Full Paper

Variations in mating-type-like (*MTL*) loci direct PCR-based tracking of *Zygosaccharomyces* strains formed by mating

(Received September 1, 2017; Accepted November 7, 2017; J-STAGE Advance publication date: March 31, 2018)

Tomoo Ogata,^{1,*} Katsuaki Kuroki,¹ Koki Ito,¹ Akifumi Kondo,¹ and Kensuke Nakamura²

¹ Department of Biotechnology, Maebashi Institute of Technology, Maebashi, Gunma 371-0816, Japan

² Department of Life Science and Informatics, Maebashi Institute of Technology, Maebashi, Gunma 371-0816, Japan

Variations of chromosomal structures and nucleotide sequences around mating-type-like (*MTL*) loci among *Zygosaccharomyces* species have been reported. We have analyzed these differences in more detail and, on the basis of PCR- and next-generation sequencing data, we describe the *MTL* loci on chromosomes C and F for *Z. rouxii* type-strain NBRC1130, *Z. rouxii* NBRC0740 and *Zygosaccharomyces* sp. NBRC1876. We developed a mating strategy for *Zygosaccharomyces* sp. NBRC1876 and *Z. rouxii* NBRC0740, and found that the mated stains could be identified from parental strains on the basis of nucleotide sequence variations of the *MTL* loci. We further obtained evidence that *Zygosaccharomyces* sp. NBRC1876 is a natural interspecies hybrid between *Z. rouxii* and a related species.

Key Words: interspecies hybrid; mating; mating-type-like (*MTL*) loci; *Zygosaccharomyces*

Introduction

Zygosaccharomyces yeast species have been traditionally used for the production of miso and soy source in Japan, although they can also cause spoilage of foods (Deak and Beuchat, 1996). Mating between different mating types of industrial yeast strains is a promising and acceptable method for breeding yeast strains. If mating between *Zygosaccharomyces* yeast strains could be confirmed by PCR or by another experimental approach, the identification of the resulting mated strains would be easier. In haploid yeast, the *MAT* locus determines the

mating type of the cell: if it contains a *Y α* sequence, the cell is of mating type *a*; otherwise, when a *Y α* sequence is present, it has mating type *α* . Besides the *MAT* locus, many yeast species contain two silent mating-type-like (*MTL*) loci (Butler et al., 2004; Gordon et al., 2011; Hanson et al., 2014; Riley et al., 2016). All three loci play a role in mating-type switching, which involves DNA repair mediated by conserved X- and Z-regions that flank the variant Y sequence (Hanson and Wolfe, 2017). According to the whole-genome sequence published by the Genolevures Consortium (2009), the sequence of *Z. rouxii* (CBS732) contains three *MTL* loci: two *Y α* regions reside on chromosome F, and one *Y α* sequence is located on chromosome C (Table 1, Fig. 1). The *MTL* loci are linked with particular genes, such as *CHAI*, *SLA2* or *DIC1*. A region of approximately 133 kb separates these two *Y α* sequences. The *Y α* sequence region on chromosome C is situated 118 kb upstream from the right arm end of the chromosome. Genetic variations in *MTL* loci are observed in *Zygosaccharomyces* (Bizzarri et al., 2016; Solieri et al., 2014; Watanabe et al., 2013). Watanabe et al. (2013) reported reciprocal chromosomal translocations between chromosomes F and C for NBRC1130 (Figs. 1(b) and (d)), although CBS732 and NBRC1130 are deemed to be identical. The original organization around the *MTL* locus on chromosome C was proposed to be identical to that of NBRC1130: *DIC1-MAT-SLA2* (Fig. 1(d)).

In this study, the *MTL* loci of three *Zygosaccharomyces* strains (NBRC1130, NBRC0740 and NBRC1876) were investigated using next-generation sequencing, and PCR strategies were developed that could help to analyze the mating results. For the mating experiment, we used *Zygosaccharomyces* sp. NBRC1876 (CBS4837) of mating type *a* and *Z. rouxii* NBRC0740 on the basis of reports that the *Y α* sequence was present in all three *MTL*

*Corresponding author: Tomoo Ogata, Department of Biotechnology, Maebashi Institute of Technology, 460-1 Kamisadori, Maebashi, Gunma 371-0816, Japan.

E-mail: tomoo.ogata@maebashi-it.ac.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Table 1. Primers used in this study.

