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Reclassification of the *Cryptococcus humicola* complex

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Ten strains of the *Cryptococcus humicola* complex were reclassified on the basis of sequence analyses of 18S rDNA and internal transcribed spacer regions and DNA–DNA reassociation experiments. They were differentiated into seven species including *C. humicola*. Five novel species are proposed: *Cryptococcus daszewskae* sp. nov. (type strain CBS 5123^T = JCM 11166^T = MUCL 30649^T), *Cryptococcus fragicola* sp. nov. (type strain JCM 1530T^T = CBS 8898^T), *Cryptococcus longus* sp. nov. (type strain CBS 5920^T = JCM 11167^T = MUCL 30690^T), *Cryptococcus musci* sp. nov. (type strain JCM 1531^T = CBS 8899^T) and *Cryptococcus pseudolongus* sp. nov. (type strain JCM 9712^T = CBS 8297^T). A syntype of *Sporobolomyces albidus* JCM 1460^T is also revealed to be a distinct species; the name *Cryptococcus ramirezgomezianus* nom. nov. is therefore proposed for *Sporobolomyces albidus* Ramírez Gómez (type strain IJFM 502^T = CBS 2839^T = JCM 1460^T = NRRL Y-2478^T), since the name *Cryptococcus albidus* (Saito) C. E. Skinner has already been recognized for a distinct species within the genus *Cryptococcus*. Strains possessing either Q-9 or Q-10 have been reported to occur in *C. humicola*; however, after reclassification, the ubiquinone type of the species in each phylogenetic group was shown to be uniform, indicating that it is a useful criterion for the taxonomy of the Trichosporonales.

Keywords: novel species, *Cryptococcus humicola* complex, reclassification, *Sporobolomyces albidus*, internal transcribed spacer (ITS)

INTRODUCTION

Cryptococcus humicola (Daszewska) Golubev has been isolated from various substrates such as plants, soil and clinical specimens (Fell & Statzell-Tallman, 1998). This species has been reported to be heterogeneous on the basis of the wide range of G+C contents of nuclear DNA, the occurrence of both ubiquinone types Q-9 and Q-10 and the results of whole-cell protein electrophoresis (Nakase & Komagata, 1971; Vancanneyt *et al*., 1994; Yamada & Kondo, 1972). Phylogenetically, the species showed a close relationship to *Cryptococcus curvatus* (Diddens *et* Lodder) Golubev and to *Trichosporon* species (Fell *et al*., 2000; Takashima & Nakase, 1999). When Fell *et al*. (2000) proposed the order Trichosporonales, they did not include this species because of its low bootstrap value to the Trichosporonales based on the D1/D2 region of 26S rDNA sequences (651 bp). However, Takashima & Nakase (1999) reported that *C. humicola* was included in the *Trichosporon* lineage with a high bootstrap value for 18S rDNA sequences (1609 bp).

Recently, we reported a high degree of intraspecific heterogeneity in *C. humicola* on the basis of the sequences of 18S rDNA and internal transcribed spacer (ITS) regions (Sugita *et al*., 2000). Of 16 strains, including the type strain, that appeared in that paper, five were confirmed to belong to *C. humicola* and two were identified as *C. curvatus* and *Cryptococcus podzolicus* (Bab'eva *et* Reshetova) Golubev. Eight strains were suspected to be undescribed species. We found

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Abbreviation: ITS, internal transcribed spacer.

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that three of these strains produced arthroconidia and two novel species in the genus *Trichosporon* have been proposed to accommodate them (Sugita *et al*., 2001). The remaining five strains were shown to be divergent on the basis of sequence analyses of the 18S rDNA and ITS regions. Furthermore, two strains were reported to contain Q-10 and one strain had Q-9 as the major ubiquinone (Yamada & Kondo, 1972; Vancanneyt *et al*., 1994). The CoQ system of the remaining two strains has not yet been determined. This report represents a systematic study to clarify the taxonomic position of these strains. In addition, a further strain of *Cryptococcus* sp., which was reported to be close to *C. humicola* on the basis of physiological and biochemical properties (Nakase *et al*., 1996), was included. These strains were shown to be different from *C. humicola* and were reclassified into six distinct species. After reclassification of the *C. humicola* complex, the ubiquinone type of each phylogenetic cluster was recognized to be uniform. A discussion is included of the taxonomic significance of the ubiquinone type in the Trichosporonales.

METHODS

Yeast strains. The strains used in this study are listed in Table 1. Nine strains were stock cultures identified as *C. humicola* in the Centraalbureau voor Schimmelcultures (CBS) collection, The Netherlands, and the Japan Collection of Microorganisms (JCM), Japan. Of these, strains CBS 5123^T, CBS 5290^T, JCM 1460^T, JCM 1530^T and JCM 1531^T were suggested in a previous paper to be undescribed species (Sugita *et al*., 2000). One strain, *Cryptococcus* sp. JCM 9712^T, which was isolated from a dead tree as a cellulolytic yeast (Nakase *et al*., 1996), was also included in this study.

Morphological, physiological and biochemical characteristics. Most of the morphological, physiological and biochemical characteristics were examined according to Yarrow (1998). The assimilation of nitrogen compounds was investigated on solid media using starved inoculum. Vitamin requirements were determined according to the method of Komagata & Nakase (1967). The maximum growth temperature was determined in YM broth (Difco) using thermoregulated metal block baths.

Major ubiquinone. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml YM broth on a rotary shaker at 150 r.p.m. at 25 °C and were harvested in the early stationary growth phase. The cells were washed with distilled water. Extraction, purification and identification of ubiquinones were carried out according to the method of Nakase & Suzuki (1986).

DNA base composition and DNA–DNA relatedness. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml YM broth on a rotary shaker at 150 r.p.m. at 25 °C and were harvested in the exponential growth phase. The cells were washed with distilled water and freeze-dried. Isolation and purification of nuclear DNA were done according to Takashima & Nakase (2000). The DNA base composition was determined by HPLC after enzymic digestion of DNA to deoxyribonucleosides as described by Tamaoka & Komagata (1984). The DNA-GC kit (Yamasa Shoyu) was used as the quantitative standard. DNA–DNA reassociation experiments were carried out using the membrane-filter method of Hamamoto & Nakase (1995).

