Reclassification of the *Sporobolomyces roseus* and *Sporidiobolus pararoseus* complexes, with the description of *Sporobolomyces phaffii* sp. nov.

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More than 50 ballistoconidium-forming yeast strains, isolated from plant leaves collected in Yunnan, China, were identified as *Sporobolomyces roseus* Kluiver & van Niel by conventional methods. However, comparison of the internal transcribed spacer (ITS) region and 26S rDNA D1/D2 domain sequences indicated that these strains represented more than one species. Type or authentic strains of the synonyms of *Sporobolomyces roseus* and the closely related species *Sporidiobolus pararoseus* Fell & Tallman were employed in the rDNA sequence comparison. *Sporobolomyces boleticola* Ramírez, *Sporobolomyces pollaccii* Verona & Ciferri, *Sporobolomyces roseus* var. *madurae* Janke and *Torulopsis somala* Verona were confirmed to be conspecific with *Sporobolomyces roseus*. Another synonym of this species, *Sporobolomyces salmonaeus* Derx, was located together with *Sporobolomyces marcillae* Santa Maria in a separate clade. Two synonyms of *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* Yamasaki & Fuji (nom. inval.) and *Sporobolomyces japonicus* Iizuka & Goto, were revealed to represent two distinct species. The name *Sporobolomyces carnicolor* is validated, with strain CBS 4215T as the type strain. A novel species represented by five of the selected Yunnan strains was confirmed, for which the name *Sporobolomyces phaffii* sp. nov. is proposed (type strain CH 2.052T = AS 2.2137T = JCM 11491T = CBS 9129T). This study also indicates that yeast species with similar ITS sequences may have quite different D1/D2 sequences.

**Keywords:** *Sporobolomyces phaffii* sp. nov., *Sporobolomyces roseus*, *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor*, *Sporobolomyces japonicus*

**INTRODUCTION**

*Sporobolomyces roseus* Kluyster & van Niel is a common ballistoconidium-forming yeast species and occurs in many different habitats, but most frequently in the phyllosphere (Derx, 1930; Tubaki, 1953; Last, 1955; Nakase, 2000). According to the recent taxonomic system of basidiomycetous yeasts (Boekhout & Nakase, 1998), more than 50 ballistoconidium-forming yeast strains isolated from various wilting leaves collected in Yunnan, China, in 1996 were identified as *Sporobolomyces roseus* by conventional methods. However, these strains varied to different degrees in carbon and nitrogen compound assimilation patterns. Comparison of the internal transcribed spacer (ITS) region and 26S rDNA D1/D2 domain sequences of representatives of these strains showed that more than one species existed among them. Therefore, the species circumscription of *Sporobolomyces roseus* should be redefined.

The authentic strains of two synonyms of *Sporobolomyces roseus* were used in this study: *Sporobolomyces boleticola* Ramírez, *Sporobolomyces pollaccii* Verona & Ciferri, *Sporobolomyces roseus* var. *madurae* Janke and *Sporobolomyces carnicolor* Yamasaki & Fuji. These synonyms were shown to be conspecific with *Sporobolomyces roseus*. Another synonym of this species, *Sporobolomyces salmonaeus* Derx, was located together with *Sporobolomyces marcillae* Santa Maria in a separate clade. Two synonyms of *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* Yamasaki & Fuji (nom. inval.) and *Sporobolomyces japonicus* Iizuka & Goto, were revealed to represent two distinct species. The name *Sporobolomyces carnicolor* is validated, with strain CBS 4215T as the type strain. A novel species represented by five of the selected Yunnan strains was confirmed, for which the name *Sporobolomyces phaffii* sp. nov. is proposed (type strain CH 2.052T = AS 2.2137T = JCM 11491T = CBS 9129T). This study also indicates that yeast species with similar ITS sequences may have quite different D1/D2 sequences.
myces roseus, Sporobolomyces ruberrimus Yamasaki & Fuji var. ruberrimus (CBS 7500) and Sporobolomyces ruberrimus var. albus Yamasaki & Fuji (CBS 7253, CBS 7501), have been shown to represent a distinct species by D1/D2 domain sequence analysis (Fell et al., 2000). The type or authentic strains of the remaining synonyms of Sporobolomyces roseus were employed in the present study, when available from culture collections. Since Sporobolomyces roseus is phenotypically similar and phylogenetically closely related to Sporobolomyces shibatanus (Okunuki) Verona & Ciferri, the anamorph of Sporidiobolus pararoseus Fell & Tallman (Boekhout, 1991; Boekhout & Nakase, 1998; Hamamoto & Nakase, 2000), the type strains of the synonyms of the latter species were also included. The taxonomic status of these taxa was clarified by ITS region and D1/D2 domain sequence analysis. The taxonomic positions of representative Yunnan strains were determined and a hitherto undescribed yeast species was found among them. We describe the latter as Sporobolomyces phaffii sp. nov., in honour of the late Herman J. Phaff.

