Consequences of cryopreservation in diverse natural isolates of *Saccharomyces cerevisiae*

Authors: Kieslana M. Wing, Mark A. Phillips, Andrew R. Baker and Molly K. Burke*

Affiliations: Department of Integrative Biology, Oregon State University**,** 3029 Cordley Hall, 2701 SW Campus Way, Corvallis, OR 97331

Author for Correspondence*: Molly Burke (molly.burke@oregonstate.edu)

Data deposition: The raw genome sequence files for this project are available through NCBI's SRA (BioProject ID: PRJNA603243). Other text files with data associated with the project will be deposited at Dryad upon final acceptance of the manuscript.

Acknowledgements: This work was supported by start-up funds to MKB at Oregon State University. Gianni Liti provided all the strains used in this study. Shawn O'Neill and Dana Gibbon with OSU's Center for Genome Research and Biocomputing assisted with genome data analysis. KMW, ARB, and MKB conducted experiments, KMW, MAP and MKB analyzed data and wrote the manuscript.

Abstract

Experimental evolution allows the observation of change over time as laboratory populations evolve in response to novel, controlled environments. Microbial evolution experiments take advantage of cryopreservation to archive experimental populations in glycerol media, creating a frozen, living "fossil" record. Prior research with *Escherichia coli* has shown that cryopreservation conditions can affect cell viability and that allele frequencies across the genome can change in response to a freeze-thaw event. We expand on these observations by characterizing fitness and genomic consequences of multiple freeze-thaw cycles in diploid yeast populations. Our study system is a highly recombinant *Saccharomyces cerevisiae* population (SGRP-4X) which harbors standing genetic variation that cryopreservation may threaten. We also investigate the four parental isogenic strains crossed to create the SGRP-4X. We measure cell viability over 5 consecutive freeze-thaw cycles; while we find that viability increases over time in the evolved recombinant populations, we observe no such viability improvements in the parental strains. We also collect genome-wide sequence data from experimental populations initially, after one freeze-thaw, and after five freeze-thaw cycles. In the recombinant evolved populations, we find a region of significant allele frequency change on chromosome 15 containing the ALR1 gene. In the parental strains, we find little evidence for new mutations. We conclude that cryopreserving yeast populations with standing genetic variation may have both phenotypic and genomic consequences, though these same cryopreservation practices may have only small impacts on populations with little or no initial variation.

Keywords: experimental evolution, pool-seq, freeze-thaw, standing genetic variation, glycerol

Significance: Investigators studying laboratory populations of microbes tend to store them indefinitely as frozen stocks at -80C, so that they can be revived for future experiments. While it is a widespread assumption that such freezing does not impact population genotypes or phenotypes, this assumption has not been generally tested, especially in cases where populations are recombinant and harbor a large amount of genetic variation. Here, we show that five cycles of freezing and thawing result in very clear changes to both fitness and genetic variation in replicated, recombinant yeast populations. Our results suggest that care should be taken to minimize the evolutionary consequences of cryopreservation in initially recombinant microbial populations.

Introduction

Experimental evolution can be generally defined as research that involves exposing replicated populations to a novel environment for many generations and observing how those populations evolve in real time (Garland and Rose 2009). This approach allows investigators to directly test hypotheses about evolutionary processes in the laboratory. When these experiments involve microbes, it is common practice to regularly archive samples of replicate populations by storing them at –80°C in media supplemented with glycerol. This practice adds value to evolution experiments by providing reserves of cells that can be revived for future use. Reasons for reviving archived cells might include recovery following an experimental error, assaying particular biochemical or physiological phenotypes, or intentionally restarting an evolutionary trajectory from a given timepoint to assess how repeatable an experimental evolutionary outcome might be (Kawecki et al. 2012). Essentially, cryopreservation in microbial experimental evolution allows researchers to produce a living "fossil" record over the course of a population's evolutionary history.

Such a record may not be perfect; as with any fossil record, the archives of an evolution experiment may be subject to preservation biases (Efremov 1940, Behrensmeyer et al. 2000). While the duration of freezing may not affect the survival of microbial cells, freeze-thaw events are known to impose reductions in viability (e.g. Gao and Critser 2000). Glycerol is one of the most widely used cryoprotectants in microbial research; it functions by lowering the freezing temperature and reducing osmotic shock to cells during freezing (e.g. Mazur 1984). While the use of glycerol for cryopreservation is widespread, it has reported toxic effects including oxidative stress, DNA damage, and alterations to gene expression (reviewed by Fahy 1986). *E. coli* strains with different genetic backgrounds have been observed to survive the process of

freezing and thawing at different rates (Sleight et al. 2006, 2008; Sleight and Lenski 2007), and natural isolates of *S. cerevisiae* have also been shown to exhibit different viabilities following glycerol exposure (Warringer et al. 2011) and freeze-thaw stress (Kvitek et al. 2008). Thus, several lines of evidence suggest that cryopreservation – the combination of glycerol exposure and freezing/thawing - may exert selective pressures on microbial populations.

Sprouffske et al. (2016) subjected three strains of *Escherichia coli* to a single freeze-thaw event across a range of glycerol concentrations and freezing methods. Not only did the strains display differences in viability across glycerol concentrations, but this single freeze-thaw event evidently resulted in the loss of rare alleles in experimental populations. The authors concluded that cryopreservation alone may influence a population's phenotypes and genotypes, and they attributed these changes to natural selection rather than genetic drift. This demonstration of the potential genomic consequences of cryopreservation is especially relevant to experimental evolution projects involving populations that harbor genetic variation. In most cases, microbial evolution experiments are initiated from single clones with little to no genetic diversity, and evolution proceeds via the sequential fixation of *de novo* beneficial mutations. Such mutations are expected to rapidly increase in frequency in a population under selection (e.g. Lang et al. 2013), so at any given timepoint few rare alleles should exist. Thus, the impacts of cryopreservation may be small if only rare alleles at frequencies < 1% are threatened (as observed by Sprouffske et al. 2016). By contrast, in populations with more genetic diversity, allele frequencies may be vulnerable to perturbations due to cryopreservation. While there are few studies of the effects of freezing on heterogeneous or genetically diverse microbial populations, Turner et al. (1996) provide a relevant example; two *E. coli* strains were mixed at different initial frequencies prior to freezing and thawing. One strain increased in frequency in

all 15 trials regardless of initial frequency, suggesting that freezing and thawing alone can change the relative frequencies of competing strains.

Here we aim to evaluate the consequences of freezing and thawing at both phenotypic and genomic levels, using *S. cerevisiae* populations that either harbor a great deal of genetic diversity or none. We assay fitness and genetic variation in five different yeast populations (one recombinant population and 4 isogenic strains) across five consecutive cycles of freezing and thawing. Glycerol is typically used at concentrations of 2-55% (Hubálek 2003) to supplement liquid cultures of the yeast *Saccharomyces cerevisiae* at -80°C for cryopreservation, and a popular laboratory manual recommends routinely archiving yeast at final concentrations of 15-20% glycerol (Dunham et al. 2015). Thus, we also implement two glycerol conditions, final concentrations of either 15% or 25% glycerol, in our experiments, and assess the effects of acute glycerol toxicity on cell viability. We analyze genome-wide sequence data from all populations collected initially, after one freeze-thaw, and after five freeze-thaws to evaluate changes in the frequencies of either pre-existing variants or *de novo* mutations over time.