Sequencing and phylogenetic analysis of 18S rDNA. The nucleotide sequences of 18S rDNA and ITS regions, in-

Table 1. Strains used in this study

Strain	Other designation(s)	Source	Major ubiquinone*
Cryptococcus humicola			
JCM 1457^T†	= ATCC 14438^T = CBS 571^T = IGC 3387^T = MUCL 29840^T = NCYC 818^T = NRRL Y-12944^T	Soil	Q9[a]
JCM 1459	= ATCC 9949 = CBS 2041 = DSM 6382 = IFO 0753 = NRRL Y-1266	Culture of *Sachsia suaveolens* Lindner	Q9[a]
JCM 1461	= CBS 4280 = MUCL 30648	Surface of *Amanita muscaria*	ND
JCM 9575	–	Soil	ND
Cryptococcus daszewskae CBS 5123^T	= JCM 11166^T = MUCL 30649^T	Skin	Q10[b]
Cryptococcus fragicola JCM 1530^T	= CBS 8898^T	Strawberry	Q10[a]
Cryptococcus longus CBS 5920^T	= JCM 11167^T = MUCL 30690^T	Radioactive solution at pH 2	Q9[d]
Cryptococcus musci JCM 1531^T	= CBS 8899^T	Moss	Q9[a]
Cryptococcus pseudolongus JCM 9712^T	= CBS 8297^T	Dead tree	Q9[c]
Cryptococcus ramirezgomezianus JCM 1460^T‡	= CBS 2839^T = IJFM 502^T = NRRL Y-2478^T	Rotten toadstool	Q9[d]

* As determined by: *a*, Yamada & Kondo (1972); *b*, Vancanneyt *et al*. (1994); *c*, Nakase *et al*. (1996); *d*, this study.

† Type strain of *Torula humicola* Daszewska.

‡ Syntype of *Sporobolomyces albidus* Ramírez Gómez.

ND, Not determined.

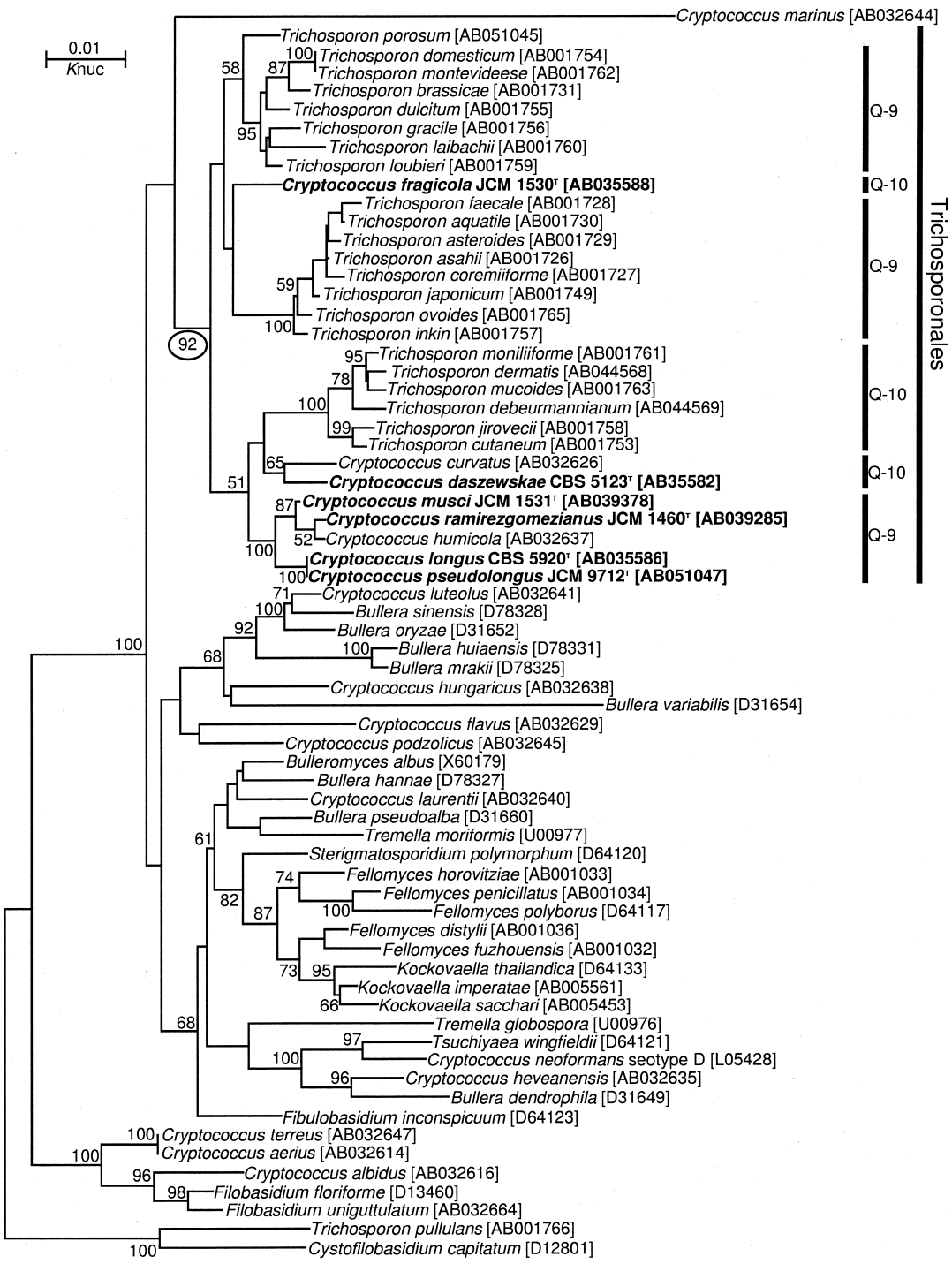

Fig. 1. Phylogenetic tree of the *Cryptococcus humicola* complex and related species based on 18S rDNA sequences. Numerals represent percentages from 100 replicate bootstrap samplings (values less than 50 % are not shown). Sequences were retrieved from the DDBJ/GenBank/EMBL databases under the accession numbers indicated.

cluding the 5.8S rDNA of *Cryptococcus* sp. JCM 9712^T and *Trichosporon porosum* JCM 1458^T, were determined directly using PCR products according to the method of Sugita & Nakase (1999). Reference sequences used for the phylogenetic study were obtained from the DDBJ/GenBank/EMBL database (Fig. 1). Sequences generated were aligned with related species using the CLUSTAL W computer program version 1.8 (Thompson *et al.*, 1994) and checked visually. The phylogenetic tree was constructed from the evolutionary distance data according to Kimura (1980) using the neighbour-joining method (Saitou & Nei, 1987) and the PHYLIP 3.573c computer program. Sites where gaps existed

in any sequences were excluded. Bootstrap analyses (Felsenstein, 1985) were performed from 100 random resamplings.

