10^4$ cells/mL; (**C**) $10^7 : 10^2$ cells/mL. Electropherograms of decreasing cell concentrations of a 1:1 mixture of *S. cerevisiae* : *P. membranifaciens*, demonstrating TRFLP detection limit: (**D**) 1×10^4 cells/mL; (**E**) 1×10^3 cells/mL; (**F**) 1×10^2 cells/mL (*S.c.*: *Saccharomyces cerevisiae*; *P.m.*: *Pichia membranifaciens*).

Previous analysis of an earlier vintage of the Dolce fermentation revealed dominance of *Saccharomyces* (Mills et al. 2002). To confirm the relative populations of *C. zemplinina* and *S. cerevisiae* observed in 2009 using TRFLP (early dominance of *C. zemplinina* and late appearance of *Saccharomyces*), we used qPCR to detect total yeast, *C. zemplinina*, and *Saccharomyces* spp. (Figure 4A). Total yeast cell concentrations gradually declined from a maximum of 9.1×10^6 cells/mL ($\pm 7.7 \times 10^5$) at day 0 to 4.7×10^4 cells/mL ($\pm 5.4 \times 10^3$) by week 66. This qPCR assay, designed to quantify wine yeasts by targeting a section of the D1/D2 loop of the 26S rRNA gene (Hierro et al. 2006) most likely detects molds and other similar fungi as well, hence the maximal detection at crush (parallel with the mold-associated TRFs detected by TRFLP). Total *Saccharomyces*, in turn, were detected at $8.1 \times 10^2 \pm 1.4 \times 10^2$ cells/mL at crush and remained at these levels for the first 2 weeks ($1.8 \times 10^3 \pm 1.8 \times 10^1$ cells/mL at day 10). Only at day 17 did this yeast reach a concentration of 2.0×10^5 cells/mL ($\pm 1.4 \times 10^2$), less than 5% of the total yeast present. *Saccharomyces* peaked at 1.3×10^6 cells/mL ($\pm 4.1 \times 10^4$) at day 32 (~50% of the total yeast) and declined thereafter, remaining at ~50 to 75% of the total yeast population detected. *C. zemplinina*, on the other hand, was detected at higher levels at crush ($2.4 \times 10^3 \pm 1.7 \times 10^4$ cells/mL) and increased from there to peak at 7.8×10^5 ($\pm 4.9 \times 10^4$) cells/mL at day 26. After day 26, this population remained stable as the total yeast count fell, accounting for a greater proportion of the yeast community from days 32 to 66. After day 49, the population continued to decline, while remaining at ~50% of the total yeast count. Brix gradually fell from 34 Brix at crush to 13.25 Brix at day 66 (Figure 4B). By the time *Saccharomyces* emerged at day 17, the fermentation had already reached 19.5 Brix.

A second vintage (2008, inoculated with a strain of *S. cerevisiae*) of the Dolce fermentation was analyzed using TRFLP, demonstrating the robust ability of this technique for describing community shifts in large-scale, vertical and lateral studies. Results show a similar cast of yeasts as observed in the 2009 vintage (Figure 3B). Two separate press pan samples (representing grapes from two different vineyards blended prior to fermentation) were tested, indicating a large presence of *B. cinerea* (33–37%), *M. pulcherrima* (14–38%), *Hanseniaspora* (3–37%), and *C. zemplinina* (1–11%) but no *Saccharomyces*. On pumping from the press pan, all samples were inoculated with *S. cerevisiae*, hence the sudden dominance of this yeast at day 1 (44%). *Botrytis cinerea, Hanseniaspora*,

