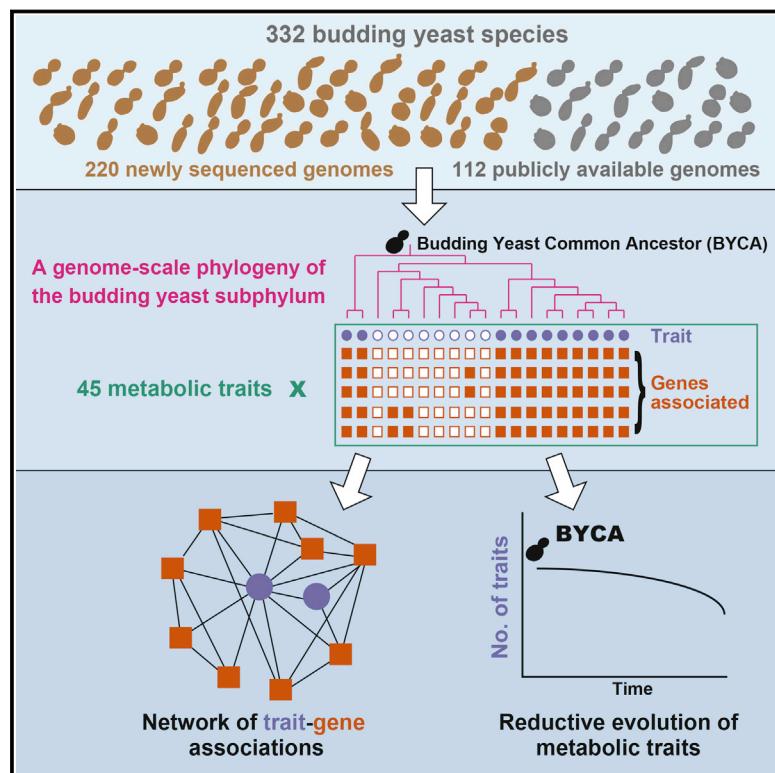


Tempo and Mode of Genome Evolution in the Budding Yeast Subphylum

Graphical Abstract



Highlights

- 332 genomes, including 220 newly sequenced, covering ~1/3 of known budding yeasts
- Genome-scale inference of robust phylogeny and time tree of budding yeast subphylum
- Reconstruction of 45 metabolic traits infers complex budding yeast common ancestor
- Reductive evolution of traits and genes is a major mode of evolutionary diversification

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In Brief

An integrated phylogeny of over 300 budding yeast species encompasses the natural diversity and history of diversification of Saccharomycotina with insights into a metabolically complex common ancestor and common reductive evolution leading to metabolic specialization.



Tempo and Mode of Genome Evolution in the Budding Yeast Subphylum

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SUMMARY

Budding yeasts (subphylum Saccharomycotina) are found in every biome and are as genetically diverse as plants or animals. To understand budding yeast evolution, we analyzed the genomes of 332 yeast species, including 220 newly sequenced ones, which represent nearly one-third of all known budding yeast diversity. Here, we establish a robust genus-level phylogeny comprising 12 major clades, infer the timescale of diversification from the Devonian period to the present, quantify horizontal gene transfer (HGT), and reconstruct the evolution of 45 metabolic traits and the metabolic toolkit of the budding yeast common ancestor (BYCA). We infer that BYCA was metabolically complex and chronicle the tempo and mode of genomic and phenotypic evolution across the subphylum, which is characterized by very low HGT levels and widespread losses of traits and the genes that control them. More generally, our results argue that reductive evolution is a major mode of evolutionary diversification.

INTRODUCTION

Yeasts—unicellular fungi that lack fruiting bodies—have evolved multiple times across fungi (Stajich et al., 2009), but most known yeast species belong to the subphylum Saccharomycotina (hereafter referred to as budding yeasts or simply yeasts). This diverse group, whose genetic diversity is on par with the plant and animal lineages (Figure 1), includes the baker's yeast and premier eukaryotic model system *Saccharomyces cerevisiae* (Peter et al., 2018), the common human commensal and opportunistic pathogen *Candida albicans*, and over 1,000 other known species with more continuing to be discovered (Dujon and Louis, 2017; Hittinger et al., 2015; Kurtzman et al., 2011).

Yeasts exhibit remarkably diverse heterotrophic metabolisms, which have allowed them to successfully partition nutrients and ecosystems to inhabit every continent and every major aquatic and terrestrial biome (Kurtzman et al., 2011; Opulente et al., 2018). Comparative genomic investigations have served as the launching pads into the evolution of budding yeast metabolism and ecological specialization in numerous clades, including the clade of *S. cerevisiae* and its close relatives (Hittinger, 2013), the genus *Lachancea* (Vakirlis et al., 2016), the *C. albicans/Candida tropicalis* clade (Butler et al., 2009), and the *Candida*



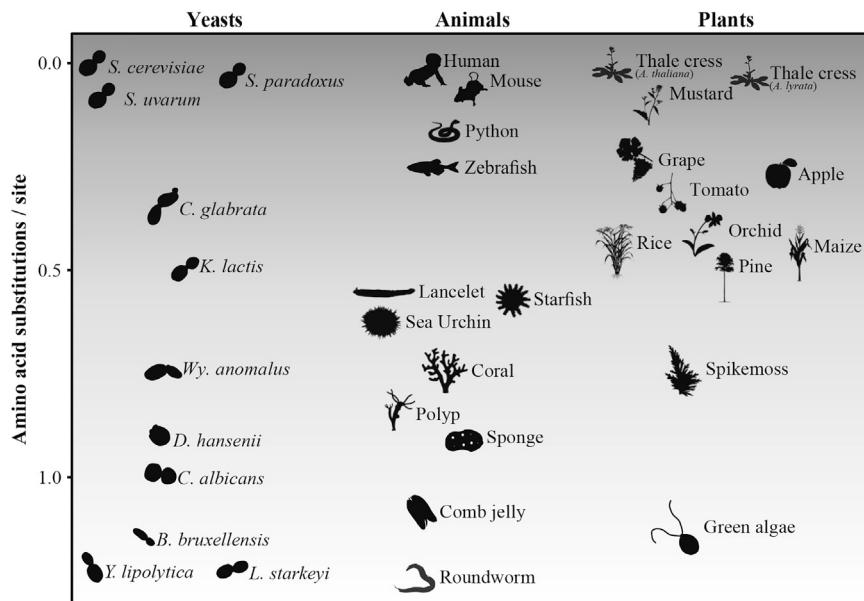


Figure 1. Levels of Evolutionary Sequence Divergence within the Budding Yeast Subphylum Are on Par with Levels Observed in Animals and Plants

The phylogenetic distance (in terms of amino acid substitutions/site) between iconic species in budding yeasts (*Saccharomyces cerevisiae*), animals (*Homo sapiens*), and plants (*Arabidopsis thaliana*) and other representative species in each lineage. For each lineage, phylogenetic distance was estimated from a concatenated ML tree inferred from analysis of 295 single-copy BUSCO genes. *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. paradoxus*, *Saccharomyces paradoxus*; *S. uvarum*, *Saccharomyces uvarum*; *C. glabrata*, *Candida glabrata*; *K. lactis*, *Kluyveromyces lactis*; *Wy. anomalous*, *Wickerhamomyces anomalous*; *D. hansenii*, *Debaryomyces hansenii*; *C. albicans*, *Candida albicans*; *B. bruxellensis*, *Brettanomyces bruxellensis*; *L. starkeyi*, *Lipomyces starkeyi*; *Y. lipolytica*, *Yarrowia lipolytica*. Images representing taxa were drawn by hand, taken from PhyloPic (<http://phylopic.org>), or modified from Google Images. The data matrix and ML tree used for calculating sequence divergence in each of the three lineages are provided in the Figshare depository.

glabrata/Nakaseomyces clade (Gabaldón et al., 2013). Although these investigations have shaped our understanding of budding yeast evolution, they have either focused on small slices of biodiversity or have been very broad investigations using collections of taxa skewed toward biomedically or industrially relevant yeasts (Gabaldón et al., 2013; Génolevures Consortium et al., 2009; Riley et al., 2016).

This sparse and sporadic sampling of genome sequences has forestalled efforts to examine the impact of different evolutionary processes or to identify major evolutionary trends across the entire subphylum. For example, several illustrative studies have shown how the presence of specific genes and pathways correlates with phenotypes, including clear cases of gains (Gonçalves et al., 2018; Hall and Dietrich, 2007) and losses (Hittinger et al., 2004; Riley et al., 2016; Slot and Rokas, 2010; Wolfe et al., 2015), but the frequency and generality of these observations remain unclear. Collectively, the use of a relatively small subset of distantly related budding yeast genomes has prevented the state-of-the-art quantification and statistical analyses that would be required to understand the tempo and mode of genomic and metabolic evolution across the subphylum.

To address this gap, we sequenced the genomes of 220 budding yeast species and coupled them with the published genomes of an additional 112 species to investigate the evolution of biodiversity of the subphylum Saccharomycotina. By interrogating genomic and metabolic trait variation, we reconstructed a robust genome-wide phylogeny, established the geological timeline of budding yeast diversification, quantified horizontal gene transfer (HGT) in budding yeast genomes, and inferred the evolution of 45 metabolic traits and their underlying genetic toolkit from the ~400 million-year-old budding yeast common ancestor (BYCA) to the present.

RESULTS AND DISCUSSION

332 Yeast Genomes Spanning the Diversity of the Yeast Subphylum

We sampled the genomes of 332 budding yeast species representing 79/92 genera across the subphylum Saccharomycotina (STAR Methods). Of the 332 yeast genomes, 112 were publicly available and previously published, 24 sequenced by RIKEN were publicly available (http://www.jcm.riken.jp/cgi-bin/nbrp/nbrp_list.cgi) but are described here for the first time, and 196 were newly sequenced by the Y1000+ Project (<http://www.y1000plus.org>) (Figures 2 and S1; Table S1). 195/196 newly sequenced Y1000+ Project genomes were from type strains (the other was from an authentic strain), as were at least 81/136 publicly available ones; in addition, 72/79 included genera were represented by their type species (Table S1).

Compared to the 136 publicly available genomes, the 196 newly sequenced genomes (Y1000+ Project genomes) had generally smaller average N50 values (new = 417.2 kb vs. public = 1,235.4 kb), but they had comparable degrees of genome assembly completeness (new = 91.3% vs. public = 94.0%), GC contents (new = 40.3% vs. public = 41.1%), and average numbers of predicted genes (new = 5,822.4 genes vs. public = 5,525.7 genes), suggesting that they are suitable for most evolutionary genomic analyses.

A Robust Phylogeny for the Subphylum Saccharomycotina

To infer the budding yeast phylogeny, we assembled 2 full data matrices (2408OG data matrix and 1292BUSCO data matrix) and 7 additional ones (by subsampling subsets of genes in the 2408OG data matrix) and analyzed them using three phylogenetic strategies (concatenation under a single partition,

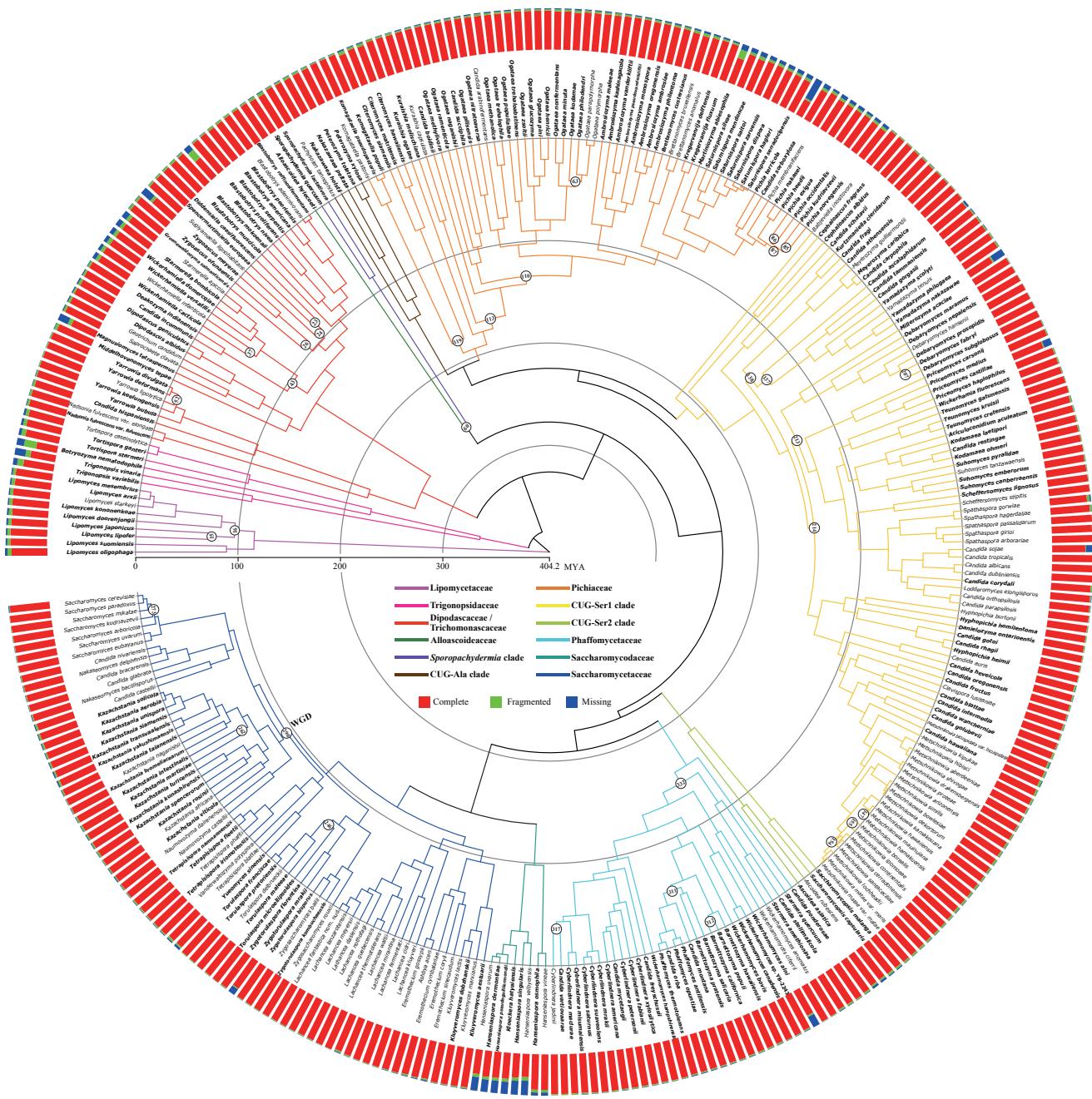


Figure 2. Time-Calibrated Phylogeny of the Budding Yeast Subphylum

Divergence times were estimated using the autocorrelated clock model of rate variation across different lineages implemented in MCMCTree (clock = 3), with a topology reconstructed from the concatenation-based maximum likelihood analysis of 2,408 amino acid orthologous groups (OGs) under a single LG+G4 model. The 32 internal branches that were not robustly recovered across our analyses are marked with circles. The 220 genomes published in this study are shown in bold. The bar plot next to each species indicates genomic quality assessed by a set of 1,759 BUSCO genes. “Complete” indicates the fraction of full-length BUSCO genes; “Fragmented” indicates the fraction of genes with a partial sequence; and “Missing” indicates the fraction of genes not found in the genome. Note that the CUG-Ser1 clade includes interspersed taxa from the families Debaryomycetaceae, Metschnikowiacae, and Cephaloasaceae; the CUG-Ser2 clade includes the families Ascoideaceae and Saccharomycopsidaceae; the newly recovered CUG-Ala clade includes several taxa in need of reassignment; and the Pichiaceae clade includes several taxa in need of reassignment; and the Dipodascaceae/Trichomonascaceae clade mainly includes interspersed taxa from these two families. mya, million years ago.

See also Figures S1, S2, and S3 and Tables S1 and S2.

concatenation under gene-based partitioning, and coalescence). These analyses produced a strongly supported and largely concordant phylogeny (Figure 2); only 32/331 yeast internodes were not recovered by all 27 phylogenies, with most instances of incongruence being due to whether the data matrix was analyzed by concatenation or coalescence (Figure S2).

Surprisingly, one of the resolved internodes concerned the placement of the CUG-Ser2 clade, which was equivocal in previous phylogenomic studies (Riley et al., 2016; Shen et al., 2016a, 2017). For example, the most recent phylogenomic analysis, in which the CUG-Ser2 clade was represented by a single taxon, showed that the clade's placement was unduly influenced by inclusion of the *DPM1* (in *S. cerevisiae*) gene (Shen et al., 2017). However, our sampling of three additional representative taxa from the CUG-Ser2 clade eliminated the gene's disproportionate influence and resolved this clade's placement in the budding yeast phylogeny (Figure S3).

