Diversity of Mating-Type Chromosome Structures in the Yeast *Zygosaccharomyces rouxii* Caused by Ectopic Exchanges between *MAT*-Like Loci

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Abstract

We investigated sex chromosome diversity in *Zygosaccharomyces rouxii* (*Z. rouxii*). In the current study, we show that the organization of the mating-type (*MAT*) locus is highly variable in the *Z. rouxii* population, indicating the *MAT*, *HML*, and *HMR* loci are translocation hotspots. Although NBRC1130 and CBS732 were originally two stocks of the type strain of the species, only NBRC1130 retains the original karyotype. A reciprocal translocation between the *MAT* and *HMR* loci appears to have occurred during the early passage culture of CBS732, which was used for genome sequencing. In NBRC1733, NBRC0686, NBRC0740 and NBRC1053, the terminal region of the chromosome containing the *HMR* locus was replaced with the chromosomal region to the left of the *MAT* or *HML* loci. The translocation events found in NBRC1733, NBRC0686, NBRC0740, and NBRC1053 were reconstructed under our experimental conditions using the DA2 background, and the reconstruction suggests that the frequency of this type of translocation is approximately $10^{-7}$. These results suggest that the *MAT* and *MAT*-like loci were the susceptible regions in the genome, and the diversity of mating-type chromosome structures in *Z. rouxii* was caused by ectopic exchanges between *MAT*-like loci.

Introduction

Chromosomal rearrangements, which include translocations and large-scale deletions, play key roles in phenotypic variation, genome evolution and genetic disease [1,2]. Multi-copy genes such as retrotransposons are known to elicit these chromosomal rearrangements. During the evolution of the Saccharomycetaceae, translocations have cycled telomere location, and the mating-type (*MAT*) locus has been a deletion hotspot [3,4].

In model yeast *Saccharomyces cerevisiae*, the *MAT* locus determines sex (mating-type). Two mating type genes (idiomorphs), either *MATa* or *MATα*, are encoded at the *MAT* locus and enable the locus to specify three cell identity types: haploid α, haploid α/α or diploid α/α. The *MAT* locus is often replaced with either *HMLα* or *HMRα*, which are silenced by chromatin modifications and are therefore called “silent cassettes” [5]. Based on extensive analysis of yeast sex evolution, mating-type switching has evolved from a two-step process: gain of silent cassettes and HO endonuclease [6]. These two-step events occurred after the ancestor of hemiascomycetes had diverged from other families, such as genus *Debaryomyces* and *Kluyveromyces*. Fabre et al. have suggested that a remnant of the HO gene resides within the *K. lactis* genome [7]. However, Butler showed that this sequence is distantly related to the other HO gene and is most similar to a second HO-like sequence present in the *S. castellii* genome [5]. Therefore, the *K. lactis* gene and the *S. castellii* gene likely evolved independently of HO, which was acquired late in the evolution of hemiascomycetes [5]. The presence of HO endonuclease enables efficient switching because HO endonuclease catalyzes DNA double-strand breaks (DSBs) at the Y-Z junction of the *MAT* locus and subsequently repairs the break using *HMLα* and *HMRα* [5]. Chaotic DSBs lead to cell cycle arrest and/or cell death; therefore, HO gene expression is strictly regulated by cell type, cell cycle and mother/daughter cell expression. However, whether the HO gene was regulated immediately after it was acquired is unknown.

*Z. rouxii* diverged from the *Saccharomyces* lineage after gaining the HO gene but before whole-genome duplication (WGD) [6]; hence, chromosomal rearrangements involving mating type conversions may be detected in its genome. The availability of complete genome sequences for hemiascomycetous yeast, including the *Z. rouxii* strain CBS732 [8], provides an ideal opportunity to analyze the consequences of HO gene acquisition. A recent study has already demonstrated that switching errors accumulate along evolutionary lineages and have a profound effect on the structure of the *MAT*-containing chromosome in post-WGD species, and that the non-WGD species (except for *Z. rouxii*) retain an organization similar to *Tulasnella delbrueckii*, which has the *MAT* organization DIC1-MAT-SLA2 [4]. We wondered why the *MAT* organization of *Z. rouxii* was different from other non-WGD species.

