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Temperature preference can bias parental genome retention during hybrid evolution

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Abstract

Interspecific hybridization can introduce genetic variation that aids in adaptation to new or changing environments. Here, we investigate how hybrid adaptation to temperature and nutrient limitation may alter parental genome representation over time. We evolved *Saccharomyces cerevisiae* x *Saccharomyces uvarum* hybrids in nutrient-limited continuous culture at 15˚C for 200 generations. In comparison to previous evolution experiments at 30˚C, we identified a number of responses only observed in the colder temperature regime, including the loss of the *S. cerevisiae* allele in favor of the cryotolerant *S. uvarum* allele for several portions of the hybrid genome. In particular, we discovered a genotype by environment interaction in the form of a loss of heterozygosity event on chromosome XIII; which species' haplotype is lost or maintained is dependent on the parental species' temperature preference and the temperature at which the hybrid was evolved. We show that a large contribution to this directionality is due to a temperature dependent fitness benefit at a single locus, the high affinity phosphate transporter gene *PHO84*. This work helps shape our understanding of what forces impact genome evolution after hybridization, and how environmental conditions may promote or disfavor the persistence of hybrids over time.

Author summary

Organisms are facing rapid alterations to their habitat due to climate change, the introduction of invasive species, habitat fragmentation, and other human mediated transformations. How species will survive and adapt to these changes is one of the biggest questions of our time. Hybridization, or the mating of different species, offers a potential solution, as it immediately introduces novel genetic variation genome wide. To investigate how hybrids adapt to new environments, and how the environment influences hybrid genomes, we created hybrid yeast in the lab and evolved them under different nutrient limited conditions and at different temperature regimes for hundreds of generations. We show that genetic variation introduced from a cold tolerant species of yeast helps a hybrid adapt to a phosphate-limited cold temperature environment, and thus that the

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environment can influence which species' genes persist and which species' genes are lost in the generations following a hybridization event.

Introduction

Comparative genomics of thousands of plants, animals, and fungi has revealed that portions of genomes from many species are derived from interspecific hybridization, indicating that hybridization occurs frequently in nature. However, the influence of processes such as selection, drift, and/or the presence or absence of backcrossing to a parental population on hybrid genome composition in incipient hybrids remains largely unknown. In some cases, hybrids will persist with both parental genomes in fairly equal proportions as new hybrid species or lineages, while in other instances, hybrid genomes will become biased towards one parent's sub-genome over time [1–9]. Untangling the genetic and environmental factors that lead to these outcomes is a burgeoning field.

Some hybrid genotypes will be unfit due to genetic hybrid incompatibilities or cytotype disadvantage; decades of work across many systems have illustrated examples of hybrid sterility and inviability [10]. Recent work has demonstrated that in hybrid genomes with a bias in parental composition like humans, in which most of the genome is comprised of modern human haplotypes with small fragments derived from archaic human, regions from the minor parent (e.g., Neanderthal or Denisovan) are decreased near functional elements and hybrid incompatibilities [11–13]. Conversely, there are examples of "adaptive introgression," in which alleles from the minor parent confer a benefit, like wing patterning in butterflies, high altitude tolerance in the Tibetan human population, and winter color morphs in the snowshoe hare [14–22]. The environment undoubtedly plays a significant role in hybrid fitness, and genotype by environment interactions will shape hybrid fitness in a similar manner as they shape non-hybrid fitness. For example, there is general acceptance that the *Saccharomyces* species complex is largely void of genic incompatibilities (with exceptions [23]), however most experiments looking for incompatibilities have used laboratory conditions. Hou *et al.* utilized different carbon sources, chemicals, and temperatures to show that over one-fourth of intra-specific crosses show condition-specific loss of offspring viability [24]. This is echoed by many examples of condition specific hybrid incompatibility in plants [25–30]. Similarly, there are numerous examples of environment dependent high fitness hybrid genotypes [31] [32–41], exemplified by classic research showing Darwin's finch hybrids with different beak shapes gained a fitness benefit during and after an El Niño event [15].

The budding yeasts in the genus *Saccharomyces* have emerged as a particularly adept system to study genome evolution following hybridization. Recent evidence supports the hypothesis that the long-recognized whole genome duplication that occurred in the common ancestor that gave rise to *Saccharomyces* resulted from hybridization [42], and led to speculation that ancient hybridization could also explain other whole genome duplications in plants and animals [43]. Introgression and hybridization have also been detected across the *Saccharomyces* clade [44–50]; most famously, the lager brewing lineage *S. pastorianus* is a hybrid between *S. cerevisiae* and *S. eubayanus* [51–56]. A bias towards one parent sub-genome was identified in the ancient hybridization event and in *S. pastorianus*, and selection is inferred to be important in this process [1,42]. Experimental evolution of lab derived hybrids has provided significant new insights into the genetic architecture and influence of the environment on hybrid genome evolution [41,57–59].

To empirically understand the genomic changes that occur as a hybrid adapts to a new environment, we previously created *de novo* interspecific hybrids between two yeast species, *S. cerevisiae* and *S. uvarum*, which are approximately 20 million years divergent and differ in a range of phenotypes, notably in preferred growth temperature. *S. uvarum* has been isolated primarily from *Nothofagus* (beech) and associated soil in Patagonia and similar habitats across the world, and is specifically known for fermentation of cider and wines at cold temperatures [60–64]. Many *S. uvarum* strains show evidence of introgression from several other yeast species, and *S. cerevisiae* x *S. uvarum* hybrids have been isolated from fermentation environments [60,65].

We previously evolved *S. cerevisiae* x *S. uvarum* hybrids in the laboratory in several nutrient-limited environments at the preferred growth temperature of *S. cerevisiae* [66]. We frequently observed a phenomenon known as loss of heterozygosity (LOH) in these evolved hybrids, in which an allele from one species is lost while the other species' allele is maintained. The outcome of such events is the homogenization of the hybrid genome at certain loci, and represents a way in which a hybrid genome may become biased toward one parent's sub-genome. This type of mutation can occur due to gene conversion or break induced replication, and as previously noted, has also been observed in organisms including *S. pastorianus*, pathogenic hybrid yeast, and hybrid plants, but its role in adaptation has been unclear [49,67,68]. We used genetic manipulation and competitive fitness assays to show that a particular set of LOH events was the result of selection on the loss of the *S. uvarum* allele and amplification of the *S. cerevisiae* allele at the high affinity phosphate transporter *PHO84* in phosphate limited conditions. By empirically demonstrating that LOH can be the product of selection, we illuminated how an underappreciated mutation class can underlie adaptive hybrid phenotypes.

This prior study described an example of how the environment (differences in nutrient availability) can bias a hybrid genome towards one parent's sub-genome. Due to many examples of genotype by temperature interaction in hybrids across many taxa, and a difference in species' temperature preference in our hybrids, we speculated that temperature is an important environmental modifier that may influence parental sub-genome representation in hybrids. Temperature can perturb fundamentally all physiological, developmental, and ecological processes, and as such, temperature is an essential factor in determining species distribution and biodiversity at temporal and spatial scales [69–71]. We hypothesized that in *S. cerevisiae* x *S. uvarum* hybrids, *S. cerevisiae* alleles may be favored at warmer temperatures, whereas *S. uvarum* alleles may be preferred at colder temperatures, giving the hybrid an expanded capacity to adapt. To test how temperature influences hybrid genome composition over time, we evolved the same interspecific hybrid yeast in the laboratory at 15˚C for 200 generations. In comparing laboratory evolution at 15˚C and 30˚C, we present evidence that temperature can indeed bias hybrid genome composition towards one parental sub-genome, and we focus on a reciprocal LOH event at the *PHO84* locus. We show that which species' allele is lost or maintained at this locus is dependent on the parental species' temperature preference and the temperature at which the hybrid was evolved, thus revealing a genotype by environment interaction. Our results are one of the first clear examples with a molecular genetic explanation of how hybrids have expanded adaptive potential by maintaining two genomes, but also how adapting to one condition may abrogate evolutionary possibilities in heterogeneous environments.

