Cryptococcus mujuensis sp. nov. and *Cryptococcus cuniculi* sp. nov., basidiomycetous yeasts isolated from wild rabbit faeces

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Two previously undescribed anamorphic yeasts, strains T-11^T and T-26^T, recovered from wild rabbit faecal pellets collected in Muju, Korea, were identified using phenotypic and molecular taxonomic methods. The isolates were characterized by the proliferation of budding cells, positive diazonium blue B and urease reactions, the presence of Q-10 as the major ubiquinone, the presence of xylose in whole-cell hydrolysates and the inability to ferment sugars. Phylogenetic analyses based on 26S rRNA gene partial sequences revealed that strain T-11^T was located in the *Bulleromyces* clade and was related to *Sirobasidium intermedium*, *Tremella exigua*, *Cryptococcus cellulolyticus* and *Bullera pseudoalba*. Strain T-26^T was located in the Mesenterica clade and was closely related to *Cryptococcus* sp. F6 and *Cryptococcus heveanensis* CBS 8976. Sequence divergence values of more than 4 % from other described *Cryptococcus* species, together with the phenotypic differences, showed that the isolated yeasts represent previously unrecognized members of this genus. Therefore, two novel yeast species are proposed: *Cryptococcus mujuensis* sp. nov., with strain T-11^T (=KCTC 17231^T=CBS 10308^T) as the type strain, and *Cryptococcus cuniculi* sp. nov., with strain T-26^T (=KCTC 17232^T=CBS 10309^T) as the type strain.

Probiotics have been defined as live microbial food supplements that affect the host beneficially by improving the intestinal microbial balance or 'living micro-organisms that, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition' (Guarner & Schaafsma, 1998). While screening wild rabbit faeces for probiotic yeasts for consumption by farm animals, a number of yeast strains were isolated, two of which exhibited very low genetic relatedness to any previously described species of the genus *Cryptococcus*. Accordingly, this paper describes the phenotypic, biochemical and genotypic characterizations of these novel strains and, based on a comparative analysis with other related species, it is proposed that they represent two novel species in the genus *Cryptococcus* Vuillemin.

Strains T-11^T and T-26^T were isolated from faecal pellets left by wild rabbits on a hill in Muju, Korea in 2002. One gram of

each sample was suspended in 9 ml sterile water and stirred for 10 min. The yeasts were isolated after picking from plates of YM agar (Difco) supplemented with 5 µg gentamicin ml⁻¹ and 16 µg chloramphenicol ml⁻¹ spread with a suitable diluted suspension from the samples. The strains were then purified by streaking an isolated colony onto YM agar without antibiotics and incubating at 25 °C. Thereafter, the strains were maintained at 4 °C. Cell suspensions were stored at -80 °C in broth cultures supplemented with 10 % (w/v) glycerol for long-term preservation.

Phenotypic tests were performed in duplicate using the standard protocols described by Yarrow (1998). Utilization of carbon and nitrogen sources in liquid yeast nitrogen base (Difco) and yeast carbon base (Difco) was examined at 25 °C on a rotary shaker rotating at a speed of 100 r.p.m., whereas growth at different temperatures was determined on YM agar. The extraction and purification of ubiquinones was carried out according to Yamada & Kondo (1973) and ubiquinone isoprenologues were identified by reverse-phase TLC (HPTLC RP-18 F254; Merck), based on comparing

The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA gene D1/D2 domain sequences of strains T-11^T and T-26^T are DQ333884 and DQ333885, respectively.

their R_F values with those of standard ubiquinones. Xylose in the cells was investigated by TLC analysis, as reported by Komagata & Suzuki (1987). Genomic DNA isolation and PCR amplification of the D1/D2 region of the 26S rRNA gene was performed using protocols described by Fell *et al.* (2000). Amplified fragments were purified using a QIAquick PCR purification kit (Qiagen) and directly sequenced using an ABI Taq Dye Deoxy Terminator cycle sequencing kit and ABI 310 DNA sequencer (Applied Biosystems). The resultant sequences were automatically aligned using the multiple-sequence alignment program CLUSTAL X (Thompson *et al.*, 1997) and subsequently manually corrected. Phylogenetic relationships were then calculated using the program package PHYLIP 3.57c (Felsenstein, 1995), a distance matrix was obtained using the program DNADIST and an unrooted phylogenetic tree was constructed using neighbour-joining (Saitou & Nei, 1987) and the program NEIGHBOR. Bootstrap values (Felsenstein, 1985) were calculated from 1000 replicates; *Tremella encephala* CBS 6968 (AF189867) was the designated outgroup in the analysis and other related sequences were obtained from GenBank.

