Changes in the relative abundance of two *Saccharomyces* species from oak forests to wine fermentations

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Abstract

Saccharomyces cerevisiae and its sibling species *S. paradoxus* are known to inhabit temperate arboreal habitats across the globe. Despite their sympatric distribution in the wild, *S. cerevisiae* is predominantly associated with human fermentations. The apparent ecological differentiation of these species is particularly striking in Europe where *S. paradoxus* is abundant in forests and *S. cerevisiae* is abundant in vineyards. However, ecological differences may be confounded with geographic differences in species abundance. To compare the distribution and abundance of these two species we isolated *Saccharomyces* strains from over 1,200 samples taken from vineyard and forest habitats in Slovenia. We isolated numerous strains of *S. cerevisiae* and *S. paradoxus* as well as small number of *S. kudriavzevii* strains from both vineyard and forest environments. We find *S. cerevisiae* less abundant than *S. paradoxus* on oak trees within and outside the vineyard, but more abundant on grapevines and associated substrates. Analysis of the uncultured microbiome shows that both *S. cerevisiae* and *S. paradoxus* are rare species in soil and bark samples, but can be much more common in grape must. In contrast to *S. paradoxus*, European strains of *S. cerevisiae* have acquired multiple traits thought to be important for life in the vineyard and dominance of wine fermentations. We conclude that *S. cerevisiae* and *S. paradoxus* currently share both vineyard and non-vineyard habitats in Slovenia and we discuss factors relevant to their global distribution and relative abundance.

Introduction

The ability to ferment sugar in the presence of oxygen originated around the time of a whole genome duplication and is shared by many yeast species (Hagman et al., 2013). Among these yeasts, *Saccharomyces* species are distinguished in being present and intentionally used by humans for the production of alcoholic beverages. While strains of *S. cerevisiae* are the most widely used, other *Saccharomyces* species and their hybrids are involved in various types of fermentations. *S. cerevisiae* x *S. eubayanus* hybrids are used for lager production (Libkind et al., 2011; Nakao et al., 2009), *S. cerevisiae* x *S. kudriavzevii* and *S. cerevisiae* x *S. uvarum* hybrids are used for low temperature wine production (Belloch et al., 2009; Borneman et al., 2012; Bradbury et al., 2006; Erny et al., 2012; Le Jeune et al., 2007; Lopandic et al., 2007; Oliva et al., 2006), and *S. uvarum* is used to ferment apple cider and wine at low temperatures (Naumov et al., 2000, 2001; Rainieri et al., 1999). One of the clearest differences among these species and one taken advantage of for certain types of fermentations is their thermal growth profile; *S. cerevisiae* and *S. paradoxus* are thermophilic and *S. kudriavzevii* and *S. uvarum* are cryophilic (Gonçalves et al., 2011; Salvadó et al., 2011). However, other aspects of the ecology and evolution of these species might also be relevant to the origin of industrial yeast strains and the predominant use of *S. cerevisiae*.

Outside human ferments, the *Saccharomyces* species have primarily been isolated from arboreal habitats. Originally noted to be associated with sap seeping from slim fluxes (Naumov et al., 1998), these yeast species have now been consistently isolated from bark, leaves and surrounding soil of *Quercus* (Oak) and other tree species. Both *S. cerevisiae* and *S. paradoxus* are widely distributed and have been isolated from temperate forests in North America (Charron et al., 2014; Hyma and Fay, 2013; Sniegowski et al., 2002; Sylvester et al., 2015), Europe (Almeida et al., 2015; Bozdag and Greig, 2014; Johnson et al., 2004; Koufopanou et al., 2006; Legras et al., 2014; Naumov, 2013; Sampaio and Gonçalves, 2008), Asia (Almeida et al., 2015; Naumov et al., 1997; Wang et al., 2012) and Oceania (Zhang et al., 2010), often sympatrically. *S. uvarum* and *S. eubayanus* have also been found to be widely distributed (Almeida et al., 2014). However, *S. kudriavzevii*, *S. arboricola* and *S. mikatae* have thus far only been found in restricted geographic regions (Naumov et al., 2013). Currently, it is unknown whether arboreal habitats are a primary habitat or just one of many environments able to sustain populations of these species (Goddard and Greig, 2015).

Vineyards are likely an important interface between wild yeast populations and those used for wine fermentations (Hyma and Fay, 2013). Grapes periodically provide an abundant source of sugar, attract

a high density of potential insect vectors, and generate exceptionally high concentrations of yeast by the end of fermentation. Furthermore, wine must is not sterilized prior to fermentations and the skins, stems and microbial sediments from fermentation are typically discarded back into the vineyard. Thus, before the now common practice of inoculating wine must, there was ample opportunity for both inter- and intraspecific competition within vineyard environments. Indeed, commercial wine yeast is found dispersed throughout vineyards in France (Valero et al., 2005), and European "wine" strains and North American "wild" strains of *S. cerevisiae* are both present on grapes and oak trees in vineyards in North America, while only North American "wild" strains are found in arboreal habitats outside of vineyards (Hyma and Fay, 2013). Mixing of various *S. cerevisiae* populations also occurs in Italy, facilitated by wasps (Stefanini et al., 2012). Finally, the above mentioned hybrids of *Saccharomyces* species have thus far only been isolated from vineyard and brewing environments.

The historical acquisition of *S. cerevisiae* but not *S. paradoxus* into human-associated fermentative environments is particularly perplexing given they are both strong fermenters and widely distributed. For example, *S. paradoxus* has only been reported as a significant contributor to wine fermentations in Croatia (Redzepović et al., 2002). Furthermore, many of the growth characteristics that give *S. cerevisiae* a competitive advantage during wine fermentations are shared with *S. paradoxus* and the two species are equally competitive in high sugar environments such as grape juice (Williams et al., 2015). Consistent with these observations, both *S. cerevisiae* and *S. paradoxus* have been isolated from vineyards in North America (Hyma and Fay, 2013).

In Europe, however, there appears to be ecological differentiation between wine strains of *S. cerevisiae* and wild populations of *S. paradoxus*. Historically, *S. paradoxus* was isolated from arboreal habitats while *S. cerevisiae* was isolated from vineyards (Naumov, 2013), which lead to the reasonable proposition that *S. cerevisiae* is a domesticated species (Martini, 1993; Mortimer, 2000). While absent from northern European arboreal habitats (Johnson et al., 2004; Koufopanou et al., 2006; Sampaio and Gonçalves, 2008), *S. cerevisiae* has now been isolated from multiple Mediterranean oak trees (Almeida et al., 2015; Sampaio and Gonçalves, 2008) and may constitute a wild stock from which European wine strains were derived. In contrast, *S. paradoxus* has been isolated from arboreal habitats throughout Europe (Boynton and Greig, 2014; Glushakova et al., 2007; Naumov, 1996, 2013; Naumov et al., 1992; Sampaio and Gonçalves, 2008). One caveat, however, is that concurrent sampling of vineyard and arboreal habitats within the same region is needed to tease apart geographic and habitat effects on the abundance and distribution of these species.

In this study, we examine the abundance of *S. cerevisiae* and *S. paradoxus* across sympatric ecological environments and fine-scale geographic locations in Slovenia. Our sampling strategy was arboreal sources, including bark and soil from oak trees, within and outside of vineyards compared to wine must, soil and berries from grapevines within vineyards. Using enrichments we find both species present within and outside the vineyard, and analyze their abundance in arboreal- and grape-associated habitats. We also quantify species abundance using enrichment free microbial profiling of bark, soil and wine must before and during fermentation. By quantifying phenotypes relevant to life in the vineyard we provide an explanation for why *S. paradoxus* is rare or absent in autochthonous wine fermentations despite its presence in the vineyard.

Materials and Methods

Sample collection and strain isolation

Samples were obtained from seven vineyards and four forest sites in Slovenia (Table S1). The majority of samples were soil, bark and berries from grapevines and soil and bark from oak trees (*Quercus robur*, *Q. petrea*, *Q. ilex*, *Q. pubescens* and *Q. cerris*). A small number of samples were from insects, fruits, cellar swabs and wine must. Oak samples were obtained by prying off bark at the base of the tree and sampling soil at the base of the tree. Samples were obtained between July of 2013 and April of 2014. Three of the forest locations were transects from the edge of Vipava Valley leading up

into the surrounding mountains and these forested areas began immediately adjacent to vineyards. One forest location was on a hill above the town of Vipava surrounding an abandoned castle.

For each sample, approximately 5-25 cm^3 of substrate was placed into a sterile falcon tube using ethanol sterilized forceps or scalpels. Twenty-five ml of enrichment medium (1% yeast extract, 2% peptone, 10% glucose and 5% of ethanol, pH 5.3) was added to each sample (Hyma and Fay, 2013; Mortimer and Polsinelli, 1999). After four to ten days of fermentation at room temperature, approximately 20-25°C, 2 µl of well mixed enrichment medium was spread on Petri dishes and incubated for 2-4 days. Bacteria-like colonies were excluded by testing for growth on chloramphenicol (100 mg/L). A single yeast colony was isolated from each enrichment and place into 3 ml of liquid YPD and incubated with 200 rpm shaking overnight. For 13 enrichments we isolated two colonies from the same enrichment corresponding to different morphology. Only one of the two isolates was used in the analysis.

A subset of 518 samples collected in October were enriched at both room temperature and 37°C. These samples were derived by thoroughly mixing each sample with enrichment media, then pouring off 10 ml of the enrichment into a sterile, 15 ml tube and incubated at high temperature. The high temperature enrichments were subsequently treated the same as those at room temperature and single colonies were obtained from both.

Species identification

Isolates were screened for *Saccharomyces* species by PCR and restriction digests as in Hyma and Fay (2013). Briefly, total DNA was extracted from yeast using lyticase and glass beads. A multiplex PCR assay was used to distinguish *Saccharomyces* and non-*Saccharomyces* species (Nardi et al., 2006). *Saccharomyces* isolates were further distinguished using restriction digests of the ITS PCR products (McCullough et al., 1998). Identification failed for 56 (6%) isolates, either because of PCR failure or digests with unexpected band sizes.

Sampling analyses

For each species, the frequency of isolation from all oak- and grapevine-associated samples was fit to a logistic model with terms for source (oak, grapevine), location type (vineyard, non-vineyard), location (11 sampling sites) and month of isolation (July, September, October, April). Significant terms were identified by dropping single terms and comparing models using a likelihood ratio test.

