

Evolution of ecological dominance of yeast species in high-sugar environments

Kathryn M. Williams,^{1,2} Ping Liu,³ and Justin C. Fay^{3,4}

¹Evolution, Ecology and Population Biology Program, Washington University, St. Louis, Missouri

²E-mail: kathrynmrwilliams@gmail.com

³Department of Genetics, Washington University, St. Louis, Missouri

⁴Center for Genome Sciences and Systems Biology, Washington University, St. Louis, Missouri

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In budding yeasts, fermentation in the presence of oxygen evolved around the time of a whole genome duplication (WGD) and is thought to confer dominance in high-sugar environments because ethanol is toxic to many species. Although there are many fermentative yeast species, only *Saccharomyces cerevisiae* consistently dominates wine fermentations. In this study, we use coculture experiments and intrinsic growth rate assays to examine the relative fitness of non-WGD and WGD yeast species across environments to assess when *S. cerevisiae*'s ability to dominate high-sugar environments arose. We show that *S. cerevisiae* dominates nearly all other non-WGD and WGD species except for its sibling species *S. paradoxus* in both grape juice and a high-sugar rich medium. Of the species we tested, *S. cerevisiae* and *S. paradoxus* have evolved the highest ethanol tolerance and intrinsic growth rate in grape juice. However, the ability of *S. cerevisiae* and *S. paradoxus* to dominate certain species depends on the temperature and the type of high-sugar environment. Our results indicate that dominance of high-sugar environments evolved much more recently than the WGD, most likely just prior to or during the differentiation of *Saccharomyces* species, and that evolution of multiple traits contributes to *S. cerevisiae*'s ability to dominate wine fermentations.

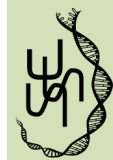
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Evolutionary innovation can promote the ecological dominance of some lineages by enabling them to occupy new niches. Although conservation of an innovation among descendent taxa reflects its contribution to their ecological success, ecological dominance may not be an immediate consequence of evolutionary innovation. Phylogenetic studies indicate that the current dominance of some lineages may result from events temporally distinct from major evolutionary transitions (Wing and Boucher 1998; Alfaro et al. 2009; Edwards et al. 2010; Near et al. 2012; Schranz et al. 2012; Bouchenak-Khelladi et al. 2014). This apparent lag between the evolution of an innovation and the rise to dominance of descendent lineages may occur because dominance depends upon certain environments, ecological communities, or the acquisition of additional traits.

In budding yeasts, evolution of the ability to ferment sugar in the presence of oxygen dramatically changed the way some species harness energy. Although most species acquire energy

through respiration in the presence of oxygen, certain species such as *Saccharomyces cerevisiae* acquire most of their energy via the less efficient process of fermentation (Pronk et al. 1996). Evolution of this fermentative lifestyle likely involved multiple steps both before and after a whole genome duplication (WGD) in the yeast lineage, including the ability to grow without mitochondrial electron transport and the transcriptional rewiring of carbon metabolizing enzymes (Ihmels et al. 2005; Merico et al. 2007; Field et al. 2009; Hagman et al. 2013; Lin et al. 2013). Although the evolutionary transition to a fermentative lifestyle began prior to the WGD, lineages that diverged after the WGD show a clear preference for fermentation in the presence of oxygen (Merico et al. 2007; Hagman et al. 2013).

Fermentation in the presence of oxygen is thought to provide WGD yeast species with a fitness advantage in high-sugar environments such as grape juice (Wolfe and Shields 1997; Piskur and Langkjaer 2004; Thomson et al. 2005; Piskur et al. 2006; Conant



and Wolfe 2007). Theoretical modeling shows that a fermentative lifestyle can yield a growth advantage in high-sugar environments due to a higher rate of sugar consumption and energy production (Pfeiffer et al. 2001; MacLean and Gudelj 2006; Conant and Wolfe 2007). Additionally, ethanol produced during fermentation may inhibit the growth of competitor species (Gause 1934; Piskur and Langkjaer 2004; Thomson et al. 2005; Piskur et al. 2006). Thus, the fermentative lifestyle is expected to enable WGD species to dominate high-sugar environments such as grape juice.

Although *S. cerevisiae* has been shown to dominate competitions with multiple non-WGD species (Holm Hansen et al. 2001; Pérez-Nevado et al. 2006), the importance of the fermentative lifestyle remains equivocal. Competition experiments between *S. cerevisiae* and several non-WGD species did not support the role of ethanol but instead implicate different factors depending upon which competitor species was used. Competitions with *Torulaspora delbrueckii* and *Lachancea thermotolerans* demonstrated that low oxygen and cell density contribute to *S. cerevisiae*'s dominance (Holm Hansen et al. 2001; Nissen et al. 2003, 2004), whereas competitions with *Hanseniaspora guilliermondii* and *H. uvarum* showed that *S. cerevisiae* produces a toxic metabolite derived from glyceraldehyde 3-phosphate dehydrogenase peptides (Pérez-Nevado et al. 2006; Albergaria et al. 2010; Branco et al. 2014). Although *S. cerevisiae* exhibits high ethanol tolerance (Pina et al. 2004; Belloch et al. 2008; Arroyo-López et al. 2010; Salvadó et al. 2011a), monoculture growth rates of various species indicate that temperature is more important to *S. cerevisiae*'s dominance than ethanol tolerance (Goddard 2008; Salvadó et al. 2011a).

Within the vineyard environment, grapes and wine musts contain hundreds of yeast species, including a number of fermentative species (Pretorius 2000; Fleet 2003, 2008; Jolly et al. 2006; Bokulich et al. 2012; Pinto et al. 2014). Yet, even without the introduction of commercial wine yeast, *S. cerevisiae* consistently dominates grape juice as it ferments to wine (Fleet 2003, 2008). Because little is known about the relative fitness of most WGD species in high-sugar environments such as grape juice, it is unclear whether *S. cerevisiae*'s dominance in wine fermentations reflects certain attributes of the grape juice environment or the yeast species present within the community, and whether dominance in high-sugar environments is a simple consequence of the fermentative lifestyle or involves the acquisition of additional traits.

The objectives of this study were to determine when the ability to dominate high-sugar environments evolved in the yeast lineage and to identify traits that confer *S. cerevisiae* with a growth advantage in these environments. To infer when dominance arose and lessen the impact of any potential strain or species outliers, we examined a taxonomically diverse sample of 18 different yeast species spanning the WGD and evolution of

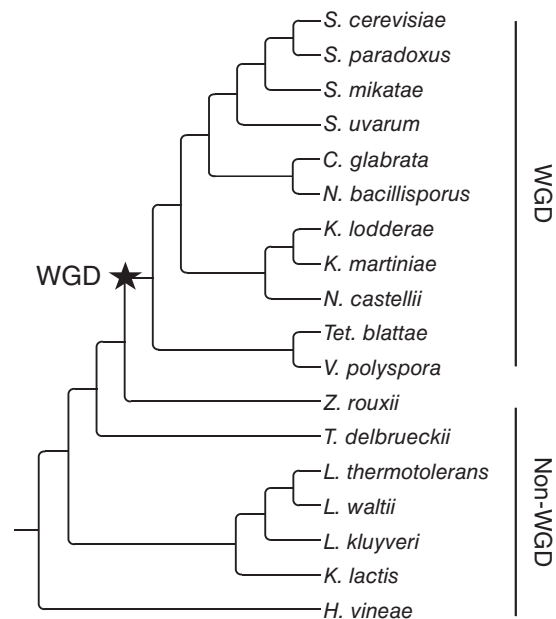


Figure 1. Phylogenetic relationship of yeast species used in this study. The phylogeny is based on two previous studies (Kurtzman and Robnett 2003; Salichos and Rokas 2013) and the placement of the *Nakaseomyces* (*C. glabrata* and *N. bacillisporus*) using chromosome rearrangements (Scannell et al. 2006). The whole genome duplication (WGD) event is shown by a star.

the fermentative lifestyle (Fig. 1). Given that the evolution of the fermentative lifestyle spanned the WGD (Hagman et al. 2013), we included non-WGD species that produce small amounts of ethanol (*Lachancea* and *Torulaspora* species) and WGD species that exhibit intermediate levels of fermentation (*Vanderwaltozyma* and *Tetrapisispora* species). To identify when dominance arose we directly competed these species with one another and found that dominance of high-sugar environments evolved much more recently than the evolution of the fermentative lifestyle. To identify traits involved in dominance, we compared species' intrinsic growth rates under a variety of conditions and found that both ethanol and the type of high-sugar environment influence fitness.