Materials and Methods

Yeast maintenance and cryopreservation selection experiment

The focal yeast population in this experiment, which is called "SGRP-4X", is a highly recombinant diploid population useful for QTL mapping (Cubillos et al. 2013). The SGRP-4X was constructed by crossing four stable heterothallic haploid strains: a European wine strain (DBVPG6765); a West African palm wine strain (DBVPG6044); a North American oak tree isolate (YPS128); and a Japanese sake strain (Y12). All of these strains belong to the Saccharomyces Genome Resequencing Panel (SGRP; Liti et al. 2009). Both the SGRP-4X and

the four isogenic "founder" strains in were used as base populations in our evolution experiments. The SGRP-4X population was originally acquired from G. Liti in January 2011, grown overnight in liquid culture, diluted to a final concentration of 15% glycerol, and frozen at -80°C until it was revived for this project in March 2017. The isogenic founder strains were obtained as haploids from the NCYC collection (SGRP Strain Set 2); crosses between *MAT* A and *MAT* α haploids produced diploids for each strain, and single diploid colonies were isolated, grown up in YPD liquid culture overnight, diluted to a final concentration of 15% glycerol, and frozen at -80°C until they were revived for this project in March 2017.

Experimental populations were first established as follows. 600 µL of a frozen archive of the SGRP-4X population was aliquoted onto a YPD agar plate to create a lawn that was allowed 48 hours of recovery growth. A wooden applicator was used to collect cells in a cross-hatch pattern that covered the entire lawn so that the substantial genetic diversity in this population was sufficiently sampled; these cells were then used to inoculate liquid YPD for overnight culture. Freezer stocks of the four diploid isogenic founder strains were sampled with wooden applicators and struck for single colonies on YPD agar plates twice before a single colony was chosen as the ancestor for this experiment and cultured in liquid YPD overnight. From these five overnight liquid cultures, 500 µl of cells were aliquoted into 4.5mL of YPD to create a more dilute population of cells, and optical density (OD_{600}) was checked to ensure that cell density was consistent for all experimental populations. This technique was used to found 12 replicate populations of SGRP-4X and 3 replicate populations of each isogenic strain for each glycerol condition. One milliliter of each diluted population was added to 1mL of either 50% glycerol or 30% glycerol, forming pre-freeze cultures of 25% and 15% final glycerol concentrations, respectively.

Serial dilutions were performed on these pre-freeze cultures for the fitness assays. One hundred microliters of the appropriate dilutions were plated in triplicate on YPD agar plates, allowed to grow at 30°C for 48 hours, and then the number of colonies were counted. After the removal of liquid required for the serial dilutions, the cultures were placed in a -80°C freezer for 48 hours. Previous studies show that there is an optimal cooling rate for the survival of eukaryotic cells (Gao and Critser 2000), though slow cooling by placing samples directly in the freezer is likely the easiest and most preferred method of freezing for microbes such as *S. cerevisiae*.

After 48 hours in the freezer, samples were then removed to initiate the next freeze-thaw cycle. Post-freeze cultures were allowed to thaw completely at room temperature (approximately 12-14 minutes to fully thaw) before the serial dilution step was repeated as before, allowing direct pre- and post-freeze fitness comparisons of cell viability as measured via colony counting. Viability was calculated as the ratio $N_{final}/N_{initial}$, where N_{final} is the average number of colonies per plate per dilution post-freeze, and $N_{initial}$ is the average number of colonies per plate per dilution pre-freeze. Revival lawns were created by aliquoting 500µL of post-freeze cultures on YPD agar plates and were allowed a 48-hour recovery and growth period before being sampled with a wooden applicator to inoculate 10mL liquid YPD for overnight growth. This cycle of freezing and thawing was repeated a total of five times before archiving all samples during the sixth freeze (Figure S1). In order to assess whether cell viability increased over time in the 12 replicate recombinant populations, we carried out ANOVAs on arcsine square root transformed viabilities treating "freeze-thaw cycle" as a continuous regressor. We used this same ANOVA approach on transformed viabilities of the initially isogenic lines over time. Finally, we carried out Kruskal-Wallis tests on viabilities of the founder strains after one freeze-thaw to determine

whether there might be initial strain-specific differences in freeze-thaw viability, for both glycerol concentrations used.

We estimate that ~100 generations have passed over the course of five freeze-thaw cycles. While it is difficult to precisely pinpoint the number of cell doublings that occurred over the course of the experiment, we can use average colony counts collected during fitness assays to provide some ball-park estimates. For example, the number of cells transferred from cryotubes to agar plates was consistently around 5 million cells. The number of cells recovered from agar lawns after 48 hours of growth was consistently between 1-10 billion cells. It follows that the number of doublings that likely occurred during this phase of noncompetitive growth on agar plates ranged between 7.64 doublings (given a minimum of 1 billion cells recovered) and 10.96 doublings (given a maximum of 10 billion cells recovered). Only a small fraction of all the cells that grew on these agar plates were transferred to 10mL of liquid YPD media for the next phase of growth, and we estimate that the number of cells transferred (via wooden applicator) ranged from 1-10 million cells. We can also use colony counts from fitness assays to extrapolate the approximate number of cells present in that 10mL of YPD after 24 hours of growth, and this was consistently ~2 billion cells. It follows that the number of doublings that likely occurred during this period of 24 hours of growth in liquid media is 7.64 on the low end (in the case of 1 million cells transferred from the plate) and 10.96 on the high end (in the case of 10 million cells transferred from the plate. Therefore, over the 5 weeks of the experiment we suggest that the number of generations ranged between 75-110 (~7.5 doublings * 2 phases of growth * 5 weeks = 75 generations on the low end and ~11 doublings * 2 phases of growth * 5 weeks = 110 generations on the high end). Of course the number of generations varied among replicate populations, and over the weeks of the experiment, but given that such variation was always less

than one order of magnitude we think it appropriate to use global means to generate these ballpark estimates. We also note that ancestral populations were maintained as lawns on YPD agar plates kept at room temperature and re-plated weekly until all fitness assays were concluded (to avoid repeatedly thawing archival stocks).

Glycerol toxicity assay

In order to distinguish between stresses induced by glycerol toxicity versus the freezing and thawing process, we evaluated how each of the populations that had endured 5 freeze-thaw cycles ("evolved" populations) responded to acute glycerol exposure. Each of these 24 evolved populations (12 evolved in 15% glycerol and 12 evolved in 25% glycerol) were compared with the SGRP-4X ancestor to determine relative fitness when exposed to acute glycerol stress. In this assay, we employed three "treatments": (i) the ancestor, (ii) populations evolved for 5 freeze-thaw cycles in 15% glycerol, and (iii) populations evolved for 5 freeze-thaw cycles in 25% glycerol. Each treatment was assayed in 12 replicates; each of the 12 experimentally-evolved replicates per glycerol condition was assayed, and the ancestor was sampled 12 times independently for a balanced design. Each replicate was assayed as 5 individual overnight cultures.