Microbiome analysis

Microbiome samples were collected after harvest from vineyards and wine must. From five vineyards we obtained twenty samples from oak bark and soil and grapevine soil and twenty must samples from uninoculated fermentations. Nine of the must samples were from pressed grapes or pomace within a day of harvest, the remainder were from within the first week of fermentation. Temporal samples were taken from fermenting must from two vineyards, Carga and Burja (previously part of the Sutor estate). From Burja, samples were taken from Malvazija pomace at harvarst (20.2 °Brix, pH 3.37, total titratable acidity 7.1 g/L, supplemented with ammonium bisulfite at 0.1 g/L) and at seven subsequent points over 18 days of fermentation in the cellar. The same must was also brought to the lab and 800 ml of must was fermented in flasks in triplicate during which we obtained 8 samples over 14 days. On the eleventh day of fermentation, must in the lab and in the cellar was pressed to remove the skins and seeds and the remaining juice continued to ferment. From Carga, juice from pressed Tokaj grapes (17.9 °Brix, pH 3.21, total titratable acidity 8.1 g/L, supplemented with potassium metabisulfite at 0.1 g/kg) was brought to the lab and 800 ml of must was fermented in flasks in triplicate during which we obtained 10 samples over 17 days.

For soil samples, DNA was extracted from 150 mg of soil using ZR Soil Microbe DNA extraction kit (Zymo Research, CA, USA). For bark, berry and juice/pomace samples, samples were immersed and shaken in water, solid material removed, and DNA was extracted from the pellet after centrifugation

using either the ZR Soil Microbe kit (bark) or a Qiagen Plant DNA kit (Hilden, Germany). ITS1 was amplified using BITS1 and B58S3 primers (Bokulich and Mills, 2013). For the BITS1 primer we included an 8 bp barcode followed by a linker (CT) at the 5' end (Table S2) in order to multiplex the samples. Illumina sequencing adaptors were added via a second round of PCR and these included a 9 bp index for further multiplexing. Amplicons were purified, quantified and pooled then sequenced using an Illumina MiSeq with single-end 250 bp reads.

Barcodes and index were identified and removed using custom perl scripts allowing 1 mismatch in each. Adaptors and low quality sequences were trimmed using ea-utils (v1.04.676 https://code.google.com/archive/p/ea-utils/) using a window size of 3 and a quality threshold of 20. Sequences less than 100 bp were removed. Sequences were aligned by blastall (v2.2.26 (Camacho et al., 2009)) using a cutoff of 1e-20 to 287,101 sequences in the UNITE+INSD database (4/7/2014 (Kõljalg et al., 2013)) after removing sequences from uncultured fungi. For classification into taxonomic groups all top hits were used. For species classification, the top hit for each sequence was retained when greater than 97% identity, resulting in the retention of 75% of all sequences. To eliminate rare and potentially spurious hits, species representatives were only kept if two or more samples had more than 10 hits each to that representative. This eliminated 3,091 out of 3,935 species representatives. After these filters, the median number of hits per sample was 46,619 with a range of 1,086 to 822,149.

Species' richness was estimated from each sample using the rarefied number of species and species' diversity was estimated by Simpson's diversity index using the vegan package in R (Oksanen et al., 2015). Species' richness and diversity were tested for association with sample substrate (bark, soil, must) using an ANOVA and pairwise differences were assessed using Tukey's method. For the fermentation time-course, species diversity and richness did not change linearly over time and so we fit a linear model to the ranked order of richness and diversity from each fermentation. Nonmetric multidimensional scaling was implemented using the metaMDS function in the vegan package of R with 20 starting points based on Bray-Curtis dissimilarity among samples.

Wine phenotypes

Strains were grown in 200 µl of complete medium (2% glucose, 2% yeast nitrogen base with ammonium sulfate) overnight in 96-well plates. Strains were then resuspended 1:20 in complete medium with sulfite, copper, ethanol, tartaric acid or unaltered and grown for 48 hrs without shaking at 30°C in 96-well plates. Sulfite medium was 0.7 and 1.5 mM Na_2SO_3 buffered to a pH of 3.5 with tartaric acid. Low pH was 5 mg/mL of tartaric acid, copper medium was 0.5 mM and 1.0 mM copper sulfate, ethanol medium was 6% and 10% (v/v) ethanol. These concentrations were selected based on preliminary assays to distinguish North American oak and commercial wine strains. Cell density (OD600) was measured (iEMS plate reader, Thermo Lab Systems, Helsinki, Finland) at 0, 19, 24, 36 and 48 hrs after treatment. Data for certain time-points, 1.4% of all the data, was interpolated due to plate reader malfunction: two of the plates for sulfite treatment at 19 hrs, one plate for tartaric acid at 24 hrs, and one plate at 48 hrs for the no stress control. Data were interpolated by taking the average of the prior and subsequent time-points. The phenotype of each strain was measured by the area under the growth curve (AUC) and we used the average AUC when growth was measured under two different stress concentrations. Commercial strains were obtained from yeast distributors. North American oak tree strains were those from Hyma et al. (2013) from which we excluded *S. cerevisiae* and *S. paradoxus* strains closely related to European strains.

Reanalysis of North American samples

From the raw data of Hyma et al. (2013) we analyzed 187 *S. cerevisiae* and 240 *S. paradoxus* isolates from 977 oak- and 492 grape-associated samples for which the same enrichment medium was used. The frequency of each species was fit to a logistic model with terms for state (MO, OR), location type (vineyard, non-vineyard), source (oak, grapes) and year of isolation (2008, 2009). Significant terms were identified by dropping single terms and comparing models using a likelihood ratio test.

Results

Isolation of Saccharomyces yeasts

To characterize the distribution and abundance of *Saccharomyces* species we sampled 1,233 substrates from 7 vineyards and 4 non-vineyard locations in Slovenia between July of 2013 and April of 2014. Substrates were primarily from oak trees (66%) and grapevines (24%). The remaining samples were from wine cellars, must, fruit, insects and other plant material (Table S3). Following enrichment of the samples, we isolated 869 strains and distinguished *Saccharomyces* species from one another and from non-*Saccharomyces* species (Methods). Our sample yield was highest for non-*Saccharomyces* species (28%), followed by *S. paradoxus* (23%), *S. cerevisiae* (12%) and *S. kudriavzevii* (2.1%) (Table S3).

To test whether enrichment at higher temperature increased our recovery of *S. cerevisiae*, we split 518 of the samples into enrichments at room temperature and 37ºC. High temperature enrichments yielded a higher ratio of *S. cerevisiae* relative to *S. paradoxus* strains (29:1 compared to 81:123, Fisher's Exact Test $P < 0.01$). However, substantially fewer high temperature enrichments yielded yeast (11%) compared to those at room temperature (80%) due to proliferation of bacteria (Fisher's Exact Test $P < 0.01$). The higher ratio of *S. cerevisiae* to *S. paradoxus* strains from high temperature enrichments was not a primary consequence of temperature since most of the corresponding low temperature enrichments from the same sample yielded the same species and in only four cases was *S. paradoxus* isolated at the low temperature when *S. cerevisiae* was isolated at the high temperature. To avoid potentially redundant samples, we removed the 55 high temperature isolates from the remainder of the analysis.

Species abundance differs by source, geographic location and time of year

As a proxy for species abundance, we compared rates of isolation from 1,055 samples associated with oak trees within the vineyard (467), oak trees outside the vineyard (316), and grapevines (272). While we found no differences between vineyard and non-vineyard locations, *S. cerevisiae* and non-*Saccharomyces* yeasts were more prevalent on grapevine- compared to oak-associated substrates and *S. paradoxus* was depleted (Figure 1, Table S4). We also found variation across sampling locations for both *S. cerevisiae* and *S. paradoxus*, but not for non-*Saccharomyces* yeast as a group (Table S4 and S5). One apparent outlier was an abandoned castle on a hill overlooking the town of Vipava; it was the only non-vineyard location with more *S. cerevisiae* than *S. paradoxus* isolates. However, removing this location still yielded equivalent ratios of *S. cerevisiae* to *S. paradoxus* from vineyard oaks and non-vineyard oaks (odds ratio [95% confidence interval] = 0.23 [0.16,0.32] and 0.13 [0.052,0.26], respectively, $P = 0.172$).

Time of year influenced the sampling rate of all species except for *S. cerevisiae* (Table S3). In September during harvest time we found the lowest rates of *S. paradoxus* and the highest rates of non-*Saccharomyces* yeast (Table S4). Except for one isolate from April, all *S. kudriavzevii* strains were obtained in October, mostly from non-vineyard oak samples.

Saccharomyces abundance within the oak- and grape-associated microbiomes

To quantify the relative abundance of *Saccharomyces* and other yeast species without enrichment, we performed ITS1 sequencing on 20 vineyard samples of oak bark and soil and 20 samples from uninoculated wine must. Bark and soil samples contained more species than must samples (Tukey $P < 0.001$), but there was no difference in Simpson's diversity index, which measures the skew towards one or a small number of abundant species (Tukey $P > 0.05$, Table S6). However, species' relative abundance differed across sample substrates. At a broad taxonomic level, five out of six classes with overall abundance above 5% differed in frequency among must, bark and soil samples (Figure 2, ANOVA $P < 0.01$), with the one exception being *Dothideomycetes* which were abundant in all samples. Must samples were enriched for *Saccharomycetes* and *Leotiomycetes*, with the most common species

being *S. cerevisiae* and the grape pathogen *Botryotinia fuckeliana*, respectively. Bark samples were enriched for *Lecanoromycetes*, with the most common species being the lichen *Physciella chlorantha*, and soil samples were enriched for *Agaricomycetes*, with the most common being the mushroom *Russula fragilis*. Multidimensional scaling of species' abundance also distinguished must from bark and soil samples, the latter two of which were more similar to one another (Figure S1).

The frequency of *Saccharomyces* species was highly variable across samples (Figure 3). Both *S. cerevisiae* and *S. paradoxus* were rare in soil and bark samples, averaging $6.9x10^{-4}$ for *S. cerevisiae* and $6.6x10^{-5}$ for *S. paradoxus*. The two species were more variable in must samples, with *S. paradoxus* constituting up to 82% and *S. cerevisiae* up to 87% of identified species (Table S6). Although *S. cerevisiae* tended to have a higher frequency than *S. paradoxus* across all samples (Mann-Whitney test, $P = 6.7x10^{-5}$), there was no significant difference among substrates in the relative abundance of *S. cerevisiae* to *S. paradoxus* (Kruskal Wallis test, P = 0.085). Another *Saccharomyces* species found, *S. kudriavzevii*, was only present in a single sample (SM56) at a frequency of $4.8x10^{-5}$.

