Incipient Balancing Selection through Adaptive Loss of Aquaporins in Natural Saccharomyces cerevisiae Populations

Jessica L. Will1, Hyun Seok Kim2*2, Jessica Clarke1, John C. Painter1*, Justin C. Fay2, Audrey P. Gasch1,3

1 Laboratory of Genetics, University of Wisconsin–Madison, Madison, Wisconsin, United States of America, 2 Department of Genetics, Washington University, St. Louis, Missouri, United States of America, 3 Genome Center of Wisconsin, University of Wisconsin–Madison, Madison, Wisconsin, United States of America

Abstract

A major goal in evolutionary biology is to understand how adaptive evolution has influenced natural variation, but identifying loci subject to positive selection has been a challenge. Here we present the adaptive loss of a pair of paralogous genes in specific Saccharomyces cerevisiae subpopulations. We mapped natural variation in freeze-thaw tolerance to two water transporters, AQY1 and AQY2, previously implicated in freeze-thaw survival. However, whereas freeze-thaw–tolerant strains harbor functional aquaporin genes, the set of sensitive strains lost aquaporin function at least 6 independent times. Several genomic signatures at AQY1 and/or AQY2 reveal low variation surrounding these loci within strains of the same haplotype, but high variation between strain groups. This is consistent with recent adaptive loss of aquaporins in subgroups of strains, leading to incipient balancing selection. We show that, although aquaporins are critical for surviving freeze-thaw stress, loss of both genes provides a major fitness advantage on high-sugar substrates common to many strains’ natural niche. Strikingly, strains with non-functional alleles have also lost the ancestral requirement for aquaporins during spore formation. Thus, the antagonistic effect of aquaporin function—providing an advantage in freeze-thaw tolerance but a fitness defect for growth in high-sugar environments—contributes to the maintenance of both functional and nonfunctional alleles in S. cerevisiae. This work also shows that gene loss through multiple missense and nonsense mutations, hallmarks of pseudogenization presumed to emerge after loss of constraint, can arise through positive selection.

Introduction

Biologists have long sought to understand the process of natural selection and the signatures left behind in extant species. Finding evidence of adaptive evolution has been a holy grail for evolutionary biologists, because it can provide insights into how and why organisms evolve. However, examples of adaptive selection from which to glean insights remain relatively scarce [1]. The recent explosion in the number of genomes available for different organisms provides an exciting opportunity to identify loci with unusual patterns of variation indicative of selection (for example [2–7]). However, even for loci with strong signatures of selection, the affected phenotypes are often a complete mystery. In contrast, mapping studies link quantitative trait variation to genomic loci that can then be interrogated for evidence of selection. The challenge in most organisms is identifying responsible SNPs within candidate regions, which are often megabases long and contain hundreds of functional elements, hindering further study [8].

Here, we used the power of yeast genetics and genomics to uncover a unique example of adaptive gene loss, involving multiple paralogous genes and several sequential evolutionary events. We previously surveyed phenotypic variation in Saccharomyces collected from diverse environments and found that relatively few of those strains (12%) could survive freeze-thaw (FT) stress [9]. Many tolerant strains were isolated from oak soil in the Northeastern United States, whereas sensitive strains were typically isolated from warm environments, often from fruit or fermentations. This suggested that FT tolerance has been selected for in strains from cold climates but lost in other isolates. Several genes have been linked to freeze-thaw tolerance in yeast and other organisms, including water transporters. The paralogous yeast aquaporins (AQYs) AQY1 and AQY2 were implicated in FT stress by the baking industry, which found that AQY over-expression increases yeast viability in frozen bread dough [10]. Rapid export of water through AQYs is thought to increase FT survival by preventing intracellular freezing due to water crystallization [10,11]. The paralogs may have arisen in the whole-genome duplication (WGD) event in the Saccharomyces lineage [12], since all post-WGD species all have two aquaporins whereas most pre-WGD species have a single ortholog (Dana Wohlbach and A.P.G., unpublished). It has been observed that laboratory and industrial strains as well as several vineyard isolates harbor non-functional alleles of AQY2, while several strains harbor a non-functional
Local adaptation is thought to be a driving force in population differentiation and the formation of new species. Yet, there are few examples of ecologically relevant phenotypes that have been mapped to individual genes, making it difficult to know what drives the evolution of such genes and contributes to the molecular mechanisms underlying divergence. Here, we provide a unique case of local adaptation through multi-gene loss. We mapped the genetic basis for natural variation in yeast freeze-thaw tolerance to two water transporters, AQY1 and AQY2. Although tolerant strains harbor functional alleles of both genes, the set of sensitive strains lost aquaporins at least 6 independent times, through missense mutations and frame-shifting deletions. Genome-wide scans reveal several signatures of recent, partial selective sweeps at the aquaporin loci, indicating positive selection for gene loss. This was likely driven by a major fitness advantage of aquaporin loss when cells grow in high sugar concentrations common to many strains’ niche. Surprisingly, strains that lost aquaporins also lost the ancestral requirement for these genes during sexual reproduction. This work provides a compelling example of how gene loss through nonsense mutations, a hallmark of pseudogenization, is caused not by loss of constraint but by positive selection.

