

Whole-Genome Sequencing of Sake Yeast *Saccharomyces cerevisiae* Kyokai no. 7

TAKESHI Akao¹, ISAO Yashiro², AKIRA Hosoyama², HIROSHI Kitagaki¹, HIROSHI Horikawa², DAISUKE Watanabe¹, RINJI Akada³, YOSHINORI Ando⁴, SATOSHI Harashima⁵, TOYOHISA Inoue⁶, YOSHIHARU Inoue⁷, SUSUMU Kajiwarara⁸, KATSUHIKO Kitamoto⁹, NORIYUKI Kitamoto¹⁰, OSAMU Kobayashi¹¹, SATORU Kuhara¹², TAKASHI Masubuchi¹³, HARUHIKO Mizoguchi¹⁴, YOSHIHIRO Nakao¹⁵, ATSUMI Nakazato¹⁶, MASAHIRO Namise¹⁷, TAKAHIRO Oba¹⁸, TOMOO Ogata¹⁹, AKINORI Ohta⁹, MASAHIRO Sato²⁰, SEIJI Shibasaki²¹, YOSHIFUMI Takatsume⁷, SHOTA Tanimoto²², HIROKAZU Tsuboi²³, AKIRA Nishimura²⁴, KOJI Yoda⁹, TAKEAKI Ishikawa²⁵, KAZUHIRO Iwashita¹, NOBUYUKI Fujita², and HITOSHI Shimoi^{1,*}

National Research Institute of Brewing, Higashi-hiroshima, Hiroshima 739-0046, Japan¹; National Institute of Technology and Evaluation, Shibuya-ku, Tokyo 151-0066, Japan²; Department of Applied Molecular Bioscience, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8611, Japan³; Kagoshima Prefectural Institute of Industrial Technology, Kagoshima 899-5105, Japan⁴; Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan⁵; Nihonsakari Co., Ltd, Nishinomiya, Hyogo 662-8521, Japan⁶; Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan⁷; Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8501, Japan⁸; Department of Biotechnology, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan⁹; Food Research Center, Aichi Industrial Technology Institute, Nagoya, Aichi 451-0083, Japan¹⁰; Central Laboratories for Frontier Technology, Kirin Holdings Co., Ltd, Yokohama, Kanagawa 236-0004, Japan¹¹; Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan¹²; Gunma Industrial Technology Center, Maebashi, Gunma 379-2147, Japan¹³; General Research Laboratory, Kiku-Masamune Sake Brewing Co., Ltd, Kobe, Hyogo 658-0026, Japan¹⁴; R&D Planning Division, Products Development Center, Suntory Holdings Ltd, Kawasaki, Kanagawa 211-0067, Japan¹⁵; Department of Fermentation, Faculty of Applied Bio-Science, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan¹⁶; Research Institute, Gekkeikan Sake Co., Ltd, Kyoto 612-8385, Japan¹⁷; Biotechnology and Food Research Institute, Fukuoka Industrial Technology Center, Kurume, Fukuoka 839-0861, Japan¹⁸; Research Laboratories of Brewing Technology, Asahi Breweries, Ltd, Moriya, Ibaraki 302-0106, Japan¹⁹; Frontier Laboratories of Value Creation, Sapporo Breweries Ltd, Yaizu, Shizuoka 425-0013, Japan²⁰; School of Pharmacy, Hyogo University of Health Sciences, Kobe, Hyogo 650-8530, Japan²¹; Food Technology Research Center, Hiroshima Prefectural Technology Research Institute, Hiroshima 732-0816, Japan²²; General Research Laboratory, Ozeki Corporation, Nishinomiya, Hyogo 663-8227, Japan²³; Research and Development Department, Hakutsuru Sake Brewing Co., Ltd, Kobe, Hyogo 658-0041, Japan²⁴ and Brewing Society of Japan, Kita-ku, Tokyo 114-0023, Japan²⁵

*To whom correspondence should be addressed. Tel. +81 82-420-8016. Fax. +81 82-420-0809.
E-mail: simoi@nrib.go.jp

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Abstract

The term 'sake yeast' is generally used to indicate the *Saccharomyces cerevisiae* strains that possess characteristics distinct from others including the laboratory strain S288C and are well suited for sake brewery. Here, we report the draft whole-genome shotgun sequence of a commonly used diploid sake yeast strain, Kyokai no. 7 (K7). The assembled sequence of K7 was nearly identical to that of the S288C, except for several subtelomeric polymorphisms and two large inversions in K7. A survey of

heterozygous bases between the homologous chromosomes revealed the presence of mosaic-like uneven distribution of heterozygosity in K7. The distribution patterns appeared to have resulted from repeated losses of heterozygosity in the ancestral lineage of K7. Analysis of genes revealed the presence of both K7-acquired and K7-lost genes, in addition to numerous others with segmentations and terminal discrepancies in comparison with those of S288C. The distribution of Ty element also largely differed in the two strains. Interestingly, two regions in chromosomes I and VII of S288C have apparently been replaced by Ty elements in K7. Sequence comparisons suggest that these gene conversions were caused by cDNA-mediated recombination of Ty elements. The present study advances our understanding of the functional and evolutionary genomics of the sake yeast.