Results

Laboratory evolution of hybrids and their parents at cold temperatures

To test whether temperature can influence the direction of resolution of hybrid genomes, we evolved 14 independent populations of a *S. cerevisiae* x *S. uvarum* hybrid in nutrient-limited

media at 15˚ C for 200 generations (phosphate-limited: 6 populations; glucose-limited: 4 populations; sulfate-limited: 4 populations; see S4 Table for strain details). Diploid *S. cerevisiae* and *S. uvarum* populations were evolved in parallel (4 populations of *S. cerevisiae* and 2 populations of *S. uvarum* in each of the three nutrient limited conditions; see S4 Table for strain details). Populations were sampled from the final timepoint and submitted for whole genome sequencing and analysis.

Loss of *S. cerevisiae* alleles in cold evolved hybrids

We detected large scale copy number variants in our cold evolved populations, including whole and partial chromosome aneuploidy and loss of heterozygosity (Table 1; S1 Table; S2 Table; S1 Fig; S2 Fig; S3 Fig; S4 Fig; S5 Fig; S6 Fig; S7 Fig). Copy number changes, and specifically amplification of nutrient transporter genes, are well-recognized paths to adaptation in laboratory evolution in nutrient limited conditions [50,66,72–81]. We find a preference for *S. cerevisiae* partial and whole chromosome amplification in hybrids evolved at both 15˚C and 30˚C, which may reflect an increased capacity for *S. cerevisiae* to acquire this type of mutation (Table 1) [82]. In contrast, we observe a bias in the direction of LOH resolution dependent on temperature. Previously, we observed more LOH events in hybrids evolved at 30˚C in which the *S. uvarum* allele was lost (5/9 LOH events) [66]. In this study, we observe 6/6 LOH events in hybrids evolved at 15˚C in which the *S. cerevisiae* allele is lost and the *S. uvarum* allele is maintained, suggestive of a *S. uvarum* cold temperature benefit. While our sample sizes are modest, together these results indicate that temperature can determine hybrid genomic composition in the generations following a hybridization event.

In line with previous studies, we find both chromosomal aneuploidy and LOH are nutrient limitation specific, with repeatable genomic changes occurring in replicate populations under the same nutrient condition, but no changes shared across nutrients. In glucose limitation, 3/4 hybrid populations experienced chromosome XV LOH, losing the *S. cerevisiae* allele for portions of the chromosome. Haploidization of one of these implicated regions on chromosome XV was previously observed in *S. cerevisiae* diploids evolved at 30˚C in glucose limitation [66,79], but it was not observed in any previously evolved hybrids, and which genes may be responsible for fitness increases are unclear. In sulfate limitation, we recapitulate previous hybrid laboratory evolution results [66], observing the amplification of the *S. cerevisiae* high affinity sulfate transporter gene *SUL1* in low sulfate conditions (4/4 hybrids, S1 Fig; S2 Fig). Amplification of *S. cerevisiae SUL1* therefore seems to confer a high relative fitness regardless of temperature (see section below, "Pleiotropic fitness costs resulting from loss of heterozygosity"). Though prior work showed highly repeatable amplification of *S. cerevisiae SUL1* at 30˚C in *S. cerevisiae* haploids and diploids [66,72,79–81], and amplification of *S. uvarum SUL2* after approximately 500 generations at 25˚C in *S. uvarum* diploids [81], we never observed amplification of *SUL1* or *SUL2* in *S. cerevisiae* or *S. uvarum* diploids at 15˚C, albeit our experiments were terminated at 200 generations.

Finally and most notably, in low phosphate conditions, we discovered a LOH event in which the *S. cerevisiae* allele is lost and the *S. uvarum* allele is amplified on chromosome XIII, which encompasses the high affinity phosphate transporter *PHO84* locus (2/6 hybrid populations; Fig 1A). The LOH tract length extends approximately 80kb from the telomere in both cold evolved populations (P1-15˚C: 0–82,283; P3-15˚C: 0–79,085), and the breakpoints are potentially due to microhomology, located in the genes *GIM5* and *VPS9*, respectively. This LOH event is of high interest, as we previously identified a repeated LOH event encompassing the same genomic region when hybrid populations were evolved at 30˚C (3/6 populations, Fig 1A). Furthermore, the directionality of this LOH event is the opposite outcome of our

Table 1. Mutations in cold-evolved hybrid populations.

Population	Location	Gene(s)	Mutation
P1-15˚C	*S. cerevisiae* chrXVI: 772650	*CLB2*	coding-nonsynonymous: D333A
	S. uvarum chrVII: 818275	*UBR1*	coding-nonsynonymous: F1634C
	S. uvarum chrXI: 5832	*JEN1*	5'-upstream
	S. cerevisiae chrXIII: 1–81102 *S. uvarum* chrXIII: 1–82283	41 genes including *PHO84*	LOH: loss of *S. cerevisiae* allele and amplification of *S. uvarum* allele
	S. cerevisiae chrXIII: 81102–168343	47 genes	CNV: Segmental amplification of *S. cerevisiae*
P2-15˚C	*S. uvarum* chrIV: 903941	*YBR259W*	coding-nonsynonymous: S624Y
P2F-15˚C	*S. uvarum* chrV: 179951	*YAT2*	coding-synonymous
P3-15˚C	*S. uvarum* chrXI: 101238	*LST4*	coding-nonsynonymous: S533A
	S. cerevisiae chrXIII: 1–79085 *S. uvarum* chrXIII: 1–80181	40 genes including *PHO84*	LOH: loss of *S. cerevisiae* allele and amplification of *S. uvarum* allele
	S. cerevisiae chrXIII:79085–168343	48 genes	Segmental amplification of *S. cerevisiae*
G7-15˚C	*S. cerevisiae* chrXI:80553	*ACP1*	5' upstream
	S. cerevisiae chrIV:651345–871844	118 genes	Segmental amplification *S. cerevisiae* allele
	S. uvarum chrII: 554234–1289935	159 genes	Segmental amplification *S. uvarum* allele
	S. cerevisiae chrXV:976083–1071297	46 genes	LOH: loss of *S. cerevisiae* allele
G8-15˚C	*S. cerevisiae* chrIV:955826	*VHS1*	5'-upstream
	S. cerevisiae chrV:321068	*AIM9*	coding-nonsynonymous: A369V
	S. cerevisiae chrXI:80553	*CCP1*	5'-upstream
	S. cerevisiae chrXV:977571	*TYE7*	coding-nonsynonymous: E167K
	S. uvarum chrXV:685069	*IKI1*	coding-nonsynonymous: A2E
	S. cerevisiae chrIII:169495–316620	82 genes	LOH: loss of *S. cerevisiae* allele
	S. cerevisiae chrXII:732111–1078177	181 genes	Segmental amplification of *S. cerevisiae* allele
	S. cerevisiae chrXV: 340969–464306,464306–594878	71 genes, 72 genes	LOH: loss of *S. cerevisiae* allele
G9-15˚C	*S. cerevisiae* chrXII:812389	*FKS1*	coding-nonsynonymous: S798Y
	S. cerevisiae chrXII:818575–1078177	136 genes	Segmental amplification of *S. cerevisiae* allele
	S. cerevisiae chrXV:976083–1071297	46 genes	LOH: loss of *S. cerevisiae* allele
G10-15˚C	*S. cerevisiae* chrXV:1034298	*GPB1*	5' upstream
S7-15˚C	*S. cerevisiae* chrXII:238463	*YLR046C*	coding-nonsynonymous: M117I
	S. cerevisiae chrXII:1047895	*FMP27*	coding-nonsynonymous: P1300S
	S. cerevisiae chrII:786030–813184	12 genes including *SUL1*	Segmental amplification of *S. cerevisiae* allele
S8-15˚C	*S. cerevisiae* chrIX:212119	*AIR1*	5' upstream
	S. cerevisiae chrIX:212130	*AIR1*	5' upstream
	S. cerevisiae chrII:786025–813184	12 genes including *SUL1*	Segmental amplification of *S. cerevisiae* allele
S9-15˚C	*S. cerevisiae* chrII:786017–813184	12 genes including *SUL1*	Segmental amplification of *S. cerevisiae* allele
S10-15˚C	*S. uvarum* chrXIII: 752118	*GTO3*	5' upstream
	S. uvarum chrXVI: 456125	*SUR1*	5' upstream
	S. cerevisiae chrII:770240–813184	21 genes including *SUL1*	Segmental amplification of *S. cerevisiae* allele