S. cerevisiae increases in abundance during wine fermentations

To capture changes in temporal dynamics that occur during fermentation we obtained juice from the Carga estate and grape pomace from the Burja estate (previously a part of Sutor), and carried out triplicate fermentations in the lab, taking 8-10 samples over 14-17 days of fermentation. In parallel, we sampled the same pomace from the Burja estate that was being fermented in the Burja winery cellar.

Species' richness and Simpson's diversity index decreased over time for both the Burja ($P = 0.02$ and $P = 3.3x10^{-5}$, respectively) and Carga ($2.9x10^{-6}$ and $4.1x10^{-9}$, respectively) experimental fermentations but not for the Burja cellar fermentation ($P > 0.05$, see Methods). Initial richness and diversity of the experimental fermentations was within the range of the 20 must samples (Table S7) and primarily consisted of *Saccharomycetes* (Figure 4). By the end of fermentation, only three species were above 5%: *S. cerevisiae*, *Starmerella bacillaris* and *Botryotinia fuckeliana*. Interestingly, *S. paradoxus* was absent from the Carga fermentation and was only present at an initial frequency of $3.8x10^{-4}$ in the Burja fermentation. In comparison, *S. cerevisiae* was at an initial frequency of $8.7x10^{-3}$ and $8.2x10^{-2}$ in the Carga and Burja fermentations, respectively.

European strains of _S. cerevisiae_ have acquired resistance to stresses applied during wine making

The presence of both *S. cerevisiae* and *S. paradoxus* within vineyard and wine must suggests *S. paradoxus* should often make it into wine fermentations. However, the Carga and Burja fermentations along with previous work (Bokulich et al., 2014b; David et al., 2014; Gayevskiy and Goddard, 2012; Pinto et al., 2014, 2015; Setati et al., 2012; Taylor et al., 2014; Wang et al., 2015) indicate that it may not often be a major contributor to fermenting wine must. One potential cause for a shift in the relative abundance of these two species going from the vineyard into the winery is the addition of copper and sulfites to the grape must. Indeed, sulfites were added to both the Carga and Burja musts before being brought to the lab. Previous studies have shown wine strains are particularly resistant to copper and sulfites (Liti et al., 2009; Warringer et al., 2011). To characterize sensitivity to the wine making environment among our isolates we measured the growth profiles of 168 *S. cerevisiae* and 263 *S. paradoxus* from Slovenia in comparison to a set of 35 reference commercial wine strains, 29 North American oak tree strains, and 34 North American *S. paradoxus* strains (Hyma and Fay, 2013). As a control we measured growth in the absence of stress and in the presence of ethanol, which has not been reported to differ between the two species.

As expected, North American *S. cerevisiae* strains are more sensitive than commercial wine strains to sulfites, copper and low pH, but not high ethanol (FDR < 0.01, Figure 5, Figure S2, Table S8). Slovenian *S. cerevisiae* strains are resistant to sulfites, copper and low pH; more so than North American *S. cerevisiae* or *S. paradoxus* (FDR < 0.01 Table S8). This high level of resistance of Slovenian *S. cerevisiae* strains is indistinguishable from that of commercial wine strains (FDR > 0.01).

Discussion

Strains of *S. cerevisiae* have been widely used for the production of beer, bread, wine and other human-associated fermentations (Sicard and Legras, 2011). Its sibling species, *S. paradoxus*, is rarely associated with human fermentations (Boynton and Greig, 2014) but is a strong fermenter and is competitive with *S. cerevisiae* in grape juice (Williams et al., 2015). The distinction between these two species is particularly well defined in Europe, where *S. cerevisiae* is most often isolated from vineyards whereas *S. paradoxus* is most often isolated outside of vineyards.

In this study we used intensive sampling and microbial profiling to show that there is not a clear cut difference in the abundance and distribution of these two species within Slovenian vineyards and forests. Similar to North American vineyards and forests (Hyma and Fay, 2013), the two species can occur sympatrically in Europe and we find they only differ in their relative abundance: *S. paradoxus* is more abundant on oak tree-associated substrates and *S. cerevisiae* is more abundant on grapevine-associated substrates. Although there are likely many factors, discussed below, that contribute to variation in the relative abundance of these two species, our results support the idea that current wine making practices greatly enrich *S. cerevisiae* within the vineyard via the acquisition of multiple traits by European wine strains.

Is *S. paradoxus* rare within vineyard environments?

Our results based on both enrichment and microbiome analysis indicate that *S. paradoxus* is not excluded from vineyard environments, including wine must, and can be as abundant as *S. cerevisiae*. While our findings differ from those of prior studies, multiple factors influence the relative abundance of these two species and may explain these differences.

Distinguishing *Saccharomyces* species

Because the *Saccharomyces* species were not clearly delineated until the 1990s (Naumov, 1996; Naumov et al., 1992), early work on yeasts present within vineyard and wine fermentation may not have distinguished between *S. cerevisiae* and *S. paradoxus*. Even so, *S. cerevisiae* remains the predominant yeast isolated from European vineyards, e.g. in Spain (Cordero-Bueso et al., 2011), Portugal (Schuller et al., 2005), Italy (Di Maio et al., 2012; Stefanini et al., 2012) and France (Valero et al., 2007). With the exception of Mediterranean regions where *S. cerevisiae* is found to co-occur with *S. paradoxus* on trees (Almeida et al., 2015; Sampaio and Gonçalves, 2008), *S. paradoxus* is the predominant *Saccharomyces* species isolated from forest environments (Bozdag and Greig, 2014; Glushakova et al., 2007; Johnson et al., 2004; Koufopanou et al., 2006; Kowallik et al., 2015), reinforcing the notion that *S. paradoxus* is a wild yeast and absent from vineyards (Boynton and Greig, 2014).

More recently, the diversity of yeasts present within fermenting wine have been examined by direct sequencing of the wine microbiome (Bokulich et al., 2014b; David et al., 2014; Gayevskiy and Goddard, 2012; Pinto et al., 2014, 2015; Setati et al., 2012; Taylor et al., 2014; Wang et al., 2015). While these microbiome studies have not reported *S. paradoxus* within the wine must, certain methods of analysis do not distinguish it from *S. cerevisiae*. One common practice is the representation of closely related sequences at the level of 97-99 percent identity by operational taxonomic unites (OTUs). In the UNITE database (Kõljalg et al., 2013), these groups are termed species hypothesis (SH) and do not distinguish *S. cerevisiae* from *S. paradoxus* even though they are readily distinguishable by their ITS1 sequence (McCullough et al., 1998). Thus, the absence of reports of *S. paradoxus* within vineyards and wine must may be partly attributed to not specifically distinguishing it from *S. cerevisiae*.

Variation across ecological niches

In our samples we found the relative abundance of *S. cerevisiae* to *S. paradoxus* is related to habitat,

grapevines or oak trees, but not whether the oak trees occur within or outside of vineyards. As such, it is not surprising that we also isolated *S. paradoxus* from grapevine-associated substrates and that the habit surrounding vineyards is relevant to the microbial community colonizing grapevines and being incorporated into wine must (Bokulich et al., 2014b; Knight et al., 2015; Setati et al., 2012).

In contrast to our enrichment samples, our microbial profiling experiments detected no significant differences in the relative abundance of *S. cerevisiae* to *S. paradoxus* among must, bark or soil samples; *S. cerevisiae* was found to be uniformly more abundant. This difference could be a consequence of the low frequency of *S. cerevisiae* and *S. paradoxus* in most bark and soil samples, close to the detection limit of 10^{-4} to 10^{-5} determined by the number of sequence reads per sample. Furthermore, the sample size was small relative to the number of enrichment samples and we observed substantial sample to sample variation in the relative abundance of the two species. Finally, we cannot exclude the possibility that our enrichment process generated a biased representation of species abundance. The presence of other microbes in a sample can influence yeast growth (Kowallik et al., 2015) and so it is possible that differences in sample abundance occurred because *S. cerevisiae* and *S. paradoxus* differ in their ability to compete with microbes that are not evenly distributed across oak and grapevine habitats.

Local geographic and temporal variation

By design we sampled multiple vineyards to help ensure our results were reflective of Slovenian vineyards and the Vipava valley. By necessity we sampled multiple times during the year, with the majority of samples being collected before (July) and after (October) harvest. Both location and time of year are associated with the relative abundance of *S. cerevisiae* to *S. paradoxus*. The most interesting deviation within our sampling locations was an abandoned castle on a hill in Vipava. While the castle itself is old (13th Century) the oak forest surrounding it consist of young oak trees, approximately 10-20 cM in diameter at the base of the tree. While not optimal for characterizing species abundance in European forests, this location highlights the importance of fine-scale variation and historical context in sampling locations.

While our finding of sympatric *S. cerevisiae* and *S. paradoxus* within and outside of vineyards in Slovenia may be a regional finding, it is consistent with certain studies. *S. paradoxus* was found in a vineyard in a region of Croatia approximately 150 km East of Vipava (Redzepović et al., 2002), and wild populations of *S. cerevisiae* associated with Mediterranean oaks have been isolate from Southern Europe (Almeida et al., 2015).

Global geographic variation

Our work establishes *S. paradoxus* as part of the vineyard environment, at least in Slovenia. While this raises the possibility that it may also occur in vineyards outside of the Balkans, *S. paradoxus* has thus far only been isolated from North American vineyards (Hyma and Fay, 2013). Because Hyma and Fay (2013) did not report rates of isolation, we analyzed the raw data for comparison with our results from Slovenia.

Similar to Slovenia, numerous isolates (130) of *S. paradoxus* were obtained from vineyards (Table S9). Highlighting the importance of geographic variation, *S. paradoxus* was almost exclusively isolated from both vineyard and forest locations in Oregon. Yet even accounting for geographic variation, *S. paradoxus* was less abundant in vineyards (OR = 0.55, P = 0.03) and not significantly different from *S. cerevisiae* when comparing oak versus grapevine samples (OR = 0.83, P = 0.73, Table S10). Thus, while both the Slovenian and United States samples show evidence of geographic variation, the United States samples differ by sample location (vineyard vs. forest) rather than sample source (grapevine versus oak). However, it should be noted that in the US only 22 isolates were obtained from grapes and 16 of these were *S. cerevisiae*.