Key words: *Saccharomyces cerevisiae*; sake yeast; genome sequence; diploid; loss of heterozygosity

1. Introduction

Sake is a traditional Japanese alcoholic beverage that is fermented from steamed rice by the concerted action of two types of microorganisms, filamentous fungi and yeast. In the production of sake, enzymes secreted by the fungus *Aspergillus oryzae*, which is grown on steamed rice, convert rice starch into glucose. Yeast cells in the sake mash then produce ethanol, higher alcohols and their esters, organic acids and amino acids, which are important components that contribute to sake aroma and taste. Hence, the choice of a yeast strain is one of the most critical factors in determining the resulting aroma and taste characteristics of sake products. Yeast strains that were originally isolated in sake breweries have been identified as *Saccharomyces cerevisiae* and are now commercially distributed as sake yeast.¹

Phylogenetic studies conducted using DNA markers have indicated that sake yeast strains are closely related, forming a sake strain cluster that belongs to a lineage distinct from other industrial and laboratory strains in the phylogenetic tree of *S. cerevisiae*.^{2–5} Comprehensive genome-wide studies of diverse *S. cerevisiae* strains have also indicated the existence of a unique sake cluster that is distinct from wine and laboratory strains.^{6,7} Consistent with their unique phylogenetic position, sake yeast strains possess characteristic traits that differ from other *S. cerevisiae* strains and are ideal for sake brewing, including ability for high ethanol productivity (reaching 20%)^{1,8,9} efficient growth and fermentation at low temperatures (below 15°C).¹ In addition, nearly all sake yeast strains generate a foam on the mash surface during the brewing process, which results from yeast cells fermenting sugars into CO₂ bubbles¹⁰ and possess biotin biosynthetic ability.¹¹

Several sake yeast-specific genes that are responsible for the desirable features of sake yeast and affect the brewing process have been identified. For example, *AWA1*, which encodes a cell-surface hydrophobic protein with a GPI anchor, was identified from a sake yeast genome and is responsible for foam formation in sake mash.¹⁰ Many *S. cerevisiae* strains are biotin

auxotrophs due to the lack of certain biotin biosynthetic pathway genes. *BIO6*, a homolog of a bacterial biotin biosynthetic pathway gene, was identified in sake yeast strains and is essential in them for the production of biotin.¹¹ In addition to these studies, several genes involved in yeast's quantitative phenotypes, including fermentability and aroma production, have been suggested.^{12,13} However, because the genetic basis for the superiority of sake yeast in sake brewing is largely unknown, genome-wide genetic approaches are required to understand these complex traits. Since the first complete genome sequencing of strain S288C in 1996,¹⁴ the genomes of several other *S. cerevisiae* strains have been sequenced.^{15–18} Strains such as K11, Y9 and Y12 were also subjected to genome sequencing and the sake cluster was consequently proposed. However, in each case, total length of sequence reads reached <0.9-fold of the haploid genome, not enough to get whole picture of the genome.⁶ In addition, these sequenced strains are not the typical industrial sake yeast strains. Accordingly, yeast strains used in sake brewing have not been subjected to whole-genome analyses, despite their importance in industry and yeast phylogenetic systematics.

In the present study, we performed the whole-genome sequencing of the sake yeast strain K7 (kyokai is the Japanese word for 'society') as the first step for subsequent functional, phylogenetic and evolutionary genomic studies. K7 has been one of the most extensively used industrial sake yeast strains over the past several decades and has also been employed in numerous genetic and biochemical studies as a model sake yeast and a parent strain for breeding.^{8,10–13,19–24} Here, we report the overall chromosome structure and remarkable features of the K7 genome.

2. Materials and methods

2.1. Strain

Saccharomyces cerevisiae K7 was distributed to Japanese sake breweries by the Brewing Society of Japan in 2004 and used as a DNA donor.

2.2. Sequencing, assembly and validation

The nucleotide sequence of the K7 genome was determined using the whole-genome shotgun sequencing approach. Genomic DNA was isolated from cells according to the method described by Hereford *et al.*²⁵ Plasmid libraries with average insert sizes of 1.6 and 5.0 kb were constructed in pUC118 (Takara Bio Inc.), while a fosmid library with an average insert size of 35 kb was constructed in pCC1FOS (Epicentre Biotechnologies), as described previously.²⁶ Raw sequence reads corresponding to a 9.1-fold coverage of the haploid genome (42 842, 88 830 and 15 227 reads from libraries with 1.6, 5.0 and 35 kb inserts, respectively) were first obtained by sequencing from both ends of the inserts on an ABI 3730xl DNA Analyzer (Applied Biosystems). Sequence reads were trimmed at a threshold quality value (Phred) of 20 and assembled using the Phrap assembler.^{27,28} We obtained a total of 712 contigs that were then put in order based on paired-end information from the constructed fosmid library to obtain supercontigs. The overall assembly was then validated and refined using Optical Mapping (OpGen, Inc.). A number of short contigs were incorporated into supercontigs with the assistance of optical maps and transposon-mediated random sequencing from fosmid clones (2112 reads). Following this analysis, the final contig number was reduced to 706.

2.3. Sequence comparison

An overall comparison of the K7 chromosomes with S288C (NC_001133–NC_001148), EC1118 (FN393058–FN393060, FN393062–FN393087, FN394216 and FN394217) and YJM789 (AAFW 2000000) strain chromosomes was performed using MUMmer 3.0 software.²⁹ Similarity-based searches of individual genes were performed using BLAST³⁰ and BLAST2.³¹ Phylogenetic analyses were carried out using CLUSTALW 1.83.³²

2.4. Extraction of heterozygous positions

To determine the positions of heterozygosity in the K7 genome, nucleotide positions containing one or more bases different from the consensus base with the Phred quality values >20 were automatically extracted as candidates for heterozygous positions. The identified positions were then manually validated on the electropherogram to validate the sequence. Non-supercontig contigs, which failed to be assembled into supercontigs, were not included in the analysis because their chromosomal positions were unknown.