LOH: loss of heterozygosity; CNV: copy number variant. No mutations were detected in populations P4-15˚C, P5-15˚C, or P6-15˚C. Breakpoints of CNV and LOH are approximate.

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observations of hybrids evolved at 30˚C, in which the *S. cerevisiae* allele was amplified and the *S. uvarum* allele was lost. We are unfortunately limited by sample size in determining if these LOH events are statistically significant; however, the repeatability and directionality in resolution of these LOH events suggest they are modulated by temperature and worthy of further investigation.

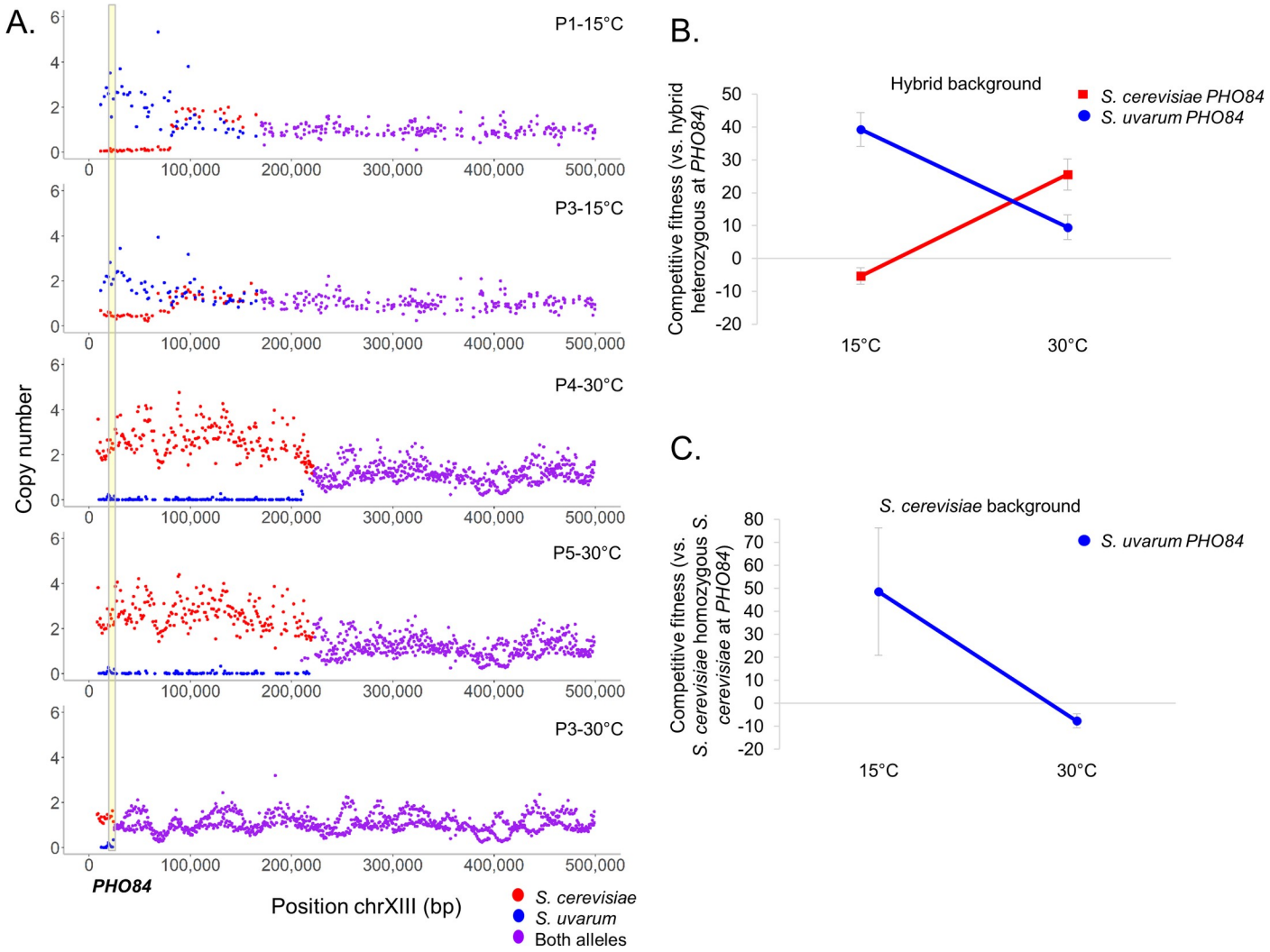

Fig 1. Loss of heterozygosity directionality results from selection on different species' alleles at different temperatures. A. Evolved hybrids exhibit reciprocal loss of heterozygosity on chromosome XIII encompassing the high affinity phosphate transporter gene *PHO84* (located in region shaded with yellow) in phosphate limited conditions at different temperatures. 2/6 independent populations lost the *S. cerevisiae* allele when evolved at 15˚C (top 2 panels, breakpoints in *S. cerevisiae* coordinates P1-15˚C: 82,283; P3-15˚C: 79,085), 3/6 independent populations lost the *S. uvarum* allele when evolved at 30˚C (bottom 3 panels, breakpoints in *S. cerevisiae* coordinates P4-30˚C: 221753; P5-30˚C: 234112; P3-30˚C: 24,562). Purple denotes a region where both alleles are present at a single copy, blue denotes a *S. uvarum* change in copy number, red denotes a *S. cerevisiae* change in copy number. Note, copy number was derived from sequencing read depth at homologous ORFs. Clone sequencing was utilized for experiments at 30˚C and population sequencing was utilized for experiments at 15˚C, so exact population frequency and copy number changes are unclear for experiments at 15˚C. **B.** Allele swap experiments in which a hybrid with one allele of *PHO84* from each species is competed against a hybrid with both copies of *PHO84* either from *S. cerevisiae* (Sc*PHO84*/Sc*PHO84*; red) or *S. uvarum* (Su*PHO84*/Su*PHO84*; blue) reveal a fitness effect dependent on temperature. Error bars denote 95% confidence intervals. **C.** Allele swap experiments in which a diploid *S. cerevisiae* homozygous for *S. cerevisiae* alleles of *PHO84* is competed against a diploid *S. cerevisiae* with both copies of *PHO84* from *S. uvarum* (Su*PHO84*/Su*PHO84*; blue) revealing a fitness effect dependent on temperature. Error bars denote 95% confidence intervals. See **S1 Data** file for data used to calculate competitive fitness.

