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Yeasts from Greenhouse Grapes Show Less Phenotypic and Genetic Diversity than Yeasts from Vineyard Grapes when Isolated from Grape Crush Cultured in Liquid Media

Brittany Goldhawke[1], Manjot Kahlon[1], Jeremy Lotto[1], and Christoph M. Deeg[2]
Department of Microbiology and Immunology, University of British Columbia

High quality and regional wines are characterized by the use of indigenous species of yeasts in co-fermentation with commercial strains, providing unique and robust flavors and aromas. Cultivation of indigenous species of yeasts of the grape berry may prove difficult with the diverse conditions required for growth, and the prevalence of commercial yeast species in the vineyard. Assessing yeast diversity on grapes in the absence of a commercial winery environment may prove useful in isolating indigenous yeasts. We hypothesized that yeasts isolated from greenhouse grapes would comprise of species not commonly associated with the vineyard environment, and would originate from outside the genus *Saccharomyces*. To test this hypothesis, 20 yeast isolates were collected from greenhouse and vineyard grapes. Qualitative growth response assays and rDNA genotyping were used to compare isolates at a phenotypic and genetic level, respectively. Among yeast isolates, vineyard isolates displayed more diversity at both a phenotypic and molecular level than the greenhouse isolates. In the vineyard, ethanol tolerant isolates belonged to the genus *Metschnikowia*, while the remaining belonged to the genus *Hanseniaspora* with few exceptions. The dominant greenhouse isolates were identified as the less ethanol tolerant species *Debaromyces hanseneii.* Through phylogenetic analysis, the *Metschnikowia* species isolated from the vineyard was found to be highly diverged from the commercial *Saccharomyces cerevisiae* strain EC1118. The species *Hanseniaspora uvarum, D. hanseneii* and *S. cerevisiae* EC1118 were most similar at the genetic level. Overall, we observed less yeast diversity than expected. We speculate that a liquid enrichment step may have selected for highly competitive species, and recommend exploring non-culture based methods to identify more species and to characterize diversity.

Each year, winemakers must carefully choose which grapes to grow, when to perform their harvest, and most importantly, the type of yeast strain to use in fermentation. The most desirable yeast strains will achieve complete fermentation of sugar to ethanol, while providing a unique flavor profile to the wine that is satisfying to the consumer (1). These properties differ amongst species of yeast and relate to their relative ability to tolerate approximately 30% glucose, the concentration of glucose in grapes at harvest (2). If complete fermentation is to be achieved, a high tolerance to ethanol is also required as levels ranging from 10- 20% are seen in table and ice wines (2). Historically, species of yeast capable of complete fermentation were collected and preserved as a starter culture for future use in wine production (3). Through human selection, commercially available wine yeasts, specifically the species *Saccharomyces cerevisiae*, have now become a staple of modern winemaking (3). One such commercial strain is the popular *S. cerevisiae* EC1118, isolated in France and known for its strong fermentation characteristics and high ethanol production (4).

The use of indigenous and non-*Saccharomyces* species of yeast can provide a unique "terroir" quality to the wine; flavors and aromas imparted by the environment in which the wine is grown and produced (5). Within each region, a unique yeast microbiome with diverse species can be found on the grape berry. The microbiome also changes during "veraison"; the onset of grape berry ripening (6). The succession of yeasts during veraison commonly begins with the genera *Aureobassidium*, *Cryptococcus*, *Rhodosporidium* and *Rhodotorula* (6). As the berry ripens and the grape skins begin to turn from green to purple, the yeast community becomes dominant in the genera *Hanseniaspora, Candida,* and *Metschnikowia* (6). Over-ripened and damaged berries further select for these yeast

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genera, as well as species of the genus *Saccharomyces* used in fermentation (6). The grape species and sugar content provide unique and necessary factors for yeast selection, metabolism and growth, and the yeast itself produces phenols, esters, acids, and alcohols that result in a depth of flavor and aroma (7, 8). Although indigenous yeasts may produce beneficial qualities in wine, their low ethanol tolerance often results in incomplete fermentation (9). Co-inoculating with non-*Saccharomyces* and *Saccharomyces* species results in complete fermentation and provides more depth in flavor than the use of *Saccharomyces* species alone (9).

Isolation and use of a diverse vineyard yeast community is therefore beneficial in wine production, but their cultivation and isolation amongst commercial strains may be a difficult task to achieve. Every harvest, the residual grape sediments from wine produced by commercial yeasts are recycled back into the vineyard, introducing the commercial *S. cerevisiae* strains into the vineyard yeast community (10). A genetic and phenotypic study comparing two novel indigenous yeast strains to the commercial *S. cerevisiae* strain EC1118 found that all three strains displayed a close genetic relationship, suggesting that commercially available yeasts disseminated into the vineyard and evolved into new genotypes not originally found in the environment (11).