To meticulously characterize yeast sex chromosome evolution, we compared the organization of the *MAT* and *MAT*-like loci in
the \( Z. \) rouxii population. In this paper, we report evidence that sex chromosome diversity abounds in \( Z. \) rouxii.

Results

Isolation of a CBS732-like haploid strain in \( Z. \) rouxii

Recent studies have shown that various strains originally identified as "\( Z. \) rouxii" in yeast culture collections comprise at least three groups: \( Z. \) rouxii-type strain group (such as CBS732), \( Z. \) pseudorouxii-type group (such as NCYC3042, and also called \( Z. \) pseudorouxii), and an allopolyploid group (such as ATCC42981 which is a hybrid between the \( Z. \) rouxii type strain group and \( Z. \) pseudorouxii-type group). Therefore, the hybrid strain possesses T and P subgenome derived from CBS732 and NCYC3042, respectively (Fig. S1A) [9, 10]. Solieri et al. studied the ploidy level and demonstrated that ATCC42981 and some wild strains isolated from traditional balsamic vinegar had a haploid DNA content [11]. More recently, Solieri et al. showed that the group of \( Z. \) rouxii-related strains is a complex of haploid and diploid heterogeneous species including: 1) the haploid species \( Z. \) rouxii, e.g., CBS732; 2) the diploid species \( Z. \) sapae, isolated from high sugar environments; and 3) a diploid mosaic lineage that includes strains retrieved from a salty environment (Fig. S1A) [12, 13]. According to their classification, the allopolyploid species, such as ATCC42981, are categorized as a diploid mosaic lineage.

To remove the diploid species \( Z. \) sapae and a diploid mosaic lineage from this study, PCR analysis was performed using a T subgenome-specific primer and a P subgenome-specific primer (Table S1). The ADE2 and SOD2 alleles encoded by the T subgenome were detected in all strains used in this study except for DA2 [14], an ade2-disrupted strain derived from UL4, an \( ura3^- \) mutant strain derived from CBS732 [15]. In contrast, \( ADE2 \) and \( SOD2 \) encoded by the P subgenome were only detected in the NBRC1876, NBRC1877, and Z3 strains (Fig. S1B). NBRC1876 is allopolyploid [9] or a diploid lineage strain [13], and NBRC1877 is a diploid lineage strain [13]. Therefore, Z3 could also be allopolyploid or a diploid mosaic lineage strain. The remaining strains (NBRC1130, DL2, DA2, NBRC0686, NBRC0740, NBRC1053, and NBRC1733) do not appear to be hybrids and could be haploid. To further confirm the ploidy status, we performed flow cytometric analysis (Fig. S2). For DA2 and DL2, we obtained two peaks corresponding to two subpopulations; one population had 2n content (G2/M phases) of the haploid genome.

Background

A clear shift of the two peaks toward a double amount of DNA was obtained for strains NBRC1876, NBRC1877, and Z3. However, we obtained two peaks corresponding to two subpopulations; one population had 2n content (G2/M phases) of the haploid genome. To further confirm the ploidy status, we performed the same PCR analysis by using specific primer sets that annealed to \( MAT \) flanking regions of the genome. Interestingly, a PCR product was detected by using the following oligonucleotide primer pairs: 1-A, 2-B, and 3-C (Fig. 1B). The PCR product confirmed CBS732 genome sequencing data. In addition, a PCR product was detected by using the primer pairs 1-A, 2-A, and 3-C in NBRC1733, and a PCR product was detected by using the primer pairs 1-C, 2-A, and 3-C in NBRC0686, NBRC0740 and NBRC1053 (Fig. 1B). These results suggest that the flanking sequences of \( MAT \), \( HML \), and \( HMR \) are highly variable in the \( Z. \) rouxii population. To interpret this result, we evaluated all possible combinations of the chromosome pieces to yield the PCR amplicons detected in Fig. 1B. As shown in Fig. 1C, the PCR results can be represented by six possible combinations, implying that chromosomal rearrangements occurred in the \( Z. \) rouxii population.

