A simple genotyping method for *RIM15* gene polymorphisms in sake yeast strains

Takashi Kuribayashi[a,b]*, Nanako Asahi[b], Hina Satone[b]*, Junpei Tanaka[a], Masamichi Sugawara[a], Keigo Sato[a], Yoshihito Nabekura[a], Toshio Joh[c], and Toshio Aoki[a]

[a] Niigata Prefectural Sake Research Institute, 2-5932-133 Suido-cho, Chuoh-ku, Niigata 951-8121, Japan
[b] Faculty of Food Industry, Niigata Agro-Food University, 2416 Hiranedai, Tainai, Niigata, 959-2702, Japan
[c] Faculty of Agriculture, Niigata University, 2-8050 Ikarashi, Nishi-ku, Niigata, 950-2181, Japan

*Corresponding author:
kuribayashi.takashi@pref.niigata.lg.jp (T. Kuribayashi),
hina-satone@nafu.ac.jp (H. Satone)

Abstract

In the process of brewing sake, *Saccharomyces cerevisiae* not only synthesizes numerous flavor compounds but also produces alcohol, which enhances fermentation efficiency. A previous study identified a sake yeast mutant with a single-nucleotide insertion in the *RIM15* protein kinase gene (*RIM15* with an insertion of adenine at position 5067: *RIM15*ins5067A). In this study, we developed primer sets (P1/P2) with engineered mismatches that introduce restriction sites for the restriction enzyme CpsCI. This allowed us to create a simple and rapid polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to identify Kyokai no. 7 (K7) group sake yeast strains carrying the *RIM15*ins5067A mutation. We validated the PCR-RFLP method in detecting the *RIM15*ins5067A mutation in the K7 group of yeast strains. This approach provides a straightforward, effective, and valuable tool for distinguishing specific yeasts used in sake production.

Keywords:
polymerase chain reaction-restriction fragment length polymorphism; *RIM15*; *Saccharomyces cerevisiae*

Sake is a popular Japanese alcoholic beverage that is made by fermenting steamed rice with *Saccharomyces cerevisiae* (Desm.) Meyen and *koji*, which is a culture of *Aspergillus oryzae* (Ahlb.) Cohn, that is grown on steamed rice (Yoshizawa, 1999). *Saccharomyces cerevisiae* synthesizes a wide range of flavor compounds and produces alcohols that enhance fermentation efficiency.

Historically, sake brewing relied on wild-house sake yeasts, which naturally develop in the fermentation environments of individual breweries. This led to a diverse array of sake varieties, as each brewery utilized its own distinct sake yeast strains. Over the past 80 y, a group of genetically similar sake yeast strains, known as the Kyokai no.7 (K7) group strains (Kyokai no. 6, 7, 9, and 10 series), have been isolated showing high fermentation rates in sake mash (Azumi & Goto, 2001). These K7 group strains have become the predominant choice for sake brewing over the past few decades, due to their effectiveness in enhancing the quality of sake and producing high-quality premium sake. However, the widespread use of these industrial yeasts may have reduced the diversity of its gustatory properties compared to when unique house sake yeast strains were used in each brewery. Consequently, interest has grown in brewing sake using alternative yeasts other than the K7 group strains to diversify the taste and flavor of sake.

The house sake yeast *S. cerevisiae* strain YS4 was isolated from the Sake Brewery "Yoshinogawa" (Settaya, Nagaoka, Japan), one of the oldest sake breweries in Niigata, established in 1548. The brewing properties of the YS4 strain differ from those of the K901 strain in the K7 group (Hatakeyama et al., 2020). Despite significant attention to house sake yeasts and sake production, few reports have focused on these characteristics, partly due to the difficulty in isolating house yeast strains given their similar physiological properties to K7 group strains.

Recent studies have identified loss-of-function mutations in genes common to the K7 group strains, specifically *RIM15*, *MSN4*, and *PPT1* loci (Watanabe, 2012). These mutations, found only in the K7 strains, offer a valuable tool for identifying them (Akao et al., 2018). Of these genes, *RIM15*, which is encoded by a protein kinase that plays a role in cell proliferation in response to nutrients, possesses a frameshift mutation that may serve as a critical genetic determinant for the increased production of ethanol in modern sake yeast strains (Watanabe & Takagi, 2016). The frameshift mutation at nucleotide position 5068 (*RIM15*ins5067A, Fig. 1) was predicted to cause a premature stop codon, which would lead to a reduction of 75 amino acids in the C-terminal region of the *RIM15* gene product (Watanabe et al., 2012). While high-resolution melting analysis can detect *RIM15*ins5067A, it requires costly equipment, which is impractical for routine use by brewers.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is useful for quickly and precisely identifying single nucleotide polymorphisms (SNPs) that create distinctive restriction sites (Zhang et al., 2005). PCR-RFLP is widely to identify sake yeast mutants that produce high levels of fatty acid esters and have mutations *FAS2*-G1250S (G3748A) (Tanaka et al., 2023) and *FAS2*-F1279Y (T3836A) (Kuribayashi et al., 2022). This study presents a simple molecular assay designed to genotype *RIM15*ins5067A polymorphisms to differentiate K7 group strains from other sake yeast strains.