Primer	Sequence (5'-3')	Comments
Chr_C_1444951	CCTTCTTTACCTGGAGCGAGCTCTTCTTGAG	Chromosome C sequence (sequence nos. 144951–144982)
Chr_C_1446049R	TTAACACCTTCCTCGTTCGTTAACTGCCGT	Chromosome C sequence (sequence nos. 1446020–1446049, complementary)
JW_2m	TGCGGCGAGCGCATGATGAAACGAAGATGCAAC	Chromosome C sequence (sequence nos. 1434872–1434906)
JW_Am	CTATAACCAACCAAGTGTAGTGTATCGATAAGTC	Chromosome F sequence (sequence nos. 1304656–1304690, complementary)
JW_1m	AAGACGAAATGCTACTCCCTCATTAAGAACATGAAA	Chromosome F sequence (sequence nos. 1301646–1301680)
JW_Bm	CCTACAATACTCTATTTCGTCCTGTTATCGTTGGT	Chromosome C sequence (sequence nos. 1437076–1437110, complementary)
JW_3m	TTCTATACTTACTGGATGAATCTTCTGTGA	Chromosome F sequence (sequence nos. 1532146–15321750)
JW_Cm	TCAGTACCAGAAAGTGGTCTTTGAAACGATTGCCGC	Chromosome F sequence (sequence nos. 1534591–1534625, complementary)
Y α _724R	AAATCTGTGATACAAATTCCTGGAACTGCACTCT	AB781020 sequence (sequence nos. 1365–1399, complementary)
Y α _686	ATATAGGATGCAGTATCCAGGAATTGTATCACAG	AB781020 sequence (sequence nos. 1361–1395)
Y α _484R	GAGATATGTTTGGGGCTTCTATGATGATTAACCT	AB781022 sequence (sequence nos. 1613–1647, complementary)
Y α _439	ATTGCAAGAAAAGGTAATACATCATAGAGGCCCA	AB781022 sequence (sequence nos. 1602–1636)
Scaffold1957_1876_128R	ATCAACCGGTAAGTGTCTTTCATAAAGTC	LC269302 sequence (sequence nos. 99–128, complementary)

loci of NBRC0740 (Watanabe et al., 2013) and that NBRC1876 could mate with strain *Z. rouxii* CBS4838 of mating type α (Solieri et al., 2013; Wickerham and Burton, 1960).

Materials and Methods

Strain and media. *Zygosaccharomyces rouxii* NBRC1130 (type strain), NBRC0740 (mating type α), and *Zygosaccharomyces* sp. NBRC1876 (mating type α , CBS4837) were used. YPD medium containing 2% glucose, 2% bacto-peptone (Difco, USA) and 1% bacto-yeast extract (Difco) was used for yeast cultivation. Yeast mutants resistant to cycloheximide were isolated on YPD containing 5 μ g/ml of cycloheximide. The abbreviations of culture collections are as follows: NBRC, Biological Research Center in NITE (National Institute of Technology), CBS, Centraalbureau voor Schimmelcultures.

Chromosomal localization of mating-type-like (*MTL*) loci. The chromosomal location of mating-type-like (*MTL*) loci was revealed by PCR analysis as described (Watanabe et al., 2013). Primer sets JW_2m/JW_Am, JW_1m/JW_Bm and JW_3m/JW_Cm were based on oligonucleotides used by Watanabe et al. (2013), that would amplify the respective loci in NBRC1130 (Fig. 2). Insertion of Y α and Y α sequences was tested using primers Y α _724R or Y α _686 and Y α _484R or Y α _439, respectively, combined with primers annealing to their flanking regions. The DNA sequences of *MTL* loci in NBRC0740 and NBRC1876 were deposited in DDBJ under accession numbers LC269303 to LC269306. PCR primers used in this study are described in Table 1.

Whole-genome sequencing by a next-generation sequencer. The genomes of *Zygosaccharomyces* strains NBRC1130, NBRC0740, NBRC1876 were sequenced on the HiSeq2500 platform (Illumina, Inc., CA, USA). Chromosomal DNA was prepared by a phenol-based method from stationary grown cells after lysis by zymolyase treatment as previously reported (Amberg et al., 2005) and paired-ended 125-bp sequences were obtained. Sequence scaffolds were constructed using the genome assembly software Platanus (Kajitani et al., 2014). The whole-genome shotgun sequence data have been deposited in DDBJ with accession numbers DDR092856 to DDR095858.

Isolation of cycloheximide resistant strains. Cells of *Z. rouxii* NBRC0740 grown on YPD medium to the middle-log phase were collected and suspended in 5 ml of 0.1 M sodium phosphate buffer, pH 7.5, at a cell density of about 10^7 cells/ml. To induce mutagenesis in the cells, 2 ml of cell suspension was treated with 60 μ l of ethyl methanesulfonate (final concentration 34.0 μ g/ml), and incubated at 25°C for 1 h. After incubation, the cell suspension was mixed with 8 ml of 5% sodium thiosulfate and the mixture was incubated at room temperature for 10 min. After this treatment, this cell was plated on YPD plate containing 5 μ g/ml of cycloheximide. The plates were incubated at 25°C for 5 days. Two cycloheximide resistant yeast mutants, designated as NBRC0740-CYR1 and -CYR2, were used in the mating experiments.

