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Diversity and molecular determination of wild yeasts in a central Washington State vineyard

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Abstract: Yeasts were isolated from grapes collected from a research vineyard at the WSU-IAREC, located at Prosser, WA. Species determination was based on cultural features, microscopic morphology, physiological tests and analysis of ITS and D1/D2 rDNA sequence data. Fifty-three species were found distributed among five fungal subphyla, a greater number than expected based on similar published studies. Within Saccharomycotina, 13 species in the genera *Candida*, *Hanseniaspora*, *Metschnikowia*, *Meyerozyma*, *Pichia*, *Wickerhamomyces* and *Yamadazyma* were determined. Isolates within the *Metschnikowia pulcherrima* clade appeared to possess considerable diversity. Pucciniomycotina was represented by 12 species, in *Curvibasidium*, *Rhodospordium*, *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces*. Five phylogenetically distinct species in the subphylum could not be assigned to any described species. Isolates in Ustilaginomycotina were placed in *Pseudozyma* except for a single strain determined to be *Rhodotorula bacarum*. Within Agaricomycotina, 17 species in the genera *Cryptococcus*,

Cystofilobasidium, *Hannaella*, *Holtermanniella* and *Mrakiella* were determined. Seven species of yeast-like Pezizomycotina were found, representing classes Leotiomycetes, Dothideomycetes and Sordariomycetes. Isolates of *Aureobasidium pullulans* represented three phylogenetically distinct subspecific lineages. Seventeen species identified in this study were previously unreported from wine grapes and 18 species were unreported from North America. Several strains appear to represent undescribed species, including the recently described *Curvibasidium rogersii*.

Key words: fungi, yeast, grape, *Vitis*, phylogeny, biodiversity, systematics, molecular determination, ITS, D1/D2, rDNA, *Aureobasidium*, *Candida*, *Cryptococcus*, *Curvibasidium*, *Cystofilobasidium*, *Hannaella*, *Hanseniaspora*, *Holtermanniella*, *Metschnikowia*, *Meyerozyma*, *Mrakiella*, *Phaeococcomyces*, *Pichia*, *Pseudozyma*, *Rhodospiridium*, *Rhodotorula*, *Sporidiobolus*, *Sydowia*, *Wickerhamomyces*, *Yamadazyma*

Introduction: Wine grapes (*Vitis vinifera*) are the third most valuable fruit crop in Washington, and the state's wine grape production is the second largest in the USA following California (Anonymous, 2012). Continued growth of the Washington wine industry has led to an interest in the native yeast flora. These naturally-occurring yeasts likely affect the flavor characteristics of wines, both through activity in the vineyard and during vinification. While cultivated strains of the yeast species *Saccharomyces cerevisiae* Meyen ex E.C. Hansen usually are the primary agents of vinification (Bisson 2004), so-called non-*Saccharomyces* yeasts are always present in inoculated and in non-inoculated fermentations and may play important roles as spoilage organisms or by making positive contributions to finished wine (Loureiro & Malfeito-Ferreira, 2003; Jolly *et al.*, 2006; Ciani & Comitini, 2011). Winemakers may benefit from knowledge of local yeast diversity and a collection of local yeast strains. This paper represents the first study of yeasts associated with wine grapes in Washington that included sound grapes and employed molecular determination methods.

Yeast systematics underwent a revolution at the end of 20th century with the adoption of the D1/D2 domain of the large subunit (LSU) nuclear ribosomal DNA (rDNA) for purposes of molecular identification and phylogenetic

inference (Kurtzman & Robnett, 1998; Fell *et al.*, 2000), resulting in a rapid increase of described species that continues today (Kurtzman *et al.*, 2011b). Many basidiomycetous yeasts and filamentous fungi may be determined more precisely based on internal transcribed spacer (ITS) rDNA sequence (Scorzetti *et al.*, 2002), and this locus has been proposed as a “universal DNA barcode marker” for the kingdom Fungi (Schoch *et al.*, 2012). The ITS and D1/D2 loci are directly adjacent within the rDNA cistron and may be amplified and sequenced as a single amplicon. Accuracy and precision of molecular determination may be improved by combining the loci, given that molecular determination requires a locus that provides the appropriate ratio of inter- to intraspecific variation (i.e. the “barcoding gap”), which is known to vary between the ITS and D1/D2 loci (Scorzetti *et al.*, 2002; Schoch *et al.*, 2012). In the current study, yeasts were characterized and determined based on cultural and microscopic morphology, ITS and D1/D2 rDNA sequences and physiological attributes.

The goal of this study was to improve our understanding of the diversity of wild yeast occurring on *Vitis vinifera* in Washington State, USA.

Materials and Methods: The primary sampling site was a research vineyard at the

Washington State University (WSU) Irrigated Agriculture Research and Extension Center (IAREC) in Prosser, WA, where samples were collected on June 30, August 31, September 14 and October 26, 2010. Some additional samples were taken from a second site in Richland, WA at the WSU Tri-Cities campus vineyard on September 14, 2010. Two cultivars, 'Chardonnay' and 'Riesling,' were sampled at the Prosser site, and six, 'Chardonnay,' 'Riesling,' 'Gewürztraminer,' 'Cabernet Sauvignon,' 'Merlot' and 'Syrah' at the Tri-Cities site. Entire grape inflorescences were collected aseptically and stored in plastic bags at 4°C until processing, up to two weeks. Cell suspensions were made from samples collected on June 30 by rinsing entire inflorescences in 1% SDS (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) solution, while suspensions from August 31 samples were made by rinsing grape berries in 1% Triton-X11 (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) solution. Suspensions of samples collected September 14 and October 26 were made from macerated inflorescences. Suspensions were diluted and 0.1 mL inoculated onto 100 mm Wallerstein Labs nutrient agar (BD, Franklin Lakes, NJ, USA) plates with 100 mg L⁻¹ streptomycin sulfate (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and grown at 20–22°C. Representative colonies of yeast-like morphotypes were subcultured onto potato dextrose agar and streaked for isolation. Isolated strains were cryopreserved in 15% glycerol solution at -80°C.

Based on differences in culture morphology and microscopic features, 121 strains were selected for molecular determination from a total of 228 isolates. An additional five strains were isolated, characterized and sequenced to aid taxonomic assessment of *Pseudozyma* isolates. Strains were cultured on potato dextrose agar until sufficient growth was observed and DNA was extracted using the "FastDNA® Spin Kit" (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer's instructions. Regions of the rDNA containing the ITS and D1/D2 or D1-D3

LSU domains were amplified using combinations of the primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) (Gardes & Bruns, 1993), ITS5 (GGAAGTAAAAGTCGTAACAAGG) (White *et al.*, 1990), ITS1 (TCCGTAGGTGAACCTGCGG) (White *et al.*, 1990), LR0R (ACCCGCTGAACCTTAAGC) (Moncalvo *et al.*, 2000), ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990), NL1 (GCATATCAATAAGCGGAGGAAAAG) (Kurtzman & Robnett, 1998), LR3 (CCGTGTTTCAAGACGGG) (Vilgalys & Hester, 1990) and TW14 (GCTATCCTGAGGGAAACTT) (Hamby *et al.*, 2008). Most commonly, the primer pair of ITS1-F and TW14 was used to produce an amplicon containing the entire ITS and D1-D3 region, approximately 1300–1700 bp in length. Reactions of 25 µL volume containing 50 ng DNA template, 1.0 µL each of forward and reverse primers (4.0 pmol µL⁻¹), 4.0 µL of dNTP (200 µmol L⁻¹ each dNTP), 5.0 µL of 5x GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 1.5 µL of MgCl₂ (25 mmol L⁻¹) and 0.5 µL of GoTaq® Taq Polymerase (5 U µL⁻¹) (Promega, Madison, WI, USA) were performed with a 4 min initial denature at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 54°C, and 1 min 72°C, and a final 10 min 72°C extension. PCR products were resolved using a 1.4% agarose gel, stained with EtBr and visualized under UV light. Amplicons were cleaned using ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA, USA) and sequenced by ELIM Biopharm (Hayward, CA, USA) using PCR primers and dye terminator methods. Most commonly, the ITS1-F–TW14 amplicon was sequenced in four reactions, two with the PCR primers and additional two using the internal primers ITS4 and LR0R. Chromatograms resulting from sequencing reactions were analyzed with the Chromaseq 1.0 plugin to Mesquite 2.75 (Maddison & Maddison, 2011a; Maddison & Maddison, 2011b), using the programs phred 0.071220.c (Green & Ewing, 2002) and phrap 1.090518 (Green 2009) to call bases, assemble contigs, and assess quality. Bases

Table 1. Yeast species identified from *V. vinifera*.