**Results**

We mapped FT tolerance using a cross between naturally FT-resistant strain YPS163, collected from Pennsylvania oak trees [17], mated to a FT-sensitive lab strain derived from S288c, by phenotyping 44 recombinant strains together genotyped at 198 markers spaced roughly every 60 kb (~30 cM) [18]. Two loci were identified: one on the left arm of chromosome 12 and one on the right arm of chromosome 16 (Figure 1, see Methods). Each contained one of two paralogous aquaporin transporters, AQY2 and AQY1, respectively, which were previously linked to freeze-thaw tolerance [10,11,19]. Together, these genes explained >90% of the phenotypic variation, with AQY2 alone explaining two-thirds of the effect (Figure 1C). This was confirmed by reciprocal translocation experiments (Figure 1D): deletion of either gene from YPS163 diminished FT tolerance according to the QTL effect plots, while deletion of both genes ablated FT survival. Introducing either gene into the S288c-derived lab strain (which harbors non-functional alleles of both genes [13–16]) donated partial FT tolerance to the otherwise sensitive strain. Thus, AQY2...
and, to a lesser extent, AQY1 are major effectors of natural variation in yeast FT tolerance.

Sequencing AQY2 and AQY1 from the population revealed a near-perfect correlation between FT tolerance and the presence of functional AQY genes. Tolerant strains contained nearly identical and known functional alleles of both genes, while several strains with an intermediate phenotype contained only one functional gene. However, FT-sensitive strains displayed several different non-functional AQY alleles (Figure 2 and Figure S4). There were three distinct frame-shifting deletions in AQY2, including a known 11-bp deletion in laboratory and vineyard strains [14,16], deletion of G at position 25 (G25) in Asian isolates and several other strains [14,16], and a G320 deletion in the Malaysian AQY2 that is unable to contribute FT tolerance in our assay (Table S2). Several coding polymorphisms were shared in the recapitulated proteins encoded by the 11 bp-deletion allele or by the G25 allele (Figure 2). There were also three different non-functional AQY1 alleles in the population, including the A881 deletion that renders AQY1 inactive in our context (see Table S2 and Figure S5C), the V121M polymorphism known to inactivate water transport [13], and a 955-bp deletion that removes the first 106 bp of AQY1 and its upstream region in Malaysian strains. The trees for Aqy2 and Aqy1 are distinct from one another, and significantly different from trees based on neutral or genomic sequence that show clear distinction between Asian strains and vineyard isolates [9,20,21]. Such discordance between gene and species trees can be a sign of non-neutral evolution. Furthermore, there were five different combinations of non-functional AQY1 and AQY2 alleles, and a higher-than-expected frequency of strains harboring both functional or both non-functional genes (p = 3.5 × 10^-4, Chi-square test). This cannot be simply explained by shared ancestry, which would have produced similar protein trees and a limited combination of alleles, and instead supports the non-random retention or loss of both AQY genes.

Signatures of selection at AQY2 and AQY1

We applied several tests to assess if loss of AQY function may have been selected for in some strains. Under the neutral model, the rate of polymorphism within strains should be similar to the rate of divergence across species. Instead, both AQY1 and AQY2 show an excess of replacement polymorphism, assessed by the McDonald-Kreitman test [22] that compares non-synonymous (A) to synonymous (S) codon changes (Table S3). AQY1 showed an A/S ratio of polymorphism (5/4 = 1.25) that was significantly higher than that of divergence (11/49 = 0.22, p = 0.026, Fisher’s exact test). AQY2 also showed an excess of polymorphic sites (A/S of 8/20 = 0.4) compared to divergent sites (3/40 = 0.075, p = 0.019), as well as an excess of deletions (3/20 versus 0/40, p = 0.045). AQY2 (but not AQY1) also deviated from neutral evolution at synonymous sites, showing an excess of SNPs compared to 8 intergenic sequences (p = 0.028, multi-locus HKA test [23], Table S4 and Table S5). For the most part, the tests were not significant if subgroups of strains, defined by AQY haplotypes in Figure 2 (see Table S1 for details), were considered separately (Table S3 and Figure 2).

Figure 2. Multiple independent losses of AQY2 and AQY1 in diverse S. cerevisiae isolates. Bayesian trees of the recapitulated AQY proteins (where gaps were treated as missing data before translation) for Aqy2 (left) and Aqy1 (right). Trees were generated using Mr. Bayes 3.1 and a mixed amino acid replacement model with invgamma rates. All nodes displayed posterior probabilities >0.92. Strains with full-length proteins are nearly identical and do not resolve in the tree. Each star represents the appearance of a different deletion, including the Malaysian G528 (grey), 11-bp (yellow), and Asian G25 (blue) deletions in AQY2, and the amino-terminal Malaysian (green) and A881 (orange) deletions in AQY1. Appearance of the aqy1-V121M polymorphism is highlighted with a pink circle. Strains are color coded according to their niche as shown in the key. Freeze-thaw tolerance scores are listed to the right of strains shown in the Aqy1 tree (with +++ for tolerance and– for sensitivity, see Methods for details).

doi:10.1371/journal.pgen.1000893.g002
Table S5). This result indicates that much of the variation is between strain groups.

Excess polymorphism can result from relaxed constraint in the species, or if local adaptation is driving divergence between populations [24,25]. To distinguish between these models, we used non-imputed genome sequence data of Liti et al. [21] to characterize sequence variation flanking the AQY genes. We applied several empirical tests, which can handle the missing data in the low-coverage genomic sequences and are less subject to the unusual features of S. cerevisiae populations (including extensive population structure, unknown population dynamics, ambiguous balance between clonal vs. sexual reproduction, and human-associated migration [21,26–29]) that can confound standard tests [2,30]. To monitor the variation surrounding AQY2, we subdivided 21 strains with data at AQY2 into strains harboring the Asian G25 deletion, the 11 bp deletion, or the full-length AQY2 (clonal Malaysian strains were not considered, see Methods). We calculated the average pairwise nucleotide differences surrounding AQY loci within and between groups, and then compared this variation to other regions across the genome. We tested for several signatures: a recent selective sweep is predicted to reduce variation flanking the selected allele in the affected population, while balancing selection can increase variation between strain groups [25]. Since much of the genome may be evolving neutrally, loci with extreme values display the strongest evidence for non-neutral evolution.

