High-Resolution Mutation Mapping Reveals Parallel Experimental Evolution in Yeast

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High-Resolution Mutation Mapping Reveals Parallel Experimental Evolution in Yeast

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Understanding the genetic basis of evolutionary adaptation is limited by our ability to efficiently identify the genomic locations of adaptive mutations. Here we describe a method that can quickly and precisely map the genetic basis of naturally and experimentally evolved complex traits using linkage analysis. A yeast strain that expresses the evolved trait is crossed to a distinct strain background and DNA from a large pool of progeny that express the trait of interest is hybridized to oligonucleotide microarrays that detect thousands of polymorphisms between the two strains. Adaptive mutations are detected by linkage to the polymorphisms from the evolved parent. We successfully tested our method by mapping five known genes to a precision of 0.2–24 kb (0.1–10 cM), and developed computer simulations to test the effect of different factors on mapping precision. We then applied this method to four yeast strains that had independently adapted to a fluctuating glucose–galactose environment. All four strains had acquired one or more missense mutations in GAL80, the repressor of the galactose utilization pathway. When transferred into the ancestral strain, the gal80 mutations conferred the fitness advantage that the evolved strains show in the transition from glucose to galactose. Our results show an example of parallel adaptation caused by mutations in the same gene.

Introduction

Characterizing the genetic changes that underlie evolutionary adaptation is important for understanding the emergence of new phenotypes. Experimental evolution makes it possible to follow the evolutionary history of populations exposed to known selective pressures. Moreover, the reproducibility of evolutionary paths can be explored by comparing identical, independent experiments. Such studies are beginning to shed light on the genetic basis of evolutionary adaptation [1–4], but many questions remain open, such as how rare gain-of-function mutations are relative to loss-of-function ones, and how often similar phenotypic adaptations are the result of similar genetic changes. A major challenge is finding the adaptive (beneficial) mutations without having to make prior assumptions about their type or site.

Several strategies have been used to search for mutations associated with evolved traits. These include sequencing candidate genes [5–7], tracking the insertion sites of mobile genetic elements [8–10], partial- or whole-genome sequencing [11,11–13], gene expression profiling [2,14], identifying large chromosomal rearrangements [8,15], and linkage analysis [16–18]. Some of these approaches rely on the assumption that mutations found repeatedly in several independently evolved populations are likely to be beneficial. Ultimately, the effects of the mutations on the evolved phenotypes have to be verified experimentally [3,4,19].

Linkage analysis is the least biased and most general method for finding adaptive mutations in a background of neutral ones. It relies on linkage between the mutations that produce the phenotype of interest and neutral genetic markers (DNA polymorphisms) that can be easily followed, and thus makes no assumptions about the nature or locations of the adaptive mutations [20,21]. Such analyses are often applied to progeny (segregants) from a cross between two strains that differ for both the selected trait and the genetic markers. Advances in genome technology have enabled simultaneous genotyping of thousands of DNA polymorphism markers by hybridizing genomic DNA to oligonucleotide arrays [22,23]. This has led to better genome coverage and mapping resolution, as demonstrated on several traits in budding yeast, including growth at high temperature and sporulation efficiency [22,24,25]. However, such quantitative trait mapping methods are laborious and expensive for mapping multiple traits or multiple strains (e.g., strains evolved in parallel experiments), as they usually require the genotyping of multiple individual segregants for each strain or trait being mapped. One solution is to mix DNA from many individuals expressing the trait of interest, and genotype it as a pool (selective DNA pooling; [26]). A variety of pooled DNA genotyping methods have been used in association studies in humans [27–30], as well as in quantitative trait locus (QTL) mapping in plants and animals, where experimental crosses are possible [31–36].

Here we map mutations in the budding yeast, Saccharomyces cerevisiae, which we use as a model organism to study the genetic basis of experimentally evolved traits ([37]; see also...
To overcome the limitations described above, we used high-density oligonucleotide arrays to genotype a single large pool of segregants that express the trait of interest, an approach also used in plants [33]. This strategy reduces the number of microarrays needed for mapping, and increases mapping resolution due to the wide variety of recombination breakpoints present in a large pool of segregants. We tested and optimized our method on five known genetic loci and developed computer simulations to test the effect of various factors on mapping precision. We then applied it to four yeast strains that have been evolved in an environment where they were exposed to a regular alternation of carbon sources. The adaptive phenotype was mapped to the same locus in all four strains. We identified the adaptive mutations in the mapped regions and experimentally verified their contribution to the evolved trait.

Results
A Bulk Segregant Mapping Method

A schematic description of the mapping method is presented in Figure 1. Briefly, a haploid yeast strain that expresses the trait of interest (target strain) is crossed to a reference strain that lacks the trait (Figure 1A). The hybrid diploid is then sporulated (undergoes meiosis), giving rise to a diverse pool of recombinant haploid progeny (segregants) that contain different combinations of their parents’ genomic DNA. A large pool of segregants that express the trait of interest is selected from the progeny (the selected pool); as a control, a random pool of similar size is collected without selecting for the trait (the control pool). DNA from each pool is hybridized to an oligonucleotide microarray that detects the polymorphic sites. If it is difficult to select simultaneously for multiple segregants that express the trait, single progeny can be screened individually for the phenotype and later pooled for the linkage analysis. For each locus, the extent of hybridization reveals the fraction of the DNA that is derived from the target strain (Figure 1B); regions where the target strain’s genotype is significantly overrepresented in the selected pool relative to the control pool are predicted to contain mutations that contribute to the trait of interest (target loci).

Genetic Map Construction for Linkage Analysis

The first step in mapping is finding loci that are polymorphic between the target and reference strains. We identified these by hybridizing the genomic DNA of the target and reference strains separately onto high-density oligonucleotide arrays. Oligonucleotides (features) that hybridized significantly more strongly to DNA of the target strain than to that of the reference strain were the polymorphic features considered in this study (single-feature polymorphisms [SFPs]). We identified SFPs with a detection algorithm that uses a one-tailed two-sample t test (see Materials and Methods) and estimated its sensitivity and specificity using

![Figure 1. Schematic Description of the Pooled Mapping Method Applied to a Single Pool of Segregants in Budding Yeast](https://www.plosbiology.org/static/image/10.1371/journal.pbio.0040256.g001)
two strains (S288c and YJM789) whose genomic DNA sequences are known (see Materials and Methods). We identified 4,438 S288c/YJM789 SFPs out of 12,602 true SFPs at a p value of $10^{-6}$, yielding a true-positive rate of 35.22% (fraction of the true polymorphic features that are scored as polymorphic) at an estimated false discovery rate (FDR) of 6.74% (fraction of detected SFPs that are not truly polymorphic) (Figure S1). At this p value, we detect 45% of the SFPs where the sequence difference between the two strains lies in the central 15 bases of the 25-base oligonucleotide on the array. We chose a p value cutoff of $10^{-6}$ for SFP identification as it gave the highest true-positive-to-false discovery rate ratio of the cutoffs tested.