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Environment-dependent loss of heterozygosity aids in temperature adaptation in hybrids

Based on previous results that demonstrated that LOH at the *PHO84* locus conferred a high competitive fitness benefit at warm temperatures (measured by direct competition of strains carrying the LOH vs. an ancestral strain labeled with a neutral GFP marker), we hypothesized that this apparent preference for the alternate species' allele in different environments is explained by a genotype by environment interaction at the *PHO84* locus itself. Pho84 is a

H^+-coupled inorganic phosphate transporter, responsible for both sensing phosphate in the environment and phosphate uptake, particularly when phosphate is scarce [83–86]. The two species' proteins have a pairwise identity of 90% and are conserved at key residues identified as essential in phosphate transport, but do differ in several residues in transmembrane domains and notably in the large loop VI in the cytoplasm (**S8 Fig**; **S9 Fig**).

To test the hypothesis that there is a genotype by environment interaction involving the *PHO84* locus, we repeated the competitive growth assays of allele-swapped strains from Smukowski Heil *et al*. (2017) at 15˚C. These strains are either homozygous *S. cerevisiae*, homozygous *S. uvarum*, or heterozygous for both species at the *PHO84* locus (including both promoter and coding sequences) in an otherwise isogenic hybrid background. Indeed, we find a fitness tradeoff dependent on temperature, in which hybrids homozygous for *S. uvarum PHO84* show a fitness increase of 39.30% (+/-5.16; 95% C.I.) at 15˚C relative to their hybrid ancestor, which carries a copy of each species' *PHO84* allele. In contrast, hybrids homozygous for *S. cerevisiae PHO84* show a slight relative fitness decrease (-5.36% +/-2.54; 95% C.I.) at this temperature (**Fig 1B**). There is a significant difference between fitness of hybrids homozygous for *S. cerevisiae PHO84* at different temperatures ($p<0.001$, Welch Two Sample t-test), and between fitness of hybrids homozygous for *S. uvarum PHO84* at different temperatures ($p<0.0001$, Welch Two Sample t-test) suggesting that both species' alleles of *PHO84* are temperature sensitive.

To further explore how genetic interactions in the hybrid influence strain fitness, we created a *S. cerevisiae* diploid homozygous for *S. uvarum PHO84* (including both promoter and coding sequence) in an otherwise isogenic background. This strain exhibits a fitness increase of 48.56% (+/-27.72, 95% C.I.) at 15˚C and a fitness decrease of -7.61% (+/-3.04, 95% C.I.) at 30˚C relative to a diploid *S. cerevisiae* homozygous for *S. cerevisiae PHO84* (**Fig 1C**; $p = 0.0071$, Welch Two Sample t-test). These results remain consistent with our previous results in the hybrid background, in which the *S. uvarum* allele is more beneficial at cold temperatures. This suggests that the *PHO84* locus alone is sufficient to confer a temperature dependent fitness benefit and that no sizable genetic interactions contribute to this effect. Technical issues prevented us from testing the reciprocal combination (*S. uvarum* diploid with *S. cerevisiae PHO84* alleles).

Pleiotropic fitness costs resulting from loss of heterozygosity

We clearly demonstrate a fitness trade-off dependent on temperature at the *PHO84* locus. To explore if other mutations in evolved hybrids show antagonistic pleiotropy at divergent temperatures, we conducted a series of competitive fitness assays at 15˚C and 30˚C. We isolated two clones from each hybrid population evolved at 15˚C, and competed the clone against a common GFP-marked unevolved hybrid ancestor in the nutrient limitation it was evolved in at both 15˚C and 30˚C. We observe that clones isolated from the same population often have differences in competitive fitness, which we attribute to genetically different subpopulations coexisting in the population. As all of our analyses are conducted using population sequencing as opposed to clone sequencing, the mutations present in an individual clone may be different than indicated in **Table 1** (and **S1 Table**; **S2 Table**). However, we are still able to detect several trends, including examples of antagonistic pleiotropy and temperature independent high fitness genotypes.

First, we sought to identify how the chromosome XIII LOH event influences competitive fitness beyond the *PHO84* locus. We genotyped clones isolated from evolved populations and selected clones that have chromosome XIII LOH. Clones evolved in phosphate limitation with the chromosome XIII LOH event (homozygous *S. uvarum PHO84*; P1-15˚C and P3-15˚C)