To investigate what level of diversity occurs amongst indigenous species of yeast when separated from a commercial winery environment, grapes can be grown in newly acquired land or in a greenhouse, where they are more frequently found (50% plants and 100% soils) than on the outside (17% of plants and soils) (12). Furthermore, 15% of fungal isolates from greenhouse *ficus* plants were of yeast origin and belonged to the genera *Cryptococcus* and *Rhodotorula*, comprising 23 different species (13). Studies on the isolation of yeasts from greenhouse environments have yet to involve those of use in wine production. In this study we aimed to assess the level of oenological and genetic diversity present among yeasts collected from grapes in a greenhouse environment, in comparison to those collected from grapes in a vineyard.

Due to previously isolated yeast strains from grapes post veraison, and the annual introduction of commercial strains into the vineyard, we hypothesized that the yeast microbiome of vineyard grapes would be largely represented by the genera: *Hansenispora, Metshcnikowia, Candida* and *Saccharomyces*. Conversely, we predicted that the yeast microbiome of greenhouse grapes after veraison would show more diversity in species outside the genus *Saccharomyces* and those not commonly associated with a vineyard. Furthermore, we hypothesized that isolates of the vineyard would display more tolerance to oenological conditions such as high glucose and ethanol, while those of the greenhouse would show a moderate tolerance to glucose, and a low tolerance of ethanol.

MATERIALS AND METHODS

Isolation of yeast from grapes. Fifty grapes of the *Vitis vinifera* Merlot variety were collected from Dr. Simone Castellarin's grape crops in a greenhouse on the University of British Columbia (UBC) campus in Vancouver, Canada. Grapes were grown without previous exposure to commercial wine yeasts, and picked after the onset of veraison. The temperature of the greenhouse at harvest was 23°C. Greenhouse grape crops had previously been treated with 3 fungicides; Rhapsody, Switch and Milstop to treat Powdery mildew infestation. Vineyard grapes of the *Vitis vinifera* Pinot Noir variety collected from an Okanagan vineyard in British Columbia, Canada, was supplied as a gift by Jay Martiniuk of the Wine Research Centre from the September 2015 harvest. The grapes from the greenhouse and the vineyard were pooled together in separate Erlenmeyer flasks with liquid yeast peptone dextrose (YPD) media containing 20 g/l peptone, 20 g/l dextrose, and 10 g/l of yeast extract (Difco Laboratories, Detroit, MI, USA), and put on a shaking platform at room temperature (20°C) for 24 hours. 50 µl of the liquid culture was spread plated onto YPD agar plates containing 20 g/l peptone, 10 g/l yeast extract, 20 g/l dextrose, 15 g/l agar (Sigma Aldrich, St Louis, MO, USA) and 50 µg/ml of chloramphenicol for bacterial inhibition. The plates were incubated for 24-72 hours at 30°C for growth of isolated colonies. After incubation, each colony was visually screened for differences in color and morphology; each unique colony was re-streaked onto YPD agar plates, and further incubated for 24-72 hours at 30°C. Lalvin™ EC1118 *Saccharomyces cerevisiae,* was purchased from BosaGrapes in Burnaby, Canada, as a control culture and rehydrated according to the package instructions.

DNA Isolation. 50 µl of the rehydrated EC1118 culture, as well as the vineyard and greenhouse cultures, were re-streaked onto YPD plates to isolate clonal colonies. DNA isolation was performed as follows: a single colony from each isolate used was aseptically picked and suspended in 100 µl of 0.2 M lithium acetate 1% SDS solution (14). Each solution was incubated in a 70°C water bath for 5 minutes. After incubation, 300 µl of 100% ethanol was added to each solution for a final concentration of 75% ethanol and vortexed. The culture was centrifuged at 15000 *g* for 3 minutes, the supernatant poured off, and the pellet washed with 70% ethanol and centrifuged at 15000 *g* for 3 minutes. The pellet was dissolved into 100 µl of sterile distilled water, and centrifuged at 15000 *g* for 15 seconds. The supernatant was collected, the DNA concentration measured in triplicate using the Nano-drop 2000™ UV-vis Spectrophotometer by Thermo Scientific, and stored at -8°C for PCR analysis.

PCR amplification of the D1/D2 region of 26S rDNA. The PCR method utilized in this study to amplify the D1/D2 domain of yeast rDNA was adopted from New England Biolabs and Lee et al., for the amplification conditions and NL1 and NL4 primer sequences respectively (2). The PCR reaction was performed in a final volume of 50 µl, with the following components: 5 µl of 10X PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of 10 µM NL1 primer 5'-GCATATCAATAAGCGGAGGAAAAG-3', 1 µl of 10 µM NL4 primer 5'-GGTCCGTGTTTCAAGACGG-3' (Integrated DNA Technologies), 200 ng of template DNA, 0.25 µl of Taq DNA polymerase and the appropriate amount of sterile H_2O. Thermo-cycler conditions were as follows: denaturation at

94°C for 1 min, 33 cycles of 95°C for 1 min, 55°C for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 6 minutes. The amplified products were run at 100 V for 1.5 hours on 1.5% agarose gels, and visualized under UV light using a UV transilluminator.