Fig. 1. Schematic overview of chromosomes C and F in *Zygosaccharomyces rouxii* CBS732 or NBRC1130.

(a) Inferred chromosome F in *Z. rouxii* CBS732 based on genome database. (b) Inferred chromosome F in *Z. rouxii* NBRC1130 from the data of Watanabe et al. (2013). (c) Inferred chromosome C in *Z. rouxii* CBS732 based on genome database. (d) Inferred chromosome F in *Z. rouxii* NBRC1130 from the data of Watanabe et al. (2013). Light-gray boxes indicate the nucleotide sequence deposited as chromosome F in the database (accession no. CU928178); the white boxes indicate the nucleotide sequence deposited as chromosome C in the database (accession no. CU928175). The dark-gray boxes indicate *MTL* (mating-type-like) loci. The genes present within and near to *MTL* loci are indicated by dark-gray arrows. Double-headed thin solid arrows indicate expected nucleotide length. Thin solid arrows lines indicate annealing sites for PCR primers. Broken arrows mark genomic scaffolds assembled on the basis of our sequencing data. The nucleotide sequences of scaffolds were deposited in DDBJ (accession numbers LC269288–LC269292).

Isolation of mated yeast strains. A single cell of NBRC1876 and a single cell of NBRC0740-CYR1 or -CYR2 were attached to each other on an agar plate by means of a micro-manipulator. Formed colonies were examined for cycloheximide resistance and the presence of gene ZYROOC18458g by PCR amplification with primer set Chr_C_1444951/Chr_C_1446049R. PCR primers used in this study are described in Table 1.

Results

Chromosomal location of mating-type-like (*MTL*) loci

Rearrangements between chromosomes C and F around mating-type-like (*MTL*) loci resulted in differences between *Z. rouxii* NBRC1130 and the type strain thought to be identical with it, *Z. rouxii* CBS732 (Fig. 1; Watanabe et al., 2013). After publication of its whole-genome sequence (The Genolevures Consortium, 2009), the genome of *Z. rouxii* CBS732 has served as a reference, but it has been argued that the original *MTL* loci are conserved only in NBRC1130, while that of CBS732 has been translocated (Watanabe et al., 2013). In short, originally one *MTL* locus with a *Ya* sequence region is present on chromosome

F, while the putative *MAT* locus on chromosome C, containing both *Yα* and *Ya* sequences, and the other *MTL* locus with a *Yα* sequence is located near the right arm end of chromosome C as found in NBRC1130 (Fig. 1; Watanabe et al., 2013). We analyzed these loci by PCR in NBRC1130 and our results are in agreement with their proposed organization. First, the same order of *DIC1* (ZYROOC18304g)-putative *MAT*-*SLA2* (ZYROOF15862g) is observed, while both *Ya* and *Yα* sequences are present in the putative *MAT* locus (Fig. 1(d)). Second, an *MTL* locus with a *Ya* region is located between *CHA1* (ZYROOF15774g) and ZYROOC18392g located on chromosome F (Fig. 1(b)) and is referred to as *HMR* by Watanabe et al. (2013). Third, an *MTL* locus with a *Yα* region is located between *CHA1* (ZYROOF18524g) and ZYROOF18634g located near the right arm end of chromosome C (Fig. 1(d)) which is called the *HML* locus (Watanabe et al., 2013).

In view of our aim to mate *Zygosaccharomyces* strains, strain NBRC0740 is of interest because analysis of the *MTL* loci indicated that *Yα* sequences are inserted into all *MTL* loci in NBRC0740 (Fig. 3; Watanabe et al., 2013). Our PCR analysis of the three *MTL* loci in NBRC0740

Fig. 2. PCR analysis of *MTL* loci in NBRC1130.

(a) The *MTL* locus flanked by *DIC1* (ZYRO0C18304g) and *SLA2* (ZYRO0F15862g) on chromosome C, containing both a *Yα* and a *Ya* sequence, as shown with primer combinations JW_2m/*Yα*_724R (lane 1), JW_2m/*Ya*_484R (lane 2), *Yα*_686/JW_Am (lane 3), and *Ya*_439/JW_Am (lane 4). (b) The *MTL* locus flanked by *CHA1* (ZYRO0C15774g) and ZYRO0C18392g on chromosome F, as shown with primer sets JW_1m/*Yα*_724R (lane 1), JW_1m/*Ya*_484R (lane 2), *Yα*_686/JW_Bm (lane 3), and *Ya*_439/JW_Bm (lane 4). (c) The *MTL* locus flanked by *CHA1* (ZYRO0C18524g) and ZYRO0C18634g on chromosome C, as shown with primer pairs JW_3m/*Yα*_724R (lane 1), JW_3m/*Ya*_484R (lane 2), *Yα*_686/JW_Cm (lane 3), and *Ya*_439/JW_Cm (lane 4). Lane M is a *Hind* III-digested λ DNA size marker.

