Cryptococcus pinus sp. nov., an anamorphic basidiomycetous yeast isolated from pine litter

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A novel species of the genus *Cryptococcus*, isolated from dead needles of *Pinus sylvestris*, was identified using mycocolotyping and rDNA sequence data. Phylogenetic analysis showed that the novel species was located in the *Kwoniella* clade of the Tremellales and was closely related to *Cryptococcus dejecticola*. The type strain of the novel species, *Cryptococcus pinus* sp. nov., is VKM Y-2958^T (=CBS 10737^T).

Basidiomycetous yeasts of the genus *Cryptococcus* Vuillemin have been isolated from different environments and substrates and have a worldwide distribution (Golubev, 2002; Rosa & Peter, 2006). Molecular studies have revealed the paraphyletic character of this genus that encompass species mainly within the hymenomycetous orders Tremellales and Filobasidiales, but also some species in the orders Trichosporonales and Cystofilobasidiales (Scorzetti *et al.*, 2002). Moreover, so called 'ubiquitous' species [e.g. *Cryptococcus albidus* (Saito) Skinner and *Cryptococcus laurentii* (Kufferath) Skinner] are also taxonomically heterogeneous and represent groups of several distinct species (Fonseca *et al.*, 2000; Takashima *et al.*, 2003).

During a survey of the yeast community colonizing dead needles of pine litter, many isolates could be identified as *C. laurentii*, *Cryptococcus flavescentis* (Saito) Skinner or *Cryptococcus magnus* (Lodder & Kreger-van Rij) Baptist & Kurtzman according to taxonomic keys and standard criteria used for differentiation (Fell & Statzell-Tallman, 1998; Barnett *et al.*, 2000). However, mycocolotyping showed that the isolates differed from the type strains of these species and from other members of the *C. laurentii* complex: *Cryptococcus aureus* (Saito) Takashima *et al.*, *Cryptococcus carnescentis* (Verona & Luchetti) Takashima *et al.* and *Cryptococcus peneaus* (Phaff *et al.*) Takashima *et al.* Subsequent comparative studies of the new isolates by sequencing of the D1/D2 domains of the large subunit (LSU) rDNA and of the ITS region revealed that they were

not conspecific with any known *Cryptococcus* species and should be assigned to a novel species.

The strains studied (isolation numbers: Ps-35, 38, 40A, 41, 51, 52, 55 and 63) were isolated from dead needles collected underneath *Pinus sylvestris* L. trees in a mixed forest (Moscow region, Russia) in October 2006. Serial dilutions of needle washings were plated onto pine needle agar and incubated at room temperature for 1–2 weeks. Needle infusion agar was prepared in the following manner: 200 g of dead pine needles was boiled in 1 l of water for 10 min. The liquid was filtered through gauze and water was added to the filtrate to make up 1 l. After adding 20 g of agar, the medium was autoclaved for 15 min at 6.8 kg overpressure. After cooling to 50–60 °C, streptomycin (500 mg) was added and the medium was poured into sterile Petri dishes. Other strains used in this study were from the Russia Collection of Microorganisms (VKM; <http://www.vkm.ru/>). For morphological and physiological characterization, standard methods currently employed in yeast taxonomy were used (Yarrow, 1998).

Genomic DNA was extracted using MasterPure yeast DNA purification kit (Epicentre Biotechnologies). The primers used for PCR amplification and sequencing of the D1/D2 region of the 26S rDNA were NL-1 (5'-GCATATCA-ATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTG-TTCAAGACGG-3'). The ITS 5.8S region was amplified and sequenced with primers ITS1 (5'-TCCGTAGGTG-AACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTG-ATATGC-3'). The reaction mixture contained 200 µM of each dNTP, 25 pmol of each primer, 2.5 U *Taq* DNA polymerase and 200 ng genomic DNA. The mixture was buffered with 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂ and 0.1 % Triton X-100. The amplification was carried out in a PTC-100 programmable thermal controller (MJ Research Inc.). The PCR reaction consisted of an initial denaturation for 5 min at 94 °C followed by 35

Abbreviation: LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 region and ITS region of the rDNA gene of *Cryptococcus pinus* sp. nov. are EF672245 and EF672246, respectively.