2.5. Gene prediction and annotation

For predicting protein-encoding genes, ORFs larger than 90 bp were comprehensively included as candidates. ORF prediction was then carried out based on a

direct comparison of S288C ORFs with the K7 genome supercontigs. When direct comparison was difficult, ORFs were predicted using the software programs CRITICA,³³ Glimmer2,³⁴ GlimmerHMM³⁵ and SIM4.³⁶ Finally, all K7 ORFs were manually validated by expert annotators. When one or more incomplete ORFs, such as those truncated by a sequence gap and lacking a start or a stop codon, were mapped to a single S288C ORF, each incomplete K7 ORF was annotated as a single ORF. Functional annotation was based primarily on the *Saccharomyces* Genome Database (SGD; <http://www.yeastgenome.org/>), secondarily on the *Saccharomyces* species database (yeast comparative genomics: http://www.broadinstitute.org/annotation/fungi/comp_yeasts/) and also on COG/KOG (<http://www.ncbi.nlm.nih.gov/COG/>) and DDBJ/EMBL/GENBANK non-redundant databases. Orthology with the S288C ORF was evaluated using the BLASTP similarity and calculated as the percent of matched amino acid residues versus the total covered region between a K7 ORF and the best-hit S288C ORF (Supplementary Table S4) as truncated by a sequence gap. Similarity was calculated by the number of matching residues in only the corresponding regions of the S288C ORF. Dubious ORFs, ORFs in Ty elements and ORFs in telomeric regions were excluded as possible protein-coding genes and were not annotated. Prediction and annotation of RNA genes, Ty elements including solo long terminal repeats (LTRs) and telomeric elements were manually performed based on the results of BLASTN searches of the K7 genome with the S288C sequences of these genes and elements as queries.

All annotated ORFs and genetic elements were given individual numbers (Supplementary Table S4). Nomenclature of the K7 genes was based on the following rules: (i) each protein-encoding or RNA gene was named according to the orthologous S288C gene using the format 'K7_' plus the S288C standard gene name (with >80% similarity) and the systematic name (with >50% similarity) given in SGD; (ii) K7 identification numbers or K7 original gene names, such as *AWA1*, were given to genes that were non-orthologous or of low similarity to S288C genes (with ≤50% similarity); (iii) each name of a gene truncated by a sequence gap or segmented by point mutations was followed by a lower case 'a', 'b' or 'c', such as 'XXX1a' and 'XXX1b', to show its correspondence to a partial region of the ortholog; and (iv) Ty elements and LTRs were independently termed according to the identical nomenclature used for S288C.

3. Results and discussion

3.1. Sequencing and assembly of the K7 genome

Genome sequencing of the diploid sake yeast strain K7 was performed by a whole-genome shotgun

method that yielded 1.49×10^5 sequence reads with an estimated 9.24-fold redundancy of the haploid genome. Following *de novo* sequence assembly, 17 supercontigs were generated. Since homologous chromosome pairs of K7 were almost indistinguishable from each other during the assembly process, nearly all the reads from each homologous chromosome pair were assembled together into a single supercontig. Consequently, the resulting consensus chromosomal sequences represented a diploid genome, although they seemed to be that of a haploid genome. The total length of the supercontig corresponded to 98.1% of the estimated K7 genome size. The sequencing results and the assembled supercontigs are summarized in Supplementary Tables S1 and S2. Comparison with the S288C genome revealed that the 17 supercontigs corresponded to the set of S288C chromosomes and mitochondrial DNA (Fig. 1; Supplementary Table S2). No DNA sequence for the yeast 2- μ g plasmid was detected from any reads, which was consistent with a previous study.³⁷

In the assembly process, only 200 of 706 contigs were used for generating the supercontigs because the remaining 506 contigs failed to align with any of the supercontigs. The total length of these non-supercontig contigs was ~604 kb. Of the 506 non-supercontig contigs, 360 (71.1%) were singletons and 395 (78.1%) contained <1000 bases (Supplementary Fig. S1A and B). The majority of these contigs seem to be excluded from the supercontigs due to the repetition of telomeric and Ty-related type sequences. The remainder seemed to be excluded as a result of considerable heterozygosity between the two homologous

chromosomes due to base substitution, in-del, Ty insertion or other such events (Supplementary Fig. S1C and D). Several non-supercontig contigs contained ORF-like sequences that were not found in the supercontigs. For example, the nucleotide sequence of *MATa* was found in a non-supercontig contig due to its considerable heterozygosity, while the supercontig corresponding to the K7 chromosome III only contained the *MATalpha* sequence, even though K7 possesses both mating-type loci, *MATalpha* and *MATa*. Contigs including the sequence corresponding to *VTH1/VTH2*, which are paralogous and nearly indistinguishable from each other, were not assembled in the supercontigs, presumably due to adjacent repetitive sequences. Similarly, the sequence for *ARR3* was also present only in the non-supercontig contigs (data not shown). The non-supercontig contigs were excluded from subsequent analyses such as comparison with related strains, gene prediction and the survey of heterozygosity and Ty elements.

To date, the genome sequences of several *S. cerevisiae* strains have been reported; therefore, we compared the K7 genome with available genomes.^{14,15,17} Pairwise nucleotide polymorphisms among four strains (K7, S288C, YJM789 and EC1118) were analyzed by sequence alignment using MUMmer 3.0 software.²⁹ The number of substitutions and small indels between K7 and the three other strains ranged from ~67 900 (5.6/kb) to 78 000 (6.5/kb) and ~19 300 (1.6/kb) to 23 500 (2.0/kb), respectively, while those among the remaining three non-K7 strains ranged from ~46 100 (3.8/kb) to 56 700 (4.7/kb) and ~14 800 (1.2/kb) to 16 700 (1.4/kb), respectively (Supplementary Fig. S2). These results indicate

