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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* Kluyver & 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 salmoneus* Derx, was located together with *Sporobolomyces marcillae* Santa Maria in a separate clade. Two synonyms of *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* Yamasaki & Fujii (nom. inval.) and *Sporobolomyces japonicus* Iizuka & Goto, were revealed to represent two distinct species. The name *Sporobolomyces carnicolor* is validated, with strain CBS 4215^T 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 2.052^T = AS 2.2137^T = JCM 11491^T = CBS 9129^T). 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 Kluyver & 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 *Sporobolo-*

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Abbreviation: ITS, internal transcribed spacer.

The GenBank accession numbers for the sequences determined in this study are AY069991–AY070006 (ITS region) and AY070007–AY070018 (26S rDNA D1/D2 domain) as indicated in Fig. 1.

myces roseus, *Sporobolomyces ruberrimus* Yamasaki & Fujii var. *ruberrimus* (CBS 7500) and *Sporobolomyces ruberrimus* var. *albus* Yamasaki & Fujii (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′-GGTCCGTGTTTCAAGACGG-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 primers ITS1 and NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and the reverse primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and NL4 using the ABI

Table 1. Yeast strains employed

Strain	Source/notes
Sporidiobolus pararoseus	
CBS 491^T	Soil
CBS 484	Type of *Sporobolomyces pararoseus*
Sporobolomyces blumeae JCM 10212^T	*Blumea* sp.
Sporobolomyces carnicolor CBS 4215^T	Unknown
Sporobolomyces japonicus CBS 5744^T	Oil brine
Sporobolomyces marcillae CBS 4217^T	Air
Sporobolomyces phaffii sp. nov.	
CH 2.049	Wilting leaf of *Ehretia corylifolia*
CH 2.052^T	Wilting leaf of *Nerium indicum*
CH 2.083	Wilting leaf of *Oxytenanthera* sp.
CH 2.091	Wilting leaf of *Eriobotrya japonica*
CH 2.304	Wilting leaf of *Oryza sativa*
Sporobolomyces roseus	
CBS 485	Type of *Sporobolomyces pollaccii*
CBS 486^T	Type of *Sporobolomyces roseus*
CBS 993	Type of *Torulopsis somala*
CBS 2646	Type of *Sporobolomyces roseus* var. *madurae*
CBS 2840	Authentic strain of *Sporobolomyces boleticola*
CBS 2841	Authentic strain of *Sporobolomyces boleticola*
CH 2.053	Wilting leaf of *Nerium indicum*
CH 2.116	Wilting leaf of *Sapindus delavayi*
CH 2.332	Wilting leaf of *Nicotiana tabacum*
Sporobolomyces ruberrimus CBS 7500^T	Air
Sporobolomyces salmoneus CBS 488^T	Etiolated grass
Sporobolomyces sp. CH 2.500	Wilting leaf of *Parthenocissus* sp.

BigDye cycle sequencing kit. Electrophoresis was done on an ABI PRISM 377 DNA sequencer.

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 484^T, mt A1) and *Sporobolomyces ruber* Yamasaki & Fujii (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 marcillae* 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 & Fujii (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 marcillae* 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* Ramírez, *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 marcillae* in the *Sporidiobolus pararoseus* clade. The type strains of *Sporobolomyces salmoneus* and *Sporobolomyces marcillae* 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 ballistoconidium-fall method (Nakase & Takashima, 1993). Approximately 100 ballistoconidium-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

