Mrakiella cryoconiti gen. nov., sp. nov., a psychrophilic, anamorphic, basidiomycetous yeast from alpine and arctic habitats

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A novel psychrophilic basidiomycetous species is described in a new anamorphic genus as *Mrakiella cryoconiti* gen. nov., sp. nov.; the type strain of *Mrakiella cryoconiti* is strain A15^T (=CBS 10834^T =DSM 21094^T). Two representatives were isolated from alpine glacier cryoconite and from northern Siberian sediment. Physiological and biochemical properties are similar to characteristics shared by members of the genus *Mrakia*, although sexual reproduction is absent. *Mrakiella cryoconiti* strains are psychrophilic and produce cold-active pectate lyase. Sequence analyses of the ITS and 26S rRNA D1/D2 regions indicated that these strains represent a distinct taxon within the *Mrakia* clade of the order Cystofilobasidiales, class Tremellomycetes and phylum Basidiomycota. On the basis of phenotypic and genotypic characteristics, *Cryptococcus aquaticus* (a member of the *Mrakia* clade) is transferred to the newly described genus as *Mrakiella aquatica* comb. nov.

INTRODUCTION

The order Cystofilobasidiales (Fell *et al.*, 1999) includes teleomorph and anamorph genera. Psychrophily, i.e. the ability to grow and reproduce well at temperatures around 0 °C (Morita, 1975; Margesin *et al.*, 2008), is a typical feature of the teliospore-producing genus *Mrakia*; the lowest growth temperature for *Mrakia* strains was reported to be –12 °C (Panikov & Sizova, 2007). Members of this genus have been isolated from numerous low-temperature environments in various regions, such as forest substrates in European Russia, glacier-preserved fossil lichens in Greenland, Antarctic soil or snow, glacial meltwater rivers in Patagonia, Argentina, and alpine Italian subglacial sediments, ice and meltwater, but also from frozen fish in Japan and frozen yoghurt in Brazil (Fell & Statzell-Tallman, 1998; Moreira *et al.*, 2001; Bab'eva *et al.*, 2002; Xin & Zhou, 2007; Turchetti *et al.*, 2008). Some strains related to the species *Mrakia frigida* produce cold-active pectinolytic enzymes (Barnett *et al.*, 2000; Nakagawa *et al.*, 2004). These enzymes could be useful for a wide range of applications, such as the food industry for juice clarification below 5 °C or low-temperature pre-treatment of wastewater contain-

ing pectic substances. Two cold-active pectate-lyase-producing strains (Margesin *et al.*, 2005) were previously assigned to *Mrakia frigida* on the basis of internal transcribed spacer (ITS) and large-subunit (LSU) rRNA gene sequences. However, detailed investigation of the strains demonstrated the absence of teliospore production, which is characteristic of the genus *Mrakia*. In addition, ITS and LSU rRNA gene D1/D2 sequences indicate that these strains belong to a hitherto unknown genus and species, for which the name *Mrakiella cryoconiti* gen. nov., sp. nov. is proposed.

METHODS

Sample collection and isolation. Yeast strain A15^T was isolated from alpine glacier cryoconite collected from the Stubai glacier near Innsbruck in Tyrol, Austria (altitude 2900 m above sea level) (Margesin *et al.*, 2002). Strain AG25 originated from a sediment sample containing mud, spring water and moss, which was collected in the Gyda peninsula in northern Siberia (Gounot, 2001). Yeasts were maintained on R2A agar (Difco) plates; long-term storage was performed in 10 % (w/v) skimmed milk at –80 °C.

Physiological and biochemical characterization. Morphological, physiological and biochemical properties were determined according to Kurtzman & Fell (1998) and Barnett *et al.* (2000). Induction of the sexual stage was tested by incubating single or mixed cultures of the each of the two strains on cornmeal agar (CMA), Sabouraud glucose agar (SabG) or R2A agar (Difco) at 1 and 10 °C for 2 months. Assimilation of carbon and nitrogen compounds and growth requirements were tested at 10–15 °C. The effect of temperature

Abbreviations: DBB, diazonium blue B; ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the ITS domain and LSU rRNA gene D1/D2 domain sequences of strains A15^T and AG25 are AJ866976 and AJ866977 (ITS) and AJ866978 and AJ866979 (D1/D2), respectively.

was examined at 1–30 °C (5 °C intervals) in liquid culture and on agar plates; pectate lyase production was determined as described previously (Margesin *et al.*, 2005).