Sub- phylum	Class (-mycetes)	Order	Species	Authority	
Saccharomycotina (14)	Saccharo-	Saccharomycetales	<i>Candida asiatica</i>	Limtong, Kaewwichian, Am-In, Nakase and Lee	a b
			<i>Candida californica</i>	(Anderson & Skinner) Bai, Wu & Robert	
			<i>Candida oleophila</i>	Montrocher	
			<i>Candida railenensis</i>	C. Ramírez & A. González	a
			<i>Candida saitoana</i>	Nakase & M. Suzuki	a b
			<i>Hanseniaspora uvarum</i>	(Niehaus) Shehata, Mrak & Phaff ex M.Th. Smith	
			<i>Metschnikowia chrysoperlae</i>	S.-O. Suh, Gibson & M. Blackwell	
			<i>Metschnikowia pulcherrima</i> clade spp.		
			<i>Meyerozyma caribbica</i>	(Vaughan-Martini, Kurtzman, S.A. Meyer & O'Neill) Kurtzman & M. Suzuki	
			<i>Meyerozyma guilliermondii</i>	(Wickerham) Kurtzman & M. Suzuki	
			<i>Pichia kluyveri</i>	Bedford ex Kudryavtsev	
			<i>Pichia membranifaciens</i>	(E.C. Hansen) E.C. Hansen	
			<i>Wickerhamomyces anomalus</i>	(E.C. Hansen) Kurtzman, Robnett & Basehoar-Powers	
			<i>Yamadazyma mexicana</i>	(Miranda, Holzschu, Phaff & Starmer) Billon-Grand	
Pucciniomycotina (12)	Cystobasidio-	Cy.	<i>Rhodotorula pallida</i>	Lodder	a b
		i.s.	<i>Rhodotorula</i> sp. (<i>aurantiaca</i> clade) (1)		
			<i>Rhodotorula</i> sp. (<i>aurantiaca</i> clade) (2)		
	Microbotryo-	Sporidiobolales	<i>Rhodosporidium babjevae</i>	Golubev	
			<i>Rhodotorula colostri</i>	(Castelli) Lodder	a b
			<i>Rhodotorula mucilaginosa</i>	(Jørgensen) F.C. Harrison	
			<i>Rhodotorula</i> sp. (<i>glutinis</i> clade)		
			<i>Sporidiobolus metaroseus</i>	Sampaio	
			<i>Sporidiobolus</i> aff. <i>metaroseus</i>		
			<i>Sporobolomyces coprosmae</i>	Hamamoto & Nakase	a b
		i.s.	<i>Curvibasidium pallidicorallinum</i>	W. Golubev, Fell & N. Golubev	a b
			<i>Curvibasidium rogersii</i>	Bourret & Glawe	a b
Ust. (3)	Ustilagino-	Us.	<i>Pseudozyma</i> sp. (1)		
			<i>Pseudozyma</i> sp. (2)		
	Exobasidio-	Mi.	<i>Rhodotorula bacarum</i>	(Buhagiar) Rodrigues de Miranda & Weijman	b

Table 1, cont. Yeast species identified from *V. vinifera*. *Candida railenensis* was only isolated from the Richland site (Appendix 1). The taxonomic authorities for genera and species are those appearing in relevant chapters of (Kurtzman *et al.*, 2011a). Exceptions include *Candida asiatica* (Limtong *et al.*, 2010), *Curvibasidium rogersii* (Bourret *et al.* 2012), *Hannaella* (Wang & Bai, 2008), *Holtermanniella* (Wuczkowski *et al.*, 2011) and *Aureobasidium pullulans* (Zalar *et al.*, 2008). The authorities for taxonomic levels of order and higher appear in (Hibbett *et al.*, 2007; Wuczkowski *et al.*, 2011). Co. = Coniochaetales, Cf. = Cystofilobasidiales, Cy. = Cystobasidiales, Do. = Dothideales, Ho. = Holtermanniella, Mi. = Microstromatales, Th. = Thelobolales, Ust. = Ustilaginomycotina, Us. = Ustilaginales, i.s. = *incertae sedis*. a = first time reported from *Vitis*, b = first time reported in North America.

Sub- phylum	Class (-mycetes)	Order	Species	Authority	
Agaricomycotina (17)	Tremello-	Cf.	<i>Cystofilobasidium infirmominiatum</i>	(Fell, I.L. Hunter & Tallman) Hamamoto, Sugiyama & Komagata	
			<i>Cystofilobasidium macerans</i>	Sampaio	
			<i>Mrakiella cryoconiti</i>	Margesin & Fell	a b
		Filobasidiales	<i>Cryptococcus adeliensis</i>	Scorzetti, Petrescu, Yarrow & Fell	
			<i>Cryptococcus albidosimilis</i>	Vishniac & Kurtzman	a b
			<i>Cryptococcus magnus</i>	(Lodder & Kreger-van Rij) Baptist & Kurtzman	
			<i>Cryptococcus saitoi</i>	Á. Fonseca, Scorzetti & Fell	a b
			<i>Cryptococcus stepposus</i>	Golubev & J. Sampaio	a b
			<i>Cryptococcus uzbekistanensis</i>	Á. Fonseca, Scorzetti & Fell	a b
		Tremellales	<i>Cryptococcus carnescens</i>	(Verona & Luchettii) Takashima, Sugita, Shinoda & Nakase	b
			<i>Cryptococcus laurentii</i>	(Kufferath) C.E. Skinner	
			<i>Cryptococcus tephrensis</i>	Vishniac	a b
			<i>Cryptococcus victoriae</i>	M.J. Montes, Belloch, Galiana, M.D. García, C. Andrés, S. Ferrer, Torres-Rodríguez & J. Guinea	a
			<i>Cryptococcus</i> sp. (<i>Bulleromyces</i> clade)		
			<i>Hannaella luteola</i>	F.-Y. Bai & Q.-M. Wang	a b
		Ho.	<i>Holtermanniella festucosa</i>	(Golubev & Sampaio) Libkind, Wuczkowski, Turchetti & Boekhout	a b
			<i>Holtermanniella takashimae</i>	Wuczkowski, Passoth, Andersson, Turchetti, Prillinger, Boekhout & Libkind	b
Pezizomycotina (7)	Dothideo-	Do.	<i>Aureobasidium pullulans</i>	(de Bary) G. Arnaud	
			<i>Dothideales</i> sp.		
			<i>Sydowia</i> aff. <i>polyspora</i>		
		i.s.	<i>Phaeococcomyces</i> aff. <i>nigricans</i>		
	Leotio-	Th.	<i>Thelobolales</i> sp. (1)		
			<i>Thelobolales</i> sp. (2)		
	Sordario-	Co.	<i>Coniochaetales</i> sp.		

manually edited or with quality scores of 50% or less were indicated by lower-case letters. The resulting sequences were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) under the accessions JX188090 - JX188248.

Taxonomic determinations were made by comparing the two loci for each strain with sequences derived from type material and authentic strains stored in GenBank (Altschul *et al.*, 1990). Molecular determination of yeasts followed criteria described by (Kurtzman & Robnett, 1998; Fell *et al.*, 2000; Kurtzman *et al.*, 2011d). Systematic authorities used for determination are authors of the various chapters of (Kurtzman *et al.*, 2011a) except where noted. Phylogenetic analyses were used to assess strains that could not be readily assigned to taxa based on similarity of aligned sequences. Sequences used for phylogenetic inference were obtained from this study as described above, GenBank and the CBS yeast database. Phylogenetic inference served two primary purposes. The first was to infer the size of the “barcode gap” for a particular clade, which assists the determination of isolates exhibiting rDNA sequences ambiguously close to, but not identical to that of type material. These alignments often contained all sequences in GenBank of sufficient quality for analysis. The second purpose was to determine phylogenetic placement of isolates that did not appear conspecific with known described species. Alignments for this purpose were often limited to sequences derived from type specimens. Alignments consisted of ITS, D1/D2 or both loci combined. Alignments were made using the rmcoffee mode of the program T-Coffee (Notredame *et al.*, 2000) and were adjusted manually. Phylogenetic trees were inferred with maximum parsimony and neighbor joining methods using version 5.05 of the program MEGA (Tamura *et al.*, 2011). For all analyses with MEGA, phylogenies were tested with 1000 bootstrap replications, and the data set used for analysis was obtained using a partial deletion method with a 95% site coverage cutoff. For

maximum parsimony analysis, the Close-Neighbor Interchange tree search method was used, with 10 initial trees and a search level of 3. For neighbor joining analysis, the Kimura 2-parameter model of nucleotide evolution was used (Kimura, 1980) with uniform rates. Alignments consisting of both D1/D2 and ITS loci were not partitioned prior to analysis with MEGA.

The program jModeltest (Guindon & Gascuel, 2003; Posada, 2008) was used to test the fitness of 88 mathematical models of nucleotide evolution for each partition. Model choice was based on Akaike information criterion (Akaike 1974) rank. For alignments consisting of both D1/D2 and ITS loci each locus was tested separately and the entire alignment was also tested. The model with the lowest Akaike score was used for maximum likelihood; the lowest-scoring model that could be implemented in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) was used for Bayesian likelihood. Models were referred to by the acronyms used in (Posada, 2009).

Maximum likelihood analysis was performed using the program GARLI 2.0 (Zwickl, 2006) implementing previously selected models and performing 1000 bootstrap replications with searchreps = 1 and genthreshfortopoterm = 10 000. The best tree was obtained by performing 100 searchreps with genthreshfortopoterm = 20 000, and bootstrap results were summarized onto the best tree using the program sumtrees.py, part of the DendroPy phylogenetic computing library (Sukumaran & Holder, 2010). Bayesian inference was performed with MrBayes 3.1.2 optimized for parallel computing (Altekar *et al.*, 2004). A minimum of 1 000 000 Bayesian generations were simulated with 1 hot and 3 cold Markov chains, and generations were increased until the standard deviation of split frequencies was consistently below 0.01. A burn-in period comprising the first ¼ of the generations was used when summarizing the data, which were

Fig. 1. Subphyla of isolates collected from the Prosser site (Appendix 1). Methods for isolation in June and August involved washing berries while September and October macerated berries. Isolates were selected based on distinctive cultural morphology and do not represent relative abundance on isolation plates. Sampling effort was not equal across dates.

only accepted if the Markov chains were observed to coalesce. Alignments consisting of both D1/D2 and ITS loci were partitioned when analyzed using likelihood methods; trees were also inferred from each individual locus.