Transition into the winery and competition during fermentation

Similar to other studies of microbial diversity (Combina et al., 2005; Mercado et al., 2007; Pinto et al., 2015), we found a diverse fungal community from harvested and/or pressed grapes before or at the initial stages of fermentation followed by a rapid decline in diversity as *S. cerevisiae* became the dominant species. While the grape must community was distinct from oak bark and soil communities, the community was also quite variable at the species level. This variability could be related to any number of differences in location, method and time of harvest or contact with winery equipment. Along with overall variation in the grape must microbiome, the relative abundance of *S. cerevisiae* and *S. paradoxus* also varied, with one sample of must from pressed grapes containing 82% *S. paradoxus* and only 14% *S. cerevisiae*.

One limitation of our grape/must samples is that we did not control for sulfite or other treatments of the grapes or must before sampling. Although we sampled from wineries that carry out autochthonous fermentations, the vineyards also spray copper sulfate as a fungicide and use sulfites to inhibit the growth of bacteria and other microorganisms. Indeed, sulfites were added by the wineries prior to deriving wine fermentations in the lab. Such treatments very likely alter initial microbial diversity or their dynamics during fermentation to wine (Bokulich et al., 2014a). Our observation that unlike *S. cerevisiae*, *S. paradoxus* does not become common during wine fermentation can be explained by differences in resistance to copper, sulfite or low pH. However, these aspects of the experimental wine fermentation were not controlled and so further work will be needed to distinguish them from other factors that could be involved.

Even after being brought into the lab we observed substantial variation in species abundance during fermentation to wine. The most notable difference was the maintenance of much higher levels of diversity in the Burja wine fermented at large volume in the cellar and in one of our laboratory replicates as compared to the other two replicates carried out in the lab. The cellar fermentation could be different due to a slower rate of fermentation, larger volume or lower temperature, but could also be due to our mixing the lab fermentation prior to every sample taken.

Resistance to copper, sulfites and tartaric acid distinguishes Slovenian strains of *S. cerevisiae* and *S. paradoxus*

Prior work has shown that both resistance to copper and sulfites are common in wine strains compared to oak strains of *S. cerevisiae* and *S. paradoxus* (Fay et al., 2004; Liti et al., 2009; Pérez-Ortín et al., 2002; Strope et al., 2015; Warringer et al., 2011; Yuasa et al., 2004), as might be expected given their frequent use in vineyards and wineries. Our phenotypic analysis of Slovenian yeast adds resistance to low pH to these two previously characterized “domestication” phenotypes and shows that these phenotypes differentiate vineyard isolates of *S. paradoxus* from European but not North American *S. cerevisiae*. Thus, we can conclude that the sensitivity of *S. paradoxus* to copper, sulfite and low pH is not because *S. paradoxus* is absent from vineyards and hasn't had the opportunity of facing selective pressures that are common in the vineyard environment.

The acquisition of copper and sulfite resistance in wine strains has been extensively studied and is known to be primarily caused by changes at *CUP1* (Adamo et al., 2012; Chang et al., 2013; Fogel and Welch, 1982; Strope et al., 2015; Zhao et al., 2014) and *SSU1* (Goto-Yamamoto et al., 1998, 1; Nardi et al., 2010; Yuasa et al., 2004, 1; Zimmer et al., 2014), respectively. However, the relationship between resistance to sulfite and tartaric acid is less clear. Sensitivity to sulfite was measured at a pH of 3.5 since there is little of the active agent sulfur dioxide at higher pH (Casalone et al., 1992). Sensitivity to low pH was measured by adding tartaric acid, since it is abundant in grapes (Kliewer et al., 1967). While resistance between the two is correlated ($r^2 = 0.44$), resistance to tartaric acid only explains 3% of variation in sulfite resistance once differences among major groups (64% of variation, Figure 5) are accounted for.

Conclusions

The history and origins of wine strains has begun to emerge with detailed studies of *S. cerevisiae* in comparison to its closest known relative *S. paradoxus* (Boynton and Greig, 2014). While certain aspects of these two species are notably different, they are sympatric in North American forests (Hyma and Fay, 2013; Sniegowski et al., 2002) and our present results demonstrate that they can inhabit the same vineyard environments. Thus, *S. paradoxus* may be similar to *S. cerevisiae* in its opportunistic colonization of certain environments (Goddard and Greig, 2015). However, one of the fundamental differences between these two species is the higher diversity and stronger geographic structure of *S. paradoxus* compared to *S. cerevisiae* (Liti et al., 2009). Not only is the spread of European wine strains relevant to *S. cerevisiae* population structure (Fay and Benavides, 2005), but there is now also evidence for the spread of wild oak populations of *S. cerevisiae* based on the clonal relatedness of isolates from North America and Japan (Almeida et al., 2015; Hyma and Fay, 2013). Thus, the current sympatric relationship between *S. cerevisiae* and *S. paradoxus* in Slovenian vineyards, and perhaps North American forests, may be a relatively recent development. Further elucidation of the history and relationship between these two species will have to meet the challenge of geographic and temporal heterogeneity while accounting for the historic use or vegetation of the habitats sampled. With sufficient fortitude or luck we may be able to better define the vectors and environmental reservoirs, humans-associated or otherwise, pertinent to these closely related but differentially exploited species.

Author Contributions

Conceived and designed the experiments: SD, LB, JP and JF. Collected and analyzed data: SD, PL, HV, LB, JP and JF. Wrote the paper: SD and JF.

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Figure Legends

Figure 1. Rates of isolation depend on sample source. The sampling frequency of each species is shown for oak-associated samples within and outside of vineyards, and for grapevine-associated samples.

Figure 2. Frequency of abundant taxonomic classes differs across samples. Boxplots are shown for six abundant classes (> 5%) grouped by substrate from which they were obtained.

Figure 3. Frequency of *S. cerevisiae* and *S. paradoxus* in soil, bark and must samples. Points shown on the x-axis had no *S. paradoxus* counts.

Figure 4. Changes in species abundance during fermentation. Twelve species with at least 5% abundance at one time-point are shown for Burja and Carga fermentations at the start (cellar) and end (lab, 3 replicates) of each time-course. Also shown is a Burja cellar sample at the start and end of fermentation. Counts of *Saccharomyces boulardii* were included in those of *S. cerevisiae*.

Figure 5. Slovenian *S. cerevisiae* strains are resistant to sulfite, copper and tartaric acid. Growth rates (area under the growth curve) in the presence of sulfite (A), copper (B) and tartaric acid (C) relative to the absence of stress for *S. cerevisiae* (Scer), *S. paradoxus* (Spar) and commercial wine strains. Black circles and bars represent the mean and its 95% confidence interval.

Supplementary Material

Figure S1. Multidimensional scaling of microbiome samples. First and second coordinates are from non-metric multidimensional scaling using Bray-Curtis dissimilarity.

Figure S2. Slovenian and North American strains' resistance to ethanol. Growth rates (AUC) in the presence of ethanol relative to its absence. Black circles and bars represent the mean and its 95% confidence interval.

Data File S1. Samples used for enrichment and species identified.
Data File S2. Species counts across microbiome samples.
Data File S3. Phenotype data.
Data File S4. Samples analyzed from North America.
Data File S5. Microbiome raw reads and metadata (this data file will be submitted to dryad once the manuscript has been accepted in accordance with dryad policy).
Data File S6. Supporting tables S1-10.