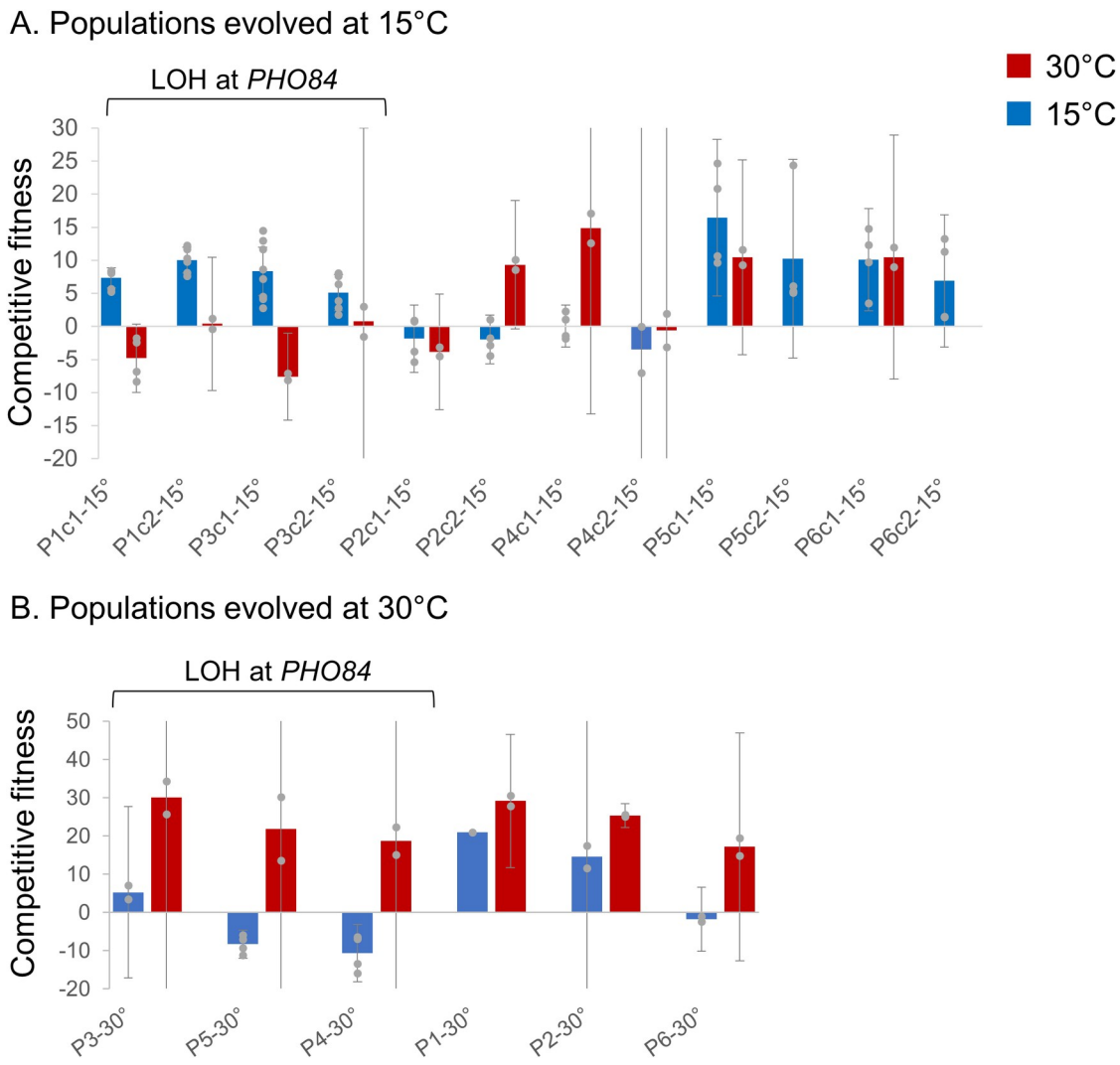

Fig 2. Fitness assays exhibit that loss of heterozygosity can result in antagonistic pleiotropy. A. One or two clones were isolated from each population evolved in phosphate limitation at 15˚C and competed against a common competitor, the fluorescently-labeled hybrid ancestor of the evolution experiments, at 15˚C (blue) and 30˚C (red) in phosphate limitation. Clones with chromosome XIII loss of heterozygosity exhibited higher fitness relative to their ancestor at 15˚C and neutral or negative fitness at 30˚C. Data for P5c2-15˚C and P6c2-15˚C competed at 30˚C was not collected. The height of the bar represents the average competitive fitness value; gray points represent independent fitness measurements used to calculate the average. Error bars denote 95% confidence intervals; note where no cap of the error bar is apparent, the error bar is truncated for visualization purposes. See S5 Table and S1 Data for exact fitness mean and 95% C.I. **B.** Clones evolved in phosphate limitation at 30˚C were competed against a common competitor, the hybrid ancestor of the evolution experiments, at 15˚C and 30˚C in phosphate limitation. Clones with chromosome XIII loss of heterozygosity exhibited higher fitness relative to their ancestor at 30˚C and neutral or negative fitness at 15˚C. The height of the bar represents the average competitive fitness value; gray points represent independent fitness measurements used to calculate the average. Error bars denote 95% confidence intervals; note where no cap of the error bar is apparent, the error bar is truncated for visualization purposes. See S5 Table and S1 Data for exact fitness mean and 95% C.I. No error bars are present for P1-30˚C competed at 15˚C as no replicates were successfully completed.

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have higher competitive fitness at 15˚C and decreased competitive fitness at 30˚C, displaying antagonistic pleiotropy (Fig 2A, although note clones isolated from the same population have different magnitudes of fitness decreases). The competitive fitness increases seen in evolved hybrid clones at 15˚C are much less extreme than the values observed for the allele swap strains (Fig 1B), suggesting that other genes included in the LOH event may have a negative fitness effect, and/or that genetic interactions dampen the magnitude of the fitness increase.

To compare these results to the reciprocal LOH event seen in hybrids evolved at 30˚C in which the evolved strains became homozygous for *S. cerevisiae PHO84*, we competed clones from populations initially evolved at 30˚C at 15˚C. Indeed, clones with the LOH event homozygous for *S. cerevisiae PHO84* (P3-30˚C, P4-30˚C, P5-30˚C) have increased fitness at 30˚C and decreased fitness at 15˚C (Fig 2B), consistent with the *PHO84* allele swap competitive fitness results. Of course, there are other mutations present in these clones, and some evidence that these fitness values may be influenced by the tract length of the LOH event, which ranges from approximately 79kb to 234kb. For example, P3-30˚C has the shortest LOH tract at approximately 25kb in length and has a higher relative fitness at 15˚C than either P4-30˚C or P5-30˚C, whose LOH tracts extend to 221kb and 234kb, respectively (Fig 1A). The LOH tract length is approximately 80kb in both cold evolved populations (P1-15˚C: 82,283; P3-15˚C: 79,085), but is made more complex by the amplification of a portion of the *S. cerevisiae* sub-genome adjacent to the LOH event (P1-15˚C: 81,105–168,345; P3-15˚C: 79,074–168,345; Fig 1A). Together, these results support a temperature sensitive fitness response at the *PHO84* locus, but also imply that there may be other genes modulating fitness in the chromosome XIII LOH events, something we hope to explore in future work.

In contrast, clones isolated from populations without chromosome XIII LOH (P2-15˚C, P4-15˚C, P5-15˚C, P6-15˚C, P1-30˚C, P2-30˚C, P6-30˚C) generally show increased fitness at the temperature they were evolved at, but have variable fitness responses at the temperature they were not evolved at. Similarly, hybrid clones evolved in other media conditions at 15˚C generally show an increase in fitness at 15˚C, but variable responses at 30˚C, with some clones having higher relative fitness at 15˚C and lower fitness at 30˚C, some clones showing the opposite trend, and some clones having similar fitness at both temperatures (Fig 3). It thus appears that temperature specific antagonistic pleiotropy, in which a clone has high fitness at one temperature and low fitness at the other temperature, is relatively rare, with the LOH encompassing *PHO84* being the only clear example (although clones P2-C2 and G9-C2 display a pattern of antagonistic pleiotropy as well). The only other distinct pattern in the fitness data is that all hybrids evolved in sulfate limitation at 15˚C show fitness gains at both 15˚C and 30˚C. All sulfate-limited hybrid clones have an increased fitness ranging from 23.66–41.01% relative to their hybrid ancestor at 30˚C, except for the clone from population S9-15˚C (Fig 3B). This result is in line with the observation of an amplification of *S. cerevisiae SUL1* at very low frequency and/or low copy number in population S9-15˚C compared to other sulfate limited evolved populations, which display high copy number *S. cerevisiae SUL1* amplification (S1 Fig). Previous work conducted at 30˚C has illustrated that *SUL1* amplification in sulfate-limited conditions is highly advantageous. These new data suggest that an amplification of *S. cerevisiae SUL1* confers a fitness benefit at both cold and warm temperatures, but is most beneficial at warm temperatures. *SUL1* thus provides a clear example of a temperature independent high fitness genotype.

Mutations in *TPK2* are likely responsible for flocculation phenotype

Through comparison of the single nucleotide variants and indels called in the hybrid populations evolved at 15˚C and 30˚C, we observed a slight, though not significant, increase in the number of mutations in the *S. cerevisiae* portion of the genome when evolved at temperatures preferred by *S. uvarum* (12/19 mutations are in the *S. cerevisiae* sub-genome at 15˚C compared to 16/30 mutations in the *S. cerevisiae* sub-genome at 30˚C, Fisher's exact test p = 0.5636; S3 Table). There was no overlap in genes with variants identified in datasets from 15˚C and 30˚C.

We suspect that the low growth temperature is a selective pressure for both the hybrids and the parental populations, and we did observe mutations in two genes (*BNA7* and *OTU1*) that