The new phylogeny divides the subphylum into 12 major clades (Figure 2), at least 2 and up to 8 more than previously recognized (Dujon and Louis, 2017; Hittinger et al., 2015; Kurtzman and Boekhout, 2017; Kurtzman et al., 2011; Shen et al., 2016a). Several families are now well circumscribed (e.g., Pichiaceae), whereas other families are not reciprocally monophyletic (e.g., Dipodascaceae/Trichomonascaceae), leading us to consider them as major clades comprised of multiple known families. One major reorganization stems from the recognition that the split between the genus *Sporopachydermia* and other budding yeasts is very deep. Although previous authors had recognized affinities to the families Dipodascaceae and Trichomonascaceae, our analyses placed the genus *Sporopachydermia* on its own long branch with deep affinities to the family Alloascoideaceae in most analyses (Figure 2). A second major reorganization stems from the placement of a group of species that all translate the CUG codon as alanine (the CUG-Ala clade) (Krassowski et al., 2018; Riley et al., 2016) as a lineage that is separate from and sister to the Pichiaceae clade. In previous studies, some members of these two clades were placed together, while the taxonomic placements of taxa in the genera *Nakazawaea*, *Pachysolen*, and *Peterozyma* were still unclear (Kurtzman and Boekhout, 2017; Kurtzman et al., 2011). Our results suggest that the CUG-Ala clade represents a new, yet-to-be-described taxonomic family.

A Timescale for Budding Yeast Diversification

To estimate divergence times of the budding yeast subphylum, we employed Bayesian relaxed clock approach (Yang, 2007) with four generally accepted calibration points (STAR Methods) on the 2408OG data matrix. We estimate the origin of the subphylum between 317 and 523 (95% credibility interval; posterior mean date: 404) million years ago (mya); the origin of the CUG-Ser1 clade, which contains the major opportunistic pathogen *C. albicans*, between 178 and 248 (210) mya, the origin of the whole-genome duplication (WGD) clade between 82 and 105 (93) mya, and the divergence of *S. cerevisiae* and *C. albicans* from their sister species (*Saccharomyces paradoxus* and *Candida dubliniensis*) between 4.0 and 5.8 (4.9) mya and 5.0 and 14.0 (8.7) mya, respectively (Figure 2; Table S2).

HGT into Budding Yeasts

To identify the tempo and mode of HGT into the budding yeast gene lineage, we investigated all 1,538,912 genes that reside in the 8,792 contigs \geq 100 kb using a robust and conservative phylogeny-based approach (STAR Methods). We found that 878 genes in 186 yeast genomes were likely acquired via 365 distinct HGT events from non-fungal (mostly bacterial) sources (Figures 3A and S4; Table S3). Gene ontology enrichment analysis of the 878 genes shows that most are associated with metabolism-related terms (Figure S5; Table S3). Furthermore, 616/878 genes (~70%) were significantly supported with the approximately unbiased (AU) test (Shimodaira, 2002), a value quite similar to the percentage supported by AU tests among previously described HGT-acquired genes (15/21, or ~71%; Table S3). Although precisely quantifying the fraction of HGT events is challenging, the approximately 0.04% (616/1,538,912) to 0.06% (878/1,538,912) of genes identified to have been putatively acquired via HGT suggests that the process is a very small contributor to overall yeast genome diversification.

From the 878 HGT-acquired genes, 333 were present in the genomes of the six representative species from the W/S clade (Figure 3A; Table S3). In contrast, the genomes of 57/103 species in the CUG group, whose genetic codes deviate from the universal one, did not contain any HGT-acquired genes (Figure 3A). The fraction of HGT-acquired genes was significantly higher in the 226 yeast genomes that use the universal genetic code than in the 103 CUG group genomes (0.071% of 1,054,747 genes vs. 0.025% of 484,165 genes; Fisher's exact test; p value = 2.2×10^{-16} ; which remained significant even after exclusion of the W/S clade species at 0.041% of 1,027,319 genes vs. 0.025% of 484,165 genes; Fisher's exact test; p value = 2.5×10^{-5}), supporting the hypothesis that altered genetic codes serve as barriers to HGT (Richards et al., 2011; Woese, 2000).

Finally, our dense genome sampling also facilitated the identification of cases involving the independent acquisition of genes participating in the same metabolic pathway into the same yeast lineage, as well as the independent acquisition of the same gene in two distantly related yeast lineages (Figures 3B and 3C). Specifically, an ancestor of *Wickerhamiella* (*Candida*) *versatilis* and *Wickerhamiella domercqiae*, two highly osmotolerant species, acquired a choline oxidase associated with the production of the osmoprotectant betaine from choline; remarkably, that same ancestor appears to have also independently acquired a choline sulfatase involved in the conversion of choline-O-sulfate into choline (Figure 3B). *W. versatilis* and *W. domercqiae* also horizontally acquired a nitronate monooxygenase likely associated with resistance to propionate 3-nitronate (P3N; a plant and fungal toxin) from genus *Pseudomonas* bacteria (Figure 3C).

Evolution of Metabolic Traits across Budding Yeasts

To examine the evolution of budding yeast metabolism, we used Bayesian inference to estimate rates of gain and loss for a compilation of 45 discrete metabolic traits in 274/332 budding yeasts across the subphylum (Kurtzman et al., 2011; Opulente et al., 2018) (STAR Methods; Table S4). We found that 39/45 traits experienced higher rates of loss, whereas the remaining 6 experienced higher rates of gain (Table 1; Figure S6). These

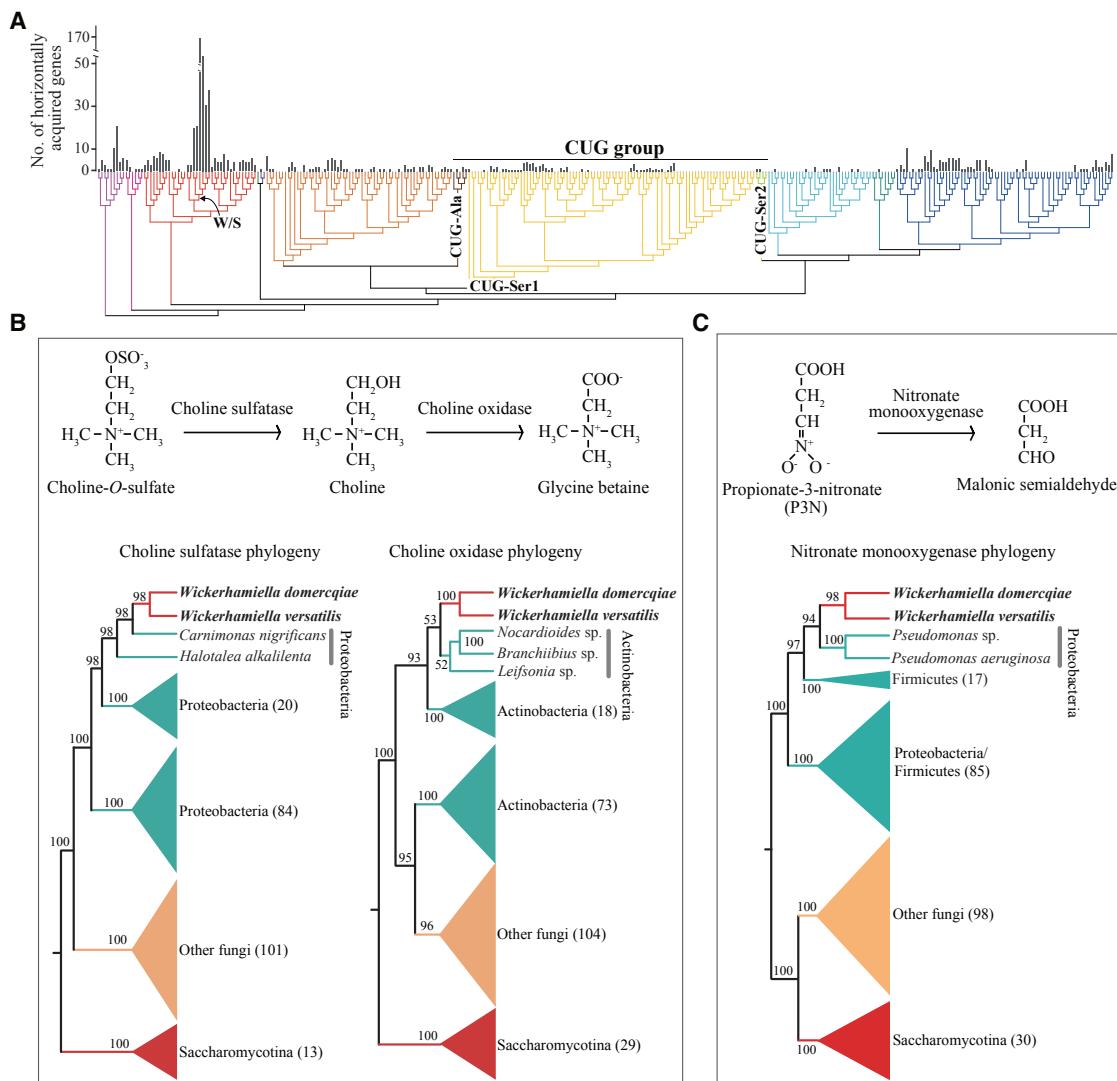


Figure 3. Very Few Budding Yeast Genes Were Acquired via HGT

(A) Mapping of the 878 putative HGT-acquired genes on the 332-taxon phylogeny of budding yeasts (Figure 2). These 878 genes were acquired through 365 distinct HGT events, of which 230 appear to be species-specific and the other 135 involve two or more species.

(B) Two independent HGT events provided osmotolerant budding yeasts the genetic machinery to produce the osmoprotectant glycine betaine. Top: the biochemical pathway for the biosynthesis of glycine betaine from choline-O-sulfate is shown. Choline-O-sulfate is first converted by the action of choline sulfatase into choline; then choline is converted by the action of choline oxidase into glycine betaine. Bottom: the phylogenies for the choline sulfatase and choline oxidase genes are shown. Note that the sequences of the sister lineages to the *W. domercqiae* and *W. versatilis* choline sulfatase sequences are from Proteobacteria, whereas the sequences of the sister lineages to the *W. domercqiae* and *W. versatilis* choline oxidase sequences are from Actinobacteria.

(C) Two independent events provided two different lineages of budding yeasts (the W/S clade and a clade of three species in the genus *Kluyveromyces*) the genetic machinery to metabolize the mitochondrial toxin propionate-3-nitronate (P3N). Top: the biochemical pathway for the oxidation of P3N is shown, where P3N is converted into malonic semialdehyde via the action of nitronate monooxygenase. Bottom: the phylogeny for the nitronate monooxygenase acquired by an ancestor of *W. domercqiae* and *W. versatilis* is shown. Note that the sequences of the sister lineages to the *W. domercqiae* and *W. versatilis* nitronate monooxygenase sequences are from Proteobacteria in the genus *Pseudomonas*; the phylogeny for the nitronate monooxygenase acquired by an ancestor of the three *Kluyveromyces* species is not displayed, but these sequences are most closely related to sequences from Proteobacteria in the genus *Acinetobacter*. The budding yeast species inferred to have been the recipients of horizontally transferred genes are shown in bold. Data matrices and phylogenies for all HGT-acquired genes are provided in the Figshare repository.

See also Figures S4 and S5 and Table S3.

trends were statistically significant for 16/39 traits where the rate of loss was higher than the rate of gain and for 1/6 traits where the rate of gain was higher than the rate of loss (Table 1). As the metabolic trait data came from multiple sources and the

strains tested sometimes differed from the strains sequenced, we also experimentally determined the growth on 13/45 metabolic traits and a control trait (glucose) (Table S4) for 328/332 strains of the budding yeast species whose genomes we

Table 1. Rates of Trait Gain, Loss, and Posterior Probabilities of Ancestral States 0 and 1 of the BYCA for 45 Metabolic Traits

Trait	q01	q10	PP (0)	PP (1)
Glucose fermentation	1.16	0.37	0.96	0.04
Galactose fermentation ^a	0.31	1.48	0.87	0.13
Sucrose fermentation	0.40	2.00	0.98	0.02
Maltose fermentation ^a	0.34	3.17	0.56	0.44
Lactose fermentation ^a	0.17	78.03	0.50	0.50
Raffinose fermentation ^a	0.27	2.35	0.76	0.24
Trehalose fermentation ^a	0.38	1.55	0.00	1.00
Inulin ^a	0.23	3.49	0.12	0.88
Sucrose	0.21	0.92	0.68	0.32
Raffinose ^a	0.23	2.13	0.00	1.00
Melibiose ^a	0.06	2.89	0.00	1.00
Galactose	0.49	0.31	0.01	0.99
Lactose ^a	0.18	2.74	0.04	0.96
Trehalose	0.62	0.83	0.28	0.72
Maltose	0.27	0.92	0.67	0.33
Melezitose	0.43	1.22	0.83	0.17
Methyl- α -D-glucoside	0.24	1.53	0.00	1.00
Soluble starch ^a	0.11	2.25	0.00	1.00
Cellobiose	0.24	0.75	0.17	0.83
Salicin	0.10	0.76	0.20	0.80
L-Sorbose	0.50	0.51	0.01	0.99
L-Rhamnose ^a	0.02	1.97	0.03	0.97
D-Xylose	0.12	0.38	0.00	1.00
L-Arabinose ^a	0.02	1.36	0.00	1.00
D-Arabinose	0.18	2.27	0.99	0.01
D-Ribose	0.17	0.96	0.99	0.01
Methanol	0.03	0.27	1.00	0.00
Ethanol	0.73	0.14	0.00	1.00
Glycerol ^a	1.95	0.23	0.40	0.60
Erythritol ^a	0.03	1.38	0.00	1.00
Ribitol	0.33	0.38	0.97	0.03
Galactitol ^a	0.13	1.85	0.09	0.91
D-Mannitol	0.11	0.27	0.00	1.00
D-Glucitol	0.04	0.23	0.00	1.00
myo-Inositol	0.01	1.43	0.00	1.00
DL-Lactate	0.68	0.89	0.66	0.34
Succinate	0.35	0.20	0.00	1.00
Citrate	0.46	0.85	0.08	0.92
D-Gluconate	0.75	0.97	0.58	0.42
D-Glucosamine ^a	0.04	1.35	0.01	0.99
N-Acetyl-D-glucosamine	0.08	0.57	0.21	0.79
Hexadecane	0.17	0.89	0.91	0.09
Nitrate ^a	0.01	2.12	0.02	0.98
Nitrite	0.16	1.18	0.95	0.05
2-Keto-D-gluconate	0.53	0.48	0.87	0.13

q01 (instantaneous transition rate of trait gain): the rate of change from state 0 (absent) to state 1 (present). q10 (instantaneous transition rate of trait loss): the rate of change from state 1 to state 0. PP (0) and PP (1) denote posterior probabilities of ancestral states 0 (absence) and 1 (presence) for the budding yeast common ancestor (BYCA). Every value in this table is derived from the largest peak of density of posterior distributions. See also Figure S6 and Table S4.

^aTraits in which rates q01 and q10 are significantly different (assessed by Bayes factors).

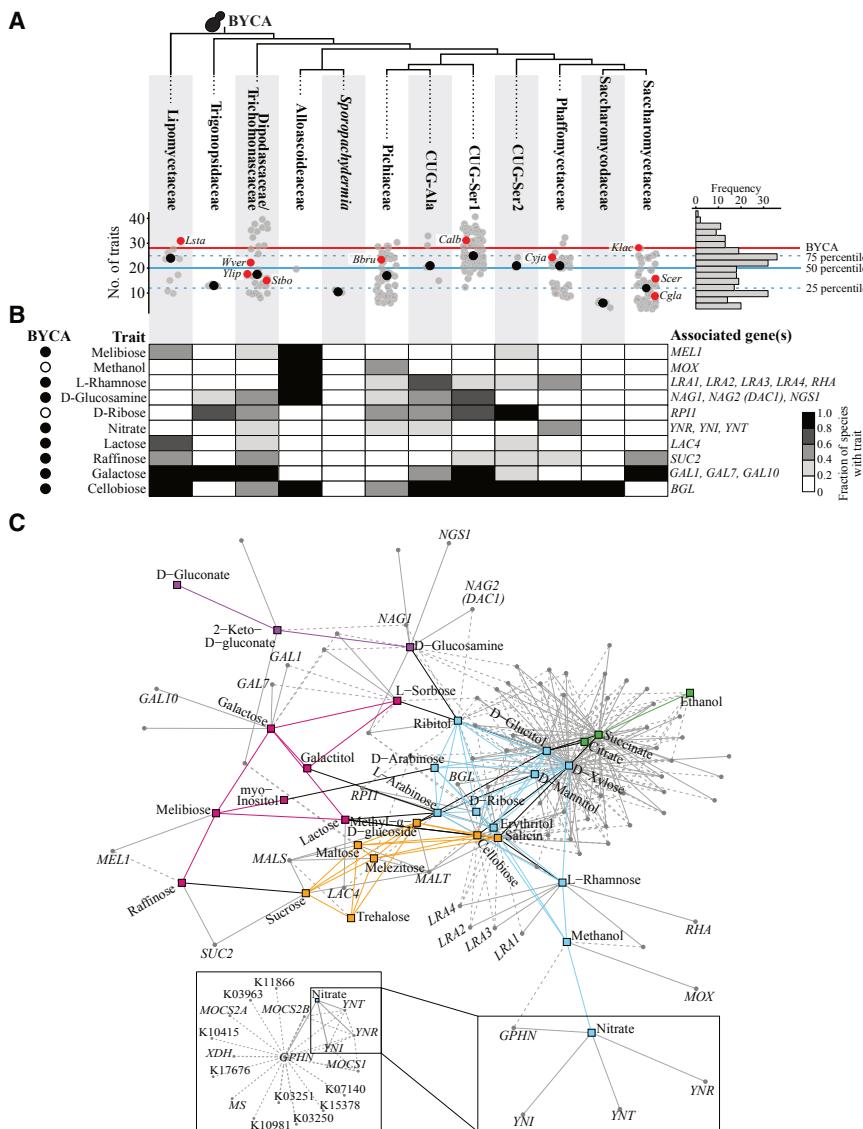


Figure 4. Evolution of Metabolic Traits across the Budding Yeast Subphylum

(A) The number of traits per major clade (columns) is depicted in a scatterplot where each gray dot corresponds to a species. Red dots indicate representative species, and black dots represent the median number of traits across each family. Right: the distribution across the subphylum Saccharomyctaceae in histogram form. The red line corresponds to the inferred number of metabolic traits present (i.e., posterior probability of trait presence > 0.5 in Table 1) in the BYCA (budding yeast common ancestor). The blue dashed and solid lines represent the 75th (25 traits), 50th (median; 20 traits), and 25th (12 traits) percentiles of the numbers of traits. Representative species names are written using a four-letter abbreviation as follows: Lsta, *Lipomyces starkeyi*; Ylip, *Yarrowia lipomyces*; Wver, *Wickerhamella versatilis*; Stbo, *Starmerella bombicola*; Bbru, *Brettanomyces bruxellensis*; Calb, *Candida albicans*; Cyja, *Cyberlindnera jadinii*; Klac, *Kluyveromyces lactis*; Cgla, *Candida glabrata*; Scer, *Saccharomyces cerevisiae*.

