Short Communication

Phylogenetic position of the yeast strain Saccharomycodes sinensis IFO10111T

Toyohiko Yamazaki,∗ Youji Nakagawa, Masayuki Hayakawa, and Yuzuru Iimura

Department of Applied Chemistry and Biotechnology, Faculty of Engineering, Yamanashi University,
Kofu 400–8511, Japan

(Received November 11, 2002; Accepted October 13, 2004)

Key Words—asccospore formation; Cladogram tree; Saccharomycodaceae; Saccharomyces sinensis
IFO10111T; 26S rDNA D1/D2; 5.8S rDNA

The original strain (HS506) of Saccharomyces sinensis Yue (1977), isolated from soil in the forest of Mount Chienfeng at Hainan Island in China, had been deposited as Sd. sinensis NRRL Y-12797T, NRRL Y-17406, CBS7075T, IFO10111T, and CCRC22324. Kurtzman and Robnett (1998) demonstrated that the nucleotide sequences of Sd. sinensis (NRRL Y-12797T and CBS7075T) and Nadsonia fulvescens var. elongata CBS2594T are identical in their D1/D2 regions (569 nucleotides). On the basis of this result, Miller and Phaff (1998a) described Sd. sinensis Yue as a synonym of N. fulvescens var. elongata in THE YEASTS, A Taxonomic Study, 4th ed. However, they also stated, “We were unable to detect ascospore formation” [of Sd. sinensis], and “We believe that further studies are required to place this species in an appropriate genus” (Miller and Phaff, 1998b).

In the present study we subjected Sd. sinensis IFO10111T to a rDNA (the D1/D2 and the 5.8S/ITS regions) sequencing study, as well as a detailed phenotypic analysis, and investigated the phylogenetic position of Sd. sinensis IFO10111T.

Strains used in this study are shown in Table 1. Morphological and physiological characteristics for identification of Sd. sinensis IFO10111T were examined with the procedures described by Yarrow (1998). The vegetative cells stored at −80°C in 10% glycerol were cultivated on YM agar medium [Difco] at 30°C for 3 days. The cultivated cells were incubated at 25°C for 14 days on Gorodkowa agar medium for sporulation. Samples for SEM were prepared according to the manufacturer’s instructions (Jeol, Tokyo, Japan). The cells coated with pt-pd for 250 s in a JEC-1100 ion sputtering device were examined in a JSM T100 electron microscope (Jeol). In the typical bipolar budding cells of Sd. sinensis IFO10111T, a few budscars were observed in a concentric circle (Fig. 1A). These vegetative cells produced ascospores without the conjugation of two cells (Fig. 1B). In particular, the freezing conservation technique was useful for the sporulation of this strain, because its sporulation was not detected in the cells that had been stored at 4°C.

The diagnostic physiological properties of Sd. sinensis IFO10111T, as shown in Table 2, were consistent with the descriptions (Yue, 1977) of the original strain.

* Address reprint requests to: Dr. Toyohiko Yamazaki, Department of Applied Chemistry and Biotechnology, Faculty of Engineering, Yamanashi University, 4 Takeda, Kofu 400–8511, Japan.
E-mail: tyamazak@ab11.yamanashi.ac.jp

Abbreviations: CBS, Centraalbureau voor Schimmelcultures, Delft/Baam, The Netherlands; CCRC, Culture Collection and Research Center, Food Industry Research & Development Institute, Hsinchu, Taiwan; IFO, Institute for Fermentation, Osaka, Japan; NRRL, ARS Culture Collection, Northern Regional Research Center, US Department of Agriculture, Peoria, USA; D1/D2, D1/D2 domain at the 5’ end of large subunit (26S) rDNA; and 5.8S/ITS, 5.8S rDNA and two ITSs.
Sd. sinensis HS506, but were distinguishable from those (Miller and Phaff, 1998a) of N. fulvescens var. elongata CBS2594T as follows: galactose fermentation and assimilation, positive; casamino acids assimilation, positive; sorbose and ammonium sulfate assimilation, negative; and growth at 30°C, positive. Besides neither strain was able to utilize sucrose, maltose, lactose, raffinose or potassium nitrate (data not shown in Table 2). Of these diagnoses described above, the "ascus formation with the conjugation of cells" and the "non-growth at 30°C" are the best for the genus Nadsonia, while the "failure to grow in the presence of ammonium sulfate" is the best for Sd. sinensis HS506, because this characteristic has not been observed except in the genus Saccharomycopsis (Van der Walt, 1970). From these physiological and morphological properties, it was revealed that Sd. sinensis IFO10111T is identical to the original strain Sd. sinensis HS506 and distant from the genus Nadsonia. We then confirmed that these two taxa should be assigned to the same clade, Saccharomycodaceae: an ascospore-forming apiculate yeast family, the vegetative cells of which are reproduced by typical bipolar bud-fission.

