Independent Evolution of Winner Traits without Whole Genome Duplication in DeKKera Yeasts

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Abstract

DeKKera yeasts have often been considered as alternative sources of ethanol production that could compete with S. cerevisiae. The two lineages of yeasts independently evolved traits that include high glucose and ethanol tolerance, aerobic fermentation, and a rapid ethanol fermentation rate. The Saccharomyces yeasts attained these traits mainly through whole genome duplication approximately 100 million years ago (Mya). However, the DeKKera yeasts, which were separated from S. cerevisiae approximately 200 Mya, did not undergo whole genome duplication (WGD) but still occupy a niche similar to S. cerevisiae.

Upon analysis of two DeKKera yeasts and five closely related non-WGD yeasts, we found that a massive loss of cis-regulatory elements occurred in an ancestor of the DeKKera yeasts, which led to improved mitochondrial functions similar to the S. cerevisiae yeasts.

The evolutionary analysis indicated that genes involved in the transcription and translation process exhibited faster evolution in the DeKKera yeasts. We detected 90 positively selected genes, suggesting that the DeKKera yeasts evolved an efficient translation system to facilitate adaptive evolution. Moreover, we identified that 12 vacuolar H+-ATPase (V-ATPase) function genes that were under positive selection, which assists in developing tolerance to high alcohol and high sugar stress. We also revealed that the enzyme PGK1 is responsible for the increased rate of glycolysis in the DeKKera yeasts. These results provide important insights to understand the independent adaptive evolution of the DeKKera yeasts and provide tools for genetic modification promoting industrial usage.

Introduction

Yeasts usually win the competition in sugar rich environment and become the predominant group in nature [1]. The baker’s yeast Saccharomyces cerevisiae was the most representative
winner which could rapidly convert sugars into ethanol even under aerobic conditions. This aerobic fermentation is also known as the Crabtree effect [2]. When sugars are abundant in the environment, S. cerevisiae transforms sugars into ethanol, which results in the inhibition of other microorganisms. S. cerevisiae also uses ethanol as a carbon source to produce energy and growth. This "make–accumulate–consume" lifestyle has benefited S. cerevisiae and its close relatives throughout history [3, 4]. Fast sugar consumption, ethanol production, accumulation and tolerance, and the ability to propagate without oxygen are the traits responsible for S. cerevisiae becoming the "winner" in nature and industrial ethanol production [5]. However, nonconventional yeast species, Dekkera yeasts, have been observed to compete with S. cerevisiae in several ethanol production plants that contain low concentrations of sugars [6].

Dekkera yeasts are a major cause of wine spoilage worldwide [7] due to the production of volatile by-products. It has been estimated that D. bruxellensis diverged from a common ancestor of S. cerevisiae 200 million years ago [5]. However, these two phylogenetically distant yeasts have independently evolved similar features beneficial for ethanol production. They are also often found in the same habitats and share several food-related traits, such as the production of higher levels of ethanol and the ability to grow without oxygen [8]. Both yeasts display the capacity to produce ethanol under aerobic conditions and the ability to tolerate high levels of ethanol, to grow under oxygen-limited conditions, and to survive without mitochondria [9–11]. These traits of S. cerevisiae are considered to be the consequence of yeast whole genome duplication (WGD) and promoter rewiring that happened approximately 100 million years ago [12–15]. It is apparent that the Dekkera yeasts did not undergo WGD. Nevertheless, Dekkera have been recognized as an alternative to S. cerevisiae in ethanol production [16, 17]. It is not clear how the Dekkera yeasts acquired the adaptive "winner" traits without WGD. The analysis of promoter sequences indicates that both Dekkera and S. cerevisiae independently underwent a massive loss of a specific cis-regulatory element in respiration genes, which contributes to the Crabtree effect [5]. However, the dysfunction of mitochondrial oxidative phosphorylation and the Crabtree effect may reinforce each other mutually. The causes and mechanisms involved in the Crabtree effect are not completely understood.