(B) Heatmap showing the fraction of species in each major clade (columns) that display a representative set of metabolic traits; values near white indicate major clades (whose species are) lacking the trait, and values near black indicate major clades with the trait. To the left of the heatmap, the presence (black) or absence (white) of a trait in BYCA (inferred from ancestral trait reconstruction) is shown. To the right of the heatmap, well-characterized genes whose distributions are significantly associated with each trait are shown.

(C) Positive association network for genes and traits. Traits and genes are represented by squares and circles, respectively. Trait communities are represented by the following colors: magenta, contains galactose; purple, modified glucose, green, respiratory; orange, glucosides; cyan, sugar alcohols and pentose phosphate pathway (Opulente et al., 2018). Associations among gene and traits were calculated using mutual information (MI) analysis, and negative associations were detected using a Jaccard index (all values less than 0.25 were considered negative associations).

and excluded). Edges connecting genes to traits are colored gray and are represented by solid lines for associations that had a MI value greater than or equal to 0.15; for genes already appearing in the figure, dashed lines representing MI values between 0.10 and 0.15 were included. The inset includes genes associated with GPHN, which encodes gephyrin.

See also Table S5.

analyzed. We found that the average consistency of the character states shared between the compilation of available metabolic trait data and our experimental measurements was 94% (Table S4), suggesting that our inferences are based on accurate data.

Metabolic trait reconstruction also allowed us to infer the ancestral state at each node in the budding yeast phylogeny (STAR Methods). For example, we inferred that BYCA was most likely a metabolically complex organism capable of assimilating 27/45 of the carbon- and nitrogen-containing compounds analyzed (average posterior probability of trait presence = 0.64; average posterior probability of trait absence = 0.36) (Figure 4A; Table 1). Using a stricter cutoff (posterior probability ≥ 0.9), we

found 21 traits that were most likely present in BYCA (e.g., nitrate, xylose, and galactose assimilation), and 8 that were most likely absent (e.g., glucose fermentation and methanol, ribose, and hexadecane assimilation) (Table 1).

Although the genetic bases for many of these metabolic traits remain unknown, the genes involved in a handful of metabolic pathways are well characterized, allowing us to not only reconstruct characterized genetic pathways, but to also use the cooccurrence of traits and genes across the budding yeast phylogeny to identify novel connections and functions. To this end, we used a mutual information-based approach to analyze the phylogenetic distribution of functional annotations of genes in yeast species, together with the metabolic trait values of those species

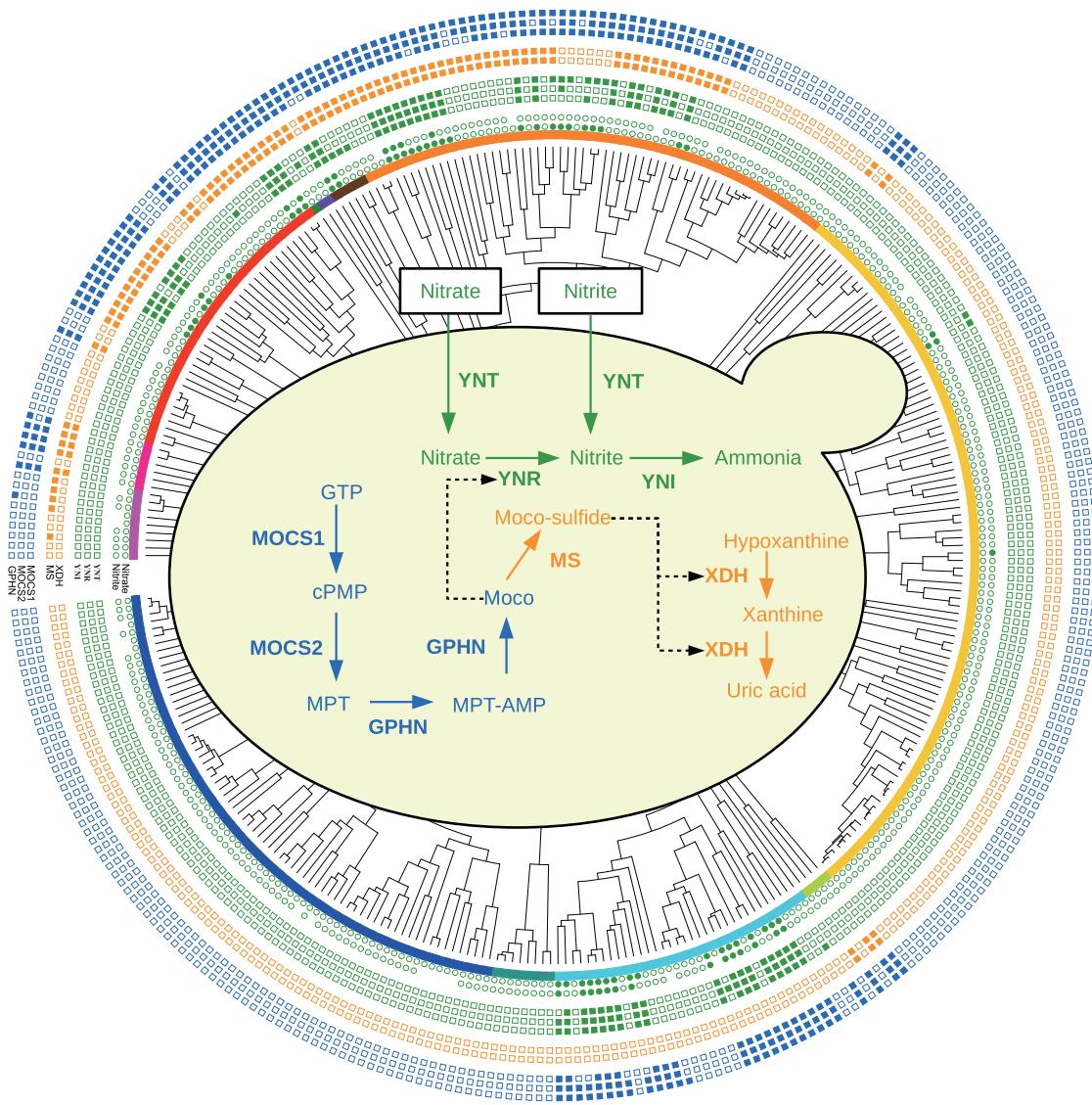


Figure 5. Interconnections and Interdependence of Nitrate Assimilation, Xanthine Assimilation, and the Molybdopterin Cofactor Biosynthesis Pathways across the Budding Yeast Subphylum

The concentric tracks on the periphery of the figure depict (from inner to outer) the phylogenetic distribution of growth phenotype on media containing nitrate or nitrite as a sole nitrogen source (inner green circles) and genes encoding proteins involved in nitrate/nitrite assimilation (green squares); hypoxanthine/xanthine assimilation (orange squares); and Moco biosynthesis (blue squares). The underlying phylogeny and distribution of taxa is the same as in Figure 2, but species names have been omitted. The central diagram depicts the individual steps of nitrate assimilation (green, top), xanthine assimilation (orange, right), and Moco biosynthesis (blue, left) pathways, with proteins involved shown in bold: nitrate transporter (YNT), nitrate reductase (YNR), nitrite reductase (YNI), cyclic pyranopterin monophosphate synthase (MOCS1), molybdopterin synthase (MOCS2), gephyrin (GPHN), molybdopterin sulfurtransferase (MS), and xanthine dehydrogenase (XDH). Molecules in the pathways: guanosine triphosphate (GTP), cyclic pyranopterin monophosphate (cPMP), molybdenum (MPT), adenylated molybdopterin (MPT-AMP), molybdenum cofactor (Moco), and thiomolybdenum cofactor (Moco-sulfide). Solid arrows indicate subsequent steps in each pathway; dashed lines indicate use of a specific cofactor by an enzyme.

See also Table S6.

(**STAR Methods**). We then overlaid our gene-to-trait scores onto a network of trait-to-trait ecological associations among 784 budding yeast species ([Opulente et al., 2018](#)). These analyses recovered multiple positive gene-to-trait associations for gene-trait pairs established in model systems, such as genes encoding beta-galactosidases (e.g., *LAC4*) with growth on lactose, alcohol oxidases (e.g., *MOX*) with growth on methanol, and the *GAL1*,

GAL7, and *GAL10* genes with growth on galactose ([Figure 4B; Table S5](#)) ([Riley et al., 2016](#)). These examples validate the utility of this analytical approach for elucidating the genetic underpinnings of metabolic trait variation.

As expected from our analyses showing a strong trend toward metabolic trait loss ([Table 1](#)) and previous studies on the losses of iconic genetic pathways ([Hittinger et al., 2004](#),

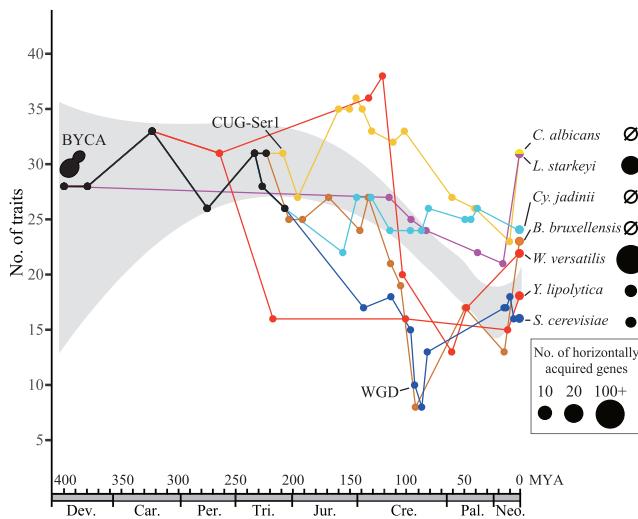


Figure 6. Evolution of the Budding Yeast Subphylum Is Characterized by Lineage-Variable HGT and Widespread Losses of Genes and Traits

The x axis depicts the posterior mean of the age of (representative) nodes in the budding yeast phylogeny, and the y axis depicts the number of metabolic traits inferred to have been present at these nodes. The lines of different colors represent the evolutionary trajectories (in the spaces of time and metabolic traits) for 7 representative yeast taxa and their common ancestors (depicted by dots). For each ancestral node, metabolic traits were considered to be present when the posterior probability of ancestral state 1 (present) was > 0.5 for the node. The gray region is the 95% confidence interval for the number of metabolic traits present across budding yeast evolution based on ancestral trait reconstruction of the distribution of inferences for 45 metabolic traits across 274 budding yeasts. The inferred numbers of HGT-acquired genes are depicted by the circles of different sizes next to each taxon's name. *C. albicans*, *Candida albicans*; *L. starkeyi*, *Lipomyces starkeyi*; *Cy. jadinii*, *Cyberlindnera jadinii*; *B. bruxellensis*, *Brettanomyces bruxellensis*; *W. versatilis*, *Wickerhamiella versatilis*; *Y. lipolytica*, *Yarrowia lipolytica*; *S. cerevisiae*, *Saccharomyces cerevisiae*; BYCA, budding yeast common ancestor; mya, million years ago; Dev., Devonian; Car., Carboniferous; Per., Permian; Tri., Triassic; Jur., Jurassic; Cre., Cretaceous; Pal., Paleogene; Neo., Neogene.

See also Figure S7.

Riley et al., 2016; Slot and Rokas, 2010; Wolfe et al., 2015), the distributions of many of the genes involved in these metabolic processes (e.g., the genes required to assimilate D-glucosamine and N-acetyl-D-glucosamine and the genes required for L-rhamnose assimilation) were consistent with widespread losses, which included both ancient losses deep within major clades and more recent losses differentiating closely related taxa.

Complex Interactions between Metabolic Pathways Affect Gene Retention

By analyzing these gene and trait networks, we also identified genes whose associations with specific metabolic traits has heretofore received only limited attention in select budding yeasts. For example, although the model yeast *S. cerevisiae* cannot assimilate nitrate or nitrite (Kurtzman et al., 2011), we found that the genomes of 50 budding yeasts that could assimilate either of these nitrogen sources were generally predicted to encode nitrate/nitrite transporters (YNTs, 39/50), nitrate reductases (YNRs, 40/50), and nitrite reductases (YNIs, 47/50) (Figures

4C and 5). These proteins are homologs of a pathway commonly deployed in filamentous fungi to import nitrate (YNTs), reduce nitrate into nitrite (YNRs), and reduce nitrite into ammonia (YNIs) for central metabolism (Slot and Hibbett, 2007), strongly suggesting that the same pathway is deployed across many budding yeasts (Pérez et al., 1997). As with most other metabolic traits (Figure 4), nitrate assimilation genes were frequently lost, both anciently (e.g., in the CUG-Ser1 clade) and more recently (e.g., in the genus *Cyberlindnera*).

Our network-based approach also enabled us to use the phylogenetic distribution of the nitrate assimilation pathway to identify additional metabolic genes, pathways, and traits that co-varied but lacked obvious ecological connections. For example, we uncovered several significant associations with the gene encoding gephyrin (Figure 4C; Table S5), an enzyme involved in the biosynthesis of the molybdenum cofactor (Moco) used by the nitrate reductase enzyme (Schwarz and Mendel, 2006). When we examined secondary associations (genes associated with genes that are, in turn, associated with traits), we identified genes predicted to encode the full Moco biosynthesis pathway (Figures 4C, inset, and 5; Table S6).

Surprisingly, the phylogenetic distribution of various components of the Moco biosynthesis and nitrate assimilation pathways revealed that YNRs were never present in the absence of the Moco biosynthesis pathway, but the reverse was often the case (Figure 5). Furthermore, most of the organisms that harbored the Moco biosynthesis pathway but lacked the nitrate assimilation genes also contained the genes encoding xanthine dehydrogenases (XDHs) (Figure 5). In filamentous fungi, XDH is responsible for the assimilation of xanthine and hypoxanthine and requires a thiolated form of Moco, which is created during an additional processing step performed by a specialized Moco sulfurtransferase (MS) (Bittner et al., 2001). The phylogenetic distributions of the genes encoding XDHs and MSs were strongly correlated; of the 83 species with either gene, 78 (or 94%) were predicted to have both genes, illustrating how the underlying metabolic architecture of genetic pathways can lead to remarkable evolutionary linkages between traits that may seem unconnected at first.

Conclusions

Genome sequencing and analyses of 332 metabolically diverse species allowed us to infer that the last common ancestor of budding yeasts, BYCA, was metabolically complex, that very few genes were acquired via HGT, and that the observed metabolic diversity of extant budding yeasts was largely achieved through repeated, extensive losses of metabolic traits through reductions in their underlying genetic toolkit (Figure 6). Thus, the portrait of BYCA that emerges is similar to an archetypal member of its sister subphylum, Pezizomycotina, which contains iconic filamentous fungi, such as *Aspergillus* and *Neurospora*. These filamentous fungi have much larger genomes ($\sim 30\text{--}80$ Mb) and gene sets ($\sim 10,000\text{--}13,000$ genes, including metabolic genes). Consequently, they exhibit a broader appetite and consume a wide array of nitrogen and carbon sources by deploying many genes not present in *S. cerevisiae* (Wisecaver et al., 2014).