The nucleotide insertion at position 5067 in the *RIM15* gene of K7 group strains did not situate near any restriction sites; therefore, we developed the reverse primer P2 with a

mismatch (AA>TG) at the second and third positions from the 3′ end (Fig. 1). This mismatch was designed to incorporate a restriction site for CspCI within the PCR amplicon when the *RIM15*ins5067A mutation was present at position 5067, resulting in 240-, 35-, and 10-bp fragments. However, in the wild-type *RIM15* gene, lacking insert A at position 5067, the CspCI restriction site was not incorporated, resulting in an undigested 284 bp product.

To validate the custom-designed primers and PCR-RFLP assay, we used the Kyokai no.1-10, shochu yeast strains S-2 and SH-4, wine yeast strain KW-1 (provided by National Research Institute of Brewing) and the wild-type strain S288C (purchased from Open Biosystems, Huntsville, AL, USA). PCR amplification was carried out in reaction volumes of 50 μL, which included 25 μL of Takara Taq HS Perfect MIX (Takara Bio, Shiga, Japan), 0.4 μM of each primer, and 20 ng of genomic DNA from yeast cells. The PCR cycling conditions included 35 cycles of denaturation at 94 °C for 5 s, annealing at 55 °C for 1 s, and extension at 68 °C for 10 s. PCR products were extracted using a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Osaka, Japan), purified (18 μL) using 10× rCutSmart Buffer (2 μL) and restriction enzyme CspCI (1 μL) from New England Biolabs (Ipswich, MA, USA), and incubated for one hour at 37 °C before purification with the FastGene Gel/PCR Extraction Kit (Nippon Genetics). The purified samples were then run on 4% agarose gels (Agarose L03, Takara Bio) for 40 min at 100 V and visualized with Midori Green Advance (Nippon Genetics) staining.

To assess the accuracy of the mismatch PCR-RFLP assay for detecting the *RIM15*ins5067A polymorphism (with the insertion) and S288C (wild-type, lacking the insertion). As shown in Fig. 1, the PCR product from S288C was 284 bp and remained undigested by CspCI, while the K7 amplicon was cleaved by CspCI into a 240 bp fragment. DNA sequence analysis confirmed the intended mismatched nucleotides (Supplementary Fig. 1).

Among the 14 yeast strains examined, the PCR-RFLP assay detected the mutation in Kyokai no. 6, 7, 9, and 10 strains (Table 1), consistent with a previous study identifying the *RIM15*ins5067A in these K7 strains (Watanabe et al., 2012). We also confirmed this mutation using DNA sequence analysis of *RIM15* (Table 1). These findings indicate that this assay identify sake yeasts possessing the *RIM15*ins5067A gene but cannot distinguish between the individual strains, such as Kyokai no. 9 and Kyokai no. 10 or SH-4 and S288C. Despite this methodological limitation, the results obtained showed strong concordance with our data (Table 1) and previously reported mutation sequences (Watanabe et al., 2012). Therefore, the PCR-RFLP is effective for distinguishing K7 group strains from other *S. cerevisiae* strains.

The *RIM15*ins5067A mutation, found exclusively in K7 group strains was used to differentiate specific yeast strains, including the house yeast strain. A previous study indicated that the house yeast strain Km67 from the Kiku-masamune brewery lacked this mutation (Takao et al., 2018). We employed the PCR-RFLP method on the house yeast strain YS4 from Yoshinnogawa (Fig. 2). The PCR product from the house yeast strain YS4 was 284 bp in length and was not digested by CspCI; therefore, the mismatch PCR-RFLP assay effectively distinguished between K7 and the house yeast strain YS4. We also confirmed that YS4 does not harbor this mutation in *RIM15* (Supplementary Fig. S1). These results suggest that our PCR-RFLP assay is helpful for the isolation of a house sake yeast strain from a sake brewery that usually produces sake using K7 group strains carrying *RIM15*ins5067A.