Glycerol toxicity assays utilized cultures that were standardized to an OD_{600} of 0.1 in 10mL of YPD media, then grown for 24hrs in a 30°C shaking incubator. For each assayed population, nine total cultures were started, and upon removal from the incubator the five with 10^{-2} diluted OD_{600} readings closest to a target value of 0.3 were selected for glycerol testing. This was done to ensure synchronized cell stages across replicates, as close to the transition from log phase to stationary phase as possible. In pilot work we observed substantial mortality in the

ancestral populations at this cell stage when exposed to high (45%) glycerol concentrations (data not shown). For the glycerol stress, 1mL of each culture from a selected population was placed in either 1mL of sterile water or 1mL of sterile 90% glycerol (for a total glycerol concentration of 45%). These test cultures were vortexed thoroughly before being allowed to sit on the bench for 20 minutes of exposure time. Following this exposure period, test cultures were vortexed again before being serially diluted to a 10^{-5} concentration. One hundred microliters of the 10^{-5} diluted test cultures were spread on each of YPD agar plates and placed in a 30°C plate incubator for 48hrs. Colonies were counted and averaged across plates, and viability was calculated as the average number of colonies surviving 45% glycerol divided by the average number of control colonies (0% glycerol). To determine whether viabilities differed between the ancestor, populations evolved in 15% glycerol, and populations evolved in 25% glycerol, we ran a Kruskal-Wallis test of viabilities versus "treatment".

DNA extraction and sequencing

DNA was extracted from 1mL of overnight liquid cultures in YPD (~10^9 diploid cells) as the experiment progressed, beginning with the ancestral generation before any planned freeze-thaws were imposed. Extractions were performed using the Qiagen Gentra Puregene Yeast/Bacteria kit to collect DNA from entire mixed populations at once. Once extracted, DNA was standardized to a quantification of between 6.25 ng/μL and 10 ng/μL using a Qubit 3.0 Flourometer. Sequencing libraries were constructed using the Nextera Library Preparation Kit (Illumina) for 41 populations: the ancestral SGRP-4X population prior to any intentional freeze-thaws; all 12 SGRP-4X derived populations after a single freeze-thaw in 15% glycerol; all 12 SGRP-4X derived populations after five freeze-thaws in 15% glycerol; the four founder strains

prior to any intentional freeze-thaws; and 12 replicates of the founder-derived strains after five freeze-thaws in 15% glycerol (3 replicates x 4 founders). Some modifications to the Nextera protocol were imposed to save costs (details of library prep available upon request).

Libraries were combined in equal molarities and run on three SE100 lanes of a HiSeq3000 instrument at the OSU Center for Genomic Research and Biocomputing. Populations derived from the SGRP-4X were run on 2 lanes to obtain high coverage, and all populations derived from the isogenic founders were run on a single lane. The Burke lab has developed a processing pipeline for estimating allele frequencies in each population directly from pooled sequence data. GATK v4.0 (McKenna et al. 2010; Poplin et al. 2018) was used to align raw data to the current *S.cerevisiae* S288C reference genome (R64-2-1) and create a single VCF file for all variants identified across all replicate populations. We also downloaded and indexed SNP information for a number of distinct natural isolates of *S. cerevisiae* (VCF file from Bergström et al. 2014); this is a recommended best practice for calibrating base quality with GATK v4.0. This VCF file was converted into a SNP frequency table with a custom python script (available upon request) suitable for downstream analysis with R. SnpEff v.4.3T (Cingolani et al. 2012) was used to extract predicted effects of individual SNPs. Only SNP positions with >10X coverage in all populations were used in subsequent analyses.

To assess variation in coverage in individual populations, we calculated the coefficient of variation (standard deviation in coverage divided by mean coverage); this metric has been used to assess coverage uniformity within a sample (Feng et al. 2015). A value less than one indicates low variance and uniform coverage across the genome (i.e. little sequencing bias), while values greater than one indicate high variance and skewed coverage distributions. We also looked more directly at patterns of coverage variation across the genome as a means of identifying potential

structural variations in our populations (i.e. regions of elevated coverage suggestive of duplications, regions of reduced coverage suggestive of deletions, etc.). Specifically, we looked at how normalized coverage, defined as site-specific coverage divided by the genome-wide mean coverage averaged across 10kb windows, varied across the genome to screen for regions where values diverge from one.

Identifying allele frequency changes over time

Following the approach of Burke et al. (2014), we used two strategies for identifying changes in SNP frequencies over time: i) a genome scan for standing genetic variants that changed dramatically from the start to the end of the experiment, as well as consistently across replicate populations; and ii) a genome scan for *de novo* mutations that might have emerged after the experiment began. The first approach only applies to the initially recombinant populations, as no standing variation exists in the initially isogenic populations. Thus, in the 12 recombinant populations we carried out linear regression on individual arcsine square root transformed allele frequencies, treating "cycle" as a continuous regressor (*c.f.* Burke et al. 2014). This model was weighted by the square root of the population allele count, or coverage, per position. The $-\log_{10}$ transformed p-values returned for individual SNPs are very similar to logarithm of the odds (LOD) scores (Broman and Sen 2009), a common metric in genome-wide association studies. To establish significance thresholds for our regression analysis, we first generated null distributions for these scores using the permutation approach featured in Burke et al. (2014). Briefly, we randomly shuffled population identifiers (replicate number and freeze-thaw cycle) and created 1000 dummy datasets. As in Burke et al. (2014), we chose not to shuffle the population identifiers for the ancestor (freeze-thaw cycle "0") as it was shared among all populations. Next,

we performed our regression analysis in each of the 1000 permuted datasets as was done with the real data. We then recorded the smallest p-value generated from the entire genome scan from each permuted dataset, to generate a null distribution against which the observed data can be evaluated.

To identify potential *de novo* mutations in the recombinant populations, we identified SNPs that were fixed in the ancestor, but had reached high (>0.20) frequency in at least one replicate from freeze-thaw cycle 5. We further curated this list by selecting only SNPs that were private to single replicate populations; any SNP at high frequency shared by more than one replicate population likely was present at in the ancestor but missed by our sequencing. For initially isogenic strains, we took the same approach; we searched for SNPs that were fixed in the respective ancestor but exceeded a frequency of 0.20 in at least one cycle 5 replicate. We again selected SNPs that were private to a single evolved replicate for each isogenic strain, narrowing the dataset to those SNPs that most likely represent true *de novo* mutations.

Estimating haplotype frequencies

As we collected sequence data from the four ancestral diploid founder strains, we can estimate which founder allele(s) introduced a SNP into the SGRP-4X ancestor. As most SNPs in this recombinant population are private to a single founder, most SNPs are expected to occur at frequencies of either ~0.25 or ~0.75 with respect to the S288C reference genome. For any small region of the genome we can integrate this information to estimate the most likely set of founder haplotype frequencies that explains the vector of observed SNP frequencies in an evolved population. Following the precedent and methodology of Burke et al. (2014), we evaluated 10kb windows of the genome with a 2kb step size; at focal positions every 2kb across the genome we

used the set of SNPs within 5 kb of either side to determine the most likely set of haplotype frequencies that would produce the observed set of SNP frequencies.