Material and Methods

YEAST STRAINS

A total of 20 yeast strains representing 18 non-WGD and WGD species were used for our experiments (Table S1 and Fig. 1). We chose a *S. cerevisiae* strain isolated from oak (YPS163) to represent *S. cerevisiae* (Sniegowski et al. 2002). As a control for population variation within *S. cerevisiae*, we also included I14, a *S. cerevisiae* strain isolated from a vineyard in Italy (Fay and Benavides 2005) and BJ20, a *S. cerevisiae* strain isolated from a tree in China (Wang et al. 2012).

GROWTH MEDIA

The primary assay media used were two high-sugar environments: Chardonnay grape juice (Vintners Reserve, Winexpert Inc., Port Coquitlam, BC, Canada), hereafter referred to as “Grape,” and high sugar rich medium (10 g/L yeast extract, 20 g/L peptone, 120 g/L dextrose), hereafter referred to as “HS.” We chose HS to reflect the glucose concentration typical of the grape juice environment, ~120 g/L (Rodicio and Heinisch 2009). Low-pH HS was made by adjusting the pH of HS from 6.7 to 3.7, the pH of our Grape medium, using tartaric acid, the predominant acid present in grape juice (Radler 1993). The media used to test for nutrient limitations in Grape included Grape with one of five nutrient supplements: YP (10 g/L yeast extract, 20 g/L peptone), CM (1.3 g/L synthetic complete with amino acids, 1.7 g/L yeast nitrogen base, and 5 g/L ammonium sulfate), AA (1.3 g/L complete amino acids), NB (1.7 g/L yeast nitrogen base), or AS (5 g/L ammonium sulfate). Assay media to test the ethanol tolerance of each yeast species was YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) with ethanol concentrations ranging from 0% to 10%. Assay media to identify unknown inhibitor compounds produced by *S. cerevisiae* during growth was YPD made using supernatant from 16 other species (Table S1) grown in monoculture and coculture with *S. cerevisiae*. We chose YPD with 2% dextrose for ethanol tolerance and supernatant assays because ethanol produced during growth by fermenting species should not attain inhibitory concentrations.

COMPETITION EXPERIMENTS

Growth conditions

To assess the relative growth of non-WGD and WGD yeast species in high-sugar environments, we performed two competition experiments in which we measured the abundance of representative strains of multiple species relative to a focal species strain of *S. cerevisiae* or *S. paradoxus* after growth in coculture. In the first competition experiment (Competition 1), we measured the abundances of six non-WGD and seven WGD species (Table S1) relative to a single *S. cerevisiae* strain (YPS163). As controls, we assessed the ability of the conspecific strain, I14, to grow relative to our reference *S. cerevisiae* strain, and grew each species in monoculture. Competitions were initiated at approximately 10^3 cells of each species per milliliter of Grape and HS media. One milliliter cultures were grown in 2 mL 96-well plates that were covered with breathable film and incubated at 30°C with shaking at 400 rpm for 48 h. To assess the effects of strain and temperature on the relative growth of WGD species in high-sugar environments, we measured the abundance of five WGD species relative to three *S. cerevisiae* strains (YPS163, I14, and BJ20) and a single strain of *S. paradoxus* (YPS152) following coculture (Competition 2). Cultures were prepared and grown as in

Competition 1, except that replicates of each culture were grown at 30°C and 22°C.

Sampling

For both competition experiments, samples were taken at the beginning and the end of the experiment and frozen at -20°C for later use. For monocultures in Competition 1, cells from *S. cerevisiae* monoculture were mixed in equal volume with cells from each of the other species' monocultures at 48 h and then frozen.

DNA extraction

DNA for Competition 1 was extracted from each sample using a protocol modified from Hoffman (2002) that included adding approximately 200 μ l of 0.5 mm diameter glass beads (BioSpec Products, Bartlesville, OK) to each sample and lysing cells in a bead beater (BioSpec Products) on high for 5 min at room temperature. For samples grown in Grape, DNA was also column purified to remove an unknown inhibitor of PCR amplification. DNA for Competition 2 was extracted using the ZR-96 Fungal/Bacterial DNA Kit (Zymo Research, Organge, CA).

Pyrosequencing

To quantify the abundance of *S. cerevisiae* relative to each competitor species in Competition 1, we pyrosequenced species-specific single nucleotide variants (SNVs). Previous studies have shown that pyrosequencing can accurately quantify the frequency of SNVs in pooled DNA samples (Lavebratt and Sengul 2006). To design pyrosequencing primers, we generated pair-wise sequence alignments of *ACT1* or *CYT1* between *S. cerevisiae* and each of the other species and identified at least one SNV for each pair and designed corresponding primer sets that included (1) forward and reverse primers for PCR and (2) a pyrosequencing primer (Table S2). Pyrosequencing was carried out using a PyroMark Q96 MD Automated pyrosequencer (Qiagen, Valencia, CA) following the protocol described by King and Scott-horton (2007) and the manufacturer's directions.

To calibrate each primer set, DNA from samples containing known ratios of cells from *S. cerevisiae* and each of the other species were also pyrosequenced and used to establish standard curves using linear and polynomial regression (Fig. S1). For two species (*Nakaseomyces bacillisporus* and *Tetrapisispora blattae*), we were not able to design sets of primers, and for three species (*Kazachstania lodderae*, *K. martiniae*, and *Kluyveromyces lactis*), we were not able to quantify abundance due to severely biased PCR or pyrosequencing identified by our control calibrations.

High-throughput sequencing

To quantify the abundance of each *S. cerevisiae* or *S. paradoxus* strain relative to each competitor species in Competition 2, we

used high-throughput sequencing due to lower cost, larger sample capacity, and a change in the availability of pyrosequencing. The internal transcribed sequence (ITS1) between the 5.8S and 18S ribosomal genes was amplified using 24 barcoded primers (Bokulich et al. 2012). Samples were further multiplexed using 18 indexed adaptors (Table S3). Following quantification and pooling at equal concentrations, the sample library was sequenced on a single 1×250 bp run of an Illumina MiSeq. Sample sequences were de-multiplexed allowing a single mismatch to each bar code or index followed by adaptor trimming and clipping of any low quality bases using ea-utils (Aronesty 2011). Species' counts in each sample were obtained by blastall (version 2.2.25) against 287,101 sequences from culturable species in the UNITE+INSD database (Kõljalg et al. 2013). Of the 11.6 million raw reads, 8.0 million were included in the analysis with a median of 4346 species tags across 578 samples.

ITS1 species counts were calibrated using DNA from samples with known ratios of cells from *S. cerevisiae* or *S. paradoxus* and each of the competitor species. Standard curves were generated using linear regression (Fig. S2). For three species with nonlinear calibration curves, *Naumovozyma castellii*, *K. lodderae*, and *Candida glabrata*, we used a modified regression:

$$y = bx / (1 + x(b - 1)),$$

where b is a bias parameter, x is the observed frequency, and y is the known frequency (Moskalev et al. 2011). For two species with particularly strong bias, *N. castellii* and *K. lodderae*, we also generated calibration curves for *ACT1* PCR fragments. *ACT1* PCR fragments showed a weaker bias, and so we used these fragments for all competitions between *S. cerevisiae* and either *N. castellii* or *K. lodderae*.

Assessing dominance

To determine the abundance of *S. cerevisiae* or *S. paradoxus* relative to each species, we adjusted the percentage of species-specific SNVs sequenced during pyrosequencing (Competition 1) or the number of species-specific counts from high-throughput sequencing (Competition 2) using our standard curves. If values were negative after this adjustment, we conservatively adjusted them to 0.005. To calculate the relative fitness of each competitor species, we used

$$\ln(C_t/R_t) - \ln(C_0/R_0) = t \ln(w_c/w_r),$$

where C_t and R_t are the frequencies of the competitor and reference at generation t and w is fitness. For all competitions, we assumed $t = 14$ based on changes in cell density.