**Mapping Method Tested on Five Known Genes**

We asked whether we could use pools of segregants from single crosses to map known genetic loci. We chose W303 and SK1 as the target and reference yeast strains as they are widely used in laboratory studies and a high level of polymorphism was reported between them [23]. Using our SFP detection algorithm, we identified 10,330 W303/SK1 SFPs at an estimated FDR of 2.9%, resulting in an average marker density of 1 SFP per $\sim 1.1$ kb or $\sim 0.4$ cm (the distribution of distances between SFPs is shown in Figure S2). These SFPs made up the genetic map used for the linkage analyses in this work.

We tested the performance of our method by mapping one metabolic and four drug resistance genes whose chromosomal locations are known (details in Materials and Methods). The target strain was a derivative of W303 that carries alleles that confer resistance to four drugs, canavanine (can1), geneticin (KANR), hygromycin (HYGR), and nourseothricin (NATR), and that can produce lysine (LYS5). It was crossed to the reference strain, a derivative of SK1 that is sensitive to all four drugs and cannot make lysine (lys5), and the hybrid diploid was sporulated. To simultaneously map LYS5 and can1, we selected approximately $10^7$ segregants that grew in liquid medium lacking lysine and containing canavanine. To map KANR, HYGR, and NATR, we selected a second pool of approximately $10^7$ segregants in liquid medium containing geneticin, hygromycin, and nourseothricin (Clonat). A control pool of segregants of comparable size was isolated in rich medium without drugs. The genomic DNA of each selected and control pool was hybridized to four identical arrays. The genomic DNA of each selected pool of segregants of comparable size was isolated in rich medium without drugs. The genomic DNA of each selected and control pool was hybridized to four identical arrays. The genomic DNA of each selected and control pool was hybridized to four identical arrays. The genomic DNA of each selected and control pool was hybridized to four identical arrays. The genomic DNA of each selected and control pool was hybridized to four identical arrays. The genomic DNA of each selected and control pool was hybridized to four identical arrays. The genomic DNA of each selected and control pool was hybridized to four identical arrays.

We tested the genetic map used for the linkage analyses in this work.

The order of the SFPs was scrambled 1,000 times, for each chromosome containing the mapped loci, and Figure S3 shows the LMS across the entire genome.

A total of six peaks were found above the cutoff: two for the can1, LYS5 pool and four for the KANR, HYGR, and NATR pools. Of the six peaks, five corresponded to the five selected genes (Figure 2). We used mapping deviation, the distance between the center of a mapped peak and the center of the linked target gene, as a measure of mapping precision. In addition, we estimated 95% confidence intervals for each mapped locus using computer simulations (see Materials and Methods). The mapping deviations of the five genes, 0.2, 1.7, 1.1, 9.5, 0.2, and 24.2, are shown in Table 1. The mapping deviations of the five genes, 0.2, 1.7, 1.1, 9.5, 0.2, and 24.2, are shown in Table 1.
A variety of factors could cause alleles that contribute to a trait to be absent from some members of a pool that had been selected to express the trait strongly. To examine the robustness of our method to such deviations, we tested whether three drug-resistance genes could be mapped when their enrichment level in the selected pool is close to 70%–75% instead of 90%–100% as with our test case. Thus, we mixed equal amounts of DNA from a pool of segregants selected for resistance to geneticin, hygromycin, and nourseothricin and a control, unselected pool, yielding an approximately 3:1 ratio of target strain polymorphisms to reference polymorphisms in regions linked to the drug resistance genes. Although the LMSs were greatly reduced, we still observed three peaks that corresponded to the three selected genes, demonstrating that we can map alleles that are absent from a substantial fraction of the selected pool (Figure S3). In addition, the signal-to-noise ratio of the LMSs was still substantially high (Figure S5).

Computer Simulations of the Mapping Process

We developed a computer model that simulates the whole mapping process (see Materials and Methods) to assess the effect of experimental design (e.g., number of arrays), intrinsic genetic factors (e.g., recombination rate), and adjustable statistical parameters (e.g., p value cutoff for SFP detection) on mapping precision. Figure 3 presents the effect of the number of array replicates, noise levels between replicate intensities (coefficient of variation, the standard deviation divided by the mean), SFP FDR, SFP density (number of SFPs per 1 kb), and recombination rate on mapping deviation. Aside from the factor being varied, the parameters for the simulations were taken from our mapping experiments. The mapping deviations obtained with simulations are consistent with the experimentally observed mapping deviations for the five test case genes. Of the factors tested, SFP density and recombination rate displayed the strongest effect on mapping precision, with higher SFP density and higher recombination rate improving mapping precision. Even in regions with a ten-fold lower SFP density than average (~99% of SFPs lie in denser regions; Figure S2) or a four-fold lower recombination rate than average, our simulations suggest that a gene can successfully be mapped, albeit with lower resolution. We tested the effect of SFP density in our experiments by excluding varying fractions of

Table 1. Mapping Precision as a Function of Segregant Pool Size

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<th>Gene</th>
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<td>KAN&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.2 (0.1) ±44 (±17.6)</td>
<td>0.2 (0.1) ±44 (±17.6)</td>
<td>4.2 (1.7) ±43 (±17.2)</td>
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<td>HYG&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.3 (3.3) ±26 (±10.4)</td>
<td>23.7 (9.5)&lt;sup&gt;a&lt;/sup&gt; ±10 (±4)</td>
<td>2.7 (1.1) ±17 (±6.8)</td>
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<tr>
<td>NAT&lt;sup&gt;6&lt;/sup&gt;</td>
<td>24.2 (9.7) ±44 (±17.6)</td>
<td>11.2 (4.5) ±45 (±18)</td>
<td>51.2 (20.5)&lt;sup&gt;a&lt;/sup&gt; ±24 (±9.6)</td>
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The mapping deviations between the predicted positions of the mapped genes and their actual centers, and the estimated 95% confidence intervals (CI) are given in kb units rounded to the nearest 0.1 kb, and their corresponding genetic distance in cM is given in parentheses.

*These mapping deviations marked fall outside their estimated 95% confidence intervals. This may be because the local recombination rate is lower than the average rate used for the simulations, which would lead to an underestimation of the confidence intervals.