Phylogenetic analysis. Phylogenetic analysis was done by sequencing the ITS region and the 5' end of the LSU rRNA gene, including the variable domain D1 and D2. For DNA isolation, cells were harvested from 5-day-old subcultures and lyophilized. DNA was isolated by the CTAB (hexadecyltrimethylammonium bromide) method (O'Donnell *et al.*, 1997). Primers used for the two distinct PCRs and sequencing of the two fragments were ITS5 (5'-GG-AAGTAAAGTCGTAACAAGG-3'), IT2 (5'-CCTCCGCTTATTGATATGCTTAAG-3'), F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LR3 (5'-TCCTCCGCTTATTGATATGC-3'). Conditions for the two PCRs were identical. DNA was amplified through 35 cycles of 30 s at 92 °C, 30 s at 52 °C and 1 min at 72 °C. DNA sequencing was performed with primers ITS5 and IT2 using a Beckman-Coulter CEQ Dye Terminator Cycle Sequencing Quick Start kit. Heuristic maximum-parsimony analysis was employed (100 rounds of heuristic search with TBR branch swapping, starting from trees obtained by random addition of sequences, multrees option on, deepest descent option off) and was validated using 1000 rounds of bootstrap analysis (Felsenstein, 1985). Maximum-parsimony, neighbour-joining and bootstrap calculations used the PAUP* software (Swofford, 2001).

RESULTS AND DISCUSSION

Phenotypic properties of strains

Two representatives of *Mrakiella cryoconiti* gen. nov., sp. nov. were isolated from remote geographical, alpine and arctic locations (European alpine glacier, northern Siberia). Their physiological and biochemical properties were almost identical. The strains utilized D-glucuronate, myo-inositol and nitrate, produced starch-like compounds and lacked the capacity to utilize aromatic compounds (phenol), which are characteristics typical of the Cystofilobasidiales (Fell *et al.*, 1999; Sampaio, 2004). The strains also shared a number of properties with representatives of the teliospore-producing genus *Mrakia*, such as psychrophilic growth (maximum growth temperature 20 °C) and positive diazonium blue B (DBB) and urease reactions (Fell & Statzell-Tallman, 1998). However, attempts to induce the sexual stage were always negative. Asexual reproduction occurred by polar budding. Differentiating phenotypic characteristics of species of the genera *Mrakiella* and *Mrakia* are shown in Table 1.

Table 1. Differentiating phenotypic characteristics of species of the genera *Mrakiella* and *Mrakia*

Data were taken from Jones & Sloof (1966), Kurtzman & Fell (1998), Bab'eva *et al.* (2002), Xin & Zhou (2007), the CBS Data Base (<http://www.cbs.knaw.nl/>) and this study. All strains are positive for the following features: aerobic growth, asexual reproduction by polar budding, DBB reaction, formation of extracellular amyloid compounds, urea hydrolysis and assimilation of D-glucose, galactose, trehalose, D-xylose, L-arabinose, D-mannitol, D-gluconate, salicin and nitrate. All strains are negative for assimilation of erythritol. +, Positive; –, negative; v, variable; w, weak; d, delayed; ND, no data available; $T_{\max}$, maximum growth temperature.

