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 A15T (=CBS 10834T = DSM 21094T). 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 containing 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 A15T 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...
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-AAGTAAAAGTGCTAAACAGG-3′), IT2 (5′-CCTCCGCTATTGA-TATGCCTAAG-3′), F63 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and LR3 (5′-TCCTCCGTTATGGATATGC-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. 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 *Mrakia cryoncini* 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 *Mrakia* and *Mrakia* are shown in Table 1.

**Table 1. Differentiating phenotypic characteristics of species of the genera *Mrakia* 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-glucuronate, salicin and nitrate. All strains are negative for assimilation of erythritol. +, Positive; −, negative; v, variable; w, weak; d, delayed; ND, no data available; $T_{\text{max}}$, maximum growth temperature.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Mrakia cryoncini</em></th>
<th><em>Mrakia aquatica</em></th>
<th><em>Mrakia curviuscula</em></th>
<th><em>Mrakia frigida</em> (type species)</th>
<th><em>Mrakia gelida</em></th>
<th><em>Mrakia psychrophila</em></th>
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</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (°C)</td>
<td>20</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;, 21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25</td>
<td>17</td>
<td>17</td>
<td>18</td>
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<tr>
<td>Growth at 25 °C</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Teliospores</td>
<td>−</td>
<td>−</td>
<td>+ (?)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Growth on (assimilation of):</td>
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<tr>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>−</td>
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<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Soluble starch</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Ribose</td>
<td>+</td>
<td>w, d, −</td>
<td>w</td>
<td>−</td>
<td>v</td>
<td>+</td>
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<tr>
<td>Glycerol</td>
<td>+</td>
<td>d</td>
<td>−</td>
<td>v</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Inositol</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>v</td>
<td>v</td>
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<tr>
<td>Methanol</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Citrate</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>v</td>
<td>−</td>
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<tr>
<td>Methyl α-D-glucopyranoside</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>v +</td>
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<tr>
<td>D-Glucosamine</td>
<td>v</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Vitamin-free medium</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>50% Glucose</td>
<td>+</td>
<td>−</td>
<td>w/−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Splitting of arbutin</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data obtained from: a, this study; b, CBS Data Base; c, Jones & Sloof (1966).
**Phylogenetic placement**

Sequence analysis of the D1/D2 domain of the LSU rRNA gene of representative strains of species in Cystofilobasidiales (Fig. 1) demonstrates seven major groups. (i) The genus *Mrakia* (*Mrakia gelida, Mrakia frigida* and *Mrakia psychrophila*) is in a strongly supported (100%) clade with strains A15<sup>T</sup> and AG25 and the type strain of *Cryptococcus aquaticus* (CBS 5443<sup>T</sup>). As discussed later, the lack of bootstrap support for the *Mrakia* clade may be due to the genetic variability within and between species. (ii) The anamorphic genera *Tausonia* and *Guehomyces* are closely related. The major differences indicated by *Tausonia* are the formation of incomplete clamp connections and chlamydospores. (iii) The anamorphic ballistoconidia-forming genus *Udeniomyces* is represented by the type species *Udeniomyces pyricola* and two additional species. (iv) *Udeniomyces pannonicus* is closely related to *Itersonilia perplexans*, with significant differences exhibited by *I. perplexans*, with the formation of pseudoclamps and appressoria. (v) *Mrakia curviuscula*, which inhabits grasslands of eastern Russia (Bab’eva et al., 2002), has an identical D1/D2 sequence to *Cryptococcus huempii*. The latter species was isolated from an evergreen forest in Chile (Ramirez & Gonzalez, 1984). *Mrakia curviuscula* differs from other *Mrakia* species in several characteristics, including environmental habitat and *T<sub>max</sub>* of 25 °C (Bab’eva et al., 2002). The other members of the genus have a *T<sub>max</sub>* of 20 °C or less and they are generally isolated from low-temperature, ice-associated environments. *Mrakia curviuscula* is reported to produce a sexual, teliosporic state; however, the photographs presented by Bab’eva et al. (2002) suggest large cells rather than the typical *Mrakia*-type of teliospores. Our limited investigations of the species have not confirmed the presence of a sexual state in either *Mrakia curviuscula* or *Cryptococcus huempii*. Consequently, *Mrakia curviuscula* may represent a synonym of the anamorphic species *Cryptococcus huempii* and a member of a genus that is distinct from *Mrakia* and *Mrakia*. (vi) *Cystofilobasidium* is a teleomorphic and teliosporic genus. An incomplete sexual cycle for *Cryptococcus macerans* was reported by Rodrigues de Miranda (1984), which indicates that additional study will result in a formal description of the species as a member of *Cystofilobasidium*. (vii) The teleomorphic genus *Xanthophyllumyces* is commercially important due to the production of astaxanthin, which is a dietary pigment source for pen-raised salmon and shrimp (Johnson, 2003). As indicated by the representatives included in Fig. 1, there is considerable genetic variability between the strains, including the anamorphic state *Phaffia rhodozyma* (Fell et al., 2007; Weber et al., 2008).