DOI: 10.1371/journal.pbio.0040256.t001

8.3, 9.5, and 24 kb (~0.1–10 cM) for KAN<sup>6</sup>, can1, HYG<sup>6</sup>, LYS5, and NAT<sup>6</sup>, respectively, all fell within their 95% confidence intervals, which ranged from ±10 to ±44 kb (±4 to ±18 cM). The mapping deviations are robust to the array preprocessing method used (see Tables S1 and S2, and Figure S4). This test case demonstrates that genes proximal to the centromere or telomere (can1 and KAN<sup>6</sup>) can be mapped at high resolution, and that two genes on the same chromosome arm can be easily separated from each other (HYGR and HYG<sup>6</sup>). The LMS is influenced by several factors, including SFP density, local recombination frequency, and the variance of the hybridization to individual probes on the array. As a result, the differences between the LMSs of the different peaks do not reflect the quantitative contribution of the different loci to the selected phenotype (see Protocol S1).

Aside from one potential false-positive peak found on Chromosome 10 at position 146.3 ± 30 kb, the signal-to-noise levels of the LMS was high (Figures S3 and S5). One possible interpretation for the extra peak is that it has uncovered real DNA differences between the target (W303) and reference (SK1) strains that contribute to the resistance to one or more of the drugs used for the selection. The failure to observe this peak in the can1, LYS5 pool supports this hypothesis, although we found no obvious candidates for genes whose polymorphisms might contribute to drug resistance in the 95% confidence interval on Chromosome 10.

To assess the consistency of our mapping precision, KAN<sup>6</sup>, HYG<sup>6</sup>, and NAT<sup>6</sup> were mapped in two additional selected pools derived from different initial W303/SK1 hybrid diploids, yielding a total of three completely independent mapping experiments (unpublished data). The genes’ mean mapping deviations and their standard deviations are 7.5 ± 6.4 kb, 4.1 ± 3.6 kb, and 14.9 ± 8.6 kb, respectively, with a total mean mapping deviation of 8.8 kb.

For some organisms or phenotypes, selecting for $10^7$ segregants that express the trait of interest is impractical. We therefore asked whether we could map the KAN<sup>6</sup>, HYG<sup>6</sup>, and NAT<sup>6</sup> alleles with $10^3$ or $10^4$ segregants. Even with $10^3$ segregants, the three drug-resistance genes were mapped to within 50 kb of their correct locations (Table 1; see Figure S5 for whole-genome maps). No significant correlation was seen between the mapping precision of the three genes and pool size ($10^0$, $10^1$, and $10^2$ segregants) ($p > 0.6$, t test).
the SFPs that lay near KAN^R from our analysis, to create an SFP density that ranged from 1.8 to 0.2 SFPs/kb. The negative trend of mapping deviation as a function of SFP density seen in simulations (Pearson’s correlation coefficient $R^2 = -0.96$ for mapping deviation versus logarithm of SFP density) corresponded closely to that observed in this manipulation of our experimental data ($R^2 = -0.95$). As increased SFP FDR has little impact on mapping precision, especially in our observed experimental range (3%–7%), choosing a more liberal cutoff for identifying SFPs might help to map genes located in regions with low SFP density. In contrast, the number of array replicates and coefficient of variation between replicate intensities show only a weak effect on mapping precision, in particular in the ranges relevant for our study (two to four arrays, and coefficient of variation of 8%–15%). The simulations suggest that duplicate arrays for the selected and control pools suffice, which is in agreement with our mapping results of the test genes (see Table S3).

Mapping an Experimentally Evolved Trait in Yeast

We used our mapping method to uncover the genetic basis of an experimentally evolved trait in yeast. We chose four W303 populations, derived from the same ancestor, that had been alternately grown in glucose- and galactose-containing media for 36 sexual cycles as part of a selection for altered mating preference [37] (Figure 4A). A single haploid clone was chosen from each population after the 36th cycle of selection. After ~700 generations all four strains had evolved to resume proliferation more rapidly than their ancestors when transferred from glucose- to galactose-containing medium (see below). GAL3, which encodes a coinducer of the galactose pathway [38], was found to be overexpressed three- to five-fold in the evolved strains compared with the ancestor when grown in medium with glucose as the sole carbon source (unpublished data).

We used GAL3 as a reporter gene to select for segregants from crosses between the evolved strains and SK1 (Ev/SK1) that express the adaptive phenotype. For each of the four evolved strains, about $10^4$ Ev/SK1 segregants that expressed high levels of Gal3 fused to a green fluorescent protein (GFP) were selected using flow cytometry (see Materials and Methods). The genomic DNA of the control and selected pools were hybridized onto two or three replicate arrays each and the LMS was calculated for the entire genome (Figure S6). In all four strains, the adaptive phenotype was mapped to the same region on Chromosome 13 with a mean peak center of 173.3 kb and a mean 95% confidence interval of ±56 kb (±14 cm; Figure 4B). An additional peak linked to GAL3-GFP on chromosome 4 was found for strain Ev2 because the Ev2/SK1 hybrid diploid was heterozygous for the GAL3-GFP with the one copy of GAL3-GFP lying on the chromosome derived from the evolved strain, while the other strains were homozygous for GAL3-GFP (Figure S6).

GAL80, which encodes the key repressor of the galactose utilization pathway [39], lies within 1 kb of the mean center of 173.3 kb and a mean 95% confidence interval of ±56 kb (±14 cm; Figure 4B). An additional peak linked to GAL3-GFP on chromosome 4 was found for strain Ev2 because the Ev2/SK1 hybrid diploid was heterozygous for the GAL3-GFP, with the one copy of GAL3-GFP lying on the chromosome derived from the evolved strain, while the other strains were homozygous for GAL3-GFP (Figure S6).

GAL80, which encodes the key repressor of the galactose utilization pathway [39], lies within 1 kb of the mean center of the linked intervals. We therefore sequenced this gene in the four evolved strains and in the ancestor. One or two missense mutations were found in GAL80 in all four strains (Figure 4C). Two of the strains carry the same mutation. One of the mutations, Q392H, has been recently identified in a screen for GAL80 mutations that cause loss of the Gal80 inhibitory activity [40]. Three other mutations, I361M, Q392H, and H36Y, lie in nuclear localization sequence regions of Gal80 [41]. The transcriptional regulation of the galactose utilization pathway, including the role of Gal80, is depicted in Figure 5A.