Characteristic	<i>Mrakiella cryoconiti</i>	<i>Mrakiella aquatica</i>	<i>Mrakia curviuscula</i>	<i>Mrakia frigida</i> (type species)	<i>Mrakia gelida</i>	<i>Mrakia psychrophila</i>
$T_{\max}$ (°C)	20	20 ^a *, 21 ^b	25	17	17	18
Growth at 25 °C	–	–	+	–	–	–
Teliospores	–	–	+	+	+	+
Glucose fermentation	–	– ^{ab} , w ^c	–	+	+	+
Growth on (assimilation of):						
Raffinose	+	+	–	+	+	+
Lactose	+	+	+	v	–	+
Maltose	+	+	+	–	+	+
Melezitose	+	+	+	–	+	+
Soluble starch	+	+	v	–	+	+
D-Ribose	+	w, d, –	w	–	v	+
Glycerol	+	d	–	v	–	+
Inositol	+	–	–	v	v	+
Methanol	–	–	–	–	–	+
Citrate	–	+	+	v	v	–
Methyl α -D-glucopyranoside	–	–	–	–	v	+
D-Glucosamine	v	–	+	–	+	+
Vitamin-free medium	–	–	–	–	–	+
50 % Glucose	+	–	w/–	–	–	+
Splitting of arbutin	+	+	ND	+	+	–

*Data obtained from: a, this study; b, CBS Data Base; c, Jones & Sloof (1966).

Fig. 2. Neighbour-joining analysis (PAUP 4.0b10) of GenBank sequence data of strains related to *Mrakia* species based on ITS rRNA gene sequence analysis. Numbers on branches represent bootstrap percentages from 1000 replicates in a full heuristic search. Unnamed CBS strains represent sequences from Thomas-Hall (2005); uncultured, sequences from DePriest *et al.* (2000); the sequence of *Cryptococcus aquaticus* H2 was reported by Birgisson *et al.* (2003). *Guehomyces pullulans* CBS 2532^T is used as the outgroup. Bar, 0.005 substitutions per site.

(2005), whose strains are indicated by the unnamed CBS strains. Other studies include a strain from Iceland (H2; Birgisson *et al.*, 2003) and cloned sequences from fossil lichens in Greenland (uncultured strains; DePriest *et al.*, 2000).

As presently viewed, the *Mrakia* clade consists of six clusters: (i) *Mrakia psychrophila* from Antarctic soil (Xin & Zhou, 2007), with two Antarctic strains (Thomas-Hall, 2005); (ii) *Mrakia frigida*, with the synonym *Mrakia nivalis* and the type strain of *Cryptococcus curiosus*, whose sexual state was reported by Fell & Stätzell-Tallman (1998); (iii) an undescribed species that is represented by Antarctic strains (Thomas-Hall, 2005) and two cloned sequences from Greenland lichens (DePriest *et al.*, 2000); (iv) *Mrakia gelida* and strains from the Antarctic and cloned sequences from fossil lichens (DePriest *et al.*, 2000); (v) *Mrakiella cryoconiti*; and (vi) *Cryptococcus aquaticus* (*Mrakiella aquatica* comb. nov.) with a strain (H2) from Iceland that has an optimal temperature for growth of 9 °C and a $T_{\max}$ of 18 °C (Birgisson *et al.*, 2003). *Cryptococcus aquaticus* CBS 5443^T is representative of several Antarctic strains with similar or identical ITS sequences (NCBI BLAST and Thomas-Hall, 2005). An NCBI BLAST comparison of D1/D2 gene sequences of CBS 5443^T (GenBank accession no. AF075470) with CBS 8924 (AY029345) demonstrated 9 position differences. A similar ITS comparison (Fig. 2)

showed 5 differences. Mating studies between CBS 5443^T and CBS 8924 and the additional Antarctic strains were negative (S. Thomas-Hall, personal communication), which suggests the presence of separate species. However, comparative phenotypic and biochemical tests must be completed prior to the formal presentation of new taxa (S. Thomas-Hall, personal communication).