How have budding yeasts survived and even thrived while undergoing this reductive evolution? Although the arc of budding

yeast evolution bends strongly toward reduced genomes and metabolisms across history, among both extant and inferred ancestral yeasts, plenty of taxa have much broader metabolic capabilities than the mean (Figures 6 and S7). One attractive hypothesis, whose testing will require even denser taxon sampling, is that generalist lineages with less reduced genomes and more metabolic capabilities are more likely to produce new species and less likely to go extinct. Evolutionary dynamics favoring generalists over specialists could conceivably play out over geological time through some form of clade selection (Stanley, 1975; Williams, 1992), by drawing from the considerable genetic variation segregating within species (Hittinger et al., 2010; Peter et al., 2018), or both.

The observed pattern of widespread metabolic trait and gene losses complements the well-established losses or reductions of several flagship eukaryotic genomic and molecular features in many budding yeasts, such as introns, the molecular machinery for RNA interference, and H3K9me2/3 heterochromatin (Dujon and Louis, 2017). Evolution by loss is well documented for parasitic and symbiotic lineages (Dujon et al., 2004; Katinka et al., 2001; Spanu et al., 2010; Vogel and Moran, 2013; Wolfe et al., 1992) and in the aftermath of the whole genome duplication events where most second copies are lost (Soltis et al., 2015; Wolfe et al., 2015). Nonetheless, the magnitude and pervasiveness of metabolic trait and gene loss across 400 million years of budding yeast evolution in organisms with widely divergent, free-living lifestyles provides unexpectedly broad support for the argument that reductive evolution is a major contributor to genome evolution (Albalat and Cañestro, 2016; Wolf and Koonin, 2013), in general, and not simply associated with specific lifestyles and genomic events.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Taxon sampling
 - Yeast growth
- METHOD DETAILS
 - Genome sequencing and assembly
 - Assessment of genome assemblies
 - Genome annotation
 - Identification of potential hybrid species
 - Phylogenomic data matrix construction
 - a) 2408OG data matrix
 - b) 1292BUSCO data matrix
 - c) 7 additional subsampled data matrices
 - Phylogenetic analyses
 - Molecular dating
 - Horizontal gene transfer analyses
 - Analyses of trait evolution
 - Validation of metabolic trait data
 - Trait and gene association network analyses
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and six tables and can be found with this article online at <https://doi.org/10.1016/j.cell.2018.10.023>.

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AUTHOR CONTRIBUTIONS

X.-X.S., D.A.O., J.K., X.Z., C.P.K., C.T.H., and A.R. designed the research; X.-X.S., D.A.O., J.K., X.Z., J.L.S., M.A.H.B., J.H.W., M.W., and J.T.B. performed the computational analyses and contributed computational and statistical scripts; D.A.O., K.V.B., R.M.S., D.T.D., Q.K.L., J.D., and A.B.H. performed the experiments; M.O., R.E., M.T., R.M., N.C., D.L., C.A.R., J.D., A.B.H., M.G., C.P.K., and C.T.H. contributed the strains and reagents; X.-X.S., D.A.O., J.K., and J.L.S. prepared the figures with input from C.T.H. and A.R.; X.-X.S., D.A.O., J.K., X.Z., J.L.S., C.T.H., and A.R. wrote the paper; and all authors provided comments and input on the draft manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Phenol:Chloroform:Isoamyl Alcohol	Sigma	Cat#P2069
Critical Commercial Assays		
NEBNext Ultra DNA Library Prep	NEB	Cat#E7370L
TruSeq DNA PCR-free Library Preparation Kit	Illumina	Cat#20015963
Nextera Mate Pair Sample Preparation Kit	Illumina	Cat#FC-132-1001
Quant-iT PicoGreen ds DNA Assay Kit	Quant-iT	Cat#P11496
Deposited Data		
De novo assemblies	DDBJ/ENA/GenBank	See Table S1
Genome annotations	This study	Figshare data repository: 10.6084/m9.figshare.5854692
Data matrices	This study	Figshare data repository: 10.6084/m9.figshare.5854692
Phylogenetic trees	This study	Figshare data repository: 10.6084/m9.figshare.5854692
Alignments and ML trees for horizontally acquired genes	This study	Figshare data repository: 10.6084/m9.figshare.5854692
Metabolic trait data	This study	Figshare data repository: 10.6084/m9.figshare.5854692
Trait ancestral character state reconstructions	This study	Figshare data repository: 10.6084/m9.figshare.5854692
Experimental Models: Organisms/Strains		
343 sampled species	This study	See Table S1
Software and Algorithms		
iWGS v1.1	Zhou et al., 2016	https://github.com/zhouxiaofan1983/iWGS/
Trimmomatic v0.33	Bolger et al., 2014	http://www.usadellab.org/cms/?page=trimmomatic
Lighter v1.1.1	Song et al., 2014	https://github.com/mourisl/Lighter/
KmerGenie v1.6982	Chikhi and Medvedev, 2014	http://kmergenie.bx.psu.edu/
ABYSS v1.5.2	Simpson et al., 2009	https://github.com/bcgsc/abyss
DISCOVAR r51885	Weisenfeld et al., 2014	https://software.broadinstitute.org/software/discover/blog/
MASURCA v2.3.2	Zimin et al., 2013	https://github.com/alekseyzimin/masurca
SGA v0.10.13	Simpson and Durbin, 2012	https://github.com/jts/sga
SOAPdenovo2 v2.04	Luo et al., 2012	https://github.com/aquaskyline/SOAPdenovo2
SPADES v3.7.0	Bankevich et al., 2012	http://cab.spbu.ru/software/spades/
QUAST v4.4	Gurevich et al., 2013	https://github.com/ablab/quast
NextClip v0.8	Leggett et al., 2014	https://github.com/richardmleggett/nextclip/
ALLPATHS-LG v51828	Gnerre et al., 2011	ftp://ftp.broadinstitute.org/pub/crd/ALLPATHS/Release-LG/
BUSCO v2.0.1	Waterhouse et al., 2017	https://busco.ezlab.org/
OrthoDB Version 9	Zdobnov et al., 2017	https://busco.ezlab.org/
AUGUSTUS v 3.2.2	Stanke and Waack, 2003	http://bioinf.uni-greifswald.de/augustus/downloads/
MAKER v2.31.8	Holt and Yandell, 2011	http://www.yandell-lab.org/software/maker.html
GeneMark-ES v4.32	Ter-Hovhannisyan et al., 2008	http://exon.gatech.edu/GeneMark/gmes_instructions.html
SNAP v2013-11-29	Korf, 2004	http://korflab.ucdavis.edu/software.html
RepeatMasker v4.0.6	Institute for Systems Biology	http://www.repeatmasker.org
Cuffcompare v2.2.1	Trapnell et al., 2012	https://github.com/gperteau/CuffCompare
MAFFT v7.299	Katoh and Standley, 2013	https://mafft.cbrc.jp/alignment/software/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pal2Nal v14	Suyama et al., 2006	http://www.bork.embl.de/pal2nal/#Download
paml v4.8	Yang, 2007	http://abacus.gene.ucl.ac.uk/software/paml.html
MCMCTree v. 4.9	Yang, 2007	http://abacus.gene.ucl.ac.uk/software/paml.html
OrthoMCL v2.0	Li et al., 2003	http://orthomcl.org/common/downloads/software/v2.0/
Phyutility v2.2.6	Smith and Dunn, 2008	https://github.com/blackrim/phyutility
FastTree v2.1.9	Price et al., 2010	http://www.microbesonline.org/fasttree/
PhyloTreePruner v1.0	Kocot et al., 2013	https://sourceforge.net/projects/phylotreepruner/
trimAl v1.4	Capella-Gutiérrez et al., 2009	http://trimal.cgenomics.org/
IQ-TREE v1.5.1	Nguyen et al., 2015	http://www.iqtree.org/
phylomeDB v4	Huerta-Cepas et al., 2014	ftp://phylomedb.org/phylomedb/phylomes/phylome_0005/
MARE v0.1.2	Zoological Research Museum Alexander Koenig	https://www.zfmk.de/de/forschung/forschungszentren-und-gruppen/mare
RAxML v8.2.3	Stamatakis, 2014	https://sco.h-its.org/exelixis/web/software/raxml/index.html
ExaML v3.0.17	Kozlov et al., 2015	https://sco.h-its.org/exelixis/web/software/examl/index.html
ASTRAL-II v4.10.2	Mirarab and Warnow, 2015	https://github.com/smirarab/ASTRAL
MEGA7	Kumar et al., 2016	https://www.megasoftware.net/
iTOL v3	Letunic and Bork, 2016	https://itol.embl.de/help.cgi#batch
BayesTraits v3	Pagel et al., 2004	http://www.evolution.rdg.ac.uk/BayesTraitsV3.0.1/BayesTraitsV3.0.1.html
R package igraph	Csárdi and Nepusz, 2006	https://cran.r-project.org/web/packages/igraph/index.html
R package infotheo	Meyer, 2008	https://cran.r-project.org/web/packages/infotheo/index.html
R package minet	Meyer et al., 2008	https://www.bioconductor.org/packages/release/bioc/html/minet.html
R package wgcna	Langfelder and Horvath, 2008	https://cran.r-project.org/web/packages/WGCNA/index.html
R package topgo	Alexa and Rahnenfuhrer, 2016	https://bioconductor.org/packages/release/bioc/html/topGO.html
GhostKOALA	Kanehisa et al., 2016a	https://www.kegg.jp/ghostkoala/
ARACNE	Margolin et al., 2006	https://bioconductor.org/packages/release/data/experiment/html/aracne.networks.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests may be directed to and will be fulfilled by the corresponding authors Chris Todd Hittinger (cthittinger@wisc.edu) and Antonis Rokas (antonis.rokas@vanderbilt.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Taxon sampling**

Detailed information about the strains and genomes of the 332 budding yeast species sampled and analyzed in this study can be found in **Table S1**. We did not include genomes associated with different strains from the same species or with known interspecies hybrids. Taxonomy, strain ID, and source information of 332 budding yeasts and the 11 outgroup taxa (9 representatives of the sub-phylum Pezizomycotina and 2 representatives of the subphylum Taphrinomycotina) used in this study are provided in **Table S1**. All sequenced strains have been publicly deposited in the NRRL, CBS, and/or JCM strain collections.

The percentage of newly sequenced 220 species (196 newly sequenced genomes from the Y1000+ Project and 24 publicly available but unpublished genomes from RIKEN) in each of the 12 major clades ranged from 41% to 100%, with an average of 78% (**Figure S1**). Specifically, these newly sequenced 220 species represent 63 different genera; 74 are representatives of 36 genera (e.g., *Ambrosiozyma*, *Saturnispora*, and *Barnettozyma*) whose genomes have not been previously sequenced; 111 are representatives of 25 genera (e.g., *Ogataea*, *Kazachstania*, *Lipomyces*, and *Cyberlindnera*) that previously had one or two publicly available

genomes; and the remaining 35 are representatives of genera (e.g., *Candida* and *Hanseniaspora*) that already had 3 or more publicly available genomes. The sampled 332 budding yeasts use three different genetic codes: 229 follow the universal genetic code and decode the CUG codon as leucine (Figure 2; Table S1); 5 in the genera *Nakazawaea*, *Pachysolen*, and *Peterozyma*, decode CUG codon as alanine (CUG-Ala clade); 94 in the families Debaryomycetaceae, Metschnikowiaceae, and Cephaloascaceae encode CUG codon as serine (CUG-Ser1 clade); and 4 in the families Ascoideaceae and Saccharomycopsidaceae also decode CUG codon as serine (CUG-Ser2 clade).

Yeast growth

Most yeast strains were obtained from the USDA Agricultural Research Service (ARS) NRRL Culture Collection in Peoria, Illinois, USA. Strains from all yeast species were initially plated from freezer stock on yeast extract peptone dextrose (YPD) plates and grown for single colonies. For all carbon and nitrogen testing, we set up 5 replicates on separate days using different colonies for each yeast species. Yeast strains from each species were inoculated into YPD and grown for 48 hours at room temperature. After 48 hours of growth, we randomized and arrayed species into a 96-well deep well plate for storage and future use. Using the same cultures, we also inoculated species into a minimal-based starvation medium containing 0.1% glucose, 5g/L ammonium sulfate, and 1.7g/L Yeast Nitrogen Base (w/o amino acids, ammonium sulfate, or carbon) and grew them overnight. After 24 hours of growth, we transferred the yeast species into carbon or nitrogen treatment plates for phenotyping.

METHOD DETAILS

Genome sequencing and assembly

For each of the 196 newly sequenced species from the Y1000+ Project (Table S1), genomic DNA (gDNA) was isolated using a modified phenol:chloroform extraction method that used a second round of phenol:chloroform to remove additional proteins, sonicated, and ligated to Illumina sequencing adaptors as previously described (Hittinger et al., 2010). The paired-end libraries were submitted for paired end sequencing (2 × 250 base pairs) on an Illumina HiSeq 2500 instrument.

Paired-end Illumina DNA sequence reads were used to generate whole genome assemblies using the meta-assembler pipeline iWGS v1.1 (Zhou et al., 2016). We first preprocessed the raw sequenced reads by trimming of adapters and low-quality bases with Trimmomatic v0.33 (Bolger et al., 2014) and Lighter v1.1.1 (Song et al., 2014). Next, we identified the optimal *k*-mer length for each genome's assembly using KmerGenie v1.6982 (Chikhi and Medvedev, 2014). We then used the processed sequence reads as input into six different *de novo* assembly tools: ABYSS v1.5.2 (Simpson et al., 2009), DISCOVAR r51885 (Weisenfeld et al., 2014), MASURCA v2.3.2 (Zimin et al., 2013), SGA v0.10.13 (Simpson and Durbin, 2012), SOAPdenovo2 v2.04 (Luo et al., 2012), and SPADES v3.7.0 (Bankevich et al., 2012). The resulting genome assemblies were assessed for quality with QUAST v4.4 (Gurevich et al., 2013); as the “best” assembly for each genome we chose the one with the highest genome size and N50 value (i.e., the contig or scaffold value above which 50% of the total length of the sequence assembly can be found).

For the genomes of the 24 species sequenced by RIKEN (Table S1), a paired-end library with an approximate insert size of 240 bp was prepared from the genomic DNA using the TruSeq DNA PCR-free Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocols. A mate-pair library with an approximate insert size of 3 kb was also prepared from DNA using the Nextera Mate Pair Sample Preparation Kit (Illumina) with some modifications. Both paired-end and mate-pair libraries were sequenced on a HiSeq 2500 (Illumina) to generate 151-base paired-end reads. Mate-pair libraries derived from the DNA of *Hyphopichia homilentoma* JCM 1507, *Candida sorboxylosa* JCM 1536, *Candida intermedia* JCM 1607, *Wickerhamia fluorescens* JCM 1821, *Cyberlindnera fabianii* JCM 3601, *Saccharomyces malanga* JCM 7620, *Candida carpophila* JCM 9396, *Candida succiphila* JCM 9445, *Wickerhamiella domercqiae* JCM 9478, *Sporopachydermia quercuum* JCM 9486, *Starmerella bombicola* JCM 9596, *Candida boidinii* JCM 9604, *Nakazawaea peltata* JCM 9829, *Scheffersomyces lignosus* JCM 9837, and *Ambrosiozyma kashinagacola* JCM 15019 were sequenced on a MiSeq (Illumina) to generate 309-base paired-end reads. All mate-pair reads were processed using NextClip software, v0.8 (Leggett et al., 2014) to trim the adaptor sequences. The estimated sequencing depths ranged from 76 × for *Yarrowia deformans* JCM 1694 to 281 × for *Wickerhamiella versatilis* JCM 5958. The ALLPATHS-LG software version 51828 (for *Cyberlindnera fabianii* JCM 3601, *Wickerhamiella domercqiae* JCM 9478, and *Starmerella bombicola* JCM 9596), version 52155 (for *Hyphopichia homilentoma* JCM 1507, *Candida sorboxylosa* JCM 1536, *Candida intermedia* JCM 1607, *Priceomyces haplophilus* JCM 1635, *Yarrowia deformans* JCM 1694, *Wickerhamia fluorescens* JCM 1821, *Ambrosiozyma monospora* JCM 7599, *Saccharomyces malanga* JCM 7620, *Candida carpophila* JCM 9396, *Candida succiphila* JCM 9445, *Sporopachydermia quercuum* JCM 9486, *Candida boidinii* JCM 9604, *Nakazawaea peltata* JCM 9829, *Scheffersomyces lignosus* JCM 9837, *Yarrowia keelungensis* JCM 14894, and *Ambrosiozyma kashinagacola* JCM 15019), and version 52488 (for *Wickerhamiella versatilis* JCM 5958, *Ascoidea asiatica* JCM 7603, *Alloascoidea hylecoeti* JCM 7604, *Ogataea methanolica* JCM 10240, and *Millerozyma acaciae* JCM 10732) were used to assemble the reads into scaffolds using the default parameters (Gnerre et al., 2011). Small contigs (< 1 kb) were not included in the final genome assemblies. The sequence library generation and sequencing were performed at the Genome Network Analysis Support Facility, RIKEN Center for Life Science Technologies (Yokohama, Japan).