Trees produced by each tree-building method were compared visually to each other, and distinct topologies retained. If trees produced by all four methods had the same topology, then the support values from all four methods were summarized onto a single tree. For partitioned likelihood analyses, trees produced by D1/D2, ITS and partitioned alignments were similarly compared. Trees were visualized and rooted using the program FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) and annotated using GIMP® (<http://www.gimp.org>). Support values for maximum parsimony, neighbor joining and maximum likelihood analysis are percentage bootstrap scores, while support values for the Bayesian likelihood analysis are percentage posterior probabilities; values above 70 are shown. Scales are

substitutions per site for neighbor joining, maximum likelihood and Bayesian likelihood trees; scales are parsimony steps for maximum parsimony trees. It is important to note that binomial names listed in dendrograms are based on names listed as part of GenBank accessions; therefore all names should be considered provisional unless accompanied by a superscript “T” indicating a type specimen.

Physiological tests were used to aid in species determination of some Pucciniomycotina, including *Curvibasidium pallidicorallinum*, *Curvibasidium rogersii*, *Rhodosporeidium babjevae* and *Sporobolomyces coprosmae*. Methods were adapted from (Kurtzman *et al.*, 2011e), based on those first outlined by (Wickerham, 1951). Carbon assimilation tests were performed with 5 mL of liquid media in polypropylene 17x100 mm test tubes. Strains obtained from the USDA ARS Culture Collection (NRRL) (Peoria, IL, USA) were used for comparison.

Results and Discussion: The cultural and microscopic morphological features and rDNA sequences of 126 yeast strains were assessed (Appendix 1). Species were determined primarily on the basis of rDNA sequences. Results revealed evidence for 53 species distributed among 24 genera (Table 1). Binomials could be applied to 39 species, while other taxa were determined provisionally on the basis of phylogenetic placement. A new species, *Curvibasidium*

rogersii was described previously based on one of the strains (Bourret *et al.*, 2012). The twenty-one species of ascomycetes and 32 of basidiomycetes represented five of the six subphyla of subkingdom Dikarya. The greatest number of species found (17 species) were members of subphylum Agaricomycotina, followed by Saccharomycotina (14 species), Pucciniomycotina (12 species), Pezizomycotina (7 species) and Ustilaginomycotina (3 species) (Hibbett *et al.*,

Fig. 2. Neighbor joining tree from a D1/D2 alignment of type strains in the *Metschnikowia pulcherrima* clade and sequences from the nine isolates obtained in the current study. Strains known to produce visible pigment have squares. *Candida picachoensis* and *C. pimensis* were used to root the tree. Haplotypes are expanded in Fig. 3.

Fig. 3. Maximum likelihood (TIM2+I+G) tree from a D1/D2 alignment of type strains in the *Metschnikowia pulcherrima* clade and sequences from the nine isolates obtained in the current study. Strains known to produce visible pigment have squares. *Candida picachoensis* and *C. pimensis* were used to root the tree.

2007; Kurtzman, 2011; Boekhout *et al.*, 2011). One additional species of *Pseudozyma* was isolated from grass samples but not grapes. At the Prosser location, yeasts in the Agaricomycotina, Pucciniomycotina and Pezizomycotina were recovered from all dates sampled (Fig. 1). All grape Ustilaginomycotina isolates were collected on August 31, while Saccharomycotina isolates were found only on the September and October sampling dates (Fig. 1, Appendix 1).

Biodiversity of isolates

The number of species encountered in the current study, 53 (Table 1), was greater than any of 13 similar studies of yeasts from wine grapes, in which a range of 8 to 31 species was reported (Sabate *et al.*, 2002; Jolly *et al.*, 2003; Combina *et al.*, 2005; Renouf *et al.*, 2005; Raspor *et al.*, 2006; Nisiotou *et al.*, 2007; Barata *et al.*, 2008; Romancino *et al.*, 2008; Brezna *et al.*, 2010; Cadez *et al.*, 2010; Francesca *et al.*, 2010; Li *et al.*, 2010; Setati *et al.*, 2012). The diversity encountered also was large compared to that reported from other surveys of yeasts from other substrates that were determined using molecular data (e.g. Wuczkowski & Prillinger 2004; Fraser

et al., 2006; Laitila *et al.*, 2006; Sláviková *et al.*, 2009; Branda *et al.*, 2010; Burgaud *et al.*, 2010; Gori *et al.*, 2011; Voglmayr *et al.*, 2011; Fernandez *et al.*, 2012). Seventeen species found in this study were not reported previously from *Vitis*, and 18 were not reported previously from North America (Table 1). An additional 14 distinct taxa could not be determined to species, many of which could represent novel species based on phylogenetic analysis.

The high degree of diversity encountered in the current study could be because the diversity of wild yeasts at the Prosser vineyard was unusually large, because of the methods used (which included holding samples refrigeration for days or weeks), or because of the timing of sampling. In the present study, 228 strains were isolated, while in previous studies 720 isolated strains represented 15 species (Jolly *et al.*, 2003), 752 isolated strains represented 13 species (Raspor *et al.*, 2006), 1463 isolated strains represented 18 species (Nisiotou *et al.*, 2007), and 2575 isolated strains represented 11 species (Romancino *et al.*, 2008). In two studies isolates were obtained by subculturing randomly-chosen strains from isolation plates (Jolly *et al.*, 2003; Nisiotou *et al.*, 2007), while in two other studies (Raspor *et al.*,

Fig. 4. Maximum likelihood tree from a D1/D2 alignment including GenBank accessions in the *aurantiaca* clade of (Hamamoto *et al.*, 2011; Sampaio, 2011a), including sequences from P34D004 and P44D004. Support values are, from left to right, maximum parsimony, neighbor joining, maximum likelihood (TIM2+G) and Bayesian likelihood (GTR+G). *Sporobolomyces gracilis* was used to root the tree.

Fig. 5. Bayesian likelihood tree of *Rhodotorula glutinis* clade (clade A of Coelho *et al.*, 2011), from an alignment of ITS and D1/D2 sequences. Support values are maximum parsimony, neighbor joining, maximum likelihood (GTR+I+G ITS, TIM3+I+G D1/D2) and Bayesian likelihood (GTR+I+G both partitions). *Rhodosporidium babjevae* and *Rhodotorula graminis* were not included. *Rhodotorula lusitaniae* was used to root the tree.

Fig. 6. Neighbor joining tree of a subset of the *Sporidiobolus johnsonii* clade (clade C of Coelho *et al.*, 2011), including ballistoconidium-producing strains from the current study, from an alignment of combined ITS and D1/D2 sequences. Support values are maximum parsimony and neighbor joining bootstrap values. *Sporidiobolus johnsonii* was used to root the tree. Haplotypes are expanded in Fig. 7.

Fig. 7. Maximum likelihood tree of a subset of the *Sporidiobolus johnsonii* clade (clade C of Coelho *et al.*, 2011) including ballistoconidium-producing strains from the current study, from a partitioned alignment of ITS and D1/D2 sequences. Support values are maximum likelihood (TIM2+I+G ITS, GTR+I D1/D2) bootstrap and Bayesian likelihood (GTR+I+G ITS, GTR+I D1/D2) posterior probability. *Sporidiobolus johnsonii* was used to root the tree.

2006; Romancino *et al.*, 2008), and the current study, isolates were chosen for subculturing on the basis of differences in culture morphology. In the current study, grape bunches sometimes were refrigerated for days or weeks before isolations were made. These varying amounts of time held at 2°C before processing may have favored isolation of rarer, psychrophilic species. Furthermore, incubating isolation plates at 18–21°C may have supported growth of some of the psychrophiles (Kurtzman *et al.*, 2011e).

The number of species (14) of Saccharomycotina that were found was not greater than that reported in similar studies (Jolly *et al.*, 2003; Renouf *et al.*, 2005; Nisiotou *et al.*, 2007; Barata *et al.*, 2008; Li *et al.*, 2010). Also, most of the species diversity found in the current study was in the form of basidiomycetous species, many of which were isolated only from unripe grapes collected on the August 31 sampling date. Most other studies focused only on the mature, ripe grape berries, suggesting that the earlier sampling in the present study may have enhanced the degree of diversity encountered. Over the duration of the study, yeasts isolated changed from primarily basidiomycetous species to ascomycetous species (Fig. 1). In other published studies (Davenport, 1976; Renouf *et al.*, 2005; reviewed by Villa & Longo, 1996; Barata *et al.*, 2012), the abundance of yeasts on berries increased as berries matured. Plant-associated, “resident” basidiomycetous species may be more rarely isolated from mature grape berries due to the relative abundance of insect-vectored, “transient” ascomycetous species that might have been introduced to the berries as the season progressed (Davenport, 1976; Villa & Longo, 1996; Barata *et al.*, 2012).

