Cryptococcus anemochoreius sp. nov., a novel anamorphic basidiomycetous yeast isolated from the atmosphere in central South Africa

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A novel yeast strain, CBS 10258^T, was isolated from the atmosphere in central South Africa. Sequence analysis of the D1/D2 domain and internal transcribed spacer region of the novel strain indicates that it represents a novel species within the *Cryptococcus laurentii* complex. Phylogenetic analyses based on the D1/D2 domain revealed that the novel strain occupies a relatively isolated position within this complex with *Papiliotrema bandonii*, *Cryptococcus perniciosus*, *Cryptococcus nemorosus* and *Cryptococcus* sp. CBS 8363 being the closest relatives. However, the novel strain could be distinguished from related species by standard physiological tests including the inability to assimilate rhamnose, methyl α -D-glucoside, salicin, lactose, erythritol, ribitol, xylitol, citrate and ethanol. In addition, no extracellular starch production was observed and the isolate was able to grow in the absence of additional vitamins. On the basis of these results, we suggest that the new strain represents a novel species for which the name *Cryptococcus anemochoreius* sp. nov. is proposed [type strain CBS 10258^T (=NRRL Y-27920^T)].

Members of the anamorphic basidiomycetous genus *Cryptococcus* Vuillemin are characterized by their ability to assimilate D-glucuronate, their lack of fermentative ability, the presence of xylose in cell hydrolysates, positive urease and Diazonium blue B reactions as well as the presence of coenzymes Q-9 and Q-10 (Fell & Statzell-Tallman, 1998). Most species produce extracellular starch and those that do not, utilize inositol. It is well known that the genus *Cryptococcus* is polyphyletic, occurring in the Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). In addition, members of this genus have been isolated from a diverse range of habitats worldwide and several reports exist for the isolation of *Cryptococcus* species, such as *Cryptococcus aerius*, *Cryptococcus albidus*, *Cryptococcus flavus*, *Cryptococcus laurentii*, *Cryptococcus luteolus* and *Cryptococcus magnus*, from the atmosphere (Fell & Statzell-Tallman, 1998). It is also known that the pathogenic *Cryptococcus* species, *Cryptococcus neoformans*, often occurs

in the air as small, respirable particles (Ruiz & Bulmer, 1981; Sukroongreung *et al.*, 1999).

Due to the prevalence of HIV in the mining workforce of South Africa, a study of the diversity of culturable airborne fungi (which may pose health risks to immunocompromised workers) was conducted in an active gold mine. During this study, comparative samples of the atmosphere outside were taken in order to establish a possible source of the fungal species found indoors. Of the isolates obtained from the atmosphere outside the mine, 25.9% were yeasts. The majority of these isolates belong to known species in the genera *Candida*, *Rhodotorula*, *Filobasidium* and *Cryptococcus*. However, a novel anamorphic basidiomycetous isolate was obtained and we propose the name *Cryptococcus anemochoreius* sp. nov. for this isolate on the basis of morphological, physiological and molecular characteristics.

Since this species was isolated from the atmosphere, it is difficult to speculate on the ecology of *C. anemochoreius*. However, trees belonging to the genera *Pinus*, *Rhus*, *Schinus* and *Eucalyptus* grow in the vicinity of the sampling site and since several reports exist regarding the association of *Cryptococcus* species with trees, especially *Eucalyptus* (Ellis & Pfeiffer, 1990; Sorrell & Ellis, 1997; Fortes *et al.*, 2001; Randhawa *et al.*, 2001; Granados & Castaneda, 2005), it is

The GenBank/EMBL/DDBJ accession number for the 26S rDNA D1/D2 and internal transcribed spacer region sequences of strain CBS 10258^T are DQ384929 and DQ830986, respectively.

An additional phylogenetic tree based on the sequences of the internal transcribed spacer region of strain CBS 10258^T and related species is available as a supplementary figure in IJSEM Online.

Fig. 1. Phylogenetic tree derived from neighbour-joining analysis based on the 26S rDNA D1/D2 region showing the placement of *Cryptococcus anemochoreius* sp. nov. in the *Cryptococcus laurentii* phylogenetic group 1 complex (Sugita *et al.*, 2000; Takashima *et al.*, 2003). Bootstrap percentage (1000 replicates) of 50 % or greater are shown. The three species of *Filobasidium* were included to root the tree. Bar, 1 % nucleotide sequence divergence.

tempting to speculate that *C. anemochoreius* may be associated with one or more of these trees.