(a) Chromosome F in NBRC0740 (Watanabe *et al.* (2013))

(b) Chromosome C in NBRC0740 (Watanabe *et al.* (2013))

Fig. 3. Schematic overview of chromosomes C and F in *Zygosaccharomyces rouxii* NBRC0740.

(a) Inferred chromosome F from the data of Watanabe *et al.* (2013). (b) Inferred chromosome C from the data of Watanabe *et al.* (2013). Light-gray boxes indicate the nucleotide sequence deposited as chromosome F in the database (accession no. CU928178); the white boxes indicate the nucleotide sequence deposited as chromosome C in the database (accession no. CU928175). Dark-gray boxes indicate *MTL* (mating-type-like) loci. The genes present near to *MTL* loci are indicated by dark gray arrows. Thin solid arrows indicate annealing sites for PCR primers. Broken arrows mark genomic scaffolds assembled on the basis of our sequencing data. The nucleotide sequences of scaffolds were deposited in DDBJ (accession numbers LC269293–LC269296).

Fig. 4. PCR analysis of *MTL* loci in NBRC0740.

(a) The *MTL* locus flanked by *DIC1* (ZYRO0C18304g) and *SLA2* (ZYRO0C15862g) on chromosome C, as shown with primer combinations JW_2m/*Yα*_724R (lane 1), JW_2m/*Ya*_484R (lane 2), *Yα*_686/JW_Am (lane 3), and *Ya*_439/JW_Am (lane 4). (b) The *MTL* locus flanked by *CHA1* (ZYRO0C15774g) and ZYRO0C18634g on chromosome F, as shown with primer sets JW_1m/*Yα*_724R (lane 1), JW_1m/*Ya*_484R (lane 2), *Yα*_686/JW_Cm (lane 3), and *Ya*_439/JW_Cm (lane 4). (c) The *MTL* locus flanked by *CHA1* (ZYRO0C18524g) and ZYRO0C18634g on chromosome C, as shown with primer pairs JW_3m/*Yα*_724R (lane 1), JW_3m/*Ya*_484R (lane 2), *Yα*_686/JW_Cm (lane 3), and *Ya*_439/JW_Cm (lane 4). Lane M is a *Hind* III-digested λDNA size marker.

(a) Chromosome F on which is deduced from our sequencing in NBRC1876

(b) Chromosome C which is deduced from our sequencing in NBRC1876

Fig. 5. Schematic overview of chromosomes C and F in *Zygosaccharomyces* sp. NBRC1876.

(a) Inferred chromosome F deduced from our sequencing data. (b) Inferred chromosome C deduced from our sequencing data. Light-gray boxes indicate the nucleotide sequence deposited as chromosome F in the database (accession no. CU928178); white boxes indicate the nucleotide sequence deposited as chromosome C in the database (accession no. CU928175). Since the part of the sequence of Scaffold1957_NBRC1876 is not identical to the sequence of chromosome F of *Z. rouxii* CBS732, the region of the right side of the *MTL* loci is not colored in dark gray. Dark-gray boxes indicate *MTL* (mating-type-like) loci. The genes present near to *MTL* loci are indicated by dark-gray arrows. Thin arrows indicate annealing sites for PCR primers; where the arrow is gray and dashed, the primer would not anneal. Broken arrows mark genomic scaffolds assembled on the basis of our sequencing data. The nucleotide sequences of scaffolds were deposited in DDBJ (accession numbers LC269297–LC269302).

Fig. 6. PCR analysis of *MTL* loci in NBRC1876.