To test whether these mutations account for the adaptive phenotype, the endogenous GAL80 gene of the ancestral cells was replaced with the three different mutant genes, and the growth curves of these haploid strains were compared to those of the ancestor and a gal80Δ strain. Compared to the ancestral allele, all the mutations in GAL80 conferred a growth advantage during the transition from using glucose as
Figure 4. Four Evolved Strains That Independently Adapted to Glucose–Galactose Transition Acquired One or Two Missense Mutations in GAL80 (A) A schematic description of the evolution experiment. In each cycle of evolution, haploid cells (light blue) were grown in glucose-containing media for 2 d, mated on YPD plates, and then transferred to galactose-containing medium (Figure S7A–S7H). The ancestral strains carrying the evolved gal80 mutations behaved similarly to the gal80A strain, suggesting that the mutations in GAL80 cause a partial or complete loss-of-function of Gal80’s repressive activity on the galactose utilization pathway (Figure 5E). Hence, the gal80 mutations explain the growth advantage observed for the four evolved strains relative to the ancestor following transfer from glucose to galactose (~1.2–1.9-fold more evolved cells than ancestral cells 6 h after transfer; Figure 5F–5I). The slower growth observed for the ancestor compared with the evolved strains in the transition from glucose to galactose is likely the result of a growth delay while the ancestral cells repress Gal80’s inhibitory effect and induce the genes required for galactose uptake and catabolism.

We compared the effects of the mutations in GAL80 in two strains: the evolved strains and the strains produced by transforming these mutations into the ancestral strain. Ancestral cells carrying only the evolved gal80 mutations have a larger growth advantage relative to the ancestral GAL80 strains than do the evolved strains from which these mutations were rescued (compare Figure 5B–5D to 5F–5H). This difference is probably due to one or more other mutations that accumulated in the evolved strains and have a fitness cost under the conditions of our experiment. The slight decrease in fitness observed for the evolved strains (Ev2 and Ev42) relative to the ancestor when transferred from one glucose-containing medium to another supports this hypothesis (Figure S7E–S7L).

During each cycle of the evolution experiment, diploid evolving cells were transferred from glucose- to galactose-containing medium (Figure 4A). This selection prompted us to ask whether a single copy of the gal80 mutations conferred any advantage on a diploid background. We compared the growth curve of ancestral diploids heterozygous for one of the three sets of gal80 mutations to that of an ancestral diploid, a gal80Agal80A diploid, and a GAL80gal80A diploid, following transfer from glucose to galactose (Figure 5J–5M).

The intermediate growth levels of the heterozygous GAL80 gal80 diploid and the GAL80gal80A diploid compared with the ancestor and the gal80Agal80A diploid show that all three sets of gal80 mutations, similar to gal80A, are phenotypically semidominant. In a molecular sense, this argues that the GAL80 gene is weakly haploinsufficient, most likely because of the sole carbon source to galactose (~2-fold increase at 6 h; Figure 5B–5D). However, no significant growth differences were observed for cells transferred from glucose- to glucose-containing medium or from galactose- to galactose-containing medium (Figure S7A–S7H). The ancestral strains carrying the evolved gal80 mutations behaved similarly to the gal80A strain, suggesting that the mutations in GAL80 cause a partial or complete loss-of-function of Gal80’s repressive activity on the galactose utilization pathway (Figure 5E). Hence, the gal80 mutations explain the growth advantage observed for the four evolved strains relative to the ancestor following transfer from glucose to galactose (~1.2–1.9-fold more evolved cells than ancestral cells 6 h after transfer; Figure 5F–5I). The slower growth observed for the ancestor compared with the evolved strains in the transition from glucose to galactose is likely the result of a growth delay while the ancestral cells repress Gal80’s inhibitory effect and induce the genes required for galactose uptake and catabolism.

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We compared the effects of the mutations in GAL80 in two strains: the evolved strains and the strains produced by transforming these mutations into the ancestral strain. Ancestral cells carrying only the evolved gal80 mutations have a larger growth advantage relative to the ancestral GAL80 strains than do the evolved strains from which these mutations were rescued (compare Figure 5B–5D to 5F–5H). This difference is probably due to one or more other mutations that accumulated in the evolved strains and have a fitness cost under the conditions of our experiment. The slight decrease in fitness observed for the evolved strains (Ev2 and Ev42) relative to the ancestor when transferred from one glucose-containing medium to another supports this hypothesis (Figure S7E–S7L).

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Figure 5. Transformation of gal80 Mutations into the Ancestral GAL80 Reconstructs the Adaptive Phenotype

(A) A schematic depiction of the regulation of the galactose utilization pathway. In the absence of galactose, Gal80 inhibits the transcriptional activator, Gal4, by binding to Gal4 in the nucleus. When galactose is present it enters the cell through Gal2 transporters and binds Gal3, a coinducer of the pathway, which in turn binds Gal80 in the cytoplasm, sequestering Gal80 away from the nucleus. This relieves the repression of Gal4 allowing it to induce the transcription of genes required for galactose uptake and catabolism (GAL genes), including GAL2, GAL3, GAL80, and the genes encoding the enzymes of galactose catabolism [38]. A similar phenotype is obtained through loss-of-function of the repressor, GAL80.

(B–E) The gal80 mutations confer a fitness advantage in transfers of exponentially growing haploid cells from glucose- to galactose-containing medium, but not in transfers in which the carbon source does not change (Figure S7). Three different sets of gal80 mutations in the coding region were transformed into the ancestral GAL80 gene in an ancestral haploid strain (A0) (Ev2 indicates the mutation is from evolved culture 2, etc.). Ev42 and Ev43 have the same mutation in the coding region. Cell density (OD) was measured for each of these strains and for the ancestor and a GAL80 knockout strain following transfer from glucose- to galactose-containing medium.

(F–I) The four evolved strains are more fit than their ancestor (A0) when transferred from glucose- to galactose-containing medium. Cell density (OD) was measured for the haploid evolved strains Ev2, Ev14, Ev43, and Ev42, and their ancestor, following transfer from medium containing only glucose to medium containing galactose.

(J–M) The gal80 mutations have a semidominant effect when present in one copy in the ancestral diploid strain following transfer from glucose- to galactose-containing medium. The cell density (OD) of ancestral diploids carrying one copy of the gal80 mutations from either Ev2, Ev14, or Ev43 were compared to that of an ancestral diploid (A0) and a diploid lacking both copies of GAL80 (gal80D/gal80D), following transfer from glucose- to galactose-containing medium. As a control, an ancestral diploid was made hemizygous for GAL80 (M; GAL80/gal80D).

Mean cell density and a standard deviation from at least three independent cultures were plotted for each datapoint for (B–M). Error bars that are not visible are smaller than the datapoint.

