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Full paper

Mrakia arctica sp. nov., a new psychrophilic yeast isolated from an ice island in the Canadian High Arctic

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ABSTRACT

Two strains of a psychrophilic basidiomycetous yeast species belonging to the genus *Mrakia* were isolated from a melt-pool mat community, on an ice island located in Disraeli Fjord, Ellesmere Island in the Canadian Arctic. Analysis of the large subunit rDNA D1/D2 domain and internal transcribed spacer region sequences indicated that these strains represent a novel species, and the name *Mrakia arctica* sp. nov. is proposed. This new species could grow at sub-zero temperatures and in vitamin-free media. Moreover, lipase and cellulase enzymes of *M. arctica* were strongly active even at −3 °C. These results suggest an important role for *M. arctica* in the biogeochemical cycle of glacial ecosystems.

1. Introduction

Cold environments cover a large part of the Earth, and many ecosystems are continuously exposed to temperatures below 5 °C (Feller and Gerday 2003). Fungi in cold environments can grow and decompose organic compounds at sub-zero temperatures, and can therefore play a role in the biogeochemical cycles of polar ecosystems (Welander 2005; Margesin et al. 2007).

Yeast species of the genus *Mrakia* have been reported from a variety of extreme, perennial cold environments including the Arctic, Siberia, the Alps, Alaska, Patagonia, and Antarctica (Margesin et al. 2005; Panikov and Sizova 2007; Thomas-Hall et al. 2010; de Garcia et al. 2012; Singh and Singh 2012; Tsuji et al. 2016b). di Menna (1966) reported that *Mrakia* spp. accounted for approximately 24% of culturable yeasts in soil from Ross Island, Antarctica. Moreover, about 35% of culturable fungi in Skarvsnes ice-free area, East Antarctica belonged to the genus *Mrakia* (Tsuji et al. 2013a). These results strongly suggested that *Mrakia* is a fungal genus that is well adapted to the polar environment. Currently, the genus *Mrakia* consists of a total of eight species: *Mrakia aquatica* (E.B.G. Jones & Slooff) X.Z. Liu, F.Y. Bai, M. Groenew. & Boekhout; *M. blollopis* Thomas-Hall; *M. cryoconiti* (Margesin & Fell) X.Z. Liu, F.Y. Bai, M. Groenew. & Boekhout; *M. frigida* (Fell, Statzell, I.L. Hunter & Phaff) Y. Yamada & Komagata; *Mrakia gelida* (Fell, Statzell, I.L. Hunter & Phaff) Y. Yamada & Komagata; *M. nicombsii* (Thomas-Hall) X.Z. Liu, F.Y. Bai, M. Groenew. & Boekhout; *M. psychrophila* M.X. Xin & P.J. Zhou; and *M. robertii* Thomas-Hall & Turchetii (Yamada and Komagata 1987; Xin and Zhou 2007; Thomas-Hall et al. 2010; Liu et al. 2015).

In this study, two yeast colonies were isolated from an ice island habitat in the Canadian High Arctic. Based on physiological testing and molecular analysis using the large subunit 26S rDNA (LSU D1/D2 domain) and the internal transcribed spacer (ITS) sequences, these strains were classified into a new basidiomycetous yeast species in the genus *Mrakia*, for which the name *Mrakia arctica* sp. nov. is proposed.

2. Materials and methods

2.1. Sampling sites and sample collection

The ice island was in Disraeli Fjord, northern Ellesmere Island, in the Canadian High Arctic (lat. 82°50′N, long. 73°40′W), and was a remnant of the Ward Hunt Ice Shelf that collapsed in 2011–12; maps and further details about this site are given in Vincent et al.

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(2011). The island was accessed by helicopter on 18 Jul 2016, and microbial mat samples from the bottom of a shallow (0.3 m depth), freshwater melt-pool were aseptically transferred to sterile 5 mL sample tubes. The mats formed a loose, several mm-thick flocculent layer over the ice at the bottom of the pool, with a thin orange surface layer that overlaid olive colored organic 'matlets', as described in Mueller et al. (2005). Within 1 h of sampling, the tubes were transferred to a −20 °C freezer, and were stored at that temperature until subsequent analysis. Total carbon and nitrogen concentrations in the mat samples were measured using a CN analyzer (SUMIGRAPH NC-220F, Sumika Chemical Analysis Service, Tokyo, Japan), and gave concentrations of 5.20 ± 0.70% C and 0.54 ± 0.09% N.

2.2. Isolation of strains

Each 0.1 g frozen ice island mat sample was directly placed on potato dextrose agar (PDA, Difco, Becton Dickinson Japan, Tokyo, Japan) containing 50 μg/mL chloramphenicol and incubated at 10 °C for a period of up to 3 wk. Yeast samples were chosen for isolation based on colony morphology. Two yeast colonies, cream in color were purified by repeated streaking on fresh PDA. The resultant pure cultures of *Mrakia arctica* were deposited at the Japan Collection of Microorganisms (JCM), Riken, Japan, at the HUT Culture Collection (HUT), Hiroshima University, Japan.