We analyzed also the rDNA sequence of Sd. sinensis IFO10111T. General treatments of DNA molecules were carried out according to standard protocols (Sambrook et al., 1989). The D1/D2 regions were amplified by the colony direct PCR method (Ling et al., 1995), in a TaKaRa PCR Thermal Cycler PERSONAL, with TaKaRa Ex Taq™ polymerase (TaKaRa, Shiga, Japan) and two primers (NL1F: 5'/H11032- GCATATCAATAAGCGAGGAAAG-3' and NL4R: 5'/H11032- GGTCCTGGTTTCAAGACGG-3'). The following internal primers were used if needed: 26S2R (5'-CGATATCAATAAGCGAGGAAAG-3') and 26S3F (5'-GCAGAGACCGTATGCCGAACA-3'). After 5 min at 95°C, the reaction mixture was subjected to 35 cycles, with de-
naturation at 94°C for 1 min, annealing at 52°C for 2 min, and extension at 72°C for 1 min. The amplified fragments were purified with a SUPREC™ PCR Kit (TaKaRa), and sequenced directly with a CEQ Dye Terminator Cycle Sequencing Quick Start Kit, following the manufacturer’s instructions, in a CEQ™ 2000XL DNA Analysis System (Beckman-Coulter, Fullerton, CA, USA). These sequence procedures were performed three times for each strain. The nucleotide sequences determined in this study have been deposited with the DNA Data Bank of Japan (DDBJ) under the accession numbers shown in Table 1. The determined nucleotide sequences of each strain were aligned with the GENETYX-MAC program (Software Development, Tokyo, Japan). A cladogram tree was constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) on the multialignment program CLUSTAL W 1.75 (Thompson et al., 1994). The evolutionary distances were calculated by the PHYLIP 3.57c program DNADIST (Felsenstein, 1995), with Kimura’s two-parameter model. We evaluated the robustness of the branches in the tree by performing a bootstrap analysis (Felsenstein, 1985) with 1,000 replicates. In the sequence analyses of the 5.8S/ITS regions, the procedures were virtually identical with those described above, except for the following: primers, ITS1F (5’-TC-CTTATTTGATATGC-3’) and ITS4R (5’-AAAAC—5.8S rDNA—5’-TTGA—26S rDNA), and is shown in Table 1.

The sequenced D1/D2 region of Sd. sinensis IFO10111T (AB127389) was aligned with those of the reference strains, and compared pairwise. The nucleotide divergences of Sd. sinensis IFO10111T from Sd. sinensis NRRL Y-12797T (U94946) and from N. fulvescens var. elongata IFO0665 (AB127390), respectively, were 17.9% (106/592). This result exceeds

#### Table 2. Diagnostic phenotypes for identification of Sd. sinensis IFO10111T.

<table>
<thead>
<tr>
<th></th>
<th>Sd. sinensis¹ IFO10111T</th>
<th>Sd. sinensis² HS506</th>
<th>N. ful. var. elongata³ CBS2594T</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell shape</td>
<td>lemon shaped</td>
<td>lemon shaped</td>
<td>lemon shaped</td>
</tr>
<tr>
<td>Cell size (µm)</td>
<td>2.8–5.0×3.9–7.8</td>
<td>2.6–5.9×3.3–9.7</td>
<td>5–8×8–16</td>
</tr>
<tr>
<td>Vegetative reproduction</td>
<td>bipolar bud-fission</td>
<td>bipolar bud-fission</td>
<td>bipolar bud-fission</td>
</tr>
<tr>
<td>Ascus formation</td>
<td>without*</td>
<td>without*</td>
<td>with*</td>
</tr>
<tr>
<td>Ascospore surface</td>
<td>smooth</td>
<td>smooth</td>
<td>warty, brownish</td>
</tr>
<tr>
<td>No. of ascospores per ascus</td>
<td>1–2</td>
<td>1–2 (rarely 4)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Physiology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fermentation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>Assimilation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sorbose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 30°C</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

¹ This study. ² Original descriptions (Yue, 1977). ³ Miller and Phaff (1998a).

* Conjugation of cells prior to sporulation.