Eight faecal pellets were collected in one sampling in March 2002. A total of 49 yeast isolates was obtained from the wild rabbit faecal samples. The temperatures of the faecal samples ranged from 15 to 18 °C. The 49 isolates could be distinguished by molecular means based on sequences of the D1/D2 domain of the 26S rRNA gene. Most of the strains present in the samples revealed a basidiomycetous affinity and were identified as *Cryptococcus victoriae* ($n=25$), *Bullera armeniaca* ($n=1$), *Rhodotorula pinicola* ($n=1$) and *Cryptococcus* species ($n=15$). The others were identified as *Wallrothiella subiculosa* ($n=7$). Two of the yeast isolates, T-11^T and T-26^T, were selected for further analysis on the basis of their 26S rRNA gene D1/D2 nucleotide sequences, which were very different and displayed 4% or greater sequence divergence from established species in comparative 26S rRNA gene partial sequencing. A tree constructed by the neighbour-joining method and depicting the phylogenetic position of these unknown yeasts among Tremellales from Hymenomycetes is shown in Fig. 1. Strain T-11^T was found in the *Bulleromyces* clade of Tremellales and its closest phylogenetic relatives were *Sirobasidium intermedium* CBS 7805 (95.8% similarity; differed by 24 nt substitutions and 2 nt indels), *Tremella exigua* RB 6623-15 (95.1% similarity; differed by 28 nt substitutions and 5 nt indels), *Cryptococcus cellulolyticus* CBS 8294^T and *Bullera pseudoalba* CBS 7227^T, which both differed by 23 nt (96.0%). Strain T-26^T grouped with *Cryptococcus* sp. F6 (96.7% sequence similarity; 19 nt differences) and *Cryptococcus heveanensis* CBS 8976 (96.0% similarity; 23 nt differences). *C. heveanensis* CBS 569^T was also related to strain T-26^T, with a sequence similarity of 94.1% (34 nt differences).

The two novel strains were characterized by their lack of ballistoconidia, the presence of xylose in cell hydrolysates and the presence of Q-10 as the major ubiquinone. No sexual states were observed in T-11^T and T-26^T cultures on

Fig. 1. Neighbour-joining tree based on D1/D2 divergent domains of large-subunit rRNA gene sequences of *Cryptococcus mujuensis* sp. nov. T-11^T, *Cryptococcus cuniculi* sp. nov. T-26^T and their closest relatives. Bootstrap values (%) above 70% from 1000 samples are shown. Accession numbers are given in parentheses. Bar, 0.01 nt substitutions per site. The outgroup sequences were from *Filobasidiella neoformans* var. *neoformans* CBS 132^T (AF075484) and *Filobasidium uniguttulatum* CBS 1730^T (AF075468).

cornmeal agar and 5% malt extract. Although their phenotypic and chemotaxonomic characteristics were consistent with those of members of the genus *Cryptococcus* (Fell & Statzell-Tallman, 1998), the isolates could be differentiated phenotypically from other *Cryptococcus* species described to date. Strain T-11^T differed from *C. cellulolyticus* CBS 8294^T in its assimilation of erythritol, growth in the presence of 0.01% cycloheximide and very weak growth at 30 °C. It also differed from *B. pseudoalba* CBS 7227^T in its ability to utilize erythritol, to grow weakly at 30 °C and to produce starch-like substances, and its inability to utilize D-glucarate and to grow in the presence of 0.1% cycloheximide.

Strain T-26^T could be distinguished from *C. heveanensis* CBS 569^T based on its inability to grow on methyl α -D-glucoside, raffinose, inulin, erythritol, DL-lactate, quinic acid and 50% (w/v) glucose and its ability to grow at 30 °C, on glycerol and D-tryptophan and in the presence of 0.01% cycloheximide. It also differed from *Bullera*

dendrophila CBS 6074^T in its inability to assimilate methyl α -D-glucoside and DL-lactate and to grow in 50 % (w/v) glucose and its ability to assimilate L-sorbose, cadaverine and D-tryptophan and to grow in 10 % (w/v) NaCl and 0.01 % cycloheximide.

Results of the polyphasic taxonomic analysis clearly showed that the yeasts isolated from the faecal pellets of wild rabbits represented hitherto unrecognized species within the genus *Cryptococcus*. Thus, based on the phenotypic and molecular data presented above, it is proposed that strains T-11^T and T-26^T be classified as representatives of novel species within the genus *Cryptococcus*: *Cryptococcus mujuensis* (type strain T-11^T=KCTC 17231^T=CBS 10308^T) and *Cryptococcus cuniculi* (type strain T-26^T=KCTC 17232^T=CBS 10309^T).