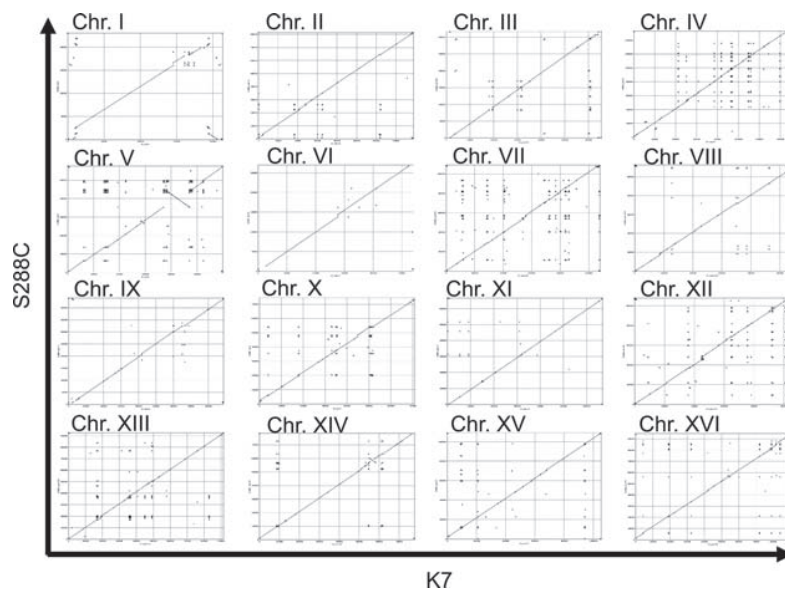


Figure 1. Dot-plot alignments of homologous chromosomes of *S. cerevisiae* strains K7 and S288C. The nucleotide sequence of each K7 supercontig was compared with that of the homologous S288C chromosome (indicated above each alignment) using MUMmer 3.0.

that the phylogenetic position of K7 is relatively distant from that of S288C, YJM789 and EC1118, as expected from previous studies.^{2,3,5–7,17}

3.2. Inversions in chromosomes V and XIV

We compared chromosomal structures between K7 and S288C using MUMmer 3.0 software,²⁹ as shown in Fig. 1. Although the overall genome structure of K7 closely resembled that of S288C, we identified two types of chromosomal rearrangements. One type involved several complicated subtelomeric rearrangements that are also observed or suggested by previous genome-wide studies of various *S. cerevisiae* strains, indicating that such rearrangements were not infrequent events.^{14,17,18,38–40} The other type of rearrangement was characterized by two large internal inverted regions. We confirmed that these inversions were homozygous by PCR analysis (data not shown). One inversion of the ~100-kb region on the right arm of chromosome V had not previously been described. Both boundary regions of this inversion on the K7 chromosome V were flanked by two Ty2 elements (K7_YERCTy2-3 and K7_YERWTy2-4) that were inverted in relation to each other and were absent in the corresponding region of S288C. Therefore, these Ty2 elements have been proposed to mediate this reciprocal inversion (Supplementary Fig. S3).¹⁵ Since Ty insertions differ by strain or lineage, this inversion would be unique to K7 or related lineages (Supplementary Fig. S3). The second identified inversion was a ~30-kb region on the left arm of chromosome XIV. This inversion was also observed in other strains.^{15,17} Inverted homologous regions located close to the breaking points on chromosome XIV (YNL018C–YNL019C and YNL033W–YNL034W) may have mediated this reciprocal inversion.

3.3. Uneven distribution of heterozygosity

The sequence obtained for the K7 genome represents the consensus haploid sequence derived from the two homologous chromosomes, although K7 is a diploid. Accordingly, we surveyed the positions of heterozygosity by carefully examining the sequence reads. A total of 1347 heterozygous sites between the homologous chromosomes were detected, and their positions were subsequently mapped (Fig. 2). Interestingly, the windows containing multiple heterozygous sites were unevenly distributed as clusters in several chromosomal regions.

Simple evolutionary accumulation of point mutations is insufficient to explain such uneven distribution of heterozygosity.⁴¹ It is more likely that a heterozygous diploid was generated by the out-crossing of two different haploid strains and subsequent loss

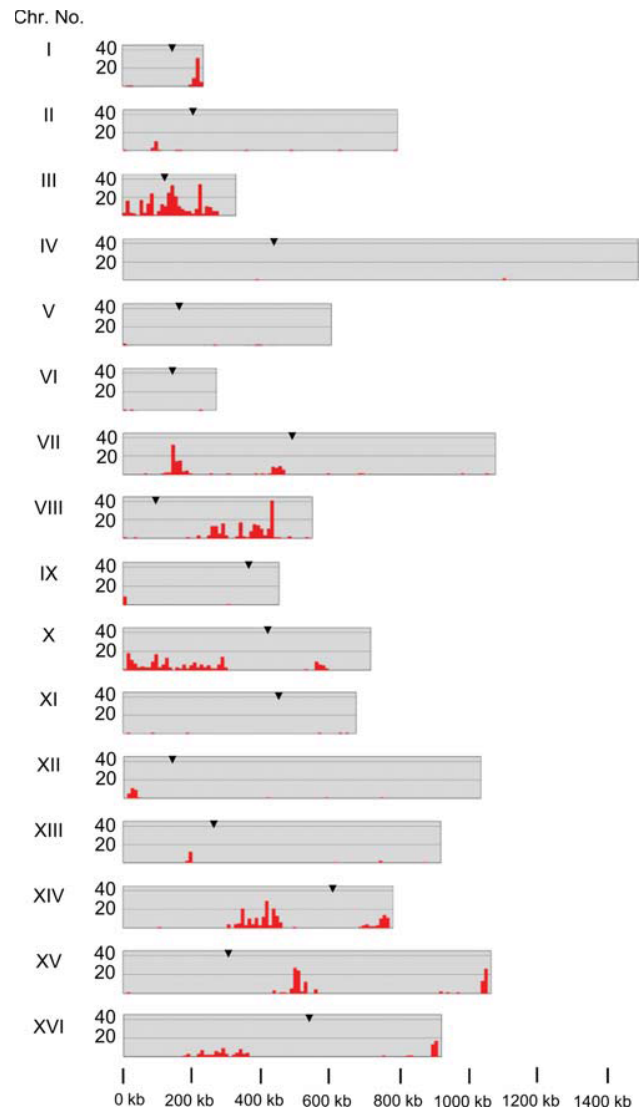


Figure 2. Genome-wide distribution of heterozygosity between homologous chromosomes of K7. Heterozygous sites were identified by manually checking sequence reads assembled within the supercontigs. The frequency of the extracted heterozygosity in homologous K7 chromosomes was plotted by each 10 kb window of the chromosomal coordinates. x-axis, chromosomal coordinates; y-axis, heterozygosity counts; arrow heads, position of centromeres.

