A genotyping method for discrimination between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* from natural environment

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Abstract

In this study, we developed a straightforward technique for differentiating between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*, thereby facilitating the isolation of *S. cerevisiae* from natural environments. Our findings suggest that this method is easy, quick, and beneficial for producing fermented foods and beverages using wild *S. cerevisiae*.

Keywords

screening; *Saccharomyces cerevisiae*; *Saccharomyces paradoxus*

Saccharomyces cerevisiae is generally used for fermenting foods and alcoholic beverages (Kitagaki and Kitamoto, 2013). *S. cerevisiae*, a budding yeast, significantly influences the aroma and taste of foods and beverages. Historically, industrial brewing yeasts have been domesticated using naturally occurring wild yeasts. However, in recent years, the use of native yeasts for sake brewing has attracted consumers' attention because wild yeasts can produce sake with a flavor different from that produced by industrial brewing yeasts. Indeed, wild *S. cerevisiae* has been isolated from the wild environment, and *Saccharomyces paradoxus* has been obtained at the same time (Dashko et al., 2016).

S. paradoxus is a wild yeast, which is closely related to *S. cerevisiae* (Nikulin et al., 2020). It predominantly resides in natural habitats such as forest bark and soil. Unlike *S. cerevisiae*, which is used for producing bread and sake, *S. paradoxus* is believed to have remained undomesticated by humans. This yeast species is widely distributed in forests across North America (Sniegowski et al., 2002), Europe (Sampaio and Gonçalves, 2008), Japan, and other regions. Genetically, it bears a strong resemblance to *S. cerevisiae*; however, it exhibits variations in fermentation capabilities and sugar assimilation. Consequently, it is rarely employed in producing fermented foods such as beer or bread.

Because of numerous similarities between *S. cerevisiae* and *S. paradoxus*, they are typically indistinguishable when assessed using conventional taxonomic approaches. These approaches emphasize the phenotypic characteristics, including the morphology of cells, spores, and asci. Various identification techniques utilizing molecular biology have been documented (Fernández-Espinar et al., 2006). Nonetheless, these techniques have been applied to identify different *S. cerevisiae* subspecies, and are not specifically tailored to detect *S. cerevisiae* coexisting with *S. paradoxus*. In this study, we developed a straightforward method for differentiating between *S. cerevisiae* and *S. paradoxus* for isolating *S. cerevisiae* from natural environments.

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is a reliable technique for rapidly and precisely identifying single nucleotide polymorphisms (SNPs) that result in distinct restriction sites (Hashim and Al-Shuhaib, 2019). In developing this assay, we focused on identifying SNP variations in the 26S rDNA D1/D2 sequence using the universal primers NL1 and NL4 (DDBJ/EMBL/GenBank database accession number LC797988 for *S. cerevisiae*, and NG_055028 for *S. paradoxus*). By analyzing these reference sequences, two of six SNPs were chosen. The nucleotide sequence at position 463 in this segment of *S. cerevisiae* includes the MslI restriction sites that are absent in *S. paradoxus*. This assay aimed at a restriction site for MslI within the PCR amplicon when template DNA from *S. cerevisiae* was used, producing 461- and 111-bp fragments (Fig. 1). Conversely, in the *S. paradoxus* gene lacking the restriction site at the same position, an undigested 572-bp product was obtained. Meanwhile, for *S. paradoxus*, the sequence at position 485 in the NL1–NL4 product contained a restriction site for Hpy188III, which was absent in *S. cerevisiae*. Therefore, PCR–RFLP targeted a restriction site for Hpy188III within the PCR amplicon when the template DNA from *S. paradoxus* was used, resulting in 480- and 92-bp fragments (Fig. 2). However, in the *S. cerevisiae* gene lacking the restriction site at the corresponding position, an undigested 572-bp product was obtained.

To confirm the effectiveness of these PCR–RFLP assays, we used a *S. cerevisiae* strain S288C (Open Biosystems, Huntsville, AL, USA), and a *S. paradoxus*-type strain NBRC

00010609 (Biological Resource Center, NITE). PCR amplification was performed in a 50 μL reaction mixture comprising 25 μL of a Takara SapphireAmp Fast PCR Master Mix (Takara Bio, Shiga, Japan), 0.4 μM of each primer, and 20 ng genomic DNA extracted from yeast cells. The PCR protocol involved 33 cycles of denaturation at 98 °C for 5 s, annealing at 55 °C for 5 s, and extension at 72 °C for 5 s. PCR products were isolated using a High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland), purified (45 μL) using a 10× rCutSmart Buffer (5 μL) and MslI or Hpy188III (1 μL) (New England Biolabs, Ipswich, MA, USA), followed by a 30-min incubation at 37 °C. The samples were subsequently subjected to electrophoresis on 3% agarose gels (Agarose S; Nippon Gene, Tokyo, Japan) for 30 min at 100 V and visualized using Novel Juice (Bio-Helix, Keelung City, Taiwan) staining.

The PCR product from *S. cerevisiae* was digested by MslI into a 461-bp fragment, whereas the *S. paradoxus* amplicon (572 bp) was not digested by MslI (Fig. 1). The 572-bp PCR product from *S. cerevisiae* remained intact when exposed to Hpy188III, whereas the *S. paradoxus* amplicon was cleaved by Hpy188III into a 480-bp fragment (Fig. 2). DNA sequencing analyses confirmed these differences. This straightforward and practical technique will be beneficial for distinguishing between *S. cerevisiae* and *S. paradoxus* during fermentation of foods and beverages using wild *S. cerevisiae* in a natural environment.

Disclosure

The authors declare no conflict of interest. All experiments complied with the current laws of the country, in which they were performed. This study was presented orally at the 69th Annual Meeting of the Mycological Society of Japan (E-12; 18th, May, 2025).