With some combination of primers, we could not detect amplicons, e.g., 2-B in NBRC0686. If the sequences of the target flanking regions are not conserved due to SNPs and/or polymorphisms among these strains, the primers could not anneal to the target sequences and would give rise to false negative results. To reduce this possibility, we performed the same PCR analysis by using \( 1^9 \), \( 2^9 \), \( 3^9 \), \( A^9 \), \( B^9 \), and \( C^9 \) primers, which were designed to the out side of the 1-A, 2-B, and 3-C primer pairs. The results were identical to those obtained above (data not shown).

To identify the idiomorph encoded at each \( MAT \)-like locus, PCR analysis was carried out using an \( a^9 \) or \( \alpha^9 \) idiomorph-specific primer together with the corresponding \( A^9 \), \( B^9 \), or \( C^9 \) primer that flanked the each \( MAT \)-like locus (Fig. S3A, Table S1). Using the \( A^9 \) primer, \( a^9 \) idiomorph-specific signals were detected in DL2, NBRC1130 (slightly), and ATCC2623, and \( \alpha^9 \) idiomorph-specific signals were detected in CBS732, DA2, NBRC1130, NBRC1733, NBRC0686, NBRC0740, and NBRC1053. Using the \( B^9 \) primer, \( a^9 \) idiomorph-specific signals were detected in CBS732, DA2, DL2, NBRC1130, and ATCC2623. Using the \( C^9 \) primer, \( \alpha^9 \) idiomorph-specific signals were detected in all of the strains used in this study (Fig. S3B). These results suggest that each \( MAT \)-like locus has the corresponding idiomorph. The inferred idiomorph was noted in Fig. 1B. This discrimination between \( a^9 \) and \( \alpha^9 \) idiomorphs was also supported by sequence data (see below).

Translocation and reciprocal translocation among \( MAT \), \( HML \), and \( HMR \) loci

To verify whether the high variability in the \( MAT \), \( HML \), and \( HMR \) flanking sequences in the studied yeast strains was accompanied by chromosomal rearrangement between the \( MAT \), \( HML \), and \( HMR \) loci, we compared the karyotype of these strains by using pulsed-field gel electrophoresis (PFGE). Although NBRC1130, DA2, DL2 and ATCC2623 share the same genetic background, their karyotypes were highly variable, suggesting that the karyotype is unstable in \( Z. \) rouxii (Fig. 2). Based on Southern blot analysis and genomic data from CBS732, PEX2 and \( \text{ZFOC18458g} \) were located on chromosome 3, and \( SIR1 \), \( VAC17 \), and \( FET4 \) were located on chromosome 6 and chromosome 5 in DA2 and DL2, respectively (Fig. 2). In NBRC1130, PEX2, \( VAC17 \) and \( FET4 \) were detected on chromosome 6, and \( \text{ZFOC18458g} \) and \( SIR1 \) were detected together on chromosome 5. In NBRC0686, NBRC0740, and NBRC1053, two copies of

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FET4, one with PEX2 and VAC17 (on chromosome 5) and another with SIR1 (on chromosomes 6, 4, and 6, respectively), were detected. ZYRO0C18458g was not detected in these strains. In NBRC1733, two copies of VAC17 and FET4, one with PEX2 (on chromosome 5) and another with SIR1 (on chromosome 6), were detected, but ZYRO0C18458g was not detected (Fig. 2). These results suggest that the high variability within the MAT-flanking region is coincident with chromosomal rearrangements, which occurred by translocation or reciprocal translocation among the MAT-like loci. To determine the interchromosomal fusion point, we sequenced the PCR fragment from DA2, NBRC1130, NBRC1733, and NBRC0686 obtained by using specific primer sets designed outside of the 1-A, 2-B, and 3-C amplicons.

Homologous recombination apparently occurred at the X or Z regions in the MAT, HML, and HMR loci; however, the precise fusion point could not be determined because these loci share nearly identical sequences in the X and Z regions. The sequences reported in this paper have been deposited in the DNA Data Bank of Japan (accession no. AB781017-AB781029), and some representative sequence data were shown in Fig. S5, S6, S7, S8, S9, and S10.