DNA sequencing. Amplified PCR products were purified using Purelink® PCR purification kit, subsequently measured for purity and concentration using a Nano-drop 2000 in triplicate, and prepared to a final concentration >15 ng/µl. Primer NL1 was prepared to a final concentration of 5 pmol/µl, and was sent along with DNA samples at a volume of 20 µl, to the Nucleic Acid Protein Service (NAPS) unit of the Michael Smith Laboratories located at UBC Vancouver, Canada for Sanger sequencing. The sequences were retrieved from the NAPS database, and chromatograms analyzed using Geneious® 9.0.4 software (15). Ambiguous nucleotides were trimmed from the ends to perform a species identity search. Using the Basic Local Alignment Search Tool (BLAST) in Geneious® 9.0.4, with Database: "nr", program: "Megablast", results; "Hit table", Retrieve: "matching region", with 100 hits, the identity of each species, and accession number was retrieved with 100% query cover. Sequences were grouped and then trimmed to the same size according to what genus and species they were identified under. Nucleotides denoted as "N" were either trimmed or reassigned the appropriate nucleotides manually according to the chromatogram. Sequences from *Metschnikowia sp*. were trimmed to a size of 465 bp, *M. fructicola* to a size of 450 bp, *H. uvarum* to a size of 506 bp, *D. hanseneii* to a size of 556 bp, and *S. cerevisiae* to a size of 441 bp (S.Fig. 6).

D1/D2 Sequence alignment and phylogeny analysis. All sequences belonging to the same species were grouped together and aligned using Geneious® 9.0.4 software, Multiple Sequence Comparison by Log-Expectation (MUSCLE) alignment with 8 iterations and default settings (15, 16). The pairwise % identity was retrieved for all sequences grouped and aligned within their species. Due to high similarity, a single representative sequence from each species was chosen with a high HQ % (the percentage of untrimmed bases in a sequence that are of high quality). Multiple alignments of the 5 sequences were aligned using Geneious® 9.0.4 software, with MUSCLE alignment algorithm, with 8 iterations and default settings (15, 16). A phylogenetic tree represented as a Maximum likelihood model of evolution was generated within Geneious® 9.0.4 using Randomized Axelerated Maximum Likelihood (RAxML) 7.2.8 plugin, and the Maximum likelihood tree algorithm (15, 17). Nucleotide substitution rates were optimized, GAMMA rate of heterogeneity and general time reversible model employed, using the nucleotide model "GTR GAMMA I". Confidence values were inferred using "Rapid bootstrapping" and validated with 1000 Bootstrap replicates. The additional command line options"-f a -x 54475" were generated and sent to RAxML executable (15, 17).

Ethanol spot assay. An isolated colony was selected from each of the vineyard and greenhouse plates, suspended into 3 ml fresh liquid YPD, and grown for 17 hours at 30°C. After 17 hours, the OD_{600} was measured and each culture was diluted into new liquid YPD to a final OD_{600} of 0.1, and grown further to an OD_{600} of 1. Each culture was then diluted with sterile distilled water by 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. Ethanol-YPD plates were made by autoclaving 70 g of YPD media in distilled water to a final volume of either 920 ml or 880 ml. Ethanol was added after autoclaving in volumes of 80 ml or 120 ml of 100% (v/v) ethanol to retrieve a final concentration of 8% or 12% (v/v) ethanol in YPD, respectively. Control YPD plates without ethanol were also prepared. 3 µl of each dilution was spotted in duplicate onto control YPD, 8% ethanol-YPD, and 12% ethanol-YPD plates, and incubated for 24 hours at 30°C.

Dextrose spot assay. YPD plates containing 30% (w/v) dextrose were made by combining 20 g/l peptone, 10 g/l yeast extract, 20 g/l agar (Difco Laboratories, Detroit, MI, USA) in distilled water to a final volume of 400 ml and autoclaved. A 50% dextrose solution was made by adding 50 g of dextrose (Fisher Scientific Company, Ottawa, Ontario, Canada) to a final volume of 100 ml in distilled water. 600 ml of the 50% (w/v) dextrose solution was sterile filtered and added to the 400 ml of yeast peptone agar media. To make 40% dextrose plates, 800 ml of 50% (w/v) dextrose solution was added to 200 ml of autoclaved yeast peptone agar media. The same yeast culture dilutions made for the ethanol spot assays were used to spot 3 µl of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions onto the YPD plates. The plates were incubated for 24 hours at 30°C until growth of colonies was observed.