Results

Viability assays over the course of cryopreservation selection

Linear regressions of viability trajectories reveal a significant increase in viability over time for the SGRP-4X in both 15% glycerol ($p = 2.2\text{x}10^{-6}$) and 25% glycerol ($p = 3.44\text{x}10^{-5}$, Figure 1). Shapiro-Wilk tests show that residual errors are normally distributed for these regressions (p=0.146 for 15% glycerol data and p=0.057 for 25% glycerol data), validating the appropriateness of arcsin-root transformation of viability estimates. We did observe some evidence of heteroscedasticity for the 15% glycerol data (Breusch-Pagan test p=0.03414), though this appears to be driven by a single outlier observation; population replicate 1 exhibited a low viability during cycle 5. Removing this outlier replicate from the analysis also removes the heteroscedasticity, so we are inclined to retain the regression approach as a framework for interpreting the time-series data. The isogenic founders displayed differences in initial viability between strains; a Kruskal-Wallis test of the cycle 1 freeze-thaw viabilities suggests a significant effect of "founder" ($p = 1.89\text{ x}10^{-3}$), but no significant effect of glycerol concentration (p = 0.4865) with the European wine strain exhibiting the lowest viability in both (Figure 2). When transformed viabilities were regressed against all 5 freeze-thaw cycles, no significant increases were observed over time in any founder, for either glycerol condition (Figure S2). The ANOVA yielding the smallest p-value was for the West African founder in 25% glycerol (p=0.0811), but interestingly the viabilities decrease rather than increase (Figure S2). All regressions of

transformed viabilities from initially isogenic strains over time exhibited normal distributions of residuals and homoscedasticity.

Glycerol toxicity assays

We found no evidence to support the hypothesis that the evolved populations had an increased ability to tolerate acute glycerol stress compared to the ancestor SGRP-4X (Figure S3). In fact, a Kruskal-Wallis test yielded a significant effect of "treatment" on viability ($p = 9.538 \times 10^{-8}$), but this effect is driven by the observation that the populations evolved in 15% glycerol exhibited *lower* viabilities than the ancestor (populations evolved in 25% glycerol performed comparably to the ancestor). Thus, the observed increases in viability following subsequent freeze-thaw stresses are unlikely to be rooted in an increased ability to withstand glycerol toxicity.

Sequence coverage assessment

We identified a total of 113,773 bi-allelic SNPs segregating in the dataset. Prior to any sort of filtering, mean SNP coverage in the raw dataset was ~60X and mean coverage per population ranged from ~28X to ~106X (with only a single sample having less than 30X; Table S1). The coefficient of variation was less than 1 for all populations, which indicates a low variance in genome-wide coverage within each population (Table S1). As a means of detecting evidence of structural variants (e.g. duplications, deletions, etc.) we examined normalized coverage across the genome averaged over 10kb windows to see if any regions were dramatically different than the genome-wide mean coverage. We found one such region on chromosome 12, which is a single 10kb window containing 9 high-coverage SNPs between positions 460,103-

460,515. Normalized coverage in this particular region was > 3 in all populations, compared to values much closer to 1 in the rest of the genome. This region was ultimately found to overlap with NTS1-2, a non-transcribed region of the rDNA repeat located between 3' ETS and RDN5. As such, the abnormally high coverage found here is likely due to the highly repetitive nature of this sequence and suggestive of some sort of structural variant in our populations. We did not observe changes in normalized coverage over time in any population.

Allele frequency changes over time

To identify standing variants that may have changed in frequency over the course of the experiment due to selection, we screened for SNPs that: i) occurred at a non-zero frequency in the SGRP-4X ancestor, and ii) changed consistently in all 12 replicate populations over time. After filtering out low-coverage sites (sites <10X in any population), this left 90,304 high-quality SNPs to evaluate. We performed linear regressions on arcsine square root transformed SNP frequencies from the ancestor, cycle 1 and cycle 5, which revealed a ~43kb region of significant allele frequency change on chromosome 15, containing 25 genes (Figure 3). The peak exceeds a significance threshold of $-\log_{10}(p)=8.622$, which corresponds to the most significant minimum value observed in any of the 1000 permuted datasets. Put another way, this threshold can be viewed as an empirically-determined genome-wide alpha of 0.001, since it is the value observed in the single most extreme null dataset of the group of 1000. The most significant SNP in this region is a synonymous mutation (Figure 4) that lies within the *ALR1* gene, which is involved in magnesium transport across the cell membrane (MacDiarmid and Gardner 1998, Graschopf et al. 2001). Five of the significant alleles in this region are predicted to lead to amino acid sequence changes, including one that causes a premature stop codon, (Table S2); these are perhaps the

most promising candidates for quantitative trait nucleotides (QTN) that might underlie the observed phenotypic increases in freeze-thaw viability. The allele frequency changes in this region were not accompanied by any significant haplotype frequency changes, though the SNPs in question largely belonged to the YPS128 founder haplotype (Figure 4).

Examining haplotype frequency changes in the recombinant populations over time may provide more power to detect genome-level changes than examining SNP frequencies alone (Burke et al. 2014a & b). However, while average haplotype frequencies across the n = 12 recombinant replicate populations implicate the same region on chromosome 15 as the SNP regression analysis, haplotype frequencies did not reveal obvious additional candidate regions (Figure S4). To visualize haplotype frequency changes directly in individual replicate populations, versus averaging across all 12, we generated correlation plots by plotting cycle 5 haplotype frequencies against the ancestral haplotype frequencies for each individual population/founder haplotype (Figures S5-S8). While these revealed a number of outlier points in the genome where haplotype blocks changed in individual populations, generally these outliers involve only single windows, and are therefore weak candidates. Strong candidate regions, such as the one on chromosome 15, will involve many linked windows; this pattern is evident in the correlation plot for the North American YPS128 haplotype (Figure S5). Notably, this reveals interesting heterogeneity among replicate populations, as we observe much higher haplotype frequency changes in 6 replicates compared to the other 6 (Figure S5).

We identified only 5 potential *de novo* beneficial mutations that went from a frequency of zero in the SGRP-4X ancestor to >0.20 in a single replicate population replicate by freeze-thaw cycle 5 (Table S3). One of these, observed in replicate population 9, is predicted to have a high impact as it leads to a premature stop codon in the gene PCA1. In the initially isogenic founders,

after filtering out sites with low (<10X) coverage in any population, we observed substantial variation in the number of potential *de novo* mutations identified per strain. We found 21 potential *de novo* mutations in the West African founder, and 3 potential *de novo* mutations in each of the other three strains (Table S4). In general, we did not observe overlap among the putative mutations, or even regions containing them, across founders. However, we find candidate mutations impacting the gene FLO9 in an evolved replicate derived from the West African replicates, and a candidate mutation impacting the gene FLO1 in an evolved replicate derived from the North American oak strain – both of these genes belong to the same family and are involved in flocculation (Table S4).