The decoding of the Dekkera genomes presented an opportunity to study the molecular evolution features in the Dekkera yeasts [8, 18]. In this study, Dekkera bruxellensis CBS 2499 and its nearest phylogenetic neighbor Dekkera anomala strain YV396, a species found to co-exist with D. bruxellensis, were chosen to represent the Dekkera yeasts [19]. We compared the Dekkera yeasts with five related non-WGD yeasts (Y. lipolytica, K. lactis, L. kluyveri, L. waltii, and E. gossypii) to investigate the independent evolution of "winner" traits in the Dekkera yeasts. The results indicated that the Dekkera yeasts and S. cerevisiae evolved independently to develop the "winner" traits with different evolutionary processes.

Results
Gene identification and annotation in the two Dekkera yeasts
We used the AUGUSTUS online gene prediction tool to identify all of the possible protein coding open reading frames (ORFs) in the genomes of Dekkera bruxellensis CBS 2499 and Dekkera anomala strain YV396 [20]. In total, 5208 and 5241 ORFs were predicted for D. bruxellensis CBS 2499 and D. anomala YV396, respectively. Next, we utilized a reciprocal best-hits (RBH) BLAST approach for the prediction of ORFs in the S. cerevisiae protein-coding genes [21]. Ultimately, we annotated 3446 and 3500 genes which were one to one orthologous gene of S. cerevisiae in D. bruxellensis and D. anomala, respectively (S1 Table). All of these genes were used for further studies. Using WEGO, a web tool for plotting GO annotations, these annotated genes
were mapped to 48 main GO categories in three domains (24 GO terms in Biological Process, 12 GO terms in Molecular Function, and 12 GO terms in Cellular Component) (Fig 1).

The loss of Cis-elements in *D. bruxellensis* and *D. anomala* mitochondrial ribosomal genes

Modification of cis-elements at conserved sites is linked to the transcriptional network and regulation of gene expression, which have an intimate relationship with phenotypic diversity [13]. For example, the AATTTT motif, known as the rapid growth element (RGE), was found in the genes involved in rapid growth and respiration [22]. Previous studies have reported the profoundly massive loss of AATTTT motifs in the respiration-associated genes in *D. bruxellensis*, *S. cerevisiae*, and their sister species [5, 8]. To verify whether AATTTT motifs were also lost in *D. anomala* compared with the other yeasts, we collected three data sets: the CRP set (the rapid growth-associated genes, including the cytoplasmic ribosomal genes, ribosomal biogenesis and nuclear export of ribosomal subunits genes, and genes related to the purine biosynthesis pathway), the rRNA set (the rapid growth-associated genes, including the rRNA processing genes, genes coding for proteins involved in RNA biogenesis and transport, t-RNA transporters, pyrimidine biosynthesis pathway), and the MRP set (including mitochondrial ribosomal genes). We searched 600 bp of sequence upstream from these genes to identify promoter regions and analyzed the presence of the AATTTT motifs.

As expected, the AATTTT motifs were enriched in the sequences which were 50–150 bp upstream from the start codon ATG. The patterns of AATTTT motifs in rRNA genes from *D. anomala* were nearly similar to those in *D. bruxellensis*. Approximately 40% of the rRNA genes...
possessed AATTTT motifs in the sequences 100 bp upstream of ATG. The AATTTT patterns of CRP genes and MRP genes in the
D. bruxellensis and D. anomala (Fig 2A and 2B) were different from those in K. lactis and L. waltii (Fig 2C and 2D). The MRP and CRP genes of the
Dekkera yeasts showed relatively lower percentages of AATTTT motifs 50–150 bp upstream of ATG compared with the rRNA genes. Nevertheless, the percentage of MRP genes in the Dekkera yeasts that contain an AATTTT motif was nearly half of that in K. lactis and L. waltii (15% versus 30%). This result illustrated that both the Dekkera yeasts lost cis-regulatory AATTTT motifs in the MRP gene promoters, similar to the post-WGD S. cerevisiae.