For strains harboring the Asian G25 allele of AQY2, we saw a high correlation in between-group variation and within-group variation across much of the genome, including the right arm of chromosome 12 (Figure 3A, right side). However, a 50 kb stretch on the left arm of chromosome 12 showed below-average variation within the strains (0.76th percentile compared to other similarly sized regions genome-wide, see Methods) but high variation between groups. There was a sharp break in this pattern at ~72 kb, which may represent the breakpoint of a selective sweep. To further explore this, we calculated the difference in between-group variation minus within-group variation, then calculated the area under contiguous peaks in the difference curve for comparison (see Figure S2 and Methods). This procedure identified a 5.6 kb region spanning the 870 bp AQY2 ORF that ranked in the top 1.2 percentile of 4,600 regions genome-wide with skewed between-group versus within-group variation.

Figure 3. Skewed patterns of variation surrounding the AQY2 locus. The average number of pairwise nucleotide differences per 1,000 bp sliding window of step size of 100 bp was plotted on Chromosome 12. Variation within (blue curve) and between (red curve) groups of (A) 4 strains harboring the Asian G25 AQY2 deletion, (B) 16 strains with the 11-bp deletion allele, and (C) 6 strains containing the full-length AQY2. Horizontal blue lines represent the genome-wide average of pair-wise variation within each group, and vertical lines represent manually defined breakpoints in trends. Regions identified with skewed between-group minus within-group variation (see Methods) are highlighted in blue. (D) A plot of FST based on the three groupings in (a–c); horizontal grey line represents the genome-wide average. Gene positions are shown above plots as black boxes, with AQY2 highlighted in yellow.

doi:10.1371/journal.pgen.1000893.g003
Strains harboring the 11-bp deletion displayed a 4,300 bp region encompassing AQY2 with a significant skew in the between-versus within-group variation (1.80th percentile of 3,235 regions genome-wide, Figure 3B) and below-average within-group variation (6.30th percentile genome-wide). Strains harboring the full-length AQY2 showed a smaller peak of 1,500 bp with high between-group variation (6.30th percentile, Figure 3C), but average within-group variation (>50th percentile). These results show that strains harboring either deletion have low variation within those strain groups, and that the high variation at AQY2 distinguishes the three groups from one another. Indeed, a genome-wide plot of FST, which measures the population differentiation based on these three groupings, identified a clear peak of 6.4 kb over AQY2 with above-average FST, ranking among the top 1.50th percentile genome-wide (Figure 3D).

A confounding feature is the extensive population structure within S. cerevisiae [21,29], which can mimic some signatures of selection. Several controls indicate that the observed patterns are unlikely due to demographics. First, these regions were among the most extreme across the genome, which is not expected if population structure is the underlying cause. However, many S. cerevisiae strains have mosaic genomes, for which large regions have distinct lineages [21,29]. To control for this, we performed a partitioning sampling: strains were partitioned at each of 1,370 randomly chosen SNPs across the genome. The difference in between-group minus within-group variation was scored surrounding the partitioning SNP and compared to the difference profile when strains were partitioned based on AQY2 allele (see Methods). The regions observed for the Asian G25 or 11-bp deletion classes remained among the most extreme (4.70th and 5.40th percentile, respectively). Thus, the profiles we observe in Figure 3A and 3B are uncommonly found at random SNPs, most of which likely reflect neutral variation. In contrast, the skew in variation found in strains with full-length AQY2 was not significant by this assessment (26th percentile).

We conclude that the observed skew in polymorphism observed in strains with the Asian G25 deletion and the 11-bp deletion in AQY2 resulted from two separate partial selective sweeps that reduced variation within each group. The high variation distinguishing strain groups is a signature of balancing selection, which may be maintaining both functional and non-functional AQY2 alleles in the population. Indeed, we observed a positive Tajima’s D at AQY2, assessed on a smaller set of high-quality sequences (D = 0.851, p<0.05, Figure S3), indicating an excess of intermediate-frequency polymorphism that is consistent with balancing selection [24].

The patterns at AQY1 were less clear. Strains harboring the aqy1 V121M allele or the A081 deletion showed reduced variation within each group and high variation between groups at the AQY1 region (Figure S4). Although these were highly significant compared to other loci across the genome (0.77th and 1.23rd percentile, respectively), they were not significant compared to random-SNP partitioning described above (16th and 40th percentile, respectively). Thus, the slight skew in between-group versus within-group variation at AQY1 could be due to demographic factors, incorrect strain groupings, or older or weaker selective sweep(s) that have since recovered variation through recombination or mutation.

Loss of AQY function provides an advantage in high osmolarity

The above results strongly suggest selective pressure to lose AQY function in some strains, perhaps driven by environmental factors. We previously reported an anti-correlation between FT survival and osmotic tolerance across a wide range of S. cerevisiae strains (R = −0.35, p = 0.006) [9]. Furthermore, a lab strain with functional AQYs was shown to be sensitive to hypo- and hyper-osmotic cycling, but not to consistently high osmolarity [13,14]. Instead, we found that loss of both AQY genes provides a major growth advantage in high osmotic conditions found in nature (Figure 4). A YPS163 mutant lacking both AQYs displayed ~1.7X greater survival in 1.5 M sorbitol, whereas introducing a functional AQY into the S288c-derived lab strain decreased survival 2–3X. Furthermore, sorbitol tolerance was anti-correlated to both freeze-thaw tolerance (R = −0.38) and the number of functional aquaporins (R = −0.31) in these strains (Table S1). The sugar concentration used here is comparable to that found in the fruit substrates of many wild strains [31]. Thus, AQY function presents a substantial fitness defect in conditions relevant in nature, likely due to passive water loss triggered by the high osmolarity of sugary substrates.