In recent years, sake manufacturers and research institutions in Japan have developed a variety of sake yeast strains. This trend may have reduced the diversity of the flavor profile of sake compared to that of sake fermented with unique house yeast strains specific to each brewery in the past. The use of non- K7 group strains has also gained interest for diversifying the taste and flavor of sake. House yeasts offer potential for creating novel sake varieties with distinct flavors (Hatakeyama et al., 2020; Takao et al., 2018). Although it has been reported that the *K7_02212*, *PPT1*, and *PHO3* genes have been targeted for the facile identification of industrial yeast strains (Hatakeyama et al., 2020; Kuribayashi et al., 2014), the addition of *RIM15* to these targets provides a more meticulous approach to distinguish K7 group strains and screen for house yeast strains.

This study demonstrates the usefulness of PCR-RFLP for identifying the *RIM15*ins5067A mutation in K7 group of yeast. This simple and effective method is valuable for distinguishing specific yeasts in sake breweries.

Disclosure

The authors declare no conflict of interest. All the experiments undertaken in this study complied with the current laws of the country in which they were performed.

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References

Akao, T., Zhou, Y., Watanabe, D., Okazaki, N., & Shimoi, H. (2018). Development of DNA markers to differentiate good Kyokai sake yeast and other yeast strains. *Journal of the Brewing Society of Japan*, *113*(10), 631–641. doi:10.6013/jbrewsocjapan.113.631.

Azumi, M., & Goto-Yamamoto, N. (2001). AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces* sensu stricto and its application to phenetic clustering. *Yeast*, *18*(12), 1145–1154. doi:10.1002/yea.767.

Hatakeyama, A., Watanabe, Y., Arimoto, K., Kuribayashi, T., Hara, T., & Joh, T. (2020). Screening and characterization of a house yeast strain for sake brewing using the loop-mediated isothermal amplification method. *Journal of the Brewing Society of Japan*, *115*(9), 537–544. doi:10.6013/jbrewsocjapan.115.537.

Kuribayashi, T., Sakurai, T., Hatakeyama, A., Joh, T., & Kaneoke, M. (2022). Genotypic analysis of the *FAS2*-F1279Y (3836T> A) polymorphism conferring high ethyl caprylate productivity in industrial sake yeast strains. *Journal of General and Applied Microbiology*, *68*(5), 248–252. doi:10.2323/jgam.2022.05.001.

Kuribayashi, T., Sato, K., Kasai, D., Fukuda, M., Kaneoke, M., & Watanabe, K. I. (2014). Differentiation of industrial sake yeast strains by a loop-mediated isothermal amplification method that targets the *PHO3* gene. *Journal of bioscience and bioengineering*, *118*(6), 661-664. doi:10.1016/j.jbiosc.2014.05.019

Takao, Y., Takahashi, T., Yamada, T., Goshima, T., Isogai, A., Sueno, K., Fujii, T., & Akao, T. (2018). Characteristic features of the unique house sake yeast strain *Saccharomyces cerevisiae* Km67 used for industrial sake brewing. *Journal of Bioscience and Bioengineering*, *126*(5), 617–623. doi:10.1016/j.jbiosc.2018.05.008.

Tanaka, J., Sugawara, M., Kuribayashi, T., Sato, K., Nabekura, Y., & Aoki, T. (2023). Study on improvement of Niigata sake yeast -Isolation of a no-urea-producing sake yeast with high ethyl caproate productivity-. *Quest for Traditional Food*, *50*, 55–60.

Watanabe, D., Araki, Y., Zhou, Y., Maeya, N., Akao, T., & Shimoi, H. (2012). A loss-of-function mutation in the PAS kinase Rim15p is related to defective quiescence entry and high fermentation rates of *Saccharomyces cerevisia*e sake yeast strains. *Applied and Environmental Microbiology*, *78*(11), 4008–4016. doi:10.1128/AEM.00165-12.

Watanabe, D. (2012). How has sake yeast acquired high alcohol fermentation ability ? *KAGAKU TO SEIBUTSU*, *50*(10), 723–729. doi:10.1271/kagakutoseibutsu.50.723.

Watanabe, D., & Takagi, H. (2016). *RIM15*, a kyokai sake yeast-specific mutated gene associated with the high alcoholic fermentation performance. *Journal of the Brewing Society of Japan*, *111*(10), 638–647. doi:10.6013/jbrewsocjapan.111.638.

Yoshizawa, K. (1999). Sake: production and flavor. *Food Reviews International*, *15*(1), 83–107. doi:10.1080/87559129909541178.