To identify significant growth differences between species, we either used a one-tailed paired Welch's t -test (Competition 1), or a mixed effect model with random effect for strain and a fixed

effect for the competitor's fitness (Competition 2). To correct for multiple comparisons, we used the method of Benjamini and Hochberg (1995) and a false discovery rate (FDR) cutoff of less than 0.01 for Competition 1 and 0.05 for Competition 2, because fewer species were competed in Competition 2.

INTRINSIC GROWTH RATE EXPERIMENTS

Growth assays

Cultures were inoculated at an optical density (OD) at 600 nm of approximately 0.25 in 1 mL of growth medium and were grown in 2 mL 96-well plates that were covered with breathable film and incubated at 30°C with shaking at 400 rpm for up to 48 h. Cell density was measured by OD at 620 nm at 0, 4, 8, 12, 18, 24, 36, and 48 h using an iEMS microplate reader (Thermo Lab Systems, Helsinki, Finland).

Analysis

The intrinsic growth rate (r) of each species was calculated for each time interval using the equation:

$$N_t = N_0 e^{rt},$$

where N_t is final cell density, N_0 is initial cell density, and t is time in hours. The average intrinsic growth rate of each species across all sampling intervals for a given medium was then used to evaluate the effect of a treatment (i.e., low pH or a nutrient supplement) on the growth of each species, ($\Delta r_{Treatment} = r_{Treatment} - r_{Control}$), or the effect of the species in a given environment ($\Delta r_{Species} = r_{non-S. cerevisiae} - r_{S. cerevisiae}$). Although the average growth rate does not distinguish between differences in lag phase, exponential growth rate, and carrying capacity, we used it because it had a smaller error among replicates.

For two sets of experiments, we only used OD up to and including 24 h in our analysis: ethanol tolerance experiments and supernatant experiments. For ethanol tolerance experiments, we observed flocculation that increased the variability of OD measurements beginning at 36 h. For inhibitor compound experiments, we observed that un-inoculated control samples registered noticeable effects on OD measurements beginning at 36 h.

Ethanol tolerance among species was measured by the ethanol concentration that inhibited growth by 50% (IC₅₀). IC₅₀ estimates were obtained by fitting dose–response curves using a three-parameter Weibull function in R (R Development Core Team 2013) using the “drc” package (Ritz and Streibig 2005). Statistical comparisons between the estimated IC₅₀ for *S. cerevisiae* and each species were made using the “comped” function (Ritz and Streibig 2005; R Development Core Team 2013) followed by the Altman and Bland method to calculate P values from confidence intervals (Altman and Bland 2011). To correct for multiple comparisons, we used the method of Benjamini

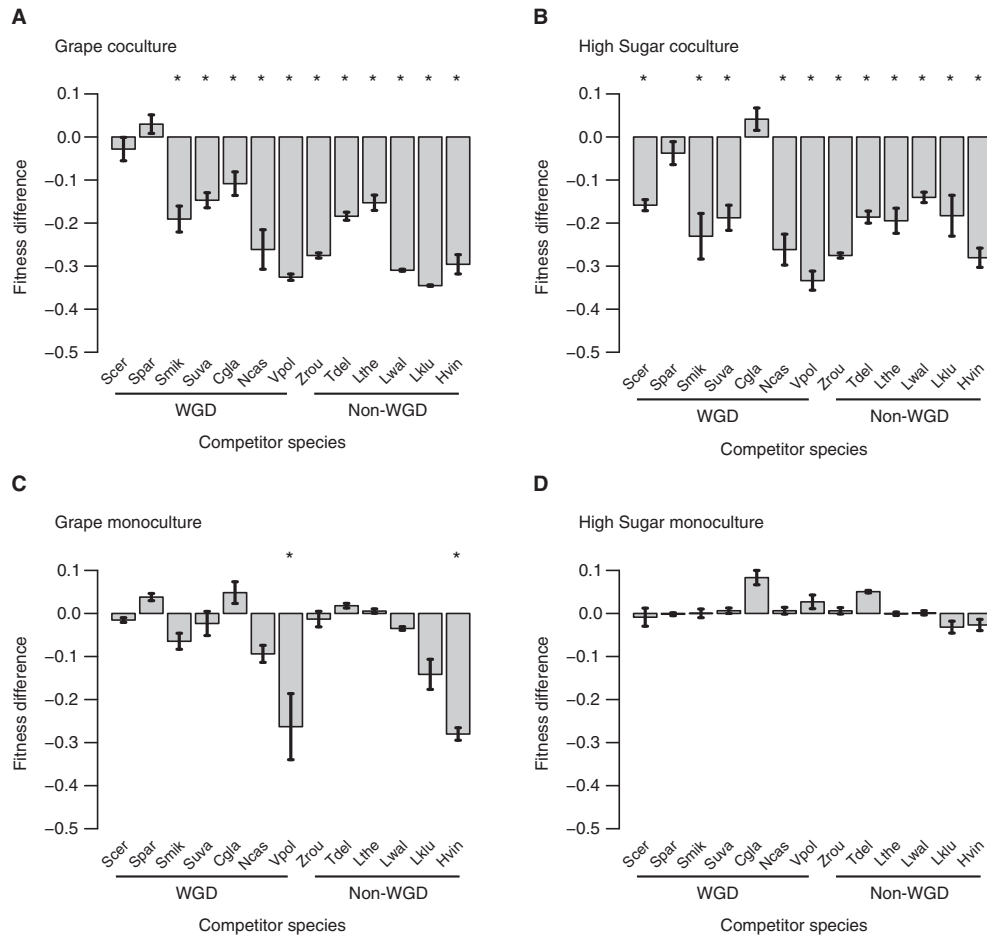


Figure 2. Fitness differences of *S. cerevisiae* relative to WGD and non-WGD yeast species after coculture or monoculture in two high-sugar environments. The fitness of each species in Grape (A) and HS (B) cocultures and Grape (C) and HS (D) monocultures. Bars and whiskers represent the mean and SE ($n = 3$) of the difference between the competitor fitness (w_c) and the *S. cerevisiae* (YPS163) reference fitness (w_r), which is set to 1. Species labels are Scer (*S. cerevisiae*), Spar (*S. paradoxus*), Smik (*S. mikatae*), Suva (*S. uvarum*), Cgla (*C. glabrata*), Ncas (*N. castellii*), Vpol (*V. polyspora*), Tdel (*T. delbruekii*), Lthe (*L. thermotolerans*), Lwal (*L. waltii*), Lklu (*L. kluyveri*), Hvin (*H. vineae*), and WGD and non-WGD species are indicated. Fitness significantly different from the reference is shown for FDR < 0.01 (*).

and Hochberg (1995) and an FDR cutoff of less than 0.01 for significance.

Results

ECOLOGICAL DOMINANCE OF YEAST SPECIES IN GRAPE JUICE EVOLVED MORE RECENTLY THAN THE FERMENTATIVE LIFESTYLE

To determine when ecological dominance of high-sugar environments evolved, we grew representative strains of multiple non-WGD and WGD yeast species in two high-sugar environments: Chardonnay grape juice (Grape) and a high sugar rich medium (HS). If dominance in high-sugar environments evolved along with the evolution of fermentation in the presence of oxygen, we expect *S. cerevisiae* will consistently exhibit higher relative fitness than all non-WGD species but not WGD species in both

high-sugar environments. Grape was chosen to represent a natural high-sugar environment, and HS was chosen to replicate the sugar concentration typical of the grape juice environment while limiting the potential influence of nutrient content and low pH. Because the ability to dominate is inherently a relative trait, we assessed the growth of each non-WGD and WGD species relative to a representative *S. cerevisiae* strain isolated from an oak tree (YPS163) using cocultures. As a control, we also grew a *S. cerevisiae* strain isolated from a vineyard (I14) in coculture with our reference *S. cerevisiae* strain. If the relative abundance of *S. cerevisiae* was significantly higher at the end compared to the start of the experiment, indicating a higher relative fitness, it was considered “dominant.”