Rapid evolution of coding sequences in the D. bruxellensis and D. anomala

Analyses of orthologous genes suggested that rapid evolution occurred in the genes mediating the adaptation to produce high levels of ethanol and grow without oxygen. Among the 3500 predicted coding genes in the Dekkera yeasts, 2429 genes were annotated as high-confidence one-to-one orthologous genes among the seven non-WGD yeasts. These genes were used to estimate the evolutionary constraints acting on the Dekkera yeasts (Fig 3A). We calculated the dN/dS value for each GO term in the Dekkera branch and the other four non-WGD branches using the free ratio model implemented in PAML4 [23]. In total, 24 GO categories showed significantly higher dN/dS values in the Dekkera branch than those in the other branch yeasts (Adjusted P < 0.05, binomial test). These categories were primarily related to gene transcription and translation, including rRNA export from the nucleus (GO:0006407, P = 0.0031), small ribosomal subunit (GO:0015935, P = 0.039), SWI/SNF complex (GO:0031225, P = 0.009), cytoplasmic translation (GO:0002181, P = 0.0013), and transcription coactivator activity (GO:0003713, P = 0.013). A few categories showed lower dN/dS values in the Dekkera yeasts, such as NADP binding (GO:0050661, P = 0.021) (S2 Table, Fig 3B). The free ratio model also
indicated that 748 (30.7%) genes evolved more than two-fold faster in the Dekkera yeasts, while 646 (26.5%) genes evolved faster in the other four non-WGD yeasts. A wide range of coding genes in the Dekkera yeasts experienced different evolutionary rates in these two branches, indicating that the Dekkera yeasts altered their physiological and metabolic processes, making them distinct from the other non-WGD yeasts.

To investigate the metabolic processes, we focused on the fermentation pathway related genes. The glycolysis pathway is the key metabolic pathway responsible for fermentation. At least one copy of the orthologous genes for the glycolytic pathway enzymes were detected among the seven yeasts examined. We used a branch model to distinguish the different rates of evolution in the D. bruxellensis and D. anomala, with the hypothesis that the Dekkera branch and the other non-WGD branches evolved at different rates. Our analysis identified 9 fermentation related genes that displayed different evolutionary rates between the Dekkera and other yeasts. Among them, HXX2, LAT1, ACS2, and PDB1 underwent significantly slower evolution in the Dekkera yeasts. The other five genes, ALD2, PYK1, PRM15, PGK1, and YOR283W (GPM1-like protein) exhibited significantly faster evolution (S3 Table). Moreover, we found that the PDC gene in the Dekkera yeasts evolved into a form closer to the PDC6 gene in S. cerevisiae. Transcription of PDC6 is glucose- and ethanol-dependent and is strongly induced during sulfur limitation [24]. The Codon Adaptation Index (CAI) value of PDC in the Dekkera yeasts was significantly higher than the genome average level (0.46 and 0.31 versus 0.136 ± 0.062 and 0.139 ± 0.062 in D. bruxellensis and D. anomala, respectively), which suggests that PDC might be highly expressed in the Dekkera yeasts. As expected, under limited sugar supply and low concentrations of oxygen, the AB SOLiD sequencing technique showed that PDC is highly expressed in Dekkera (RPKM = 1330, rank 68) [25]. These results indicate that the PDC6-like gene may play a critical role in the adaptive evolution of the Dekkera yeasts.

Positive selection in the D. bruxellensis and D. anomala
The branch-site model executed in PAML4 [23] identified 90 positively selected genes among the 2429 orthologous genes (3.7%) possibly associated with the adaptation of Dekkera yeasts.
Table 1. Classification of genes under positive selection in the *D. bruxellensis* and *D. anomala*.