A phylogenetic tree of *Cryptococcus pinus* sp. nov. and closely related species based on the alignment of ITS sequences is available as a supplementary figure with the online version of this paper.

cycles of 1 min denaturation at 94 °C, annealing for 30 s at 55 °C and extension for 1 min at 72 °C. The final extension step was carried out at 72 °C for 5 min. The PCR products were purified with the High Pure PCR product purification kit (Roche Diagnostics). Sequencing of both strands was performed with an ABI 3100 sequencer (Applied Biosystems). The sequence data were deposited in the National Center for Biotechnology Information (NCBI) online service database.

The phylogenetic position of the yeast under study was estimated using the maximum-parsimony, neighbour-joining and Bayesian phylogenetic reconstruction methods based on the D1/D2 and ITS1-5.8S-ITS2 sequences, independently. DNA sequences were aligned using CLUSTAL_X v1.83 (Thompson *et al.*, 1994). The effect of alignment (removing ambiguous regions, manual corrections) on phylogenetic inference was tested by comparing tree topologies obtained by independent reconstructions. The topology was generally not affected by the inclusion or exclusion of unalignable regions or gaps as separate characters. The results of the Bayesian reconstruction are presented. The symmetrical model with a gamma rate distribution across sites (SYM+G, Zharkikh, 1994) was suggested as an appropriate model of molecular evolution (MODELTEST v3.7, Posada & Crandall, 1998) for both regions. Bayesian phylogenetic inference was performed with MrBayes v3.1 (Ronquist & Huelsenbeck, 2003). For both data matrices, each search was performed in two independent runs with four simultaneous chains initializing with a flat prior distribution. Chains were run for two million generations where every 100 generations were sampled after a burning of 750 000 generations. Suitable convergence was achieved in all cases. Trees were rooted to *Filobasidiella neoformans* var. *neoformans* ATCC 32045, mating type α , serotype D (D1/D2 GenBank accession no. AF335984; ITS GenBank accession no. AF162916).

The procedure for determining mycocin sensitivity patterns has been described previously (Golubev *et al.*, 2006).

The strains studied were almost identical in cultural, morphological and physiological characteristics. Pairwise mixing of strains on cornmeal agar did not result in any mating reactions. The following properties of the novel isolates placed them in the genus *Cryptococcus*: asexual reproduction by budding, ballistoconidia and arthroconidia not produced, no alcoholic fermentation, a positive urease test, the assimilation of i-inositol and D-glucuronate, and the production of starch-like compounds. The strains did not utilize nitrate and resembled species of the *C. laurentii* complex in carbon assimilation profiles. However, significant differences in sensitivity to mycocins were found between the novel isolates and other species (Table 1). The taxonomic specificity of sensitivity to mycocins has already been shown (Golubev, 1998; Golubev & Golubeva, 2006) and the differences observed in this study suggested that the new strains represent a novel species.

Molecular sequence data confirmed this suggestion, further indicating that the new taxon is a sister group of *Cryptococcus dejecticola* Thanh *et al.* differing by six mismatches (out of 591 nucleotides) in the D1/D2 domain of the LSU rDNA (Fig. 1). More discrepancies (eight gaps and 21 nucleotide mismatches in 554 positions) were observed in the ITS region (see Supplementary Fig. S1. in IJSEM Online). Alternative sequence alignments affected only the placement of *Bullera dendrophila* but the *C. dejecticola*–*Cryptococcus pinus* group was supported by high posteriori probability in all cases.

Our isolates were also distinct from *C. dejecticola* (Thanh *et al.*, 2006) in the assimilation of inulin, erythritol, salicin, arbutin, soluble starch, ethanol and lactate, in the utilization of glucosamine as nitrogen source, and in the

Table 1. Mycocin sensitivity patterns of phenotypically resembling *Cryptococcus* species

Taxa: 1, *C. carnescens* VKM Y-720^T; 2, *C. pinus* sp. nov. VKM Y-2958^T; 3, *C. perniciosus* VKM Y-2905^T; 4, *C. nemorosus* VKM Y-2906^T; 5, *C. magnus* VKM Y-1550^T; 6, *C. peneaus* VKM Y-1291^T; 7, *C. aureus* VKM Y-328^T; 8, *C. flavescens* VKM Y-1595^T; 9, *C. laurentii* VKM Y-1665^T; 10, *C. heveanensis* VKM Y-1457^T. +, Sensitive; w, weakly sensitive; –, non-sensitive; x, not applicable.