Discussion

We report evidence that cryopreservation has significant effects on initially recombinant populations at both phenotypic and genomic levels, but we did not observe this phenomenon in initially isogenic strains. Recombinant populations displayed increased abilities to survive a freeze-thaw cycle over the course of 5 cycles, while isogenic lines showed no notable differences in viability under the same conditions. The magnitude of these viability changes in recombinant populations is surprising, given that we conservatively estimate only ~100 generations to have elapsed over the course of these five freeze-thaw cycles. We chose to replicate the experiment in two commonly used final glycerol concentrations, 15% and 25%, to evaluate potential phenotypic consequences between those concentrations, as glycerol toxicity might impact cell viability (Fahy 1986, Warringer et al. 2011, Sprouffske 2016). We observed similar consequences on cell viability in both glycerol treatments with respect to ability to survive a freeze-thaw stress.

To test the hypothesis that the observed viability increases might be attributable to selection for increased tolerance to glycerol rather than increased tolerance to freezing/thawing, we assayed both evolved and ancestral populations in high concentrations of glycerol. We find no evidence to support this hypothesis; in fact, the ancestral population generally exhibited higher viability following exposure to 45% glycerol than any of the replicate populations that were repeatedly frozen. We generally interpret this as evidence that the viability increases we saw following repeated freeze-thaws are indeed a response to the specific selective pressure of freezing/thawing, rather than an evolved response to glycerol toxicity.

Historically, experimental evolution using initially isogenic yeast populations has allowed us to address a wide variety of major evolutionary questions (reviewed by Fisher and Lang 2016). A smaller but important category of evolution experiments have tested questions using populations harboring standing genetic variation (e.g. Cubillos et al. 2013, Burke et al. 2014b, McDonald et al. 2016, Kosheleva and Desai 2018) to model eukaryotic evolution and address questions about how evolution proceeds in the presence of standing genetic variation and sex. McDonald et al. (2016) find that recombination makes natural selection more efficient at fixing beneficial mutations and purging deleterious hitchhikers. While this experiment does not involve mating or sex, we expected similar results among recombinant populations, since these underwent several rounds of intercrossing before this cryopreservation experiment to effectively break up large blocks of linked haplotypes (Cubillos et al. 2013). Thus, we expected – and observed – larger phenotypic shifts in response to repeated freeze-thaw stress due to the potential for selection to act on variants in that have been decoupled from deleterious hitchhikers. By contrast, we expected the initially isogenic strains to evolve solely by the accumulation of new mutations or structural rearrangements in the genome due to the lack of initial genetic variation

for selection to act on. Given the relatively small number of generations that passed here, we did not necessarily expect to observe dramatic phenotypic differences in resistance to freeze-thaw stress in the isogenic lines, and indeed we did not observe this.

Because we found no dramatic fitness differences when populations were cryopreserved in 15% vs. 25% glycerol, we carried out population-level whole-genome sequencing (Pool-SEQ) in evolved populations that experienced just one of these conditions – repeated freezing/thawing in 15% glycerol. Pool-SEQ data from initially isogenic lines revealed some potential genetic consequences of freeze-thaw cycles. Examining SNP frequency changes over time revealed a number of putative *de novo* SNPs in each of the isogenic lines (21 in DBVPG6044, 3 in YPS128, 3 in Y12, and 3 in DBVPG6765; Table S4). However, given that viability did not meaningfully change over repeated freeze-thaw cycles, we argue that these putative mutations likely have few or no phenotypic consequences related to freezing and thawing. Notably, we observed evidence of potential *de novo* beneficial mutations in two different flocculation genes in evolved populations from different genetic backgrounds: FLO9 in DBVP6044-derived replicate 3, and FLO1 in YPS128-derived replicate 3 (Table S4). Yeast flocculation, or cell aggregation, is an important mechanism of stress resistance and known to evolve in response to long-term laboratory experiments (e.g. Hope et al. 2017). Thus it is possible that one or both of these mutations promotes flocculation, and therefore general stress resistance, in the replicates bearing them; however, the absence of phenotypic changes observed in any initially isogenic line makes this speculation rather lackluster.

Examining data from the recombinant populations, we found 5 examples of potential *de novo* beneficial SNPs that might underlie our observed viability increases; these alleles were fixed in the recombinant ancestor but present at intermediate frequency in an evolved population.

All of these are in gene coding regions on chromosome 2 (in gene PCA1) or chromosome 4 (in genes SED1 and NUM1). Each of these mutations is specific to one of the 12 replicate populations and reached a frequency >0.20 over the course of the experiment. Most of these are predicted to have low or moderate impact (see Table S3), but one T -> A mutation at position 793934 on chromosome 2 results in a premature stop codon, suggesting a high potential impact on gene function for PCA1. PCA1 encodes a cadmium transporter, and expression of this gene has been associated with a fitness trade-off in natural *S. cerevisiae* populations (Chang and Leu 2011). Thus it is interesting to speculate that this (presumably) loss-of-function mutation might confer higher fitness in the low-cadmium environment of laboratory culture. On the other hand, previous research suggests that *de novo* mutations play a small role in the first several hundred generations of evolution when standing variation is present (Burke et al. 2014b) and given the relatively few generations we estimate to have elapsed during this study, it is unlikely that these putative *de novo* SNPs drive the large-scale phenotypic changes we observed. Since these SNPs were unique to individual replicate populations, no single one can be responsible for the dramatic shift in viability under freeze-thaw stress that we observed in all twelve replicate populations. On the other hand, examining SNP frequency changes over time in the recombinant lines provides compelling evidence of dramatic change at a region on chromosome 15 (Figures 3 & 4). Because the alleles in this region consistently changed in the same direction in all twelve replicate populations, we invoke selection rather than drift as the driving evolutionary force; this region on chromosome 15 may be involved in a freeze-thaw-resistance phenotype. The most significant SNP in this region lies within the *ALR1* gene, which is involved in magnesium transport across the cell membrane (MacDiarmid and Gardner 1998, Graschopf et al. 2001).

We also examined sequence coverage for evidence of structural variation within and between populations. Because coverage remained consistent within genomes, and over time, we report no evidence of large-scale deletions or duplications in our populations. At first glance this may seem surprising, given that aneuploidy is a common outcome of evolution experiments utilizing yeast as a model (Gerstein et al. 2006, Hong and Gresham 2014, Voordeckers et al. 2015, Fisher et al. 2018). These studies reveal whole-genome duplications in evolved lines relative to initially haploid ancestral strains. Gerstein et al. (2006) also showed that tetraploid cells lost genetic material. Together, these results suggest a tendency for *S. cerevisiae* to evolve towards diploidy in laboratory experiments. As our populations – both initially isogenic and recombinant – were diploid as ancestral populations, the lack of large-scale structural changes is consistent with previous work in yeast experimental evolution.

Finally, we examined haplotype frequencies for the recombinant lines across all 12 replicate populations. Haplotype frequency changes can sometimes allow for better detection of candidate regions than tracking individual SNP frequency change (Burke et al 2014a; 2014b), but in this experiment we did not find this to be true. On the other hand, haplotype-level analysis reveals that the region of significantly diverged SNPs belong to the North American oak strain haplotype (YPS128), which is notable because a previous study showed that strains collected from oak soil are highly resistant to freeze-thaw stress, and suggest that this might be important for surviving harsh winters (Kvitek at al. 2008). Notably, YPS128 is the only founder strain of the four we used to be isolated from a natural, as opposed to commercial fermentation environment.