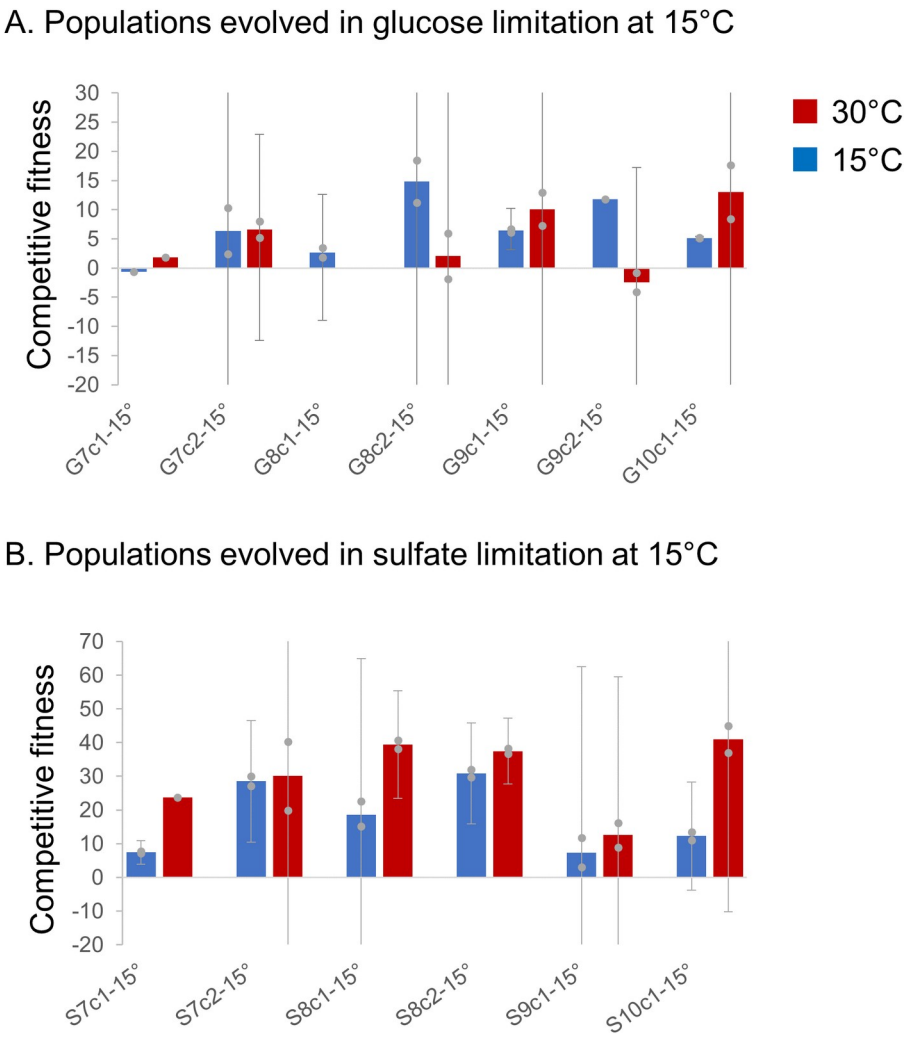

Fig 3. Fitness assays in glucose and sulfate limited media. A. One or two clones were isolated from each population evolved in glucose limitation at 15˚C and competed against a common competitor, the fluorescently-labeled hybrid ancestor of the evolution experiments, at 15˚C (blue) and 30˚C (red) in glucose limitation. The height of the bar represents the average competitive fitness value; gray points represent independent fitness measurements used to calculate the average. Error bars denote 95% confidence intervals; note where no cap of the error bar is apparent, the error bar is truncated for visualization purposes. See S5 Table and S1 Data for exact fitness mean and 95% C.I. Where no error bars are shown, no replicates were successfully completed. **B.** One or two clones were isolated from each population evolved in sulfate limitation at 15˚C and competed against a common competitor, the fluorescently-labeled hybrid ancestor of the evolution experiments, at 15˚C (blue) and 30˚C (red) in sulfate limitation. The height of the bar represents the average competitive fitness value; gray points represent independent fitness measurements used to calculate the average. Error bars denote 95% confidence intervals; note where no cap of the error bar is apparent, the error bar is truncated for visualization purposes. See S5 Table and S1 Data for exact fitness mean and 95% C.I. Where no error bars are shown, no replicates were successfully completed.

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were previously identified in a study of transcriptional differences of *S. cerevisiae* in long-term, glucose-limited, cold chemostat exposure [87]. We found no overlap with genes previously identified to be essential for growth in the cold [88,89], or differentially expressed during short-term cold exposure [90,91], though our screen is not saturated and growth conditions differ between these studies. Additionally, we observed some mutations in genes that are members of the cAMP-PKA pathway, which has previously been implicated in cold and nutrient-limitation adaptation [87,92].

Based on the mutations observed in populations evolved at 30˚C, we previously hypothesized that an intergenomic conflict between the nuclear and mitochondrial genome of *S. cerevisiae* and *S. uvarum* could be an important selection pressure during the evolution of these hybrids [66]. We find further circumstantial evidence for the possibility that mitochondrial conflicts are influential in hybrid evolution as 3/19 point mutations in the hybrids are related to mitochondrial function, whereas 1/20 are related in the parental species populations (for a total of 7/46 point mutations in hybrids and 1/46 point mutations in parentals when both temperatures are considered; p = 0.0585, Fisher's exact test).

Finally, we did observe one recurrent mutational target. Eight independent *S. cerevisiae* diploid lineages had a substitution occur at 1 of 3 different amino acid positions in Tpk2, a cAMP-dependent protein kinase catalytic subunit. Previously, it has been reported that Tpk2 is a key regulator of the cell sticking phenotype known as flocculation through inactivation of Sfl1, a negative regulator of *FLO11*, and activation of *FLO8*, a positive regulator of *FLO11* [93,94]. This mutation was detected exclusively in flocculent populations. We and others have previously established that flocculation evolves quite frequently in the chemostat, likely as an adaptation to the device itself, but we have not previously observed a flocculation phenotype caused by these mutations in other evolved populations of *S. cerevisiae* [95]. While we have not definitively demonstrated causation, prior literature links *TPK2* to flocculation, and all evolved clones bearing a *TPK2* mutation flocculated within seconds of resuspension by vortexing (**S10 Fig**). Most mutations were heterozygous, but within several lineages, we observed evidence of a LOH event that caused the *TPK2* mutation to become homozygous. Clones bearing a homozygous mutation in *TPK2* showed a faster flocculation phenotype than their heterozygous counterparts. We similarly observed one lineage with a mutation causing a premature stop and subsequent LOH in *SFL1*, whose isolated clones displayed a robust flocculation phenotype. We suspect that our previous lack of detection is likely due to the well-established genetic differences in the *FLO8* gene between the strains used in this study and previous studies, which would alter whether a *FLO8* dependent flocculation phenotype is possible [96].

Discussion

In summary, we evolved populations of interspecific hybrids at cold temperatures and show that temperature can influence parental representation in a hybrid genome. We find a variety of mutations whose annotated function is associated with temperature or nutrient limitation, including both previously described and novel genes. Most notably, we discover a temperature and species specific gene by environment interaction in hybrids, which empirically establishes that temperature can influence hybrid genome evolution.

Growth temperature appears to be one of the most definitive phenotypic differences between species of the *Saccharomyces* clade, with *S. cerevisiae* being exceptionally thermotolerant, while many other species exhibit cold tolerance [97–99]. Significant work has focused on determining the genetic basis of thermotolerance in *S. cerevisiae* with less attention devoted to cold tolerance, though numerous genes and pathways have been implicated [88–91,100–104]. Hybrids may offer a unique pathway for coping with temperatures above or below the optimal growing temperature of one parent [47,105,106], and may aid in the identification of genes important in temperature tolerance. For example, it has long been speculated that the allopolyploid hybrid yeast *S. pastorianus* (*S. cerevisiae* x *S. eubayanus*) tolerates the cold temperatures utilized in lager beer production due to the sub-genome of the cold adapted *S. eubayanus* [51–56,107–109]. Indeed, creation of *de novo* hybrids between *S. cerevisiae* and cold tolerant species *S. uvarum*, *S. eubayanus*, *S. arboricola*, and *S. mikatae* all show similar ability to ferment at 12˚C [109]. A pair of recent studies show that mitochondrial inheritance in hybrids is also

important in heat and cold tolerance, with the *S. cerevisiae* mitotype conferring heat tolerance and *S. uvarum* and *S. eubayanus* mitotypes conferring cold tolerance [106,110]. The hybrid ancestor used for our laboratory evolution experiments at both 15˚C and 30˚C has *S. cerevisiae* mitochondria, but exploring how this has influenced the evolution of these hybrids is worthy of further work.