| Category                                      | Genes                                                                 |
|------------------------------------------------|----------------------------------------------------------------------|
| Metabolism (28)                               | PTC7, BG1, RKM3, MEC1, FAT1, YBR056W, CYC8, MNN2, TDP1, PGK1, PHO2, SN2, SSY1, PPM1, EDC3, YER134C, BST1, MSB2, STR2, SWI5, TRZ1, SET3, RIX7, CAR2, RHN1, CDC5, DSS1, ORT1 |
| Transcription (24)                             | TFC3, CYC8, TFC1, PIM1, PHO2, PRP42, SN2, SSY1, MIG2, PCF11, HAT2, VHR1, TA03, STS1, SWI3, PRI2, TRZ1, SET3, DSS1, LE01, CLP1, CTR9, HDA3, NAB3 |
| Vacuolar function (12)                         | BST1, END3, SEC16, HC1, COG6, SEC10, SEC15, GYP5, LTE1, PE03, NVV1, BRO1 |
| Cell cycle and DNA processing (31)             | SPC97, BI01, LTE1, MEC1, KAP104, TDP1, MRC1, CDC1, CYK3, HAT2, BEM2, SEC15, CUL3, STS1, DBP11, RAD7, SWI3, PRI2, SET3, RIX7, SEC10, BUD6, RH11, CDC5, CLU1, NDC1, DSS1, END3, CTR9, HDA3, GYP5 |
| Protein with binding function or cofactor requirement (48) | LTE1, MEC1, FAT1, UMP1, KAP104, PBY1, CYC8, PIM1, PGK1, PRP42, SSY1, MSN5, PCF11, HAT2, BEM2, YER134C, SEC15, CHC1, CUL3, TA03, SU2, RAD7, SWI3, PRI2, TRZ1, SET3, RPL38, PEPE, SP2A, RIX7, CAR2, CTR9, SEC10, BUD6, MVP1, RH11, CDC5, DSS1, LIP1, END3, MTR10, CLP1, CTR9, BRO1, GYP5, SRP72, NAB3, SEC16 |
| Regulation of metabolism and protein function (14) | PTC7, LTE1, MEC1, UMP1, CYC8, HAT2, BEM2, YER134C, STE2, SU2, CDC5, WHI2, BRO1, GYP5 |
| Cell rescue, defense and virulence (13)         | MEC1, UMP1, TDP1, PIM1, PHO2, MSB2, SU2, RAD7, PRI2, BUD6, DSS1, WHI2, BRO1 |
| Interaction with the environment (13)           | PIM1, CDC1, SSY1, NAB3, HAT2, STE2, MSB2, SYG1, SWI3, SP2A, END3, CTR9, BRO1 |
| Biogenesis of cellular components (25)          | SPC97, BI01, MEC1, TFC1, MNN2, CDC1, HAT2, BEM2, BST1, SEC15, MSB2, CHC1, SWI3, SET3, PEPE, SP2A, RIX7, CRN1, BUD6, RH11, CLU1, NDC1, END3, WHI2, GYP5 |
| Energy (4)                                      | FAT1, PGK1, MSB2, RIX7 |

(S4 Table). Using the MIPS and the SGD databases, we classified these genes into 10 functional categories (Table 1). These results demonstrated that the highest number of genes (53.9%) represented proteins with a binding function or cofactor requirement. Moreover, the metabolism and cell cycle DNA processing classes were over represented. Consistent with our rapid evolution study, 24 positive selection genes were mapped into the transcription functional class. For example, CLU1, NIP1, and SUI2 were subunits of translation initiation factors; and TCF1 and TFC3 were subunits of the RNA polymerase III transcription initiation factor complex. This suggested that although the *Dekkera* yeasts have a single copy of the genome, they may have acquired the "winner" traits by evolving an efficient translation system to improve protein levels. This could explain why glycolysis genes are expressed at high levels in the *Dekkera* yeasts. In addition, we found that 12 genes were represented in vacuolar function, which is required for tolerance to alcohol stress [26]. Genes, such as END3, CHC1, and PEP3, are required for vacuolar biogenesis; MNN2, which encodes mannosyltransferases, is involved in maintaining cell wall integrity. One of the three rate-limiting enzymes in glycolysis, 3-Phosphoglycerate Kinase (*PGK1*), was found to be under positive selection. *PGK1* catalyzes the reversible transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate (1,3-BPG) to ADP, producing 3- phosphoglycerate (3-PG) and ATP. The catalytic rate of the *PGK1* enzyme determines the flux of carbon degradation in the direction of pyruvate or glycerol. Because efficient anaerobic fermentation needs a higher glycolysis flux (GF), we speculate that the positive selection sites in these two glycolysis rate limiting enzymes represent adaptive evolution to improve enzyme activities in the *Dekkera* yeasts.
Aligned amino acid sequences of the seven yeast species showed that the Y74L, E127T, K242V, V279Y, and P315E mutations are simultaneously retained in the Dekkera PGK1 (Fig 4A). Then, we analyzed the three-dimensional structure of *S. cerevisiae* PGK1 to predict the impact of selective mutations on the rate of enzyme catalysis (Fig 4B). Yeast PGK1 exists as a 415-residue monomer containing two nearly equal-sized domains that correspond to the N- and C-termini of the protein [27]. 3-PG and 1,3-BPG bind to the N-terminal domain, while the nucleotide substrates, MgATP or MgADP, bind to the C-terminal domain of the enzyme. This extended two-domain structure is associated with large-scale "hinge-bending" conformational changes, similar to those found in hexokinase [28]. Binding of either substrate triggers a conformational change, and the transfer of the phosphate group occurs when both substrates are bound [29]. PGK1 catalyzes the glycolytic direction in the glycolysis pathway and catalyzes its reverse direction in the gluconeogenic pathway. In our study, the five positive selection sites were found to be located at both N- and C-termini of PGK1. The three C-terminal selective sites, located in the region surrounding the 6-stranded parallel beta-sheet and alpha helices, may help the C-terminal domain fold into a specific conformation. Y74L and E127T, which are located in the N-terminus, are situated very close to the binding pocket of 3PG and 1,3-BPG. Particularly, the negatively charged side chain of the E127 residue was at a distance of merely 4 Å to the positively charged side chain of R121 in *S. cerevisiae*. The R121 residue plays a key role in the stabilization of the phosphate groups of 3-PG and 1,3-BPG. However, in the *D. bruxellensis* and *D. anomala*, as the negatively charged E127 residue is mutated to a non-charged T127, the influence of reverse charge for R121 would disappear. Therefore, the electrostatic interaction between R121 and the phosphate group of 3PG would be enhanced. However, as
1,3-BPG has two phosphate groups and the R121 of PGK1 enhances the non-reaction phosphate group, the reaction group would be pulled down from 1,3-BPG more easily. Thus, the catalytic activity of PGK1 would be redirected to the glycolytic pathway in the Dekkera yeasts as a consequence of positive selection.