RESULTS AND DISCUSSION

Reclassification of the *C. humicola* complex

The *C. humicola* strains used in this study occupied diverged positions in the order Trichosporonales (Fig. 1). Strain JCM 1530^T was also placed in the Trichosporonales, but its phylogenetic position was not reliable because of a low bootstrap value. This strain was isolated from a strawberry (Nakase & Komagata, 1971) and contained Q-10 as the major ubiquinone (Yamada & Kondo, 1972). Since no other strains have been reported to be phylogenetically close to this strain, we concluded that strain JCM 1530^T represents a distinct species.

Strain CBS 5123^T constituted a cluster with *C. curvatus* (bootstrap 65%). One of the *Trichosporon* clusters, which included *Trichosporon cutaneum* (de Beurmann *et al.*) Ota, occurred at a position adjacent to this cluster, but its bootstrap value was low. The similarity of 18S rDNA sequences between CBS 5123^T and *C. curvatus* JCM 1532^T was 98·8%. The similarity in the ITS region was reported to be less than 90% (Sugita *et al.*, 2000). In view of these facts and because of physiological and biochemical differences, we concluded that strain CBS 5123^T represented a species distinct from *C. curvatus*. With respect to the major ubiquinone, this cluster is a ubiquinone Q-10 cluster.

Strains JCM 1460^T, JCM 1531^T, CBS 5920^T and JCM 9712^T were clustered with *C. humicola* JCM 1457^T. This cluster contains only Q-9 strains, since CBS 5920^T and JCM 1460^T were investigated and found to be Q-9 in this study. With respect to sequence analysis of the 18S rDNA, a close relationship was detected between JCM 1460^T and JCM 1531^T. Moreover, CBS 5920^T and JCM 9712^T were also found to be closely related. In the ITS region, the ITS2 sequences of JCM 1460^T and JCM 1531^T were identical and five base substitutions and ten base deletions or insertions were detected in the ITS1 region (total 115 bp; similarity, 87·0%), as shown in Fig. 2. Thus, the overall similarity of the ITS region between JCM 1460^T and JCM 1531^T was 95·8%, as discussed previously (Sugita *et al.*, 2000). In the case of CBS 5920^T and JCM 9712^T, the sequence similarity of ITS1, ITS2 and the overall ITS region was 100, 85·4 and 90·6%, respectively (Fig. 3). According to the species concept of Sugita *et al.* (1999), these strains would be separate species. To confirm this, we carried out DNA–DNA reassociation experiments and found that the strains examined represented five distinct species (Table 2).

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JCM 1460T  GTGATTGGCCTTAGTGCCTAAAAACTATATCCCAAACACCTGTGAACTGTTGAACCGAAA
JCM 1531T  GTGATTGGCCTTAGTGCCTAAAAACTATATCCCAAACACCTGTGAACTGTTGAATTGCGT
           ************************************************** *

           ----------GGTTCTTTTACAAACATTGTGTAATGAACGTCATAACATTATAAA
           CTTCGGATGCGGTTCTTTTACAAACATTGTGTAATGAACGTCATAACATTATAAA
                     *******************************************
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Fig. 2. Primary sequences of the ITS1 regions of JCM 1460^T and JCM 1531^T. Gaps are indicated by dashes. Asterisks indicate identical nucleotides between the two aligned sequences.

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JCM 9712T  GATCTCTCAACCAATAGAGTTTTCTATTGGCTTGGATCTGGGTGTTGCGAG-CGATCGCT
CBS 5920T  GATCTCTCAACCAATAGAGTTTTCTATTGGCTTGGATCTGGGTGCTGCGAAACAATCGCT
           ********************************************** *****  * ******

           CACCTTAAAGGAGTTAGCATAT-AGC-ATGTCGTTTGGCGTAATAAGTTTCGCTTAGTAA
           CACCTTAAAGGAGTTAGCAACTAAGCGATGTCGTCCGACGTAATAAGTTTCGTCTGGTAA
           ******************  * *** *******  * **************  * ****

           ATCGACAAGGCT--TTGCTTCTAATCGTCTTTTGACTTTTTTGAC
           TTCGACTGAGCCAATTGCTTCTAATTGTCTTTTGACTTTTTTGAC
            *****   **   *********** ********************
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Fig. 3. Primary sequences of the ITS2 regions of CBS 5920^T and JCM 9712^T. Gaps are indicated by dashes. Asterisks indicate identical nucleotides between the two aligned sequences.

On the basis of D1/D2 sequences of the 26S rDNA, Fell *et al.* (2000) showed that *C. humicola* was closely related to the Trichosporonales, but did not include this species in this order because of the low bootstrap value. However, on the basis of 18S rDNA sequences, Takashima & Nakase (1999) reported the inclusion of this species in the *Trichosporon* lineage. In this paper, we also obtained a high bootstrap value (92%) for this lineage after the reclassification of the *C. humicola* complex and confirmed the inclusion of *C. humicola* in the Trichosporonales. The order Trichosporonales contains the *Trichosporon* species [with the exception of *Trichosporon pullulans* (Lindner) Diddens *et* Lodder], *C. curvatus* and *C. humicola*. The diagnostic criterion between the genera *Trichosporon* and *Cryptococcus* in the order is whether or not arthroconidia are produced. Furthermore, strain JCM 1460^T, which is a syntype of *Sporobolomyces albidus* Ramírez Gómez and was reported to produce ballistoconidia, has been treated as belonging to the genus *Cryptococcus*, because the ballistoconidium-forming activity of this species is supposed to have been lost (Fell & Statzell-Tallman, 1998). The taxonomic significance of ballistoconidium-forming activity was discussed in several papers (Fell *et al.*, 2000; Hamamoto & Nakase, 2000; Takashima & Nakase, 1999). Based on these aspects and also because our novel species do not produce arthroconidia, we tentatively place these species in the genus *Cryptococcus* and here propose five novel species, *Cryptococcus daszewskae*, *Cryptococcus fragicola*, *Cryptococcus longus*, *Cryptococcus musci* and *Cryptococcus pseudolongus*. Concerning a syntype of *S. albidus* (JCM 1460^T), since the name *Cryptococcus albidus* (Saito) C. E. Skinner has been recognized for a distinct species within the genus *Cryptococcus*, the name *Cryptococcus ramirezgomezianus* nom. nov. is proposed for *S. albidus* Ramírez Gómez. These species are not phylogenetically close to the authentic *Cryptococcus* species, since the neotype species of the genus, *Cryptococcus neoformans*, occurs in another lineage

Table 2. DNA relatedness among *C. humicola* and phylogenetically closely related species

Strain	DNA G+C content (mol%)	Relative binding (%) of labelled DNA from:				
		1	5	6	7	8
1. *C. humicola* JCM 1457^T	58·7	100	33	27	21	11
2. *C. humicola* JCM 1459	59·8	88	–	–	–	–
3. *C. humicola* JCM 1461	58·7	92	–	–	–	–
4. *C. humicola* JCM 9575	59·8	98	–	–	–	–
5. *C. longus* CBS 5920^T	60·6	41	100	25	35	13
6. *C. musci* JCM 1531^T	58·8	46	36	100	28	32
7. *C. pseudolongus* JCM 9712^T	59·1	40	46	26	100	32
8. *C. ramirezgomezianus* JCM 1460^T	60·7	35	20	32	15	100

(Fell *et al*., 2000; Takashima & Nakase, 1999). These novel species will be transferred to the appropriate genus when it is eventually proposed.