In ATCC2623, all signals from PEX2, ZYRO0C18458g, SIR1, VAC17, and FET4 were unexpectedly detected at the same position (Fig. 2). These results suggest that these genes are located on the same chromosome or on different chromosomes that are similar in length. The PCR amplification pattern in ATCC2623 verified that the MAT organization in the ATCC2623 strain corresponded to the MAT organization found in NBRC1130 (Fig. 1B).

The original organization of the MAT locus in Z. rouxii

One of the significant findings from this study is the high variability in the MAT locus detected in NBRC1130, DL2, DA2 and ATCC2623, despite the fact that these strains originated from the same strain. To determine the original organization of the MAT locus in Z. rouxii, we compared the MAT organization with the gene organization in related species. Gordon et al. inferred the gene order and core genomic structure of the ancestral species that existed immediately before WGD [16]. This ancestral strain and Lachancea kluyveri, which diverged from the Saccharomyces lineage before the divergence of Z. rouxii, have the same gene order around the MAT locus in NBRC1130; DIC1 is located to the left of MAT, and SLA2 is located to the right of MAT in both species. A similar organization (DIC1-MAT-SLA2) is also observed in Torulaspora delbrueckii, which has a closely related genome to Z. rouxii [4]. CBS732 and its derivative also have SLA2 to the right of MAT, whereas CHA1 is located on the left side of MAT (Fig. 3A, B). These results suggest that the organization of the MAT locus in NBRC1130 is original, unlike the organization of CBS732, which was used for genomic sequencing. The chromosomal organization...
of CBS732 could have been caused by reciprocal translocation between MAT and HMR (Fig. 4). We designated the MAT-like locus (dark green-orange junction) in CBS732 as the putative MAT locus because mating type switching was observed at the locus (Fig. 1, Fig. S3).

The organization of NBRC0686, NBRC0740, NBRC1053, and NBRC1733 could have been caused by interchromosomal rearrangement accompanying ectopic recombination between the MAT and HMR loci or between the HML and HMR loci (Fig. 4). Accordingly, the region containing ZYRO0C18458g to the right of the HMR locus is missing in NBRC0686, NBRC0740, NBRC1053, and NBRC1733.

Translocation frequency

Why does Z. rouxii have a highly variable MAT organization? To determine the translocation frequency between MAT and HMR or between HML and HMR, we constructed the DA2AU strain, which has ScADE2 and ScURA3 at the ZYRO0C18458g locus in the DA2 and DA2 hoD backgrounds. If the right side of HMR is lost and replaced by the right side of MAT (designated DA2AU-M or DA2AU-L mutants, respectively), we can positively select the mutants as red colonies on 5-FOA plates (Fig. 5).

We obtained a total of 175 5-FOAr red colonies from 3.96×10^8 screenings in HO cells and 169 5-FOA r red colonies from 8.17×10^8 screenings in hoD cells. The resulting colonies were classified by PCR using the primer set used in Fig. 1. In HO cells, a total of 106 DA2AU-M and 61 DA2AU-L mutant colonies were identified, and the remaining 4 colonies could not be classified. In hoD cells, a total of 92 DA2AU-M and 76 DA2AU-L mutant colonies were identified, and the remaining 1 colony could not be classified. In HO cells, the frequencies of spontaneous mutation were 2.44×10^-7 and 1.23×10^-7 for DA2AU-M and DA2AU-L, respectively (Fig. 5). No significant differences were observed between the DA2 and DA2 hoD backgrounds. To verify that the HO gene catalyzed DSBs and enabled efficient switching, semi-quantitative PCR was carried out using an a or a idiomorph-specific primer together with a primer from the common flanking sequence (Table S1). The flanking primer resided outside the W and Z regions shared with the HMR and HML cassettes and, therefore, will only amplify a product from the MAT locus. The a idiomorph-specific PCR products were detected at cycles 27 and 33 in DA2 and DA2 hoD, respectively. From these results, the mating type conversion ratio in DA2 was at least 8-fold higher than the conversion ratio in DA2 hoD (Fig. S4).