Figure 1

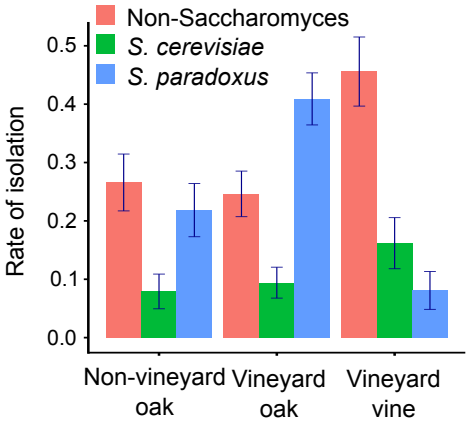


Figure 2

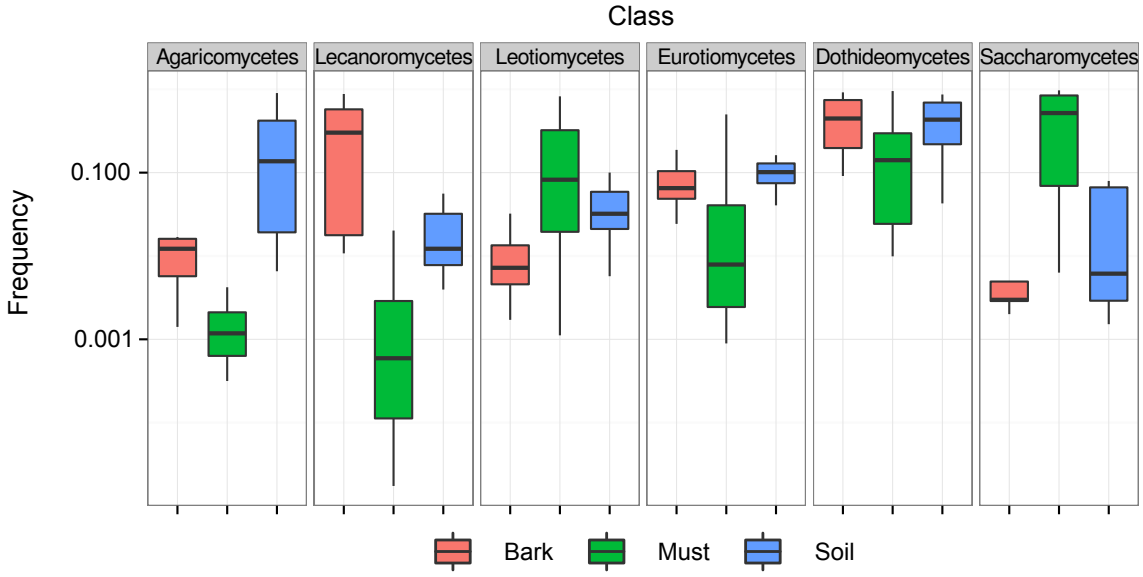


Figure 3

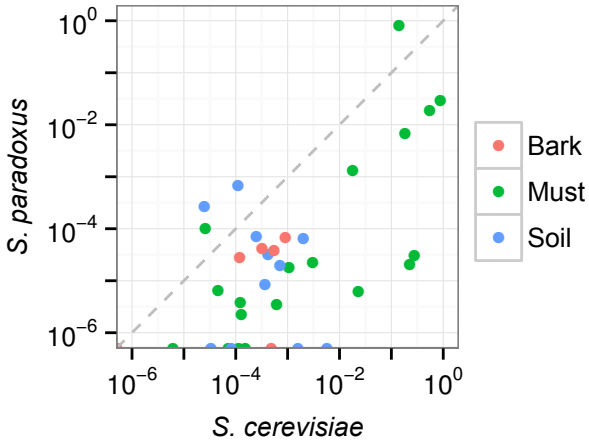


Figure 4

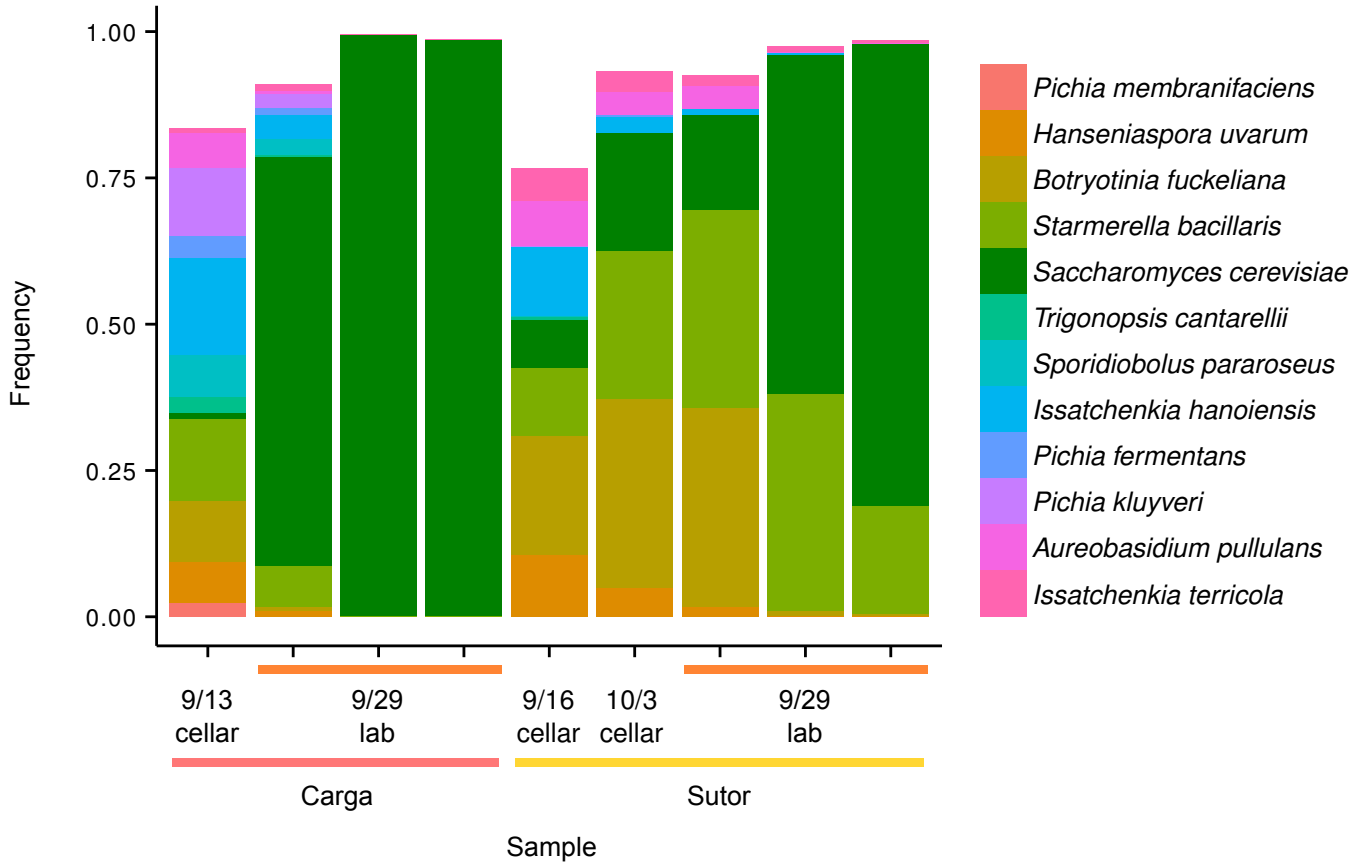
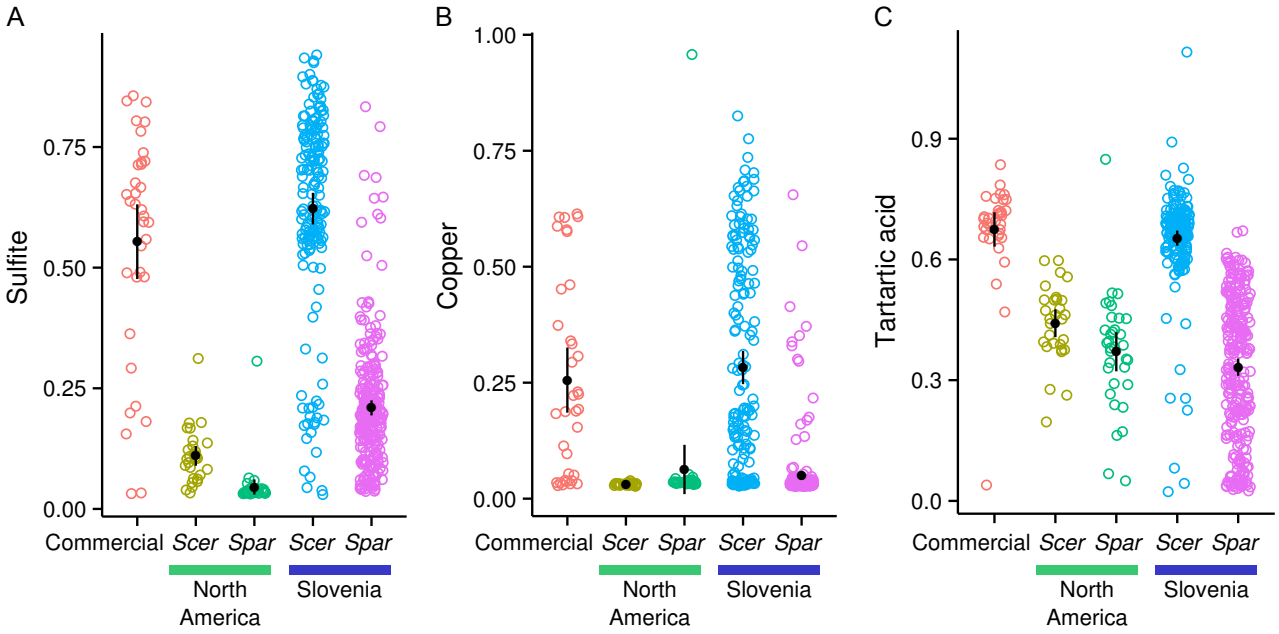


Figure 5



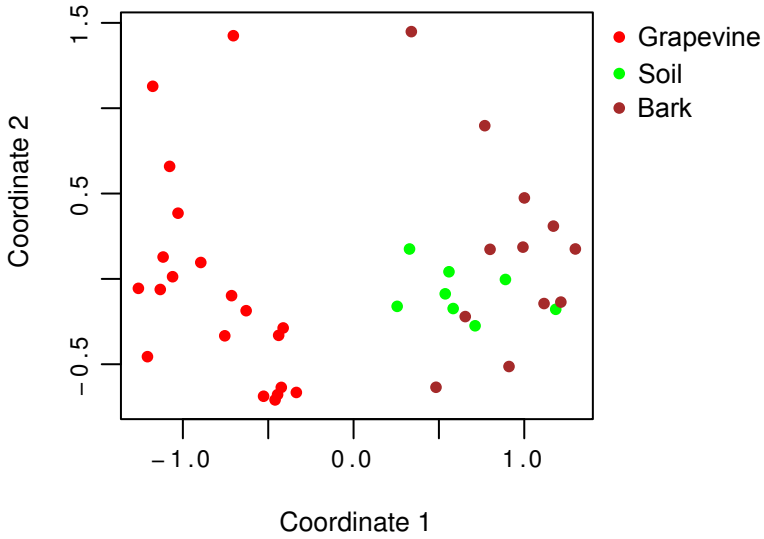


Figure S1. Multidimensional scaling of grapevine, soil and bark microbiome samples. First and second coordinates are from non-metric multidimensional scaling using Bray-Curtis dissimilarity.

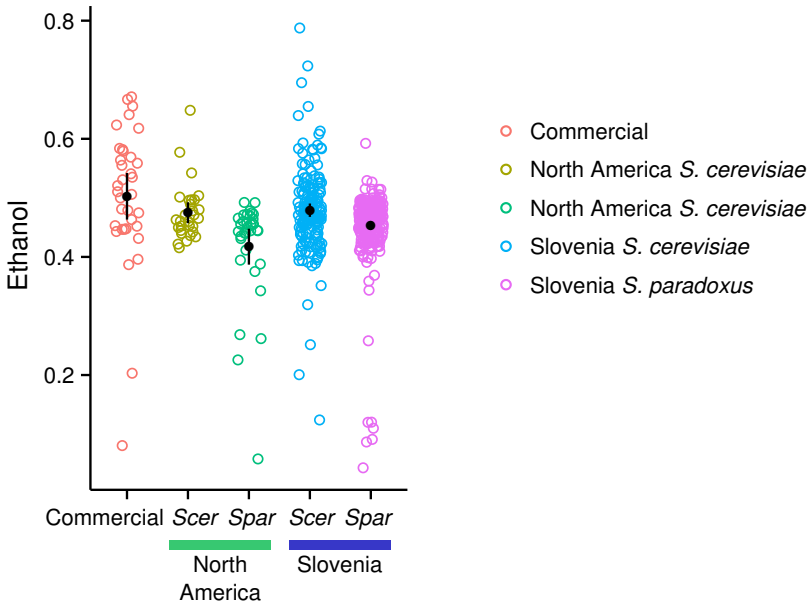


Figure S2. Slovenian and North American strains' resistance to ethanol. Growth rates (AUC) in the presence of ethanol relative to its absence. Black circles and bars represent the mean and its 95% confidence interval.

Table S1. Sampling locations.