This ITS analysis (Fig. 2) shows that strains A15^T and AG25 represent a separate genotype (species) within the *Mrakia* clade. Due to the lack of an observed sexual state, the species should not be included within *Mrakia*; therefore, the species should be described as the anamorphic state. The genus *Cryptococcus* is the traditional nomenclature selected for anamorphic species in the Tremellomycetes. However, the type species, *Cryptococcus neoformans*, is a member of the order Tremellales. Therefore, in our opinion, the nomenclatural designation of *Cryptococcus* for species within the Cystofilobasidiales is not appropriate. As previously discussed and depicted in Fig. 1, the Cystofilobasidiales represents a diverse group of anamorphic and teleomorphic genera with strong bootstrap support for these generic groups. Consequently, we believe that *Mrakiella* should be confined to the *Mrakia* clade.

As demonstrated by D1/D2 and ITS rRNA gene sequence analyses (Figs 1 and 2; Fell *et al.*, 2000; Scorzetti *et al.*, 2002), *Cryptococcus aquaticus* is an anamorphic species in the *Mrakia* clade. The type strain (CBS 5443^T) was isolated from a freshwater lake in Great Britain. That strain was originally described (Jones & Sloof, 1966) with a $T_{\max}$ of 25 °C; however, our own investigations demonstrated the absence of growth at 25 °C but growth at 20 °C both in liquid culture and on agar plates in different media. The absence of growth at 25 °C is also indicated by the CBS Data Base (<http://www.cbs.knaw.nl/>) and Barnett *et al.* (2000). This discrepancy from the original strain description could be explained by culture methodology (unfortunately not indicated by Jones & Sloof, 1966), since microbial $T_{\max}$ can be influenced by a number of factors within the culture conditions, such as medium composition or cultivation in liquid or on solid media (Bowman *et al.*, 1998). As discussed previously, the *Cryptococcus aquaticus* cluster may represent several species. The relationship of this cluster and *Cryptococcus aquaticus* to the *Mrakia* clade demonstrates that *Cryptococcus aquaticus* should be included in the genus *Mrakiella*.

Latin diagnosis of *Mrakiella* Margesin et Fell gen. nov.

Fungi Cystofilobasidialium asexuales, affinitas cum Mrakia. Hyphae non formantur, pseudohyphae formentur. Flosculi sunt polares. Coloniae cremeae ad subfuscae in colouri. Nitratum assimilatur, materia amyloidea iodophila formatur. Reactiones DBB et ureasi positivae. Incrementum psychrophilicum (incrementum ad 1 °C respondet, incrementum ad 25 °C non respondet). Typus Mrakiella cryoconiti Margesin et Fell.

Description of *Mrakiella* Margesin & Fell gen. nov.

Mrakiella (Mra.ki.el'la. N.L. fem. n. *Mrakiella* referring to Professor E. M. Mrak).

Asexual members of the Cystofilobasidiales, which are closely related to the teleomorphic genus *Mrakia*. True hyphae are not produced, but pseudohyphae may be produced. Reproduction occurs by polar budding. Colonies are cream-coloured to light tan. Nitrate is assimilated, starch-like compounds are produced, DBB and urease reactions are positive. Psychrophilic growth characteristics (good growth at 1 °C, absence of growth at 25 °C). The type species is *Mrakiella cryoconiti* Margesin & Fell.

Latin diagnosis of *Mrakiella cryoconiti* Margesin et Fell sp. nov.