Practically, *C. fragicola* differs from all other *Cryptococcus* species by its incapacity to assimilate cadaverine dihydrochloride. *C. daszewskae* is distinguished from *C. curvatus* because it is able to utilize melibiose and saccharate and unable to assimilate methyl α-D-glucoside or salicin. *C. humicola* and the related species *C. longus*, *C. musci*, *C. pseudolongus* and *C. ramirezgomezianus* can be discriminated by their responses to several tests, namely assimilation of raffinose, hexadecane, butane-2,3-diol and sodium nitrite and growth at 30 and 35 °C, as shown in Table 3.

Taxonomic significance of ubiquinone in the Trichosporonales

In the basidiomycetous yeasts, most species contain Q-10 as the major ubiquinone and relatively few species are characterized by Q-8 or Q-9. Major ubiquinone types have been used as a chemotaxonomic criterion at the genus level; however, they have occasionally been shown to be inconsistent with the delimitation of some species. *C. humicola* and *Leucosporidium scottii* Fell *et al*. were reported as examples of such inconsistency (Yamada & Kondo, 1972).

In this study, we determined the major ubiquinones of JCM 1460^T and CBS 5920^T. Although *C. humicola* was reported to include both Q-9 and Q-10 strains, the phylogenetic tree (Fig. 1) clearly showed that the Q-9 strains in the *C. humicola* complex were phylogenetically separated from the Q-10 strains. Sugita & Nakase (1998) reported that the ubiquinone types were uniform in each phylogenetic cluster of *Trichosporon* species and that these types correlated with the serotypes of *Trichosporon* species. Our result also showed that the ubiquinone type of the species that make up the four major clusters were uniform, as shown in Fig. 1. These results suggest that the ubiquinone type, which corresponds to the phylogenetic relationships detected so far, is relevant as a taxonomic criterion in the Trichosporonales. In view of the phylogeny of the Trichosporonales, this order will probably be divided into several genera. The major ubiquinone type should be used as an important taxonomic criterion for this revision in combination with other characteristics.

The ITS region as a marker for identification

Sugita *et al*. (1999) observed that more than 99% sequence similarity of the overall ITS region (ITS1+ITS2) indicated the same species. Between phylogenetically closely related species, the sequence similarity of ITS1 was shown to be similar or slightly lower than that of ITS2 (e.g. Sugita *et al*., 2000; Takashima & Nakase, 2000). This region was believed to contain intervening sequences without function, but Musters *et al*. (1990) and van der Sande *et al*. (1992) reported that ITS2 was involved in the processing of rRNA. ITS1 was also reported to be involved in rRNA maturation (Lalev *et al*. 2000).

In this study, we found that the ITS2 sequences of JCM 5920^T and JCM 9712^T were identical, but 85·4% similarity was detected in ITS1 (overall similarity 90·6%). In the case of JCM 1460^T and JCM 1531^T, the sequence similarity of ITS1, ITS2 and the overall ITS region was 87·0, 100 and 95·8%, respectively. The DNA relatedness values obtained indicated that each of these strains represented a separate species. Based on this approach, we concluded that identification based on ITS1 or ITS2 individually might be insignificant and that involvement of the 'overall' (total) ITS sequence is essential for correct identification as described by Sugita *et al*. (1999).

Latin diagnosis of *Cryptococcus daszewskae* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

In liquido 'YM', *post dies* 3 *ad* 25 °C, *cellulae ovoideae*, *ellipsoideae aut elongatae*, 2–8 × 3–12 μm, *singulae*, *binae*, *aut in fasciculis*, *propagantes gemmarum blasticarum*. *Sedimentum formatur*. *Post unum mensem ad* 17 °C, *annulus repens*, *pellicula fragilis et completa*, *et sedimentum formantur*. *In agaro* 'YM', *post unum*

Table 3. Salient characteristics of the newly proposed species and related species

Species are identified as: 1, *C. fragicola*; 2, *C. daszewskae*; 3, *C. curvatus*; 4, *C. longus*; 5, *C. musci*; 6, *C. pseudolongus*; 7, *C. ramirezgomezianus*; 8, *C. humicola*. Characters are scored as: +, positive; −, negative; L, latent; W, weak; LW, latent and weak; V, variable. NA, Not available.

Characteristic	1	2	3	4	5	6	7	8
Assimilation of carbon compounds								
L-Sorbose	+	L	LW	+	+	+	+	+
Trehalose	+	+	LW	+	+	+	+	+
Melibiose	+	+	−	+	+	+	+	+
Raffinose	+	+	+	L	−	−	L	V
Melezitose	+	+	L	+	+	+	+	+
D-Arabinose	+	+	LW	+	+	+	+	+
L-Rhamnose	+	+	L	+	+	+	+	+
Ethanol	L	+	+	+	+	+	+	LW/W/+
Erythritol	+	+	L	+	+	+	+	+
Ribitol	+	+	L	+	+	+	+	+
Methyl α-D-glucoside	+	−	L	+	+	+	+	+
Salicin	+	−	+	+	+	+	+	+
Glucono-δ-lactone	−	−	LW	+	+	+	+	+
Inositol	+	+	LW	+	+	+	+	+
Hexadecane	−	−		L	L	−	−	−/LW
Saccharate	+	+	−	L	+	+	+	+
Xylitol	L	+	LW	+	+	+	+	+
L-Arabitol	L	+	L	+	+	+	+	+
Butane-2,3-diol	LW	L	−	+	+	+	−	LW/W/L
Assimilation of nitrogen compounds								
Sodium nitrite	−	−	NA	−	+	−	−	V
Ethylamine hydrochloride	+	−	NA	+	+	+	+	+
Cadaverine dihydrochloride	−	+	NA	+	+	+	+	+
Growth temperature (°C)	30+, 35−	35+	NA	30+, 35−	30+, 35−	30+, 35−	30+, 35−	35+
Major ubiquinone	Q-10	Q-10	Q-10	Q-9	Q-9	Q-9	Q-9	Q-9