(a) The *MTL* locus flanked by *DIC1* (ZYRO0C18304g) and *SLA2* (ZYRO0C15862g) on chromosome C, as shown with primer combinations JW_2m/Yα_724R (lane 1), JW_2m/Yα_484R (lane 2), Yα_686/JW_Am (lane 3), and Yα_439/JW_Am (lane 4), lane 5 shows confirmation of insertion of a unique *MTL* locus in NBRC1876 (PCR primers are JW_2m and Scaf1957_1876_128R). (b) The *MTL* locus flanked by *CHA1* (ZYRO0F15774g) and ZYRO0C18392g on chromosome F, as shown with primer sets JW_1m/Yα_724R (lane 1), JW_1m/Yα_484R (lane 2), Yα_686/JW_Cm (lane 3), and Yα_439/JW_Cm (lane 4). (c) The *MTL* locus flanked by *CHA1* (ZYRO0C18524g) and ZYRO0C18612g on chromosome C, as shown with primer pairs JW_3m/Yα_724R (lane 1), JW_3m/Yα_484R (lane 2), Yα_686/JW_Cm (lane 3), and Yα_439/JW_Cm (lane 4). Lane M is a *Hind* III-digested λDNA size marker.

confirms this (Fig. 4). The *MTL* loci on chromosome C in NBRC0740 were amplified with primers JW_2m or JW_Am, and JW_3m or JW_Cm, and the *MTL* locus on chromosome F with primers JW_1m or JW_Cm. On chromosome C in NBRC0740, the same order of genes as in NBRC1130 was found for two *MTL* loci. However, chromosome F in NBRC0740 differs from that in NBRC1130. The *MTL* locus on chromosome F is located between *CHA1* (ZYRO0F15774g) and ZYRO0F18634g (Fig. 3(a)). The right sequence of this *MTL* locus is the same as the right sequence of the *MTL* locus near the right arm end of chromosome C (Fig. 3(b)).

Zygosaccharomyces sp. NBRC1876 was reported to mate with strain *Z. rouxii* CBS4832, which has been inferred to be of mating type α (Solieri et al., 2013; Wickerham and Burton, 1960). In the absence of sequence information for NBRC1876, we based our PCR analysis of its *MTL* loci on that for NBRC1130 (Fig. 6). The *MTL* loci on chromosome C in NBRC1876 were amplified with primers JW_2m and Scaffold1957_1876_128R, and JW_3m or JW_Cm, and the *MTL* locus on chromosome F with primers JW_1m or JW_Bm. This analysis demonstrated the presence of an *MTL* locus on chromosome F containing a Yα sequence flanked by *CHA1* (ZYGO0F15774g) and

Fig. 7. Presence of the gene ZYRO0C18458g in *Zygosaccharomyces* strains.

A region of ZYRO0C18458g was amplified from NBRC0740-CYR1 (lane 1), NBRC1876 (lane 2), and the strain resulting from mating between NBRC0740-CYR1 and NBRC1876 (lane 3). Lane M is a *Hind* III-digested λDNA size marker.

Fig. 8. Cycloheximide resistance of *Zygosaccharomyces* strains.

Ten-fold serial dilutions of the indicated yeast cultures were spotted on YPD with (a) or without (b) cycloheximide. The incubations were carried out for 7 days at 25°C.

ZYGO0C18392g (Fig. 6(b)), and a $Y\alpha$ sequence in the *MTL* locus on chromosome C that was flanked by *CHAI* (ZYGO0F18524g) and ZYGO0F18634g (Fig. 6(c)). No PCR products were obtained with primers JW_2m or JW_Am (Fig. 6(a), lanes 1 to 4), indicating that in addition the *MTL* locus flanked by *DIC1* (ZYRO0C18304g) and *SLA2* (ZYRO0F15862g) on chromosome C, would be different from that of NBRC1130.

Whole-genome sequencing of *Zygosaccharomyces* strains

The PCR results described above provided information on the *MTL* loci in the genomes of the three yeast strains but did not directly test the proposed association of these regions with either chromosome C or F. Furthermore, lack of sequence information precluded analysis of all the *MTL* loci in NBRC1876 and in putative mated strains after mating. For this reason, we obtained genomic sequences from *Zygosaccharomyces* strains NBRC1130, NBRC0740 and NBRC1876 by means of next-generation sequencing. Number of reads for NBRC1130, NBRC0740 and NBRC1876 were 41.4M, 33.6M and 30.7M, respectively. Total called bases for NBRC1130, NBRC0740 and NBRC1876 were 5,170 Mbp, 4,206 Mbp and 3,837 Mbp, respectively. Total numbers of scaffolds for NBRC1130, NBRC0740 and NBRC1876 after being assembled were 1,349, 2,672 and 2,649, respectively. The total length of scaffolds was about 9.8 Mb for both NBRC1130 and NBRC0740, which is consistent with the total genome length of CBS732. On the other hand, the total length of scaffolds for NBRC1876 was 19.2 Mb, suggesting the presence of another genome.