In agar SabG et CMA post 5 dies ad 15 °C, cellulae ovoideae (3–4 × 5–8 µm in agar SabG; 2–3 × 3–6 µm in agar CMA). Flosculi sunt polares. In agar CMA et SabG, coloniae cremeae in colori, rotundae, convexae et margine toto. Pseudohyphae et hyphae non formantur. Fermentatio (glucosum) nulla. Assimilat D-arabinosum, L-arabinosum, cellobiosum, D-galactosum (typus: exigue), D-glucosum, D-lactosum, maltosum, melezitium, palatinosum, raffinatum, D-ribosum, L-sorbose, sucrosum, trehalosum, D-xylosum, D-sorbitolum (=D-glucitolum), D-mannitolum, glycerolum, inositolum, ribitolum (=adonitolum), ethanolum, N-acetylglucosaminum, potassium 2-ketogluconatum, potassium 5-ketogluconatum, potassium gluconatum, sodium glucuronatum, succinatum, salicinum, nitratum, nitritum, L-lysinum. Non assimilatur erythritolum, galactitolum (=dulcitolum), DL-lactatum, methanolum, acidum laevulinicum, methyl- α -D-glucopyranosidum, phenolum (1 mM), cadaverinum. Thiaminum externum ad crescentiam necessarium est. Incrementum respondet in medio 50 % glucosum aut ampicillino (50 µg ml⁻¹). Non crescit in medio 1 % acido acetico aut 0.01 % cycloheximido. Materia amyloidea iodophila formatur. Reactiones ureasi, lipasi, proteasi, pectatum-lyasi, β -lactamasi, β -glucosidasi positivae sunt. Arbutinum finditur. Incrementum ad 1–20 °C respondet, ad 25 °C non respondet. Typus A15^T isolatus ex cryoconito glacialis, Stubai Glacier, Austria, depositus in collectione zymotica Centraalbureau voor Schimmelcultures, Utrecht, Nederland, CBS 10834^T (=DSM 21094^T).

Description of *Mrakiella cryoconiti* Margesin & Fell sp. nov.

Mrakiella cryoconiti (cry.o.co.ni'ti. N.L. gen. n. *cryoconiti* from cryoconite, referring to glacier cryoconite, where the type strain was found).

After 5 days of growth at 15 °C on SabG agar and CMA, the cells are ovoid (3–4 × 5–8 µm on SabG agar; 2–3 × 3–6 µm on CMA) (Fig. 3). Budding is polar. Colonies are creamy white on CMA and SabG agar. Colonies are round

Fig. 3. Yeast cells of *Mrakiella cryoconiti* A14^T after 9 days at 15 °C in SabG broth. Bar, 5 µm.

and convex, with entire margins. No pseudohyphae or true hyphae are formed. Fermentation ability (glucose) is negative. The following compounds are assimilated: D-arabinose, L-arabinose, cellobiose, D-galactose (type strain: weak), D-glucose, D-lactose, maltose, melezitose, palatinose, raffinose, D-ribose, L-sorbose, sucrose, trehalose, D-xylose, D-sorbitol (=D-glucitol), D-mannitol, glycerol, inositol, ribitol (=adonitol), ethanol, N-acetylglucosamine, potassium 2-ketogluconate, potassium 5-ketogluconate, potassium gluconate, sodium glucuronate, succinate, salicin, nitrate, nitrite and L-lysine. Erythritol, galactitol (=dulcitol), methanol, DL-lactate, laevulinic acid, methyl α -D-glucopyranoside (=methyl α -D-glucoside), phenol (1 mM) and cadaverine are not assimilated. Amino acids are not required for growth, but thiamine is required for growth. Growth occurs on 50 % glucose agar and in presence of ampicillin (50 µg ml⁻¹). No growth in the presence of 1 % acetic acid or cycloheximide (0.01 %). Formation of extracellular amyloid compounds, activities of urease hydrolysis, lipase (Tween 80), protease (skimmed milk), pectate lyase, β -lactamase, β -glucosidase (hydrolysis of aesculin) and splitting of arbutin are present. Growth occurs at 1–20 °C but not at 25 °C.

The type strain is A15^T (=CBS 10834^T =DSM 21094^T). Strains were isolated from alpine glacier cryoconite collected from the Stubai glacier near Innsbruck in Tyrol, Austria (A15^T), or a sediment sample containing mud, spring water and moss, collected in the Gyda peninsula in northern Siberia (AG25).

Mrakiella aquatica (Jones & Sloof) Margesin & Fell comb. nov.

Basionym: *Candida aquatica* Jones & Sloof. *Antonie van Leeuwenhoek* 32, 224, 1966.

Synonyms: *Vanrija aquatica* (Jones & Sloof) R. T. Moore (1980); *Cryptococcus aquaticus* (Jones & Sloof) Rodrigues de Miranda & Weijman (Weijman *et al.*, 1988).

Type strain: CBS 5443.

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