Acknowledgments

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Figure legends

Fig. 1. PCR–RFLP assay for identifying *S. cerevisiae*. Gel electrophoresis analysis of PCR amplicons in the PCR–RFLP assay at the bottom left; lane 1, laboratory yeast strain S288C, *S. cerevisiae*; lane 2, PCR products of lane 1 digested with MslI; lane 3, *S. paradoxus*; lane 4, PCR products of lane 3 digested with MslI; M, Violamo DNA Ladder Marker (Violamo; AS ONE, Osaka, Japan).

Fig. 2. PCR–RFLP assay for *S. paradoxus* identification. Gel electrophoresis analysis of PCR amplicons in the PCR-RFLP assay at the bottom left; lane 1, laboratory yeast strain S288C, *S. cerevisiae*; lane 2, PCR products of lane 1 digested with Hpy188III; lane 3, *S. paradoxus*; lane 4, PCR products of lane 3 digested with Hpy188III; M, Violamo DNA Ladder Marker (Violamo; AS ONE, Osaka, Japan).

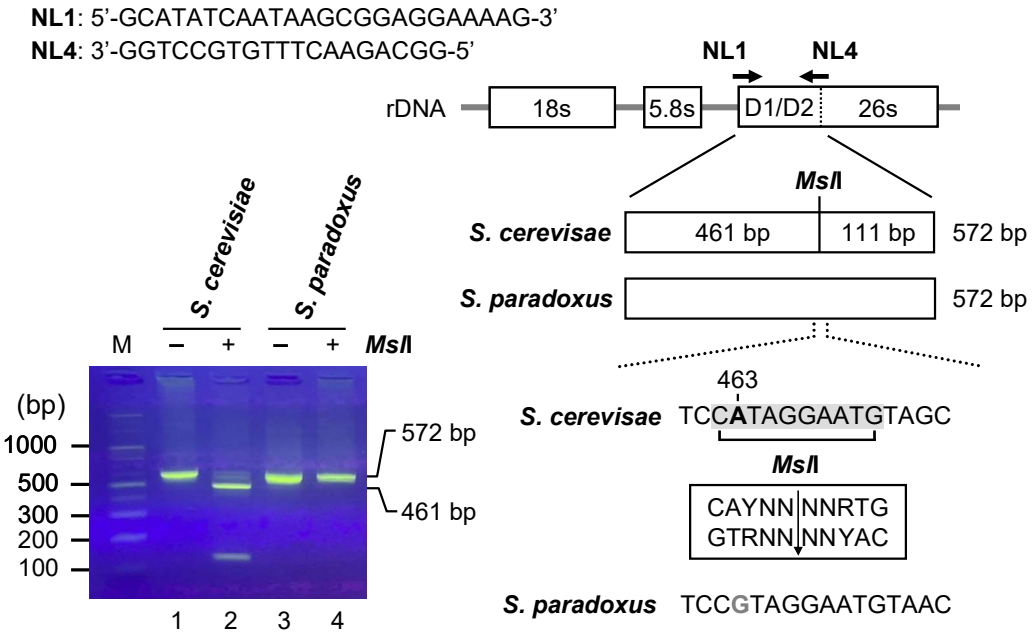

Figure 1 F. Sawaguchi

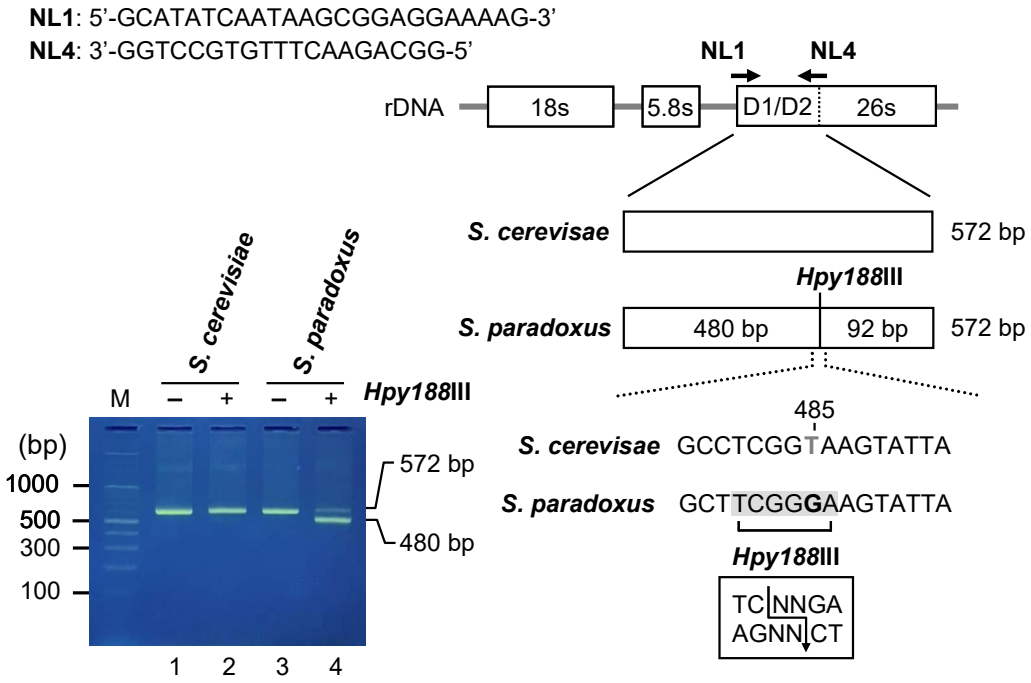

Figure 2 F. Sawaguchi