of heterozygosity (LOH), resulting in the observed pattern. Alternatively, a complex history of out- and/or back-crossings of the ancestral strain in natural environments could have also caused this pattern. However, repeated backcrossing is unlikely to have occurred in nature since the sporulation efficiency of sake yeast, including K7, is markedly low. Thus, we speculate that sequential LOH events have resulted in the uneven distribution of heterozygosity in K7. Conversely, it is reasonable to presume that isolated heterozygosities were introduced by point mutations independent of LOH events. Genome-wide LOH was

also proposed for the diploid strain YJM128.⁴¹ However, LOH in K7 was far more extensive than that found in YJM128, resulting in 82.7% of the entire genome being almost homologous. As LOH is mainly caused by mitotic recombination, the probability of LOH events is dependent on the number of clonal generations during asexual proliferation. Therefore, it is likely that K7 passed through more mitotic generations than YJM128 after the out-crossing event. A sporulation defect of K7 may have contributed to a long-term clonal proliferation that allowed extensive LOH events without meiosis. LOH events also result in the selection of one haplotype between two homologous chromosomes whose haplotypes differ from each other. Consequently, LOH can be a major driving force in the diversification and microevolution of diploid strains, such as K7, which lost a meiotic life cycle, as observed in *Candida albicans*.⁴²

3.4. Gene predictions

Following the sequencing and assembly of the K7 genome, we predicted and annotated 5815 ORFs on 16 nuclear chromosomes and mitochondrial DNA (Supplementary Table S4). We observed many incomplete ORFs interrupted by a sequence gap between contigs: 39 ORFs had a truncated terminal at one end and 124 ORFs (62 pairs) had internal gaps. When compared with the S288C genome, frame shifts caused by small indels and single-nucleotide changes at the start or the stop codons resulted in many ORF polymorphisms, including terminal disagreement, such as extension or truncation (132 genes), segmentation of a single S288C ORF into multiple K7 ORFs (89 genes corresponding to 43 orthologs in S288C) and the fusion of ORFs (13 genes corresponding to 26 orthologs in S288C; Supplementary Table S4). The influence of these polymorphisms on each respective gene function is unclear and remains to be elucidated, although for several cases, such as the K7 ortholog of *MSN4*, polymorphism appears to influence the characteristic features of K7.⁴³

The average BLAST similarity between K7 ORFs and the most similar S288C ORFs was greater than 95% (95.7 and 95.8% at the nucleotide and amino acid levels, respectively). More than 90% of the K7 ORFs displayed a similarity exceeding 97%, with the corresponding S288C ortholog at the amino acid level (Fig. 3). Genes for tRNAs and other non-coding RNA were also annotated (Supplementary Table S3), which revealed that they corresponded to an almost complete set of S288C RNA genes. However, K7 lost three tRNA genes and one copy of two *RUF5* ncRNA genes (data not shown). Their absence would not be

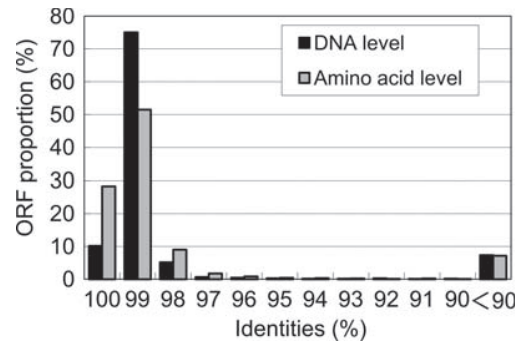


Figure 3. Identities of K7 ORFs with the top-hit S288C ORF. Each K7 ORF was compared with the corresponding orthologous S288C ORF using BLASTN (nucleotide level) and BLASTP (amino acid level). The ORF proportion based on the observed identities was then plotted.

expected to have an effect on the cellular functions of K7 because tRNA genes corresponding to a specific codon are highly redundant and one copy of *RUF5* was still present in the K7 genome.

3.5. Differentially present genes between K7 and S288C

The comparison of ORFs between K7 and S288C genomes disclosed 97 differentially present genes: 48 ORFs unique to K7 and 49 ORFs unique to S288C (Supplementary Tables S5 and S6). In this analysis, we excluded differences in subtelomeric multicopy gene families such as *PAU*, *COS*, *DAN*, *SNO* and *MAL*. Many of the unique genes were located at subtelomeric plastic regions of K7 and S288C, indicating that they may have been acquired or lost by chromosomal rearrangement events. Most differentially present genes located in internal chromosomal regions resulted from small mutations such as frame shifts or gene duplication events in a single strain.

3.5.1. K7 genes absent in S288C The genes predicted as present in K7 that are absent in S288C are listed in Supplementary Table S5. Several K7 genes already demonstrated involvement in the characteristic features of sake yeast, including *K7_AWA1* (K07_06182) and a paralogous set of *K7_BIO6* genes (*K7_BIO6-1/BIO6-2a/BIO6-2b/BIO6-3/BIO6-4a/BIO6-4b*: K07_11198/11203/11204/03384/11206/11207), which are unique to sake yeast strains.^{10,11} *BIO1*, which is absent in S288C, is also required for biotin biosynthesis in yeast.⁴⁴ As expected from the biotin prototrophy displayed by K7, *BIO1* orthologs were also found in K7 (*K7_BIO1-1/BIO1-2/BIO1-3*: K07_00624/03376/03381).^{11,45}

Three paralogous genes that are not found in S288C, K07_00009/11194/04100 (named *K7_EHL1/EHL2/EHL3* in this study), were predicted to encode proteins similar to bacterial epoxide hydrolase. Numerous

bacterial orthologs to *K7_EHL1/EHL2/EHL3* have been identified in the DDBJ/EMBL/GenBank non-redundant database, whereas eukaryotic orthologs have only been found in *S. paradoxus* to date (Supplementary Fig. S4). Thus, these epoxide hydrolase genes may have been horizontally transferred from bacteria to a common ancestor of these yeasts. *K7_EHL1/EHL2/EHL3* should be involved in the detoxification of harmful epoxide compounds. However, the actual substrates are unknown, and to date, no epoxide compounds have been identified from sake or fermenting sake mash.