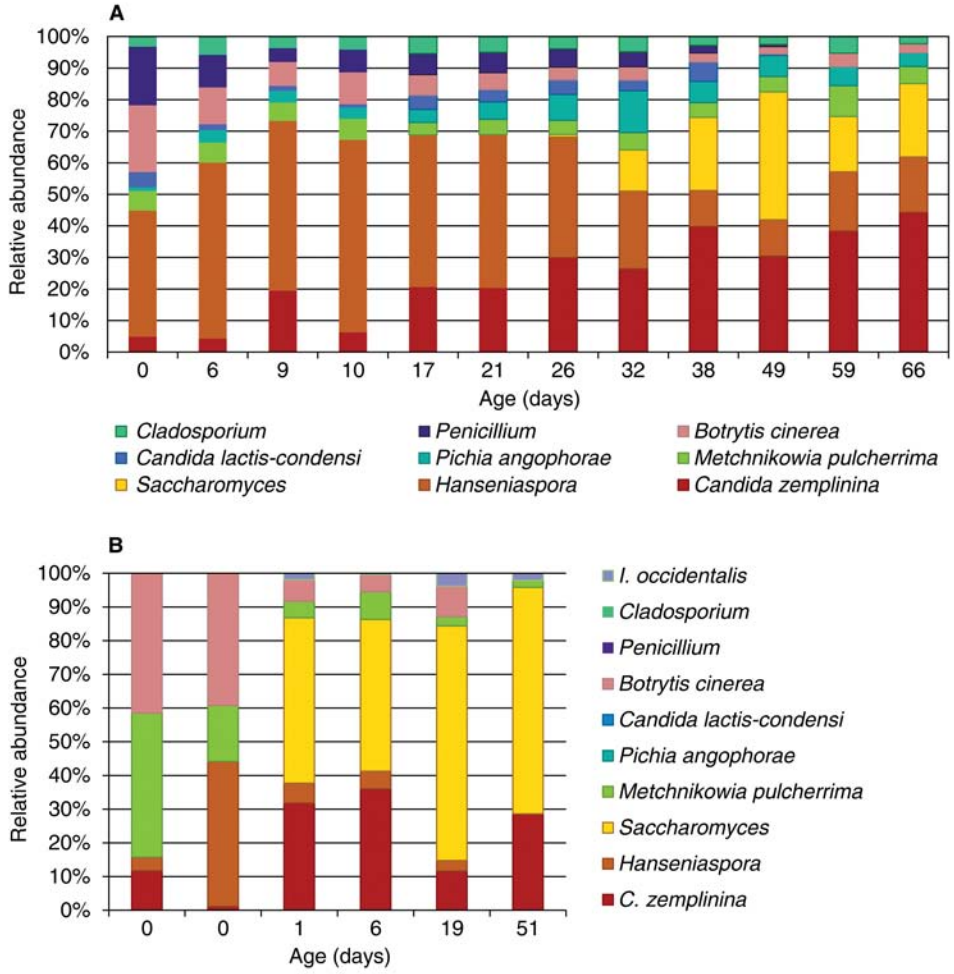

Figure 3 Relative populations of yeasts identified in the Dolce fermentation over time determined by TRFLP. Relative populations are determined as ratios of specific peak fluorescence to total filtered peak fluorescence. All samples were tested in duplicate and data are average values. (**A**) 2009 vintage; (**B**) 2008 vintage.

and *M. pulcherrima* constituted minor constituents from day 1 through day 51, but *C. zemplinina* still thrived in this fermentation even following inoculation, growing to 28% of the total population at day 1 and fluctuating from 11 to 34% of the total population through day 51. *Issatchenkia occidentalis* was also found at very low levels (0.4–3.0%) in this vintage from day 1 through 51 (but not in the press pan).

qPCR was again used to confirm the relative populations of yeasts observed using TRFLP, and corroborated this trend (Figure 4C), detecting 2.7×10^7 cells/mL ($\pm 1.0 \times 10^6$) of total yeasts in one of the press pan samples, but only 3.2×10^3 cells/mL ($\pm 6.0 \times 10^1$) of *Saccharomyces*. Following inoculation, total yeasts remained >10^7 cells/mL through day 6, ~50% of which was *Saccharomyces*, before dropping into the 10^6 cells/mL range from day 19 through 51. *Saccharomyces* was detected at 28% of the total population at day 19 and 60% at day 51. *Candida zemplinina* was detected at 4.6×10^4 cells/mL ($\pm 1.6 \times 10^2$) in the press pan and increased dramatically to 1.4×10^6 cells/mL ($\pm 2.9 \times 10^4$) after day 1 of fermentation. After day 1, this population slowly declined, but remained a significant contingent of the yeast community, detected at 3.0×10^5 cells/mL ($\pm 2.0 \times 10^3$) on day 51. The sugar concentration measured at crush was 36 Brix and, after six days of lag, gradually declined to a final concentration of 12.25 Brix (Figure 4D).

Discussion

The yeast TRFLP database described in this work was a necessary prerequisite for the application of TRFLP for accurate identification of yeasts in wine and, indeed, any other environment, due to the lack of yeast ITS sequence data deposited in public databases and to the inclusion of numerous, variable ITS copies within the genomes of individual yeast strains, leading to a disparity between empirical TRF patterns and *in silico* predictions (Anderson and Cairney 2004). The ITS spacers are noncoding regions, accumulating mutations more quickly than vital coding regions, such as the flanking 18S and 26S rRNA genes that are also commonly used for identification of fungal isolates. For this reason, the ITS region was chosen as a target for this TRFLP assay, as the 18S and 26S rRNA are not sufficiently divergent to allow restriction-enzyme-based discrimination of closely related yeast species using TRFLP. As shown in this work, the ITS region presents an optimal site for TRFLP analysis, containing enough intraspecies similarity to afford reliable identification but enough intragenus heterogeneity to effectively separate most genera into species.