METHODS

Yeast strains and characterization. The yeast strains examined are listed in Table 1. The strains from Yunnan were isolated by the improved ballistoconidium-fall method (Nakase & Takashima, 1993). Type and authentic strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), The Netherlands, and the Japan Collection of Microorganisms (JCM), Japan.

Most of the morphological, physiological and biochemical characteristics were examined according to standard methods commonly employed in yeast taxonomy (Yarrow, 1998). Assimilation of nitrogen compounds was investigated on solid media with starved inocula as described by Nakase & Suzuki (1986). Vitamin requirement tests were performed according to Komagata & Nakase (1967).

Extraction, purification and identification of ubiquinones were carried out according to Nakase & Suzuki (1986). Xylose in the cell hydrolysate was analysed by HPLC as described by Suzuki & Nakase (1988).

ITS region and 26S rDNA D1/D2 domain sequencing. Nuclear DNA was extracted using the method of Makimura et al. (1994). The DNA fragment covering the ITS region and 26S rDNA D1/D2 domain was amplified with the primers ITS1 (5'-GTCGTAACAAGGTTTCCGTAGGTG-3') and NL4 (5'-GGTCGGTATTGAAGACGGG-3'). PCR was performed for 36 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1.5 min. Cycle sequencing was performed with the forward primer ITS1 and NL1 (5'-GCAATACATAAAGCGGAGAACAAG-3') and the reverse primers ITS4 (5'-TCCTCCGTTATGATGC-3') and NL4 using the ABI

Table 1. Yeast strains employed

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source/notes</th>
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<tbody>
<tr>
<td>Sporobolomyces pararoseus</td>
<td></td>
</tr>
<tr>
<td>CBS 491T</td>
<td>Soil</td>
</tr>
<tr>
<td>CBS 484</td>
<td>Type of Sporobolomyces pararoseus</td>
</tr>
<tr>
<td>Sporobolomyces blumeae JCM 10212</td>
<td>Blumea sp.</td>
</tr>
<tr>
<td>Sporobolomyces carnicolor CBS 4215</td>
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</tr>
<tr>
<td>Sporobolomyces japonicus CBS 5744</td>
<td>Oil brine</td>
</tr>
<tr>
<td>Sporobolomyces marcellae CBS 4217</td>
<td>Air</td>
</tr>
<tr>
<td>Sporobolomyces phaffii sp. nov.</td>
<td>Wilting leaf of Ehretia coryllifolia</td>
</tr>
<tr>
<td>CH 2.049</td>
<td>Wilting leaf of Nerium indicum</td>
</tr>
<tr>
<td>CH 2.052T</td>
<td>Wilting leaf of Oxytenanthera sp.</td>
</tr>
<tr>
<td>CH 2.083</td>
<td>Wilting leaf of Eriobotrya japonica</td>
</tr>
<tr>
<td>CH 2.091</td>
<td>Wilting leaf of Oryza sativa</td>
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<tr>
<td>CH 2.304</td>
<td></td>
</tr>
<tr>
<td>Sporobolomyces roseus</td>
<td></td>
</tr>
<tr>
<td>CBS 485</td>
<td>Type of Sporobolomyces pollaccii</td>
</tr>
<tr>
<td>CBS 486T</td>
<td>Type of Sporobolomyces roseus</td>
</tr>
<tr>
<td>CBS 993</td>
<td>Type of Torulopsis somala</td>
</tr>
<tr>
<td>CBS 2646</td>
<td>Type of Sporobolomyces roseus var. madurae</td>
</tr>
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<td>CBS 2840</td>
<td>Authentic strain of Sporobolomyces boeticola</td>
</tr>
<tr>
<td>CBS 2841</td>
<td>Authentic strain of Sporobolomyces boeticola</td>
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<td>CH 2.053</td>
<td>Wilting leaf of Nerium indicum</td>
</tr>
<tr>
<td>CH 2.116</td>
<td>Wilting leaf of Sapindus delavayi</td>
</tr>
<tr>
<td>CH 2.332</td>
<td>Wilting leaf of Nicotiana tabacum</td>
</tr>
<tr>
<td>Sporobolomyces ruberrimus CBS 7500</td>
<td>Air</td>
</tr>
<tr>
<td>Sporobolomyces salmones CBS 488</td>
<td>Etiolated grass</td>
</tr>
<tr>
<td>Sporobolomyces sp. CH 2.500</td>
<td>Wilting leaf of Parthenocissus sp.</td>
</tr>
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</table>
Molecular phylogenetic analysis. The sequences of the ITS regions or 26S rDNA D1/D2 domains of the strains determined in this study and the reference sequences were aligned with the program CLUSTAL X (Thompson et al., 1997) and adjusted manually. Reference sequences were obtained from DDBJ/EMBL/GenBank, where they had been deposited by other authors (Fell et al., 2000; Takashima & Nakase, 2000). Phylogenetic trees were constructed from the evolutionary distance data calculated from Kimura’s two-parameter model (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses (Felsenstein, 1985) were performed on 1000 random resamplings.