We find that *S. cerevisiae* dominates nearly all non-WGD and WGD yeast species in Grape and HS cocultures (Fig. 2A, B). In both Grape and HS, *S. cerevisiae* increased in abundance relative

to 10 of 12 yeast species (FDR < 0.01, Table S4). In the majority of these cocultures, *S. cerevisiae* was greater than 90% of the population at 48 h. Notably, *S. cerevisiae* remained a significant proportion of the population even when it did not dominate. These data show that *S. cerevisiae* is able to dominate in multiple high-sugar environments, and they suggest that the ability to dominate high-sugar environments arose recently in yeast evolution.

In support of a more recent evolution of ecological success in high-sugar environments, *S. paradoxus* is the only species that persists along with *S. cerevisiae* in Grape and HS cocultures. Two other strains, *S. cerevisiae* (I14) and *C. glabrata*, also competed well with our *S. cerevisiae* reference. However, their persistence depended upon the environment: *S. cerevisiae* dominated *C. glabrata* in Grape (FDR = 0.0072) but not in HS, whereas it dominated I14 in HS (FDR = 0.0010) but not in Grape. Thus, *S. paradoxus* was the only species able to compete well with *S. cerevisiae* in both high-sugar environments.

One explanation for *S. cerevisiae*'s dominance in our Grape and HS cocultures is that it has a greater carrying capacity than other species in these environments, even when they are grown individually. As a control for our coculture experiments, we also measured the density of each species grown in monoculture by mixing it with a *S. cerevisiae* monoculture after 48 h and quantifying the proportion of each species.

S. cerevisiae has a carrying capacity similar to the majority of yeast species in Grape and HS (Fig. 2C, D). In Grape, the abundance of *S. cerevisiae* was significantly greater than only 2 of 12 species after 48 h of monoculture (Fig. 2C, FDR < 0.01). Species that obtained significantly lower carrying capacities included the non-WGD species *H. vineae* and the WGD species *Vanderwaltozyma polyspora*, which were 1% and 3% of *S. cerevisiae*'s abundance after 48 h in monoculture. The relative population size of *S. cerevisiae* was also not significantly greater than 12 of 12 species tested in HS. These data imply that *S. cerevisiae*'s dominance in Grape and HS cocultures is not due to differences between species in their individual carrying capacities.

Dominance of high-sugar environments appears to be a trait shared by *S. cerevisiae* and *S. paradoxus*. However, we only used one representative strain of *S. cerevisiae* and did not compete *S. paradoxus* with other species. To test whether *S. cerevisiae*'s dominance is a property of the strain used or the species, we repeated a subset of the Grape and HS competitions using two additional strains of *S. cerevisiae*: a soil isolate (I14) from Italy that is closely related to other European strains (Fay and Benavides 2005) and a tree isolate from China that is diverged from both the European strain and our reference strain YPS163 from North America (Wang et al. 2012). The fitness of the three *S. cerevisiae* strains together in relation to each competitor species indicates that *S. cerevisiae* dominates *S. mikatae*, *S. uvarum*, *N. castellii*, and *K. lodderae* in both Grape and HS, *C. glabrata* in Grape but not

HS, and has no fitness differences in comparison to *S. paradoxus* (FDR < 0.05, Fig. 3A, B). These results confirm our previous results based on a single *S. cerevisiae* reference strain and add *K. lodderae* to the list of species dominated by *S. cerevisiae*.

Using *S. paradoxus* as the reference species, we find that *S. paradoxus* dominates the same yeast species as *S. cerevisiae* in Grape (Fig. 3A). In HS, *S. paradoxus* dominates fewer species than *S. cerevisiae*; it does not dominate *S. mikatae* and *K. lodderae* (Fig. 3B). Thus, the results of our competition experiments with *S. paradoxus* show that dominance in Grape is shared by both *S. cerevisiae* and *S. paradoxus* but that the ability of *S. paradoxus* to dominate in HS is not as strong as that of *S. cerevisiae*.

TEMPERATURE AFFECTS ECOLOGICAL DOMINANCE OF CERTAIN YEAST SPECIES IN HIGH-SUGAR ENVIRONMENTS

The *Saccharomyces* species have differentiated in both their optimal and maximum growth temperature (Belloch et al. 2008; Gonçalves et al. 2011; Kurtzman et al. 2011; Salvadó et al. 2011b). Because *S. cerevisiae*'s optimal growth temperature (32°C) is higher than that of *S. paradoxus* (30°C), *S. mikatae* (29°C), and *S. uvarum* (26°C) (Salvadó et al. 2011b), its dominance of high-sugar environments could depend on the high temperature (30°C) of our initial competition experiment. To examine this possibility, we competed a subset of species at a lower temperature (22°C).

Similar to the results of our competition experiments performed at high temperature, at 22°C both *S. cerevisiae* and *S. paradoxus* dominated *S. uvarum* and *N. castellii* in Grape and HS, and dominated *C. glabrata* in Grape but not HS (Fig. 3C, D). However, at the lower temperature neither *S. cerevisiae* nor *S. paradoxus* dominated *S. mikatae* or *K. lodderae* in Grape or HS, and *K. lodderae* dominated *S. cerevisiae* and *S. paradoxus* in HS. Thus, ecological dominance of certain yeast species in high-sugar environments depends on temperature, which implicates the evolution of thermal tolerance among the *Saccharomyces* species in the evolution of ecological dominance.

SACCHAROMYCES CEREVISIAE HAS A DISTINCT COMPETITIVE ADVANTAGE IN GRAPE JUICE

Our finding that many WGD yeast species compete poorly with *S. cerevisiae* in high-sugar environments suggests that the fermentative lifestyle is not sufficient to confer ecological success in these environments. Because the majority of the species we tested are capable of achieving similar carrying capacities to *S. cerevisiae* in these environments when grown individually, *S. cerevisiae*'s dominance in our Grape and HS cocultures must be related to either differences in intrinsic growth rates or interference competition. To investigate these two modes of ecological dominance, we measured the intrinsic growth rate of each species in monoculture. If *S. cerevisiae* does not exhibit a greater intrinsic growth rate than