It is possible that insects acted as vectors of the “transient” vineyard yeasts, a group comprising nearly all members of Saccharomycotina associated with grapes. A recent study of yeasts associated with ovipositors of *Drosophila suzukii* (Hamby *et al.*, 2012) found yeasts in 11 genera, 8

of which were also found in the current study. The ecology of *Saccharomyces cerevisiae* has been linked to wasps in the Vespidae family (Stefanini *et al.*, 2012). Results of the current study are consistent with the hypothesis that members of Saccharomycotina are vectored to ripe grapes, as members of that suphyllum were not isolated at the first two sampling dates. Alternatively, it may be that immature grape surfaces are inhospitable to colonization by species of *Saccharomyces* and related genera.

Methods used in this study cannot differentiate actively growing yeasts on grapes from spores or cells deposited on berries by wind or animal vectors, but persisting in a dormant state. Regardless of their origin, all fungi isolated in the current study are present, if not active, on grapes and in must.

Subphylum Saccharomycotina

Nearly all strains in Saccharomycotina could be determined based on degrees of similarity included in BLAST results, excepting only strains representing the *Metschnikowia pulcherrima* clade (Lachance, 2011). ITS sequences were not available for the type strains of *M. andauensis*, *M. fructicola* and *M. pulcherrima*, so phylogenetic studies were carried out using D1/D2 data. Tree topology was not conserved among the four phylogenetic inference methods used. Results indicated that eight of the nine strains studied are genetically distinct, but only one strain could be assigned to a described species (Appendix 1, Figs. 2 & 3), suggesting that species definitions in the clade could benefit from further revision. Furthermore, double-peaks observed in direct sequencing chromatograms (results not shown) suggest that some members of the clade contain distinct rDNA sequences within a single organism. Ambiguous nucleotides, potentially due to rDNA polymorphisms are present in D1/D2 sequence data submitted by (Kurtzman & Droby, 2001; Molnár & Prillinger, 2005) for the type strains of *M. fructicola* and *M.*

Fig. 8. Maximum parsimony tree of an ITS alignment of the *Ustilago hordei* clade. Haplotypes are expanded in Table 2. *Ustilago cynodontis* was used to root the tree.

Fig. 9. Neighbor-joining tree of an ITS alignment of the *Ustilago hordei* clade. Haplotypes are expanded in Table 2. *Ustilago cynodontis* was used to root the tree.

andauensis. Lack of homogeneity in rDNA regions in species of *Metschnikowia* appears more common than previously thought, complicating or preventing taxonomic separation of taxa but offering interesting clues about evolution of lineages (Sipiczki *et al.*, 2013).

Subphylum Pucciniomycotina

Only five of the twelve species in Pucciniomycotina could be determined based on

similarity in BLAST results. Some species, such as *Curvibasidium pallidicorallinum*, *Rhodosporeidium babjevae* and *Sporobolomyces coprosmae* exhibited few to no D1/D2 or ITS substitutions compared to related species and physiological tests aided determinations.

Cystobasidiomycetes: Two strains in the Cystobasidiomycetes could not be assigned to any species with confidence. Strain P44D004 appears closely related to *Rhodotorula aurantiaca* (Fig.

Fig. 10. Maximum likelihood tree of a combined ITS and D1/D2 alignment of the *Ustilago hordei* clade. Haplotypes are expanded in Table 2. Support values are maximum likelihood (TIM2 ITS, TPM3uf D1/D2) and Bayesian likelihood (GTR ITS, HKY D1/D2). *Ustilago cynodontis* was used to root the tree.

Table 2. Isolates with identical ITS sequences, appearing in Figs. 8-10. *P.* = *Pseudozyma*, *U.* = *Ustilago*, *V.* = *Vitis*, *Br.* = *Bromus*, *Pi.* = *Pinus*, *Ho.* = *Hordeum*, *Av.* = *Avena*.

Strain/Voucher	Species	Host/Substrate	Location	Accession(s)
Haplotype A				
P44A002	<i>P. sp.</i> (1)	<i>V. vinifera</i>	Prosser	JX188212
P01A021	<i>P. sp.</i> (1)	<i>V. vinifera</i>	Prosser	JX188209
P25A002	<i>P. sp.</i> (1)	<i>V. vinifera</i>	Prosser	JX188211
P45A004	<i>P. sp.</i> (1)	<i>V. vinifera</i>	Prosser	JX188213
BRTEA	<i>P. sp.</i> (1)	<i>Br. tectorum</i>	Pullman	JX188214
BRTEB	<i>P. sp.</i> (1)	<i>Br. tectorum</i>	Pullman	JX188215
PICO22B	<i>P. sp.</i> (1)	<i>Pi. contorta</i>	Pullman	JX188217
PICO23	<i>P. sp.</i> (1)	<i>Pi. contorta</i>	Pullman	JX188218
Haplotype B				
Kellner 2011	<i>U. tritici</i>	Unknown	Unknown	JN367309/JN367336
T-2	<i>U. tritici</i>	<i>Triticum</i> sp.	Canada	AF135424
PDSUT2	<i>U. tritici</i>	<i>Tr. aestivum</i>	China	JN114419
NYUT1	<i>U. tritici</i>	<i>Tr. aestivum</i>	China	JN114418
Haplotype C				
Ust.Exs.784	<i>U. hordei</i>	<i>Ho. vulgare</i>	Iran	AY345003/AF453934
83-138	<i>U. nigra</i> (= <i>avenae</i>)	<i>Ho. vulgare</i>	Canada	AF135428
Uh362	<i>U. hordei</i>	<i>Ho. vulgare</i>	Canada	AF135427
WS98	<i>U. hordei</i>	<i>Ho. vulgare</i>	USA	AF105224
Haplotype D				
F947	<i>U. hordei</i>	<i>Av. sativa</i>	Spain	AY740068/AY740122
A-60	<i>U. avenae</i>	<i>Av. sativa</i>	USA	AF135425
Haplotype E				
HUV 17782	<i>U. nuda</i>	<i>Ho. leporinum</i>	Unknown	AY740069/AJ236139
96-253	<i>U. nuda</i>	<i>Ho. vulgare</i>	Canada	AF135430

4), but results suggest that the name *R. aurantiaca* be restricted only to strains with D1/D2 Haplotype B (Fig. 4), identical to the type.

It is unclear if P34D004 (Haplotype F, Fig. 4) represents a distinct species from *Sporobolomyces salicinus*.

Figure 11. Neighbor joining tree of Microstromatales, from a D1/D2 alignment. Support values are maximum parsimony, neighbor joining, maximum likelihood (GTR+I+G) and Bayesian likelihood (GTR+I+G). *Tilletiopsis pallescens* was used to root the tree. Haplotypes are expanded in Table 3.

Table 3. Expanded haplotypes from Microstromatales phylogeny of Fig. 11.

Haplotype A		Haplotype E	
<i>Quambalaria pitereka</i> CMW6707 DQ317620		<i>Rhodotorula bacarum</i> ZIM 666 AM748543	
<i>Quambalaria pitereka</i> CMW5318 DQ317621		<i>Microstroma album</i> R.B.2072 AF352052	
<i>Quambalaria pitereka</i> WAC12956 DQ823435		Haplotype F	
<i>Quambalaria pitereka</i> WAC12958 DQ823436		<i>Rhodotorula bacarum</i> DBVPG 4739 EF643721	
<i>Quambalaria pitereka</i> WAC12957 DQ823437		<i>Rhodotorula bacarum</i> CBS 8977 AF406891	
<i>Quambalaria pitereka</i> DAR 19773 DQ823438		<i>Rhodotorula bacarum</i> HB 919 AJ508235	
<i>Quambalaria pitereka</i> QP45 DQ823439		<i>Rhodotorula sp.</i> HB 1141 AM039678	
Haplotype B		Haplotype G	
<i>Quambalaria coyrecup</i> WAC12947 DQ823444		<i>Microstroma juglandis</i> KR 0015442 EU069497	
<i>Quambalaria coyrecup</i> WAC12949 DQ823445		<i>Microstroma juglandis</i> FO 39211 AF009867	
<i>Quambalaria coyrecup</i> WAC12948 DQ823446		<i>Microstroma juglandis</i> RB2042 DQ317617	
<i>Quambalaria coyrecup</i> WAC12950 DQ823447		Haplotype H	
<i>Quambalaria coyrecup</i> WAC12951 DQ823448		<i>Rhodotorula phylloplana</i> CBS 8079 DQ832196	
Haplotype C		<i>Rhodotorula phylloplana</i> IGC AF352056	
<i>Quambalaria eucalypti</i> CMW1101 DQ317618		<i>Rhodotorula phylloplana</i> ZIM 662 AM748546	
<i>Quambalaria eucalypti</i> CMW11678 DQ317619		clone baso7052 HQ433174	
<i>Quambalaria cyanescens</i> WAC12953 DQ823443		Haplotype I	
Haplotype D		<i>Rhodotorula phylloplana</i> CBS 8073 ^T AF190004	
<i>Quambalaria simpsonii</i> CBS 124772 GQ303321		<i>Rhodotorula phylloplana</i> CBS 8079 AF190003	
<i>Quambalaria simpsonii</i> CBS 124773 GQ303322		Haplotype J	
<i>Quambalaria cyanescens</i> CBS357.73 DQ317615		<i>Jaminala angkorienensis</i> C5b EU587489	
<i>Quambalaria cyanescens</i> CBS876.73 DQ317616		<i>Rhodotorula sp.</i> FN1L09 EU523595	
<i>Quambalaria cyanescens</i> MK 742 AM261920		<i>Rhodotorula sp.</i> NN5L03 HQ623520	
<i>Quambalaria cyanescens</i> MK 617 AM261923		Haplotype K	
<i>Quambalaria cyanescens</i> CBS 357.73 AM261925		<i>Sympodiomyces paphiopedili</i> CBS 7429 ^T DQ832238	
<i>Quambalaria cyanescens</i> MK617 AM262975		<i>Sympodiomyces paphiopedili</i> ATT 271 FJ743630	
<i>Quambalaria cyanescens</i> MK742 AM262976		Haplotype L	
<i>Quambalaria cyanescens</i> WAC12952 DQ823440		<i>Sympodiomyces kandeliae</i> BCRC 23075 FJ426334	
<i>Quambalaria cyanescens</i> WAC12955 DQ823441		<i>Sympodiomyces kandeliae</i> BCRC 23165 GU047881	
<i>Quambalaria cyanescens</i> WAC12954 DQ823442