RESULTS AND DISCUSSION

Taxonomic status of the synonyms of *Sporobolomyces roseus* and *Sporidiobolus pararoseus*

Boekhout (1991) and Boekhout & Nakase (1998) listed 15 taxonomic synonyms under *Sporobolomyces roseus*. The type or authentic strains are available from culture collections for only eight of them. The sequences of the ITS regions and D1/D2 domains of these strains were determined in the present study, except for the D1/D2 sequence of the type strain of *Sporobolomyces ruberrimus*, which had been determined by Fell et al. (2000).

Among the taxonomic synonyms of *Sporidiobolus pararoseus* listed by Boekhout (1991) and Boekhout & Nakase (1998), *Sporobolomyces pararoseus* Olson & Hammer (CBS 484T, mt A1) and *Sporobolomyces ruber* Yamasaki & Fuji (nom. inval., CBS 4216, mt A2) have been confirmed to be conspecific with the former by mating compatibility and D1/D2 sequencing (Boekhout, 1991; Statzell-Tallman & Fell, 1998; Fell et al., 2000). *Sporobolomyces marcellae* Santa Maria was shown to be a distinct species by D1/D2 sequencing (Fell et al., 2000). The type or authentic strains of the remaining two synonyms of *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* Yamasaki & Fuji (nom. inval.) and *Sporobolomyces japonicus* Iizuka & Goto, were used in this study for ITS and D1/D2 sequencing. In addition, the ITS sequence of the type strain of *Sporobolomyces marcellae* was determined.

The relationships among the taxa studied are depicted by the phylogenetic trees drawn from the sequences of the ITS region (Fig. 1a) and the D1/D2 domain (Fig. 1b). *Sporidiobolus johnsonii* and *Sporidiobolus salmonicolor* were used as outgroups. The recently described species *Sporobolomyces blumeae* Takashima & Nakase (2000) formed a basal branch in both trees. The other taxa clustered around *Sporobolomyces roseus* and *Sporidiobolus pararoseus*. In the ITS tree (Fig. 1a), both clades were supported strongly (99–100%) by bootstrap analysis. In the D1/D2 tree (Fig. 1b), the *Sporobolomyces roseus* clade was strongly supported (bootstrap value 100%), but the *Sporidiobolus pararoseus* clade was not (bootstrap value < 50%).