Latin diagnosis of *Cryptococcus mujuensis* Shin et Park sp. nov.

In agar YM post dies 3 ad 25 °C cellulae ovoideae aut ellipsoideae (2.8–5.4 × 2.1–4.0 µm), singulae, aut binae, per germinationem multipolarem reproducentes. In YM (Difco) liquido post unum mensem ad 20 °C, pellicula et sedimentum formantur. Cultura pallida, albida vel crenea, glabra, nitida, butyrosa et margine glabra. Assimilantur D-glucosum, D-galactosum, D-glucosaminum, D-ribosum, D-xylosum, L-arabiosum, D-arabiosum, L-rhamnosum, sucrosum, maltosum, trehalosum, methyl α -D-glucosidum, cellobiosum, salicinum, arbutinum, melibiosum (lente), lactosum, raffinolum, melezitolum, amyllum solubile, glycerolum (lente), erythritolum, ribitolum, xylitolum, L-arabitolum, D-glucitolum, D-mannitolum, galactitolum, myo-inositolum, glucono- δ -lactonum, acidum 2-keto-D-gluconicum, acidum 5-keto-D-gluconicum, acidum gluconicum, acidum glucuronicum, acidum galacturonicum, acidum DL-lacticum (lente, infirme) et acidum succinicum. Non assimilantur L-sorbosum, inulinum, methanolum, propane-1,2-diolum, butane-2,3-diolum, acidum quini et acidum saccharicum. Assimilantur ethylaminum, L-lysinum (lente), cadaverinum et glucosaminum. Non assimilantur kallii nitratum, sodii nitritum, creatinum, creatininum, imidazolium et D-tryptophan. Vitaminae externae ad crescentiam necessariae sunt. Crescit in 0.01 % cycloheximido et 10 % NaCl/5 % glucosum. Non crescit in 50 % glucosum, 0.1 % cycloheximido et 16 % NaCl/5 % glucosum. Urea finditur. Diazonium caeruleum B positivum. Materia amyloidea iodophila formantur. Crescit in 30 °C; non crescit in 35 °C. Ubiquinonum majus: Q-10. Typus depositus in collectione Korean Collection for Type Cultures (T-11^T).

Description of *Cryptococcus mujuensis* Shin & Park sp. nov.

Cryptococcus mujuensis (mu.ju.en'sis. N.L. masc. adj. *mujuensis* of Muju, referring to the name of the region where the type strain was isolated).

After growth on YM agar for 3 days at 25 °C, cells are ovoid to ellipsoid (2.8–5.4 × 2.1–4.0 µm) and occur singly or in

pairs. Budding is multilateral. In YM broth, after 1 month at 20 °C, sediment and a pellicle are present. Streak culture is pale, white to cream, smooth, glistening and butyrous, with an entire margin. D-Glucose, galactose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, trehalose, methyl α -D-glucoside, cellobiose, salicin, arbutin, melibiose (delayed), lactose, raffinose, melezitose, soluble starch, glycerol (delayed), erythritol, ribitol, xylitol, L-arabitol, D-glucitol, D-mannitol, galactitol, myo-inositol, glucono- δ -lactone, 2-ketogluconic acid, 5-ketogluconic acid, D-gluconate, D-glucuronate, D-galacturonic acid, DL-lactate (delayed, weak) and succinic acid are assimilated. L-Sorbose, inulin, methanol, propane-1,2-diol, butane-2,3-diol, quinic acid and saccharate are not assimilated. Ethylamine hydrochloride, L-lysine (delayed), cadaverine and glucosamine are assimilated. Potassium nitrate, sodium nitrite, creatine, creatinine, imidazole and D-tryptophan are not assimilated. Does not grow in vitamin-free medium. Grows in 0.01 % cycloheximide and 10 % NaCl/5 % glucose. Does not grow in 50 % glucose, 0.1 % cycloheximide and 16 % NaCl/5 % glucose. Positive for urease activity and diazonium blue B reaction. Produces starch-like compounds. Grows (very weakly) at 30 °C, but does not grow at 35 °C. The major ubiquinone is Q-10.

The type strain is T-11^T (= KCTC 17231^T=CBS 10308^T), isolated from wild rabbit faecal samples collected on a hill in Muju, Korea.

Latin diagnosis of *Cryptococcus cuniculi* Shin et Park sp. nov.