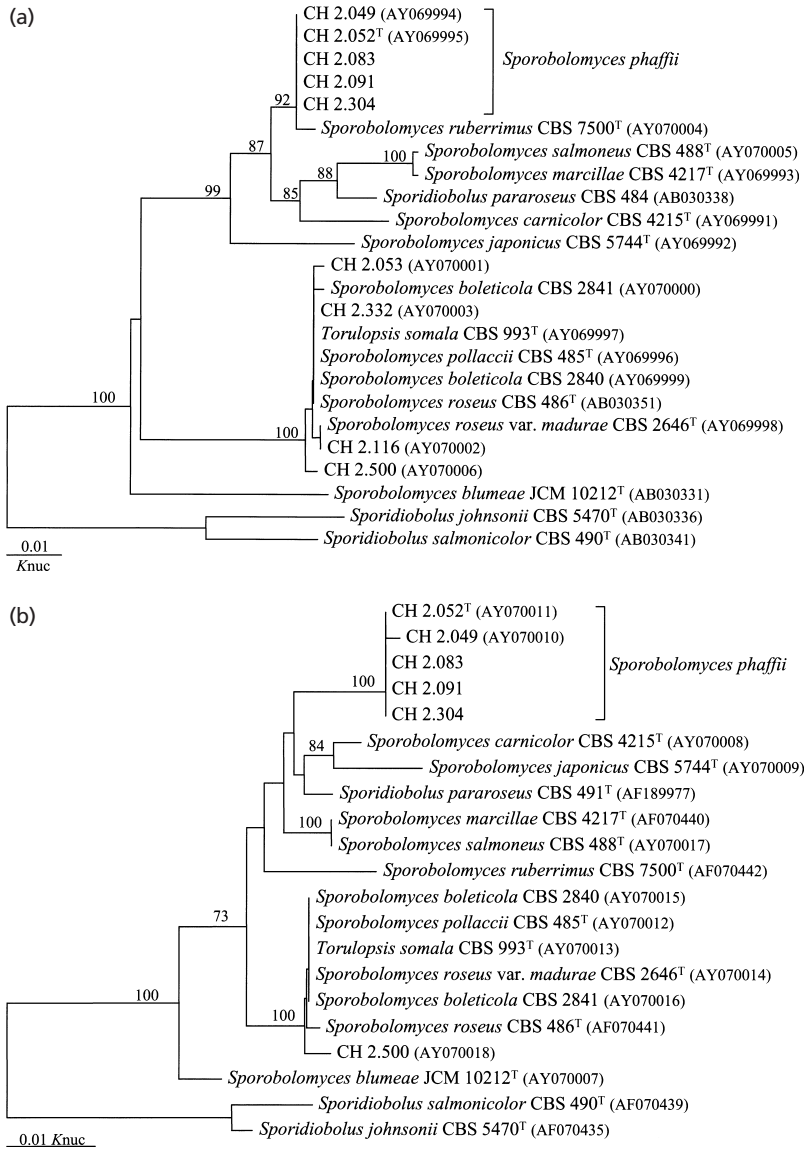

Fig. 1. Phylogenetic trees drawn from neighbour-joining analysis based on sequences of (a) the ITS region (including 5·8S rDNA) and (b) the D1/D2 domain, depicting the relationships of taxa in the *Sporobolomyces roseus* and *Sporidiobolus pararoseus* complexes, as well as the novel isolates from Yunnan, China. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown.

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 marcillae* 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 marcillae* can not. 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 salmoneus* 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

In YM *liquido post dies* 3 *ad* 25 °C, *cellulae vegetativae ovoideae*, *ellipsoideae vel elongatae*, 2·5–6·2 × 3·5–17 μm, *singulae*, *binae vel aggregatae*. *Post unum mensem ad* 20 °C, *annulus*, *pelliculum et sedimentum formantur*. *In agaro* YM *post unum mensem ad* 20 °C, *cultura rubro-aurantiaca*, *rugosa*, *margine erosa*. *Mycelium et pseudomycelium non formantur*. *Ballistosporae ovoideae vel ellipsoideae*, 2·5–3·7 × 6·2–10·2 μm. *Fermentatio nulla*. *Glucosum*, *galactosum* (*lente*), L-*sorbosum* (*lente*), *saccharosum*, *maltosum*, *cellobiosum*, *trehalosum*, *raffinosum*, *melezitosum*, *inulinum* (*lente*), *amylum solubile* (*lente*), D-*xylosum* (*lente*), D-*arabinosum*, D-*ribosum*, *ethanolum*, *glycerolum*, *ribitolum* (*lente*), D-*mannitolum*, *glucitolum*, *methyl* α-D-*glucosidum* (*lente*), *salicinum*, *glucono-δ-lactonum*, *acidum succinicum et acidum citricum assimilantur at non lactosum*, *melibiosum*, L-*arabinosum*, L-*rhamnosum*, *erithritolum*, *galactitolum*, *acidum* 2-*ketogluconicum*, *acidum* 5-*ketogluconicum*, *acidum* DL-*lacticum*, *inositolum*, *acidum* D-*glucuronicum nec acidum* D-*galacturonicum*. *Ammonium sulfatum*, L-*lysinum et cadaverinum assimilantur at non kalium nitricum*, *natrum nitrosum nec ethylaminum*. *Ad crescentiam vitaminum non necessarium est*. *Materia amyloidea iodophila non formantur*. *Urea finditur*. *Diazonium caeruleum* B *positivum*. *Ubiquinonum majus*: Q-10. *Typus*: CBS 4215^T, *depositus in collectione* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