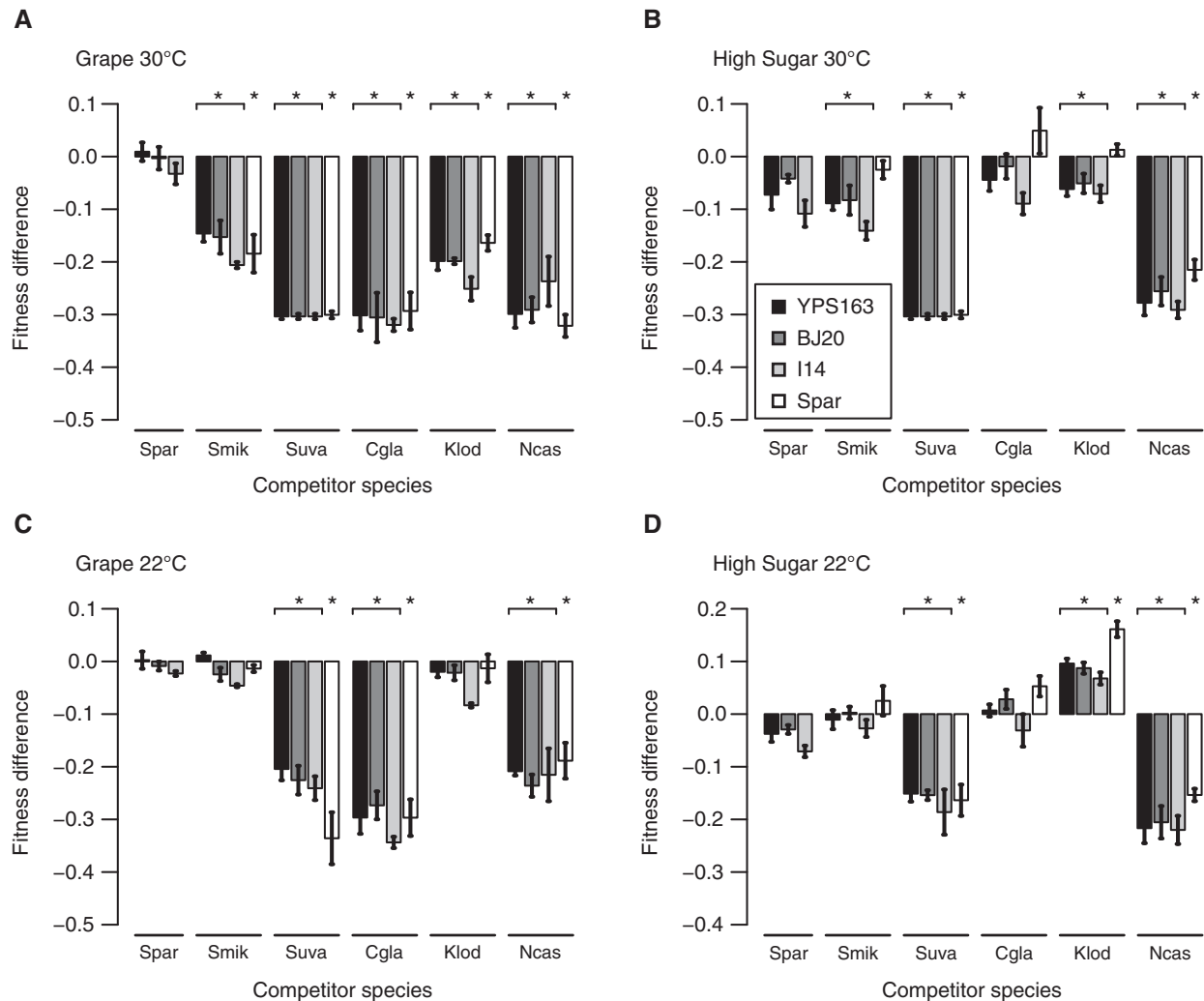


Figure 3. Fitness differences depend on temperature and growth medium. Fitness differences between each competitor species and a reference strain are shown for cocultures at 30°C in Grape (A) or HS (B) and at 22°C in Grape (C) or HS (D) medium. Reference strains are three *S. cerevisiae* strains (YPS163, BJ20 and I14) and *S. paradoxus* (Spar). Bars and whiskers represent the mean and its SE ($n = 3$). Significant differences in fitness are shown for $FDR < 0.05$ (*). Species abbreviations are the same as in Figure 2.

other species, then its ability to dominate in these environments can be attributed to interference competition.

S. cerevisiae has a greater intrinsic growth rate than nearly all yeast species in the grape juice environment (Fig. 4). Compared to *S. cerevisiae*, 16 of 17 species exhibited a significantly lower intrinsic growth rate in Grape ($FDR < 0.01$, Table S5). The one notable exception to this pattern was *S. paradoxus*, which had a lower growth rate but did not meet our cutoff for significance ($FDR = 0.028$). In stark contrast to our finding in Grape, when we compared the intrinsic growth rate of *S. cerevisiae* to the intrinsic growth rate of each of the other species in HS, we did not observe any significant difference for all 17 species (Fig. 4B). These results suggest different or multiple mechanisms contribute to the dominance of *S. cerevisiae* in high-sugar environments. Furthermore, they support the recent evolution of

traits required for ecological success in the grape juice environment. In the following sections, we examine factors that may contribute to the dominance of *S. cerevisiae* in both HS and Grape.

EVOLUTION OF ETHANOL TOLERANCE AND ITS POTENTIAL ROLE IN INTERFERENCE COMPETITION

S. cerevisiae's ability to produce and tolerate ethanol is one way in which it may dominate other species in high-sugar environments. Although previous studies showed that *S. cerevisiae* tolerates higher ethanol concentrations than many yeast species, they only included 4 of 17 of the species used in this study (Pina et al. 2004; Belloch et al. 2008; Arroyo-López et al. 2010; Salvadó et al. 2011a). To examine the potential impact of ethanol on the growth of each species, we measured the intrinsic growth rate of each species in YPD supplemented with ethanol at concentrations

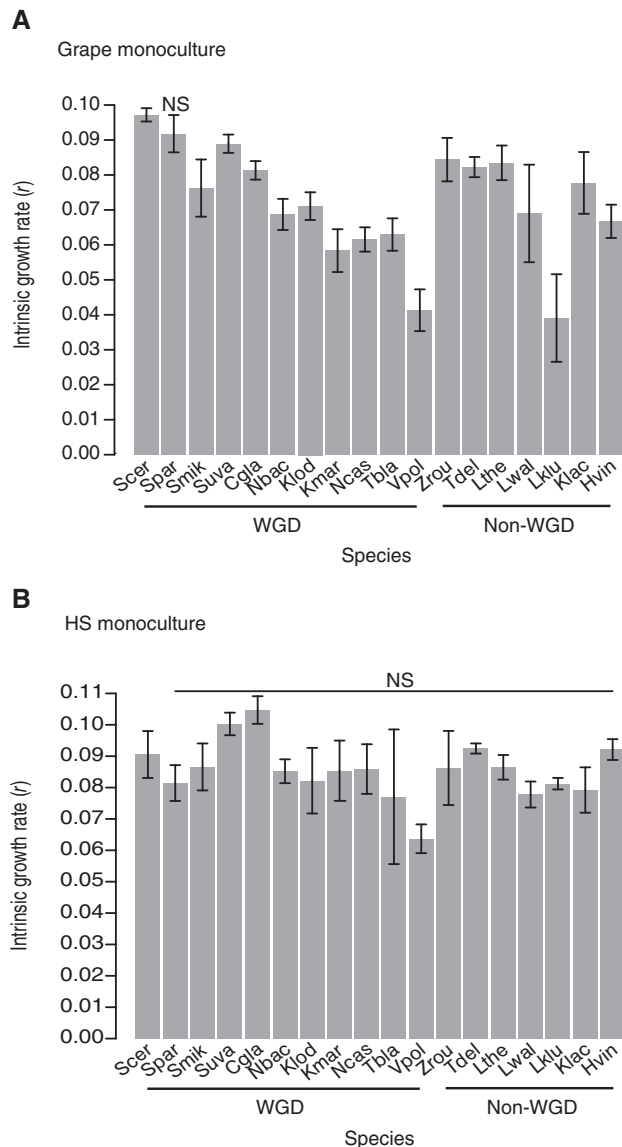


Figure 4. Intrinsic growth rate differences in high-sugar environments. The intrinsic growth rate of each species in Grape (A) and HS (B). WGD and non-WGD species are indicated. Bars and whiskers represent the mean and SD of the growth rate ($n = 3$). Species that did not differ significantly from *S. cerevisiae* at an FDR cutoff of 0.01 are indicated (NS).

ranging from 0% to 10% and calculated the ethanol concentration that inhibited growth rate by 50% (IC_{50}) for each species by fitting dose–response curves to the growth rate (see Materials and Methods).

S. cerevisiae had an IC_{50} greater than 15 of 17 yeast species (Fig. 5 and Table S6). The two exceptions to this pattern were *S. cerevisiae*'s closest relative, *S. paradoxus* (FDR = 0.0502) and *C. glabrata* (FDR = 0.0320). *C. glabrata* grew as well as *S. cerevisiae* at moderate ethanol concentrations, and it grew better than *S. cerevisiae* at low ethanol concentrations

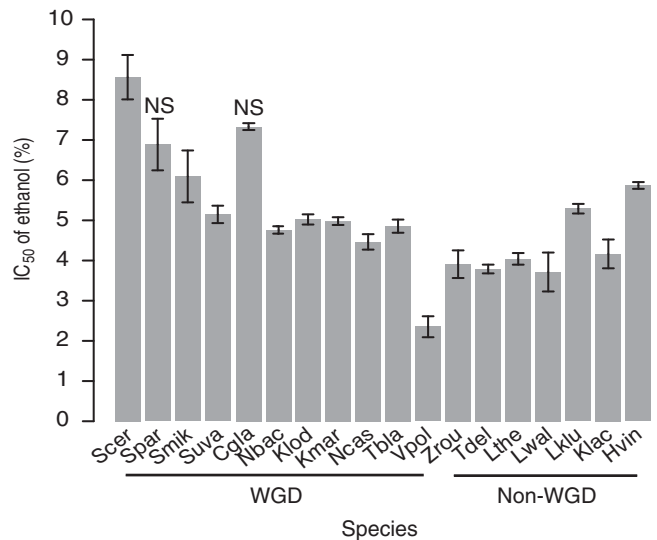


Figure 5. Species differences in ethanol tolerance. Mean (bars) and SE (whiskers) of the concentration of ethanol (%) that inhibits growth by 50% (IC_{50}) of each yeast species ($n = 3$). WGD and non-WGD species are indicated. Species with an IC_{50} that did not differ significantly from *S. cerevisiae* at an FDR cutoff of 0.01 are indicated (NS).