While it is possible that the significant region on chromosome 15 might directly drive the large phenotypic consequences observed here, assays such as RNA-seq or allele replacement

experiments would be necessary to fully validate any potentially functional consequences of the variants in this region, and such assays are beyond the scope of this study. Allele-swapping experiments are not especially straightforward in recombinant populations such as the ones in this study; the results of such assays could be confounded by differences in the genetic backgrounds among the millions of segregating haplotypes in each population and epistatic interactions have been shown to play important roles in evolutionary trajectories (McDonald et al. 2016).

This study resembles in many aspects the work presented by Sprouffske et al. (2016), who used *E. coli* to test the prediction that the freeze/thaw of a glycerol stock might alter genomic diversity. They found no evidence for fitness or genomic changes in isogenic *E. coli* stocks after a freeze-thaw, and our results with initially isogenic *S. cerevisiae* are consistent with this. However, using a stock of hypermutable *E. coli*, Sprouffske et al. (2016) demonstrated that when standing variation is present, a freeze-thaw can result in the loss of alleles that occur at very low frequencies. Notably, in a hypermutable strain of bacteria, one expects to observe many polymorphic alleles, but each should be at low frequency. The authors attribute this loss of rare alleles to selection, based on statistical resampling of their initial allele frequencies to model expected outcomes in drift-based scenarios, and as a result cautioned against using thawed populations in future experiments. Here, we work with a recombinant *S. cerevisiae* population with a large number of common alleles (99.5% of polymorphic alleles in the SGRP4X ancestor occur at frequencies between 0.05-0.95), therefore we do not face the same complication of having large numbers of rare alleles in our experimental populations. And yet, we observe that in this population, common variants can change dramatically in frequency after a small number of freeze-thaw events. We also observe a large difference in cell viability between populations

that had experienced no freeze-thaw events versus those that had experienced several. Thus, we advise experimental evolution researchers using genetically diverse populations to practice special caution when creating a frozen archive. We interpret our results as evidence that repeated freezing and thawing exerts selective pressure that may complicate future assays of allelic diversity, fitness, or other phenotypes. The very strong signal we observed at only a single locus (ALR1) suggests there may be value to "domesticating" recombinant laboratory populations to several freeze-thaw cycles prior to initiating an evolution experiment. While generally considered best practice, extra care should be taken to highly replicate population archives, such that any one cryotube is never completely thawed more than once. Notably, we do not observe the same potential impact with isogenic strains, and therefore argue that the impacts of cryopreservation on experiments involving populations with little or no standing genetic variation are likely small. On the other hand, there are many aspects of cryopreservation bias that we have left unexamined. For example, it is widely assumed that the duration of freezing has few, if any, biological consequences for microbial samples. Given that investigators rely on archival populations that have been in the freezer for years, or even decades, such assumptions are worth scrutinizing within the experimental evolution context.

References

Behrensmeyer AK, Kidwell SM, Gastaldo RA. 2000. Taphonomy and paleobiology. Paleobiology 26:103–147.

Bergström A, Simpson JT, Salinas F, Barré B, Parts L, Zia A, Nguyen Ba AN, Moses AM, Louis EJ, Mustonen V, Warringer J, Durbin R, Liti G. 2014. A high-definition view of functional variation from natural yeast genomes. Molecular Biology and Evolution 31(4):872-888.

Burke MK, King EG, Shahrestani P, Rose MR, Long AD. 2014. Genome-wide association study of extreme longevity in *Drosophila melanogaster*. Genome Biology and Evolution 6:1-11.

Burke MK, Liti G, Long AD. 2014. Standing genetic variation drives repeatable experimental evolution in outcrossing populations of *Saccharomyces cerevisiae*. Molecular Biology and Evolution, 31(12):3228-3239.

Chang SL and Leu JY. 2011. A tradeoff drives the evolution of reduced metal resistance in natural populations of yeast. PLoS Genetics, 7(3) e1002034.

Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-3. Fly 6:80-92.

Cubillos FA, Parts L, Salinas F, Bergström,A., Scovacricchi, E, Zia A, Illingworth CJR, Mostonen V, Ibstedt S, Warringer J, Louis, EJ. 2013. High-resolution mapping of complex traits with a four-parent advanced intercross yeast population. Genetics, 195(3):1141-1155.

Dhaoui M, Auchère F, Blaiseau PL, Lesuisse E, Landoulsi A, Camadro JM, Haguenauer-Tsapis R, Belgarah-Touzé N. 2011. Gex1 is a yeast glutathione exchanger that interferes with pH and redox homeostasis. Mol Biol Cell 22(12):20154-67.

Dunham, M, Gartenberg M, Brown G. 2015. Methods in Yeast Genetics and Genomics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Press.

Efremov IA. 1940. Taphonomy: new branch of paleontology. Pan-Am Geol. 74: 81–93.

Fahy GM. 1986. The relevance of cryoprotecant "toxicity" to cryobiology. Cryobiology 23(1): 1-13.

Feng Y, Chen D, Wang GL, Zhang VW, Wong LJ. 2015. Improved molecular diagnosis by the detection of exonic deletions with target gene capture and deep sequencing. *Genet Med* 17:99-107.

Fisher KJ, Buskirk SW, Vignogna RC, Marad DA, Lang GI. 2018. Adaptive genome duplication affects patterns of molecular evolution in *Saccharomyces cerevisiae*. PLOS Genetics 14(5): 1007396

Gao D, Critser JK. 2000. Mechanisms of cryoinjury in living cells. ILAR journal, 41(4): 187-196.

Garland T, Rose MR. 2009. Experimental evolution: concepts, methods, and applications of selection experiments. University of California Press.

Gerstein AC, Chun HE, Grant A, Otto SP. 2006. Genomic convergence toward diploidy in *Saccharomyces cerevisiae*. PLOS Genetics, 2(9):145.

Graschopf A, Stadler JA, Hoellerer MK, Eder S, Sieghardt M, Kohlwein SD, Schweyen RJ, 2001. The yeast plasma membrane protein Alr1 controls Mg^{2+} homeostasis and is subject to Mg^{2+}- dependent control of its synthesis and degradation. Journal of Biological Chemistry 276:16216-16222.

Hong J, Gresham D. 2014. Molecular specificity, convergence and constraint shape adaptive evolution in nutrient-poor environments. PLoS Genetics 10(1):1004041.

Hope EA, Amorosi CJ, Miller AW, Dang K, Smukowski Heil CS, Dunham MJ. 2017. Experimental evolution reveals favored adaptive routes to cell aggregation in yeast. Genetics 206:1153-1167.

Hubálek Z. 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology, 46(3):205-229.

Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC. 2012. Experimental evolution. TREE 27(10):547-560.

Kosheleva K, Desai MM. 2018. Recombination alters the dynamics of adaptation on standing variation in laboratory yeast populations. Molecular Biology and Evolution 35(1):180-201.