mensem ad 17 °C, *cultura albo-flava aut luteola*, *glabra*, *nitida*, *mollis*, *margo glabra*. *In cultura* Dalmau plate, *post dies* 27 *ad* 17 °C, *pseudomycelia et mycelia flexuosa formantur*. *Fermentatio nulla*. *Glucosum*, *galactosum*, L-*sorbosum* (*lente*), *sucrosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *raffinosum*, *melezitosum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, D-*glucosaminum*, *N-acetylum* D-*glucosaminum*, *ethanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *acidum* D-*gluconicum*, *acidum* 2-*ketogluconicum*, *acidum* 5-*ketogluconicum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *acidum saccharicum*, *xylitolum*, L-*arabitolum*, 1,2-*propanediolum*, 2,3-*butanediolum* (*lente*), *acidum* D-*glucuronicum et acidum* D-*galacturonicum assimilantur*, *autem inulinum*, *methanolum*, *methylum* α-D-*glucosidum*, *salicinum*, *glucono-δ-lactonum et hexadecanum non assimilantur*. L-*Lysinum et cadaverinum assimilantur*, *autem kalium nitricum*, *natrium nitrosum et ethylaminum non assimilantur*. *Maxima temperatura crescentiae*: 36–37 °C. *Ad crescentiam thiaminum necessarium est*. *Diazonium caeruleum* B *positivum*. *Proportio molaris guanini+cytosini in acido deoxyribonucleico*: 59·4 mol%. *Ubiquinonum majus*: Q-10. *Xylosum in cellulis presens*. *Typus* CBS 5123^T (= JCM 11166^T = MUCL 30649^T) *ex cute conservatur in collectionibus culturarum quas* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum*, The Netherlands, *sustentat*.

Description of *Cryptococcus daszewskae* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

Cryptococcus daszewskae (das.zew′ska.e. N.L. gen. n. *daszewskae* in honour of W. Daszewska for his contribution to yeast taxonomy, especially the original description of *C. humicola*).

After 3 d of growth in YM broth at 25 °C, vegetative cells are oval, ellipsoidal or elongate, 2–8 × 3–12 μm, single, in pairs or in groups, reproducing by budding (Fig. 4a). A sediment is formed. After 1 month at 17 °C, a creeping and complete ring, a complete fragile pellicle and a sediment are present. On YM agar, after 1 month at 17 °C, streak cultures are yellowish white to pale yellow, shining to semi-shining, smooth and soft and have an entire margin. In Dalmau plate culture on

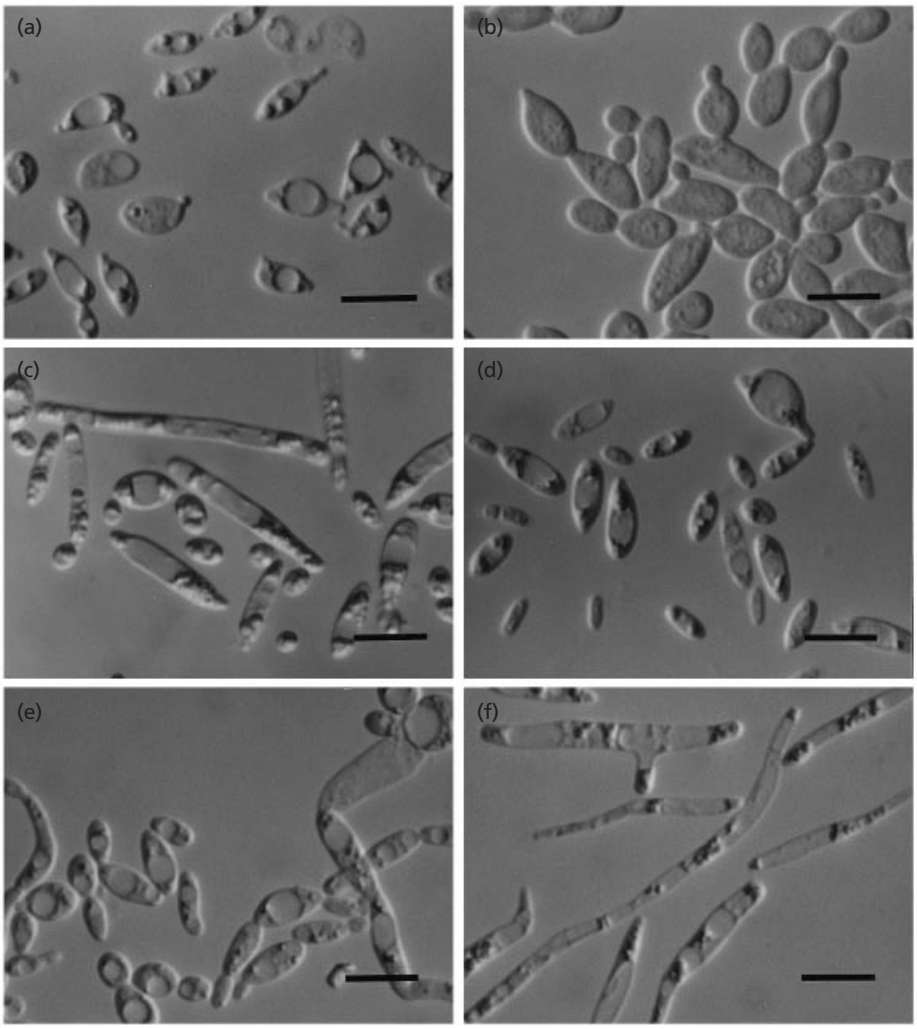

Fig. 4. Vegetative cells grown in YM broth for 3 d at 25 °C. (a) *Cryptococcus daszewskae* CBS 5123^T; (b) *Cryptococcus fragicola* JCM 1530^T; (c) *Cryptococcus longus* CBS 5920^T; (d) *Cryptococcus musci* JCM 1531^T; (e) *Cryptococcus pseudolongus* JCM 9712^T; (f) *Cryptococcus ramirezgomezianus* JCM 1460^T. Bars, 10 μm.

cornmeal agar, after 27 d at 17 °C, pseudomycelia and mycelia are formed. Mycelia are flexunose and branched at wide angles. The physiological and chemotaxonomic characteristics are summarized in Table 4.