Fig. 12. Bayesian likelihood tree of *Mrakia* and *Mrakiella* species from an alignment of ITS sequences. Support values are maximum parsimony, neighbor joining, maximum likelihood (TPM3uf+G) and Bayesian likelihood (GTR+G). *Guehomyces pullulans* was used to root the tree.

Microbotryomycetes: Several strains within the Microbotryomycetes could not be assigned to any described species. P34D001 was found to be related to *Rhodotorula araucariae* Grinb. & Yarrow and *R. hamamotoiana* (referred to in (Coelho *et al.*, 2011) but still a *nomen nudum*) (Fig. 5). Strain P34D001 appears distinct from those two species, and is likely a new species.

From the five strains producing ballistoconidia, two distinct ITS and D1/D2 haplotypes were formed, though the strains could not be separated based on morphology. An alignment of sequences representing members of the *Sporidiobolus johnsonii* clade (clade C of Coelho *et al.*, 2011) was analyzed (Figs. 6-7); topology of the clade varied between methods and between ITS and D1/D2 loci (see Bourret, 2012 Figs. 5.6-5.9). The ITS and D1/D2 rDNA sequences of CBS 5541 have been published twice (Fell *et al.*, 2002; Valerio *et al.*, 2008), and the two ITS sequences differ by 4 bp. In the combined ITS and D1/D2

alignment, P26D003 and the CBS 5541 sequence obtained by (Fell *et al.*, 2002) had identical haplotypes (Figs. 6-7). Though currently considered conspecific with *S. metaroseus*, CBS 5541 might represent a distinct species. CBS 7683^T, the type strain of *S. metaroseus* was one of only two of the 27 strains in clade C in which a pheromone receptor gene was not detected, while a receptor gene was detected in CBS 5541 (Coelho *et al.*, 2011). DNA-DNA reassociation values between the two strains are 23-33% (Valerio *et al.*, 2008), well below the value of 70% suggesting conspecificity (Kurtzman *et al.*, 2011d). Germinating teliospores produce two-celled basidia in CBS 5541, whereas the other three sexual strains in *S. metaroseus* (CBS 7683-7685) produce four-celled basidia (Valerio *et al.*, 2008). Mating trials between other asexual strains in the complex have not been successful, and all sexual strains are self-fertile (Sampaio, 2011b). If CBS 5541 were separated from *S. metaroseus*, P26D003 and P42A004 would

appear conspecific with the new species. The significance of the observed differences between the haplotype representing P34Doo6, P4oDoo6 and P43Coo2 and the other members of the clade remains to be determined.

Strain P34Coo4 resembled both *S. coprosmae* and *S. oryzaicola*, species that are known only from single strains (Hamamoto *et al.*, 2011). The type strains of the two species differ in their physiological profiles, notably in assimilation of glucono- δ -lactone (+ for *S. coprosmae*, - for *S. oryzaicola*) and growth at 30°C (- for *S. coprosmae*, + for *S. oryzaicola*). The results of both tests for P34Coo4 (-, -) indicate an ambiguous placement, with the absence of growth on glucono- δ -lactone indicating an affinity with *S. oryzaicola*, and the absence of growth at 30°C indicating an affinity with *S. coprosmae*. *Sporobolomyces coprosmae* and *S. oryzaicola* yield identical D1/D2 sequences, differing from that of P34Coo4 by a single substitution, but differ in terms of DNA complementarity. P34Coo4 differs from the type of *S. coprosmae* at the ITS locus by a single substitution, while it differs from the type of *S. oryzaicola* by 6 bp. These results suggest that P34Coo4 is more closely related to *S. coprosmae* than *S. oryzaicola*.

These results suggest the possibility that *S. coprosmae* and *S. oryzaicola* are conspecific, with somewhat variable ITS regions and physiological attributes. GenBank accessions with ITS sequences intermediate between the two named species (e.g. AM160645, JF449781 and FR799501) are consistent with this possibility. Further work will be necessary to clarify this situation. The determination of P34Coo4 in this study based on the ITS sequence similarity conforms to the current practice of regarding *S. coprosmae* as a distinct species.

Subphylum Ustilaginomycotina

None of the three species placed in the Ustilaginomycotina could be determined solely on BLAST results. Evidence suggests that these strains are indistinguishable from anamorphic state of plant pathogenic smut fungi, but these relationships remain unresolved.

Ustilaginomycetes: Six strains obtained in the current study resembled morphologically the anamorphic yeast genus *Pseudozyma*, forming fusiform to elongated cells, true hyphae and dimorphic colonies (Boekhout, 2011).

Pseudozyma species phylogenetically are Ustilaginales, where they are grouped with the

Fig. 13. Bayesian likelihood tree from a partitioned ITS (GTR+I) and D1/D2 (GTR+I+G) alignment of type strains in the *Cryptococcus albidus* clade, including strains from the current study. Support values are neighbor-joining bootstrap values from a combined ITS and D1/D2 analysis followed by Bayesian posterior probabilities for the current tree. *Filobasidium uniguttulatum* was used to root the tree. Other topological variants are in (Bourret, 2012).

smut genus *Ustilago* (Begerow *et al.*, 2000; Fell *et al.*, 2000; Boekhout, 2011). Several *Ustilago* species, including *U. maydis* (DC.) Corda, are known to exhibit yeast-like states in culture. No anamorph-teleomorph connections linking *Ustilago* or *Pseudozyma* have been reported (Boekhout, 2011) although certain *Ustilago* and *Pseudozyma* species, such as *U. maydis* and *P. prolifica* are closely related (Begerow *et al.*, 2000; Boekhout, 2011) and may have identical ITS and D1/D2 sequences. For example, *P. prolifica* CBS 319.87 [AF294700] and *U. maydis* ATCC 10819 [EU853845] are identical at 705/705 bp at the ITS locus.

The isolates obtained in this study exhibited ITS sequences that were not identical to any previously published sequences and were not closely related to any named species of *Pseudozyma*. Instead, strains appeared closely related to several species of *Ustilago*. The conserved ITS sequence from strains P01A021, P25A002, P44A002 and P45A004 was equally distant from species of *U. pamirica* and *U. tritici*. To investigate these relationships, an alignment was created of ITS sequences corresponding to the *U. hordei* clade of (Stoll *et al.*, 2005), containing *U. avenae* (Pers.) Rostr., *U. bromivora* (Tul. & C. Tul.) A.A. Fisch. Waldh., *U. bullata* Berk., *U. hordei* (Pers.) Lagerh., *U. nuda* (C.N. Jensen) Kellerman & Swingle, *U. pamirica* Golovin, and *U. turcomanica* Tranzschel. Sequences from *Ustilago tritici* (Pers.) Rostr. were not included in the phylogeny of Stoll *et al.* (2005), but the species was included in the current analysis (Table 2, Figs. 8-10). The analysis did not resolve relationships within the *hordei* clade. The *Ustilago hordei* clade appeared to consist of a group of ambiguously placed, mostly wheat and *Bromus*-infecting isolates, also containing the grape haplotypes, and a more derived, well supported clade, infecting barley and *Avena* spp.

Isolates obtained in the current study could represent the anamorph of *Ustilago* species,

suggesting several avenues for further investigation. To provide additional information about these fungi, yeast isolates were cultured from samples of two smut-infected species of grass (*Bromus tectorum* and *Bromus hordeaceus*) collected from a site on the WSU Pullman campus in September of 2011. *Bromus*-infecting *Ustilago* species are considered to be *U. bromivora* according to (Vánky, 2012), although the author notes the species may represent a complex. The isolates obtained from *B. tectorum* exhibited ITS and D1/D2 sequences 100% identical to the grape isolates of haplotype A. These results suggest that the grape isolates of *Pseudozyma* sp. (1) might be conspecific with a *B. tectorum*-infecting *Ustilago*. Two additional conspecific strains, PICO22B and PICO23 were isolated from needles of *Pinus contorta* collected on WSU Pullman campus, September 2011. The strain obtained from *B. hordeaceus* appeared morphologically and phylogenetically distinct from the grape and *B. tectorum* isolates.