Name	Type	Location	Latitude	Longitude	Samples
Castle	Forest	Vipava	45.850334	13.964775	110
Lijak	Forest	Lijak	45.959051	13.719193	82
Nanos	Forest	Nanos	45.796672	14.002717	68
Sinji Vrh	Forest	Sinji Vrh	45.907952	13.935454	97
Carga	Vineyard	Brda	46.02785	13.518732	200
Guerila	Vineyard	Planina	45.84935	13.909625	101
Rencelj	Vineyard	Dutovlje	45.752125	13.830302	25
Sutor	Vineyard	Podraga	45.811386	13.943568	160
Sveti Martin	Vineyard	Brje	45.860662	13.812358	114
Tilia	Vineyard	Dobravlje	45.892136	13.833961	120
UNG	Vineyard	Manče	45.816556	13.941429	156

Table S2. Primers used for microbiome analysis.

Name	Adaptor	Barcode/Index	Linker	Primer
BITS1.1	ACGACGCTCTTCCGATCT	ACAGTACC	CT	ACCTGCGGARGGATCA
BITS1.2	ACGACGCTCTTCCGATCT	ATGGTGTC	CT	ACCTGCGGARGGATCA
BITS1.3	ACGACGCTCTTCCGATCT	CCAAGACT	CT	ACCTGCGGARGGATCA
BITS1.4	ACGACGCTCTTCCGATCT	CTGAGGTT	CT	ACCTGCGGARGGATCA
BITS1.5	ACGACGCTCTTCCGATCT	TGTCATGG	CT	ACCTGCGGARGGATCA
BITS1.6	ACGACGCTCTTCCGATCT	TACCACAG	CT	ACCTGCGGARGGATCA
BITS1.7	ACGACGCTCTTCCGATCT	GGTTCTGA	CT	ACCTGCGGARGGATCA
BITS1.8	ACGACGCTCTTCCGATCT	GACTCCAA	CT	ACCTGCGGARGGATCA
BITS1.9	ACGACGCTCTTCCGATCT	ATGCCCTT	CT	ACCTGCGGARGGATCA
BITS1.10	ACGACGCTCTTCCGATCT	TTCCGAGT	CT	ACCTGCGGARGGATCA
BITS1.11	ACGACGCTCTTCCGATCT	GATGGTTC	CT	ACCTGCGGARGGATCA
BITS1.12	ACGACGCTCTTCCGATCT	CTGTGTGA	CT	ACCTGCGGARGGATCA
BITS1.13	ACGACGCTCTTCCGATCT	TACACGTC	CT	ACCTGCGGARGGATCA
BITS1.14	ACGACGCTCTTCCGATCT	CAAACGCA	CT	ACCTGCGGARGGATCA
BITS1.15	ACGACGCTCTTCCGATCT	CAATCCTG	CT	ACCTGCGGARGGATCA
BITS1.16	ACGACGCTCTTCCGATCT	CATTGAGG	CT	ACCTGCGGARGGATCA
BITS1.17	ACGACGCTCTTCCGATCT	GATTCTCC	CT	ACCTGCGGARGGATCA
BITS1.18	ACGACGCTCTTCCGATCT	GGGGATAA	CT	ACCTGCGGARGGATCA
BITS1.19	ACGACGCTCTTCCGATCT	CCTCTAAC	CT	ACCTGCGGARGGATCA
BITS1.20	ACGACGCTCTTCCGATCT	GCTGATTG	CT	ACCTGCGGARGGATCA
BITS1.21	ACGACGCTCTTCCGATCT	ACAGACTG	CT	ACCTGCGGARGGATCA
BITS1.22	ACGACGCTCTTCCGATCT	AGACTCTC	CT	ACCTGCGGARGGATCA
BITS1.23	ACGACGCTCTTCCGATCT	TCTCTCCT	CT	ACCTGCGGARGGATCA
BITS1.24	ACGACGCTCTTCCGATCT	GAACATGC	CT	ACCTGCGGARGGATCA
B58S3	AGACGTGTGCTCTTCCGATCT			GAGATCCRTTGYTRAAAGTT
Forward Adaptor				
Index1019		AGCGCTTAG		
Index1020		GACTGATAC		
Index1021		ATTCAATTC		
Index1022		CTGAAACCG		
Index1025		CAAGCGAGC		
Index1026		TCCCGACCG		
Index1027		GAGGATGAT		
Index1028		AAATCGAAT		
Index1029		CCTCTCGAA		
Index1030		GGTCACTAA		
Index1031		CTCAGCACC		
Index1032		CACATGGCG		
Index1033		GCCGTATGC		
Index1034		ACCCACGCA		
Index1035		GAGGCCCGA		
Index1036		GGAGACTAG		
Index1037		GCTCATGGT		
Index1038		ACACCGGAT		

Full oligo

ACGACGCTCTTCCGATCTACAGTACCCTACCTGCGGARGGATCA
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CAAGCAGAAGACGGCATACGAGATCTGAAACCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGA
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CAAGCAGAAGACGGCATACGAGATACCCACGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGA
CAAGCAGAAGACGGCATACGAGATGAGGCCCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGA
CAAGCAGAAGACGGCATACGAGATGGAGACTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGA
CAAGCAGAAGACGGCATACGAGATGCTCATGGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGA
CAAGCAGAAGACGGCATACGAGATACACCGGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGA

Table S3. Isolated species by sample source.

Source	Samples	S. cerevisiae	S. paradoxus	S. kudriavzevii	Non-Saccharomyces	Failed identification
Cellar	19	18	0	0	1	0
Fruit	16	3	2	0	5	0
Insect	47	1	1	0	11	6
Must	36	14	0	0	7	2
Oak	809	69	260	25	199	26
Vine	294	46	22	1	125	19
Yeast	2	2	0	0	0	0
Other	10	0	2	0	2	0
All	1233	153 (12.3%)	287 (23%)	26 (2.1%)	350 (28%)	53 (4.2%)

Other = other types of plant material, Yeast = commercial yeast used by vineyard

Table S4. Factors influencing rates of isolation.

Value	Term	S. cerevisiae	S. paradoxus	S. kudriavzevii	Non-Saccharomyces
P-value	Source (oak, grapevine)	0.003	0.000	0.024	0.000
	Location type (vineyard, non-vineyard)	0.858	0.420	0.743	0.739
	Location name (11 sites)	0.000	0.000	0.187	0.102
	Month (July, September, October, April)	0.178	0.000	0.000	0.000
Odds Ratio	(Intercept)	1.07 [0.88-1.31]	1.53 [1.17-2.00]	1.02 [0.93-1.13]	1.08 [0.81-1.45]
	Source (grapevine)	1.07 [1.02-1.12]	0.75 [0.70-0.79]	0.97 [0.95-1.00]	1.20 [1.12-1.28]
	Location type (vineyard)	0.98 [0.82-1.18]	1.11 [0.86-1.43]	0.98 [0.90-1.08]	1.05 [0.79-1.38]
	Location (Castle)	1.07 [0.88-1.31]	0.77 [0.58-1.01]	1.00 [0.90-1.11]	1.07 [0.79-1.44]
	Location (Guerila)	0.98 [0.73-1.07]	0.83 [0.64-1.07]	0.98 [0.89-1.07]	1.18 [0.89-1.57]
	Location (Lijak)	0.89 [0.74-1.10]	0.91 [0.70-1.19]	1.04 [0.94-1.15]	1.19 [0.89-1.59]
	Location (Nanos)	0.90 [0.78-1.17]	0.88 [0.66-1.16]	1.02 [0.92-1.13]	1.19 [0.88-1.62]
	Location (Rencelj)	1.07 [0.91-1.06]	0.79 [0.71-0.89]	1.05 [1.01-1.09]	1.05 [0.93-1.19]
	Location (Sinji vrh)	0.95 [0.92-1.24]	0.67 [0.55-0.83]	1.02 [0.94-1.10]	1.21 [0.97-1.52]
	Location (Sutor)	0.97 [0.90-1.04]	1.05 [0.96-1.16]	1.00 [0.97-1.04]	1.12 [1.01-1.25]
	Location (Sveti Martin)	1.11 [1.03-1.21]	0.88 [0.79-0.98]	1.00 [0.96-1.04]	1.10 [0.98-1.24]
	Location (Tilia)	1.12 [1.04-1.21]	0.89 [0.80-0.99]	1.00 [0.96-1.04]	1.02 [0.91-1.15]
	Location (UNG)	0.92 [0.86-0.99]	0.95 [0.86-1.04]	1.01 [0.97-1.04]	1.15 [1.04-1.28]
	Month (July)	1.03 [0.96-1.11]	0.99 [0.90-1.09]	0.98 [0.95-1.02]	0.98 [0.88-1.08]
	Month (October)	1.06 [0.99-1.13]	0.96 [0.88-1.05]	1.04 [1.00-1.07]	1.10 [1.00-1.21]
	Month (September)	1.00 [0.93-1.08]	0.80 [0.72-0.89]	1.00 [0.96-1.05]	1.16 [1.03-1.30]

P-values are from dropping terms from a logistic generalized linear model with additive terms (Species ~ Source + Location.Type + Location.Name + Month) and odds ratios followed by 2.5% and 97.5% confidence intervals are from a logistic model.

Table S5. Sample size and number of isolates by month, source and location.

Category	Value	Sample size	S. cerevisiae	S. paradoxus	S. kudriavzevii	Non-Saccharomyces
Month	July, 2013	319	31	99	0	69
	September, 2013	127	11	14	0	57
	October, 2013	490	64	122	25	170
	April, 2014	119	7	47	1	27
Source	Oak	783	69	260	25	199
	Grapevine	272	44	22	1	124
Location Name	Carga	158	17	58	1	38
	Guerila	88	10	12	5	27
	Rencelj	18	3	1	0	6
	Sutor	130	9	52	1	48
	Sveti Martin	94	22	25	1	31
	Tilia	105	24	23	1	31
	UNG	135	3	39	2	54
	Castle	101	18	14	4	19
	Lijak	74	0	15	1	23
	Nanos	65	1	20	5	20
	Sinji vrh	87	6	23	5	26
Location Type	Non-vineyard	316	25	69	15	84
	Vineyard	739	88	213	11	239

Isolates are from 1,055 samples from oak or grapevine, excluding three grapevine samples from non-vineyard locations.

Table S6. Microbiome samples, diversity and taxonomic counts.