Mycocinogenic strains	1	2	3	4	5	6	7	8	9	10
<i>Bullera hanna</i> VKM Y-2832 ^T	–	–	–	–	–	–	+	–	+	–
<i>Filobasidium capsuligenum</i> VKM Y-1439	–	–	–	–	–	–	–	–	w	+
<i>Cryptococcus podzolicus</i> VKM Y-2249	–	–	–	–	–	–	–	+	+	+
<i>Cryptococcus nemorosus</i> VKM Y-2906 ^T	–	–	–	x	–	–	–	+	+	+
<i>Bullera unica</i> VKM Y-2830 ^T	–	–	–	–	–	w	w	–	+	+
<i>Bullera alba</i> VKM Y-2829	+	–	–	–	–	+	–	+	+	+
<i>Cryptococcus laurentii</i> VKM Y-1628	–	–	+	w	+	–	–	+	–	w
<i>Cryptococcus podzolicus</i> VKM Y-2247	+	+	+	w	–	–	–	+	+	+
<i>Bullera sinensis</i> var. <i>lactis</i> VKM Y-2826 ^T	w	–	+	+	+	–	w	+	+	+
<i>Cystofilobasidium bisporidii</i> VKM Y-2700 ^T	–	+	–	+	w	+	+	+	–	+
<i>Cryptococcus laurentii</i> VKM Y-1665 ^T	–	+	+	+	+	w	+	+	x	+
<i>Cryptococcus perniciosus</i> VKM Y-2905 ^T	+	+	x	–	w	+	+	+	+	+

Fig. 1. Bayesian phylogenetic tree of *Cryptococcus pinus* sp. nov. and closely related species based on the alignment of D1/D2 LSU rDNA sequences. Posterior probabilities are also shown. Tree was rooted to *Filobasidiella neoformans*. The GenBank accession numbers of the sequences are indicated after the strain numbers. Bar, 0.1 substitutions per site.

ability to grow in vitamin-free medium. All these findings have led us to conclude that the strains under study cannot be assigned to any known species of the genus *Cryptococcus*. Consequently, the novel species *Cryptococcus pinus* sp. nov. is proposed.

Phylogenetically *C. pinus* is a close relative of the teleomorphic yeast *Kwonilella mangroviensis* Statzell-Tallman *et al.* (2008) (Fig. 1). Beside *C. pinus*, the *Kwonilella* clade includes the anamorphic tremellaceous species *Cryptococcus bestiolae* Thanh *et al.*, *C. dejecticola*, *Cryptococcus heveanensis* (Groeneweg) Baptist & Kurtzman and the ballistoconidium-forming yeast *Bullera dendrophila* van der Walt & Scott, serving as one more example of the mutual polyphyly of *Cryptococcus* and *Bullera* Derx species (Scorzetti *et al.*, 2002). It should be noted that cultures of *Bullera* spp. easily lose the ability to produce ballistoconidia, their main diagnostic characteristic. A search for clear-cut phenotypic discriminatory criteria that are congruent with sequence-based phylogenies is currently of prime importance for yeast taxonomy.

Latin diagnosis of *Cryptococcus pinus* Golubev et Pfeiffer sp. nov.

In aqua glucosum et peptonum et extractum fermenti continente, post dies 3 cellulae globosae, subglobose et ovoidae (2.6–8.5 × 3.4–12.8 µm), incapsulate, singulae vel binae. Post unum mensem sedimentum, annulus et insulae formantur. In agar morphologico (Difco) post unum mensem cultura in striis griseo-cremea, glabra, nitida, mucosa; margo integra. Ballistoconidia nullae. Nec mycelium nec pseudomycelium formatur. Status sexualis non apparet. Non fermentat. Glucosum, galactosum, α-methylglucosidum (lente), ribosum, xylosum, L-arabinosum, D-arabinosum (lente), L-rhamnosum (lente), sucrosum, maltosum, trehalosum (lente), cellobiosum, lactosum, melezitum, raffinum (lente), arbutinum (lente), salicinum (lente), amyllum (lente), ethanolum (lente), glycerolum, xylitolum (lente), arabitolum (lente), glucitolum, mannitolum, galactitolum, inositolum, glucuronatum, gluconatum, 2-ketogluconatum,