K7_02354, which was located in a subtelomeric region, is orthologous to *YJM-GNAT* of YJM789, which encodes a gene similar to bacterial GCN5-related *N*-acetyltransferase, suggesting that a common ancestor of *K7* and YJM789 acquired their ancestral gene by horizontal transfer.¹⁵ Sequences of *K7_02354* and *YJM-GNAT* were almost identical (over 99%) at both the nucleotide and the amino acid levels (Supplementary Fig. S5).

K7_KHR1 (K07_03550), which encodes a previously identified heat-resistant killer toxin,⁴⁵ was located in an internal region of chromosome IX and wedged between two solo LTRs. Although a similar structure was observed in the EC1118 genome,¹⁷ S288C does not possess *KHR1*, and only a solo LTR (YILCdelta3) is located in the corresponding locus. This suggests that the loss of *KHR1* in S288C was caused by LTR-mediated recombination, as predicted in a previous study.¹⁷ A large proportion of genes unique to *K7* (Supplementary Table S5) have not been characterized, and their involvement and function in the characteristic features of sake yeast remain to be explored.

3.5.2. S288C genes absent in K7 Forty-nine genes predicted as present in S288C but absent in *K7* are listed in Supplementary Table S6. We confirmed that these genes are not present in *K7* non-supercontigs. Notably, two subtelomeric paralogous blocks in the S288C genome, containing *HXT15-SOR2-MPH2* on chromosome IV and *HXT16-SOR1-MPH3* on chromosome X, were not identified in the *K7* genome. The paralogous pairs of *HXT15* and *HXT16*, *SOR1* and *SOR2* and *MPH2* and *MPH3* encode nearly identical hexose transporters, sorbitol dehydrogenases and maltose transporters, respectively.^{46–48} It is likely that non-reciprocal chromosomal recombinations in subtelomeric regions caused duplication of these sequences in S288C, but resulted in their loss in the *K7* lineage. Another subtelomeric gene, *AIF1*, which is located on S288C chromosome XIV and encodes a mitochondrial cell death factor,⁴⁹ was also lost in *K7*. *CWP1*, encoding a cell-wall protein linked to glucan chains,⁵⁰ was disrupted by

a frame-shift mutation in *K7*. Although the effects of the loss of this protein are unclear, it is possible that the cell-wall properties of *K7* are affected.

PPT1, which encodes a protein phosphatase,⁵¹ is located at an internal region of chromosome VII in S288C, whereas the corresponding 2.6-kb region was lost and replaced with a Ty element (*K7_YGRCTy2-2*) in the *K7* genome (Fig. 4A). The effect of the loss of *PPT1* on cellular function or the sake brewing character of *K7* is unclear. A tRNA gene tR(UCU)G3 on the left side of *PPT1* was also absent in *K7*. However, the tI(AAU)G gene located on the right side of *PPT1* in S288C was present in *K7*. Sequences located several hundred bases upstream of a tRNA gene can serve as potential target sites for Ty integration. Consequently, multiple Ty insertion and subsequent excision events may have resulted in the two solo LTRs on both sides of *PPT1* in a *K7* ancestral genome. Recombination, including insertion or gene conversion, between a Ty cDNA and yeast chromosome with LTRs, was reported in laboratory experiments.⁵² Therefore, we speculate that gene conversion between a double-stranded Ty cDNA and two solo LTRs pre-existing on the chromosome VII may have occurred in the *K7* ancestor, resulting in the replacement of *PPT1* with Ty2 (Fig. 4, Supplementary Fig. S6). Alternatively, double reciprocal crossing-over may have resulted in the observed structure; however, this is unlikely, as *PPT1* was not found in either the supercontigs or the non-supercontig contigs of *K7*. A similar structure was also observed in the *K7* right arm of chromosome I (Fig. 4B), involving the replacement of two tRNA genes tL(CAA)A and tS(AGA)A with Ty2, which we speculate may have involved a similar gene conversion mechanism. In previous studies, Ty cDNA-mediated gene conversions were only observed in genetically modified strains and were thought to be an artificially induced phenomena.⁵² The structures identified in the *K7* genome would represent the first example of the spontaneous direct Ty-mediated gene conversion in a wild-type strain.

Two tandemly-duplicated acid phosphatase genes, *PHO3* and *PHO5*, are located on chromosome II of the S288C genome.⁵³ However, *K7* possessed only *PHO5* (K07_00381), consistent with the observation that *K7* shows repressive, but not constitutive, acid phosphatase activity.⁵⁴ These tandemly arranged acid phosphatase genes are also present in YJM789, EC1118 and several other *Saccharomyces* species.^{15,17,55} The prevalence of these genes suggests that *PHO3* was looped out of the *K7* genome by homologous recombination. In the *K7* genome, no copies of *ASP3* and its neighboring ORFs, which encode cell-wall L-asparaginase II and putative proteins of unknown function, were found, even though S288C