Yeast isolation and characterization

Air samples were taken in June 2004 in the Free State province in central South Africa using a SAS Super 90 single stage impactor. The sampling time was 30 seconds at 30 l min⁻¹. In order to facilitate the isolation of yeasts, rose Bengal agar (5.0 g l⁻¹ peptone, 10.0 g l⁻¹ glucose, 1.0 g l⁻¹ potassium phosphate, 0.5 g l⁻¹ magnesium sulphate, 0.05 g l⁻¹ rose Bengal, 0.1 g l⁻¹ chloramphenicol, 15.0 g l⁻¹ agar) was used. Plates were incubated at 25 °C and yeast colonies were purified on 2 % yeast-malt extract (YM) agar. Morphological and physiological tests were performed in duplicate according to standard techniques

(Yarrow, 1998). In addition, scanning electron microscopy was performed on the isolate using a JEOL 6400 WIN-SEM instrument (Van Wyk & Wingfield, 1994).

rDNA sequence analyses

The 26S rDNA D1/D2 and internal transcribed spacer regions were PCR amplified and sequenced using primer pair NL1 (5'-GCATATCAATAAGCGGAGGAAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman & Robnett, 1998) and primer pair ITS4 (5'-TCCTCCG-CTTATTGATATGS-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al.*, 1990), respectively. The resulting sequence was compared with sequences obtained from GenBank. Sequences for phylogenetic analysis were aligned using CLUSTAL_X 1.83 (Thompson *et al.*, 1997).

Table 1. Salient characteristics of *Cryptococcus anemochoreius* sp. nov. and related species

Strains: 1, *Cryptococcus anemochoreius* sp. nov. CBS 10258^T; 2, *Cryptococcus nemorosus* VKM Y-2906^T (data from Golubev *et al.*, 2003); 3, *Cryptococcus perniciosus* VKM Y-2905^T (Golubev *et al.*, 2003); 4, *Cryptococcus laurentii* (Fell & Statzell-Tallman, 1998); 5, *Papiliotrema bandonii* PYCC 5473 (Sampaio *et al.*, 2002). +, Positive; –, negative; D/W, delayed or weak; ND, not determined.

Characteristic	1	2	3	4	5
Assimilation of:					
Citrate	–	+	D/W	+	+
Erythritol	–	–	–	+	–
Ethanol	–	+	D/W	+	D/W
Lactose	–	+	+	+	+
Methyl α -D-glucoside	–	D/W	D/W	+	+
Rhamnose	–	+	D/W	+	D/W
Ribitol	–	+	D/W	+	+
Salicin	–	+	+	+	+
Xylitol	–	+	+	+	+
Starch formation	–	+	+	+	+
Growth:					
Without vitamins	+	–	–	–	–
With glucose (50 %)	–	+	+	–	ND

Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 3.1 (Kumar *et al.*, 2004) using the neighbour-joining method with the Kimura two-parameter distance measure. Confidence values were estimated from bootstrap analysis of 1000 replicates. The three species of *Filobasidium* were used as outgroup.

Species delineation and identification

The D1/D2 sequence was compared with related sequences and phylogenetic analyses (Fig. 1) indicated that *Cryptococcus anemochoreius* represents a novel species in the *Cryptococcus laurentii* phylogenetic group 1 as described

Fig. 2. Scanning electron micrograph of cells of *Cryptococcus anemochoreius* indicating polar budding of globose to elongate cells. Bar, 1 μ m.

by Sugita *et al.* (2000) and Takashima *et al.* (2003). Within the *C. laurentii* phylogenetic group 1, *C. anemochoreius* occupies a relatively isolated position with *Papiliotrema bandonii* (20 nucleotide differences), *Cryptococcus perniciosus* (25 nucleotide differences), *Cryptococcus nemorosus* (21 nucleotide differences) and *Cryptococcus* sp. CBS 8363 (17 nucleotide differences) forming a subclade of nearest relatives. The relatively low bootstrap value between *C. anemochoreius* and the *P. bandonii* subclade (of 68 %) may increase with the isolation of more strains related to *C. anemochoreius*. Sequence data and phylogenetic analysis of the internal transcribed spacer region confirmed the positioning of *C. anemochoreius* in the *C. laurentii* phylogenetic group 1 and supported the recognition of *C. anemochoreius* as a novel isolate (a phylogenetic tree based on the internal transcribed spacer sequences is available as Supplementary Fig. S1 in IJSEM Online). Physiologically *Cryptococcus anemochoreius* can be distinguished from these related taxa as indicated in Table 1. Based on these differences, we conclude that the new isolate represents a novel species, *Cryptococcus anemochoreius* sp. nov.