Symbols: +, positive; –, negative.
1% remarkably, a value suggested by Kurtzman and Robnett (1998) to be the borderline of species separation. Between two strains, IFO0665 (AB127390) and CBS2594T (U94942), of *N. fulvescens* var. *elongata*, in this connection, their nucleotide sequences were identical. These results substantiate that *Sd. sinensis* IFO10111T is not a synonym of *N. fulvescens* var. *elongata*, as do those results from the phenotypic diagnoses described above. Consequently, we cannot readily accept that the generic position of *Sd. sinensis* Yue should be assigned to the genus *Nadsonia* (Miller and Phaff, 1998a). We, however, reconfirmed that each taxon is a member of the Saccharomycodaceae family that is now recognized in yeast systematics.

Thereupon we investigated the phylogenetic position of *Sd. sinensis* IFO10111T in order to reestablish it in an appropriate genus within the family Saccharomycodaceae. From the known genera *Saccharomycodes*, *Nadsonia*, *Hanseniaspora*, and *Wickerhamia* within the family, the five reference strains listed in Table 1 and their related strains were used. To confirm the reliable dendrogram construction, if needed, we retrieved the sequence data of each strain by searching the DDBJ database.

The constructed cladogram tree (Fig. 2) showed that the phylogenetic position of *Sd. sinensis* IFO10111T is completely separated from any of those reference genera, with a bootstrap value of 995 for the D1/D2 region.

To verify the phylogenetic position of *Sd. sinensis* IFO10111T based on the D1/D2 sequence analysis, we then sequenced the 5.8S/ITS regions, because these regions had not been determined yet in this strain. The divergence in size (155 to 157 bp) of the aligned 5.8S rDNA regions of those reference strains from *Sd. sinensis* IFO10111T was far less than that (409 to 660 bp) of the 5.8S/ITS regions (Table 1). This result suggests that the 157 bp fragment of the 5.8S rDNA is a highly conserved region. Kurtzman and Robnett (2003) used a dataset containing the 5.8S/alignable ITS sequences in order to resolve 75 species of the "Saccharomyces complex" into 14 clades, five of which were proposed as new genera (Kurtzman, 2003). The alignable ITS regions among the strains used in this study were scarcely observed (data not shown). Thus we analyzed the 5.8S rDNA sequences in order to resolve the phylogenetic relationships of *Sd. sinensis* IFO10111T with each reference strain.

The 5.8S regions of the five reference strains had between six and 11 (3.8 to 7.0%) nucleotide substitutions with that of *Sd. sinensis* IFO10111T. Mitchell et al. (1992) demonstrated that each genus among the basidiomycetous yeast genera *Cystofilobasidium*, *Filobasidium*, and *Filobasidiella* is separated from any of the others by at least three (2.1%) nucleotides in a 143 bp fragment containing the relatively conserved 5.8S rDNA. Accordingly, our results strongly indicate the generic separation between *Sd. sinensis* IFO10111T and those reference strains.

The constructed 5.8S tree (data not shown), as well as the D1/D2 tree, showed that the phylogenetic position of *Sd. sinensis* IFO10111T is completely separated from any of those reference genera, with a bootstrap value of 979. These phylogenetic relationships were invariable in any cases where the sequences of other related strains were added in the tree, and were confirmed by the unweighted pair group method with averages (UPGMA). Therefore, our phylogenetic analyses on the basis of the D1/D2 and the 5.8S rDNA sequences provide no support for including *Sd. sinensis* IFO10111T in any of the known genera, *Saccharomycodes*, *Nadsonia*, *Hanseniaspora*, and *Wickerhamia*, within the family Saccharomycodaceae.

Our results for *Sd. sinensis* IFO10111T add strong support for Yamada et al.'s (1992) proposal that "[From the 18S rRNA sequencing,] a separate genus..."
can be set up for *Sd. sinensis* [IFO10111T]." In order to reinvestigate the systematic position of *Sd. sinensis* Yue, however, the identity of the culture collections deposited as *Sd. sinensis*, HS506, IFO10111T, NRRL Y-12797T, NRRL Y-17406, CBS7075T, and CCRC22342, need to be verified, as a matter of priority.

In conclusion, *Saccharomycodes sinensis* IFO10111T is naturally assigned to the family Saccharomycodaceae, but is phylogenetically independent from any of the known genera within the family.

**Acknowledgments**

We are indebted to Dr. Satoshi Harashima and Dr. Yoshinobu Kaneko, Osaka University, Osaka, Japan, for useful advice on phylogenetic analyses, and Hideki Yamamura, Yamanashi University, Kofu, Japan, for technical advice on DNA sequencing analyses.

**References**