Assessment of genome assemblies

The qualities of the genome assemblies of the 196 newly sequenced (Y1000+ Project genomes) and 136 publicly available (RIKEN plus previously published genomes) budding yeast species were assessed by quantifying their completeness based on the expected gene content of the Benchmarking Universal Single-Copy Orthologs (BUSCO), version 2.0.1 (Waterhouse et al., 2017), as described previously (Shen et al., 2016a). We used a set of 1,759 BUSCO genes inferred to be saccharomyceta-specific (“saccharomyceta” is an informal taxonomic rank used by many databases, including NCBI: <https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=716545>) and single-copy in at least 90% of 60 genomes in the OrthoDB Version 9 database (Zdobnov et al., 2017) to evaluate the completeness of the 332 genome assemblies. Briefly, for each BUSCO gene, its consensus orthologous protein sequence among the 60 reference genomes was used as query in a tBLASTN search against each genome to identify up to three putative genomic regions, and the gene structure of each putative genomic region was predicted by AUGUSTUS v 3.2.2 (Stanke and Waack, 2003). Next, the sequences of these predicted genes were aligned to the HMM-profile of the BUSCO gene, and the ones with alignment bit-scores higher than a pre-set cutoff (90% of the lowest bit-score among the 60 reference genomes) were kept. If only one predicted gene from a genome was retained and its aligned sequence length in the HMM-profile alignment was $\geq 95\%$ of the aligned sequence lengths of genes in the 60 reference genomes, the BUSCO gene was classified to be present in the genome examined as single-copy, “full-length.” If two or more predicted genes from a genome were retained and their aligned sequence lengths in the HMM-profile alignments were $\geq 95\%$ of the aligned sequence lengths of genes in the 60 reference genomes, the BUSCO gene was classified as duplicated, “full-length.” If one or more predicted genes from a genome were retained but their aligned sequence lengths in the HMM-profile alignment were $< 95\%$ of the aligned sequence lengths of genes in the 60 reference genomes, the BUSCO gene was classified as “fragmented.” If no predicted gene from a genome was retained, the BUSCO gene was classified as “missing.” For each genome, we then calculated the fractions of single-copy (full-length) genes, duplicated (full-length) genes, fragmented genes, and missing genes, which in turn provided us with a measure of the completeness of gene content in each of the 332 genomes (Shen et al., 2016a).

Genome annotation

With the exception of *Saccharomyces cerevisiae* and *Candida albicans*, whose genome annotations are of exceptionally high quality, for consistency we annotated all the other 330 budding yeast genomes analyzed in our study using the MAKER genome annotation pipeline v2.31.8 (Holt and Yandell, 2011). Genome annotation using MAKER occurs in an iterative manner and relies on multiple inputs, some of which were universal to all genomes (e.g., homology evidence), whereas the others were species-specific (e.g., parameters for *ab initio* gene predictors). The procedure described below was followed for the annotation of all 330 genomes.

The homology evidence used in our genome annotation consists of fungal protein sequences in the SwissProt database (release 2016_11; ftp://ftp.uniprot.org/pub/databases/uniprot/previous_releases/release-2016_11/knowledgebase/uniprot_sprot-only2016_11.tar.gz) and annotated protein sequences of select yeast species from Mycocosm (Grigoriev et al., 2014), a web portal developed by the US Department of Energy Joint Genome Institute for fungal genomic analyses. Three *ab initio* gene predictors were used with the MAKER pipeline, including GeneMark-ES v4.32 (Ter-Hovhannisyan et al., 2008), SNAP v2013-11-29 (Korf, 2004), and AUGUSTUS v 3.2.2 (Stanke and Waack, 2003), each of which was trained for each individual genome. For GeneMark-ES, repeats in the genome sequence were first soft-masked using RepeatMasker v4.0.6 (<http://www.repeatmasker.org>) with the library Repbase library release-20160829 and the “-species” parameter set to “saccharomycotina” for all genomes. GeneMark-ES was then trained on the masked genome sequence using the self-training option (“-ES”) and the branch model algorithm (“-fugus”), which is optimal for fungal genome annotation. On the other hand, the training of both SNAP and AUGUSTUS requires pre-existing gene models as training data. Therefore, we carried out an initial MAKER analysis where gene annotations were generated directly from homology evidence (the “protein2genome” option in the “maker_opts.ctl” control file was set to 1) without using any *ab initio* gene predictors. The resulting gene annotations supported by homology evidence (“keep_preds” set to 0) were then used to train SNAP and AUGUSTUS.

Once all three *ab initio* gene predictors were trained, they were used together with homology evidence to conduct a first round of full MAKER analysis; it should be noted that, here, the homology evidence was only used to inform gene predictions (“protein2genome” set to 0). Resulting gene models supported by homology evidence (“keep_preds” set to 0) were used to re-train SNAP and AUGUSTUS. A second round of MAKER analysis was conducted using the newly trained SNAP and AUGUSTUS parameters, and once again the resulting gene models with homology supports were used to re-train SNAP and AUGUSTUS. Finally, a third round of MAKER analysis was performed using the new SNAP and AUGUSTUS parameters. All resulting gene models were reported (“keep_preds” set to 1), and these comprise the final set of annotations for the genome.

Thirty-nine out of the 136 published genomes analyzed in our study have previously been annotated. To evaluate the quality of our genome annotations, we performed direct comparisons between our annotations and existing ones for the same species. Genome annotations of 39 budding yeast species were downloaded from their respective sources and compared to the corresponding annotations generated in our study using Cuffcompare v2.2.1 (Trapnell et al., 2012). Using the existing annotations as the reference, our annotations achieved high levels of specificity (ranging from 92% to 99.2%, with an average of 97.3%) and sensitivity (ranging from 85.3% to 99.3%, with an average of 94.9%) at the base pair level, as well as contained few missing exons (the fraction of exons missing ranged from 2.7% to 15%, with an average of 4.8%) and genes (the fraction of genes missing ranged from 1.1% to 11.9%, with an average of 3.9%) across the 39 budding yeast species. Note that all of these calculations assume that the previously published annotations contain no errors, which might inflate estimates of inaccuracies in our annotations.

Identification of potential hybrid species

Although we excluded the genomes of known hybrid species from our study, the possibility exists that some of the 332 budding yeast genomes are the products of hybridization (Hittinger et al., 2015). To test whether this was the case, for each of the 332 budding yeast genomes, we examined the genome-wide distribution of K_s (number of synonymous substitutions per synonymous site) values determined from pairs of orthologous genes identified in the species of interest and its closest relative in the budding yeast phylogeny depicted in Figure 2 (following Ortiz-Merino et al. [2017]). To calculate the K_s distribution for a species of interest, for each gene in the species of interest we identified its closest homolog in the genome of their closest relative using BLASTP with an e-value cut off of 10^{-10} . The protein sequences of the gene pairs were then aligned using MAFFT, version 7.299 (Katoh and Standley, 2013), with options “–genafpair–maxiterate 1000.” DNA/codon alignments were then generated by threading the DNA sequence onto the protein alignment using PAL2NAL (Suyama et al., 2006). Using the DNA/codon alignments, K_s was calculated using the LWL85m method implemented in the yn00 module of PAML4 (Yang, 2007).

In known hybrid species, such as *Saccharomyces pastorianus* and *Zygosaccharomyces parabailii* (Dunn and Sherlock, 2008; Ortiz-Merino et al., 2017), the genome-wide distribution of K_s values is bimodal, reflecting the fact that some genes in the hybrid genome are most closely related to one parental species and some to the other more divergent parent. In contrast, non-hybrid species have a unimodal K_s distribution, reflecting the fact that genes originated from a single parental species. We visually inspected the resulting K_s distributions and found that the newly sequenced genomes of *Citeromyces siamensis* and *Martiniozyma abiesophila* had bimodal distributions of K_s similar to those of *S. pastorianus* and *Z. parabailii* (Dunn and Sherlock, 2008; Ortiz-Merino et al., 2017), suggesting that *Ci. siamensis* and *Ma. abiesophila* are of potentially hybrid origin. Consistent with this hypothesis, *Ci. siamensis* and *Ma. abiesophila* also had the highest numbers of predicted genes (12,786 and 12,589, respectively), the highest numbers of gene duplicates (24.1% and 23.6%, respectively), and the highest genome sizes (24.8 Mb and 14.5 Mb, respectively) in our dataset (Table S1). Both hybrid taxa were not associated with any of the 32 incongruent internodes (Figure 2).

Phylogenomic data matrix construction

We generated two complete data matrices (2408OG data matrix and 1292BUSCO data matrix) from the genomes of 332 budding yeasts and 11 outgroups, as well as 7 additional data matrices by subsampling subsets of the orthologous groups of genes (OGs) in the 2408OG data matrix.

a) 2408OG data matrix

The 2408OG orthologous group data matrix was constructed based on a 5-step workflow.

In step 1, we used all protein sequences of the 2,012,541 genes present in the 332 budding yeasts and 11 outgroups to perform an all-versus-all search using BLASTP with an e-value cutoff of 10^{-10} . We then used the BLASTP results to cluster homologous protein sequences using the Markov Cluster (MCL) algorithm implemented in OrthoMCL, version 2.0 (Li et al., 2003); we adopted the widely used inflation parameter of 1.5 for two reasons. First, this inflation parameter value was found to be the optimal one in a previous evaluation of the effects of different inflation parameter values on orthology assignment in the budding yeasts (Salichos and Rokas, 2011). Second, our examination of a range of different inflation parameter values (from 1.2 to 2.0, with a step of 0.1) showed that all values led to the generation of nearly sets of clusters of homologous genes. OrthoMCL clustering resulted in the identification of 171,715 singleton clusters that contain a single protein and 61,763 clusters that contain two or more proteins. Plotting of the distributions of the lengths of genomic contigs that contain the 171,715 singleton clusters and 61,763 clusters with two or more proteins showed that the two distributions were very similar. Retaining only those clusters with gene occupancy $\geq 50\%$, that is those clusters that were present in at least half (≥ 172) of the 343 genomes (332 budding yeasts and 11 outgroups), resulted in the identification of 4,036 putative OGs.

In step 2, we inspected these 4,036 putative OGs for the presence of two or more sequences (i.e., paralogous sequences) from a taxon. For each putative OG, we first aligned its protein sequences using the program MAFFT, version 7.299 (Katoh and Standley, 2013), with the parameters “–auto” and “–maxiterate 1000” and removed columns whose site occupancy was less than 0.01 from the resultant alignment using the program Phyutility, version 2.2.6 (Smith and Dunn, 2008), with the parameters “–aa” and “–clean 0.01.” We then used each trimmed OG alignment to reconstruct a quick but “approximate” maximum likelihood (ML) tree using the program FastTree, version 2.1.9 (Price et al., 2010), with the LG model of amino acid substitutions (Le and Gascuel, 2008), a discrete gamma approximation with 20 categories (-gamma), 4 rounds of minimum-evolution subtree-prune-regraft moves (-spr 4), and the more exhaustive ML nearest-neighbor interchange option enabled (-mlacc 2 -slownni). Whenever there were 2 or more protein sequences from a specific taxon in a given OG, we identified the best (i.e., putatively orthologous) one by using a tree-based method (maximally inclusive subtree) implemented in PhyloTreePruner, version 1.0 with a minimum internal support value of 0.95 (Kocot et al., 2013). This resulted in the retention of 2,908 OGs with gene occupancy $\geq 50\%$.

In step 3, we performed multiple sequence alignment for each of the 2,908 OGs using the E-INS-i strategy (–genafpair–maxiterate 1000) as implemented by the program MAFFT, version 7.299 (Katoh and Standley, 2013), and excluded ambiguously aligned regions using trimAl v1.4 with the “gappyout” option on (Capella-Gutiérrez et al., 2009). We then examined all the resulting alignments and removed protein sequences whose lengths were shorter than 50% the length of the trimmed multiple sequence alignment length of

the OG to which they belonged. We also removed OGs whose total trimmed multiple sequence alignment length was < 167 amino acid sites. These filters resulted in the retention of 2,424 OGs, each of which had $\geq 50\%$ gene occupancy and ≥ 167 amino acid site alignment length.

In step 4, to minimize the inclusion of potentially spurious sequences, we inferred an ML phylogram for each OG using IQ-TREE 1.5.1 (Nguyen et al., 2015) with an automatic detection for the best-fitting model of amino acid evolution and then used the ML phylogram to identify and to remove all protein sequences that resulted in terminal branch lengths that were at least 20-times longer than the median of all terminal branch lengths across the phylogram. This step led to the removal of 421 potentially spurious sequences from 292 OGs; the remaining 2,132 OGs did not contain any spurious sequences.

In the final step (step 5), we redid multiple sequence alignment and trimming for those 292 OGs that we removed as spurious sequences as part of step 4. From the 292 OGs, 276 OGs retained $\geq 50\%$ gene occupancy and ≥ 167 amino acid site alignment length and, thus, were kept; the remaining 16 were discarded.

Retention of the 2,132 OGs from step 4 and the 276 OGs from step 5 yielded a final data matrix consisting of 2,408 orthologous groups (OGs) (1,162,805 amino acid sites) of genes from the 332 budding yeast taxa and 11 outgroups.

b) 1292BUSCO data matrix

As BUSCO genes are a set of reliable markers for phylogenomic inference of diverse lineages (Waterhouse et al., 2017), including the budding yeasts (Shen et al., 2016a), we used 1,757 / 1,759 single-copy, full-length BUSCO genes from 332 budding yeasts and 11 outgroups to construct a data matrix (the EOG09344D43 and EOG09344ST8 BUSCO genes were excluded because we were unable to consistently recover them from our genomes). The number of BUSCO genes whose protein sequences are all present in the same orthologous group (OG) identified by OrthoMCL with the inflation value of 1.5 is 1,650 ($\sim 94\%$ out of 1,757). Multiple sequence alignment; trimming of ambiguously aligned regions; removal of short, spurious, or paralogous sequences; and filtering for $\geq 50\%$ gene occupancy and ≥ 167 amino acid site alignment length were done in the same way as described above for the 2408OG data matrix. Application of these filters resulted in a data matrix of 1,292 BUSCO genes (527,069 amino acid sites), each of which had $\geq 50\%$ gene occupancy and ≥ 167 amino acid site alignment length.

c) 7 additional subsampled data matrices

To explore the stability of phylogenetic relationships among the 332 budding yeasts, we constructed 7 additional data matrices by subsampling subsets of OGs from the 2408OG data matrix as follows:

1. OG2BUSCO data matrix: This data matrix was constructed by retaining only those OGs that were present in both the 2408OG data matrix and the 1292BUSCO data matrix. Overlapping OGs between the two data matrices were determined by BLASTP (e-value cutoff of 10^{-10}). Briefly, a query 2408OG sequence was considered to be overlapping to a subject 1292BUSCO sequence only if they were from the same taxon and were at least 95% similar at the protein sequence level. OGs between the two data matrices were considered overlapping only if all their sequences were overlapping, resulting in a data matrix of 1,081 OGs (545,300 amino acid sites), each of which had $\geq 50\%$ gene occupancy and ≥ 167 amino acid site alignment length.
2. OG2PHYLOME data matrix: This data matrix was constructed by retaining only those OGs that were present in both the 2408OG data matrix and the phylomeDB, version 4 (Huerta-Cepas et al., 2014). First, 1,838 orthologs from the *S. cerevisiae* phylome (P21) containing at least 11 taxa were selected. The *S. cerevisiae* phylome was curated using 21 budding yeast genomes (8 are representatives from the CUG-Ser1 clade; 12 are representatives from the Saccharomycetaceae; 1 is a representative from the Dipodascaceae/Trichomonascaceae clade). Overlap between the two sets of OGs was determined in the same way as described above for the OG2BUSCO data matrix, resulting in a data matrix of 819 OGs (317,158 amino acid sites), each of which had $\geq 50\%$ gene occupancy and ≥ 167 amino acid site alignment length.
3. Top500_ABS data matrix: This data matrix was constructed by retaining the 500 OGs (from the 2408OG data matrix) with the highest average bootstrap support (ABS) value of all internal branches on the ML gene tree (Shen et al., 2016b) and contains 472,241 amino acid sites.
4. Top500_completeness data matrix: This data matrix was constructed by retaining the 500 OGs (from the 2408OG data matrix) with the highest gene occupancy (Shen et al., 2016b) and contains 295,429 amino acid sites.
5. Top500_informativeness data matrix: This data matrix was constructed by retaining the 500 OGs (from the 2408OG data matrix) with the highest information content and contains 174,183 amino acid sites. Information content was calculated based on quartet-mapping (Nieselt-Struwe and von Haeseler, 2001) implemented in MARE, version 0.1.2 (<https://www.zfmk.de/de/forschung/forschungszentren-und-gruppen/mare>), with default settings.
6. Top500_length data matrix: This data matrix was constructed by retaining the 500 OGs (from the 2408OG data matrix) with the longest trimmed multiple sequence alignment lengths (Shen et al., 2016b), and contains 494,658 amino acid sites.