In the course of these experiments, we also discovered that YPS163 lacking either aquaporin had a major defect in spor formation during meiosis (Figure 5). Although AQY1 had been previously implicated in a late step in spore maturation [32], our phenotype is distinct in that it affects spor production. Whereas >70% of the parental YPS163 formed full tetrads within 2 days, only 18–24% of the double or single mutants produced full tetrads. After 9 days, the mutant produced more spores but was still defective compared to the parental strain (<60% full tetrads compared to ~85%, Figure S5). The AQY requirement is ancestral, since an S. paradoxus aqy1A mutant displayed an identical defect (Figure 5A). In contrast, strains without functional AQY genes produce full tetrads (albeit with lower efficiency than YPS163 [33]), consistent with a previous report showing AQY1 is not required for sporulation in vineyard strains [34]. More importantly, introducing the functional YPS163 allele of AQY1 into strains with different combinations of non-functional AQY alleles (including strains M22, K1, SK1, and S288c) did not significantly improve spor production (Figure 5B). Thus, strains lacking AQY function have also lost the ancestral need for AQY during spor production.

Discussion

This work provides the first clear evidence for adaptive loss of AQY function in subgroups of wild S. cerevisiae isolates. The excess polymorphism at AQY genes (McDonald-Kreitman and HKA

Figure 4. Strains lacking AQY genes show a fitness advantage under high osmolarity. Cells were grown on solid agar plates containing 1.5 M sorbitol for 2 days and the number of colony-forming units (CFU) was compared to a no-stress control plate. (A) CFU for YPS163 and YPS163 aqy1Δ aqy2Δ grown on rich medium plus 1.5 M sorbitol; (B) S288c-derivative BY4741 harboring the empty vector or a plasmid expressing the YPS163 allele of AQY1 or AQY2, grown on selective medium with 1.5 M sorbitol. The average and standard deviation of biological triplicates is shown. doi:10.1371/journal.pgen.1000893.g004
tests), high between-group variation surrounding \( AQY2 \) that distinguishes strain groups (group variation and \( F_{ST} \) plots, Figure 3), and skew in the frequency spectrum toward intermediate-frequency \( AQY2 \) alleles (Tajima’s D) are all consistent with non-neutral evolution. Furthermore, \( AQY \) paralogs have been lost at least 6 independent times, through 2 partial selective sweeps at \( AQY2 \) and possibly others at \( AQY1 \). The high variation between strain groups, and the non-random retention or loss of both paralogs in diverse strains, is consistent with the establishment of balanced polymorphism. We propose that the antagonistic pleiotropy of aquaporin function, coupled with spatial differences in selective pressures, provide pressure to maintain both functional or both non-functional alleles in distinct subpopulations of \( S. cerevisiae \).

FT tolerance may be crucial for survival in cold climates, and along with sporulation efficiency may impart strong pressure to retain \( AQY \) genes in strains from wintry niches. Indeed, the ratio of non-synonymous to synonymous differences in \( YPS163 \) compared to \( S. paradoxus \) is 2 - 6X lower for \( AQY2 \) and \( AQY1 \), respectively, versus 0.1 across all genes [35]. This is consistent with purifying selection acting to remove deleterious codon changes. The oak strains likely represent the ancestral state, since close relatives \( S. paradoxus \) and \( S. mikatae \) are also recovered from tree exudates and soil [17,36], display high FT tolerance [9], and require aquaporins for sporulation (Figure 5 and data not shown). Interestingly, Northeastern-US oak strains display unique phenotypes suggestive of other evolutionary forces as well. \( AQY2 \) is expressed on average 14-fold higher in \( YPS163 \) compared to 17 other surveyed strains [9,37]; those levels are doubled in \( YPS1009 \), which underwent a duplication of the entire chromosome 12 [9]. Although further studies will be needed, that over-expression of \( AQY2 \) is known to enhance FT tolerance in industrial strains [10] hints that the elevated expression may have been selected for, further underscoring the importance of \( AQY \) function in these strains.

In contrast, many other strains exist in warm environments that never experience freezing. Most of these were sampled from fruit substrates and distillations, which typically consist of ~25% sugars [31], in contrast to oak soil [38,39] from which many cold-climate strains have been isolated. Thus, the significant advantage in osmo-tolerance due to \( AQY \) loss likely played a major role in selection at this locus. It is unclear which came first–loss of aquaporin requirement during sporulation, or loss of aquaporin function that drove subsequent loss of the sporulation role. Loss of sporulation dependency on aquaporins, coupled with migration to warmer climates, would have relaxed constraint on the genes and facilitated their adaptive loss when cells moved to high-sugar substrates. This model could have involved a single loss of sporulation requirement followed by multiple independent losses of aquaporin function. Alternatively, strong selective pressure to lose aquaporins could have forced multiple independent losses of the sporulation requirement, just as it lead to multiple independent losses of aquaporin function.

\( S. cerevisiae \) strains are thought to have migrated globally through human association, after two domestication events produced sake/distillation strains and vineyard/wine-making lines 10,000 years ago [20,21,26,27,29,40]. Human-facilitated migration may have significantly increased exposure of \( S. cerevisiae \) to diverse climates, which may have imposed new selective pressures when strains

**Figure 5. AQY function is required for sporulation in YPS163.** (A) Denoted strains were sporulated as described in Methods for 2 days, and the number of events with 4, 3, 2 spores or ‘other’ (representing unsporulated or unscorable cells) was recorded. Each plot represents the average and standard deviation of biological triplicate. Results from \( S. paradoxus \) strain NRRL Y-17217 are shown here. (B) Vineyard strain M22, sake strain K1, SK1, and S228c derivative BY4743 (BY) harboring empty vector or \( pYPS\_AQY1 \) plasmid were sporulated as described for 2–3 d before scoring. doi:10.1371/journal.pgen.1000893.g005
encountered new niches. Increased migration may also have facilitated outcrossing of domesticated strains with natural strains, allowing several of these alleles to spread through natural populations. It is important to note that Malaysian strains, not previously associated with domestication events, show unique non-functional AQY alleles, revealing that loss of aquaporins is not strictly driven by domestication.