Figure 2. Mapping of PEX2, ZYRO0C18458g, SIR1, VAC17, and FET4 by PFGE and Southern blot analysis. Chromosomal DNA was isolated from the following strains: ATCC2623, NBRC1130, DA2, DL2, NBRC0686, NBRC0740, NBRC1053, and NBRC1733. For Southern blot analysis, each ORF probe was used to map target genes. S. c. indicates Saccharomyces cerevisiae chromosome DNA marker. Chromosomal numbers are indicated by roman numerals.

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Figure 3. The original organization around the MAT locus in *Z. rouxii*. (A) Phylogenetic relationships among the species and the position of the reconstructed ancestral genome. The phylogenetic tree is not to scale, and the topology is derived from a referenced study [29]. (B) The gene organization around the MAT locus from representative yeasts before and after HO gene acquisition. doi:10.1371/journal.pone.0062121.g003

Figure 4. Inferred comparative organization around the MAT-like locus in *Z. rouxii*. Coloring indicates gene position in NBRC1130: pink, left side of MAT; orange, between MAT and HML; wine red, right side of HML; dark green, left side of HMR; and blue, right side of HMR. The vertical bars are telomeres. The gene order was predicted from CBS732 except for around the fusion points. doi:10.1371/journal.pone.0062121.g004
These results indicate that the acquisition of HO endonuclease increased the frequency of genotypic switching in *Z. rouxii*; however, the interchromosomal rearrangement accompanying ectopic recombination between MAT and HMR or between HML and HMR is independent of HO endonuclease at least under our experimental conditions.

**Discussion**

We suggest that the original organization of the MAT locus is represented in NBRC1130, and the organization of the MAT locus in CBS732 was made by reciprocal translocation between MAT and HMR during the recovery from occasional accidents that occurred during mating-type switching. The reciprocal translocation events may resemble the fusion of MAT with HMRα (Hawthorne’s deletion) and of MAT with HMLα (alpha-ring chromosome) in *S. cerevisiae* [17,18]. Perhaps this reciprocal translocation occurred in the early passage culture of CBS732 during the past 80 years because the type strain, deposited in the Centraalbureau voor Schimmelcultures (CBS) collection and distributed to other collections, was originally isolated by Sacchetti in 1932 [19]; the MAT locus organization of these other collections (ATCC2623 and NBRC1130) is now different from that of CBS732. Unfortunately, the CBS732 strain was used for genomic sequencing of *Z. rouxii* [8]; however, this reciprocal translocation is a CBS732-specific event and is not common in the *Z. rouxii* population.

PCR products amplified using oligonucleotide primer pair 1-A were α and α in DA2 and DL2, respectively (Fig. 1B, S2B). In addition to the α-specific PCR product, an α-specific PCR product was also detected in DA2 (Fig. S4), indicating that mating type conversion occurred at the “new” MAT-like locus (dark green-orange junction) in CBS732 and its derivatives. This fact leads us to infer that the new MAT-like locus is the MAT locus. In this scenario, one of the products (dark green-orange junction) must become active, and the other one (pink-blue junction) must become silenced. How do the loci change between an active and a silenced state? We hypothesize 3 possible reasons: 1) the distance from the telomere determines the silencing state, i.e., the locus labeled MAT (putative) is active because its location is far from the telomere, and the locus labeled HMR (putative) is silenced because its location is within a subtelomerers where genes are transcriptionally silenced, 2) cis-acting silencers, known as E and I in *S. cerevisiae*, lie in to the right side of HMR (blue region), or 3) both situations are true. To obtain solid evidence to demonstrate that the MAT-like locus (dark green-orange junction) is MAT, we tried to obtain a zygote between DA2 (ura3Δ ade2Δ) and DL2 (ura3Δ leu2Δ) many times; however, we could not obtain a zygote with only uracil auxotrophy. Taken together, we have no direct evidence to conclude that the locus labeled MAT is actually active MAT, and thus, a study that is more detailed is needed.