We aligned homologous regions that overlapped in the various scaffolds and found a high degree of identity between chromosomes C and F in the three strains. The nucleotide sequences that we obtained, in combination with the PCR results, helped to construct the chromosomal organizations as depicted in Figs. 1, 3 and 5. The presence of gene ZYRO0C18458g in NBRC1130 and NBRC1876 was confirmed with Scaffold505_NBRC1130 and Scaffold1060_NBRC1876, respectively (Figs. 1(b) and 5(a)), and supported by PCR analysis (Fig. 7 lane 2). Both the presence of the annealing site for primer JW_Bm in Scaffold505_NBRC1130, and the span of

Fig. 9. Presence of *MTL* loci in mated strains.

Shown are the *MTL* loci on chromosome C flanked by *DIC1* (ZYRO0C18304g) and *SLA2* (ZYRO0F15862g) derived from NBRC0740 using primer set JW_2m/JW_Am, or derived from NBRC1876 with primer set JW_2m/Scaffold1957_1876_128R, the *MTL* locus flanked by *CHAI* (ZYRO0F15774g) and ZYRO0C18634g derived from NBRC0740 using primer set JW_1m/JW_Cm, and the *MTL* locus flanked by *CHAI* (ZYRO0F15774g) and ZYRO0C18392g derived from NBRC1876 using primer set JW_1m/JW_Bm. The templates were genomic DNA isolated from parental strains NBRC0740-CYR1-1 and NBRC1876, and from mated strains NBRC0740-CYR1-1 and NBRC0740-CYR1-2. Lane M is a *Hind* III-digested λ DNA size marker.

Scaffold1060_NBRC1876, demonstrated that this gene would lie on the right side of the *MTL* locus on chromosome F. Gene ZYRO0C18458g, however, was not found among the scaffold sequences of NBRC0740. The region covered by Scaffold2210_NBRC0740 replaced the corresponding region in chromosome F (Fig. 3(a)), although it was also present at the *MTL* locus at the end of the right

arm of chromosome C (Figs. 1(d) and 5(b)) in all three strains.

Comparison of *MTL* loci

Because the nucleotide sequences of the *MTL* loci have high sequence identity among each *MTL* loci, there must be sequence gaps among the scaffolds from each strain corresponding to *MTL* loci. Furthermore, the *MTL* locus on chromosome C in NBRC1876 was not identified by our PCR experiments (Fig. 6(a) lanes 1–4). The nucleotide sequence of Scaffold1957_NBRC1876 (accession no. LC269302) has 81% identity with that of *SLA2* (ZYRO0F15862g) on chromosome C in CBS732; however, the nucleotide sequence corresponding to the primer JW_Am is not identical. A PCR-amplified product was obtained from NBRC1876 genomic DNA as a template using primer set JW_2m/Scaffold1957_1876_128R, which was designed based on the sequence of Scaffold1957_NBRC1876 (Fig. 6(a) lane 5). The nucleotide sequence of this PCR product was determined (accession no. LC269306), showing that sequences of the X region and Z region share a high identity (100% and 98%, respectively) with those of the *Z. rouxii* putative *MAT* locus sequence (accession no. AB781020); however, the nucleotide sequence of the Y region has a relatively low identity (82%) with that of AB781020. Because homologous sequences to $Y\alpha 1$ and $Y\alpha 2$ exist in this sequence, it is inferred that the $Y\alpha$ sequence is inserted in the *MTL* locus on chromosome C in NBRC1876 (Fig. 5(b)). The *Mat* $\alpha 1$ amino acid sequence from NBRC1876 shows 87%, 87%, 99%, 82% identity to other *Zygosaccharomyces* *Mat* $\alpha 1$ orthologs (accession nos. XP002497889, HG931712, HG931713, and HG931714, respectively). Similarly, the *Mat* $\alpha 2$ amino acid sequence from NBRC1876 shows 80%, 80%, 100% and 69% identity to other *Zygosaccharomyces* *Mat* $\alpha 2$ orthologs (accession nos. XP002497888, HG931712, HG931713 and HG931714, respectively). The identity of *Mat* $\alpha 1$ and *Mat* $\alpha 2$ of the *MTL* locus on chromosome C in NBRC1876 is highest for HG931713, while a 27-bp deletion is observed in the X-region of HG931713.

The *MTL* locus on chromosome C in NBRC0740 was amplified by PCR using primers JW_2m or JW_Am (Fig. 4(b)). The nucleotide sequence of the resulting PCR product (accession no. LC269304), shows a 99% sequence identity to the *MAT* locus in NBRC1130 (accession no. AB781020). Collectively, therefore, these findings reveal that the nucleotide sequences of the *MTL* locus on chromosome C are different in NBRC0740 and NBRC1876.