Spot assay data analysis. Yeast growth on 8% and 12% ethanol, as well as 30% and 40% dextrose, was determined using the control YPD plates for comparison. Growth inhibition was determined in a qualitative manner by observing presence of colonies in the spotted cultures after 24 hours of growth. Growth at 10^{-1} was denoted as "1", and considered minimal growth, 10^{-2} as "2" and considered low growth. Growth at 10^{-3} was denoted as "3" and considered moderate growth, and growth at 10^{-4} dilutions as "4", and considered complete growth (Refer to S.Figs. 1-4). Complete inhibition of growth compared to the YPD control plate was determined by the complete absence of cells, and denoted as "0".

RESULTS

Culture of presumptive yeast isolates from grapes of a greenhouse and vineyard environment. To culture yeast species of the grape berry, and to promote growth of those present in very small population densities, we utilized liquid YPD as an enrichment media. To isolate yeast colonies and to select against growth of bacteria, the liquid culture was plated onto YPD agar plates containing chloramphenicol. We collected isolates displaying non-filamentous fungal morphology as previously described (18). YPD plates spread with vineyard grape cultures resulted in either opaque/cream or rust/red colored colonies. The greenhouse culture plates contained colonies that were opaque/cream colored only (Fig. 1). 40 presumptive yeast colonies were isolated from YPD-chloramphenicol plates containing the vineyard and greenhouse grape crush samples.

PCR amplification of D1/D2 domain of yeast rDNA. To ensure the isolates were of yeast origin and to further assess species diversity, PCR was used to amplify the D1/D2 region of yeast rDNA ranging from 500-700 bp in size. PCR amplification using primers NL1 and NL4 resulted in bands within 500-700 bp in size of all isolates. All greenhouse isolates produced bands at approximately 600 bp (Fig. 2a-b). Vineyard isolates V2, V4, V9, V11 and V18 produced bands 500 bp in size, while the remaining

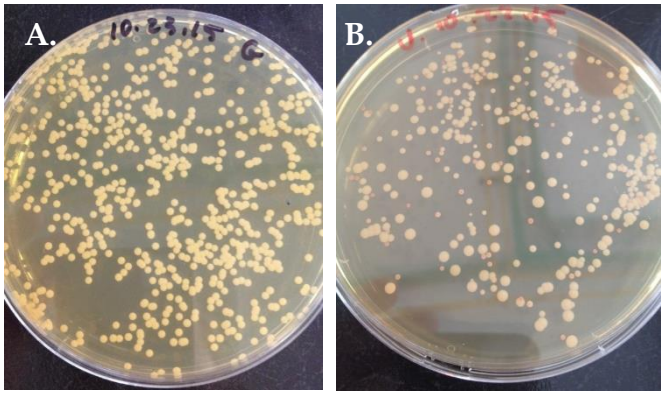


FIG. 1. Greenhouse and vineyard crush plated on YPD – Chloramphenicol yeast selection plates, incubated for 24 hours at 25°C. (A) Greenhouse presumptive yeast isolates cultured after incubation on yeast growth media. (B)Vineyard presumptive yeast isolates cultured after incubation on yeast growth media

vineyard isolates produced similar bands close to 600 bp in size (Fig. 2c-d). Lalvin EC1118™ *S. cerevisiae* produced a band close to 600 bp in size as a positive control (Fig. 2a, lane 3). Bands were not observed in the negative control conditions lacking template. PCR amplification of the D1/D2 domain in presumptive yeast isolates resulted in bands with sizes corresponding to that of the D1/D2 domain, indicating the isolates were likely of yeast origin.

Vineyard yeast isolates tolerate higher levels of ethanol and glucose and exhibit greater diversity than yeast isolates from a greenhouse. To assess phenotypic diversity and the ability of the yeast isolates to tolerate levels of ethanol and dextrose seen in wine fermentation, each cultured isolate was spotted onto YPD plates containing 8% or 12% (v/v) ethanol, and 30% or 40% (w/v) dextrose. Serial dilutions of each culture were prepared to compare the level of growth inhibition under each condition. Complete growth inhibition was scored as the absence of cells in all dilutions. Complete growth was denoted as presence of growth at all 4 dilutions and in relation to the YPD plates lacking ethanol and dextrose. The intermediate dilutions were used to qualitatively evaluate partial growth inhibition. Full growth in all dilutions was observed for all vineyard and greenhouse isolates on control YPD plates, while isolates from the greenhouse G15 and G20 exhibited moderate growth (Fig. 3a, S.Fig. 1c-d). Exposing the greenhouse and vineyard isolates to 8% ethanol resulted in complete inhibition of growth for all greenhouse isolates and all vineyard isolates except vineyard isolates V1, V3, V5, V11, V12 and V14, which exhibited minimal amounts of growth, and isolates V2 and V4 which showed low amounts of growth (Fig. 3a-b, S.Figs. 3-4). Spotting the isolates on 12% ethanol plates resulted in growth inhibition of all greenhouse and vineyard isolates (Fig. 3a-b, S.Figs. 3-4). For the 30% dextrose spot assays, complete growth was seen in all greenhouse isolates except for G15 and G20, which showed moderate growth (Fig. 3a, S.Fig. 1b). All vineyard isolates exhibited complete growth on 30% dextrose except for isolate V6 which showed moderate growth (Fig. 3b, S.Fig. 2b). 40% dextrose spot assays resulted in complete growth for greenhouse isolates G1-G10, moderate amounts of growth for isolates G11-19, and minimal amounts of growth of isolate G20 (Fig. 3a, S.Fig. 1a-d). Complete growth of the vineyard isolates V2, V9, V10, V17 and V20, was observed on 40% dextrose while all other isolates exhibited moderate amounts of growth (Fig. 3b, S.Fig. 2a-d). Taken together, these data suggest that yeast isolates from vineyard grapes are more tolerant of high levels of ethanol and glucose than the greenhouse yeast isolates. Moreover, the vineyard yeast isolates showed more growth diversity compared to yeast isolated from the greenhouse (Fig. 3a-b).