The selective sweeps of nonfunctional aquaporin alleles appear to have been recent events, given the strength of the signal at AQY2, and may reflect an ongoing process. A remaining question is the fate of the emerged balance in polymorphism. Given sufficient migration of strains between the two niches and unequal fitness costs of the opposing haplotypes (i.e. two functional or two nonfunctional AQY alleles), one haplotype may eventually win out to fixation, eliminating the balanced alleles. On the other hand, long-term balancing selection could result if equivalent selective constraints are maintained in each respective niche. In the extreme case, strongly opposing selective forces could restrict yeast migration between environments to promote ecological speciation [41]. Little is known about S. cerevisiae migration between tree soil and fruits, although oak-soil strains are genetically well separated from vineyard/fermentation isolates [21,29,40,42]. The antagonistic forces driving aquaporin loss at the cost of freeze-thaw sensitivity may be one factor that has limited gene flow between these niches.

Methods

Strains and plasmids

Strains and plasmid constructs are described in Table S6. Two S. cerevisiae strains (DY8 and DY9) were isolated from oak-tree soil from Maribel, Wisconsin using the method of [17], and typed by a mating/sporulation assay with a tester S. cerevisiae strain (Dan Kvitek and APG, unpublished). Gene deletions were created by homologous recombination, replacing AQY1 and/or AQY2 with KanMX3 or NatMX3 drug-resistance cassettes, respectively. Homothallic wild strains capable of mating-type switching (including YPS163, M22, and S. paradoxus) were sporulated and dissected, and drug-marked colonies were selected as homozygous diploids. In all cases, homozygous gene deletions were confirmed by diagnostic PCR. The region corresponding to the 870 bp full-length AQY2 ORF plus 971 bp upstream and 395 bp downstream sequence was cloned from YPS163 or BY4741, by homologous recombination replacing a GFP-ADH1-terminator cassette in plasmid BA1924 (provided by P. Kainth and B. Andrews), which is derived from pRS315-based CEN plasmid BA1926 [43] but with the NatMX3 cassette replacing the LEU2 marker. The region corresponding to the 918 bp full-length AQY1 ORF with the flanking 947 bp upstream and 747 bp downstream was similarly cloned. All clones were verified by sequencing. To assess functionality of the different alleles, AQY1 ORFs representing M22, BY4741, or Y55 alleles (identical to the YPS163 allele but harboring the A881 deletion) or the Malaysian AQY2 allele (identical to YPS163 except for the G528 deletion) were cloned between the native upstream and downstream AQY1 sequence from YPS163. This was done to prevent confounding influences on expression through variation in the flanking regulatory regions. Plasmids were introduced into YPS163 or BY4741, or other naturally AQY1-minus strains, and complementation of spore production in the YPS163 aqy1Δ mutant or of FT tolerance in BY4741 was scored (Table S2).

Phenotyping

Yeast strains were grown at 30°C in YPD medium to an optical density at 600 nm (OD600) of 0.3–0.4 in 24-well plates. To measure freeze-thaw tolerance, 200 µl of cells was transferred to 1.5 ml tubes and frozen in a dry ice/ethanol bath (<−50°C) for two hours or on ice as control. Viability was measured by scoring serial dilutions spotted onto agar plates as previously described [9], or using LIVE/DEAD stain (Invitrogen, Carlsbad, CA) read on a Guava EasyCyte Plus flow cytometer (Millipore, Billerica, MA). Scores in Figure 2 correspond to high (>80% of YPS163 viability; three pluses), medium (50–80% viability, two pluses), low (<50% viability, one plus), or no detectible (minus sign) FT tolerance. Osmotic tolerance was measured by plating cells onto agar plates containing 1.5 M sorbitol. Percent viability was scored as the number of colony-forming units compared to the no-stress control plate.

Spore analysis

Cells were grown in YPD rich medium to OD600 nm of 1.0, harvested by centrifugation, resuspended in 1% potassium acetate, and incubated at 25°C for 2 or 9 days. Cells were harvested, diluted and the number of spores per tetrad was counted on a hemocytometer.

QTL mapping and sequence analysis

QTL mapping strains and analysis were as previously described [18], using the Haley-Knott algorithms implemented in R-QTL [44]. Two additional peaks in Figure 1B (left arm of Chromosome 2 and right arm of Chromosome 8) were not significant when Chromosome 12 and 16 QTL were held as fixed terms, suggesting the additional peaks may be false positives. Sequencing using BigDye (Applied Biosystems, Carlsbad, CA) scored at least 3 reads (including forward and reverse) per basepair from 2 independent genomic preparations (GenBank accessions GQ848552-74 and GQ870433-54). The vast majority of sequence data represented homozygous sites. The few base pairs with evidence of heterozygocity were represented by one of the alleles, randomly chosen. MK-tests, Tajima’s D, and Ka/Ks were calculated in DNASP 5.0 [45] and ML-HKA was done as in [23] using sequence data from [9,20] and here.