Name	Substrate	Source	Location	Description	Rarefied Spe	Simpon's Divei	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fuk	k__Fu
BBD1	bark	oak	Carga	oak_bark_Ca	139.7960286	0.7593414335	0	###	###	89	2480	2321	31	167	833	1986	3393	851	10	4	5	0
BBD10	bark	oak	Sutor	oak_bark_Su	111.2876819	0.7500643765	0	###	###	1586	###	1381	14	24	546	2573	1113	1973	2	1	0	3
BBD13	bark	oak	SvetiMartin	oak_bark_Sv	109.6687341	0.7507020089	2	###	###	374	###	1144	16	7	452	915	292	3558	12	1	1	0
BBD16	bark	oak	SvetiMartin	oak_bark_Sv	105.3756793	0.7876558501	0	###	6924	389	###	1011	2	144	1856	311	5298	###	0	1	0	0
BBD19	bark	oak	Guerilla	oak_bark_Gu	80.00442353	0.5309011815	24	###	###	712	###	1022	0	52	895	1180	604	2029	0	1	0	1
BBD22	bark	oak	Guerilla	oak_bark_Gu	51.04738915	0.5883333472	1	###	###	441	###	1750	31	4	2832	628	122	1440	1	0	0	0
BBD4	bark	oak	Carga	oak_bark_Ca	166.3218041	0.723042887	0	###	###	911	3997	6142	35	295	565	11138	1381	3220	3	3	8	16
BBD7	bark	oak	Sutor	oak_bark_Su	181.3193868	0.7319824252	0	###	###	529	1694	5016	36	259	###	###	1051	2168	8	15	2	0
BD11	soil	oak	Sutor	oak_soil_Sut	117.3418544	0.7507724667	0	###	###	1271	###	835	13	16	635	2106	824	1676	3	3	0	5
BD14	soil	oak	SvetiMartin	oak_soil_Sve	214.2176371	0.9513255734	0	###	###	685	1903	9102	207	1746	555	###	531	###	0	0	0	0
BD17	soil	oak	SvetiMartin	oak_soil_Sve	79.76592616	0.2144259135	1	###	###	302	1526	3670	12	106	574	1343	256	###	0	0	0	0
BD2	soil	oak	Carga	oak_soil_Car	135.7706762	0.7876338458	0	###	###	236	###	6895	12	415	706	808	967	###	2	2	1	1
BD20	soil	oak	Guerilla	oak_soil_Gue	160	0.8411993361	0	621	181	7	199	570	1	3	555	31	7	5343	0	0	0	0
BD23	soil	oak	Guerilla	oak_soil_Gue	206.9318536	0.9187259993	0	1778	783	129	365	591	8	63	518	134	17	2482	0	0	0	0
BD5	soil	oak	Carga	oak_soil_Car	124.9098578	0.6250650681	0	###	###	847	3377	6492	2	3956	369	6181	1740	2791	7	3	15	8
BD8	soil	oak	Sutor	oak_soil_Sut	171.8100126	0.7515262084	0	###	###	621	1586	3870	59	250	###	###	1320	1185	14	3	1	0
BD18	soil	vine	SvetiMartin	vine_soil_Sv	135.3175984	0.8214710368	0	###	8825	5612	458	3361	40	20	1002	3174	864	###	1	1	0	0
BD3	soil	vine	Carga	vine_soil_Car	163.5120728	0.7011698524	0	###	###	267	2230	9589	60	949	806	3860	1263	###	1	0	9	0
BD6	soil	vine	Carga	vine_soil_Car	247.9376039	0.9447356	0	###	7208	889	351	6511	72	###	2119	###	558	5163	1	1	0	25
BD9	soil	vine	Sutor	vine_soil_Sut	259.9143652	0.9441337032	0	###	###	1286	1457	3615	29	55	7288	###	579	2385	2	13	13	0
Ca100	must	grape	Carga	Lab_press_n	57.44474589	0.6163513563	24	###	335	6	6	###	0	2	2353	111	19	227	0	0	0	0
Ca96	must	grape	Carga	Lab_press_n	68.03702911	0.8132849285	172	###	352	6	226	###	0	6	2969	278	52	277	1	0	0	0
Ca97	must	grape	Carga	Lab_press_n	69.56640201	0.7025387503	195	###	311	9	13	9954	0	27	2558	162	49	338	0	0	0	0
Ca98	must	grape	Carga	Lab_press_n	70.66791976	0.7956003451	111	###	447	4	69	###	0	448	3280	213	43	509	0	0	0	1
Ca99	must	grape	Carga	Lab_press_n	47.36683643	0.3660809136	38	###	397	4	14	###	1	0	2807	153	10	605	0	0	0	0
Gu100	must	grape	Guerilla	Spontaneous	52.08078713	0.7158406077	0	1535	962	5	1719	173	0	3	###	20	3	49	0	0	0	0
Gu35	must	grape	Guerilla	Pinot_noir_1_	46.9463242	0.854995415	21	###	###	1	114	2262	0	292	###	128	14	763	0	1	0	1
Gu38	must	grape	Guerilla	Merlo_same_	79.5230037	0.890183243	105	###	###	4	83	###	0	3	###	316	20	1215	0	3	0	1
Gu40	must	grape	Guerilla	Pinella_same	72.12240662	0.8470252862	6	6835	1257	10	3	6548	1	1	###	93	5	53	0	0	0	5
Gu42	must	grape	Guerilla	Pinella_same	74.20866484	0.7257365396	139	###	###	3	89	###	0	1	###	83	18	107	0	2	0	0
Gu98	must	grape	Guerilla	Spontaneous	75.41738519	0.7552133547	8	6452	###	3	2093	1882	1	169	###	86	6	437	0	0	0	0
Gu99	must	grape	Guerilla	Zelen_10_da	45.15886101	0.289197659	0	722	343	7	1350	754	1	2	###	38	3	267	0	0	0	0
M32	must	grape	Tilia	grapes_must	41.25148896	0.3265133154	0	678	178	7	901	485	0	0	###	23	3	19	0	0	0	0
M65	must	grape	Tilia	Starter_Tilia	88.57590158	0.9133576178	11	###	6029	2	110	9457	1	0	###	191	8	158	0	65	0	0
M69	must	grape	Tilia	Pomace_Tilia	83.07269474	0.8398380458	82	###	###	14	301	###	9	1	###	58	8	1588	0	0	0	20
SM50	must	grape	SvetiMartin	Barbera_1_d	81.78853355	0.9355705609	47	###	###	4	1261	###	0	5	###	108	11	466	0	0	0	0
SM53	must	grape	SvetiMartin	Rebula_1_we	47.76783802	0.5778558498	1	4006	3671	3	222	8557	0	0	###	32	3	35	0	0	0	1
SM56	must	grape	SvetiMartin	Pinella_1_we	88.06544447	0.894834899	11	9553	###	1	15	3812	0	11	###	116	8	84	0	0	0	1
Sut38	must	grape	Sutor	Malvazija_no	96.31588474	0.7945416596	8	###	1143	2	515	###	0	1	###	407	99	1241	0	0	0	2
Sut39	must	grape	Sutor	Malvazija_no	105.2869325	0.8253489471	73	###	1735	24	1109	###	0	7	###	403	63	989	1	2	0	9

k__Fuk	__Fuk	__Fuk	__Fuk	__Fuk	__Fuk	__Fuk	__Fuk	__Fuk	__Fuk	__Fu	S. cer	S. par	S. kudriavzev	Species tags
0	24	267	448	3	2	0	10	5	831	23	23	0	0	47476
0	16	410	306	11	4	0	98	0	###	20	0	0	0	105848
0	49	114	235	6	3	0	98	16	1543	39	0	0	0	138579
1	56	1700	279	14	0	0	56	7	1466	15	95	7	0	104724
1	0	1	###	30	6	1	1	3	854	3887	44	3	0	80028
0	10	339	483	5	0	0	0	7	1025	1372	55	13	0	461658
0	58	37	1066	1	5	70	22	6	1802	141	0	0	0	101470
0	143	7	4092	1	7	3	465	2	###	223	46	6	0	142881
0	10	270	256	3	0	0	67	0	9075	23	7	0	0	84581
0	878	23	6241	19	108	2	1094	52	3091	###	184	6	0	90752
0	30	3	4489	31	4	1	194	1	999	476	132	3	0	357518
0	19	80	8988	9	14	1	12	24	887	344	0	0	0	253666
0	6	0	19	0	3	0	2	0	24	50	24	0	0	4179
0	36	2	33	0	0	0	18	0	131	410	8	0	0	5053
0	50	49	439	2	8	3	26	7	1556	61	75	2	0	104302
1	165	12	5760	3	14	1	656	1	###	444	4	44	0	162070
0	105	1	###	509	2	1	###	10	###	630	19	116	0	171410
0	34	14	###	8	38	166	33	1	831	1140	7	0	0	210819
1	600	3	###	13	244	21	1575	0	5184	1016	39	3	0	92847
0	93	1	###	22	411	22	952	1	###	6387	32	9	0	127193
0	8292	0	3297	12	1	0	26	1	569	4	9	35	0	347345
1	###	1	5788	24	3	0	0	0	1231	0	0	0	0	400002
2	###	0	2205	12	2	0	4	0	1225	13	174	1	0	282287
62	###	14	4487	6	1	0	0	0	863	5	32	1	0	261801
1	6972	0	2168	2	1	0	2	0	379	1	57	1	0	440755
0	25	0	31	0	1	0	0	1	35	12	###	2188	0	115367
17	155	3	179	0	2	0	1	0	211	2	2495	18	0	822149
1	105	0	1417	8	2	0	1	0	867	2	409	7	0	384269
120	294	0	61	0	0	0	52	0	154	3	1150	83	0	63702
2	122	1	438	6	9	0	3	0	570	17	47	0	0	304584
1	78	3	150	1	4	0	3	0	142	10	###	1374	0	200384
0	11	0	33	0	0	0	0	0	16	3	###	1842	0	62908
0	14	0	49	1	0	0	14	0	76	2	7316	###	0	52231
114	1114	1	496	11	114	0	1	0	89	15	###	2	0	98963
3	2447	1	1849	19	4	0	0	0	1072	0	25	0	0	345654
7	215	0	1148	7	4	0	0	0	438	4	7621	2	16	329049
0	105	0	62	1	1	0	1	0	30	0	38	0	0	334966
1	98	0	301	0	0	0	1	8	71	14	###	3	0	98240
0	703	0	1673	6	0	0	0	0	1529	1	2	0	0	324632
0	1081	0	2082	22	7	1	0	0	1364	12	14	2	0	308976