DNA sequencing of the D1/D2 rDNA PCR amplified fragments identifies individual yeast isolates. To identify the species and assess the diversity of yeasts isolated from grapes of the vineyard and greenhouse environment, PCR amplified yeast DNA was sequenced. To ensure correct species identification, each chromatogram was trimmed using Geneious® software 9.0.4. Sequences with single nucleotide polymorphisms called as ambiguous "N" were excised. Nucleotides labelled as ambiguous in the presence of minor baseline noise were reassigned the appropriate nucleotide corresponding to the peak with the highest signal (S.Fig. 5). A Geneious® BLAST nucleotide search of all sequences resulted in identification of all species isolated from both the greenhouse and vineyard environments. 19 out of 20 greenhouse isolates were identified as *Debaryomyces hansenii.* The remaining greenhouse isolate (G17) was identified as *Hanseniaspora uvarum*. Vineyard isolates V2 and V4 were identified as *Metschnikowia fructicola*, and isolates V9, V11, and V18 were identified as an undescribed species of the genus *Metschnikowia.* The remaining vineyard isolates (V1, V3, V5-V8, V10, 12, V13, V15-V17, V19, and V20) were identified as *H. uvarum*, except for V14 which was found to be *D. hanseneii.* Sequencing the DNA of all presumptive yeast isolates identified them to the genus and species level.

Ribosomal DNA sequence cluster analysis reveals more phylogenetic diversity in yeasts isolated from a vineyard compared to a greenhouse. To assess the evolutionary relationship of the species of yeasts isolated from both environments, phylogenetic analysis was performed. The commercial wine yeast strain *S. cerevisiae* EC1118, one of the most common and globally used strains in wine fermentation, was used as a comparator (19). All trimmed yeast rDNA sequences within their own species were aligned using Geneious® 9.0.4 software and MUSCLE alignment to assess pairwise percent identity between sequences (S.Fig. 5). All sequences within each species had