Genome-wide sequence analysis was performed using unimputed, aligned data from [21] with quality scores > = 40 (generously provided by Alan Moses), treating all gaps as missing data to avoid alignment errors. Strains were grouped according to AQY2 or AQY1 alleles (see Table S1 for details), and the average number of pairwise SNPs was calculated every 1000 bp with a 100 bp step size, for all pairs of strains within each group and for all strains in a given group compared to each strain outside that group. Within-group variation was scored for all 50 kb regions across the genome with a step size of 20 kb, and for all 5 kb regions with step size of 2 kb. These regions were compared to the 50 kb region highlighted in the text (position 22,000–72,000 in Figure 3) for strains with the G25 AQY2 allele and to a 5 kb region centered on AQY2 for other strain groups. All regions were ranked based on the average pairwise within-group variation to calculate the percentile rank of regions in question.

To monitor the skew variation within and between groups, a difference profile of between-group variation minus within-group variation (calculated as described above) was taken across the genome, and all contiguous regions (“peaks”) where the difference value was >1.5X the chromosome-wide average were identified (see Figure S2 and Table S7). The area under each peak was estimated by the trapezoidal method, and compared to the area under the peaks in Figure 3A and 3B spanning AQY2. For the partitioning sampling, we scanned for SNPs with at least 3 strains harboring the minor allele, every 10,000 bp across each of the 16 yeast chromosomes. Strains were partitioned based on that SNP.
then the between-group and within-group variation was measured for 20,000 bp centered on the partitioning SNP, based on the average-pairwise differences every 1,000 bp with a 100 bp step size as above. A profile of the between-group variation minus the within-group variation was taken in every window. For each partitioning SNP, a peak in the difference profile was identified by walking outward until the difference value was <3.54, the cutoff used the genomic scan shown in Figure 3B. The area under the curve was calculated as above and compared to that measured at AQY2 by an identical procedure except that strains were partitioned by Asian G25 allele vs. all others strains or by 11-bp deletion vs. all other strains. Very similar percentile rankings were obtained if we scored 5 kb windows centered on each SNP (data not shown).

**Supporting Information**

**Figure S1** Polymorphisms in AQY2 and AQY1. The plot shows AQY2 (top) and AQY1 coding sequences, arranged 5’ (left) to 3’ (right). Blue bars indicate SNPs and orange represents verified gaps in the AQY coding sequences compared to the YPS163 allele for strains in different groups (rows). Strains are organized as shown in Figure 2. Complete sequence data is available through GenBank accession numbers GQ848352-74 and GQ870433-54. Found at: doi:10.1371/journal.pgen.1000893.s001 (0.41 MB TIF)

**Figure S2** Difference profiles of between-group minus within-group variation. To identify regions with a skew in between-group and within-group variation, we calculated the difference profile as described in the text. Peaks where values were >1.5X the chromosome-wide average were identified and compared to peaks identified at the AQY2 locus. Blue windows highlight identified peaks over AQY2; ORF positions are shown above the figure as described in Figure 3. Found at: doi:10.1371/journal.pgen.1000893.s002 (1.54 MB TIF)

**Figure S3** Tajima’s D at AQY loci. Tajima’s D was measured at 12 different loci with high-quality sequence data from [9,20] and here, in 11 or 12 of 12 strains. Values for the AQY2 (red) and AQY1 (orange) coding sequences were compared to other intergenic loci. The 95% confidence interval (mean of non-AQY loci plus two standard deviations) is shown with a horizontal red line. Many genes show negative D values, consistent with previous genome wide estimates for *S. cerevisiae* [29]. Found at: doi:10.1371/journal.pgen.1000893.s003 (0.22 MB TIF)

**Figure S4** Within-group versus between-group variation at AQY1. As shown in Figure 3 for strains harboring the V121M allele, A881 deletion, or full-length AQY1. Found at: doi:10.1371/journal.pgen.1000893.s004 (3.16 MB TIF)

**Figure S5** Sporulation defects in mutant strains. (A) Sperorulation efficiency as shown in Figure 5 but measured at 9 days. (B) Haploinsufficiency is seen for heterozygous YPS163 AQY1/aqy1A but not YPS163 AQY2/aqy2A, suggesting AQY1 plays a more significant role in YPS163 sporulation. (C) Complementation experiments show that the YPS163 aqy1A sporulation defect is not complemented by AQY1 ORFs from S288c derivative BY4741 (BY), M22, or the YPS163 coding sequence with the A881 deletion. To avoid defects due to regulatory differences, each ORF was cloned between the 947-bp upstream and 747-bp downstream sequences from YPS163, exactly as for the pYPS_AQY1 clone that was able to complement the sporulation defect. Further confirming that these alleles are non-functional in our context, we found that none of these AQY1 versions contributed FT tolerance to BY4741 (data not shown), unlike the AQY1 allele from YPS163 (Figure 1C). Although the A881 allele of Aqy1 has been shown to produce a functional water transporter in an in vitro system, it was also shown to dramatically reduce protein levels [13], which may explain why it is not relevant in our in vivo analysis. Found at: doi:10.1371/journal.pgen.1000893.s005 (0.97 MB TIF)

**Table S1** Summary of AQY2 and AQY1 alleles and phenotypes across strains. The table lists strain names, source of sequences analyzed, allele types, number of functional AQY alleles per strain, and strain phenotypes (as described in Methods). Found at: doi:10.1371/journal.pgen.1000893.s006 (0.02 MB XLS)

**Table S2** Summary of major-allele phenotyping. The table lists the allele tested, functional prediction based on sequence polymorphism, strain from which gene was cloned, and the ability to donate freeze-thaw tolerance to the lab strain or complement the sporulation defect of YPS163 aqy1A. Found at: doi:10.1371/journal.pgen.1000893.s007 (0.02 MB XLS)

**Table S3** McDonald-Kreitman tables and p-values. McDonald-Kreitman tests were performed using DNASP on high-quality sequence data from this study, using *S. paradoxus* strain Q69.8, which had the best high-quality sequence coverage [21], as the outgroup. P-values were estimated using Fisher’s exact test in DNASP 5.0 [45]. Significant tests are indicated with an asterisk. Found at: doi:10.1371/journal.pgen.1000893.s008 (0.06 MB DOC)