Discussion

The Dekkera yeasts represent an alternative to ethanol production using S. cerevisiae. The Dekkera yeasts independently evolved "winner" traits, such as high glucose and ethanol tolerance, aerobic fermentation, and rapid ethanol fermentation rate, similar to S. cerevisiae. Previous phylogenetic analysis suggests that the S. cerevisiae and Dekkera groups separated at least 200 Mya, which took place long before the WGD in S. cerevisiae. Promoter rewiring, URA1 horizontal transfer, and ADH duplication events are thought to be involved in the "make-accumulate-consume" strategy in S. cerevisiae [9, 15]. The WGD event provided the S. cerevisiae with extremely rich evolutionary resources for adapting to the pressure of high levels of sugars 100 mya. The Dekkera yeasts independently accomplished this adaptation without a duplicated genome, and often occupy a similar niche to the S. cerevisiae yeasts in nature.

In this study, we analyzed the genomes of two Dekkera yeasts: Dekkera bruxellensis and Dekkera anomala. By comparison with five closely related non-WGD yeasts, we revealed that the AATTTT motifs of MRP genes are lost on a large scale not only in D. bruxellensis, which was consistent with previous studies, but also in D. anomala. This result indicated that the loss of the AATTTT motif occurred in a common ancestor of D. bruxellensis and D. anomala. However, the global promoter rewiring can only explain the change in the expression pattern of respiration-associated genes. How did Dekkera adapt to the high levels of glucose and ethanol and produce ethanol efficiently? In a quest to unravel the potential genetic mechanisms underlying these adaptations, we found, surprisingly, that a multitude of coding genes showed significantly different evolutionary rates in the Dekkera yeasts compared with the other non-WGD yeasts. GO enrichment analysis showed that genes involved in the transcription and translation processes exhibited faster rates of evolution in the Dekkera yeasts. In addition, approximately 3.7% of coding genes contained at least one positively selected amino acid in the D. bruxellensis and D. anomala. Positive selection of CLU1, NIP1, SU12, TCF1, and TFC3 indicated that the Dekkera yeasts evolved a very efficient transcription and translation system. Positively selected genes in the cell rescue, defense and virulence, and vacuolar function classes provide a reasonable explanation for the tolerance of the Dekkera yeasts to high levels of alcohol and glucose. For example, the SPC97, MNN2, CHC1, NYV1, and MVP1 genes, which are important for vacuolar H⁺-ATPase function, may play a key role in high alcohol tolerance. Moreover, more than half of the positively selected genes were found to encode enzymes related to metabolism. These findings indicated that the metabolic process in the Dekkera yeasts acquired several new traits through adaptive evolution.