DOI: 10.1371/journal.pbio.0040256.g005
its need to titrate Gal4, the transcriptional activator. In an evolutionary sense, our observations suggest that the gal80 mutations could have been initially selected for when only one copy of the mutations was present in the diploid.

Discussion

We present an optimized method that maps adaptive mutations in yeast with higher precision and less work than previous linkage-based mapping methods [22-24,25]. One advantage of our method is the capacity to predict where the linked locus is most likely to lie within a mapped region, which helps prioritize sequencing and candidate gene testing. Therefore, even though our estimated 95% confidence intervals (20–88 kb [8–35 cM]) are comparable with mapping intervals identified in previous SFP-based methods that analyze ∼20 segregants individually in yeast (8–72 kb [3–29 cM]) [22,24,25], the centers of our mapping predictions are typically much closer to the actual mutations. By pooling at least 10,000 segregants from a single cross, we obtained mapping deviations of test genes that ranged from 0.2–24 kb (0.1–10 cM). In four independently evolved populations, mutations in the same gene (GAL80) were mapped with a mean mapping deviation of 5.7 kb, and the average position of the four predicted positions was 1 kb from the center of the GAL80 gene. Furthermore, pooling makes mapping easier and cheaper than analyzing single segregants. We can analyze more than 10^7 selected segregants simultaneously using fewer arrays (a minimum of four) than are needed to individually analyze ∼20 single segregants (20 arrays). In both methods, additional arrays (at least two) are needed for the initial, one-time prediction of SFPs. If necessary, our method can be applied to pools that contain as few as 100 segregants. This is important for organisms that produce few progeny, or for phenotypes that must be assessed by assaying individually selected segregants, which are then assembled into pools, rather than directly selecting on pooled meiotic progeny.

Since our method can simultaneously map multiple genes with high efficiency, including genes lying on the same arm (Hyg and Nat) and genes affecting a quantitative phenotype (GAL80), our method could be useful for multigenic or QTL mapping. This combination of high-throughput genotyping with oligonucleotide arrays [22] and pooling [27] has also been applied in plants [33,34], and should accelerate QTL detection compared with traditional single-segregant mapping methods in a wide variety of organisms [20,21,42,43]. Our method has advantages and disadvantages compared to other forms of QTL mapping. We do not make assumptions on the number of contributing QTLs or the type of interactions between them, as multiple QTL and composite interval mapping methods must do [21]. By selecting and genotyping pools with extreme phenotypic values, we gain mapping power, but we cannot estimate the relative effect of individual QTLs on a trait. Previous studies show that QTL effect can be estimated by genotyping pools with broader phenotypic values from the lower and upper tails of the phenotypic distribution and associating the differences in phenotypic means of the two pools to differences in their marker allele frequencies [35,44,45]. Another issue is that pools lack information on the phase between genetic markers (e.g., haplotypes) and QTLs, making it hard to learn about the type of interactions between QTLs (e.g., additive or epistatic) or to recognize distinct subsets of QTLs that can independently give rise to the same trait [27]. Since pool genotyping is commonly used in human association studies [27,30], it would be interesting to explore whether our method and its statistical framework could be extended to such studies [46].

We developed a computer model that simulates the mapping process to better understand the effects of various factors on mapping precision, and to improve our experimental protocols. The parameters of the model can be adjusted so that the simulations can be applied to other experimental designs, such as backcrosses, and to different organisms. Our simulations suggest that marker density and recombination rate are the major factors affecting mapping precision. While we have generated a very dense genetic map of about 10,300 DNA markers (on average ∼1 SFP/kb), the model predicts that with tighter genetic marker spacing (two to four markers per kb) our method could reach even higher mapping resolutions, corresponding to a few genes in yeast (∼1–2 cM). Tiling arrays that contain oligonucleotides that cover the whole genome and that are available for some organisms (recently including yeast [47]) will provide such high SFP coverage. Alternatively, different reference strains with different polymorphism distributions compared with the target strain can be used to increase genome coverage and marker density.

We showed that four independently evolved strains found the same genetic solution to repeated transitions from glucose- to galactose-containing medium and two of the strains independently acquired the same mutation. All three sets of mutations in GAL80 reduced its ability to repress genes involved in galactose metabolism. Thus, we observed parallel evolution at the genetic level, as has been seen in viruses, bacteria, and yeast that have been experimentally adapted to stressful conditions [1,2,4], and in fish with pelvic and armor plate reduction, and albinism [17,48,49]. Mutations in GAL3 or GAL4 have been shown to lead to constitutive expression of the galactose utilization pathway [50,51]. We did not find gain-of-function mutations in these genes, most likely because the target size for loss-of-function mutations in GAL80 is much larger than that for gain-of-function mutations in GAL3 or GAL4. All the missense mutations we found in GAL80 lie in residues that are highly conserved across yeast species from Saccharomyces cerevisiae to Kluyveromyces lactis (Figure S8). Our results, together with other studies [2,17], support the notion that mutations in regulatory genes may lead to large benefits in populations subjected to changing environments.

Although much effort has gone into studying evolutionary changes in experimental and natural populations [3,52], many questions remain. Is there correlation between the number, effect, and nature of the adaptive mutations and the molecular pathways that are subjected to the selective pressure? To what extent do evolutionary paths overlap at the genetic level between populations subjected to identical selective pressures and how does such overlap depend on the underlying network? The high-throughput and high-resolution aspects of our mapping method (freely available at http://www.cgr.harvard.edu/MutationMapping) make it amenable for such large-scale studies in yeast or higher eukaryotic organisms, as well as for studying the genetic basis of quantitative traits.
Materials and Methods

Genomic DNA hybridizations.

Selection for segregants expressing adaptive phenotype.

SFP identification.

SFP verification and optimization.

Mutation Mapping of Evolved Traits

Evolution experiment.

Materials and Methods

Selection for segregants expressing adaptive phenotype.

SFP identification.

SFP verification and optimization.
with a prefact match to a unique sequence in S288c and no perfect match in YJM789. We hybridized the genomic DNA of S288c and YJM789 onto eight arrays each and SFPs were predicted using the array preprocessing and normalization, and SFP identification algorithms described above. The false-positive rate calculated for S288c/YJM789 can be used to estimate an FDR between any two populations. The linkage disequilibrium-based algorithm assumes that recombination rate is proportional to the distance between two loci, and that two loci that are more than 50 cM apart are unlinked. The simulations are done at the level of the array hybridization intensities. The replicate hybridization intensities of the target strain and control pool, $L_t(i)$, onto an SFP indexed $i$ are sampled from a normal distribution with a mean intensity, $\mu(i)$, and standard deviation, $\sigma(i)$. These values are taken from observed intensity data of sample a at SFP i (Equation 6).