7. Top500_treeness2RCFV data matrix: This data matrix was constructed by retaining the 500 OGs (from the 2408OG data matrix) with the highest ratio of treeness to relative composition frequency variability (RCFV) (Phillips and Penny, 2003; Shen et al., 2016b), and contains 328,534 amino acid sites. Treeness is defined as the ratio (sum of lengths of all internal branches) / (total tree length) (Phillips and Penny, 2003; Shen et al., 2016b). RCFV (Phillips and Penny, 2003) is defined as:

$$RCFV = \sum_{i=1}^s \sum_{j=1}^t |F_{ij} - \bar{F}_i| / t$$

Where s is the number of the character states (here the s is 20 for amino acids) and t is the number of taxa in a given trimmed OG alignment. F_{ij} is the frequency of state i for the j th taxon, and \bar{F}_i is the average frequency of state i across t taxa.

Phylogenetic analyses

For each of these 9 data matrices, we inferred individual gene trees, as well as three estimates of the species phylogeny; two species phylogeny estimates were obtained by concatenation (concatenation under a single partition and concatenation under gene-based partitioning) (Rokas et al., 2003) and one by coalescence (Mirarab and Warnow, 2015).

Individual gene tree inference

Individual gene trees were reconstructed using maximum likelihood (ML) analysis. For each gene, we conducted 10 independent tree searches (5 used starting trees inferred by parsimony and the other 5 used random starting trees) to obtain the best-scoring ML tree using RAxML, multithread version 8.2.3 (Stamatakis, 2014), under the best-fitting model of amino acid substitution selected by the IQ-TREE program (option -m TEST -mrate G4) with the Bayesian information criterion (BIC).

Concatenation-based ML species tree inference

Our recent evaluation of 19 empirical phylogenomic data matrices showed that IQ-TREE and RAxML/ExaML typically recovered the ML trees with the highest-observed likelihood scores (Zhou et al., 2018). Moreover, our pilot concatenation-based ML tree inferences under a single “LG (Le and Gascuel, 2008) +G4 (Yang, 1996)” model of amino acid substitutions using the multi-threaded version of IQ-TREE v1.5.1 (Nguyen et al., 2015) and the MPI parallel version of ExaML v3.0.17 (Kozlov et al., 2015) for the 2408OG and 1292BUSCO data matrices, showed that IQ-TREE and ExaML produced topologically identical ML trees with very similar likelihood scores, but IQ-TREE did so faster (IQ-TREE: ~3,000 CPU hours / single tree search; ExaML: ~4,200 CPU hours / single tree search). Therefore, we adopted the program IQ-TREE to infer concatenation-based ML trees under a single partition (LG+G4) and gene-based partitions (i.e., model parameters were unlinked across genes with the -q option in IQ-TREE), respectively. Branch support for each internode in the ML tree was evaluated with 100 rapid bootstrapping replicates using RAxML, hybrid version 8.2.3 (Stamatakis, 2014) with the CAT model with 25 categories instead of G4 model.

Coalescence-based species tree inference

For each data matrix, we used the set of individual ML gene trees (see section on individual gene tree inference above) to infer the coalescence-based phylogeny with ASTRAL-II, version 4.10.2 (Mirarab and Warnow, 2015). This is a summary species tree method that aims to account for individual gene tree heterogeneity due to incomplete lineage sorting (ILS). The reliability of each internal branch in the coalescence-based species tree was evaluated using the local posterior probability (LPP) measure.

Quantification of incongruence

For each of 27 species phylogenies inferred from the 9 data matrices (2 original data matrices + 7 subsampled data matrices) under the three different approaches (two concatenation-based and one coalescence-based), we used internode certainty (ICA) to quantify the degree of incongruence for every internode by considering all prevalent conflicting bipartitions among individual ML gene trees (Salichos and Rokas, 2013). The (partial) internode certainty (ICA) values were calculated as implemented in RAxML, multithread version 8.2.3 (option -f i).

Molecular dating

We used the Bayesian method MCMCTree in the paml4.9e package (Yang, 2007) to estimate divergence times among the 332 budding yeasts using the 2408OG data matrix. The input tree was derived from the concatenation-based ML analysis under a single LG+G4 model (Figure 2). Since budding yeasts lack a reliable fossil record, we adopted four well-estimated ranges of divergence from four internodes of the budding yeast phylogeny as our calibrations (Kumar et al., 2017; Marcet-Houben and Gabaldón, 2015). These were: the *Saccharomyces cerevisiae* –*Saccharomyces uvarum* split (lower bound: 14.3 MYA – upper bound: 17.94 MYA), the *Saccharomyces cerevisiae* – *Kluyveromyces lactis* split (103 MYA – 126 MYA), the *Saccharomyces cerevisiae* – *Candida albicans* split (161 MYA – 447 MYA), and the origin of the subphylum Saccharomycotina (304 MYA – 590 MYA).

Because Bayesian molecular dating is computationally intractable for data matrices that contain hundreds of species and thousands of genes (Irisarri et al., 2017), such as the 2408OG one, we created 50 replicate data matrices, each of which comprised of a different, randomly chosen subset of 100 OGs, to infer the budding yeast timetree (inference of each of the replicates took ~500 CPU hours). For each replicate, we first estimated branch lengths under a single LG+G4 model with codeml in the paml4.9 e package (Yang, 2007) and obtained a rough mean of the overall mutation rate. Next, we applied the approximate likelihood method (dos Reis and Yang, 2011) to estimate the gradient vector and Hessian matrix with Taylor expansion (option usedata = 3). Last, we assigned (a) the gamma-Dirichlet prior for the overall substitution rate (option rgene_gamma) as G(1, 12.5), with a mean of 0.08 (meaning 8×10^{-10} amino acid substitutions per site per year), (b) the gamma-Dirichlet prior for the rate-drift parameter (option sigma2_gamma) as G(1, 10), and (c) the parameters for the birth-death sampling process with birth and death rates $\lambda = \mu = 1$ and

sampling fraction $\rho = 0$. We employed the autocorrelated-rate model (option `clock = 3`) to account for the rate variation across different lineages and used soft bounds (left and right tail probabilities equal 0.025) to set minimum and maximum values for the four calibration splits mentioned above. The MCMC run was first run for 100,000 iterations as burn-in, then sampled every 500 iterations until a total of 3,000 samples was collected. Lastly, the divergence time estimate for each internal branch was calculated as the average across the timetrees produced by the 50 replicates.

In addition to the Bayesian MCMCTree method, we also used the non-Bayesian RelTime method, as implemented in the command line version of MEGA7 (Kumar et al., 2016). As RelTime is computationally much less demanding than MCMCTree (Mello et al., 2017), we conducted divergence time estimation using the complete 2408OG data matrix. We used the same ML tree and calibrations as we did for the MCMCTree analysis (see above).

Comparison of divergence time estimates between RelTime and MCMCTree revealed that they were broadly consistent (Pearson's correlation coefficient $r = 0.87$, P -value $< 2.2e-16$; average time deviation = $\sim 19.5\%$), which is in agreement with a previously published comparison of the two methods based on analyses of 8 empirical phylogenomic data matrices (Mello et al., 2017). Furthermore, both our study and that of Mello et al. (2017) found that the RelTime estimates were generally older than MCMCTree estimates for the deep internodes of the budding yeast phylogeny (e.g., for the internodes between 12 major clades) and were generally younger than the MCMCTree estimates for shallower internodes (e.g., within the families Pichiaceae, Saccharomycodaceae, Saccharomyctaceae, Phaffomycetaceae, the CUG-Ala clade, and the CUG-Ser1 clade).

Horizontal gene transfer analyses

Identification of HGT events

To detect genes in budding yeast genomes that may have been horizontally acquired from non-fungal organisms, we employed a robust and conservative phylogeny-based approach (Husnik and McCutcheon, 2018; Marcet-Houben and Gabaldón, 2010; Richards et al., 2011; Wisecaver et al., 2016). Briefly, for a given budding yeast gene, we inferred it to have been acquired by HGT if there was substantial topological disagreement between the gene tree and its associated species tree and the budding yeast gene sequence was robustly nested within the donor lineage in the gene tree.

To avoid spurious results due to the presence of small genomic fragments of contaminant organisms in our genome assemblies (Schönknecht et al., 2014), we limited our analyses to those genes that resided in genomic contigs or scaffolds that were ≥ 100 kb. This filter resulted in the analysis of 1,538,912 predicted genes (out of a total of 1,892,694 genes) in 329 yeast genomes. The remaining three genomes (*Botryozyma nematodiphila*, *Blastobotrys nivea*, and *Citeromyces siamensis*) did not contain genomic contigs that were ≥ 100 kb.

For each gene, we evaluated whether it had been horizontally acquired using a two-step workflow.

In step 1, we first carried out a BLASTP search against a custom database (nr+) consisting of the NCBI non-redundant (nr) protein database (last accessed January 20, 2017) and all predicted protein sequences from 329 yeasts genomes, with an e-value cutoff of 10^{-10} . We next used custom Perl scripts to: (a) assign taxonomic information to each BLAST hit rating with the dump files downloaded from the NCBI Taxonomy database (nodes.dmp, merged.dmp, and names.dmp; <ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy>), and then (b) parse the BLAST hits, based on their taxonomic information, into three different lineages (RECIPIENT: Saccharomycotina; GROUP: Fungi; OUTGROUP: non-fungal) so as to obtain three values: **bbhO** (BLAST bitscore of the best hit in OUTGROUP lineage), **bbhG** (bitscore of the best hit in GROUP lineage but not in RECIPIENT lineage), and **maxB** (bitscore of the query to itself).

Using this information, we next calculated: (a) the Alien Index (AI) value (Wisecaver et al., 2016), which is a normalized difference of bitscore between the best hit in OUTGROUP lineage and the best hit in GROUP lineage but not in RECIPIENT lineage: $AI = bbhO/maxB - bbhG/maxB$, and (b) the percentage of species from OUTGROUP lineage (outg_pct) in the list of the top 300 hits that have different taxonomic species names (so that we avoid over-representation of multiple strains of the same species) (Marcet-Houben and Gabaldón, 2010). From the 1,538,912 genes analyzed, we found 1,806 genes with AI value ≥ 0.1 and outg_pct $\geq 90\%$.

In step 2, we retrieved the 300 most similar homologs that have different taxonomic species names from the nr+ database (see above), aligned them by the MAFFT, version 7.299 (Katoh and Standley, 2013), with “-auto” option, and trimmed ambiguously aligned regions using trimAl v1.4 (Capella-Gutiérrez et al., 2009) with “-automated1” option. We then used the resulting alignment to infer the ML tree using IQ-TREE 1.5.1 (Nguyen et al., 2015) with its best-fitting model of amino acid evolution and 1000 ultrafast bootstrapping replicates (Minh et al., 2013). Lastly, we rooted each ML tree at the midpoint using the ape and phangorn R packages and visualized it using the command version of iTOL v3 (Letunic and Bork, 2016). After manually inspecting all 1,806 ML trees, we identified 878 genes in 186 / 329 budding yeast genomes whose phylogenies indicated they were putatively acquired via HGT. A summary table file that contains species name, gene name, genomic contig ID, genomic contig length, HGT status, and Gene Ontology (GO) term for each of 1,538,912 genes in 329 yeast genomes that we examined has been deposited on the Figshare data repository.

Analysis of HGT-acquired genes

For each of 878 genes inferred to have been horizontally transferred into budding yeast genomes, we used the gene sequence and taxon names of their closest relatives on the ML tree to infer the gene name, gene function, and likely donor lineage of the HGT-acquired gene (Table S3). To examine the biological processes, cellular components, and molecular functions that these 878 HGT-acquired genes are associated with, we conducted gene ontology (GO) enrichment analysis using topGO 2.28.0 (Alexa and Rahnenfuhrer, 2016). We found that these genes were significantly enriched in metabolism-related terms, such as metabolic process, oxidation-reduction process, carbohydrate metabolic process (terms in Biological process), beta-galactosidase complex, integral

component of membrane (terms in Cellular component), acetyltransferase activity, and catalytic activity (terms in Molecular function) ([Figure S5](#)). In the list of 878 putative HGT-acquired genes ([Table S3](#)), the largest number of horizontally acquired genes, 169, was found in the genome of *W. versatilis*, one of the six representative species from the W/S clade ([Figure 3A](#)). Transcriptome data in NCBI (SRA accession numbers: SRR5942408, SRR5942407, SRR5942426, SRR5942425, SRR5942428, SRR5942427, SRR5942422, SRR5942421) showed that at least 91 of its 169 horizontally acquired genes had expression values ≥ 5 FPKM (Fragments Per Kilobase per Million mapped fragments), suggesting that most are likely functional. Gene Ontology (GO) enrichment analysis of 169 HGT-acquired genes in the genome of *W. versatilis* show they are significantly enriched in GO term functions and processes, such as oxidation-reduction process, metabolic process, catalytic activity, cofactor binding, and oxidoreductase activity.

Examination of the 878 HGT-acquired gene phylogenies showed that they stem from 365 distinct HGT events, 230 species-specific ones and 135 that involve two or more species ([Figure S4](#); [Table S3](#)). The average age of these 365 HGT events was 66.3 MYA (95% credibility interval: 56.6–76.0), the average protein sequence identity between the HGT-acquired yeast gene and the most closely related non-fungal donor gene was 58%, and the average percentage of descendant species that retained a given HGT-acquired gene was 40.7%. These results suggest that most HGT events tend to affect one or a few species, they are relatively ancient or divergent, and are frequently lost.

Finally, the examination of the genomic locations of the 878 HGT-acquired genes showed that 77 genes were physically linked in 13 contiguous clusters of 3 or more genes each. Of these 13 horizontally acquired gene clusters, 4 were shared by several budding yeast species, while the other 9 were species-specific.

Robustness of HGT inference

Inference of HGT can be noise-prone ([Husnik and McCutcheon, 2018](#); [Martin, 2018](#); [Roger, 2018](#)). To gauge the robustness of inference of HGT-acquired genes, we performed five additional sets of analyses.

First, we compared the list of 878 putative HGT-acquired genes to the list of 30 previously identified instances of HGT in budding yeasts. We found that 21 / 30 (70%) of these previously detected instances of HGT were included in our set of 878 HGT-acquired genes, corroborating the sensitivity and conservativeness of our high-throughput approach for identifying HGT ([Table S3](#)).

Second, to gauge whether setting the values of AI value ≥ 0.1 and outg_pct $\geq 90\%$ in step 1 was too strict, we also examined the top 1,806 candidates with the highest AI scores for outg_pct $\geq 10\%$. Examination of the overlap between the set of 1,806 genes recovered with AI value ≥ 0.1 and outg_pct $\geq 90\%$ and the set of the top 1,806 genes recovered with outg_pct $\geq 10\%$ showed that that 605 / 878 strongly supported cases of HGT were present in both sets.

Third, we examined diverse properties of contigs containing HGT-acquired genes and compared to those of contigs lacking HGT-acquired genes. Examination of the distribution of sequence lengths of the 480 genomic contigs that contain 878 HGT-acquired genes, alongside the 8,312 genomic contigs that do not contain any HGT-acquired genes, showed that contigs containing the HGT-acquired genes were typically longer than contigs that lack them. We further used different cutoff values of contig length (from ≥ 100 kb to $\geq 1,500$ kb with steps of 100 kb) to examine the percentage of HGT-acquired genes over total number of gene examined and found that it slightly increased with contig size.

Fourth, we compared the percentages of HGT-acquired genes in the 220 newly sequenced genomes and in the 112 publicly available genomes and found them to be similar. In contrast, the percentage of HGT-acquired genes in the 164 genomes with the highest N50 values was significantly higher than the percentage in the 165 genomes with the lowest N50 values.

Finally, to test whether the maximum likelihood (ML) gene tree (i.e., the tree with the highest ML score, which shows that the horizontally acquired yeast gene was nested within a clade of non-fungal donor genes) was statistically different from a constrained ML tree in which all fungal and yeast genes were forced to be monophyletic, we applied the approximately unbiased (AU) ([Shimodaira, 2002](#)) test in the software package CONSEL ([Shimodaira and Hasegawa, 2001](#)), version 0.20. We found that 616 out of 878 putative HGT-acquired genes are significantly supported with the AU test (AU test; p values < 0.05) ([Table S3](#)).

Analyses of trait evolution

In this study, we reconstructed the evolution of 45 discrete metabolic traits in 274 budding yeasts representing the 12 major clades. All metabolic trait data were obtained from *The Yeasts: A Taxonomic Study* and pertain to budding yeasts' abilities to grow on different substrates ([Kurtzman et al., 2011](#)).

For each discrete metabolic trait, we scored each taxon for its ability to grow (hereafter scored as "1"), not grow (hereafter scored as "0"), show variation in growth / absence of growth across different strains (hereafter scored as "v"), or lack of information / missing data (hereafter scored as "n") ([Table S4](#)).

To conduct analyses of trait evolution and ancestral state inference, we used Bayes MultiState module in the BayesTraits, version 3 ([Pagel et al., 2004](#)), because of its ability to apply reversible jump MCMC (rjMCMC) to optimize model uncertainty with different parameters. To infer ancestral states and the rates of trait gain and loss, BayesTraits takes as input the species phylogeny, the trait states, and a model of trait evolution. For the input species phylogeny, we used the ML tree with branch lengths inferred from the 2408OG data matrix using a single LG+G4 model ([Figure 2](#)), which was then pruned to keep the 274 budding yeast species for which there were metabolic trait data.