Among the synonyms of *Sporobolomyces roseus*, *Sporobolomyces boleticola* Ramirez, *Sporobolomyces pollaccii* Verona & Ciferri, *Sporobolomyces roseus* var. *madurae* Janke and *Torulopsis somala* Verona were confirmed to be conspecific with *Sporobolomyces roseus*, because they have ITS and D1/D2 sequences that are identical or very similar (only one nucleotide substitution) to those of the type strain of *Sporobolomyces roseus*.

*Sporobolomyces salmoneus* Derx, a synonym of *Sporobolomyces roseus*, was clearly separated from the *Sporobolomyces roseus* clade in both trees, and clustered together with *Sporobolomyces marcellae* in the *Sporidiobolus pararoseus* clade. The type strains of *Sporobolomyces salmoneus* and *Sporobolomyces marcellae* have identical D1/D2 sequences and differ by only 1 nt in their ITS regions.

Two synonyms of *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* and *Sporobolomyces japonicus*, respectively differed from the type strain by 19–24 and 7–8 nt in the ITS region and D1/D2 domain. They also differed from one another remarkably (Fig. 1). *Sporobolomyces carnicolor* and *Sporobolomyces japonicus* should therefore be reinstalled as two distinct species. *Sporobolomyces carnicolor* was proposed by Yamasaki & Fujii (1950) without a Latin description or type designation, and therefore needs to be validated.

Taxonomic status of the *Sporobolomyces roseus* strains from Yunnan

A total of 670 yeast strains were isolated from 43 wilting leaf samples collected in Yunnan, China, in 1996 using the improved balistocandidium-fall method (Nakase & Takashima, 1993). Approximately 100 balistocandidium-forming yeast strains were initially selected for phenotypic characterization. Of them, a total of 55 strains were identified as *Sporobolomyces roseus* according to Boekhout (1991) and Boekhout & Nakase (1998). Of nine representative strains (Table 1) selected for ITS sequencing, strains CH 2.053, CH 2.116 and CH 2.332 were confirmed to belong to *Sporobolomyces roseus* (Fig. 1a). The sequence of strain CH 2.500 differed from that of the type by 5 nt (1.4%).

The other five strains, CH 2.049, CH 2.052, CH 2.083, CH 2.091 and CH 2.304, had identical ITS sequences and the sequence differed from that of the type strain of *Sporobolomyces ruberrimus* by 3 nt (1.0%). Previous studies on basidiomycetous yeasts have indicated that conspecific strains usually have less than 1% nucleotide divergence in the ITS1 and ITS2 regions overall (Sugita et al., 1999a, b; Takashima & Nakase, 2000). However, Bai et al. (2001a, b) found that conspecific strains might have up to 5–7 nt differences (approx. 2%) in the ITS regions. Therefore, the taxonomic relationships of these five strains with
Sporobolomyces ruberrimus and that of strain CH 2.500 with Sporobolomyces roseus were examined further by D1/D2 sequencing.

Strains CH 2.052, CH 2.083, CH 2.091 and CH 2.304 had identical D1/D2 sequences and differed from CH 2.049 by only 1 nt. They differed from Sporobolomyces ruberrimus in as many as 18–19 nt (3–0%) in the D1/D2 domain (Fig. 1b). These results indicate that these strains represent a distinct species, for which the name Sporobolomyces phaffii sp. nov. is proposed.

Strain CH 2.500 differed from the type strain of Sporobolomyces roseus by 3 nt in the D1/D2 domain, suggesting that this strain probably represents a distinct species. A definite taxonomic decision for strain CH 2.500 should be supported by additional data, for example, DNA–DNA reassociation, and perhaps by the identification of additional strains.

The significance of phenotypic and phylogenetic comparison

The novel species Sporobolomyces phaffii is indistinguishable from Sporobolomyces roseus by conventional characterization. The three species Sporobolomyces carnicolor, Sporobolomyces japonicus and Sporidiobolus pararoseus also have almost identical phenotypes. Sporobolomyces roseus and Sporobolomyces phaffii can be differentiated from the latter three species in their ability to assimilate nitrate and nitrite. Though the type strains of Sporobolomyces salmoneus and Sporobolomyces marcellae have identical D1/D2 sequences and very similar ITS sequences, their nitrogen assimilation patterns are completely different. Sporobolomyces salmoneus can utilize nitrate, nitrite and ethylamine hydrochloride as sole sources of nitrogen, whereas Sporobolomyces marcellae cannot. This explains why the former was previously regarded...
as a synonym of *Sporobolomyces roseus* and the latter a synonym of *Sporidiobolus pararoseus* (Boekhout, 1991). The taxonomic relationship between *Sporobolomyces salmonensis* and *Sporobolomyces marcillae* should be studied further.