5-ketogluconatum, glucaratum (lente), succinatum, citratum (exiguum), lactatum (lente) et glycinum (exiguum) assimilantur neque glucosaminum, N-acetylglucosaminum, L-sorbosum, melibiosum, erythritolum, acidum quinicum et allantoinum. Assimilatio inulinum variabilis (exiguum aut nulla). Kalium nitosum, lysinum, ethylaminum (lente), glucosaminum, cadaverinum (lente) et tryptophanum assimilantur, kalium nitricum, creatinum et creatininum non assimilantur. Urea finditur. Vitamina externa at crescentiam necesseria sunt. Materia amyloidea iodophila formantur. Maxima temperatura crescentiae: 32 °C. Typus Ps-38^T isolates ex foliis acicularibus Pinus sylvestris L. delapsis (Rossia), conservatur in collectione micro-organismorum (=VKM Y-2958^T) Acad. Sci. Rossicum (Pushczino, Rossia) et in collectione zymotica (=CBS 10737^T) Centraalbureau voor Schimmelcultures (Utrecht, Hollandia).

Description of *Cryptococcus pinus* Golubev & Pfeiffer sp. nov.

Cryptococcus pinus (pi'nus. L. gen. n. *pinus* of pine, referring to the origin of the strains, which were isolated from pine litter).

After 3 days in glucose-yeast extract-peptone broth, cells are globose, subglobose and oval [width/length ratio 1.0–2.2 (mean 1.3)], 2.6–8.5 × 3.4–12.8 µm (mean 4.0 × 5.4 µm) with capsules and single or in pairs (Fig. 2). After 1 month, there is a sediment, a ring and islets. After 1 month, the streak culture on yeast morphology agar (Difco) is greyish cream, smooth, glistening and slimy with an entire margin. No ballistoconidia are observed. After 10 days in slide cultures on cornmeal agar, neither true mycelium nor pseudomycelium is produced. Fermentation is absent. The following carbon compounds are assimilated: D-glucose, galactose, methyl α-D-glucoside (slow), D-ribose, D-xylose, L-arabinose, D-arabinose (slow), L-rhamnose (slow), sucrose, maltose, trehalose (slow), cellobiose, lactose, melezitose, raffinose (slow), arbutin (slow), salicin (slow), soluble starch (slow), ethanol (slow), glycerol,

Fig. 2. Cells of *Cryptococcus pinus* sp. nov. VKM Y-2958^T grown on malt agar at 20 °C for 3 days. Phase-contrast. Bar, 5 µm.

xylitol (slow), L-arabitol (slow), sorbitol, mannitol, galactitol, i-inositol, D-glucuronate, D-gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, glucarate (slow), succinate, citrate (weak), lactate (slow) and glycine (weak). No growth occurs on D-glucosamine, N-acetylglucosamine, L-sorbose, melibiose, erythritol, quinic acid or allantoin. Assimilation of inulin is variable (weak or negative). Utilization of nitrogen compounds: positive for nitrite, L-lysine, ethylamine (slow), D-glucosamine, cadaverine (slow) and D-tryptophan, negative for nitrate, creatine and creatinine. Positive for urease activity. Does not grow in vitamin-free medium. Produces starch-like compounds. Maximum temperature for growth is 32 °C. Strains are sensitive to the mycocins produced by *Cryptococcus laurentii* VKM Y-1665, *Cryptococcus perniciosus* VKM Y-2905, *Cryptococcus podzolicus* VKM Y-2247 and *Cystofilobasidium bisporeidii* VKM Y-2700, but are resistant to the mycocins of *Bullera alba* VKM Y-2829, *Bullera hanna* VKM Y-2832, *Bullera sinensis* var. *lactis* VKM Y-2826, *Bullera unica* VKM Y-2830, *Cryptococcus laurentii* VKM Y-1628, *Cryptococcus nemorosus* VKM Y-2906, *Cryptococcus podzolicus* VKM Y-2249, *Cystofilobasidium infirmominiatum* VKM Y-2897 and *Filobasidium capsuligenum* VKM Y-1439.

The type strain, Ps-38^T (=VKM Y-2958^T = CBS 10737^T), was isolated from dead needles of *Pinus sylvestris* L. in the Moscow region (Russia) and has been deposited in the Russia Collection of Microorganisms (Pushchino, Russia) and in the Yeast Collection of the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands).

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