For each trait, v and n values were recoded to be "01" (this means that the character's state can be either "0" or "1") and "-" in the BayesTraits analyses, respectively. We calculated the rate of gain (q01: rate of change from 0 to 1) and the rate of loss (q10: rate of change from 1 to 0) across the budding yeast phylogeny. To test whether the rates of trait gain and loss were significantly different or

not, we compared estimates of marginal likelihoods under a model of trait evolution in which the rates of trait gain and loss were unconstrained against those obtained under a model in which the rates of gain and loss were constrained to be equal. We determined whether the two models were significantly different using log Bayes Factors ($\ln BF$): $2(\log \text{marginal likelihood [unconstrained]} - \log \text{marginal likelihood [constrained]})$; the rates of gain and loss for a given trait were considered to be significantly different when $\ln BF > 2$.

For each metabolic trait, we also inferred the posterior probability of each of the two character states (0 and 1) at the root (i.e., the budding yeast common ancestor [BYCA]) and at each of 272 internodes across the phylogeny. Each analysis was run for 10 million generations, sampling parameters every 4,000 generations until 2,000 samples were collected. The stepping stone sampler was used to estimate marginal likelihoods, sampling 200 stones in which each stone was run 1,000 generations.

Finally, we plotted the kernel density of the posterior distribution for the rates of trait gain and loss, as well as posterior probabilities (PP) of states 0 and 1 at each internode, and identified the largest peak values from their densities. To visualize the ancestral state at each internode across the budding yeast phylogeny, we used the pie chart function in iTOL v3 (Letunic and Bork, 2016).

Validation of metabolic trait data

As the metabolic trait data in *The Yeasts: A Taxonomic Study* (Kurtzman et al., 2011) comes from multiple sources, contains missing data, and the strains tested sometimes differ from the sequenced strains, we also experimentally determined the growth for 328 / 332 strains of the budding yeast species whose genomes we used on 13 / 45 discrete metabolic traits, 11 carbon-based and 2 nitrogen-based (Table S4). As a control, we also experimentally determined yeast species growth on glucose, a substrate on which all sampled strains are known to grow (Kurtzman et al., 2011).

Carbon treatment plates contained a minimal media base with ammonium sulfate and one of twelve carbon sources at a 2% concentration (Kurtzman et al., 2011). The 12 carbon sources tested in our analyses included glucose, sucrose, raffinose, galactose, lactose, maltose, cellobiose, L-rhamnose, D-xylose, glycerol, D-glucosamine, and *N*-acetyl-D-glucosamine. Nitrogen treatment plates contained a minimal media base with either potassium nitrate or sodium nitrite as the nitrogen source and 2% glucose (Kurtzman et al., 2011). Nitrogen treatment plates required two rounds of growth to accurately measure growth of species; therefore, after a week of growth, we pinned the yeast species into a second round of the respective nitrogen source. After a week of growth, we visually scored yeast species for growth on each carbon or nitrogen source. We then compared growth across replicates for each species and treatment; a species was determined to grow on a carbon source if it showed growth $\geq 50\%$ of the time across replicates. Our results show that the average consistency of the character states shared between the compilation of available metabolic trait data and our experimental measurements was 94% (Table S4), suggesting that our inferences are based on accurate data.

Trait and gene association network analyses

Calculations of positive associations among carbon and nitrogen assimilation traits were obtained from a recently published study (Opulente et al., 2018). A positive association network was created in the R package igraph v. 1.0.1 (Csárdi and Nepusz, 2006), and trait communities in these networks were determined through the Clauset-Newman-Moore algorithm (fast.greedy community), an approach that determines communities by maximizing the total network's modularity.

The input data matrix consisted of species-specific presence and absence data for functional annotations of genes, together with the species-specific qualitative information about their capabilities to grow in the conditions used in the ancestral trait reconstruction analyses. The functional annotations were Kyoto Encyclopedia of Genes and Genomes (KEGG) database entries or annotations (Kanehisa et al., 2016b) obtained through GhostKOALA (Kanehisa et al., 2016a). Mutual information coefficients were calculated for each pairwise combination of KEGG annotation and trait using the R packages infotheo, minet (Meyer, 2008; Meyer et al., 2008), and WCGNA (Langfelder and Horvath, 2008), and the resulting association network was trimmed with the ARACNE algorithm (Margolin et al., 2006). Strong associations were defined by a cutoff of 0.15 nats or higher, and weak associations were considered between 0.10 and 0.15 nats. The positive or negative character of the associations was determined by calculating the Jaccard index for each pairwise combination, with a cutoff at 0.25.

Data tracks shown on Figure 5 were subject to the following adjustments: (1) the nitrite growth track contained exclusively data from validation experiments performed in this study (Table S4), due to limited data in the literature (Table S4); (2) for the nitrate growth track, positive growth was imputed in cases of disagreement between the validation and literature; and (3) due to partial annotation redundancy for specific steps of the Moco biosynthesis pathway, MOCS1 and MOCS2 data tracks were collated from multiple annotations, with MOCS1 activity being considered present if either the K03637, K03639, or K20967 KEGG annotation was found in a genome and with MOCS2 activity being considered present if either the K03635, K03636, or K21232 KEGG annotation was found in a genome.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses and quantification approaches associated with genome sequencing, accuracy and completeness of assembly, accuracy and completeness of annotation, phylogenetic data matrix reconstruction, horizontal gene transfer (HGT) inference, and metabolic trait evolution can be found in the relevant sections of the Method Details.

The Fisher's exact tests were conducted in R version 3.4.0.

DATA AND SOFTWARE AVAILABILITY

Raw sequence read and genome assembly data for all 220 new genomes have been deposited at DDBJ/ENA/GenBank and their accession numbers are provided in [Table S1](#). All budding yeast genome assemblies and annotations, phylogenetic data matrices, multiple sequence alignments, phylogenetic trees, the metabolic trait data matrix, trait ancestral character state reconstructions, additional figures and tables, R codes, and the custom Perl scripts used in this study are available on the Figshare data repository (10.6084/m9.figshare.5854692; https://figshare.com/articles/Tempo_and_mode_of_genome_evolution_in_the_budding_yeast_subphylum/5854692). All sequenced strains have been publicly deposited in the NRRL, CBS, and/or JCM strain collections.

Supplemental Figures

Cell

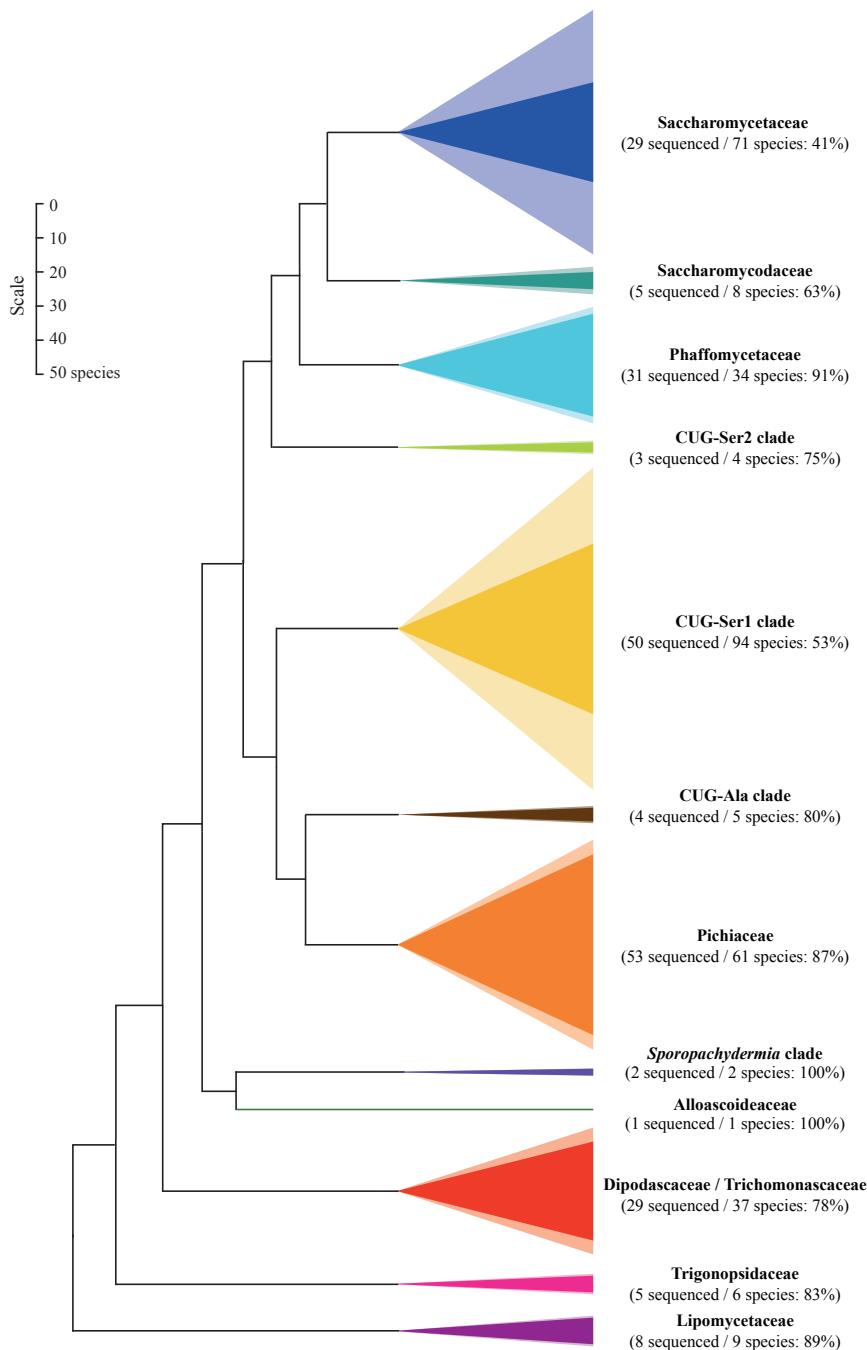


Figure S1. Distribution of 332 Budding Yeast Species with Sequenced Genomes across the 12 Major Clades of the Subphylum Saccharomycotina, Related to Figure 2

In each clade, the size of the lightly colored triangle is proportional to number of budding yeast species sampled; the size of the darkly colored triangle is proportional to number of yeast species whose genomes were newly sequenced by the Y1000+ Project (<http://www.y1000plus.org>) and RIKEN (unpublished but publicly available at http://www.jcm.riken.jp/cgi-bin/nbrp/nbrp_list.cgi). The percentage of (number of newly sequenced species) / (number of all sequenced species) for each clade is shown in parentheses. The cartoon phylogeny is based on Figure 2.

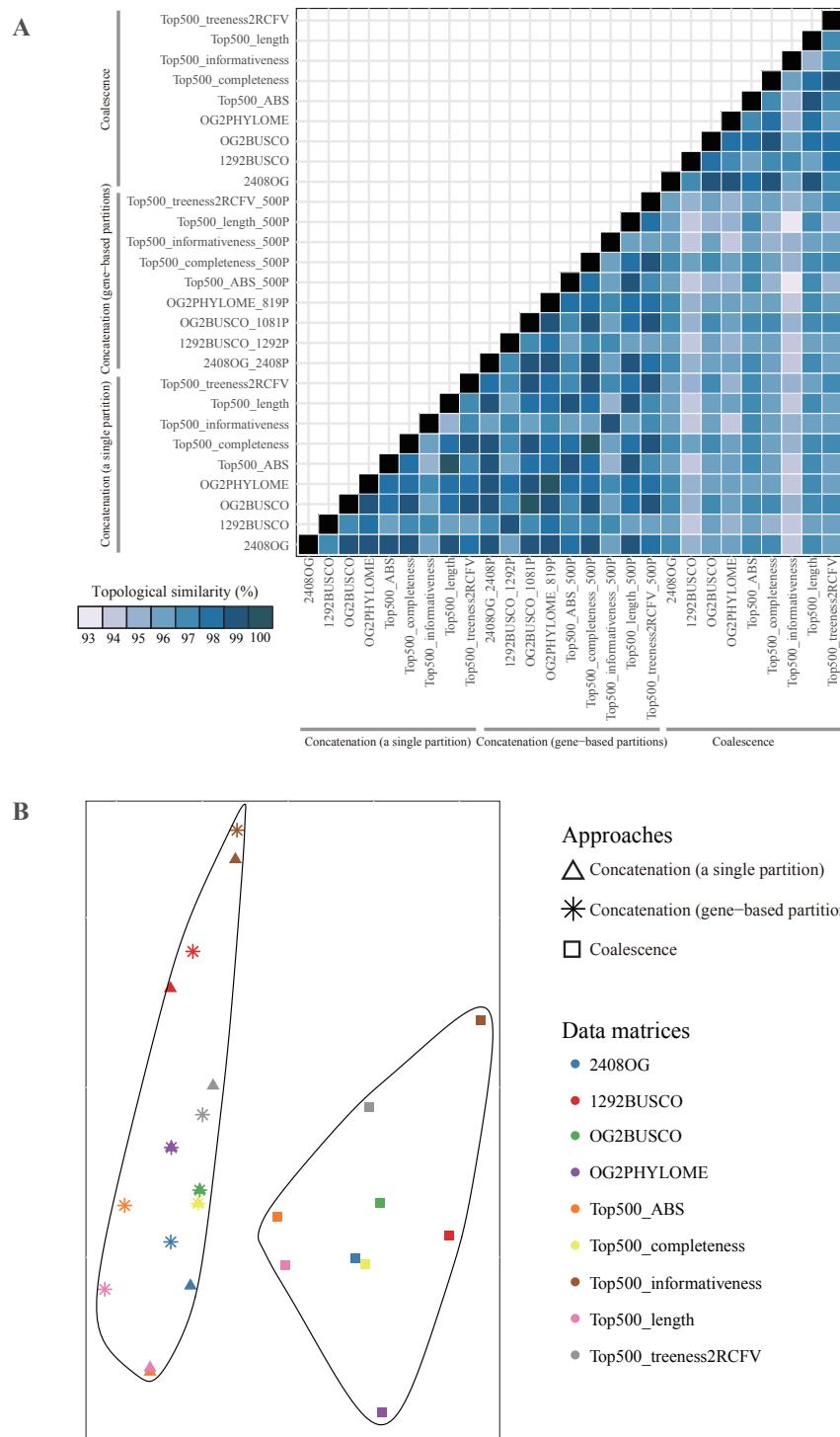


Figure S2. The Degree of Topological Similarity among 27 Phylogenies Reconstructed from Analyses of 9 Different Data Matrices, Related to Figure 2

(A) Heatmap of topological similarities for all pairwise comparisons among the 27 phylogenies reconstructed from analyses of 9 different data matrices (2408OG, 1292BUSCO, and the 7 data matrices constructed from subsamples of the 2408OG data matrix) using three different approaches (concatenation under a single

(legend continued on next page)

partition, concatenation under gene-based partitioning, and coalescence). The topological similarity between each pair of phylogenies was calculated using RAxML with the option '-f r'.
(B) The multidimensional-scaled visualization of tree space based on the pairwise Robinson-Foulds distances among the 27 phylogenies was built with R 3.4.0 (see <https://www.r-bloggers.com/multidimensional-scaling-mds-with-r/>). Most topological disagreements are between concatenation-based and coalescence-based analyses.