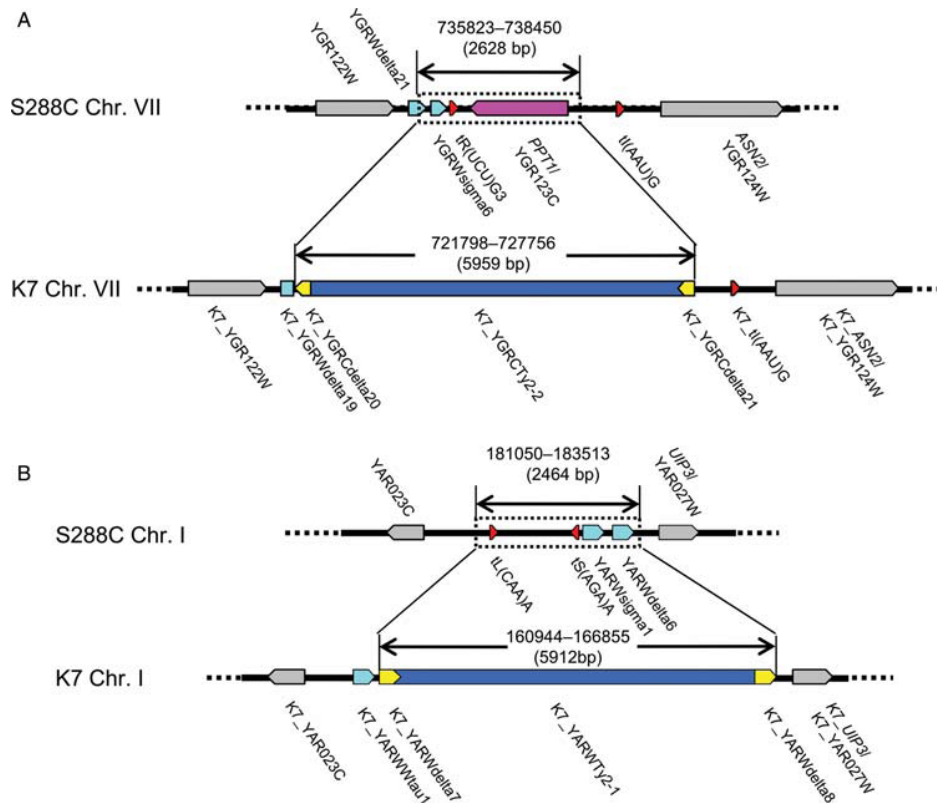


Figure 4. Loss of genomic regions by Ty2 replacement in the K7 genome. Each region within the dotted-line box in the S288C genome was replaced by a Ty element (Ty2) in the K7 genome. (A) A 2628-bp region containing the tRNA gene tR(UCU)G3 and *PPT1/YGR123C* in S288C chromosome VII was replaced by *K7_YGRCTy2-2* in K7 chromosome VII. (B) A 2464-bp region containing two tRNA genes, tL(CAA)A and tS(AGA)A, in S288C chromosome I was replaced by *K7_YARWTy2-1* in K7 chromosome I.

chromosome XII contains at least four copies of *ASP3* adjacent to rDNA repeats.⁵⁶ These sequences are also absent in numerous other *S. cerevisiae* strains.^{15–17,39} Although tandemly triplicated *ENA1/ENA2/ENA5*, encoding P-type ATPases are located on chromosome IV in S288C,⁵⁷ only one copy was identified in K7 (*K7_ENA1/K7_01190*), as observed in many other strains.^{15–17,39} The triplication is a relatively specific feature of S288C and its related strains. The K7 genome also contained one copy of *CUP1* (*K7_CUP1/K07_03116*), encoding a metallothionein, whereas S288C contains two copies in tandem,⁵⁸ consistent with the lower copper resistance of K7 than that of X2180, an isogenic diploid of S288C (data not shown).

3.6. Hexose transporter and *GAL* genes

Transport of sugars across the membrane is one of the key steps in ethanol fermentation. The S288C genome contains 20 genes that encode hexose transporter family proteins (*HXT1–HXT17* and *GAL2*) and glucose sensors (*SNF3* and *RGT2*) as shown in Fig. 5. The products of these genes show different glucose affinities and are differentially expressed in order to

coordinately control glucose uptake in environments with a broad range of glucose concentrations.⁵⁹ Many of these genes, including two glucose sensors, were highly conserved among S288C and K7. In particular, both the low-affinity glucose transporter genes *HXT1/HXT3* were conserved, implying that they may be responsible for glucose uptake during the sake brewing process, as reported in wine yeast.⁶⁰ *HXT5/HXT6/HXT7* were located at contig ends, and the DNA sequences of these regions in K7 were not completely analyzed. In S288C, *HXT6* and *HXT7* encode nearly identical proteins and are arranged in tandem; however, they may have been combined into a chimeric gene (*HXT6/7*) in K7, as observed in other strains.⁵⁹

We revealed that two distinct classes of S288C *HXT* genes displayed almost altered gene structures in K7. The first contains *HXT9/HXT11* and *HXT12*, which is annotated as a possible pseudogene due to a frame-shift mutation in *SGD*. In the K7 genome, although one of the duplicated *HXT9* orthologs was conserved (*K7_HXT9-2/K07_02205*: 98% identical to *HXT9* at the amino acid level), all other *HXT9/HXT11* orthologs were divide into two ORFs due to frame shifts