None of the three distinct ITS haplotypes obtained in the current study could be reliably assigned to a species. The inferred tree topologies were consistent with the ITS tree of the *Ustilago hordei* clade inferred by Bakkeren *et al.* (2000). AFLP trees included in the same report (Bakkeren *et al.*, 2000) also suggested that sequence divergence in the ITS region might be correlated with significant genomic changes, consistent with the possibility that the three haplotypes observed in the current study represent separate species. Each haplotype differed from all GenBank sequences by 2–3 bp, distinct enough from other sequenced fungi in this clade to be considered novel taxa (Bakkeren *et al.*, 2000; Fell *et al.*, 2000).

Exobasidiomycetes: Based on BLAST searches, strain P34A003 was found to be distinct from all published sequences, but related to strains of both *Rhodotorula bacarum* and *Microstroma album*. Though currently accepted species in the genus *Rhodotorula*, *R. bacarum* and *R.*

Fig. 14. Bayesian likelihood tree from partitioned ITS (SYM+I+G) and D1/D2 (K80+I+G) alignment within the *Bulleromyces* clade, including strains from the current study. *Cryptococcus* sp. APSS 862 was used to root the tree. Other topological variants are in (Bourret, 2012).

phylloplana are members of the Microstromatales (Ustilaginomycotina); all other *Rhodotorula* species are members of Pucciniomycotina (Sampaio, 2011a). The close phylogenetic association of *Rhodotorula bacarum* with the teleomorphic plant pathogenic species *Microstroma album* (Fig. 11, Table 3) suggests that it may represent an anamorph of *M. album*, but this has not been proven (Sampaio, 2011a). Similarly, *R. phylloplana* appears closely related to *M. juglandis* (Fig. 11) (Sampaio, 2011a). P34A003 was placed in a well-supported clade containing *R. bacarum* strains and *Microstroma album*, with the type strain of *R. bacarum*, CBS 6526^T, in the least derived position (Fig. 11).

Subphylum Agaricomycotina

Cystofilobasidiales: Strain P40Coo2 was determined based on the phylogenetic analysis of ITS sequence data. In the resulting trees it occurred in a clade with strong support corresponding to *Mrakiella cryoconiti* (Fig. 12). P40Coo2 and the type strain, CBS 10834^T, were found to be most closely related.

Filobasidiales: ITS and D1/D2 sequences of type strains and strains obtained in the current study belonging to the *Cryptococcus albidus* clade (Fonseca *et al.*, 2011) were aligned and trees were inferred (Fig. 13). Significant differences (5 bp)

Haplotype A
Teratosphaeria stellenboschiana | CPC 10886 | EU019295
Readeriella gauchensis | CBS 120303 | EU019290

Haplotype B
Teratosphaeria dimorpha | CPC 14132 | FJ493215
Colletogloeopsis dimorpha | CBS 120085 | DQ923529

Haplotype C
Batcheloromyces proteae | CBS 110696 | EU019247
Batcheloromyces leucandrii | CBS 110892 | EU019246
Batcheloromyces leucandrii | CBS 119344 | EU552103
Teratosphaeria alistairii | CBS 120035 | DQ885901

Haplotype D
cf. *Ramichloridium* sp. | CPC 10672 | GU214688
Mycosphaerella sp. M16 | HM595586

Haplotype E
Dothideomyces sp. | TRN 456 | GU323986
clone FunN5_12G | EU861749.1

95 | 99 | 82 | 95 | *Pseudeurotium zonatum* | AFTOL-ID 1912 | DQ470988
 74 | 85 | 79 | 96 | *Pseudeurotium zonatum* | CBS 329.36 | AF096198
 90 | 99 | 92 | 100 | clone asc 07101 | HQ433055
 clone OTU97-94 | JQ311759
 clone OTU97-980 | JQ311803
Geomyces pannorum | UAMH 2583 | GU951694
Geomyces destructans | MYC80282 | GU951686
 Haplotype A
 clone BPAGMIT0_E9 | EU691297
Geomyces sp. | 797_3_CZ01 | AB470544
Geomyces pannorum | UAMH 1030 | GU951690
Geomyces sp. | 795_3_Y01 | AB470548
Chrysosporium pseudomerarium | UAMH 3089 | GU951696
 clone FunN4_03H | EU861608
Geomyces pannorum | UAMH 1568 | GU951693
 clone G23_OTU72 | EF433996
 clone TH20_OTU72 | EF434103
 88 | 98 | 71 | *Leuconeurospora pulcherrima* | AFTOL-ID 1397 | FJ176884
Leuconeurospora pulcherrima | CBS 343.76 | AF096193
 85 | 87 | 75 | 100 | *Hyphozyma variabilis* | CBS 523.79 | AF353596
 Haplotype B
 clone OTU97-967 | JQ311789
 * | * | * | 100 |
 95 | 96 | 95 | 98 | *Candida davisi* | LANTA 101 | JN172921
 98 | 100 | 71 | * | clone FunN4_10G | EU861656
 clone FunN4_07F | EU861634
Leuconeurospora sp. | UFMGCB ANT 62 | HQ184181
 Haplotype D
Pseudeurotium sp. | 795_3_CY02 | AB470537
Bettisia alvei | voucher A.A. Wynns 5154(C) | HQ540516
 90 | 92 | * | 100 | *Collophora* sp. | TP-Snow-Y69 | JN400810
 * | * | * | 83 | Haplotype E
 0.02
 Haplotype A
Geomyces pannorum | UAMH 733 | GU951689
Geomyces sp. | FMCC-4 | DQ520622
Aleurodiscus farlowii | HHHB14354 | AY039323
 clone BPASol2T8_4D | EU691732
 clone OTU97-92 | JQ311737
 clone C5_OTU3 | EF433976
 clone FunN5_03H | EU861687
 Haplotype B
Thelebolus ellipsoideus | AFTOL-ID 5005 | FJ176895
Thelebolus globosus | AFTOL-ID 5016 | FJ176905
 Haplotype C
Thelebolates sp. (2) | P43C017 | JX188243
Candida davisi | MO-Y18 | GU373794
 Fungal yeast sp. | E1-14 | EU194463
 Haplotype D
Candida davisi | LANTA 107 | JN172922
Candida davisi | LANTA 114 | JF739992
Candida davisi | AF536563
Thelebolates sp. (1) | P41C001 | JX188242
Candida davisi | TEMD-40 | JQ277257
Sporobolomyces lactosus | CBS 7701 | (from CBS database)
 Haplotype C
 Haplotype E
Crinula caliciiformis | AFTOL-ID 272 | AY544680
Holwaya mucida | B 70 0009352 | DQ257356
Bulgaria inquinans | AFTOL-ID 916 | DQ470960

Fig 17. Bayesian likelihood tree derived from D1/D2 alignment of sequences related to P41C001 and P43C017. Support values are maximum parsimony, neighbor joining, maximum likelihood (TrN+I+G) and Bayesian likelihood (GTR+I+G).

Fig. 18. Bayesian likelihood tree from a D1/D2 alignment of fungi related to P45A009. Support values are maximum parsimony, neighbor joining, maximum likelihood (TIM2ef+G) and Bayesian likelihood (SYM+I+G). *Barrina polyspora* was used to root the tree.

across the combined ITS and D1/D2 alignment were observed between the ITS-D1/D2 haplotype obtained from P42C010 and P45A008 and the type of *C. uzbekistanensis* (CBS 8683^T), suggesting that the two strains might represent a separate species. Pairs of other accepted species in the clade appeared to differ by similar distances: *Cryptococcus saitoi* and *C. friedmannii* differed by 6 bp in the alignment and *Cryptococcus diffluens* and *C. liquefaciens* differed by 4 bp. One strain of both *C. adeliensis* and *C. albidosimilis* exhibited slightly different ITS-D1/D2 haplotypes from their respective type strains but were considered conspecific.

Tremellales: Phylogenetic placement of two strains in the Tremellales was inferred from a combined ITS and D1/D2 alignment of strains within the clade corresponding to the teleomorph genus *Bulleromyces* (Fonseca *et al.*, 2011) (Fig.

14). P43A004 varies considerably from the type strain of *Cryptococcus laurentii* at the ITS locus, but according to (Fonseca *et al.*, 2011) it should be considered conspecific until further systematic work is done. The relationships of the type strain of *C. aureus* (CBS 318^T), NRRL Y-30213, NRRL Y-30215 and P40A001 were not resolved, though the four strains appear closely related.

Subphylum Pezizomycotina

Dothideomycetes: Analysis of sequences from 14 isolates placed them in the Dothideales. All exhibited cultural characteristics similar to *Aureobasidium*, producing yeast-like colonies followed by filamentous growth. Colonies ranged from faintly pink to dark black, and lighter colonies tend to darken over time. Anamorphs in this clade are often classified in *Aureobasidium* or *Hormonema*, (Seifert *et al.*, 2011).