The molds detected in the 2009 vintage Dolce fermentation, *B. cinerea*, *Penicillium* sp., and *Cladosporium* sp., were putatively identified via *in silico* predictions based on restriction mapping, the same approach used for OTU identification

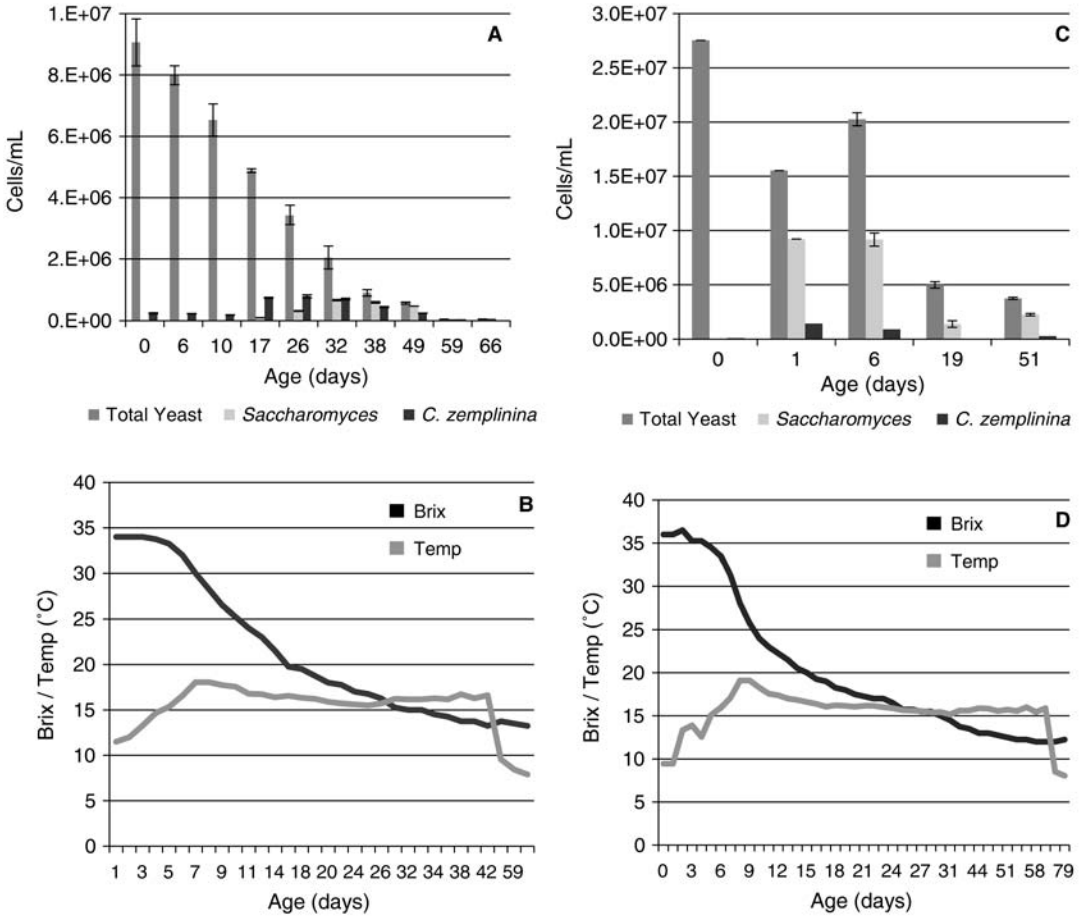

Figure 4 Quantitative PCR of Dolce fermentation over time in 2009 (**A**) and 2008 (**C**). All samples were tested in triplicate and their mean values (± standard deviation) are presented. Brix and temperature measurements for the 2009 (**B**) and 2008 (**D**) vintages.

of bacterial TRF profiles (Liu et al. 1997). The TRF profiles associated with these molds were abundant at crush (together ~42% of the total population) but rapidly diminished once fermentation began. However, even though no other taxonomic group could be matched to these TRF profiles, these are not definitive identification, as these organisms are not present in the ITS TRFLP database. This database was initially constructed to identify only organisms actively involved in wine fermentations; as most molds and nonfermentative yeasts presumably go dormant/die once grapes are crushed, fermentation begins, and oxygen availability declines, they were not considered in this category and were not included in the database. This is one major limitation of the current method, which will be corrected as the database continues to expand.