(Fig. S3 and Table S6). However, most of *S. cerevisiae*'s growth advantage occurred at ethanol concentrations at or above 4% (Fig. S3 and Table S6). Thus, although all species tolerate low concentrations of ethanol (< 4%), *S. cerevisiae* exhibits a growth advantage compared to most species at high ethanol concentrations.

NO EVIDENCE FOR INTERFERENCE COMPETITION MEDIATED BY OTHER TOXIC METABOLITES

Previous studies found that *S. cerevisiae* produced toxic metabolites other than ethanol that inhibit the growth of competitor species (van Vuuren and Jacobs 1992; Magliani et al. 1997; Musmanno et al. 1999; Pérez-Nevaldo et al. 2006; Albergaria et al. 2010; Rodríguez-Cousiño et al. 2011; Branco et al. 2014). However, other studies either did not find any evidence that *S. cerevisiae* produced an inhibitory compound (Torija et al. 2001; Nissen et al. 2003; Arroyo-López et al. 2011) or found that the ability to produce killer toxins varied among *S. cerevisiae* strains (Gutiérrez et al. 2001; Sangorrín et al. 2007; Maqueda et al. 2012). To determine whether the *S. cerevisiae* strain we used during our assays produces an inhibitor compound, we grew each species in the supernatant obtained from YPD monocultures and cocultures with *S. cerevisiae*. We chose YPD, which contains 2% dextrose, because ethanol concentrations should not attain inhibitory concentrations during growth. In no instance did the supernatant inhibit the subsequent growth of each species (Fig. S4 and Table S7).

LOW-pH AND NUTRIENT LIMITATIONS CONTRIBUTE TO *S. CEREVISIAE*'S INTRINSIC GROWTH RATE ADVANTAGE IN GRAPE JUICE

Grape juice differs from high sugar rich medium in that it has a lower pH (pH = 3.7 vs. pH = 6.7) and reduced levels of nutrients, most notably yeast assimilable nitrogen (Henschke and Jiranek 1993). To determine whether *S. cerevisiae*'s higher intrinsic growth rate in grape juice is related to pH or nutrient deficiencies, we measured the effects of altered pH of HS and nutrient content of Grape for each species.

To test the effect of pH on the intrinsic growth rate of each species, we grew each species in low-pH HS, HS adjusted to the same acidity level as our Grape medium. As a control, we compared each species' growth in low-pH HS to its growth in HS. If *S. cerevisiae*'s intrinsic growth rate is greater than other species in Grape due to low-pH, then *S. cerevisiae* should also exhibit a higher intrinsic growth rate than other species in low-pH HS.

S. cerevisiae has an intrinsic growth rate advantage in low-pH HS (Fig. 6). When grown in low-pH HS, 4 of 18 species exhibited a significantly lower intrinsic growth rate when compared to growth in HS (Fig. 6A and Table S8). Notably, only three species, including *S. cerevisiae*, were not affected by low-pH at a nominal level of significance ($P < 0.05$) compared to an FDR cutoff of 0.01. Additionally, when we compared the intrinsic growth rate of *S. cerevisiae* in low-pH HS to each of the other species in this environment, *S. cerevisiae*'s intrinsic growth rate was greater than 8 of 17 species (Fig. 6B), compared to 0 of 17 species observed in HS (Fig. 4B).

To test the effect of nutrient deficiency on the intrinsic growth rate of each species, we grew each species in Grape supplemented with one of several different nutrient sources that varied in complexity: YP, CM, NB, AA, and AS. YP is the rich nutritive base of the HS environment, CM contains vitamins and minerals, amino acids, and a single good nitrogen source, and NB, AA, and AS are the vitamins and minerals, amino acids, and nitrogen source (ammonium sulfate) components of CM, respectively. As a control, we compared each species' growth in Grape with a nutrient supplement to its growth in Grape without the nutrient supplement. If nutrient limitations contribute to intrinsic growth rate differences between species, then nutrient supplements in Grape should increase each species' growth rate and reduce or eliminate intrinsic growth rate differences between species.

Most yeast species are nutrient limited in grape juice. Of the 18 species we assayed, 12 exhibited a significant increase in intrinsic growth rate with the addition of one or more nutrient supplements, including *S. cerevisiae* (Fig. 7, Fig. S5A–D, and Table S9). However, which nutrients elicited a significant increase in growth varied by species. For example, *S. uvarum* was positively affected by the addition of YP and NB, whereas *C. glabrata*

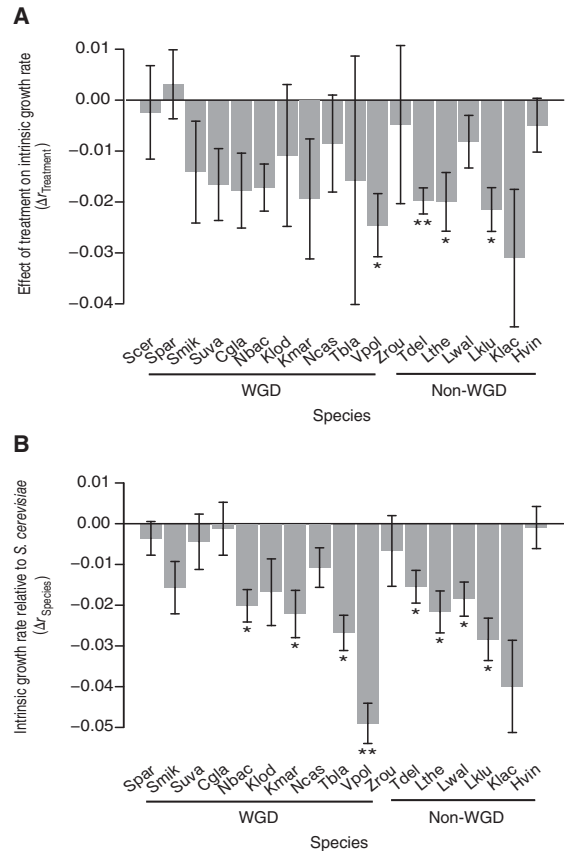


Figure 6. Intrinsic growth differences in response to low pH. (A) The effect of low-pH treatment on the intrinsic growth rate (r) of each species in HS ($\Delta r_{\text{Treatment}} = r_{\text{Treatment}} - r_{\text{HS}}$), and (B) the difference in the intrinsic growth rate between *S. cerevisiae* and each species in low-pH HS ($\Delta r_{\text{Species}} = r_{\text{non-*S. cerevisiae*}} - r_{\text{*S. cerevisiae*}}$). WGD and non-WGD species are indicated. Whiskers for each bar show 95% confidence intervals ($n = 3$). Significant differences in the growth rate of each species with or without low pH and differences between *S. cerevisiae* and each species in low pH are shown for FDR < 0.01 (*) and FDR < 0.001 (**).

was positively affected by YP, CM, and AA. Overall, YP positively affected the intrinsic growth rate of the most species (11), followed by NB (7), CM (6), and AA (3). However, none of the species we assayed grew significantly better with the addition of AS, a good nitrogen source.