The *MTL* locus on chromosome F in NBRC0740 was amplified by PCR using primers JW_1m or JW_Cm (Figs. 3(a) and 4(b)). The nucleotide sequence of the resulting PCR product (accession no. LC269303) shows a 99% sequence identity to the *HML* locus in NBRC0686 (accession no. AB781028). On the other hand, the *MTL* locus on chromosome F in NBRC1876 was amplified by PCR using primer set JW_1m/JW_Bm (Fig. 6(b)). The nucleotide sequence of this *MTL* locus in NBRC1876 was also determined (accession no. LC269305), showing that the nucleotide sequence identity is 99% to the *HML* locus in NBRC1130 (accession no. AB781022). From this result,

the nucleotide sequences of the *MTL* locus on chromosome F are also different in NBRC0740 and NBRC1876.

Isolation of mated yeast strains

We set up a mating experiment with *Zygosaccharomyces* sp. NBRC1876 and *Zygosaccharomyces rouxii* NBRC0740-CYR1 because these strains were expected to be of opposite mating types. For the identification of mating, genetic markers were needed that would be unique for the parental strains. As described above, being absent in NBRC0740-CYR1 (Fig. 7 lane 1), gene ZYRO0C18458g could be used as a marker for NBRC1876, while for NBRC0740 we isolated cycloheximide-resistant mutants, NBRC0740-CYR1 and -CYR2 (Fig. 8). Mating was carried out by means of a micro-manipulator, which yielded colonies that were both resistant to cycloheximide (Fig. 8) and contained the ZYRO0C18458g gene (Fig. 7 lane 3). Thus, using the nucleotide sequences of *MTL* loci in NBRC0740 and NBRC1876, we designed a PCR-strategy to obtain more evidence for successful mating.

MTL loci in mated yeast strains derived from NBRC0740 and NBRC1876

Because the nucleotide sequences of the *MTL* loci on chromosome C and chromosome F differ in NBRC0740 and NBRC1876, we designed PCR primers to test whether after mating, all the variant loci that would be indicative of the parental strains could be detected in genomic DNA isolated from two mated yeast strains (Fig. 9). Indeed, both the *MTL* loci on chromosome C that are derived from NBRC0740 (Fig. 9, lanes 1 and 5) and those on chromosome C that are derived from NBRC1876 (Fig. 9 lanes 2 and 6), were present. In addition, the *MTL* locus on chromosome F derived from NBRC0740 (Fig. 9 lanes 3 and 7), as well as the *MTL* locus on chromosome F derived from NBRC1876 (Fig. 9 lanes 4 and 8) were detected. The nucleotide sequences of these four PCR products were identical to those derived from NBRC0740 and NBRC1876 (not shown). These results show that PCR analysis of divergent mating-type loci is a powerful strategy to confirm the successful breeding of *Zygosaccharomyces* strains by mating.

Discussion

Zygosaccharomyces yeasts are commonly used to produce miso and soy source, and mating is a promising and acceptable method of breeding novel yeast strains in the food industry. Some reports about the mating type loci of yeast strains have been published (Butler et al., 2004; Gordon et al., 2011; Hanson et al., 2014; Riley et al., 2016). Furthermore, the whole genome sequence of *Zygosaccharomyces rouxii* type strain CBS732 (NBRC1130) has been published (The Genolevures Consortium, 2009), and three *MTL* (mating-type-like) loci were identified (Watanabe et al., 2013). However, which *MTL* locus is involved in the expression of the mating type has not been revealed precisely.

Natural interspecies hybrid strains between *Z. rouxii* and related species have been reported for *Zygosaccharomyces*

yeast strains (Gordon and Wolfe, 2008; James et al., 2005). Such allopolyploids are expected to be very osmo- and halotolerant. Chromosomal structures located near the *MTL* loci in *Zygosaccharomyces* interspecies hybrid strains have not previously been revealed. Our next-generation sequencing (HiSeq 2500) approach revealed that *Zygosaccharomyces* sp. NBRC1876 is a natural interspecies hybrid between *Z. rouxii* and a related species. The whole-genome sequence of *Zygosaccharomyces* sp. NBRC1876 further revealed that this strain has at least three *MTL* loci, and the nucleotide sequences located near the *MTL* locus on chromosome C and the *MTL* locus on chromosome F in NBRC1876 are different from those in NBRC0740. We used NBRC1876 in our mating experiment (Fig. 8), and confirm mating between NBRC0740 and NBRC1876 by using the nucleotide sequence differences in the *MTL* loci on chromosome C and chromosome F.