The type strain, CBS 5123^T (= JCM 11166^T = MUCL 30649^T), isolated from skin and deposited by E. Friedrich (strain no. 652) in June 1962, is maintained in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Latin diagnosis of *Cryptococcus fragicola* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

In liquido 'YM', *post dies* 3 *ad* 25 °C, *cellulae ovoideae*, *ellipsoideae aut elongatae*, 2–12 × 2–18 μm, *singulae*, *binae*, *aut in fasciculis*, *propagantes gemmarum blasticarum*. *Sedimentum formatur*. *Post unum mensem ad* 17 °C, *annulus incompleta*, *insula*, *et sedimentum formantur*. *In agaro* 'YM', *post unum mensem ad* 17 °C, *cultura luteola*, *glabra*, *nitida*, *mollis*, *margo fimbriata*. *In cultura* Dalmau plate, *post dies* 13 *ad* 17 °C, *pseudomycelia*, *et mycelia flexuosa et conidiogenae formantur*. *Fermentatio nulla*. *Glucosum*, *galactosum*, L-*sorbosum*, *sucrosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *raffinosum*, *melezitosum*, *inulinum* (*lente et exiguum*), *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, D-*glucosaminum*, *N-acetylum* D-*glucosaminum*, *ethanolum* (*lente*), *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *methylum* α-D-*glucosidum*, *salicinum*, *acidum* D-*gluconicum*, *acidum* 2-*ketogluconicum*, *acidum* 5-*ketogluconicum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *acidum saccharicum*, *xylitolum* (*lente*), L-*arabitolum* (*lente*), 1,2-*propanediolum*, 2,3-*butanediolum* (*lente et exiguum*), *acidum* D-*glucuronicum et acidum* D-*galacturonicum assimilantur*,

Table 4. Physiological and chemotaxonomic characterization of the newly proposed species

All strains listed are negative for assimilation of methanol. All strains listed are positive for assimilation of glucose, galactose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, *N*-acetyl D-glucosamine, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, D-gluconate, 2-ketogluconic acid, 5-ketogluconic acid, DL-lactic acid, succinic acid, citric acid, inositol, propane-1,2-diol, D-glucuronic acid and D-galacturonic acid. Characters are scored as: +, positive; −, negative; L, latent; LW, latent and weak.

Character	*C. daszewskae* CBS 5123^T	*C. fragicola* JCM 1530^T	*C. longus* CBS 5920^T	*C. musci* JCM 1531^T	*C. pseudolongus* JCM 9712^T
Fermentation	−	−	−	−	−
Assimilation of:					
L-Sorbose	L	+	+	+	+
Raffinose	+	+	L	−	−
Inulin	−	LW	−	−	−
Ethanol	+	L	+	+	+
Methyl α-D-glucoside	−	+	+	+	+
Salicin	−	+	+	+	+
Glucono-δ-lactone	−	−	+	+	+
Hexadecane	−	−	L	L	−
Saccharate	+	+	L	+	+
Xylitol	+	L	+	+	+
L-Arabitol	+	L	+	+	+
Butane-2,3-diol	L	LW	+	+	+
Ammonium sulfate	+	+	+	+	+
Potassium nitrate	−	−	−	−	−
Sodium nitrite	−	−	−	+	−
Ethylamine hydrochloride	−	+	+	+	+
L-Lysine hydrochloride	+	+	+	+	+
Cadaverine dihydrochloride	+	−	+	+	+
Vitamins required	Thiamin	Thiamin	Thiamin	Thiamin	Thiamin
Maximum growth temperature (°C)	36–37	30–31	31–32	33–34	32–33
Production of starch-like substances	+	+	+	+	+
Growth in 50 % (w/w) glucose/yeast extract agar	−	−	−	−	−
Urease reaction	+	+	+	+	+
Hydrolysis of fat	−	−	−	−	−
Acid production from glucose	−	−	−	−	−
Diazonium blue B reaction	+	+	+	+	+
Liquefaction of gelatin	−	−	−	−	−
G+C content of nuclear DNA (mol%)*	59·4[a]	58·8[b]	60·6	58·8	59·1
Major ubiquinone	Q-10	Q-10	Q-9	Q-9	Q-9
Xylose in the cell	Present	Present	Present	Present	Present

* Data not determined in this study were taken from: *a*, Nakase & Komagata (1971); *b*, Vancanneyt *et al*. (1994).

autem methanolum, *glucono-δ-lactonum et hexadecanum non assimilantur*. *Ethylaminum et* L-*lysinum assimilantur*, *autem kalium nitricum*, *natrium nitrosum et cadaverinum non assimilantur*. *Maxima temperatura crescentiae*: 30–31 °C. *Ad crescentiam thiaminum necessarium est*. Diazonium *caeruleum* B *positivum*. *Proportio molaris guanini+cytosini in acido deoxyribonucleico*: 58·8 mol %. *Ubiquinonum majus*: Q-10. *Xylosum in cellulis presens*. *Typus* JCM 1530^T (= CBS 8898^T), *ex fragis*, Akihabara, Tokyo, *Japonia*, 1962, T. Nakase *et* K. Komagata (*originaliter ut* YV-170), *conservatur in collectionibus culturarum quas* Japan Collection of Microorganisms, Saitama, *Japonia*, *sustentat*.

Description of *Cryptococcus fragicola* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

Cryptococcus fragicola (fra.gi′co.la. L. n. *fragum* the strawberry; L. suff. *-cola* inhabitant, dweller; N.L. n. *fragicola* inhabitant of strawberries, referring to the isolation of the type strain).

After 3 d of growth in YM broth at 25 °C, the vegetative cells are oval, ellipsoidal or elongate, 2–12 × 2–18 μm, single, in pairs or in groups, reproducing by budding (Fig. 4b). A sediment is formed. After 1 month at 17 °C, an incomplete ring, islets and a sediment are present. After 1 month of growth on YM agar at 17 °C, the streak culture is pale yellow,

shining to semi-shining, smooth and soft and has a fimbriate margin. In Dalmau plate culture on cornmeal agar, after 13 d at 17 °C, pseudomycelia and mycelia are formed. Mycelia are flexunose and produce conidia. Physiological and chemotaxonomic characteristics are summarized in Table 4.

The type strain, JCM 1530^T (= CBS 8898^T) (originally YV-170), isolated from a strawberry collected at a market in Akihabara, Tokyo, Japan, by T. Nakase and K. Komagata in April 1962, is maintained in the Japan Collection of Microorganisms, Saitama, Japan.