**Table S4** Input data for ML-HKA test. a Sample size (number of strains), b number of segregating sites, c number of divergent sites, *S. paradoxus* strain Q69.8 was used as the outgroup [21]. We applied the multi-locus HKA method of Wright and Charlesworth [23] to test for selection at AQY2 and AQY1, compared to 7 intergenic sequences with data for both *S. cerevisiae* and *S. paradoxus* [9]. Each fragment is denoted by the chromosome and start position on that chromosome. Due to a large deletion removing the front part of the gene in Malaysian strains, the Malaysian AQY1 allele started at position 100, removing of an upstream, intramate ATG and 30 additional basepairs that were clearly not orthologous to the full-length AQY1 from other strains. Intergenic regions were analyzed after removing two clearly non-orthologous regions from all strains (350 bp from the chr2 fragment and 387 bp from the chr16 fragment), the result of apparent recombination in subgroups of strains. This dataset amounted to 183 and 150 silent positions in AQY2 and AQY1, respectively, and 3,337 scorable sites across 7 intergenic fragments. Found at: doi:10.1371/journal.pgen.1000893.s009 (0.07 MB DOC)

**Table S5** Results of ML-HKA analysis. ML-HKA tests were run under a model in which all loci were evolving neutrally and compared to models in which AQY2, AQY1, or AQY2 and AQY1 were under selection. The program was run with chain length 100,000 in all cases. P-values were calculated based on the chi2 distribution of the likelihood statistic [28] for selection model -ln Lneutral model listed degrees of freedom (df), as described (Wright and Charlesworth 2004). There was no increase in significance when all sites in AQY2 and AQY1 were treated as silent [justified since the genes are non-functional] (data not shown). We also ran the analysis separately for only strains in Full-length group, Asian G25 deletion group, or 11 bp-deletion group, Variation at AQY2 and AQY1 within each group was compared to variation at other loci only for strains in that group. The results were the same when the genes in question were scored against intergenic sequences from all available strains. None of the tests were significant in any case.
Acknowledgments

Special thanks to Alan Moses for critical suggestions and providing data. Thanks also to Brett Payseur, Dan Kvitik, and members of the Gasch Lab for useful discussions and comments on the manuscript.

Author Contributions

Conceived and designed the experiments: APG. Performed the experiments: JKW JC JCP JCF. Analyzed the data: JWL HS KJ JCJ APG. Wrote the paper: APG.