From our results, it remains unclear which *MTL* locus is functional and is involved in the expression of mating type in NBRC1876; further study is necessary to answer these questions. *Zygosaccharomyces* interspecies hybrid yeast strains have not only a T-type subgenome derived from *Z. rouxii* but also a P-type subgenome derived from related species (Gordon and Wolfe, 2008; James et al., 2005). Further research is necessary to reveal to which subgenome the three *MTL* loci in NBRC1876 identified in this study belong, and whether NBRC1876 has more than three *MTL* loci. While we were preparing this manuscript, a report on the draft sequencing of the whole genome of *Zygosaccharomyces* sp. NBRC1876 was published (Sato et al., 2017). A search for sequence identity between our sequence of the *MTL* locus on chromosome C in NBRC1876 (accession no. LC269306) and their whole genome sequence of NBRC1876 has revealed a high identity with the 3'-prime end sequence of BBQV01000028; however, the sequences are not 100% identical. Further investigation will be required to clarify this.

Acknowledgments

We thank Dr. Jun Watanabe for valuable discussions and Dr. Satoshi Yoshida for the use of his micro-manipulator. This work was supported by grants for the Miso Central Research in 2015 and 2016.

References

- Amberg, D. C., Burke, D., and Strathern, J. (2005) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, Cold Spring Harbor Laboratory Press.
- Bizzarri, M., Giudici, P., Cassanelli, S., and Solieri, L. (2016) Chimeric sex-determining chromosomal regions and dysregulation of cell-type identity in sterile *Zygosaccharomyces allodiploid* yeast. *PLoS ONE*, **11**, e0152558.
- Butler, G., Kenny, C., Faga, A., Kurischko, C., Gaillardin, C. et al. (2004) Evolution of the *MAT* locus and its Ho endonuclease in yeast species. *Proc. Natl. Acad. Sci. USA*, **101**, 1632–1637.
- Deak, T. and Beuchat, L. (1996) *Handbook of Food Spoilage Yeasts*, CRC Press.
- Gordon, J. and Wolfe, K. H. (2008) Recent allopolyploid origin of *Zygosaccharomyces rouxii* strain ATCC42981. *Yeast*, **25**, 449–456.
- Gordon, J. L., Armisen, D., Proux-Wera, E., OhEigeartaigh S. S., Byrne, K. P. et al. (2011) Evolutionary erosion of yeast sex chromosome by mating-type switching accidents. *Proc. Natl. Acad. Sci. USA*, **108**, 20024–20029.
- Hanson, S. J. and Wolfe, K. H. (2017) An evolutionary perspective on yeast mating-type switching. *Genetics*, **206**, 9–32.
- Hanson, S. J., Byrne, K. P., and Wolfe, K. H. (2014) Mating-type switching by chromosomal inversion in methylotrophic yeasts suggests an origin for the three-locus *Saccharomyces cerevisiae* system. *Proc. Natl. Acad. Sci. USA*, **111**, E4851–E4858.
- James, S. A., Bond, C. J., Stratford, M., and Roberts, I. N. (2005) Molecular evidence for the existence of natural hybrids in the genus *Zygosaccharomyces*. *FEMS Yeast Res.*, **5**, 747–755.
- Kajitani, R., Toshimoto, K., Noguchi, H., Toyoda, A., Ogura, Y. et al. (2014) Efficient de novo assembly of highly heterozygous genomes from whole-genome shotgun short reads. *Genome Res.*, **24**, 1384–1395.
- Riley, R., Haridas, S., Wolfe, K. H., Lopes, M. R., Hittinger, C. T. et al. (2016) Comparative genomics of biotechnologically important yeasts. *Proc. Natl. Acad. Sci. USA*, **113**, 9882–9887.
- Sato, A., Matsushima, K., Oshima, K., Hattori, M., and Koyama, Y. (2017) Draft genome sequencing of the highly halotolerant and allopolyploid yeast *Zygosaccharomyces rouxii* NBRC1876. *Genome Announcements*, **5**, e01610-16.
- Solieri, L., Dakal, T. C., and Giudici, P. (2013) *Zygosaccharomyces sapae* sp. nov., isolated from Italian traditional balsamic vinegar. *Int. J. Syst. Evol. Microbiol.*, **63**, 364–371.
- Solieri, L., Dakal, T. C., Giudici, P., and Cassanelli, S. (2014) Sex-determination system in the diploid yeast *Zygosaccharomyces sapae*. *G3 Genes Genomes Genetics*, **4**, 1011–1025.
- The Génolevures Consortium (2009) Comparative genomics of protoploid *Saccharomycetaceae*. *Genome Res.*, **19**, 1696–1709.
- Watanabe, J., Uehara, K., and Mogi, Y. (2013) Diversity of mating-type chromosome structures in the yeast *Zygosaccharomyces rouxii* caused by ectopic exchanges between *MAT*-like loci. *PLoS ONE*, **8**, e62121.
- Wickerham, J. and Burton, K. A. (1960) Heterothallism in *Saccharomyces rouxii*. *J. Bacteriol.*, **80**, 492–495.