Name	Substrate	Source	Location	Timepoint	Description	Simpon's Dive	Rarefied Spec	S. cerevisiae	S. paradoxus	Species tags
Mal_c_16.09	grape	must	Sutor	1	Malvazija pon	0.902466473	63.42876133	2761	129	33702
Mal_c_17.09	grape	must	Sutor	2	Malvazija pon	0.76372716	41.72811831	109	2	56351
Mal_c_18.09	grape	must	Sutor	3	Malvazija pon	0.860019629	57.04053237	597	6	52279
Mal_c_19.09	grape	must	Sutor	4	Malvazija pon	0.901765271	80.17055639	1115	41	19237
Mal_c_23.09	grape	must	Sutor	8	Malvazija pon	0.831755851	49.22578633	5206	4	43560
Mal_c_26.09	grape	must	Sutor	11	Malvazija pon	0.791129612	49.96050122	6405	10	68813
Mal_c_1.10	grape	must	Sutor	16	Malvazija juic	0.817850707	44.16941975	6333	6	33124
Mal_c_3.10	grape	must	Sutor	18	Malvazija juic	0.784598808	40.74812508	6702	4	33392
Mal1_18.09	grape	must	Sutor	3	Malvazija pon	0.759753924	34.69938338	178	11	3972
Mal1_19.09	grape	must	Sutor	4	Malvazija pon	0.801719439	22.95698925	682	0	1308
Mal1_20.09	grape	must	Sutor	5	Malvazija pon	0.77221892	34.71228332	200	2	4845
Mal1_22.09	grape	must	Sutor	7	Malvazija pon	0.911712892	34.43100747	185	0	3160
Mal1_24.09	grape	must	Sutor	9	Malvazija pon	0.713684067	26.90535661	200	0	4296
Mal1_26.09	grape	must	Sutor	11	Malvazija pon	0.578332504	31.38605661	608	0	5298
Mal1_29.09	grape	must	Sutor	14	Malvazija juic	0.75087029	39.86624402	652	0	4031
Mal2_17.09	grape	must	Sutor	2	Malvazija pon	0.809787192	48.11294027	180	3	49629
Mal2_18.09	grape	must	Sutor	3	Malvazija pon	0.760455157	42.6528087	182	22	32926
Mal2_19.09	grape	must	Sutor	4	Malvazija pon	0.744509841	37.96532772	548	231	34435
Mal2_20.09	grape	must	Sutor	5	Malvazija pon	0.800961833	49.43372437	672	65	36786
Mal2_22.09	grape	must	Sutor	7	Malvazija pon	0.550296502	39.70251164	883	6	41389
Mal2_24.09	grape	must	Sutor	9	Malvazija pon	0.612846732	37.51941649	1555	5	33593
Mal2_26.09	grape	must	Sutor	11	Malvazija pon	0.622560028	33.1845296	1661	12	22743
Mal2_29.09	grape	must	Sutor	14	Malvazija juic	0.623013897	23.01947453	9577	9	16550
Mal3_17.09	grape	must	Sutor	2	Malvazija pon	0.866550886	67	366	1	1331
Mal3_18.09	grape	must	Sutor	3	Malvazija pon	0.765699592	47.87803589	194	9	29226
Mal3_19.09	grape	must	Sutor	4	Malvazija pon	0.705571853	39.23357392	293	2	44960
Mal3_20.09	grape	must	Sutor	5	Malvazija pon	0.664146905	39.11522535	612	8	40479
Mal3_22.09	grape	must	Sutor	7	Malvazija pon	0.653510717	39.53520836	1429	55	38506
Mal3_24.09	grape	must	Sutor	9	Malvazija pon	0.684892294	43.4362827	2781	38	45109
Mal3_26.09	grape	must	Sutor	11	Malvazija pon	0.578365642	18.65151764	17953	6	26127
Mal3_29.09	grape	must	Sutor	14	Malvazija juic	0.501468271	14.67168006	15655	4	19856
TOK_sf_13.0	grape	must	Carga	1	Tokaj spontan	0.909102178	50.30000187	37	2	4255
TOK_sf1_14.	grape	must	Carga	2	Tokaj spontan	0.898940668	56.08057894	90	2	4442
TOK_sf1_15.	grape	must	Carga	3	Tokaj spontan	0.890059332	53.52606697	43	2	3305
TOK_sf1_16.	grape	must	Carga	4	Tokaj spontan	0.911841973	50.74142306	259	7	9901
TOK_sf1_17.	grape	must	Carga	5	Tokaj spontan	0.89599148	58.62741475	152	4	2199
TOK_sf1_18.	grape	must	Carga	6	Tokaj spontan	0.87200411	49.6815389	701	8	2239
TOK_sf1_20.	grape	must	Carga	8	Tokaj spontan	0.532485972	43	738	5	1086
TOK_sf1_22.	grape	must	Carga	10	Tokaj spontan	0.560911525	36.30737098	931	5	1433
TOK_sf1_26.	grape	must	Carga	14	Tokaj spontan	0.477501934	42.66091455	1387	6	1930
TOK_sf1_29.	grape	must	Carga	17	Tokaj spontan	0.507852109	38.297088	1338	2	1917
TOK_sf2_14.	grape	must	Carga	2	Tokaj spontan	0.890208137	45.75511079	816	4	104799
TOK_sf2_15.	grape	must	Carga	3	Tokaj spontan	0.874879225	44.95603416	365	0	77033
TOK_sf2_16.	grape	must	Carga	4	Tokaj spontan	0.8850714	53.1792266	999	0	103401
TOK_sf2_17.	grape	must	Carga	5	Tokaj spontan	0.549799061	35.5614763	21580	0	32524
TOK_sf2_18.	grape	must	Carga	6	Tokaj spontan	0.030799317	8.737391211	37426	0	37842
TOK_sf2_20.	grape	must	Carga	8	Tokaj spontan	0.033052552	7.443911498	39280	0	39719
TOK_sf2_22.	grape	must	Carga	10	Tokaj spontan	0.02802413	7.304533252	42403	0	42820
TOK_sf2_26.	grape	must	Carga	14	Tokaj spontan	0.025416444	6.728378443	32602	0	32868
TOK_sf2_29.	grape	must	Carga	17	Tokaj spontan	0.02979842	6.998642011	34412	0	34700
TOK_sf3_14.	grape	must	Carga	2	Tokaj spontan	0.890693491	48.95179923	795	0	136042
TOK_sf3_15.	grape	must	Carga	3	Tokaj spontan	0.888571612	54.72738924	1751	1	115365
TOK_sf3_16.	grape	must	Carga	4	Tokaj spontan	0.889652392	56.70604004	2844	0	112067
TOK_sf3_17.	grape	must	Carga	5	Tokaj spontan	0.681305765	30.51625915	23060	4	44807
TOK_sf3_18.	grape	must	Carga	6	Tokaj spontan	0.176215682	8.2663803	29223	1	32164
TOK_sf3_20.	grape	must	Carga	8	Tokaj spontan	0.148315564	6.533891665	43074	4	46619
TOK_sf3_22.	grape	must	Carga	10	Tokaj spontan	0.131332274	6.824969104	41596	0	44525
TOK_sf3_26.	grape	must	Carga	14	Tokaj spontan	0.093179707	6.59469246	54260	7	56812
TOK_sf3_29.	grape	must	Carga	17	Tokaj spontan	0.040491666	6.261738361	36120	1	36717

Table S8. Pairwise comparisons of differences in mean levels of resistance among groups.

Phenotype	Group	Commercial	NA Scer	NA Spar	Slovenia Scer
Sulfite	NA Scer	2.00E-16	-	-	-
Sulfite	NA Spar	2.00E-16	0.1224	-	-
Sulfite	Slovenia Scer	0.0285	2.00E-16	2.00E-16	-
Sulfite	Slovenia Spar	2.00E-16	0.0027	1.10E-07	2.00E-16
Copper	NA Scer	5.40E-08	-	-	-
Copper	NA Spar	9.80E-07	0.54	-	-
Copper	Slovenia Scer	0.51	8.10E-14	2.00E-12	-
Copper	Slovenia Spar	3.70E-12	0.63	0.63	2.00E-16
Low pH	NA Scer	4.60E-09	-	-	-
Low pH	NA Spar	3.60E-15	0.08424	-	-
Low pH	Slovenia Scer	0.45103	3.90E-11	2.00E-16	-
Low pH	Slovenia Spar	2.00E-16	0.00043	0.19182	2.00E-16
Ethanol	NA Scer	0.14657	-	-	-
Ethanol	NA Spar	1.60E-05	0.00393	-	-
Ethanol	Slovenia Scer	0.11862	0.76721	4.20E-05	-
Ethanol	Slovenia Spar	0.00062	0.14657	0.01212	0.0009

Significance is shown by the false discovery rate based on pairwise t-tests assuming unequal variances.

Table S9. North American isolates of S. cerevisiae and S. paradoxus.

Class	Site	S. cerevisiae	S. paradoxus	Sample size
MO		181	87	1074
OR		6	153	395
Vineyard		98	130	941
Non-vineyard		89	110	528
Grape		16	6	492
Oak		171	234	977
2008		88	205	947
2009		99	35	522
Non-vineyard	Watrud, personal property, MO	25	25	117
Non-vineyard	Tyson Research Center, MO	60	34	228
Vineyard	Chaumette Vineyard, MO	88	21	588
Vineyard	Mount Pleasant Vineyard, MO	8	7	140
Non-vineyard	Chip Ross Park, OR	0	41	103
Non-vineyard	Bollman, personal property, OR	4	10	79
Vineyard	Tyee Vineyard, OR	2	39	96
Vineyard	Whistling Dog Vineyard, OR	0	63	118

Table S10. Relative abundance of S. paradoxus to S. cerevisiae in North America.

Term	OR	CI 2.5%	CI 97.5%	P value
intercept	0.9111176	0.2826117	2.6856696	
state (OR)	51.468924	21.7839968	144.2824317	<2e-16
location type	0.5523316	0.3133832	0.9554568	0.0336
source (oak)	0.8300457	0.3036179	2.5209741	0.7285
year (2009)	0.6405627	0.3724502	1.0953692	0.1038

The odds ratio (OR) and confidence interval (CI) of S. paradoxus relative to S. cerevisiae. Terms in parentheses indicate their effect on relative abundance.