$\mu(i) = N(\mu(i), \sigma(i))$ (Equation 6)

The mean intensity of the selected pool onto SFP $i$ is calculated according to Equation 7:

$\mu^S(i) = \frac{(1 - d_{ij}) \times \mu(i) - \mu^C(i)}{U}$, if $d > 1 \Rightarrow d = 1$

where $d_{ij}$ is the relative genetic distance between an SFP at position $X_i$ and a simulated target locus at position $X_j$, and $U$ denotes the average unlinked physical distance. We assume $d_{ij}$ is proportional to the recombination rate between two loci, and $U = 125$ kb ($\sim 50$ cM) based on an average recombination rate of 1 cM per 2.5 kb in yeast. A relative distance ($d_{ij}$) of 1 represents no linkage, while a relative distance of 0 represents 100% linkage, $T$ represents the target strain, $C$ the control pool, and $S$ the selected pool. The standard deviation of the hybridization intensities of the selected pool onto SFP $i$ is derived from the coefficient of variation ($\mu(i)$) of the control pool, as shown in Equation 8:

$\sigma(i) = \sqrt{(\frac{\mu^S(i)}{\mu^C(i)}) \times \mu(i)}$

Finally, the replicate hybridization intensities of the selected pool onto SFP $i$ are sampled from a normal distribution with a mean intensity, $\mu(i)$, and a standard deviation, $\sigma(i)$ (Equation 6). The linkage analysis procedure described in the previous section is applied to the simulated hybridization intensities of replicate arrays. The absolute value of the distance between the estimated center of the detected significant peak and the actual position of the simulated target locus is recorded for each simulation. The mapping deviation is defined as the 95th percentile of the ranked deviations calculated from $n$ simulation runs. In this work, $n = 1,000$ simulation runs were done for each set of parameters tested. Aside from the varying factor, parameters were chosen according to those used or observed in our mapping test case, including an average recombination rate of 1 cM per 2.5 kb in yeast, SFP FDR of 0.6%, mean SFP density of 0.91 SFP/kb, four array replicates for the selected and control pools and eight replicates for the target strain, smoothing window size of 50 SFPs, SFP mean intensity and standard deviations of the target strain and control pool were randomly sampled without replacement from the distribution of replicate SFP intensities from our test case experiments. For each simulation run, a different subset of SFPs was randomly flagged as false positive, and the intensities of the false SFPs were randomly sampled from the observed intensities of the target strain at nonpolymorphic features.

### 95% confidence interval estimation.

To estimate the 95% confidence intervals for a predicted linked region, a simulated target locus was positioned at the predicted center, and the 95th percentile of the ranked mapping deviations from 1,000 simulation runs was recorded. The distribution of 95% confidence intervals was used in our mapping experiments for a given chromosome was used for the simulations, as well as the observed mean intensities and coefficients of variation of the target strain and control pool at the corresponding SFPs. The
estimated 95% confidence intervals are rough estimates, as a uniform mean recombination rate is assumed across the whole genome due to lack of detailed data on the local variation in recombination rate. As a result, when local recombination rates are higher than average, the 95% confidence intervals are placed too far away from the predicted position of the target locus, and when local rates are lower than average the intervals are placed too close to the predicted position.

**GAL80 sequencing.** To sequence GAL80, we amplified a fragment on Chromosome 13 from position 171,100 to 173,315 bp. We designed sequencing primers ~400 bp apart on the same strand with ~200-bp separations between primers on opposite strands. Both strands of the PCR product were sequenced using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California, United States) and the product read with an ABI3100 Genetic Analyzer. The sequence readouts were assembled into a single contig using ContigExpress (part of VectorNTI software; Invitrogen); 2–4× coverage was obtained.

**Mutant reconstruction and growth curve assays.** To reconstruct the evolved mutations in the ancestral strain, the mutant gall80 genes and flanking sequences were amplified by PCR from the evolved strains and transformed into an ancestral strain whose GAL80 gene was replaced by a URA3 gene. The transformants were then plated on 5-fluoro-orotic acid-containing medium which selects against the URA3 gene and thus for cells where gall80A::URA3 has been replaced by the mutant gall80 genes. The structure of the GAL80 locus in each of the transformants was checked by PCR, and the genomic DNA was sequenced to show that the mutant allele had been properly integrated without introducing any further mutations. For growth curve, cells were grown in YPD (2% glucose) or YEP (2% galactose) for 4 hours and then transferred to (in the galactose-to-galactose transfers) overnight, diluted and refreshed in the same medium for 4 hours and then transferred to YPD or YEP + galactose medium. Cell numbers were estimated from the optical densities of the cell cultures using a spectrophotometer (DU640B, Beckman Coulter, Marseille, France). In each assay, at least three independent cultures were set up and their average and standard deviation is shown at each sample point.

### Supporting Information

**Figure S1.** Sensitivity and Specificity of SFP Identification Evaluated Using Two Yeast Strains with Known Sequences

(A) The true-positive, false-positive, and false discovery rates of S288c/YJM789 SFP identification are presented in percentages for a wide range of \( p \) value cutoffs (10^{-1} to 0.1). True-positive rate refers to the fraction of features that are truly polymorphic that are scored as polymorphic; false-positive rate refers to the fraction of non-polymorphic features that are scored as polymorphic; and FDR refers to the fraction of features that are scored as polymorphic that are actually non-polymorphic. The true-positive rate was determined by blasting the Affymetrix probe sequences that were derived from the S288c genome, against the YJM789 genome. Of the 120,050 Affymetrix probes tested, 107,448 were found to be nonpolymorphic between the strains, and 12,602 were found to be polymorphic (SFPs). The rates presented here were calculated using a one-tailed two-sample \( t \) test between eight replicate arrays for each strain. When considering only the SFPs whose polymorphisms lie in the central 15 bp of the 25mer probe (7,588 SFPs), the true-positive rate of SFP identification increased by 15%–30% compared with using all SFPs (third column). The true-positive rate is plotted as a function of \( p \) value cutoff rate (B) and FDR (C) in percentages over a \( p \) value cutoff range of 10^{-8} to 0.1. The dots correspond to the \( p \) values given in (A) from \( p = 10^{-8} \), the far left point, to \( p = 0.1 \), the far right point. Of the \( p \) value cutoffs in (A), \( p = 10^{-6} \) gave the maximum true-positive–false discovery rate ratio (labeled in red in panels [A], [B], and [C]). We used the FDR and not the false-positive rate to evaluate the specificity of our SFP identification, as the fraction of SFPs that are false is more relevant for mapping.