Zhang, R., Zhu, Z., Zhu, H., Nguyen, T., Yao, F., Xia, K., Liang, D., & Liu, C. (2005). SNP Cutter: a comprehensive tool for SNP PCR–RFLP assay design. *Nucleic acids research*, *33*(suppl_2), W489-W492. doi:10.1093/nar/gki358.

Figure legends

Fig. 1. Mismatch PCR-RFLP assay for the identification of the *RIM15*ins5067A mutation. A: Single nucleotide insertion (A) at position 5067 of the *RIM15* gene of the Kyokai no. 7 strain (highlighted in black). The PCR-RFLP method uses a reverse primer (P2) with two mismatches (indicated by asterisks [*]) that create a CspCI restriction site (displayed in a gray box and highlighted in gray in the *RIM15* sequence) when the *RIM15*ins5067A mutation is present. At bottom right, gel electrophoresis analysis of PCR-RFLP; lane 1, PCR amplicons of laboratory yeast strain S288C; lane 2, PCR products from lane 1 after digestion with CspCI; lane 3, PCR amplicons of the sake yeast strain Kyokai no. 7; lane 4, PCR products from lane 3 after fragmentation with CspCI; M represents the Gene Ladder 100 (Nippon Gene, Tokyo, Japan).

Fig. 2. Mismatch PCR-RFLP pattern for the house yeast strain YS4 isolated from Yoshinogawa in Niigata prefecture, Japan. Agarose gel electrophoresis analysis of PCR-RFLP: lane 1, PCR amplicons of sake yeast Kyokai no. 7; lane 2, PCR products from lane 1 after fragmentation with CspCI; lane 3, PCR amplicons of the house yeast strain YS4; lane 4, PCR fragments from lane 3 after digestion with CspCI; M represents the Gene Ladder 100 (Nippon Gene, Tokyo, Japan).

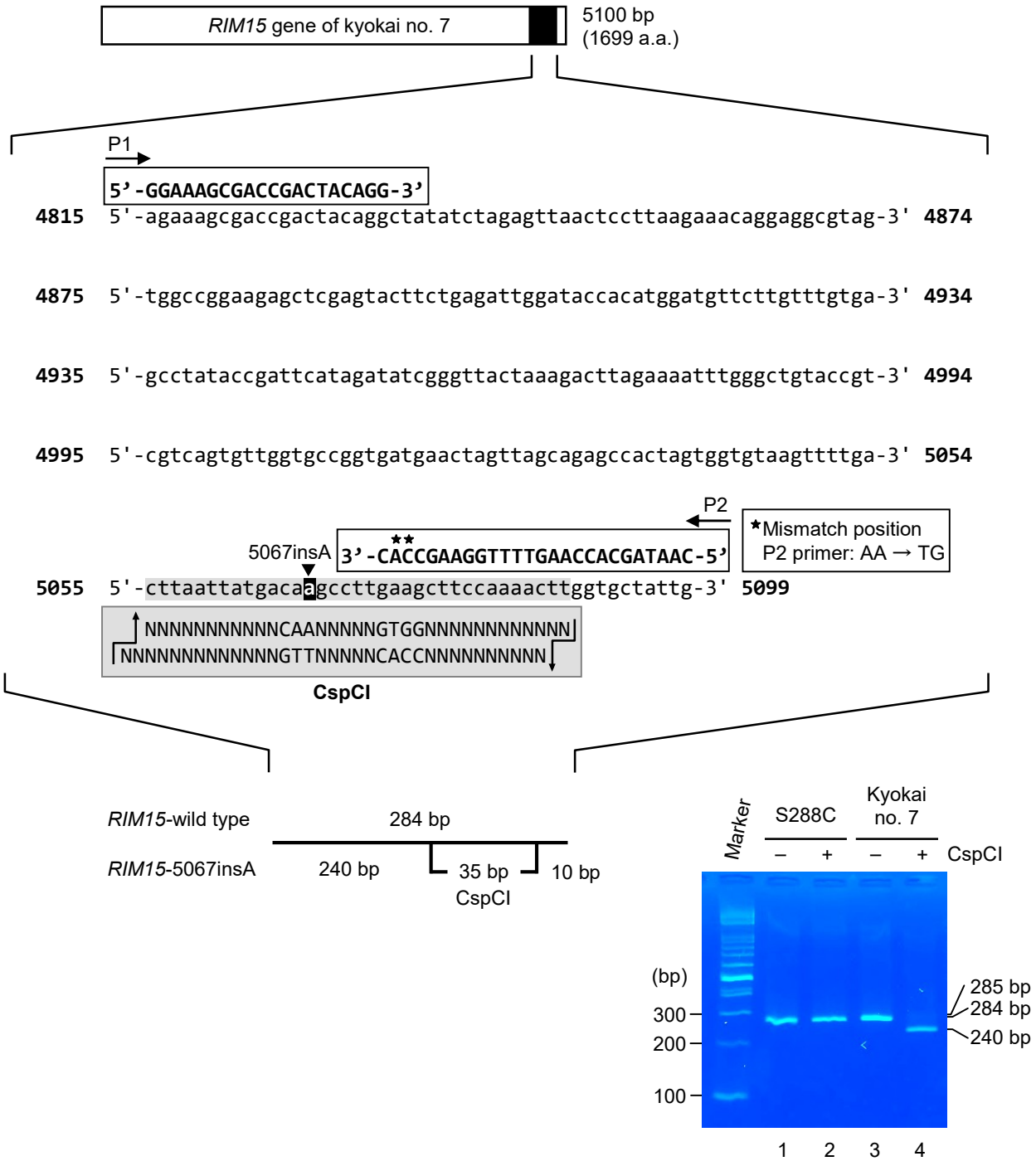
Supplementary Fig. S1. Alignment of nucleotide sequences from the PCR product of the mismatch PCR-RFLP assay.