PCR products from 2-A in NBRC1130 were α/α, indicating either diploids or a mixture of MATα and MATα. However, the α/α...
The ratio estimated from PCR signal intensity was not reproducible in NBRC1130; PCR analysis detected only a amplicons some of the time, only a amplicons some of the time, or an a/a mixture (or diploid) some of the time. Perhaps, the a/a ratio in the single colony, which was precultured for genomic DNA extraction, caused the poor reproducibility. We tried to identify a stable diploid from the colony which gave a mixture of equal parts of a and τ using PCR analysis; however, we failed to recover a stable diploid. In any case, CBS732 and NBRC1130 were potentially homothallic, in accordance with the classical observation that sugar-tolerant strains have a mating response in each culture without mixing with any other strains [20]. Interestingly, NBRC0686, NBRC0740, NBRC1053, and NBRC1733 lost a information, indicating that these strains were obligate heterothallic. Natural a,a and τ,τ,τ isolates of S. cerevisiae are found at a low frequency [21], and an a,τ,τ isolate of Candida grabata has been described [22]. In召 rouxii, an τ,τ,τ isolate (NBRC0686, NBRC0740 and NBRC1053) and an a,τ,τ,τ isolate (NBRC1733) could be caused by the interchromosomal rearrangement accompanying ectopic recombination between MAT and HMR or between HML and HMR and/or subsequent mating-type switching.

Under our experimental condition, the frequency of switching in HO cells was at least 8 times higher than in hoΔ cells in召 rouxii (Fig. S1). These results indicate that the acquisition of HO endonuclease increased the frequency of genotypic switching in召 rouxii. However, the frequency of switching is 106-times higher in HO than in hoΔ cells in S. cerevisiae [23], indicating that the contribution of HO endonuclease to genotypic switching varies among yeast species. The genotype switching from MATα to MATα still occurred at low frequency in hoΔ cells. In the genus Kluyveromyces, it is known that the genotype switching from MATα to MATα is induced by τ3 gene excision-dependent DSBs and the opposite direction switching from MATα to MATα is occurred by a different but uncharacterized mechanism [24,25]. It is therefore that the switching observed in hoΔ cells in this study may be occurred by an uncharacterized mechanism similar to genus Kluyveromyces.

We analyzed spontaneous translocation events in DA2AU strains that led to functional inactivation of a URA3 and ADE2 marker inserted to the right of the HMR locus. The frequencies of spontaneous translocation were approximately 10^{-7} and no significant differences were observed between HO cells and hoΔ cells. The HO endonuclease recognition site of HMR is blocked by chromatin modification in many hemiascomycetous yeast [26]. Therefore, the translocations observed in NBRC0686, NBRC0740, NBRC1053 and NBRC1733 are caused by interchromosomal rearrangement using homologous recombination and crossing over at the X and Z regions in the MAT-like locus.

In this study, we analyzed diversity at the mating-type locus in the召 rouxii population and demonstrated that the MAT and MAT-like loci were susceptible regions in the召 rouxii genome. To explore the precise evolution of the MAT locus, investigation of the intraspecies mutations at the MAT locus in various yeast species is needed.

Materials and Methods

Strains, media, and genetic methods

The yeast strains used in this study are listed in Table 1.召 rouxii cells were transformed using an improved electroporation method [27]. Standard YPD medium containing G418 and synthetic complete dextrose (SCD) medium lacking adenine and/or uracil for transformant selection were used.

PCR and sequence analysis

MAT-like loci were amplified from the chromosomal DNA by using primers 1, 2, 3, A, B, and C, or 1’, 2’, 3’, A’, B’, and C’ (Table S1). Each PCR reaction mixture (50 μl) consisted of 1 μl of DNA (approximately 10 ng), 25 μl of 2X KOD-FX neo buffer (Toyobo, Osaka, Japan), 0.4 mM of each deoxynucleoside triphosphate (Toyobo), 25 pmol of each primer, and 1 unit of KOD-FX neo DNA polymerase (Toyobo). PCR was performed for 33 cycles after a pre-incubation at 94°C for 2 min using the following conditions: denaturation at 98°C for 10 s; annealing at 57°C for 15 s; extension at 68°C for 2 min. The amplicons were analyzed by agarose gel electrophoresis.