Found at DOI: 10.1371/journal.pbio.0040256.sgn001 (230 KB PDF).

**Figure S2.** Distribution of the Distances between Consecutive W303/SK1 SFPs along the Genome

At a \( p \) value cutoff of 10^{-5}, 10,350 W303/SK1 SFPs were identified using a one-tailed two-sample \( t \) test on eight replicate arrays for each strain. (A) The percentage of SFP pairs is plotted as a function of the distance between consecutive SFPs along all 16 chromosomes in kb units, in 1-kb bins centered around the bin points. Note the logarithmic scale of the y-axis. The mean SFP spacing is 1.14 kb (0.5 cM). (B) A cumulative distribution of the percentage of SFP pairs that are less than \( x \) kb apart is plotted as a function of the distance between all consecutive SFPs (X). About 75% of the SFP pairs are less than 1 kb (0.4 cM) apart, and 99% of the SFP pairs are less than 11 kb (4.4 cM) apart. Note the y-axis scale.

**Figure S3.** Whole-Genome Mapping of Five Test Case Genes Using Different Pool Sizes

The LMS is plotted across all 16 yeast chromosomes for five selected W303/SK1 segregant pools: (A) \( 10^7 \), (B) \( 10^8 \), (C) \( 10^9 \) segregants resistant to geneticin, hygromycin, and nourseothricin \((\text{KAN}^R, \text{NAP}^R, \text{HYG}^R)\), and (D) \( 10^7 \) segregants resistant to canavaine that are prototrophic to lysine \((\text{can}1)\) and \(\text{LYS}5\). The five peaks that located the five selected genes all fell above the peak cutoffs estimated for each selected pool separately at 95% confidence (horizontal dashed lines drawn only in [B], [D], [F], [H], and [J]). The peaks are labeled a through e according to the gene they map: a, \(\text{KAN}^R\); b, \(\text{NAP}^R\); c, \(\text{HYG}^R\); d, \(\text{can}1\); and e, \(\text{LYS}5\). See Figure 2E and Table 1 for mapping deviations of predicted peak centers from the corresponding linked genes and estimated 95% confidence intervals. (B), (D), (F), and (H) are y-axis close-ups of (A), (C), (E), and (G), respectively. A single false-positive peak on Chromosome 10 was detected in the pools selected for resistance to geneticin, hygromycin, and nourseothricin \((\text{A}–\text{D}; \text{labeled with a green asterisk})\). See text for possible explanation for observing this peak. The signal-to-noise level drops dramatically as the number of segregants decreases (in the galactose-to-galactose transfers) overnight, diluted and refreshed in the same medium for 4 hours and then transferred to YPD or YEP + galactose medium. Cell numbers were estimated from the optical densities of the cell cultures using a spectrophotometer (DU640B, Beckman Coulter, Marseille, France). In each assay, at least three independent cultures were set up and their average and standard deviation is shown at each sample point.

**Figure S4.** Whole-Genome Mapping of Five Test Case Genes Using a Different Array Preprocessing Method

Whole-genome mapping plots of our five test case genes are presented using a different array preprocessing method that considers only the PM probes and not the MM ones, as does the method used in this paper (PM minus MM). The preprocessing method includes taking the logarithm on base 2 of each PM value and dividing it by the median log_{10} PM of the local invariant probes on the array, similar to an approach used in previous single segregant mapping methods in yeast \([22, 24, 25]\). The arrays were processed using this method for both the SFP identification at a \( p \) value cutoff of 10^{-6} and for the linkage analyses. The LMS is plotted across all 16 yeast chromosomes for five different selected segregant pools: (A) \( 10^7 \), (B) \( 10^4 \), and (C) \( 10^2 \) segregants resistant to geneticin, hygromycin, and nourseothricin \((\text{KAN}^R, \text{NAP}^R, \text{HYG}^R)\); (D) \( 10^7 \) segregants resistant to canavaine that are prototrophic to lysine \((\text{can}1)\) and \(\text{LYS}5\); and (E) \( 10^7 \) segregants resistant to geneticin, hygromycin, and nourseothricin \((\text{A}–\text{D}; \text{labeled with a green asterisk})\). The significant peak cutoff is drawn in (E) (horizontal dashed lines drawn only in [B], [D], [F], [H], and [J]). The peaks are labeled a through e according to the gene they map: a, \(\text{KAN}^R\); b, \(\text{NAP}^R\); c, \(\text{HYG}^R\); d, \(\text{can}1\); and e, \(\text{LYS}5\). To test whether we could map genes that are not fully mapped, we analyzed a subset of segregants selected in the pools selected for resistance to geneticin, hygromycin, and nourseothricin \((\text{A}–\text{D}; \text{labeled with a green asterisk})\). We used the FDR and not the false-positive rate to evaluate the specificity of our SFP identification, as the fraction of SFPs that are false is more relevant for mapping.

Found at DOI: 10.1371/journal.pbio.0040256.sgn003 (1.1 MB PDF).
which are shown to scale. The mapping method appears to be robust to the array preprocessing method used, although the PM minus MM method seems to yield slightly better mapping precisions on average than the PM only (log_{10} PM) method (see Table S1). We estimated the mapping deviations of 23 loci (including several mapping repetitions of drug resistance genes, different pool sizes, 75% enrichment of test genes, and different copy numbers) using either the MM mapped 16 of 23 loci with higher precision than log_{10} PM. Assuming the two methods are equally good, and thus each method has a probability of 0.5 of yielding a smaller mapping deviation for each measurement, the probability of seeing a bias of 16 to 7 or larger using the binomial distribution is < 0.001.

Found at DOI: 10.1371/journal.pbio.0040256.sg004 (545 KB PDF).