Kvitek DJ, Will JL, Gasch AP. 2008. Variations in stress sensitivity and genomic expression in diverse *S. cerevisiae* isolates. PLoS Genetics, 4(10): e1000223

Liti G, ####### Louis EJ. 2009. Population genomics of domestic and wild yeasts. Nature 458(7236):337-341.

MacDiarmid CW, Gardner RC. 1998. Overexpression of the *Saccharomyces cerevisiae* magnesium transport system confers resistance to aluminum ion. Journal of Biological Chemistry 273: 1727-1732.

Mazur P. 1984. Freezing of living cells: mechanisms and implications. American Journal of Physiology-Cell Physiology 247(3):C125-C142.

McDonald, MJ, Rice DP, Desai MM. 2016. Sex speeds adaptation by altering the dynamics of molecular evolution. Nature 531: 233-236.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo M. 2010. The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Research 20:1297-1303.

Poplin R, Ruano-Rubio V, DePristo M, Fennell TJ, Carniero MO, Van der Auwera G, Kling DE, Gauthier LD, Levy-Moonshine A, Roazen D, Shakir K, Thibault J, Chandran S, Whelan C, Lek M, Gabriel S, Daly MJ, Neale B, MacArthur DG, Banks E. 2018. Scaling accurate genetic variant discovery to tens of thousands of samples. bioRxiv (doi**:** https://doi.org/10.1101/201178)

Sleight SC, Wigginton NS, Lenski RE. 2006. Increased susceptibility to repeated freeze-thaw cycles in *Escherichia coli* following long-term evolution in a benign environment. BMC Evolutionary Biology 6(1):104.

Sleight SC, Lenski RE. 2007. Evolutionary adaptation to freeze-thaw-growth cycles in *Escherichia coli.* Physiological and Biochemical Zoology 80(4):370-385.

Sleight SC, Orlic C, Schneider D, Lenski RE. 2008. Genetic basis of evolutionary adaptation by *Escherichia coli* to stressful cycles of freezing, thawing and growth. Genetics 180(1):431-443.

Sprouffske K, Aguilar-Rodríguez J, Wagner A. 2016. How archiving by freezing affects the genome-scale diversity of *Escherichia coli* populations. Genome Biology and Evolution 8(5): 1290-1298.

Turner PE, Souza V, Lenski RE. 1996. Tests of ecological mechanisms promoting the stable coexistence of two bacterial genotypes. Ecology 77(7):2119-2129.

Voordeckers K, Kominek J, Das A, Espinosa-Cantu A, De Maeyer D, Arslan A, Van Pee M, van der Zande E, Meert W, Yang Y, Zhu B, Marchal K, DeLunca A, Van Noort V, Jelier R, Verstrepen KJ. 2015. Adaptation to high ethanol reveals complex evolutionary pathways. PLoS Genetics 11(11):1005635.

Warringer J, Zörgö E, Cubillos FA, Zia A, Gjuvsland A, Simpson JT, Forsmark A, Durbin R, Omholt SW, Louis EJ, Liti G, Moses A, Blomberg A. 2011. Trait variation in yeast is defined by population history. PLoS Genetics 7(6):e1002111.

Figures and Figure Captions

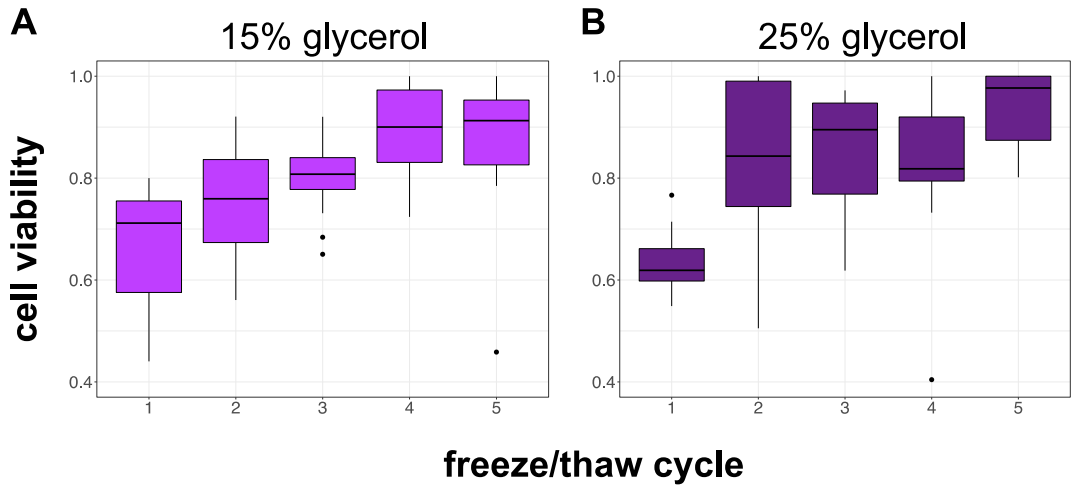

Figure 1. Relative viability of recombinant SGRP-4X-derived populations supplemented with A) 15% and B) 25% glycerol over 5 freeze-thaw cycles. Boxplots represent average viabilities measured in each of 12 replicate populations over 5 consecutive rounds of freezing and thawing. Linear regressions of viability trajectories reveal a significant increase in viability over time for the populations in 15% glycerol ($p = 2.2\text{x}10^{-6}$) and 25% glycerol ($p = 3.44\text{x}10^{-5}$).

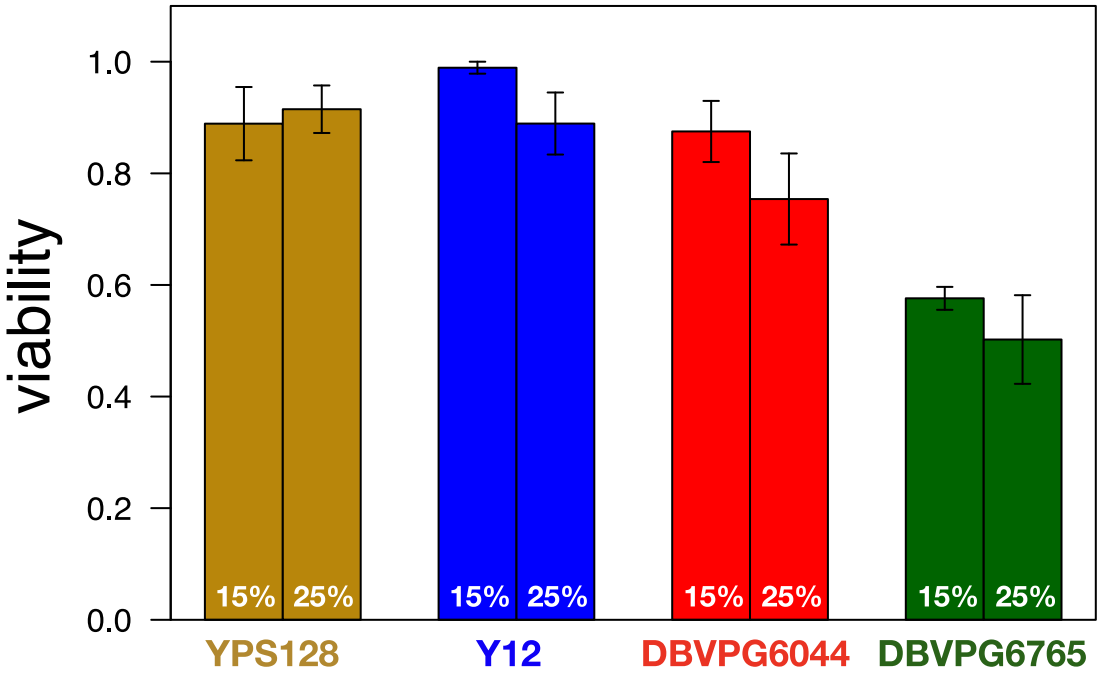

Figure 2. Relative viability of SGRP-4X founder strains supplemented with 15% (left bars) and 25% glycerol (right bars) following a single freeze-thaw event. A Kruskal-Wallis test suggests that strain identity predicts freeze-thaw viability ($p = 1.89 \times 10^{-3}$) with the European wine strain DBVPG6765 exhibiting the lowest viability. Error bars represent SEM of three independent replicates.