Phylogeny of isolates of Dothideales was studied using aligned D1/D2 sequences (Fig. 15). The strains fell into five distinct clades, three of which fit within *Aureobasidium pullulans* sensu Zalar *et al.* (2008). One group corresponded to the variety *pullulans* and the two appeared to represent different subspecific groups. Two of intraspecific groups (var. *pullulans* and var. aff. *namibiae*) may be further subdivided based on rDNA sequences, and the sequence differences appear to be correlated with morphological differences, especially the degree of pigmentation. This conclusion is consistent with results of a previous study (Manitchotpisit *et al.*, 2009) presenting evidence of subspecific clades in tropical *Aureobasidium pullulans* isolates. That study did not include D1/D2 sequences, and so no results were available to include in our analysis. The placement of the clade represented by P25A005 & P44C001 was not well supported by phylogenetic analysis (Fig. 15). The clade occupied a basal position within the well-supported clade containing *Aureobasidium*, and was distinct from the clade containing *Dothiora* and *Dothidea*. Closely related strains included CBS 124776, *Selenophoma australiensis*; CPC 14028, *Sydowia eucalypti* (Cheewangkoon *et al.*, 2009); YFL7.6d, identified as *Dothichiza* sp. (Fernandez *et al.*, 2012); and *Dothichiza pithyophila* (Zalar *et al.*, 2008).

P40D010 was determined to be *Phaeococcomyces* aff. *nigricans* based on phylogenetic analysis of D1/D2 sequence data.

The results of phylogenetic analysis placed P40D010 as a close relative of strain MZ107, identified as *Phaeococcomyces nigricans*, isolated from polyvinyl plastic in the UK (Webb *et al.* 2000) (Fig. 16). P40D010 is also part of a larger, well supported clade containing CBS 652.76a, determined as an isotype of *P. nigricans*, and CRUB 1760, determined as *Phaeococcomyces* sp. However, the genus *Phaeococcomyces* is currently considered to be a member of the Herpotrichiellaceae in the Eurotiomycetes. The placement of P40D010 appears to lie within Dothideomycetes, and is related to the Teratosphaeriaceae (Schoch *et al.*, 2009).

Leotiomyces: The determination of P41C001 and P43C017 as *Thelobolales* spp. was based on phylogenetic analysis of D1/D2 sequence data (Fig. 17).

Sordariomycetes: There were no close matches to the ITS or D1/D2 sequences derived from P45A009 in GenBank. Phylogenetic analysis of the most similar D1/D2 sequences (Fig. 18) placed P45A009 in the Coniochaetales clade of (Zhang *et al.*, 2006), also occupied by two isolates isolated from *Nothofagus nervosa* seeds and determined as *Coniochaetales* sp. The colonies of P45A009 possess the same distinctive color as *Lecythophora*, the anamorph of *Coniochaeta*, and the yeast-like cells of P45A009 bear resemblance to the phialides and ameroconidia of *Lecythophora* (Seifert *et al.*, 2011). *Lecythophora* is often associated with wood, but P45A009 was isolated from the surface of a 'Chardonnay' grape berry.

Conclusion: The present study was the first attempt to characterize the yeast biota associated with grapes, a major crop in Washington state, and the first molecular characterization of the populations of yeasts in the region. Results are consistent with the idea that Washington is host to a great diversity of yeasts that likely play ecologically-significant roles that need further

investigation. Further work in progress is examining interactions of these yeasts with other organisms, including plant pathogenic fungi. Results also provide evidence that naturally-occurring yeasts may play an important role in winemaking in the state, possibly interfering with fermentation in some cases but offering a new resource to explore for wine makers interested in using non-*Saccharomyces* yeasts to make unique, premium wines.

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Appendix 1: List of strains from which sequences were obtained

Isolate #	Determination	GenBank #	Date Collected	Site	Cultivar / Isolation Source
P01A006	<i>Aureobasidium pullulans</i> var. <i>pullulans</i>	JX188090	06/30/10	Prosser	Riesling
P45D001	<i>Aureobasidium pullulans</i> var. <i>pullulans</i>	JX188091	09/14/10	Prosser	Chardonnay
RGA003	<i>Aureobasidium pullulans</i> var. <i>pullulans</i>	JX188092	09/14/10	Richland	Gewurztraminer
RSA001	<i>Aureobasidium pullulans</i> var. <i>pullulans</i>	JX188093	09/14/10	Richland	Syrah
P34D008	<i>Aureobasidium pullulans</i> var. aff. <i>namibiae</i>	JX188095	09/14/10	Prosser	Chardonnay
P42C015	<i>Aureobasidium pullulans</i> var. aff. <i>namibiae</i>	JX188096	09/14/10	Prosser	Riesling
RChB006	<i>Aureobasidium pullulans</i> var. aff. <i>namibiae</i>	JX188099	09/14/10	Richland	Chardonnay
RGA001	<i>Aureobasidium pullulans</i> var. aff. <i>namibiae</i>	JX188100	09/14/10	Richland	Gewurztraminer
P01A025	<i>Aureobasidium pullulans</i> var. nov.	JX188094	09/14/10	Prosser	Riesling
P44A008	<i>Aureobasidium pullulans</i> var. nov.	JX188097	09/14/10	Prosser	Riesling
RChB004	<i>Aureobasidium pullulans</i> var. nov.	JX188098	09/14/10	Richland	Chardonnay
P40B001	<i>Candida asiatica</i>	JX188101	10/26/10	Prosser	Riesling
P01C003	<i>Candida californica</i>	JX188102	10/26/10	Prosser	Riesling
P25B003	<i>Candida californica</i>	JX188103-4	10/26/10	Prosser	Chardonnay
P43C013	<i>Candida californica</i>	JX188105	10/26/10	Prosser	Riesling
P40C006	<i>Candida oleophila</i>	JX188106	10/26/10	Prosser	Riesling
P40C007	<i>Candida oleophila</i>	JX188107	10/26/10	Prosser	Riesling
RCaA001	<i>Candida railenensis</i>	JX188108	09/14/10	Richland	Cab. Sauvignon
P45A002	<i>Candida saitoana</i>	JX188109-10	09/14/10	Prosser	Chardonnay
P45A009	<i>Coniochaetales</i> sp.	JX188111	08/31/10	Prosser	Chardonnay
P02B003	<i>Cryptococcus adeliensis</i>	JX188112-3	06/30/10	Prosser	Riesling
P34C003	<i>Cryptococcus adeliensis</i>	JX188114	08/31/10	Prosser	Chardonnay
P42C006	<i>Cryptococcus adeliensis</i>	JX188115	09/14/10	Prosser	Riesling
P44A007	<i>Cryptococcus adeliensis</i>	JX188116	10/26/10	Prosser	Riesling
P45C006	<i>Cryptococcus adeliensis</i>	JX188117	10/26/10	Prosser	Chardonnay
P40D008	<i>Cryptococcus albidosimilis</i>	JX188118-9	09/14/10	Prosser	Riesling
P46D001	<i>Cryptococcus carnescens</i>	JX188120	10/26/10	Prosser	Chardonnay
P43A004	<i>Cryptococcus laurentii</i>	JX188121-2	08/31/10	Prosser	Riesling
P01A018	<i>Cryptococcus magnus</i>	JX188123	08/31/10	Prosser	Riesling
P25B001	<i>Cryptococcus magnus</i>	JX188124	08/31/10	Prosser	Chardonnay
P44B001	<i>Cryptococcus magnus</i>	JX188125	10/26/10	Prosser	Chardonnay
P45A003	<i>Cryptococcus magnus</i>	JX188126	09/14/10	Prosser	Chardonnay
P01D003	<i>Cryptococcus saitoi</i>	JX188127	09/14/10	Prosser	Riesling
P01A020	<i>Cryptococcus stepposus</i>	JX188128	08/31/10	Prosser	Riesling
P25B002	<i>Cryptococcus stepposus</i>	JX188129	08/31/10	Prosser	Chardonnay
P01D005	<i>Cryptococcus tephrensis</i>	JX188130-1	09/14/10	Prosser	Riesling
P43C016	<i>Cryptococcus tephrensis</i>	JX188132	10/26/10	Prosser	Riesling
P43D003	<i>Cryptococcus tephrensis</i>	JX188133	10/26/10	Prosser	Riesling
P45C008	<i>Cryptococcus tephrensis</i>	JX188134	10/26/10	Prosser	Chardonnay
P42C010	<i>Cryptococcus uzbekistanensis</i>	JX188135	09/14/10	Prosser	Riesling
P45A008	<i>Cryptococcus uzbekistanensis</i>	JX188136	09/14/10	Prosser	Chardonnay
P25D002	<i>Cryptococcus victoriae</i>	JX188137-8	06/30/10	Prosser	Chardonnay

Appendix 1, cont.