To obtain sequence information of the MAT-like loci, the PCR products were purified using the Wizard SV Gel and PCR Clean-Up system (Promega, Fitchburg, USA) according to the supplier’s instructions. Single-extension sequencing from both strands of DNA products was performed by a commercial DNA sequencing service provider (Greiner-Japan, Tokyo, Japan).

To identify the genotype (a or τ), a fragment containing partial a or τ idiomorphs was amplified by PCR from the chromosomal DNA using the primers MITLα and MITLτα, and A’, B’, or C’. PCR was performed for 33 cycles after a pre-incubation at 94°C for 2 min using the following conditions: denaturation at 98°C for 10 s; annealing at 58°C for 15 s; extension at 68°C for 30 s.

Flow cytometry

Flow cytometry was carried out as described by Solieri et al. [11] with little modification. Briefly, the strains were grown to the mid-log phase (OD_{600} of 0.5 to 0.6) in SCD (5% glucose) medium at 28°C with shaking. Cell samples were induced to arrest at the G1 phase by treatment with 6-hydroxyquinoline at a final concentration of 100 mg/l for 24 h. Cells were harvested by centrifugation (3,000 x g for 3 min at 4°C) and fixed overnight with 70% (v/v) ethanol at 4°C. After fixation, cells were harvested, washed, and resuspended at a concentration of 5 x 10^6 cells/ml in 50 mM sodium citrate buffer, pH 7.0. The cell suspension was treated for 120 min at 50°C with RNaSe A and proteinase K at final concentrations of 0.25 and 2 mg/ml, respectively. A 2.5 x 10^6 cells/ml cell suspension was stained overnight at 4°C with propidium iodide at a final concentration of 4 μg/ml. To remove clumps and debris, sonic pulses were applied. Before FCM analysis, cells were filtered through a 35 μm mesh cell-strainer (Becton, Dickinson and Company, Franklin Lakes, NJ). FCM experiments were performed on an Accuri™ C6 flow cytometer (Becton, Dickinson and Company). A minimum of 20,000 cells per sample was acquired at a low flow rate. Offline data were analyzed with the CFlow plus software (Becton, Dickinson and Company).

Chromosomal DNA isolation and PFGE

Chromosomal DNA in a plug was prepared according to the protocol used in a previous study with minor modifications [28]. Zymolyase was used instead of lyticase, and cell treatment was performed overnight at 30°C. One percent agarose gels were prepared using pulsed-field certified agarose (Bio-Rad, Hercules, CA) and run on a CHEF apparatus (Bio-Rad) in 0.5 X TBE buffer at 10°C. The following running conditions were used: switch time, 300–400 s or 400 s; run time, 120 h or 180 h; angle, 120°; and voltage, 3 V/cm.

Southern blot analysis

Chromosomal DNAs separated by PFGE were transferred onto a Hybond-N' membrane (GE Healthcare, Buckinghamshire, UK) by upward capillary transfer. Open-reading frame regions of
PEX2, ZYRO0C18458g, SIR1, VAC17, and FET4 genes were then used to probe the membranes. These experiments were performed using a PCR digoxigenin probe synthesis kit (Roche, Basel, Switzerland) and Detection Starter Kit II (Roche) according to the manufacturer's instructions.

Estimation of switching frequency

The frequency of switching was estimated by the semi-quantitative PCR. A fragment containing partial a or z idiomorphs was amplified by PCR from the chromosomal DNA of DA2 and DA2 hoΔ cells using the primers MTLaF or MTLzF and TypingR. PCR was performed for 39 cycles after a pre-incubation at 94°C for 2 min using the following conditions: denaturation at 98°C for 10 s; annealing at 58°C for 15 s; extension at 68°C for 30 s.