Although TRF abundance has been shown to correlate strongly with relative population abundance within a sample (Lueders and Friedrich 2003), giving a reproducible perspective of community structure (pseudo-quantitatively), it should not be assumed that TRFLP can be used as a stand-alone method for absolute cell quantification. Like any PCR-based method, TRFLP is prone to errors such as primer binding bias (mitigated by combined, replicate PCRs and increased DNA template concentration; Polz and Cavanaugh 1998) and copy number heterogeneity (Crosby and Criddle 2003), which can skew the apparent abundance of community members. Reliable quantification requires the use of qPCR or other quantitative methods targeting populations of interest identified by TRFLP data. Thus, qPCR was used to confirm the accuracy of the pseudo-quantitative TRFLP results, specifically the relative populations of *S. cerevisiae* and *C. zemplinina*, the most abundant yeasts observed. As presented above, qPCR recovered relative population abundance data comparable to those revealed by TRFLP, indicating that TRFLP can reliably represent yeast community structure in wine. These data correlate fairly well with studies of older vintages of the Dolce fermentation using DGGE (Cocolin et al. 2001, Mills et al. 2002), in which *C. zemplinina* was the dominant yeast throughout the early fermentation and persisting throughout, with *S. cerevisiae* starting to appear only later in the fermentation. However, this vintage (2009) displayed a much more dominant population of *C. zemplinina* and much less prominent population of *S. cerevisiae* than previous years, as shown by both TRFLP and qPCR.

There is abundant enology research using culture-dependent methods to describe complex fermentation communities and enological treatments. Such methods are innately problematic, biasing for those organisms which are best cultured in the microbiological medium of choice. Additionally, they can be extremely time-consuming and laborious, typically requiring a molecular method for identification of an organism once it is fully cultured. Over the past decade, molecular profiling methods have slowly gained recognition for their greater power, speed, and accuracy for enology studies compared to traditional methods, although they are not without their own flaws and the most current methods are often prohibitively expensive for such studies. This is where TRFLP can prove advantageous as a rapid, culture-independent, sensitive, and above all low-cost technique for yeast community profiling. TRFLP is not an error-free method, but this method would be beneficial applied to small- and large-scale studies involving time- and/or treatment-based variables because of its low cost and adaptability for high-throughput processing. Expansion of the database to include yeasts common to other fermentations would enable adaptation to almost any food or beverage scenario. Indeed, with appropriate additions to the database this method could be used to profile fungal communities in almost any environment, such as clinical or host-associated specimens. Grape- and vine-associated fungi could also be added to enable analysis of viticultural specimens, such as vine/berry surface microbiota or grapevine pathogens.

A key attribute of this method is its technical ease, relying on basic research laboratory procedures and tools, namely PCR and restriction digests, to generate data, so could be performed with access to a PCR thermal cycler. Subsequent analysis is carried out at standard DNA sequencing facilities (routinely available) for fragment size determination. In this regard, TRFLP could be integrated at wine-testing service laboratories and could even be applied at wineries capable of performing PCR and with external access to commercial fragment analysis. The capacity of this technique for sensitively identifying complex, mixed microbiota may be useful in commercial scenarios, enabling detection of potential spoilage organisms. TRFLP is best used for resolving mixed culture fermentations, but could even be applied to spoilage yeast detection in finished wines, especially if the microbiological status is unknown and multiple contaminants may be present (a possibility in unfiltered wines). This would enable detection and identification of microbes without time-consuming culturing and isolation steps. qPCR is better suited for enumeration of specific, targeted organisms (e.g., if *Brettanomyces* is the only contaminant of interest), but, while more quantitatively accurate, will miss those populations not targeted. Therefore, pairing qPCR with a community profiling method such as TRFLP provides a mechanism for catching the "missed" populations in a standard qPCR analysis.

Conclusion

TRFLP presents a valuable technique for profiling the yeast community of wine and other fermentations. This method, using the database described herein, can sensitively distinguish multiple populations in wine and their relative population sizes. TRFLP is a rapid, powerful, pseudo-quantitative technique for revealing community structure in complex, mixed-microbial fermentations and can detect minor populations in diverse, complex fermentations to a relatively low limit of detection (1×10^2 cells/mL). The speed, low cost, and sensitivity of this technique makes it a promising option for community profiling of wine and other fermentations, with direct applications to commercial wine-testing scenarios and to enological and viticultural research.

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