Research in low-temperature environments has resulted in numerous reports of *Mrakia* in snow and soils. These strains are often identified as *Mrakia gelida* or *Mrakia frigida*, as the characterizations are by phenotypic or small-subunit or D1/D2 LSU rRNA gene sequence analyses. Because of the similarity in these characteristics, species separations are not successful. In contrast, ITS analysis (Diaz & Fell, 2000) provides the ability to define species and illustrate within-species variability. Research in polar and other cold environments has demonstrated the widespread occurrence of members of the *Mrakia* clade. Many of the strains from these ecological studies were sequenced and deposited for public use. Our analysis of ITS GenBank data (Fig. 2) should provide a guide for the phylogenetic placement of strains for on-going and future studies. The synonyms of *Mrakia frigida* and *Mrakia gelida* are included in this analysis to expand the synopsis. A major source of variability was provided by an unpublished doctoral dissertation of polar yeasts by Thomas-Hall.

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**Fig. 1.** Maximum-parsimony analysis (PAUP<sup>4.0b10</sup> of LSU rRNA gene D1/D2 domain sequences of representative strains of species in the Cystofilobasidiales. *Cryptococcus fuscescens* CBS 7189<sup>T</sup>, *Cryptococcus aerius* CBS 155<sup>T</sup> and *Cryptococcus terricola* CBS 6435<sup>T</sup> represent an outgroup. Numbers at branch nodes represent bootstrap percentages from 1000 replicates in a full heuristic search. Bar, 10 changes.
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 curvisus, whose sexual state was reported by Fell & Statzell-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) Mrakia cryoconiti and; and (vi) Cryptococcus aquaticus (Mrakia cryoconiti comb. nov.) with a strain (H2) from Iceland that has an optimal temperature for growth of 9 °C and a Tmax of 18 °C (Birgisson et al., 2003). Cryptococcus aquaticus CBS 5443T 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 5443T (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 5443T 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 A15T 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 teleomorph genera with strong bootstrap support for these generic groups. Consequently, we believe that Mrakia should be confined to the Mrakia clade.

As demonstrated by D1/D2 and ITS rRNA 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 5443T) was isolated from a freshwater lake in Great Britain. That strain was originally described (Jones & Sloof, 1966) with a Tmax 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 Tmax 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 Mrakia.

Latin diagnosis of Mrakia Margesin et Fell gen. nov.

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


Asexual members of the Cystofilobasidiales, which are closely related to the teleomorphic genus *Mrakia*. True hyphae are not produced, but pseudo hyphae 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 occurs 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.**


**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 x 5–8 μm on SabG agar; 2–3 x 3–6 μm on CMA) (Fig. 3). Budding is polar. Colonies are creamy white on CMA and SabG agar. Colonies are round and convex, with entire margins. No pseudo hyphae 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-xyllose, D-sorbitol (=D-glucitol), D-mannitol, glycerol, inositol, ribitol (=adonitol), ethanol, N-acetyl-glucosamine, potassium 2-ketogluconate, potassium 5-ketogluconate, potassium gluconate, sodium gluconate, 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 A15T (=CBS 10834T =DSM 21094T). Strains were isolated from alpine glacier cryoconite collected from the Stubaier glacier near Innsbruck in Tyrol, Austria (A15T), 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.

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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REFERENCES