In agar YM post dies 3 ad 25 °C, cellulae sphaeroidae, ovoideae aut ellipsoideae, 2.1–5.8 × 1.8–5.0 µm, singulae vel binae; gemmatione bipolari reproducentes. Cultura cremae aut xanthoalba, glabra, nitida, butyrosa et margine glabra. In medio liquido YM post unum mensem ad 20 °C, annulus tenuis et sedimentum formantur. In agar farina Zeae maydis confecto pseudomycelium rudimentarium. D-Glucosum, D-galactosum, L-sorbosum (lente), D-ribosum, D-xylosum, L-arabiosum, D-arabiosum, L-rhamnosum, sucrosum, maltosum, trehalosum, cellobiosum, arbutinum, lactosum, melezitolum, amyllum solubile, glycerolum (lente), xylitolum, L-arabitolum (lente), D-glucitolum, D-mannitolum, galactitolum, myo-inositolum, glucono- δ -lactonum, acidum 2-keto-D-gluconicum, acidum 5-keto-D-gluconicum, acidum gluconicum, acidum glucuronicum, acidum galacturonicum, acidum succinicum, ethanolum, acidum saccharicum assimilantur, at non D-glucosaminum, methyl α -D-glucosidum, melibiosum, raffinolum, inulinum, erythritolum, acidum DL-lacticum, methanolum, propane-1,2-diolum, butane-2,3-diolum nec acidum quini. Ethylaminum, L-lysinum, cadaverinum et D-tryptophanum (lente) assimilantur, at non kallii nitratum, sodii nitritum, creatinum, creatininum, glucosaminum nec imidazolium. Vitaminae externae ad crescentiam necessariae sunt. Diazonium caeruleum B positivum. Urea finditur. In medio cum 50 % glucose crescit. Amyli instar

composite creantur. Crescit in 30 °C; non crescit in 35 °C. Systema coenzymatis Q-10 adest. Typus depositus in collectione Korean Collection for Type Cultures (T-26^T).

Description of *Cryptococcus cuniculi* Shin & Park sp. nov.

Cryptococcus cuniculi (cu'ni.cu.li. L. gen. n. *cuniculi* of a rabbit, referring to the animal from faecal pellets of which the type strain was isolated).

After growth on YM agar for 3 days at 25 °C, cells are spherical, ovoid to ellipsoidal, 2·1–5·8 × 1·8–5·0 µm, single or in pairs. Budding is bipolar. Streak culture is cream to yellowish-white, smooth, glistening and butyrous, with an entire margin. In YM broth after 1 month at 20 °C, sediment and a ring are formed. In Dalmau plate cultures on cornmeal agar after 2 weeks, no hyphae or pseudohyphae are formed. The following carbon compounds are assimilated: D-glucose, galactose, L-sorbose (delayed), D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, trehalose, cellobiose, arbutin, lactose, melezitose, soluble starch, glycerol (delayed), xylitol, L-arabitol, D-glucitol, D-mannitol, galactitol, *myo*-inositol, glucono-δ-lactone, 2-ketogluconic acid, 5-ketogluconic acid, D-gluconate, D-glucuronate, D-galacturonic acid, succinic acid, ethanol and saccharate. No growth occurs on D-glucosamine, methyl α-D-glucoside, melibiose, raffinose, inulin, erythritol, DL-lactate, methanol, propane-1,2-diol, butane-2,3-diol or quinic acid. Does not assimilate potassium nitrate, sodium nitrite, creatine, creatinine, glucosamine or imidazole. Assimilates ethylamine hydrochloride, L-lysine, cadaverine and D-tryptophan (delayed). Does not grow in vitamin-free medium. Growth in the presence of 10 % NaCl/5 % glucose is delayed and weak. Positive for the diazonium blue B reaction and urease activity. Growth in the presence of 0·01 % cycloheximide is delayed and positive. Does not grow on 50 % (w/w) glucose/yeast extract agar. Produces starch-like compounds. Grows at 30 °C (very weak), but not at 35 °C on YM agar. The ubiquinone type is Q-10.

The type strain is T-26^T (=KCTC 17232^T=CBS 10309^T), isolated from rabbit faecal samples collected on a hill in Muju, Korea.

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References

- Fell, J. W. & Statzell-Tallman, A. (1998). *Cryptococcus* Vuillemin. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 742–767. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1995). PHYLIP (phylogeny inference package), version 3.57c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Guarner, F. & Schaafsma, G. J. (1998). Probiotics. *Int J Food Microbiol* **39**, 237–238.
- Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–206.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Yamada, Y. & Kondo, K. (1973). Coenzyme Q system in the classification of the yeast genera *Rhodotorula* and *Cryptococcus*, and the yeast-like genera *Sporobolomyces* and *Rhodospiridium*. *J Gen Appl Microbiol* **19**, 59–77.
- Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.