Tolerance to high levels of glucose and ethanol and the efficient rate of ethanol production point to the adaptive evolution of the fermentation pathway in the Dekkera yeasts. Positively selected and differentially evolved genes in the fermentation pathway may explain, in part, the molecular genetic mechanism for adaptive evolution. The rapid rate of ethanol production needs a relatively higher fermentation flux. Previous studies illustrated that the Dekkera yeasts express a high number of sugar transporter genes, indicating a higher rate of sugar uptake [25]. In our study, we suggest that glycolysis for sugar degradation would be accelerated in the Dekkera yeasts as a consequence of positive selection in PGK1. We surmise that the selection sites in PGK1 make pyruvate the preferred product of sugar degradation compared with glycerin. After analysis of the crystal structure of PGK1, we found that the positive selection site E127T
in \textit{PGK1} enhanced its catalytic activity in the glycolytic direction in preference to gluconeogenesis. In addition, we found that the \textit{PDC} gene in the \textit{Dekkera} yeasts is unique and closer to \textit{PDC6} in \textit{S. cerevisiae}. In contrast, \textit{PDC} in the other non-WGD yeasts was more akin to \textit{PDC1}. Expression of \textit{PDC6} is induced by glucose in conditions of high glucose. The \textit{Dekkera} yeasts express high levels of this unique \textit{PDC6}-like protein to convert pyruvate to acetaldehyde rapidly. Former studies also indicated that the \textit{ADH} genes are duplicated in both \textit{Dekkera} and \textit{S. cerevisiae}, so \textit{Dekkera} can easily convert acetaldehyde to ethanol [8]. These genetic features illuminate the evolution of a high efficiency sugar transport and degradation system in the \textit{Dekkera} yeasts to produce ethanol rapidly. When glucose is depleted and ethanol levels are high, another gene that underwent fast evolution, \textit{ALD2}, would be highly expressed in \textit{Dekkera} and convert acetaldehyde to the acetic acid. The acetic acid could convert into acetyl-CoA by \textit{ACS}, and then acetyl-CoA could be used in the TCA cycle in aerobic respiration. Moreover, the \textit{GRR1} gene and the \textit{TDH2}-like gene may also be helpful for the adaptation to grow in high levels of glucose (S1 Table) [30–32].

In conclusion, by analyzing the genome of \textit{D. bruxellensis} and \textit{D. anomala} and five non-WGD yeasts, we uncovered some molecular bases for the "winner" traits in the \textit{Dekkera} yeasts, which may explain the adaptive evolution of \textit{Dekkera}. Some mutations in \textit{Dekkera} may have contributed to the increased metabolic capacity to degrade sugar to ethanol. These findings may be useful in an industrial setting. For example, modification of \textit{PGK1} can be used to improve ethanol production by bio-engineering \textit{PGK1} in \textit{S. cerevisiae}. Thus, our study unraveled some of the molecular mechanisms underlying the adaption evolution of the \textit{Dekkera} yeasts and provides a blueprint for genetic modification for industrial usage.

\section*{Materials and Methods}
\subsection*{1. Acquisition and annotation of the sequence data}
The genomes of \textit{Yarrowia lipolytica}, \textit{Kluyveromyces lactis}, \textit{Lachancea kluyveri}, and \textit{Lachancea waltii} were obtained from the Yeast Gene Order Browser (http://ygob.ucd.ie/) [33]. The genomes of \textit{Dekkera bruxellensis} CBS 2499 and \textit{Dekkera anomala} strain YV396 were downloaded from the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome/). The genome of \textit{Eremothecium gossypii} was downloaded from the Yeast Genomes database [34] (http://www.genolevures.org/). We downloaded the detailed latest release version sequence and annotation information of \textit{Saccharomyces cerevisiae} (S288C), as a reference to annotate known genes in the sequenced species, from the Saccharomyces Genome Database (http://yeastgenome.org/) [35]. We utilized the AUGUSTUS program to predict ORFs to annotate and identify orthologous genes between \textit{Saccharomyces cerevisiae} and the other yeasts. Then, a reciprocal best-hits (RBH) BLAST approach and the inparanoid4.1 software package were used to detect orthologs between any two yeasts [36]. We removed all reduplicated genes from the analysis, for example one orthologous cluster containing more than one ortholog [33]. We retained orthologous clusters that had only one gene from each species.