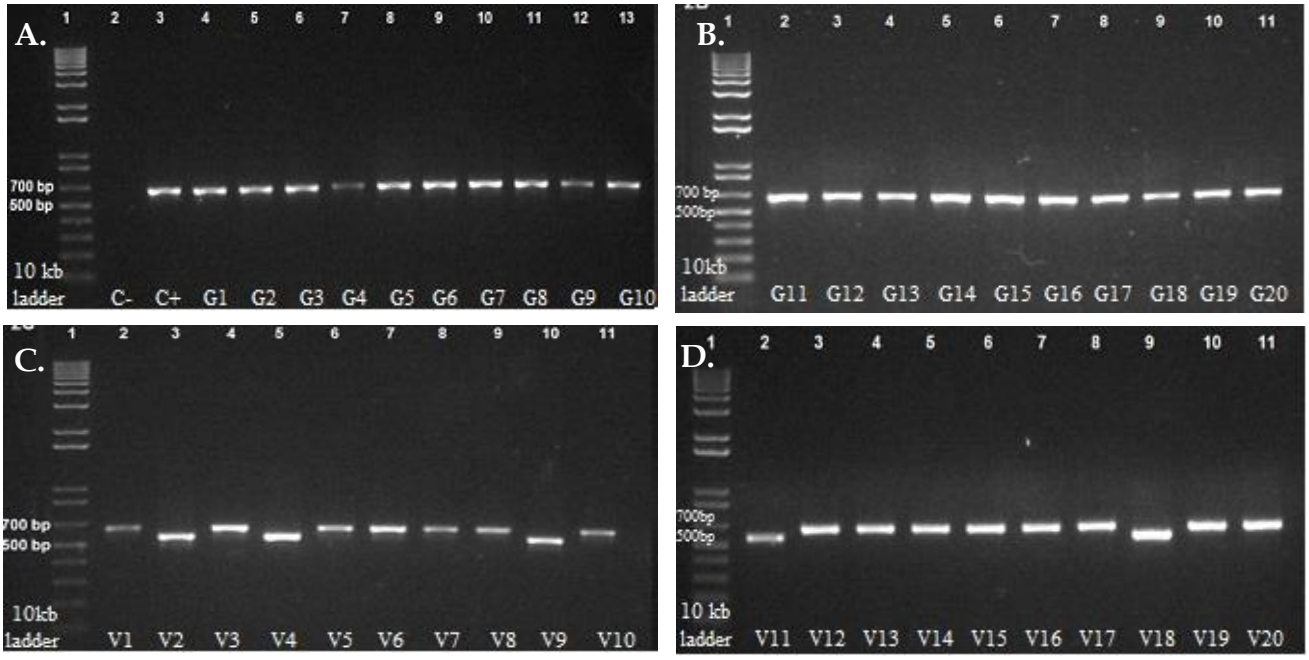


FIG. 2. 1.5% agarose gel electrophoresis of D1/D2 PCR amplified products using primers NL1, NL4, run for 1.5 hours at 100 volts. (A) Lane 1: 10 kb plus Purelink® DNA ladder; lane 2: negative control; lane 3: positive control (*S. cerevisiae* EC1118™); lanes 4-13: Greenhouse isolates 1-10. (B) Lane 1: 10 kb plus Purelink® DNA ladder; lanes 2-11: Greenhouse isolates 11-20. (C) Lane 1: 10 kb plus Purelink® DNA ladder; lanes 2-11: Vineyard isolates 1-10. (D) Lane 1: 10 kb plus Purelink® DNA ladder; lanes 2-11: Vineyard isolates 11-20.

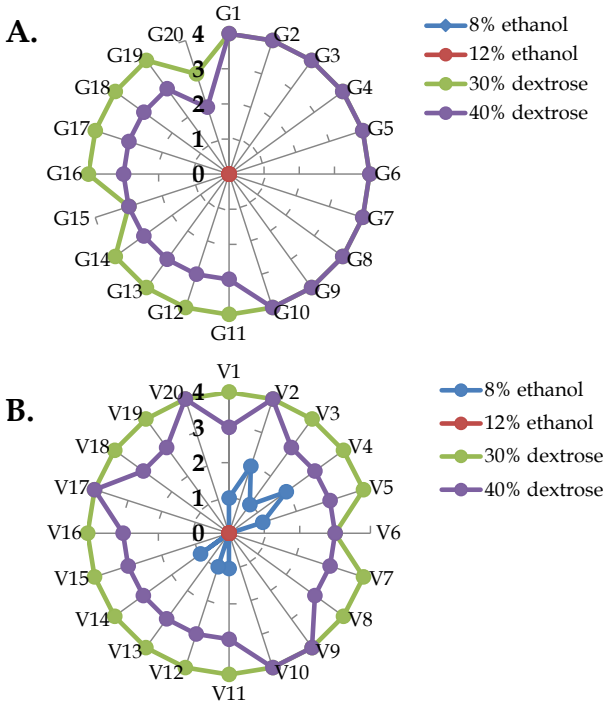
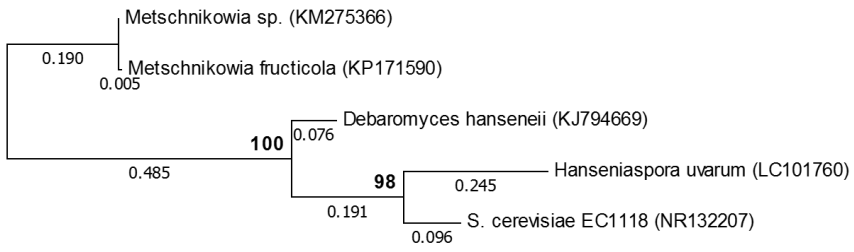


FIG.3. Growth response of Presumptive yeast isolates on Ethanol and Dextrose yeast growth media, incubated for 17 hours at 30°C. Greenhouse (A) , and vineyard (B) isolates growth response to 8%, 12%, ethanol and 30%, 40% dextrose, spotted in 3µl drops in duplicate, and in 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions. Growth at 10^{-4} denoted as "4", 10^{-3} as "3", 10^{-2} as "2", and 10^{-1} as "1" respectively. No growth is denoted as "0" (S.FIG.1-4, S.FIG.6)