References

1. Mitchell-Olds T, Willis JH, Goldstein DB (2007) Which evolutionary processes influence natural genetic variation for phenotypic traits? Nat Rev Genet 8: 845–856.
2. Akey JM, Zhang G, Zhang K, Jin L, Shriver MD (2002) Interrogating a high-density SNP map for signatures of natural selection. Genome Res 12: 1005–1015.
3. Clark AG, Glanowski S, Nielsen R, Thomas PD, Krjaávald A, et al. (2003) Inferring nonneutral evolution from human-chimp-mouse orthologous gene trios. Science 302: 1960–1963.
4. Nielsen R, Bastani C, Khorsand G, Clark AG, Glanowski S, Sackton TB, et al. (2005) A scan for positively selected genes in the genomes of humans and chimpanzees. PLoS Biol 3: e170. doi:10.1371/journal.pbio.0003170.
5. Saberi PC, Reich DE, Higgins JM, Levine HZ, Richter DJ, et al. (2002) Detecting recent positive selection in the human genome from haplotype structure. Nature 419: 892–897.
6. Voight BF, Kudaravalli S, Wen X, Pritchard JK (2006) A map of recent positive selection in the human genome. PLoS Biol 4: e72. doi:10.1371/journal.pbio.0040147.
7. Yamazaki M, Tenaillon ML, Bi IV, Schroder SG, Sanchez-Villega H, et al. (2005) A large-scale screen for artificial selection in maize identifies candidate agronomic loci for domestication and crop improvement. Plant Cell 17: 2595–2672.
8. Mackay TF, Stone EA, Ayroles JF (2009) The genomics of domestication and Saccharomyces: Characterization of a second functional water channel protein. PLoS Genet 5: e1000523.
9. Liaw V, Gobin R, Rousselet G, Badger C, Hohmann S, et al. (2002) Aquaporin expression correlates with freeze tolerance in baker’s yeast, and overexpression improves freeze tolerance in industrial strains. Appl Environ Microbiol 68: 5981–5989.
10. Tanghe A, Van Dijck P, Dumortier F, Teunissen A, Hohmann S, et al. (2002) Aquaporin expression correlates with freeze tolerance in baker’s yeast, and overexpression improves freeze tolerance in industrial strains. Appl Environ Microbiol 70: 3337–3342.
11. Wolfe KH, Shields DC (1997) Molecular evidence for an ancient duplication of the entire yeast genome. Nature 387: 708–713.
12. Bonhivers M, Carbery JM, Gould SJ, Agre P (1998) Aquaporins in Saccharomyces. Genetic and functional distinctions between laboratory and wild-type strains. J Biol Chem 273: 27565–27572.
13. Carbery JM, Bonhivers M, Boeke JD, Agre P (2001) Aquaporins in Saccharomyces: Characterization of a second functional water channel protein. Proc Natl Acad Sci U S A 98: 1000–1005.
14. Lisitzin V, Gobin R, Rousselet G, Badger C, Hohmann S, et al. (1999) Molecular and functional study of AQP1 from Saccharomyces cerevisiae: role of the C-terminal domain. Biochem Biophys Res Commun 257: 139–144.
15. Luize V, Taucet F, Ripoche P, Hohmann S (2000) Polymorphism of Saccharomyces cerevisiae aquaporins. Yeast 16: 897–903.
16. Sniegowski PD, Bombrowicz PG, Fingerman F (2002) Saccharomyces cerevisiae and Saccharomyces paradoxus coexist in a natural woodland site in North America and display different levels of reproductive isolation from European comparatives. FEMS Yeast Res 1: 299–306.
17. Kim HS, Fay JC (2007) Genetic variation in the cystine biosynthesis pathway causes sensitivity to pharmacological compounds. Proc Natl Acad Sci U S A 104: 19387–19391.
18. Tanghe A, Van Dijck P, Thevelein JM (2006) Why do microorganisms have aquaporins? Trends Microbiol 14: 76–85.
19. Fay JC, Benavides JA (2005) Evidence for domestication and wild populations of Saccharomyces cerevisiae. PLoS Genet 1: e5. doi:10.1371/journal.pgen.0010003.
20. Liti G, Carter DM, Moses AM, Warringer J, Parts L, et al. (2009) Population genomics of domestic and wild yeasts. Nature 458: 337–341.
21. MacDonald JH, Kreitman M (1991) Adaptive protein evolution at the Adh locus in Drosophila. Nature 351: 652–654.
22. Wright SI, Charlesworth B (2004) The HKA test revisited: a maximum-likelihood-ratio test of the standard neutral model. Genetica 118: 1071–1076.
23. Kreitman M (2006) Methods to detect selection in populations with applications to the human. Annu Rev Genomics Hum Genet 1: 339–359.
24. Charlesworth D (2006) Balancing selection and its effects on sequences in nearby genome regions. PLoS Genet 2: e64. doi:10.1371/journal.pgen.0020064.
25. Wright SI, Charlesworth B (2004) The HKA test revisited: a maximum-likelihood-ratio test of the standard neutral model. Genetica 118: 1071–1076.
26. Schachter J, Shapiro JA, Rudfer DM, Kruglyak L (2009) Comprehensive polymorphism survey elucidates population structure of Saccharomyces cerevisiae. Nature 458: 342–345.
27. Replinsky T, Kousipanou V, Greig D, Bell G (2008) Saccharomyces sensu stricto as a model system for evolution and ecology. Trends Ecol Evol 23: 545–550.
28. Rudfer DM, Pratt SC, Seible HS, Kruglyak L (2006) Polymorphism genomic analysis of outcrossing and recombination in yeast. Nat Genet 38: 1077–1081.
29. Schachter J, Shapiro JA, Rudfer DM, Kruglyak L (2009) Comprehensive polymorphism survey elucidates population structure of Saccharomyces cerevisiae. Nature 458: 342–345.
30. Teshima K, Coop G, Przeworski M (2006) How reliable are empirical genomic scans for selective sweeps? Genome Res 6: 702–712.
31. Catharino RR, Cunha JB, Fogaça AO, Faccio EM, Godoy HT, et al. (2006) Characterization of must and wine of six varieties of grapes by direct infusion electrospray ionization mass spectrometry. J Mass Spectrom 41: 185–190.
32. Sidoux-Walter F, Pettersson N, Hohmann S (2004) The Saccharomyces cerevisiae aquaporin Aqy1 is involved in sporulation. Proc Natl Acad Sci U S A 101: 17422–17427.
33. Gerke JP, Chen GT, Cohen BA (2006) Native isolates of Saccharomyces cerevisiae display complex genetic variation in sporulation efficiency. Genetics 174: 985–997.
34. Karpe J, Bissin LF (2006) Aquaporins in Saccharomyces cerevisiae wine yeast. FEMS Microbiol Lett 257: 117–123.
35. Dzmitry SV, Kim HS, Swain D, Courea D, Williams M, et al. (2008) A catalog of neutral and deleterious polymorphism in yeast. PLoS Genet 4: e1000183. doi:10.1371/journal.pgen.1000183.
36. Naumov GI, James SA, Naumova ES, Louis EJ, Roberts IN (2000) Three new species in the Saccharomyces sensu stricto complex: Saccharomyces cariocanus, Saccharomyces kudriavzevi and Saccharomyces mikatae. Int J Syst Evol Microbiol 50 Pt 5: 1931–1942.
37. Ray JC, McCollough HI, Sniegowski PD, Ezen MB (2004) Population genetic variation in gene expression is associated with phenotypic variation in Saccharomyces cerevisiae. Genomic Biol 5: R26.
38. Sampaio JP, Goncalves P (2008) Natural populations of Saccharomyces kudriavzevi in Portugal are associated with oak bark and are sympatric with S. cerevisiae and S. paradoxus. Appl Environ Microbiol 74: 2144–2152.
39. Xue Z, Leininger T, Lee AW, Tainter F (2001). Chemical properties associated with bacterial wetwood in red oaks. Wood Fiber Sci 33: 76–83.
40. Aa E, Townsend JP, Adams RI, Nielsen KM, Taylor JW (2006) Population structure and gene evolution in Saccharomyces cerevisiae. FEMS Yeast Res 6: 762–715.
41. Schieler D (2009) Evidence for ecological speciation and its alternative. Science 323: 737–741.
42. Diermann S, Dietrich FS (2009) Saccharomyces cerevisiae: population divergence and resistance to oxidative stress in clinical, domesticated and wild isolates. PLoS ONE 4: e5317. doi:10.1371/journal.pone.0005317.
43. Kainth P, Sassi HE, Pena-Castillo L, Chua G, Hughes TR, et al. (2009) Comprehensive genetic analysis of transcription factor pathways using a dual reporter gene system in budding yeast. Methods 48: 250–264.
44. Broman KW, Wu H, Sen S, Churchill GA (2003) R/qtl mapping in experimental crosses. Bioinformatics 19: 889–890.
45. Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.