Figure S5. Distribution of Whole-Genome LMSs following Different Enrichment Levels of Target Alleles in the Selected Pool

The signal-to-noise ratio of the whole-genome LMS is high even with only 75% enrichment of the target alleles in the selected pool. The distribution of peak heights of the three mapped genes is presented for the mapping of the three drug-resistance genes, KAN^{\text{\textregistered}}, HYG^{\text{\textregistered}}, and NAT^{\text{\textregistered}}, that are represented in either (A) 90%–100% (from Figure S3A) or (B) 70%–75% (from Figure S3J) of the segregants in the selected pool. The red arrows mark the 95th percentile of the ranked LMS values, and the black arrows mark the range of the peak heights of the three mapped genes. Note the x-axis is on a log scale and on the same scale for the two panels. Although the LMS values are much smaller with 75% enrichment, the signal-to-noise ratio is still high. With 75% enrichment, the peak heights are 35- to 39-fold larger than the 95th percentile of the ranked LMS values. In order not to lose true-positive peaks that fall below an estimated cutoff, peaks can be ranked according to height or area, and lower ranked peaks that fall below the cutoff can be tested later. To increase mapping sensitivity, a control pool could be made up of segregants from the opposite extreme tail of the phenotype distribution to that of the selected pool (i.e., segregants that do not express the trait of interest or that express it to a low phenotypic extent) [45] instead of segregants randomly sampled from the array. If segregants that do not express the trait of interest or that express it to a low phenotypic extent are used, the mapping deviation of the predicted target locus position from its simulated position in kb, the filtering method seems to yield slightly better mapping precisions on average than the PM method (log_{10} PM) method (see Table S1). We estimated the mapping deviations of 11 different target loci positioned in 10-kb increments from positions 90 to 110 kb along the chromosome. For each window size the standard deviation between the mapping deviations of the eleven loci is represented with error bars. Aside from the smoothing window size variable, parameters were set according to those used or observed in our mapping test case (for more details see the Mapping Simulations section in Materials and Methods). Mapping deviation decreases as a function of smoothing window size in the range of 10–30 SFPs per window, while in the range of 30–50 SFPs, mapping deviation is at best weakly dependent on window size. Found at DOI: 10.1371/journal.pbio.0040256.sg009 (201 KB PDF).

Protocol S1. Factors That Affect LMS

Found at DOI: 10.1371/journal.pbio.0040256.sd001 (25 KB DOC).

Table S1. Mapping Precision as a Function of Different Array Preprocessing Methods

We tested the effect of different array preprocessing methods on mapping precision and found that our method is fairly insensitive to the method used. The mapping deviations of the predicted positions of the five test case genes from their real centers are given in kb. The 10,330 W303-SK1 SFPs identified using the PM-MM preprocessing method at a p value cutoff of 10^{-6} were used for the comparison. The mean mapping deviations and standard deviations for KAN^{\text{\textregistered}}, HYG^{\text{\textregistered}}, and NAT^{\text{\textregistered}} were calculated from three separate mapping experiments. To calculate a local background (b1, b2, b3) we divided the array into 10 x 10 squares and subtracted the following values from each PM: (1) median of the MM values lying in the square encompassing the given PM (b1); (2) mean of the lower 2% of ranked PM and MM values in the corresponding square (b2); and (3) mean of the lower 2% of ranked PM values in the corresponding square (b3). These methods are similar to those used by the Affymetrix GeneChip software (http://www.affymetrix.com/support/technical/whitepapers.affx; Statistical Algorithms Description Document [57]) and Li and Wong’s dChip software [41]. All intensities were normalized by a median of a spatially local set of invariant PM values. For log_{10} PM the logarithm of PM was divided by the median log_{10} PM of the local invariant probes, similar to the approach used in previous single segregant mapping methods in yeast [22,24,25]. In the software of our mapping method, the user will have the option of choosing between different array preprocessing methods, including those that do not use the MM, and will be able to adjust tunable parameters, such as the percentile of ranked intensities used for background subtraction (http://www.cgr.harvard.edu/MutationMapping). This will allow users to find the optimal method for their mapping system.

Found at DOI: 10.1371/journal.pbio.0040256.st001 (43 KB DOC).

Table S2. The Effect of Using Different Array Preprocessing Methods for Identifying SFPs on Mapping Precision

The mapping precision of our method is robust to the array
preprocessing method used for calling features polymorphic (SFPs) at a p value cutoff of 10^{-6}. Different probe preprocessing methods were used to identify SFPs, and the linkage analysis was then done using the PM-MM preprocessing method. This allowed us to isolate the effect of the preprocessing method used to find SFPs on mapping precision. The mapping deviations of the predicted positions of the five test case genes from their real centers are given in kilobases. Although the true-positive–to–false discovery rate ratios may vary between the different methods (unpublished data), this does not appear to have a significant effect on the final outcome of the mapping. This is in accordance with the predictions of our simulations, that false SFPs do not have a significant effect on mapping precision (Figure 3C). The local backgrounds (b1, b3) are defined in Table S1. The mean mapping deviations and standard deviations for KAN^R, HYG^R, and NAT^W were calculated from three separate mapping experiments.

Found at DOI: 10.1371/journal.pbio.0040256.s002 (36 KB DOC).

Table S3. Mapping Precision of Test Case Genes as a Function of Array Replicate Number

The mapping deviations of the predicted gene locations from their actual centers are presented here as an average of the absolute deviations for each gene across all three replicates. The number of array replicates refers to the selected pool, control pool, triplicate, and quadruplicate arrays out of four replicates from a single mapping experiment. Mapping deviation is given in kb. The array replicate numbers refer to the selected pool, control pool, and target strain, W303. R^2 denotes the coefficient of determination for mapping deviation as a function of replicate number.

Found at DOI: 10.1371/journal.pbio.0040256.s003 (31 KB DOC).

Table S4. Genotype of Yeast Strains

The National Center for Biotechnology Information (NCBI) accession numbers for the genes and gene products discussed in this paper are S288c genome (NC_001133–NC_001148) and YJM789 genome (AAFW01000001–AAFW01000150). The Saccharomyces Genome Database (http://www.yeastgenome.org) accession numbers for genes and gene products discussed in this paper are gal1 (S000000789), LYS2 (S000000789), GAL3 (S000002416), GAL80 (S0000004514), GAL4 (S0000006169), and Gal2 (S000004071).

Acknowledgments

We thank G. Church, N. Ingolia, A. Regev, D. Segré, and W. Wong for computational and statistical advice, and N. Barkai, G. Lahav, B. Stern, and members of the Murray lab for helpful discussions and comments on the manuscript. We thank the Baser Center for Genomics Research for microarray support, L. Shumway for assistance with flow cytometry cell sorting, and the Davis lab for providing YJM789.

Author contributions. AVS, JYL, and AW conceived and designed the experiments. AVS and JYL performed the experiments. AVS analyzed the data. AVS contributed reagents/materials/analysis tools. AVS wrote the paper.

Competing interests. The authors have declared that no competing interests exist.

Funding. This work was supported by grants from the National Institute of General Medical Sciences, Center of Excellence on Evolution: ES1 P50 GM068763 (AVS, JYL), a Merck Genome-Research Award (JYL), and a Michael and Anna Vranos Graduate Fellowship Fund in Life Sciences (AVS).

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