Table 1. Specificity of PCR-RLFP analysis to detect *RIM15* gene and a single nucleotide insertion in *RIM15* across different yeast strains.

No.	Yeast strain	PCR-RFLP method[a]	Insertion of a single nucleotide in *RIM15* as the target for PCR-RFLP[b]
	Sake yeast		
1	Kyokai no. 1	-	-
2	Kyokai no. 2	-	-
3	Kyokai no. 3	-	-
4	Kyokai no. 4	-	-
5	Kyokai no. 5	-	-
6	Kyokai no. 6	+	A
7	Kyokai no. 7	+	A
8	Kyokai no. 8	-	-
9	Kyokai no. 9	+	A
10	Kyokai no. 10	+	A
	Shochu yeast		
11	S-2	-	-
12	SH-4	-	-
	Wine yeast		
13	KW-1	-	-
	Laboratory yeast		
14	S288C	-	-

[a] Marks: +, cutting of PCR product with CspCI; -, no-cutting of PCR product CspCI.

[b] Marks: A, adenine; -, no insertion.



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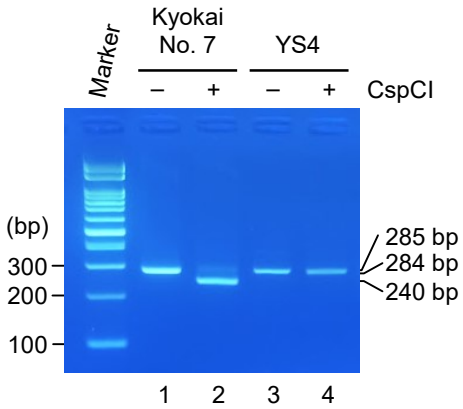

Fig.2 Kuribayashi et al.

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                     *        20         *        40         *        60         *        80
Kyokai no.7 : GGAAAGCGACCGACTACAGGCTATATCTAGAGTTAACTCCTTAAGAAACAGGAGGCGTAGTGGCCGGAAGAGCTCGAGTA : 80
YS4         : GGAAAGCGACCGACTACAGGCTATATCTAGAGTTAACTCCTTAAGAAACAGGAGGCGTAGTGGCCGGAAGAGCTCGAGTA : 80

                     *       100         *       120         *       140         *       160
Kyokai no.7 : CTTCTGAGATTGGATACCACATGGATGTTCTTGTTTGTGAGCCTATACCGATTCATAGATATCGGGTTACTAAAGACTTA :160
YS4         : CTTCTGAGATTGGATACCACATGGATGTTCTTGTTTGTGAGCCTATACCGATTCATAGATATCGGGTTACTAAAGACTTA :160

                     *       180         *       200         *       220         *       240
Kyokai no.7 : GAAAATTTGGGCTGTACCGTCGTCAGTGTTGGTGCCGGTGATGAACTAGTTAGCAGAGCCACTAGTGGTGTAAGTTTTGA :240
YS4         : GAAAATTTGGGCTGTACCGTCGTCAGTGTTGGTGCCGGTGATGAACTAGTTAGCAGAGCCACTAGTGGTGTAAGTTTTGA :240

                     *       260         *       280
Kyokai no.7 : CTTAATTATGACAAGCCTTGTGGCTTCCAAAACTTGGTGCTATTG :285
YS4         : CTTAATTATGACA-GCCTTGTGGCTTCCAAAACTTGGTGCTATTG :284
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Supplementary Fig.S1 Kuribyashi et al.