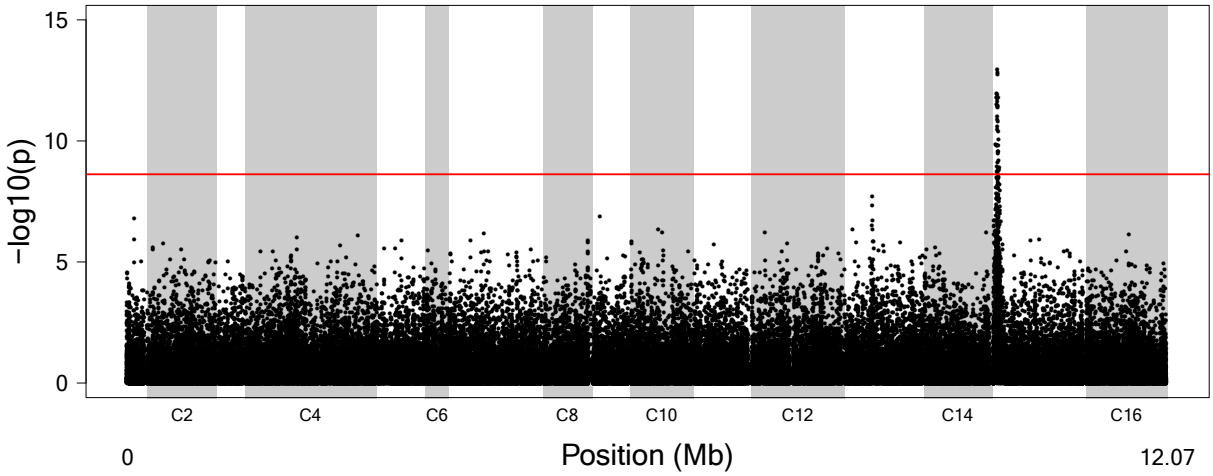

Figure 3. A genome scan for regions of significant allele frequency change in this experiment. Points represent individual -$\log_{10}$(p-values) from linear regressions of SNP frequency changes over time, from the ancestor through freeze-thaw cycle 5. We observe a region of dramatic allele frequency change on chromosome 15 across all replicate populations that exceeds the significance threshold (-$\log_{10}$(p) > 8.622).

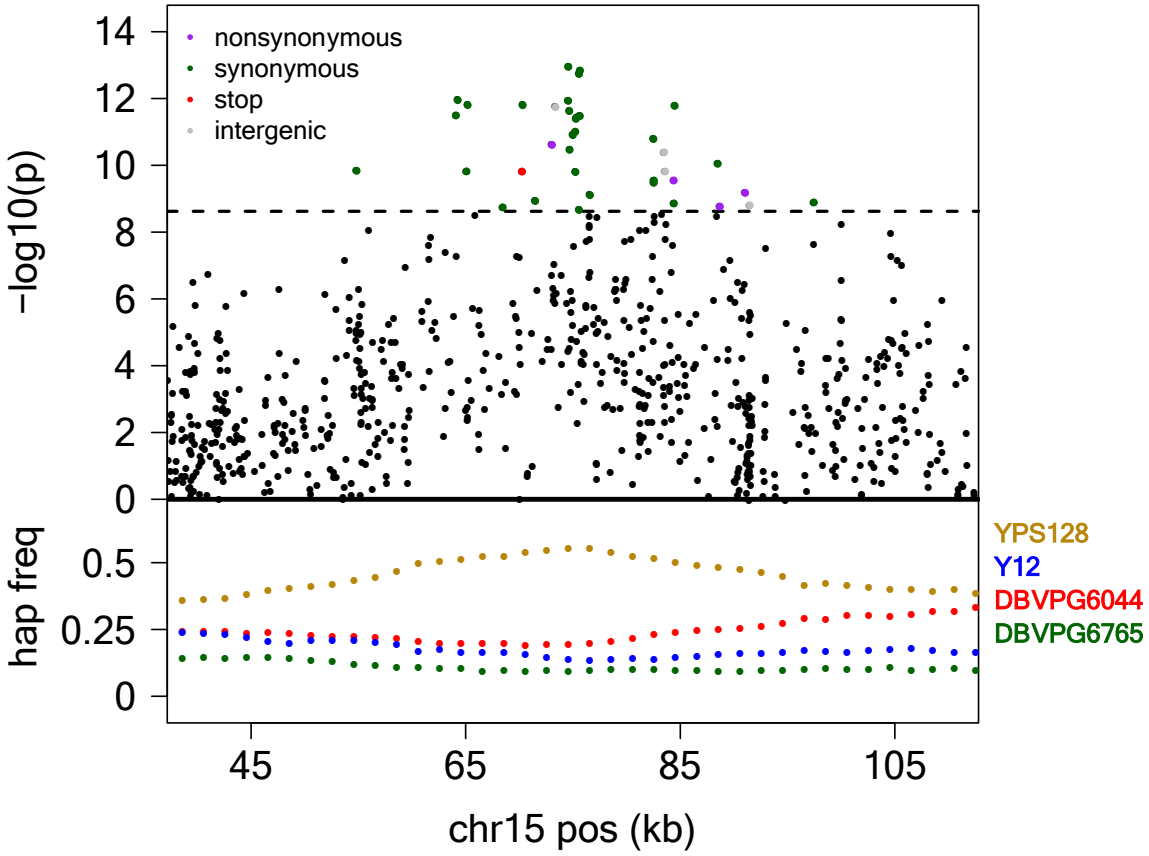

Figure 4. A close-up look at the alleles that changed the most in this experiment, with their associated haplotypes. (Top) Points represent -$\log_{10}$(p-values) for the chromosome 15 peak in recombinant evolved populations. Colors indicate the predicted effect of each SNP exceeding the significance threshold: purple = nonsynonymous, green = synonymous, red = premature stop codon, gray = intergenic. (Bottom) Points represent the estimated haplotype frequencies associated with these SNPs, with each founder haplotype shown in a different color (YPS128 = yellow, Y12 = blue, DBVPG6044 = red, DBVPG6765 = green. The highly differentiated SNPs are associated with the YPS128 haplotype.