a pairwise percent identity greater than 99% (S.Fig. 5) A single representative sequence from each species was chosen for further analysis. 5 sequences were further aligned using MUSCLE (S.Fig. 6) and then RaXML 7.2.8 to construct a phylogenic tree (Fig. 4a). The phylogenetic tree resulted in 3 monophyletic clusters arising from a single inner node, with Bootstrap support >99%. The species *H. uvarum*, *S. cerevisiae* EC1118, and *D. hanseneii* belonged to one group with bootstrap support of 100%. Within this clade, *D .hanseneii* occupied one branch, while the other branch was the stem of another monophyletic group containing *S. cerevisiae* EC1118 and *H. uvarum*. Within this subgroup, *S. cerevisiae* EC1118 and *H. uvarum* had a percent identity between their sequences at each aligned position of 85%, while *D. hanseneii* and *S. cerevisiae* EC1118 was 84% (Fig. 4b). The branch supporting species of *Metschnikowia* contained the longest branch distance from *S. cerevisiae,* and also resulted in a percent identity of 64% (Fig. 4b). *Metschnikowia* exhibited less than 66% identity with all other sequences (Fig. 4b). This analysis suggests that the yeast species isolated from the vineyard cluster into 2 separate monophyletic groups exhibiting more phylogenetic diversity than yeast isolates from the greenhouse. *H. uvarum* of the vineyard was phylogenetically most similar to the commercial yeast strain *S. cerevisiae* EC1118.

A.



B.

	Metschnikowia sp.	*M. fructicola*	*D. hanseneii*	*H. uvarum*	*S. cerevisiae*
Metschnikowia sp.		94.839%	65.756%	60.461%	63.656%
M. fructicola	94.839%		63.964%	59.309%	62.996%
D. hanseneii	65.756%	63.964%		80.078%	84.305%
H. uvarum	60.461%	59.309%	80.078%		85.202%
S. cerevisiae	63.656%	62.996%	84.305%	85.202%	

FIG. 4. Cluster analysis of the D1/D2 regions of yeast rDNA. Vineyard and greenhouse D1/D2 rDNA sequences were aligned using Geneious® software and MUSCLE alignment (S.FIG.6). (A) Unrooted phylogenetic tree constructed using Geneious® 9.0.4 software, and evolutionary distances were computed using RAxML: Randomized Axelerated Maximum Likelihood method, rapid bootstrapping with 1000 bootstrap replicates. Percent bootstrap support shown at each node. Species *Metschnikowia sp*. represents isolates V11, V9, and V18. *M.fructicola* represents vineyard isolates V2, V4. *D.hanseneii* represents greenhouse isolates G1-G20 and vineyard isolate V14. *H.uvarum* represents vineyard isolates V1, 3, 5, 6, 7, 8, 10, 12, 15, 16, 17, 19, 20, and greenhouse isolate G17. Branch length is shown in the middle of each branch in base substitutions per site. Scale bar = 0.02 base substitutions per site. NCBI database accession numbers indicated in brackets next to the species. (B) Distance matrix displayed as % Identity (percentage of bases/nucleotides that are identical), between the 5 different species *Metschnikowia sp.*, *Metschnikowia fructicola*, *Debaromyces hanseneii*, and *Hanseniaspora uvarum*, isolated in the greenhouse and the vineyard, and the commercial *S. cerevisiae* strain EC1118. Distances calculated between all species are displayed as % Identity using Geneious software 9.0.4 and MUSCLE multiple alignment with default settings and 8 iterations (S.FIG.6)

DISCUSSION

In this study we aimed to assess the level of yeast species diversity from an environment isolated from a commercial vineyard or winery. To address this, we isolated yeasts from skins of greenhouse or vineyard grapes. We assessed genetic diversity utilizing rDNA sequencing to identify and phylogenetically characterize the isolates. We further studied the yeast isolates using biochemical spot assays to characterize growth in the presence of glucose and ethanol.

In characterizing 40 yeast isolates, we found that those from the greenhouse displayed less phenotypic diversity in growth assays and exhibited lower tolerance to ethanol in comparison to the vineyard yeast isolates. Further, the greenhouse isolates were almost all the non-*Saccharomyces* species *D. hanseneii*. The other isolate from the greenhouse was *H. uvarum*. The vineyard isolates were identified as *Metschnikowia sp., M. fructicola, H. uvarum* and 1 isolate of *D. hanseneii*. Through phylogenetic analysis, we found that species of the vineyard were more genetically diverse and occupied two monophyletic groups. Combining data from the phenotypic and genetic assays, we found that the isolated species of *Metschnikowia* (which showed high tolerance to ethanol), are distantly related to *S. cerevisiae*. In comparison, *H. uvarum* isolated from the vineyard (which showed low tolerance to ethanol) was more closely related to *S .cerevisiae*.

Through phylogenetic analysis of D1/D2 rDNA, we sought to find the genetic similarities between all isolates, specifically *Metschnikowia* of high ethanol tolerance, to the commercial strain *S. cerevisiae* EC1118. *D. hanseneii* was found to belong to a monophyletic group with *S. cerevisiae* EC1118 and *H. uvarum,* suggesting the three species share a recent common ancestor. *H. uvarum* of the vineyard not only belonged to the same subclade as *S. cerevisiae* EC1118, but was also the most genetically similar exhibiting the shortest branch length. *S .cerevisiae* showed fewer nucleotide changes compared to *H. uvarum* since splitting from the common ancestor; possibly reflective of the consistent growth conditions and limited selective pressure on *S. cerevisiae* used in wine yeast cultures (3). Interestingly, the species *H. uvarum* and *D. hanseneii* were more closely related to *S. cerevisiae* than the *Metschnikowia* species with higher tolerance to ethanol. Taken together, these observations suggest that high ethanol tolerance is not a trait limited to the genus *Saccharomyces.* Likewise, low

ethanol tolerance in yeasts does not indicate a distant genetic relationship to *S. cerevisiae*.