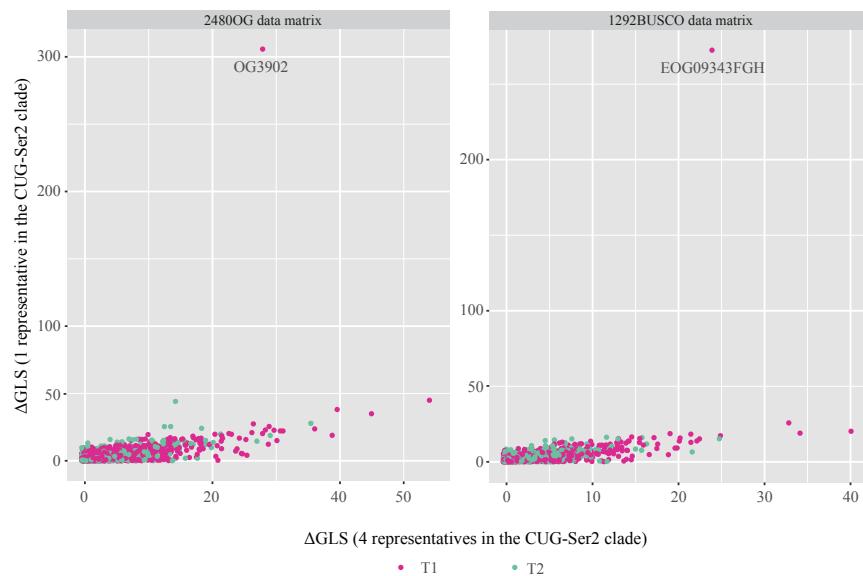


Figure S3. The Effect of Taxon Sampling on Gene-Wise Phylogenetic Signal for the CUG-Ser2 Clade Branch in Two Data Matrices (Left Panel: 2408OG Data Matrix; Right Panel: 1292BUSCO Data Matrix), Related to Figure 2

We previously showed that removal of a single gene (named *DPM1* in *S. cerevisiae*), which has the strongest gene-wise phylogenetic signal in a 1,233-gene, 86-taxon yeast data matrix, switched the ML tree's support from T1 (CUG-Ser2 as sister to a clade consisting of Phaffomycetaceae + Saccharomycodaceae + Saccharomycetaceae) to T2 (CUG-Ser2 as sister to a clade consisting of Pichiaceae + CUG-Ala clade + CUG-Ser1 clade + Phaffomycetaceae + Saccharomycodaceae + Saccharomycetaceae) for the CUG-Ser2 clade branch. In that data matrix, *Ascoidea rubescens* is the single representative of the CUG-Ser2 clade. We tested whether inclusion of three additional representatives (*Ascoidea asiatica*, *Saccharomyces malanga*, and *Saccharomyces capsularis*) for the CUG-Ser2 clade in the 2408OG and 1292BUSCO data matrices influenced estimates of the phylogenetic signal of the *DPM1* OG. For each of the two data matrices, we calculated the gene-wise phylogenetic signal by measuring the difference in gene-wise log-likelihood scores (ΔGLS) for T1 versus T2 across all genes with and without these three additional CUG-Ser2 clade representatives (*Ascoidea asiatica*, *Saccharomyces malanga*, and *Saccharomyces capsularis*). The x axis shows the gene-wise phylogenetic signal when all 4 CUG-Ser2 clade representatives are included, and the y axis shows the signal when only a single CUG-Ser2 clade representative (*A. rubescens*) is included. Pink dots denote genes supporting T1 in the full data matrix, whereas green dots denote genes supporting T2 in the full data matrix. The two dots marked OG3902 (in 2408OG data matrix) and EOG09343FGH (in 1292BUSCO data matrix) denote the two OGs containing the *DPM1* gene. Although the gene-wise phylogenetic signal values between the two analyses are highly concordant, the *DPM1* gene has very strong phylogenetic signal when only a single CUG-Ser2 clade representative is included; addition of 3 more CUG-Ser2 clade representatives dramatically reduces the gene's phylogenetic signal. This behavior is consistent with our previous analyses suggesting that the *DPM1* gene's phylogenetic signal in favor of T1 over T2 when only a single CUG-Ser2 clade representative is used stems from a poor fit between the models of sequence evolution employed and the gene's observed sequence variation.

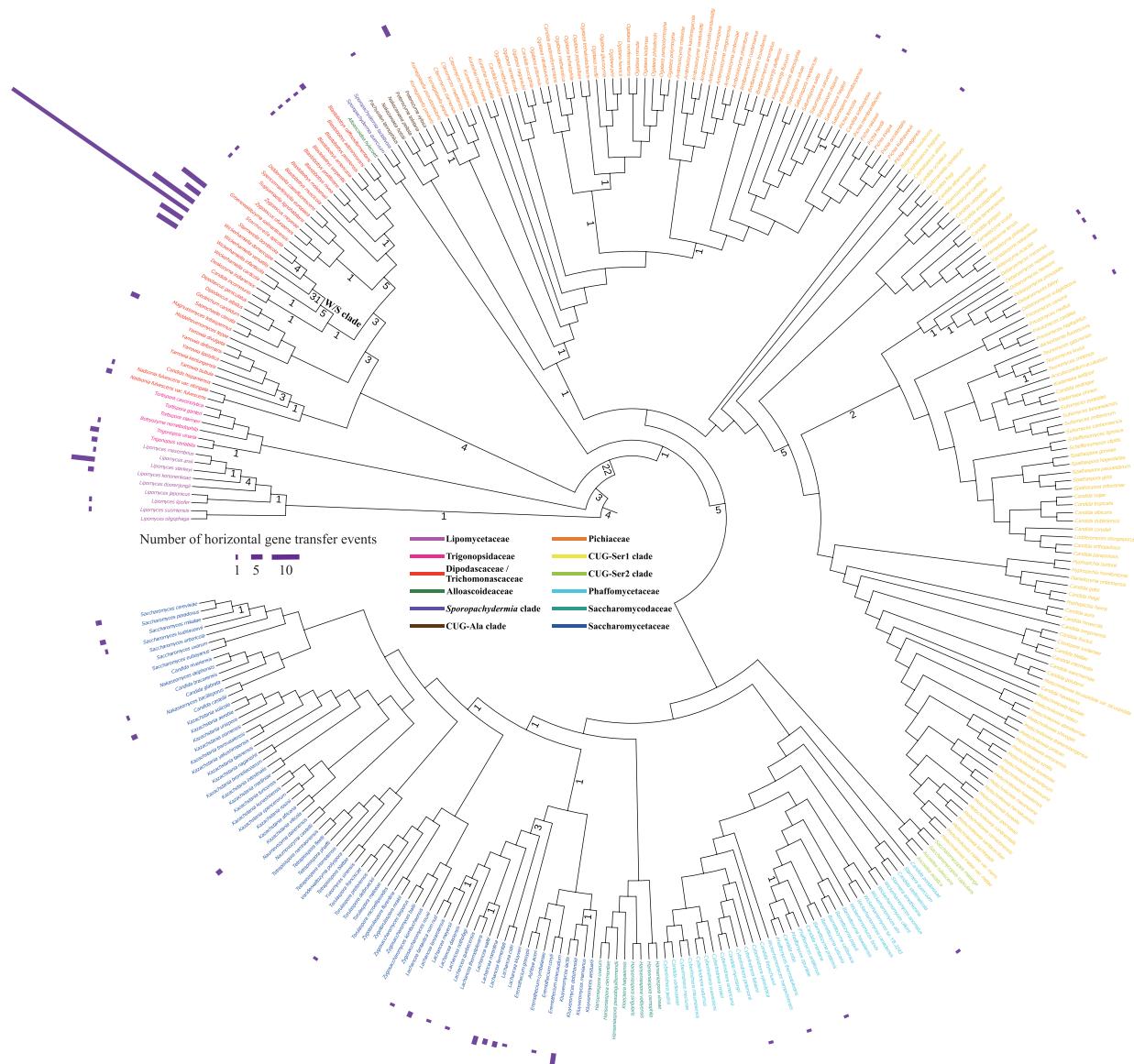


Figure S4. Distribution of 365 HGT Events on the Budding Yeasts Phylogeny, Related to Figure 3 and Table S3

Examination of the phylogenetic trees of the 878 HGT-acquired genes showed that they stem from 365 distinct transfer events. 230 of these transfer events appear to be species-specific, whereas the remaining 135 involve two or more species. Bars next to species names denote numbers of species-specific HGT events. Numbers near internodes denote numbers of HGT events that led to HGT-acquired genes found in two or more species. The budding yeast phylogeny is from Figure 2. The multiple sequence alignments and gene trees of the 878 HGT-acquired genes are provided in the Figshare depository.

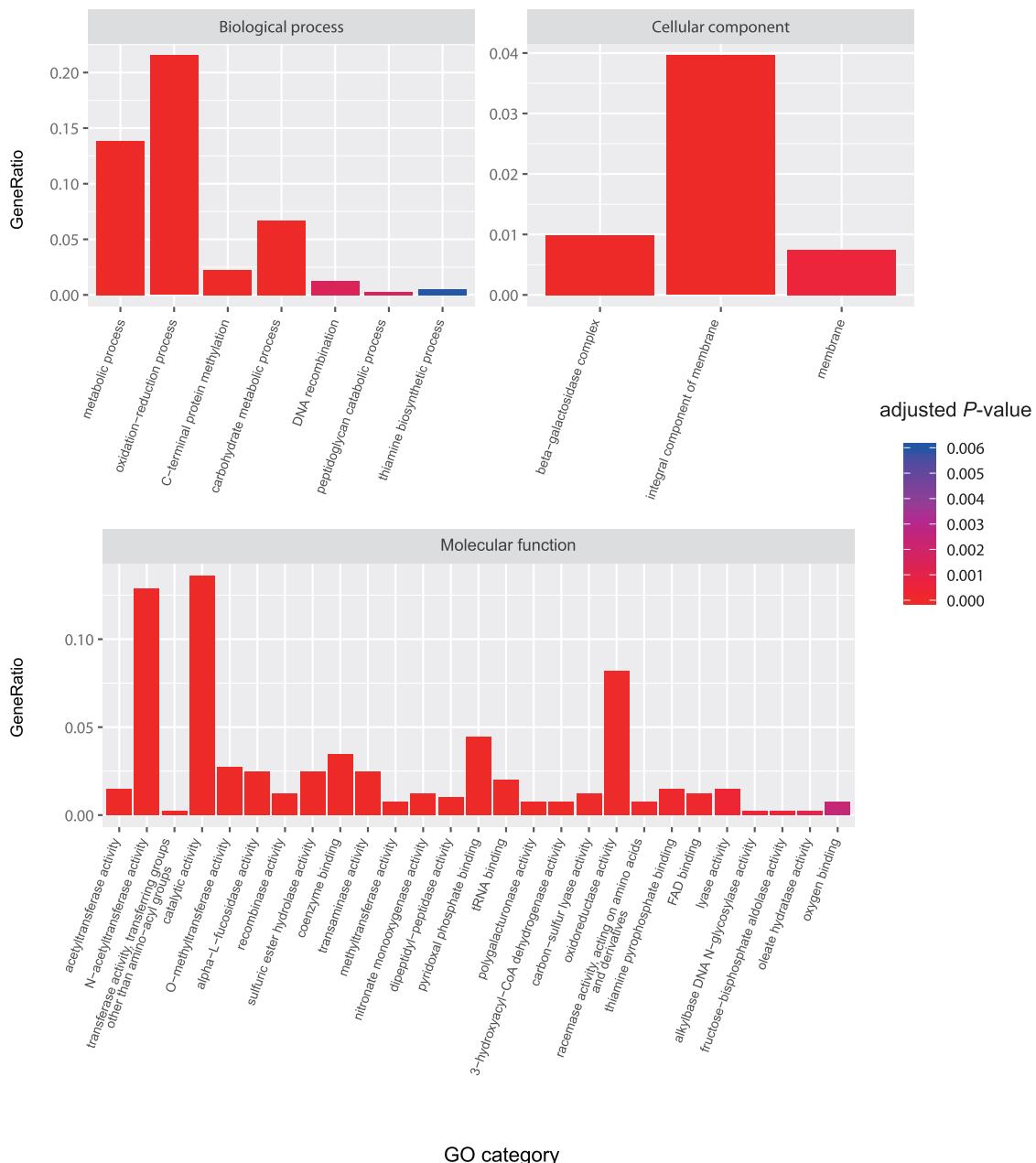
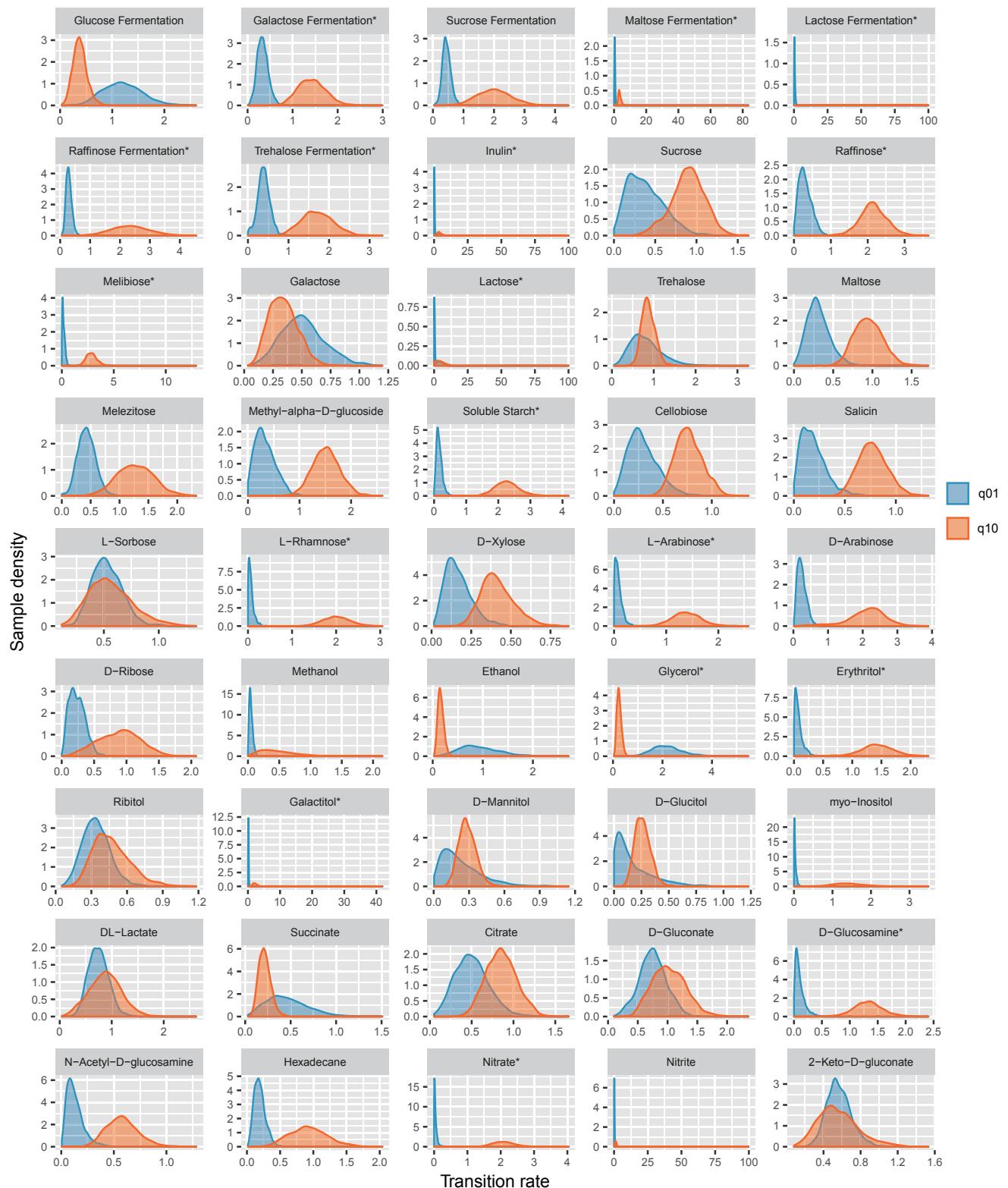


Figure S5. Gene Ontology Term Enrichment Analysis of the 878 HGT-Acquired Genes, Related to Figure 3 and Table S3

Only significantly enriched GO terms ($\text{adjusted } P\text{-value} \leq 0.01$) are shown. The GeneRatio value corresponds to the ratio of the number of genes assigned to the respective GO term to the total number of genes examined. GO terms are arranged along the x axis in ascending order of the adjusted P -values.



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Figure S6. Posterior Distribution of Rates of Trait Gain and Loss for 45 Metabolic Traits across the Budding Yeast Phylogeny, Related to Table 1

q01: rate of trait gain or rate of change from state 0 (absence of metabolic trait) to state 1 (presence of metabolic trait). q10: rate of trait loss or rate of change from state 1 to state 0. Each of the panels corresponds to a different metabolic trait. Trait evolution was reconstructed on the budding yeast species phylogeny (Figure 2) after it was pruned down to the 274 budding yeast species for which there are metabolic trait data. Traits that exhibited significantly different rates of trait gain and loss measured by log Bayes Factors (lnBF) are indicated by asterisks (*).

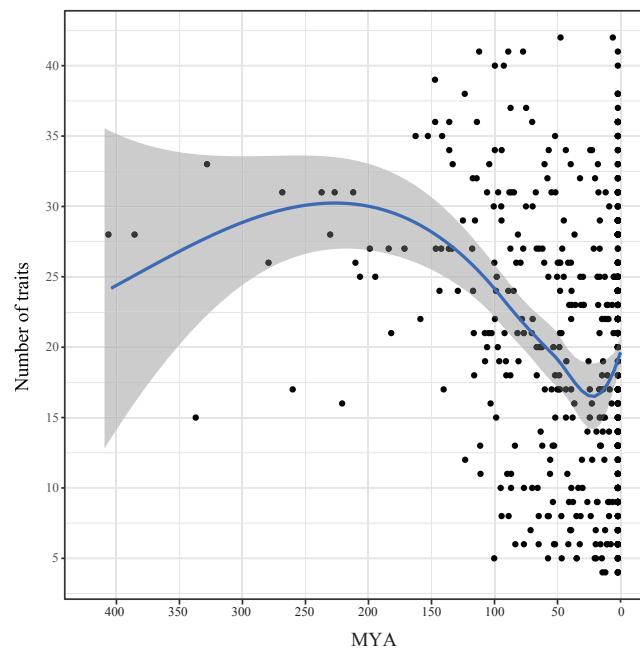


Figure S7. Distribution of 45 Metabolic Traits on the Budding Yeast Phylogeny, Related to Figure 6

There are 547 dots in the scatterplot, which correspond to the 273 internodes and 274 tips on the 274-budding yeast phylogeny. The x axis is the posterior mean of the node age, and the y axis is the number of traits with posterior probabilities of > 0.5 for being present at the node.