Though our work here is complicated by utilizing multiple selection pressures (nutrient limitation and cold temperature), several patterns are suggestive of temperature specific adaptations in evolved hybrids. We observe LOH events exclusively favoring the retention of the *S. uvarum* allele, and we demonstrate a fitness advantage of the *S. uvarum* allele compared to the *S. cerevisiae* allele at *PHO84*. The temperature sensitivity of the *PHO84* allele is a curious phenomenon for which we do not yet have a clear understanding. One potential connection is the need for inorganic phosphate for various processes involved in stress response, including heat shock and activation of the PKA pathway, for which *PHO84* is required [86,111–113]. At the level of the protein, one potential region for further investigation of causal temperature sensitivity is the cytoplasmic loop VI of the Pho84 protein, from amino acid residues 283–324, a region which contains 13 radical substitutions between *S. cerevisiae* and *S. uvarum* (**S8 Fig**; **S9 Fig**). These substitutions include changes from hydrophobic residues to charged residues, which could change temperature sensitivity. The promoter, which is quite divergent between the two species, may also be important. A genetic screen for *S. cerevisiae* growth at low temperatures found that uptake of phosphate is a growth limiting factor and implicated the overexpression of the genes *PHO84*, *PHO87*, *PHO90*, and *GTR1* in growth at 8˚C [114]. More specifically, the authors found that both *PHO84* and *GTR1* (which are located close together on chromosome XIII) must be overexpressed to produce a growth phenotype at low temperatures. While we show that *S. uvarum PHO84* alone is sufficient to produce a fitness benefit at cold temperatures, we did not assay expression nor conduct promoter swaps, which could shine light on the basis of *PHO84* temperature sensitivity as well. However, our results do suggest that the introduction of *S. uvarum PHO84* into *S. cerevisiae* strains may prove useful for industrial applications of which growth at low temperatures is required. Overall, *PHO84* provides an interesting example of identifying a gene and pathway previously not appreciated for a role in temperature adaptation, and highlights using multiple environments to better understand parental species' preferences and potentially environment specific incompatibilities.

More broadly, through the lens of *PHO84*, we establish LOH as an important molecular mechanism in hybrid adaptation, but we also show that this mutation type has fitness tradeoffs. The selection of a particular species' allele may confer a fitness advantage in a given environment, but at a risk of extinction if the environment changes. Furthermore, such mutations rarely affect single genes, and instead operate on multigenic genomic segments, leading to a further pleiotropic benefit and/or risk even in environments unrelated to the initial selective regime. Relatively constant environments such as those found in the production of beer and wine may offer fewer such risks, where hybrids may find a particular niche that is less variable than their natural environment. Future efforts are warranted to explore how variable environments influence hybrid evolution and the extent of antagonistic pleiotropy in hybrid genomes. However, because LOH has been documented in a variety of different genera and taxa that experience a range of environments, it's likely that our results have broad implications.

In conclusion, we illuminate pathways in which hybridization may allow adaptation to different temperature conditions. Mounting evidence suggests that anthropogenic climate change and habitat degradation are leading both to new niches that can be occupied by hybrids, as well as to new opportunities for hybridization due to changes in species distribution and breakdown of prezygotic reproductive isolation barriers [115–117]. Some researchers have speculated that this process is particularly likely in the arctic, where numerous hybrids have

already been identified [118]. Our work supports the idea that portions of these hybrid genomes can be biased in parental representation by the environment in the initial generations following hybridization, and that this selection on species' genetic variation may be beneficial or detrimental as conditions change.

Materials and methods

Strains

Strains used to inoculate the laboratory evolution experiments were: *S. cerevisiae* diploid (YMD139, YMD140), *S. uvarum* diploid (YMD366), and a lab-derived diploid hybrid *S. cerevisiae* x *S. uvarum* (YMD129, YMD130). These strains and those used to gauge relative fitness of *PHO84* allele replacements in competition assays were previously utilized by Smukowski Heil et al. [66]. All strains are listed in **S4 Table**.

Evolution experiments

Continuous cultures were established using media and conditions previously described with several modifications to account for a temperature of 15˚C [66,72]. Individual cultures were maintained in a 4˚C room in a heated water bath such that the temperature the cultures experienced was 15˚C, as monitored by a separate culture vessel containing a thermometer. The dilution rate was adjusted to approximately 0.08 volumes per hour (for 20 mL chemostats, 1.6 mL/hour), equating to about 3 generations per day. Samples were taken twice a week and measured for optical density at 600 nm and cell count; microscopy was performed to check for contamination; and archival glycerol stocks were made. By 200 generations, 2/16 hybrid populations, 10/12 *S. cerevisiae* diploid populations, and 0/6 *S. uvarum* diploid populations had evolved a cell-cell sticking phenotype consistent with flocculation. The experiment was terminated at 200 generations and flocculent and non-flocculent populations were sampled from the final timepoint and submitted for whole genome sequencing (40 populations total, some cultures had only a flocculent or non-flocculent population while some cultures had both subpopulations). Populations from vessels that experienced flocculation were isolated as described in [95], and are denoted with "F". Briefly, 1 mL of each flocculent population was pipetted directly from the vessel upon the termination of the experiment and archived in glycerol stocks. Colonies were struck out from glycerol, inoculated into liquid culture and grown overnight at room temperature. From overnight cultures that displayed a clumping, and/or settling, phenotype, new glycerol stocks were made and one clone from each evolved population was selected for sequencing.

Genome sequencing and analysis

DNA was extracted from each population using the Hoffman–Winston protocol (Hoffman and Winston 1987) and cleaned using the Clean & Concentrator kit (Zymo Research). Nextera libraries were prepared following the Nextera library kit protocol and sequenced using paired end 150 bp reads on the Illumina NextSeq 500 machine. The reference genomes used were *S. cerevisiae* v3 (Engel et al. 2014), *S. uvarum* (Scannell et al. 2011), and a hybrid reference genome created by concatenating the two genomes.

Variant calling was conducted on each population using two separate pipelines. For the first pipeline, we trimmed reads using trimmomatic/0.32 and aligned reads to their respective genomes (*S. cerevisiae*., *S. uvarum*, or a concatenated hybrid genome) using the mem algorithm from BWA/0.7.13, and manipulated the resulting files using Samtools/1.7. Duplicates were then removed using picard/2.6.0, and the indels were realigned using GATK/3.7.

Variants were then called using Samtools (bcftools/1.5 with the–A and–B arguments), freebayes and lofreq/2.1.2. The variants were then filtered using bcftools/1.5 for quality scores above 10 and read depth above 20. For the second pipeline, reads were trimmed using Trimmomatic/0.32 and aligned using Bowtie2/2.2.3, then preprocessed in the same manner as the first pipeline. Variants were then called using lofreq/2.1.2 and freebayes/1.0.2-6-g3ce827d (using the—pooled-discrete—pooled-continuous—report-genotype-likelihood-max—allele-balance-priors-off—min-alternate-fraction 0.05 arguments from bcbio (https://github.com/bcbio/bcbio-nextgen)). Variants were then filtered using bedtools/2.25.0 and the following arguments (**S6 Table**). In both variant calling pipelines, variants were filtered against their sequenced ancestors and annotated for gene identity, mutation type, and amino acid change consequence [119]. Final variant calls were manually confirmed through visual inspection in the Integrative Genomics Viewer [120] (1550 mutations checked in total).