Latin diagnosis of *Cryptococcus longus* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

In liquido 'YM', *post dies* 3 *ad* 25 °C, *cellulae ovoideae*, *ellipsoideae*, *elongatae aut cylindraceae*, 2·2–7·5 × 2·5–30 μm, *singulae*, *binae*, *aut in fasciculis*, *propagantes gemmarum blasticarum*. *Sedimentum formatur*. *Post unum mensem ad* 17 °C, *annulus repens*, *pellicula fragilis et completa*, *et sedimentum formantur*. *In agaro* 'YM', *post unum mensem ad* 17 °C, *cultura luteola aut griseoflava*, *rugosa aut cerebriformis*, *semi-nitida aut hebetata*, *mollis*, *margo fimbriata*. *In cultura Dalmau plate*, *post dies* 13 *ad* 17 °C, *pseudomycelia*, *et mycelia flexuosa et cymosa formantur*. *Fermentatio nulla*. *Glucosum*, *galactosum*, L-*sorbosum*, *sucrosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *raffinosum* (*lente*), *melezitosum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, D-*glucosaminum*, *N-acetylum* D-*glucosaminum*, *ethanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *methylum* α-D-*glucosidum*, *salicinum*, *glucono-δ-lactonum*, *acidum* D-*gluconicum*, *acidum* 2-*ketogluconicum*, *acidum* 5-*ketogluconicum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *hexadecanum* (*lente*), *acidum saccharicum* (*lente*), *xylitolum*, L-*arabitolum*, 1,2-*propanediolum*, 2,3-*butanediolum*, *acidum* D-*glucuronicum et acidum* D-*galacturonicum assimilantur*, *autem inulinum et methanolum non assimilantur*. *Ethylaminum*, L-*lysinum et cadaverinum assimilantur*, *autem kalium nitricum et natrium nitrosum non assimilantur*. *Maxima temperatura crescentiae*: 31–32 °C. *Ad crescentiam thiaminum necessarium est*. Diazonium *caeruleum* B *positivum*. *Proportio molaris guanini+cytosini in acido deoxyribonucleico*: 60·6 mol%. *Ubiquinonum majus*: Q-9. *Xylosum in cellulis presens*. *Typus*: CBS 5920^T (= JCM 11167^T = MUCL 30690^T), *ex solutione vegeto radio* pH 2·0 *conservatur in collectionibus culturarum quas* 'Centraalbureau voor Schimmelcultures', *Trajectum ad Rhenum*, The Netherlands, *sustentat*.

Description of *Cryptococcus longus* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

Cryptococcus longus (lon′gus. L. adj. *longus* long, referring to the distinctive morphology of the cells).

After 3 d of growth in YM broth at 25 °C, the vegetative cells are oval, ellipsoidal, elongate or cylindrical, 2·2–7·5 × 2·5–30 μm, single, in pairs or in groups, reproducing by budding (Fig. 4c). A sediment is formed. After 1 month at 17 °C, a creeping and complete ring, a complete fragile pellicle and a sediment are present. On YM agar, after 1 month of growth at 17 °C, the streak culture is pale yellow to greyish yellow, matt to semi-shining, wrinkled and cerebriform near the bottom, soft and has a fimbriate margin. In Dalmau plate culture on cornmeal agar, after 13 d at 17 °C, pseudomycelia and mycelia are formed. Mycelia are flexunose and cymose branching and produce conidia. Physiological and chemotaxonomic characteristics are summarized in Table 4.

The type strain, CBS 5920^T (= JCM 11167^T = MUCL 30690^T), isolated from radioactive solution at pH 2, Delft, The Netherlands, and deposited by R. Kokke, no. 070668 Co9, in July 1968, is maintained in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Latin diagnosis of *Cryptococcus musci* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

In liquido 'YM', *post dies* 3 *ad* 25 °C, *cellulae ovoideae*, *ellipsoideae*, *aut elongatae*, 2–10 × 2–15 μm, *singulae*, *binae*, *aut in fasciculis*, *propagantes gemmarum blasticarum*. *Sedimentum formatur*. *Post unum mensem ad* 17 °C, *annulus repens*, *pellicula fragilis et completa*, *et sedimentum formantur*. *In agaro* 'YM', *post unum mensem ad* 17 °C, *cultura griseoflava*, *farinosa et cerebriformis*, *hebetata*, *mollis*, *margo fimbriata*. *In cultura* Dalmau plate, *post dies* 13 *ad* 17 °C, *pseudomycelia*, *et mycelia flexuosa et cymosa formantur*. *Fermentatio nulla*. *Glucosum*, *galactosum*, L-*sorbosum*, *sucrosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *melezitosum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, D-*glucosaminum*, *N-acetylum* D-*glucosaminum*, *ethanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *acidum* D-*gluconicum*, *methylum* α-D-*glucosidum*, *salicinum*, *glucono-δ-lactonum*, *acidum* 2-*ketogluconicum*, *acidum* 5-*ketogluconicum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *hexadecanum* (*lente*), *acidum saccharicum*, *xylitolum*, L-*arabitolum*, 1,2-*propanediolum*, 2,3-*butanediolum*, *acidum* D-*glucuronicum et acidum* D-*galacturonicum assimilantur*, *autem raffinosum*, *inulinum et methanolum non assimilantur*. *Natrium nitrosum*, *ethylaminum*, L-*lysinum et cadaverinum assimilantur*, *autem kalium nitricum non assimilantur*. *Maxima temperatura crescentiae*: 33–34 °C. *Ad crescentiam thiaminum necessarium est*. Diazonium *caeruleum* B *positivum*. *Proportio molaris guanini+cytosini in acido deoxyribonucleico*: 58·8 mol%. *Ubiquinonum majus*: Q-9. *Xylosum in cellulis presens*. *Typus*: JCM 1531^T (= CBS 8899^T), *ex musco*, Hase-dera, Kurayoshi, Tottori, *Japonia*, 1963, T. Nakase *et* K. Komagata (*originaliter ut* M-9-2), *conservatur in collectionibus culturarum quas* 'Japan Collection of Microorganisms', Saitama, *Japonia*, *sustentat*.

Description of *Cryptococcus musci* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

Cryptococcus musci (mus′ci. L. gen. n. *musci* of moss, from which the type strain was isolated).

After 3 d of growth at 25 °C in YM broth, vegetative cells are oval, ellipsoidal or elongate, 2–10 × 2–15 μm, single, in pairs or in groups, reproducing by budding (Fig. 4d). A sediment is formed. After 1 month at 17 °C, a creeping and complete ring, a complete fragile pellicle and a sediment are present. On YM agar, after 1 month of growth at 17 °C, the streak culture is greyish yellow, matt, farinose and cerebriform, soft and has a fimbriate margin. In Dalmau plate culture on cornmeal agar, after 13 d at 17 °C, pseudomycelia and mycelia are formed. Mycelia are flexunose and cymose branching and produce conidia. Physiological and chemotaxonomic characteristics are summarized in Table 4.