The relationships among the taxa in the *Sporidiobolus pararoseus* complex revealed by the ITS sequences are discordant with those revealed by the D1/D2 sequence data, especially for the relationship between *Sporobolomyces phaffii* sp. nov. and *Sporobolomyces ruberrimus* (Fig. 1). Analysis of 18S rDNA sequences may be helpful to confirm the phylogenetic relationships among these species. Fell et al. (2000) found that some basidiomycetous yeast species with identical or similar D1/D2 sequences could be separated by ITS sequence data. On the other hand, the present study indicates that species with similar ITS sequences may have quite different D1/D2 sequence data.

**Latin diagnosis of Sporobolomyces carnicolor**
Yamasaki & Fujii ex Bai & Boekhout


**Typus**: Isolatus ex folio Nerii indici Mill., AS 2,2137® (originaliter ut CH 2.052® depositus in collectione China General Microbiological Culture Collection Center, Academia Sinica, Beijing.

**Description of Sporobolomyces phaffii Bai, Takashima & Nakase sp. nov.**

*Sporobolomyces phaffii* (phaf’fii. N.L. gen. n. phaffii in honour of the late Herman J. Phaff, USA).

In YM broth, after 3 days at 25 °C, the cells are ovoid at 20–40 × 40–80 µm (Fig. 2a). A ring, pellicle and sediment are formed. After 1 month at 17 °C, a ring, pellicle and sediment are present. On YM agar, after 3 days at 25 °C, the streak culture is butyrous, smooth, glistering with orange to orange-red colour. After 1 month at 20 °C, the culture is butyrous and becomes mucoid or reticulate in some areas, orange-red, with the margin entire to eroded. Mycelia and pseudomyecilia are not formed on Dalmau plate culture on corn meal agar. On corn meal agar, ballistoconidia are formed on short stigmata, ellipsoidal or ovoid, 20–22 × 80–90 µm (Fig. 2b). *Glucose* is not fermented. The following carbon compounds are assimilated: glucose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, soluble starch, ethanol (delayed), D-mannitol (delayed), methyl α-D-glucoside, salicin, glucono-δ-lactone, succinic acid and citric acid. The following are not assimilated: galactose (or weak and delayed), l-sorbose, lactose, inulin, d-xylose, l-arabinose, d-arabinose, d-ribose, l-rhamnosum, glycerol, erythritol, ribitol, galactitol, glucitol (or weak and delayed), 2-ketogluconic acid, 5-ketogluconic acid, dl-lactic acid, citric acid, inositol, d-glucuronic acid and D-galacturonic acid. KNO₃, NaNO₃, ethylamine (variable) and l-lysine (or weak) are utilized as sole sources of nitrogen; cadaverine is not utilized or is utilized weakly. Growth in vitamin-free medium is positive.

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Fig. 2. Sporobolomyces phaffii sp. nov. CH 2.052T. (a) Vegetative cells grown in YM broth for 5 days at 17 °C. (b) Ballistoconidia produced on corn meal agar after 5 days at 17 °C. Bars, 10 µm.

Maximum growth temperature is 32–33 °C. Starch-like compounds are not produced. Urease activity is positive. Diazonium blue B reaction is positive. The major ubiquinone is Q-10. Xylose is absent in the whole-cell hydrolysate.

The type strain, strain CH 2.052T (= JCM 1149)T = CBS 9129T, was isolated in 1996 from a wilting leaf of Nerium indicum Mill. collected in Yunnan, China. This strain has also been deposited in the China General Microbiological Culture Collection Center (CGMCC), Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, as strain AS 2.2137T.

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REFERENCES