Nutrient supplements eliminate the intrinsic growth rate differences between *S. cerevisiae* and nearly all other yeast species. Of the 17 species that grew significantly slower than *S. cerevisiae* in Grape (Fig. 4A), only two species, *N. castellii* and *V. polyspora*, grow significantly slower than *S. cerevisiae* in spite of all of the nutrient supplements used in our experiments (Table S9). Overall, Grape supplemented with CM had the fewest number of species that still grew significantly slower than *S. cerevisiae*, followed by Grape supplemented with AS (7), NB (12), YP (14), and AA (16).

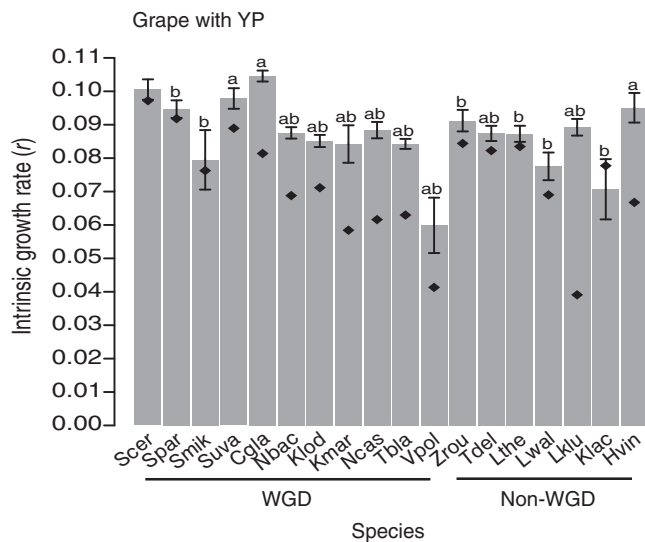


Figure 7. Intrinsic growth rate in Grape supplemented with nutrients. The mean intrinsic growth rate of each species in Grape supplemented with nutrients (YP). WGD and non-WGD species are indicated. Bars and whiskers represent the mean and SD of the growth rate ($n = 3$). Diamonds represent the mean growth rate in Grape without YP. Significant differences in the growth rate of each species with or without YP (a) and differences between *S. cerevisiae* and each species in YP (b) are labeled above each bar for FDR < 0.01.

Discussion

The diversion of more sugar to fermentation than respiration in the presence of oxygen, that is, the fermentative lifestyle, provides many yeast species the opportunity to exploit novel environments and ecological strategies. One such species, *S. cerevisiae*, consistently dominates wine fermentations and has become widely used to ferment beer, bread, and wine. In this study, we investigated when *S. cerevisiae*'s ability to dominate high-sugar environments evolved and whether its dominance is a simple consequence of the fermentative lifestyle. We find that dominance evolved much more recently than the evolution of the fermentative lifestyle and for certain species depends on the temperature and growth medium. Our results suggest that *S. cerevisiae*'s frequently observed dominance of grape juice fermentations is mediated by the evolution of multiple traits that build on an ancient change in metabolism.

EVOLUTION OF DOMINANCE IN RELATION TO THE FERMENTATIVE LIFESTYLE

Our study indicates that dominance in high-sugar environments evolved much more recently than the WGD and the transition to the fermentative lifestyle (Hagman et al. 2013). Although previous studies showed that *S. cerevisiae* dominates multiple other non-WGD species (Holm Hansen et al. 2001; Fleet 2003, 2008; Pérez-Nevedo et al. 2006; Goddard 2008), our findings

demonstrate that *S. cerevisiae* and its closest relative, *S. paradoxus*, also dominate most other WGD yeast species in high-sugar environments and that the temperature and growth medium are also important. The ability of *S. cerevisiae* and *S. paradoxus* to dominate representatives of multiple taxonomically diverse species suggests that dominance arose quite recently. However, the change in dominance relationships across environments implies that (1) multiple traits underlie dominance, (2) these traits have changed on lineages other than that leading to the ancestor of *S. cerevisiae* and *S. paradoxus*, and (3) dominance cannot be ascribed to a single lineage.

Multiple, distantly-related WGD lineages grow well in nutrient rich, high-sugar environments, but few species grow as well as *S. cerevisiae* in grape juice. Although *S. cerevisiae* and *S. paradoxus* dominate most WGD yeast species in HS medium, they do not dominate *C. glabrata* in this environment, and depending upon the temperature, they also lose some or all of their competitive advantage over *S. mikatae* and *K. lodderae*. Remarkably, *K. lodderae*'s higher relative fitness in HS compared to Grape contributes to its unique ability to dominate *S. cerevisiae* and *S. paradoxus* in HS at low temperature. One explanation for the exceptional growth observed for these species is that the ability to compete in nutrient-rich, high-sugar environment evolved independently along the lineages that gave rise to *K. lodderae*, *C. glabrata*, and the *Saccharomyces* species. Alternatively, the ability to compete well in nutrient-rich, high-sugar environments may have evolved early during the transition to the fermentative lifestyle, followed by multiple, independent losses. However, our findings suggest that the more parsimonious explanation is that the ability to dominate evolved much more recently than the transition to the fermentative lifestyle.

Evolution of temperature preferences affects the dominance of certain species. Among the *Saccharomyces* species, *S. cerevisiae*, *S. paradoxus*, and *S. mikatae* can grow at 37°C, whereas *S. arboricolus*, *S. uvarum*, and *S. kudriavzevii* cannot and the latter two are considered cryophilic (Belloch et al. 2008; Gonçalves et al. 2011; Salvadó et al. 2011b). Outside the *Saccharomyces* species, *K. lodderae*, *K. martiniae*, *T. blattae*, *L. waltii* and *H. vineae* cannot grow at 37°C (Kurtzman et al. 2011), indicating that growth at high temperature has been gained and/or lost multiple times. Given the species tree (Fig. 1), we attribute *S. cerevisiae*'s and *S. paradoxus*' dominance of *S. mikatae* at 30°C but not 22°C to evolution of thermal tolerance. Similarly, *S. cerevisiae*'s dominance of *K. lodderae* in Grape at 30°C but not 22°C may also depend on the evolution of thermal tolerance. Although we did not specifically assay dominance below 22°C, the outcome of competitions performed at even cooler temperatures may change. *Saccharomyces uvarum* dominates some wines at low temperatures (Torriani et al. 1999; Naumov et al. 2000; Sipiczek et al. 2001; Rementeria et al. 2003; Demuyter et al. 2004), and *S. kudriavzevii*

does not dominate but competes better with *S. cerevisiae* at low temperatures (Arroyo-López et al. 2011). However, *S. cerevisiae*'s dominance of *S. uvarum* may not be a simple consequence of thermal tolerance; *S. uvarum*'s growth rate in grape juice increased with supplementation of rich medium (YP) to a rate equivalent to that of *S. cerevisiae*. Furthermore, we did not observe a significant intrinsic growth rate difference between these species in high sugar rich medium when grown at 30°C (Fig. 4).

The thermal differentiation of *Saccharomyces* species raises the possibility that the ability to dominate high-sugar environments evolved prior to their differentiation. Dominance of high-sugar environments may have evolved progressively such that WGD yeast species can outcompete non-WGD yeast species even though they lose to *S. cerevisiae*. However, more pair-wise competitions between other non-*Saccharomyces* WGD species and non-WGD species are needed to test that hypothesis. Although the evolution of dominance cannot be pinned to any particular lineage, we see no clear progression to higher ethanol tolerance among WGD yeast species or evidence for higher intrinsic growth rate in grape juice outside of the *Saccharomyces* species. Thus, in addition to thermal differentiation, other traits likely to contribute to dominance evolved among the *Saccharomyces* species.

One limitation of our ability to make inferences about the evolution of dominance and how the environment influences it is that most species were only represented by a single strain. Although the fitness of three diverse strains of *S. cerevisiae* is generally quite similar to one another and to *S. paradoxus*, competitor strains may not always be representative of the species. This possibility places some limits on the interpretation of dominance relationships specific to a single species, for example, *C. glabrata* and *K. lodderae*, but is unlikely to explain overall patterns of dominance.