For comparisons with clones evolved at 30˚C which were analyzed using a different pipeline [66], we called variants on the previously published 30˚C sequencing data using the same computational pipelines described here, and completely recapitulated the previous true positive variant calls.

Flocculation assays

Overnight cultures were resuspended by vortexing for three seconds. Highly flocculant clones settled out of solution within seconds of vortexing, complicating controlled quantitative measurement of the phenotype. Instead, we relied on visual observation within the first few seconds after vortexing.

Fitness assays

We utilize competitive growth as a measurement of strain fitness. The pairwise competition experiments were performed in replicate in 20 ml chemostats as previously described [66,121]. Briefly, a *S. cerevisiae* x *S. uvarum* hybrid tagged with a neutral GFP marker is grown to steady state in parallel with a query strain. When cultures have achieved steady state (approximately 10–15 generations), the GFP and non-GFP cultures are mixed at a 50:50 concentration. The proportion of GFP to non-GFP cells is monitored approximately every 2 generations for a total of five sampling points (approximately 10 generations, 25 total generations) using a BD Accuri C6 flow cytometer. Competitive fitness is calculated as the slope of the linear region of ln [dark cells/GFP+ cells] versus generations. Efforts were made to have at least two replicates for each fitness measurement, but technical errors in the running of the fitness assays resulted in some clones having no replicates. Data used to estimate fitness can be found in **S1 Data**. The competition experiments performed at 15˚C were modified as described above for the evolution experiments. For all cold-evolved hybrid populations, one to two clones were isolated for use in competition experiments. Clones from P1-15˚C and P3-15˚C were PCR validated to have the chromosome XIII LOH event, but no other LOH, CNV, or single nucleotide variants were screened in these or any other clone tested.

Supporting information

S1 Table. Mutations in cold-evolved *S. cerevisiae* diploid populations.
(PDF)

S2 Table. Mutations in cold-evolved *S. uvarum* diploid populations.
(PDF)

S3 Table. Comparison of single nucleotide variants called in 15˚C and 30˚C experimental evolution.
(PDF)

S4 Table. Strain list.
(PDF)

S5 Table. Competitive fitness of evolved hybrid strains.
(PDF)

S6 Table. Filters used in variant calling.
(PDF)

S1 Data. Compilation of data used to calculate competitive fitness measurements.
(XLSX)

S1 Fig. Copy number plots of cold-evolved hybrid populations. Genome wide copy number is plotted for evolved hybrid populations at 15˚C. Nutrient limitation is indicated in the upper right corner (G = glucose, S = sulfate, P = phosphate), numbers indicate independent populations. Purple denotes a region where both alleles are present at a single copy (alternating purple indicates different chromosomes from chrI—chrXVI), blue denotes a *S. uvarum* change in copy number, red denotes a *S. cerevisiae* change in copy number. Note, copy number was derived from population sequencing read depth at homologous ORFs.
(PDF)

S2 Fig. Amplification of *S. cerevisiae SUL1* in hybrids evolved at 15˚C and 30˚C. Copy number is plotted across chrII in a representative hybrid clone evolved in sulfate limitation at 30˚C and a population evolved in sulfate limitation at 15˚C. Copy number was derived from sequencing read depth at homologous ORFs. A region containing the *S. cerevisiae* allele of the high affinity sulfate transporter *SUL1* is amplified in 7/7 populations evolved at 30˚C and 4/4 populations evolved at 15˚C, suggesting that the locus is not temperature sensitive, and instead that the *S. cerevisiae* allele is more fit at both temperatures.
(PDF)

S3 Fig. Copy number plots of cold-evolved *S. cerevisiae* diploid populations. Copy number is plotted across the genome for *S. cerevisiae* evolved populations. Alternating grey and red indicate different chromosomes (from chrI–chrXVI). Copy number was derived from average population sequencing read depth in 1000 bp intervals. Nutrient limitation is indicated in the upper right corner (G = glucose, S = sulfate, P = phosphate), numbers indicate independent populations.
(PDF)

S4 Fig. Copy number plots of cold-evolved, flocculent *S. cerevisiae* diploid populations. Copy number is plotted across the genome for *S. cerevisiae* evolved, flocculent populations that were isolated separately from populations dispersed in the culture. Alternating grey and red indicate different chromosomes (from chrI–chrXVI). Copy number was derived from average population sequencing read depth in 1000 bp intervals. Nutrient limitation is indicated in the upper right corner (G = glucose, S = sulfate, P = phosphate), numbers indicate independent populations.
(PDF)

S5 Fig. Loss of heterozygosity plots of cold-evolved *S. cerevisiae* diploid populations. Alternate allele (e.g., non-reference allele) frequency is plotted across the genome for *S. cerevisiae*

evolved populations. The unique pattern of heterozygosity is produced by a strain history of crossing FL100 to S288C to produce GRF167, which was crossed to S288C to produce the diploid strain used here. This produced regions of heterozygosity and regions of homozygosity (regions that appear blank because no alternate allele is called). This also allows the detection of loss of heterozygosity (LOH), where regions that were heterozygous become homozygous for the reference or non-reference allele. Alternating grey and red indicate different chromosomes (from chrI–chrXVI). Nutrient limitation is indicated in the upper right corner (G = glucose, S = sulfate, P = phosphate), numbers indicate independent populations. Note that LOH events are not at fixation in the population, so these events are instead indicated by allele frequencies approaching zero or one.
(PDF)

S6 Fig. Loss of heterozygosity plots of cold-evolved, flocculent *S. cerevisiae* diploid populations. Alternate allele (e.g., non-reference allele) frequency is plotted across the genome for *S. cerevisiae* evolved, flocculent populations that were isolated separately from populations dispersed in the culture. The unique pattern of heterozygosity is produced by a strain history of crossing FL100 to S288C to produce GRF167, which was crossed to S288C to produce the diploid strain used here. This produced regions of heterozygosity and regions of homozygosity (regions that appear blank because no alternate allele is called). This also allows the detection of loss of heterozygosity (LOH), where regions that were heterozygous become homozygous for the reference or non-reference allele. Alternating grey and red indicate different chromosomes (from chrI–chrXVI). Nutrient limitation is indicated in the upper right corner (G = glucose, S = sulfate, P = phosphate), numbers indicate independent populations. Note that LOH events are not at fixation in the population, so these events are instead indicated by allele frequencies approaching zero or one.
(PDF)

S7 Fig. Copy number plots of cold-evolved *S. uvarum* diploid populations. Copy number is plotted across the genome for *S. uvarum* evolved populations. Alternating grey and blue indicate different chromosomes (from chrI–chrXVI). Copy number was derived from average population sequencing read depth in 1000 bp intervals. Nutrient limitation is indicated in the upper right corner (G = glucose, S = sulfate, P = phosphate), numbers indicate independent populations.
(PDF)

S8 Fig. Protein alignment of Pho84. *S. cerevisiae* and *S. uvarum* alleles of Pho84 were aligned using Clustal Omega. Black indicates shared identity, white indicates a radical substitution, and grey indicates a conservative substitution.
(PDF)

S9 Fig. Predicted protein structure of Pho84. A 2D depiction of the structure of Pho84 was created by using UniProt protein accession P25297 (*S. cerevisiae* Pho84) with the program Protter [122]. Radical (blue square) and conservative (orange diamond) substitutions between *S. cerevisiae* and *S. uvarum* amino acids were annotated using a protein alignment (**S8 Fig**).
(PDF)

S10 Fig. Flocculation assay of several flocculent clones isolated from *S. cerevisiae* cold-evolved populations. Overnight cultures were resuspended by vortexing for three seconds. Flocculent clones were photographed immediately after vortexing and then again after 5 minutes.
(PDF)

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