Isolate #	Determination	GenBank #	Date Collected	Site	Cultivar / Isolation Source
P26D001	<i>Cryptococcus victoriae</i>	JX188139	06/30/10	Prosser	Chardonnay
P26D004	<i>Cryptococcus victoriae</i>	JX188140	06/30/10	Prosser	Chardonnay
P27D001	<i>Cryptococcus victoriae</i>	JX188141	06/30/10	Prosser	Chardonnay
P34D007	<i>Cryptococcus victoriae</i>	JX188142-3	08/31/10	Prosser	Chardonnay
P41A001	<i>Cryptococcus victoriae</i>	JX188144	08/31/10	Prosser	Chardonnay
P40A001	<i>Cryptococcus</i> sp. (<i>Bulleromyces</i>)	JX188145	08/31/10	Prosser	Riesling
P25A004	<i>Curvibasidium pallidicorallinum</i>	JX188146	10/26/10	Prosser	Chardonnay
P40B003	<i>Curvibasidium pallidicorallinum</i>	JX188147	10/26/10	Prosser	Riesling
P45C001	<i>Curvibasidium pallidicorallinum</i>	JX188148	09/14/10	Prosser	Chardonnay
P45C004	<i>Curvibasidium pallidicorallinum</i>	JX188149	10/26/10	Prosser	Chardonnay
P45C007	<i>Curvibasidium pallidicorallinum</i>	JX188150	10/26/10	Prosser	Chardonnay
P42A007	<i>Curvibasidium rogersii</i>	JX188232	09/14/10	Prosser	Riesling
P34B005	<i>Cystofilobasidium infirmominatum</i>	JX188151	09/14/10	Prosser	Chardonnay
P40C004	<i>Cystofilobasidium infirmominatum</i>	JX188152-3	09/14/10	Prosser	Riesling
P45C003	<i>Cystofilobasidium infirmominatum</i>	JX188154	10/26/10	Prosser	Chardonnay
P41D001	<i>Cystofilobasidium macerans</i>	JX188155	10/26/10	Prosser	Riesling
P25A005	<i>Dothideales</i> sp.	JX188156	10/26/10	Prosser	Chardonnay
P44C001	<i>Dothideales</i> sp.	JX188157	10/26/10	Prosser	Chardonnay
P42C011	<i>Hannaella luteola</i>	JX188158	09/14/10	Prosser	Riesling
P34A006	<i>Hanseniaspora uvarum</i>	JX188159-61	10/26/10	Prosser	Chardonnay
P43C011	<i>Hanseniaspora uvarum</i>	JX188162-3	10/26/10	Prosser	Riesling
P43C012	<i>Hanseniaspora uvarum</i>	JX188164-5	10/26/10	Prosser	Riesling
RChB002	<i>Hanseniaspora uvarum</i>	JX188166	09/14/10	Richland	Chardonnay
P40D005	<i>Holtermanniella festucosa</i>	JX188167	09/14/10	Prosser	Riesling
P34B009	<i>Holtermanniella takashimae</i>	JX188168	10/26/10	Prosser	Chardonnay
P34B007	<i>Metschnikowia chrysoperlae</i>	JX188169-70	10/26/10	Prosser	Chardonnay
P34A004	<i>Metschnikowia</i> aff. <i>chrysoperlae</i> (1)	JX188171	10/26/10	Prosser	Chardonnay
P34A005	<i>Metschnikowia</i> aff. <i>chrysoperlae</i> (1)	JX188172	10/26/10	Prosser	Chardonnay
P40A002	<i>Metschnikowia</i> aff. <i>chrysoperlae</i> (2)	JX188173-4	10/26/10	Prosser	Riesling
P44A006	<i>Metschnikowia</i> aff. <i>chrysoperlae</i> (3)	JX188175-6	10/26/10	Prosser	Riesling
P01C004	<i>Metschnikowia</i> aff. <i>pulcherrima</i> (1)	JX188183-4	10/26/10	Prosser	Riesling
P01A016	<i>Metschnikowia</i> aff. <i>pulcherrima</i> (2)	JX188181-2	09/14/10	Prosser	Riesling
P40B006	<i>Metschnikowia</i> aff. <i>pulcherrima</i> (3)	JX188185-6	10/26/10	Prosser	Riesling
P34D002	<i>Metschnikowia</i> sp. (<i>pulcherrima</i>)	JX188187-8	09/14/10	Prosser	Chardonnay
P43D001	<i>Meyerozyma caribbica</i>	JX188189	09/14/10	Prosser	Riesling
P46A001	<i>Meyerozyma caribbica</i>	JX188190	10/26/10	Prosser	Chardonnay
P34D003	<i>Meyerozyma guilliermondii</i>	JX188191	09/14/10	Prosser	Chardonnay
P40D002	<i>Meyerozyma guilliermondii</i>	JX188192	09/14/10	Prosser	Riesling
P40C002	<i>Mrakiella cryconiti</i>	JX188193	09/14/10	Prosser	Riesling
P40D010	<i>Phaeococcomyces</i> aff. <i>nigricans</i>	JX188194	08/31/10	Prosser	Riesling
P01C002	<i>Pichia kluyveri</i>	JX188195-6	10/26/10	Prosser	Riesling
P25B004	<i>Pichia kluyveri</i>	JX188197-8	10/26/10	Prosser	Chardonnay

Appendix 1, cont.

Isolate #	Determination	GenBank #	Date Collected	Site	Cultivar / Isolation Source
P40A003	<i>Pichia kluyveri</i>	JX188199-200	10/26/10	Prosser	Riesling
P43C008	<i>Pichia kluyveri</i>	JX188201-2	10/26/10	Prosser	Riesling
P43C009	<i>Pichia kluyveri</i>	JX188203-4	10/26/10	Prosser	Riesling
P43C007	<i>Pichia membranifaciens</i>	JX188205-6	10/26/10	Prosser	Riesling
P43C010	<i>Pichia membranifaciens</i>	JX188207-8	10/26/10	Prosser	Riesling
P01A021	<i>Pseudozyma</i> sp. (1)	JX188209	08/31/10	Prosser	Riesling
P25A002	<i>Pseudozyma</i> sp. (1)	JX188211	08/31/10	Prosser	Chardonnay
P44A002	<i>Pseudozyma</i> sp. (1)	JX188212	08/31/10	Prosser	Riesling
P45A004	<i>Pseudozyma</i> sp. (1)	JX188213	08/31/10	Prosser	Chardonnay
P01A022	<i>Pseudozyma</i> sp. (2)	JX188210	08/31/10	Prosser	Riesling
P01C001	<i>Rhodosporidium babjevae</i>	JX188219	08/31/10	Prosser	Riesling
P44D001	<i>Rhodosporidium babjevae</i>	JX188220	08/31/10	Prosser	Chardonnay
P34A003	<i>Rhodotorula bacarum</i>	JX188221	08/31/10	Prosser	Chardonnay
P41D003	<i>Rhodotorula colostri</i>	JX188222	10/26/10	Prosser	Riesling
P42A002	<i>Rhodotorula colostri</i>	JX188223-4	09/14/10	Prosser	Riesling
P45C002	<i>Rhodotorula colostri</i>	JX188225	10/26/10	Prosser	Chardonnay
P01D002	<i>Rhodotorula mucilaginoso</i>	JX188226-7	08/31/10	Prosser	Riesling
P40C005	<i>Rhodotorula pallida</i>	JX188228	09/14/10	Prosser	Riesling
P43A001	<i>Rhodotorula pallida</i>	JX188229	08/31/10	Prosser	Riesling
P44D004	<i>Rhodotorula</i> sp. (<i>aurantiaca</i>) (1)	JX188233	08/31/10	Prosser	Chardonnay
P34D004	<i>Rhodotorula</i> sp. (<i>aurantiaca</i>) (2)	JX188231	08/31/10	Prosser	Chardonnay
P34D001	<i>Rhodotorula</i> sp. (<i>glutinis</i>)	JX188230	08/31/10	Prosser	Chardonnay
P26D003	<i>Sporidiobolus metaroseus</i>	JX188234-5	06/30/10	Prosser	Chardonnay
P42A004	<i>Sporidiobolus metaroseus</i>	JX188236	09/14/10	Prosser	Riesling
P34D006	<i>Sporidiobolus</i> aff. <i>metaroseus</i>	JX188237	09/14/10	Prosser	Chardonnay
P40D006	<i>Sporidiobolus</i> aff. <i>metaroseus</i>	JX188238	09/14/10	Prosser	Riesling
P43C002	<i>Sporidiobolus</i> aff. <i>metaroseus</i>	JX188239	09/14/10	Prosser	Riesling
P34C004	<i>Sporobolomyces coprosmae</i>	JX188240	08/31/10	Prosser	Chardonnay
P42C016	<i>Sydowia</i> aff. <i>polyspora</i>	JX188241	08/31/10	Prosser	Riesling
P41C001	<i>Thelobolales</i> sp. (1)	JX188242	08/31/10	Prosser	Riesling
P43C017	<i>Thelobolales</i> sp. (2)	JX188243	10/26/10	Prosser	Riesling
P01A017	<i>Wickerhamomyces anomalus</i>	JX188244	09/14/10	Prosser	Riesling
P42B001	<i>Wickerhamomyces anomalus</i>	JX188245	10/26/10	Prosser	Riesling
RChB001	<i>Wickerhamomyces anomalus</i>	JX188246-7	09/14/10	Richland	Chardonnay
P45C009	<i>Yamadazyma mexicana</i>	JX188248	09/14/10	Prosser	Chardonnay
BRTEA	<i>Pseudozyma</i> sp. (1)	JX188214	09/01/11	Pullman	<i>Bromus tectorum</i>
BRTEB	<i>Pseudozyma</i> sp. (1)	JX188215	09/01/11	Pullman	<i>Bromus tectorum</i>
BRHO	<i>Pseudozyma</i> sp. (3)	JX188216	09/01/11	Pullman	<i>Bromus hordeaceus</i>
PICO22B	<i>Pseudozyma</i> sp. (1)	JX188217	09/01/11	Pullman	<i>Pinus contorta</i>
PICO23	<i>Pseudozyma</i> sp. (1)	JX188218	09/01/11	Pullman	<i>Pinus contorta</i>