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

In YM (Difco) *liquido post dies* 3 *ad* 25 °C, *cellulae vegetativae ovoideae vel ellipsoideae*, 2·0–4·0 × 4·0–4·8 μm, *singulae aut binae*. *Post unum mensem ad* 20 °C, *annulus*, *pelliculum et sedimentum formantur*. *In agaro* YM *post unum mensem ad* 20 °C, *cultura rubro-aurantiaca*, *glabra*, *nitida*, *et margine glabra*. *Mycelium et pseudomycelium non formantur*. *Ballistosporae ovoideae vel ellipsoideae*, 2·0–2·2 × 8·0–9·0 μm. *Fermentatio nulla*. *Glucosum*, *saccharosum*, *maltosum*, *cellobiosum*, *trehalosum*, *melibiosum*, *raffinosum*, *melezitosum*, *amylum solubile*, *ethanolum* (*lente*), D-*mannitolum* (*lente*), *methyl* α-D-*glucosidum*, *salicinum*, *glucono-δ-lactonum et acidum succinicum assimilantur at non galactosum* (*vel exigue et lente*), L-*sorbosum*, *lactosum*, *inulinum*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, *glycerolum*, *erithritolum*, *ribitolum*, *galactitolum*, *glucitolum* (*vel exigue et lente*), *acidum* 2-*ketogluconicum*, *acidum* 5-*ketogluconicum*, *acidum* DL-*lacticum*, *acidum citricum*, *inositolum*, *acidum* D-*glucuronicum nec acidum* D-*galacturonicum*. *Ammonium sulfatum*, *kalium nitricum*, *natrum nitrosum assmilantur*, *ethylaminum* (*variabile*) *et* L-*lysinum* (*fortasse exigue*) *assimilantur at non cadaverinum* (*vel exigue*). *Ad crescentiam vitaminum non necessarium est*. *Maxima temperatura crescentiae*: 32–33 °C. *Materia amyloidea iodophila non formantur*. *Urea finditur*. *Diazonium caeruleum* B *positivum*. *Ubiquinonum majus*: Q-10. *Xylosum in cellulis absens*.

Typus: *Isolatus ex folio Nerii indici* Mill., AS 2.2137^T (*originaliter ut* CH 2.052^T) *depositus in collectione* China General Microbiological Culture Collection Center, *Academia Sinica*, Beijing.

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

Sporobolomyces phaffii (phaf'fi.i. 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 to ellipsoidal, 2·0–4·0 × 4·0–8·0 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, glistening 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 pseudomycelia are not formed on Dalmau plate culture on corn meal agar. On corn meal agar, ballistoconidia are formed on short sterigmata, ellipsoidal or ovoid, 2·0–2·2 × 8·0–9·0 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-rhamnose, 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. KNO_3, $NaNO_2$, 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.

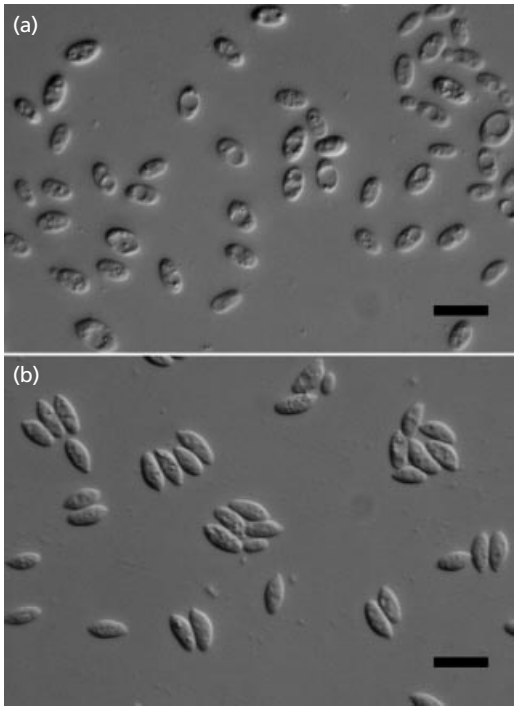

Fig. 2. *Sporobolomyces phaffii* sp. nov. CH 2.052^T. (a) Vegetative cells grown in YM broth for 5 days at 17 °C. (b) Ballistoconidia produced on corn meal agar after 5 days at 17 °C. Bars, 10 μm.

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 2.052^T (= JCM 11491^T = CBS 9129^T), 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 2.2137^T.

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