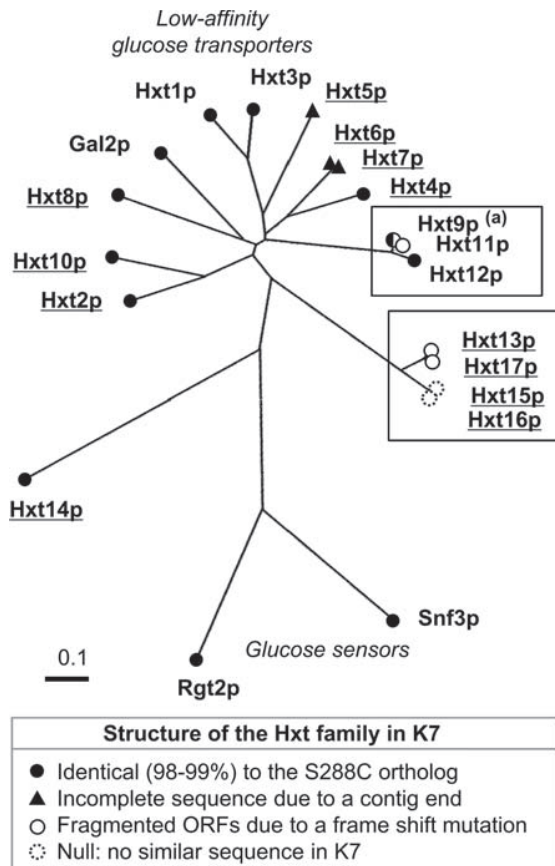


Figure 5. Phylogenetic tree of hexose transporter family proteins identified in S288C. The amino acid sequences of S288C *HXT* gene products were clustered by CLUSTALW 1.83, and the dendrogram was plotted using the TreeView program. Symbols at the end of the branches indicate the structural class of K7 orthologous gene products, as shown in the box under the dendrogram. Two groups of Hxts, whose functional structures were disrupted in K7, are framed with rectangles. Underlines indicate genes whose expression is repressed under high-glucose conditions. The letter (a) denotes each of the two K7 orthologs is indicated as a half round.

(*K7_HXT9-1a/HXT9-1b/HXT11a/HXT11b*: K07_03662/03663/06179/06180). The disrupted structure of *HXT12* was also conserved in K7 (*K7_HXT12a/HXT12b*: K07_03394/03395) as well as in S288C (Supplementary Table S4). Four genes *HXT13/HXT15/HXT16/HXT17* are classified into the second *HXT* class. In the K7 genome, the *HXT13/HXT17* orthologs (*K7_HXT13a/HXT13b/HXT17a/HXT17b*: K07_01805/1807/06162/06164) contained frame-shift mutations that may have resulted in the loss of function (Supplementary Table S4), while the sequences corresponding to *HXT15/HXT16* were absent in the K7 genome (Supplementary Table S6). Thus, in K7, the functions of gene products in this class appeared to be completely lost, although their molecular functions were unclear.

In the K7 genome, we also identified frame-shift mutations in the *GAL3* and *GAL4* genes that divided

each gene into two ORFs (K07_01156 and K07_01157 for *GAL3* and K07_06847 and K07_06846 for *GAL4*). Thus, both Gal3p and Gal4p in K7 are shorter at their C termini than their orthologs in S288C, suggesting that their molecular functions may be impaired. Since Gal3p and Gal4p function as an inducer and activator, respectively, which constitute the transcriptional induction system of galactose assimilating genes,⁶¹ the loss of Gal3p and Gal4p functions may lead to defective *GAL* gene induction in response to galactose. Consistent with this speculation, the assimilation and fermentation of galactose are remarkably weakened in several sake yeast strains, including K7.⁶²

Our analyses suggest that K7 possesses different sugar uptake and assimilation properties from those of S288C. It is likely that sake yeast cells could tolerate a functional loss from these genes without a negative selective force due to the growth of these strains in the glucose-rich environment specific to sake mash.

3.7. Structure of the *HO* gene

As K7 is a heterothallic diploid, *HO* involved in mating-type switching was predicted as non-functional in K7.⁶³ Indeed, a homozygous mutation of A1424T (H475L), which was reported to cause a loss of function in S288C,⁶⁴ was observed in the K7 *ho* allele. Moreover, a 36-amino acid deletion at 524–559 was also present in K7, as was reported in the heterothallic bioethanol strain JAY291.¹⁸ Collectively, these differences are likely to be responsible for the observed K7 heterothallism.

3.8. Comparison of *Ty* elements and LTRs

The chromosomal insertion of a *Ty* element can result not only in the loss or alteration of gene function, but may also modify gene expression levels. The annotated *Ty* elements and solo LTRs from K7 are summarized in Supplementary Tables S7 and S8. Nearly all *Ty* and solo LTR insertions followed the target-site-selection rule, displaying preferential insertion within a 1-kb upstream region of RNA polymerase III-target genes (Supplementary Table S8). We compared *Ty* insertion events, which included intact *Ty* elements and solo LTRs, between K7 and S288C. If flanking regions of two *Ty* elements, a *Ty* element and a solo LTR or two solo LTRs, were identical between the two strains, we estimated that these insertion sequences should be derived from the same *Ty* element insertion event in the common ancestral strain. A total of 198 *Ty* insertion events were estimated to be identical between K7 and S288C, while 121 and 137 insertion events were unique to K7 and S288C, respectively (Supplementary Tables S7B and S8). Interestingly, among the same 198 insertion events, only one pair

maintained intact Ty structures in both strains: K7_YCLWTy5-1 and YCLW Ty5-1. In addition, two pairs kept intact Ty structures in one of the strains: YARCTy1-1 in S288C and K7_YERWTy3-1 in K7. All other insertion events were observed as solo LTRs, representing trace sequences of Ty elements (Supplementary Table S8). This observation supports the idea that same insertion events may occur in the much more distant past than the unique insertion events. In our analyses, we were unable to locate K7 genes that were interrupted by a Ty insertion.

4. Conclusions

We revealed the sequence and structure of the K7 genome that represents the first such study of a sake yeast lineage within *S. cerevisiae* and provides the basis for future studies on the brewing characteristics, genealogy and evolution of sake yeast. The phenotypic effects of the identified structural polymorphisms between K7 and S288C genomes are largely unknown and remain to be explored. In addition, the uneven heterozygosity distribution found in the K7 genome is suggestive of the microevolution of K7 and related sake yeast strains. Future genetic studies encompassing a wide range of *Saccharomyces* strains are necessary to resolve the genetic basis for the characteristics and evolution of sake yeast strains in greater detail.

The sequences and annotations reported in this paper have been deposited at DDBJ/EMBL/GenBank under the accession no. BABQ01000001–BABQ01000705, DG000037–DG000052 and AP012028. Information of the sequence and gene annotation are also available on the sake yeast genome database (<http://nrifb1.nrif.go.jp/SYGD/>) and database of the genomes analyzed at NITE (DOGAN; <http://www.bio.nite.go.jp/dogan/top/>).

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Supplementary data: Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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