\subsection*{2. Cis-element regulator motif analysis}
The genes included in this study were divided into three sets: the CRP and rRNA sets, associated with the rapid growth-associated genes, and the MRP set, associated with the mitochondrial ribosomal genes. We used the proposed datasets of \textit{Saccharomyces cerevisiae} (the CRP, rRNA, and MRP sets contain 174, 61 and 72 genes, respectively). Genes from the three sets were mapped to the genomes of the four yeast species (all of the sequenced DNA databases were annotated using \textit{S. cerevisiae}) to extract genes belonging to the three sets from the different yeast genomes. We defined promoter sequences by searching sequences 600 bp upstream
of the genes in every set. 600 bp of the promoter sequences were divided into 12 ordered groups (according to the position of nucleotides) and the presence of AATTTT motifs and reverse complements were identified separately. All promoter sequences belonging to the same set were counted as the total number of motifs present in each ordered 50 bp window. Then, we calculated the percentage of genes with AATTTT motifs in each 50 bp window and portrayed a broken line graph. We used python scripts developed in-house to define the CRP, rRNA, and MRP sets and analyze the occurrence of regulatory AATTTT motifs. The significance of motif distribution between the four species was detected using the repeated-measures analysis of variance.

3. Calculation of evolutionary rate

We used the dN/dS ratio to measure the evolutionary rate along a lineage. The values of dN, dS, and the dN/dS ratio were calculated using the free ratio model (Parameters of one rate model: model = 1, NSsites = 0, fix_omega = 0, omega = 1) in PAML4 for the Dekkera branch and the other branches. Lineage-specific median values were estimated by concatenated alignments from all orthologs. GO term data were downloaded from the Gene Ontology Consortium [37], and GO categories with more than 5 orthologs were included in our analyses. GO terms experiencing relatively accelerated evolution were identified using a binomial test [38]. The evolution rate for the glycolysis pathway was estimated using the branch model (Parameters of one rate model, background: model = 0, NSsites = 0, fix_omega = 0, omega = 0.2; Different rate model, foreground: model = 2, NSsites = 0, fix_omega = 0, omega = 0.2). The accepted species-tree (Y. lipolytica, ((E. gossypii, (K. lactis, (L. kluyveri, L. waltii)), (D. bruxellensis #1, D. anomala #1) #1)) was generated using the MEGA6 program [39] and was used as the guide tree. Ultimately, the likelihood ratio test (LRT) was performed to find genes with remarkable difference between the foreground and background. Level of significance was set at P < 0.05, i.e., the value of chi-square is equal to or greater than 3.84. Amino acid sequences were aligned using ClustalW2 [40] and the pal2nal [41] software was used to match the nucleotide sequences.

4. Identification of positively selected genes

We calculated the ratio of nonsynonymous (Ka or dN) to synonymous (Ks or dS) substitution rate (ω = Ka/Ks or dN/dS) using CODEML in the PAML4 package. Using the guide tree, the branch-site model (Parameters: Null hypothesis: model = 2, NSsites = 2, fix_omega = 1, omega = 1; Alternative selective hypothesis: model = 2, NSsites = 2, fix_omega = 0, omega = 1) was used to detect positively selected genes. A likelihood ratio test compared the alternative hypothesis of positive selection on the foreground branch to a null hypothesis with no positive selection on the background branch for each orthologous gene. Positively selected genes were inferred only if their P values were less than 0.05. Positively selected sites were deduced using Bayes Empirical Bayes (BEB) analyses [42]. Specifically, we edited the aligned amino acid sequences using the BioEdit software and drew protein conformation using the PyMOL software. The structure of PGK was downloaded from the protein data base (PDB) with PDB id: 1QPG.

Supporting Information

S1 Table. Predicted one2one orthologous gene of S. cerevisiae genes in 7 non-WGD species. (XLS)
S2 Table. The evolutionary rate of GO categories between the Dekkera and other non-WGD yeasts.

(XLS)

S3 Table. Genes with significantly different evolution rates in the fermentation pathway related genes.

(XLS)

S4 Table. Positive selection sites in the Dekkera yeasts.

(XLS)

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Author Contributions

Conceived and designed the experiments: YCG LZ GHL JFH. Performed the experiments: YCG LZ WXL SXD JJZ. Analyzed the data: YCG LZ SXD WXL. Contributed reagents/materials/analysis tools: YCG LZ SXD JJZ GHL JFH. Wrote the paper: YCG LZ SXD GHL JFH.

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