Latin diagnosis of *Cryptococcus anemochoreius* Pohl, J. L. F. Kock, P. W. J. van Wyk et Albertyn sp. nov.

In medio liquido extracto fermenti et extracto malti confecto, post dies tres ad 25 °C, cellulae globosae ad cylindricae (2.8–6.4 μ m $\times$ 2.3–3.7 μ m). Cellulae polariter gemmantes, binae cohaerentes. In agar extracto fermenti et extracto malti confecto, post dies septem ad 25 °C, cultura glabra et salmonea, margine integra. In agar Dalmau extracto fermenti et extracto malti confecto nec pseudohyphae nec hyphae formantur. Non fermentat. D-Glucosum, D-galactosum, L-sorbosum, D-glucosaminum, D-ribosum (lente), D-xylosum, L-arabinosum, D-arabinosum, sucrosum, maltosum, trehalosum, cellobiosum, arbutinum, melibiosum, raffinose, melezitosum, inulinum, amylosum solubile, D-glucitol, D-mannitol, galactitol, inositol, D-gluconat, D-glucuronat, D-galacturonat, DL-lactat (lente) et succinat (lente) assimilantur. Rhamnosum, α -methyl-D-glucosidum, salicinum, lactosum, glycerolum, erythritolum, ribitol, xylitol, L-arabinitol, citratum, propan-1,2-diol, butan-2,3-diol, acidum malatum et acidum butyratum non assimilantur. Non crescit in methanolo vel ethanolo. L-Lysin, cadaverin et ethylaminum assimilantur neque nitratum, nitritum, creatininum, creatinum, glucosaminum et imidazolium. Ureum finditur. Materia amyloidea non formatur. Vitaminum externum crescentiae non necessarium. In medio 0.01 % cycloheximidum vel 1 % acidum aceticum vel 50 % glucosum continente non crescit. Non crescit 37 °C. Typus praeservatus in collectione zymotica Centraalbureau voor Schimmelcultures, Utrecht, Neerlandia (holotypus lyophilizatus CBS 10258^T) et in collectione zymotica Agricultural Research Services United States Department of Agriculture, Peoris, Illinois (isotypus NRRL Y-27920^T).

Description of *Cryptococcus anemochoreius* Pohl, J. L. F. Kock, P. W. J. van Wyk & Albertyn sp. nov.

Cryptococcus anemochoreius (a.ne.mo'cho.rei.us. Gr. n. *anemos* wind; Gr. adj. *choreios* of, or belonging to, a dance; N.L. masc. adj. *anemochoreius* referring to the windblown nature of the isolate).

In yeast-malt extract (YM) broth after 3 days of growth at 25 °C, cells are globose to elongate (2·8–6·4 µm × 2·3–3·7 µm). Polar budding is observed and cells occur as parent bud pairs (Fig. 2). On YM agar after 7 days at 25 °C, colonies are smooth with an entire margin and salmon coloured. In Dalmau plate cultures on YM agar, no pseudohyphae or true hyphae can be observed. No fermentation is observed. Able to assimilate D-glucose, D-galactose, L-sorbose, D-glucosamine, D-ribose (weakly), D-xylose, L-arabinose, D-arabinose, sucrose, maltose, trehalose, cellobiose, arbutin, melibiose, raffinose, melezitose, inulin, starch, D-glucitol, D-mannitol, galactitol, *myo*-inositol, D-gluconate, D-glucuronate, D-galacturonate, DL-lactate (weakly) and succinate (weakly), but not L-rhamnose, methyl α-D-glucoside, salicin, lactose, glycerol, erythritol, ribitol, xylitol, L-arabinol, citrate, propane-1,2-diol, butane-2,3-diol, malic acid or butyric acid. No growth is observed on methanol and ethanol as carbon sources. L-Lysine, cadaverine and ethylamine are utilized as nitrogen sources, but not nitrate, nitrite, creatinine, creatine, glucosamine or imidazole. Urea is hydrolysed, but no extracellular starch is produced. Vitamins are not necessary for growth, however, no growth is observed in the presence of cycloheximide (0·01 %), 1 % acetic acid or 50 % glucose. No growth is observed at 37 °C.

The type strain, CBS 10258^T (=NRRL Y-27920^T), is deposited in the yeast culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, with a copy at the yeast culture collection at the Agricultural Research Services United States Department of Agriculture, Peoria, Illinois.

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