Estimation of spontaneous translocation frequency

The DA2AU strain was constructed by inserting S. cerevisiae URA3 and ADE2 into the ZYROI0C18458g locus of DA2. The ZYROI0C18458g locus was amplified from DA2 using the primers C18458gF and C18458gR, and the resulting fragment was cloned into pGEMblue (Takara Bio, Siga, Japan), which was designated pZEU [14]. The orange and green bars encoded in the T and P subgenomes have been referred to as Zr-SOD2 and Z-SOD2, respectively [30,31].

Supporting Information

Figure S1 Isolation of a CBS732-like haploid strain in Z. rouxii. (A) Summary of natural hybridization events between haploid Z. rouxii and Z. pseudorouxii. The orange and green bars indicate the T and P subgenomes, respectively. (B) Selective amplification of the ADE2 and SOD2 genes. The SOD2 gene encoded in the T and P subgenomes has been referred to as Zr-SOD2-22 and Z-SOD2-22, respectively [30,31].

Figure S2 Fluorescence histograms of various Z. rouxii strains after propidium iodide staining. The y-axis in the graphs represents the total cell counts, and the x-axis indicates the relative fluorescence intensity of the samples.

Figure S3 Idiomorph carrying the MAT-like loci. (A) Gene organization around the MAT-like locus. Small arrows indicate primers. (B) PCR amplification of MAT-like loci from Z. rouxii CBS732, DA2, DL2, NBCR1130, ATCC2623, NBCR1733, NBCR0686, NBCR0740, and NBCR1053. PCR was performed by using idiomorph-specific primers and primers specific to each locus (Table S1).
Figure S4  Semi-quantitative PCR amplification of idiomorph-specific products from Zygosaccharomyces rouxii DA2 and DA2 hoA. PCR was performed by using idiomorph-specific primers, and genomic DNA from DA2 and DA2 hoA was used as a template.

(TIF)

Figure S5  Nucleotide sequence of the putative MAT locus amplified by using primer pair 1'-A' from DA2 (AB781017). Uppercase lettering indicates putative open reading frame. Arrows indicate gene direction. Coloring indicates gene position in NBRC1130: dark green, left side of HMR; gray, X and Z region; and orange, between MAT and HML.

(TIF)

Figure S6  Nucleotide sequence of the HMR locus amplified by using primer pair 1'-B' from NBRC1130 (AB781022). Uppercase lettering indicates putative open reading frame. Arrows indicate gene direction. Coloring indicates gene position in NBRC1130: dark green, left side of HMR; gray, X and Z region; and light blue, right side of HMR.

(TIF)

Figure S7  Nucleotide sequence of the HML locus amplified by using primer pair 1'-C' from NBRC0686 (AB781028). Uppercase lettering indicates putative open reading frame. Arrows indicate gene direction. Coloring indicates gene position in NBRC1130: dark green, left side of HMR; gray, X and Z region; and wine red, right side of HML.

(TIF)

Figure S8  Nucleotide sequence of the MAT locus amplified by using primer pair 2'-A' from NBRC1130 (AB781020). Uppercase lettering indicates putative open reading frame. Arrows indicate gene direction. Coloring indicates gene position in NBRC1130: pink, left side of MAT; gray, X and Z region; and orange, between MAT and HML.

(TIF)

Figure S9  Nucleotide sequence of the putative HMR locus amplified by using primer pair 2'-B' from DA2 (AB791018). Uppercase lettering indicates putative open reading frame. Arrows indicate gene direction. Coloring indicates gene position in NBRC1130: pink, left side of MAT; gray, X and Z region; and light blue, right side of HMR.

(TIF)

Figure S10  Nucleotide sequence of the putative HML locus amplified by using primer pair 3'-C' from DA2 (AB781019). Uppercase lettering indicates putative open reading frame. Arrows indicate gene direction. Coloring indicates gene position in NBRC1130: orange, between MAT and HML; gray, X and Z region; and wine red, right side of HML.

(TIF)

Table S1  Primers used in this study.

(XLS)

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Author Contributions

Conceived and designed the experiments: JW. Performed the experiments: JW. Analyzed the data: JW KU YM. Contributed reagents/materials/analysis tools: JW. Wrote the paper: JW KU YM.

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