MULTIPLE MECHANISMS OF ECOLOGICAL DOMINANCE

S. cerevisiae's dominance of high-sugar environments cannot be explained by a single mechanism. In addition to thermal tolerance discussed above, we also find evidence for ethanol tolerance and a high intrinsic growth rate in grape juice. Interference competition through the production of ethanol provides one explanation for *S. cerevisiae*'s dominance of high sugar rich medium. Consistent with previous studies (Pina et al. 2004; Belloch et al. 2008; Arroyo-López et al. 2010; Salvadó et al. 2011a), we found that *S. cerevisiae* exhibits greater ethanol tolerance than most species. In support of the role of ethanol tolerance in dominance, *S. paradoxus* and *C. glabrata* exhibited ethanol tolerance similar to *S. cerevisiae* and were the only two species that were not dominated by *S. cerevisiae* in high sugar rich medium at high temperature.

However, previous studies showed that oxygen, cell density, and an inhibitory peptide affect *S. cerevisiae*'s dominance of var-

ious non-WGD species (Holm Hansen et al. 2001; Nissen et al. 2003, 2004; Pérez-Nevedo et al. 2006; Albergeria et al. 2010; Branco et al. 2014). These studies excluded the effects of ethanol because non-WGD species initiated cell death by some other mechanism before ethanol reached inhibitory concentrations. Although we only measured competitions with two of the species used in earlier studies, *T. delbrueckii* and *L. thermotolerans*, we cannot exclude the possibility that these species were dominated for reasons other than ethanol inhibition. One difference between our experiments and those of prior studies is that they were carried out with low or no agitation, whereas we performed our competitions under high agitation (400 rpm). Agitation is expected to increase dissolved oxygen and might eliminate cell density and confinement effects (Nissen et al. 2003, 2004; Arneborg et al. 2005).

Multiple lines of evidence suggest that *S. cerevisiae*'s dominance in grape juice is influenced by its high intrinsic growth rate in this environment. *S. cerevisiae* exhibited the highest rate of growth in grape juice, significantly higher than all species except *S. paradoxus*. The absence of any difference in growth rate in high sugar rich medium implies that *S. cerevisiae*'s intrinsic growth advantage in grape juice is specific to grape juice or similar environments. Furthermore, lowering the pH of high sugar rich medium did not affect *S. cerevisiae* but affected the growth of other species, and supplementation of nutrients to grape juice increased the growth of many species but had little to no effect on *S. cerevisiae*. Notably, *S. uvarum*, *C. glabrata*, and *H. vineae* grew as well as *S. cerevisiae* in low-pH medium and in grape juice supplemented with rich nutrients (YP), indicating that low nutrients alone may explain their slow growth in grape juice.

The relative importance of temperature, intrinsic growth rate, and ethanol inhibition to *S. cerevisiae*'s dominance of grape juice is uncertain because their effects are difficult to disentangle from one another. In support of temperature, fermentation is exothermic and can increase the temperature of wine must by as much as 10°C (Boulton 1979; Goddard 2008). However, we found that nutrient supplementation increased most species' intrinsic growth rate in grape juice at 30°C. Ethanol inhibition is not likely to be important until the later stages of fermentation because most species were not significantly inhibited by ethanol concentrations below 5%, similar to previous reports (Goddard 2008; Salvadó et al. 2011a). As such, we favor intrinsic growth rate in grape juice as a driver of dominance as it likely acts earlier and throughout the competition.

Interactions between factors may also contribute to *S. cerevisiae*'s dominance. Ethanol and high temperature act synergistically to decrease growth due to their overlapping effects on lipid membrane integrity (Piper 1995). Lipid membrane integrity importantly affects proton (H⁺) transport across the cell membrane, and the combined effects of ethanol and high temperature increase the lipid membrane's H⁺ permeability (Madeira et al.

2010). Increased H⁺ permeability can also result in reduced intracellular pH, particularly in acidic environments such as grape juice. Although we did not measure any interaction effects, Goddard (2008) found interactions between the effects of temperature, ethanol, and media, including grape juice, on the growth rate of *Saccharomyces* versus non-*Saccharomyces* species.

ECOLOGY OF HIGH-SUGAR ENVIRONMENTS

The fermentative lifestyle is hypothesized to coincide with the evolution of flowering plants due to the abundance of diverse high-sugar environments (Wolfe and Shields 1997; Piskur and Langkjaer 2004; Thomson et al. 2005; Conant and Wolfe 2007). Although the ecology of fermentative species is not well known, many have been isolated from insects and may be transported to high-sugar environments (Kurtzman et al. 2011). However, the recent evolution of traits that contribute to *S. cerevisiae*'s dominance of high-sugar environments is perplexing. Although both *S. cerevisiae* and *S. paradoxus* can be found in vineyards (Redzepović et al. 2002; Hyma and Fay 2013), these and other *Saccharomyces* species are commonly associated with tree bark, soil, and decaying leaves (Naumov et al. 1998; Sniegowski et al. 2002; Zhang et al. 2010; Wang et al. 2012; Hyma and Fay 2013). Given their abundance in arboreal habitats, it seems unlikely that their exceptional fitness in grape juice is due to adaptation to grape juice fermentations and thereby provides evidence for exaptation (Larson et al. 2013). One way in which these species may have become adapted to high-sugar but low-nutrient environments is through associations with insect-honeydew, which is high in sugar (>10 g/L) but low in amino acids (Douglas 1993; Fischer and Shingleton 2001; Fischer et al. 2002). Although a variety of insects and other animals exploit honeydew for its sugar resources (Beggs and Wardle 2006), and a recent investigation revealed that many taxonomically diverse fungi compete for honeydew (Dhami et al. 2013), no *Saccharomyces* species were found associated with aphid honeydew from Black Beach trees in New Zealand (Serjeant et al. 2008). It is also possible that *S. cerevisiae* is not adapted to a particular niche but is a generalist that happens to be particularly fit in high-sugar environments (Goddard and Greig 2015).

In addition to dominance relationships we studied, a variety of other factors may contribute to *S. cerevisiae*'s observed dominance of grape juice fermentations in vineyards. Many WGD species may not be present within vineyard environments. Of the non-*Saccharomyces* species used in this study, only the non-WGD species *Zygosaccharomyces rouxii*, *T. delbrueckii*, *L. thermotolerans*, and *H. vineae* have been reportedly isolated from grapes or wine must (Kurtzman et al. 2011). Furthermore, sulfites are frequently added to wine must, and wine strains of *S. cerevisiae* are known to exhibit higher levels of sulfite resistance than nonwine strains (Pérez-Ortín et al. 2002; Yuasa et al. 2004). Interspecific differ-

ences in sulfite resistance (Engle and Fay 2012), copper resistance (Warringer et al. 2011), and other environmental conditions that we did not examine may thus contribute to *S. cerevisiae*'s observed dominance of wine fermentations. Although we find *S. paradoxus* to be competitive with *S. cerevisiae* in controlled laboratory settings, it has been reported to be the dominant yeast species in only a few fermentations (Redzepović et al. 2002; Valero et al. 2007). Complicating such comparisons, however, is the possibility that *S. paradoxus* may not have always been distinguishable from *S. cerevisiae*, particularly in earlier studies of dominant yeast species from spontaneous wine fermentations.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Yeast strains used in this study.

Table S2. PCR and pyrosequencing primers.

Table S3. PCR primers, barcodes, and adaptors used for high-throughput sequencing.

Table S4. Significance of changes in the relative abundance of *S. cerevisiae* compared to other yeast species in high-sugar environments.

Table S5. Significance of intrinsic growth rate differences between *S. cerevisiae* and other yeast species in high-sugar environments.

Table S6. Significance of IC50 and intrinsic growth rate differences between *S. cerevisiae* and other yeast species in ethanol (%).

Table S7. Significance of intrinsic growth rate differences between each species' own supernatant and supernatant from *S. cerevisiae* monoculture and coculture.

Table S8. Significance of low-pH on the intrinsic growth rate of *S. cerevisiae* and other species in HS.

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Figure S1. Pyrosequencing calibration.

Figure S2. High-throughput sequencing calibration.

Figure S3. The effect of ethanol on the intrinsic growth rate of each species.

Figure S4. Intrinsic growth rate in YPD made with supernatant.

Figure S5. Intrinsic growth rate in Grape supplemented with various nutrients.