The type strain, JCM 1531^T (= CBS 8899^T) (originally M-9-2), isolated from moss collected at Hase-dera, a temple in Kurayoshi, Tottori, Japan, in December 1963 by T. Nakase and K. Komagata, is maintained in the Japan Collection of Microorganisms, Saitama, Japan.

Latin diagnosis of *Cryptococcus pseudolongus* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

In liquido 'YM', *post dies* 3 *ad* 25 °C, *cellulae ovoideae*, *ellipsoideae*, *elongatae aut cylindraceae*, 1·5–17 × 2–23 μm, *singulae*, *binae*, *aut in fasciculis*, *propagantes gemmarum blasticarum*. *Sedimentum formatur*. *Post unum mensem ad* 17 °C, *annulus repens*, *pellicula fragilis et completa*, *et sedimentum formantur*. *In agaro* 'YM', *post unum mensem ad* 17 °C, *cultura luteola*, *glabra*, *semi-nidida*, *mollis*, *margo fimbriata*. *In cultura* Dalmau plate, *post dies* 13 *ad* 17 °C, *pseudomycelia*, *et mycelia flexuosa et cymosa formantur*. *Fermentatio nulla*. *Glucosum*, *galactosum*, L-*sorbosum*, *sucrosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *melezitosum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, D-*glucosaminum*, *N-acetylum* D-*glucosaminum*, *ethanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *acidum* D-*gluconicum*, *methylum* α-D-*glucosidum*, *salicinum*, *glucono-δ-lactonum*, *acidum* 2-*ketogluconicum*, *acidum* 5-*ketogluconicum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *acidum saccharicum*, *xylitolum*, L-*arabitolum*, 1,2-*propanediolum*, 2,3-*butanediolum*, *acidum* D-*glucuronicum et acidum* D-*galacturonicum assimilantur*, *autem raffinosum*, *inulinum*, *methanolum et hexadecanum non assimilantur*. *Ethylaminum*, L-*lysinum et cadaverinum assimilantur*, *autem natrium nitrosum et kalium nitricum non assimilantur*. *Maxima temperatura crescentiae*: 32–33 °C. *Ad crescentiam thiaminum necessarium est*. Diazonium *caeruleum* B *positivum*. *Proportio molaris guanini+cytosini in acido deoxyribonucleico*: 59·1 mol%. *Ubiquinonum majus*: Q-9. *Xylosum in cellulis presens*. *Typus*: JCM 9712^T (= CBS 8297^T), *ex ligno emortuo*, Myooh-in-ji, Kusado, Fukuyama, Hiroshima, *Japonia*, 1990, T. Hatano *et* S. Fukui (*originaliter ut* KO-7), *conservatur in collectionibus culturarum quas* 'Japan Collection of Microorganisms', Saitama, *Japonia*, *sustentat*.

Description of *Cryptococcus pseudolongus* Takashima, Sugita, Shinoda *et* Nakase sp. nov.

Cryptococcus pseudolongus (pseu.do.lon′gus. Gr. adj. *pseudes* false; L. adj. *longus* long; N.L. adj. *pseudolongus* a false *longus*, referring to the similarity of the morphology and other characters to those of *Cryptococcus longus*).

After 3 d of growth in YM broth at 25 °C, vegetative cells are oval, ellipsoidal, elongate or cylindrical, 1·5–17 × 2–23 μm, single, in pairs or in groups, reproducing by budding (Fig. 4e). A sediment is formed. After 1 month at 17 °C, a creeping and complete ring, a complete fragile pellicle and a sediment are present. After 1 month of growth on YM agar at 17 °C, the streak culture is pale yellow, semi-shining, smooth, soft and has a fimbriate margin. In Dalmau plate culture on cornmeal agar, after 13 d at 17 °C, pseudomycelia and mycelia are formed. Mycelia are flexunose and cymose branching and produce conidia. Physiological and chemotaxonomic characteristics are summarized in Table 4.

The type strain, JCM 9712^T (= CBS 8297^T) (originally KO-7), isolated from a dead tree in Myooh-in temple, Kusado, Fukuyama-shi, Hiroshima by T. Hatano and S. Fukui in 1990, is maintained in the Japan Collection of Microorganisms, Saitama, Japan.

Description of *Cryptococcus ramirezgomezianus* Takashima, Sugita, Shinoda *et* Nakase nom. nov.

Cryptococcus ramirezgomezianus (ra.mi′rez.go′me.zi.a.nus. N.L. gen. n. *ramirezgomezianus* in honour of C. Ramírez Gómez for his contributions to yeast taxonomy, especially his original description of *Sporobolomyces albidus*).

≡ *Sporobolomyces albidus* Ramírez Gómez *in Microbiol Esp* **10**, 238, 1957 [*non Cryptococcus albidus* (Saito) C. E. Skinner *in Am Midl Natur* **43**, 249, 1950].

The epithet *albidus* can not be used under the genus *Cryptococcus* because of the presence of *C. albidus* (Saito) C. E. Skinner. Therefore, the new name *Cryptococcus ramirezgomezianus* is herein given.

A micrograph of vegetative cells grown in YM broth for 3 d at 25 °C is shown in Fig. 4(f). The following characteristics are added to the description of Ramírez Gómez (1957). Assimilates L-sorbose, cellobiose, trehalose, lactose, melibiose, raffinose (latent), melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, *N*-acetyl D-glucosamine, ethanol, glycerol, erythritol,

ribitol, galactitol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, glucono-δ-lactone, D-gluconate, 2-ketogluconic acid, 5-ketogluconic acid, DL-lactic acid, succinic acid, citric acid, inositol, saccharate, xylitol, L-arabitol, propane-1,2-diol, D-glucuronic acid and D-galacturonic acid. Does not assimilate inulin, methanol, hexadecane or butane-2,3-diol. Assimilates ethylamine hydrochloride, L-lysine hydrochloride and cadaverine dihydrochloride. Does not assimilate sodium nitrite. Thiamin is required for growth. Starch-like substances are produced. Growth does not occur on 50 % (w/w) glucose/yeast extract agar. Urease-positive. Does not liquefy gelatin. Does not hydrolyse fat. Does not produce acid from glucose. The diazonium blue B reaction is positive. The G+C content of nuclear DNA is 60·7 mol % (by HPLC). The major ubiquinone is Q-9. Xylose is present in the cells. Maximum growth temperature is 33–34 °C. Physiological and chemotaxonomic characteristics are summarized in Table 4.

The type strain, IJFM 502^T (= CBS 2839^T = JCM 1460^T = NRRL Y-2478^T), was isolated in France by C. Ramírez Gómez from a rotten toadstool (Fell & Statzell-Tallman, 1998).

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