We found that the yeast isolates belonging to the clade *Metschnikowia* exhibited considerable evolutionary distance from all other yeasts in our study. Furthermore, *M. fructicola* of this group occupied its own subclade but did not exhibit notable evolutionary change from the undescribed species of *Metschnikowia*, suggesting a very recent divergence. Although displaying minimal divergence, they exhibited different levels of ethanol tolerance in the 8% ethanol assay. *M. fructicola* displayed low growth, while *Metschnikowia sp*. only displayed minimal growth.

Although our phenotypic and genetic analyses suggest that a higher level of species diversity is present in the vineyard environment, we must consider the possibility that our results may not comprehensively reflect the native yeast diversity in these environments. Previous yeast culturing methods by Renouf et al. compared the use of direct plating of grape wash water, as well as an initial liquid enrichment step to promote the growth of yeast species present in very low population densities (20). They concluded that a liquid enrichment prior to plating resulted in growth of species of yeasts not isolated via direct plating of the grape wash water samples on solid agar (20). In our study, we incubated grapes with liquid YPD media over 24 hours to encourage growth of small populations of yeasts. As a result, we may have decreased diversity and enriched for those more accustomed to liquid yeast media nutrient conditions. Furthermore, *Metschnikowia* and *Hanseniaspora* species, common residents of the vineyard berries, were isolated while the other commonly isolated yeasts *Candida* and *Saccharomyces* were not found. Species of the genus *Metschnikowia* species are known for their yeast inhibitory compounds, specifically *M. pulcherrima*, which produces an iron scavenging pigment called Pulcherrimin (21). Interestingly, only *S. cerevisiae* and *S. uvarum* were found to be inhibited by the Pulcherrimin in the vicinity of *M. pulcherrima,* whereas *C. stellata*, *H. uvarum*, *S. pombe*, and *S. japonicas*, showed normal growth (21). Culturing species of *Metschnikowia* overnight with the other yeasts present had no effect on *H. uvarum* as seen in the previous study, but may have inhibited the growth of *S. cerevisiae* and species of *Candida*, the yeasts we hypothesized would be found in the vineyard (21). Furthermore, several yeast isolates we cultured from the vineyard displayed a rust-colored pigment, indicating that species of *Metschnikowia* may have indeed been producing Pulcherrimin (Fig. 1b). While rDNA sequencing and a BLAST species search could not identify the *Metschnikowia* isolates to the species level, our observations of the rust-colored yeast colonies and possible yeast inhibition during culturing suggest that the undescribed species may be closely related to *M. pulcherrima*.

D. hanseneii also produces and tolerates several toxins, specifically the toxin myocin found to inhibit the growth of other yeast organisms when cultured together (22). In our study, 19 of 20 greenhouse isolates were *D. hanseneii*, which may be the result of liquid enrichment and toxin production by the yeast. The use of YPD media to isolate species of yeast from the greenhouse may have also promoted the over growth of *D. hanseneii*, while out-competing the growth of other, less cultivable species of yeast (20). Conversely, a single *D. hanseneii* species was isolated from the vineyard. This may be indicative of the low population density of the species originally present and also out-competed by other yeasts in found in the liquid culture.

In conclusion, we observed less phenotypic and genetic diversity of yeast isolated from the greenhouse than those isolated from the vineyard. In comparison, the vineyard displayed more diversity in yeast species, and more distance in their evolutionary relationship to one-another. Through phylogenetic analysis, *D. hanseneii*, *H. uvarum*, and the commercial strain *S. cerevisiae* EC1118, all belong to one monophyletic clade and exhibit a close genetic relationship regardless of the distant geographical regions they were isolated from. Lastly, the lower level of diversity seen in the greenhouse yeasts may not be indicative of the existing diversity of yeast populations in that environment, but rather a consequence of succession and competition between yeasts during culturing. Future studies may involve isolation and plating of yeasts without initial culturing in liquid broth to minimize selection for highly competitive yeast species. To ensure a full representation of yeast species, grapes can be collected before and after veraison for isolation of yeasts present during different stages of grape berry ripening. Further, the use of different nutrient media may be utilized. Lastly, to pursue yeast population dynamics, the vineyard yeast isolates of this study may be co-cultured with other species of yeasts to assess their inhibitory properties. Their usefulness in wine production may serve as natural microbial inhibitory agents.

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