2.3. Sequencing and phylogenetic analysis

DNA was extracted from yeast colonies, using an ISOPLANT II kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocol. The extracted DNA was amplified by polymerase chain reaction (PCR), using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The fragment covering the ITS region and LSU D1/D2 domain was amplified using the following primers: ITS1F (5′-GTAACAAGGTTTCCGT) and NL4 (5′-GGTCCGTGTTTCAAGACGG). The conditions for PCR were as described previously (Tsuji et al. 2016a). The amplified DNA fragments were purified using Sephacryl S-400HR (Sigma–Aldrich Japan, Tokyo, Japan). Sequences were determined using an ABI Prism 3130xl Sequencer (Applied Biosystems, Life Technologies Japan, Tokyo).

The concatenated ITS and LSU D1/D2 region sequences were aligned with the MAFFT program ver. 7.273 (Katoh and Standley 2013) using the L-INS-I algorithm. The alignments were deposited in TreeBASE (Submission ID: S20820). Maximum likelihood (ML) with an HKY+G+I model and maximum parsimony (MP) analysis with a TBR model were performed using MEGA 7 (Kumar et al. 2016). Bayesian inference (BI) was constructed using MrBayes 3.2.5 (Ronquist et al. 2012) with a GTR+I+G model and 5,000,000 generations, two independent runs, and four chains. The other parameters were set as the default values. We discarded 25% of these trees, with the remainder used to compute a 50% majority rule consensus tree to estimate posterior probabilities. A bootstrap analysis with 1000 replicates was performed to estimate the confidence of the tree nodes and a bootstrap percentage (BP) of ≥50% or Bayesian posterior probability (BPP) of ≥0.9 was considered supportive in all constructed trees in this study.

We also determined the sequence similarity and nucleotide variation in the ITS region and LSU D1/D2 domain among the species most closely related with *M. arctica*, using the EMBOSS water alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html).

2.4. Physiological characteristics

The effect of temperature on the growth of fungi on PDA plates was determined for the range −3 to 37 °C. The assessment of carbon assimilation was performed in glass vials with yeast nitrogen base liquid media for carbon assimilation tests according to standard methods (Kurtzman et al. 2011), with incubation for 2 wk at 15 °C. Assimilation of nitrogen and other physiological tests were also carried out in glass vials according to the protocols described by Kurtzman et al. (2011). Strains were examined for a sexual state after growth on the following media, which were incubated at 15 °C: YM agar (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, and 20 g/L agar), 5% malt extract agar (5% MA, 50 g/L malt extract and 30 g agar/L), and corn meal agar (CMA, Difco) for up to 8 wk. All experiments were carried out independently in three vials or on three plates.

2.5. Extracellular enzymes secretion tests

Three extracellular enzyme activities (cellulase, protease and lipase) were tested on agar plates at −3 °C, 4 °C, 10 °C, 15 °C, and 20 °C, for 3 wk. The ability to decompose cellulose was checked on yeast peptone dextrose agar (Difco) supplemented with 5.0 g/L of carboxymethylcellulose (CMC). Cellulolytic activity was observed as the formation of a clear zone after Congo red staining. Protease activity was assessed on PDA plates containing 10 g/L of skim milk (Difco, Becton Dickinson); a clear zone around a colony on the plate was indicative of protease activity. The ability to degrade long chain esters was evaluated using Tween-80 agar plates (10 g/L Tween-80, 10 g/L peptone, 5 g/L NaCl, 0.10 g/L $CaCl_2 \cdot 2H_2O$, and 20 g/L agar); an opaque halo around a colony indicated lipase production.

The diameter of each clearance zone was measured and enzyme activities were calculated according to the following formula:

$$\begin{aligned}&(\text{extracellular enzyme secretion ability})\\&= \{(\text{clear or opaque zone diameter}) - (\text{colony diameter})\}\\&\quad \times /(\text{colony diameter})\end{aligned}$$

The ability was assessed as follows: strongly positive, for values > 2.0; positive, for values between 1.0 and 2.0; weakly positive, for values < 1.0; and negative, no clear zone. It was determined based on the average from three individual experiments.

3. Results and discussion

A total of 85 fungal strains were isolated from the five different ice island mat samples collected in Disraeli Fjord, Ellesmere Island, Canada. Ellesmere Island is located near Greenland. Among these fungal strains, two strains were classified as the genus *Mrakia* (classification: Basidiomycota, Agaricomycotina, Tremellomycetes, Cystofilobasidiales) by sequence similarity of the ITS region and the LSU D1/D2 domain. Based on phylogenetic analysis of the ITS region and LSU D1/D2 domain sequences, *M. arctica* strains are branched from *M. cryoconiti* and this branch is supported with 97% BP, 83% BP, and 1.0 BPP by ML, MP, and BI analyses, respectively (Fig. 1).

From the result of the ITS region and LSU D1/D2 domain phylogenetic analysis, *M. cryoconiti*, *M. frigida* and *M. gelida* were determined as the most closely related species to *M. arctica*. Therefore, the LSU D1/D2 domain and ITS region sequences of the new *Mrakia* species isolated from the Canadian Arctic were compared with those of the closest species. The interspecific nucleotide substitutions in the LSU D1/D2 domain totaled 7, 7 and 7 nucleotide substitutions between *M. arctica* and *M. cryoconiti*,

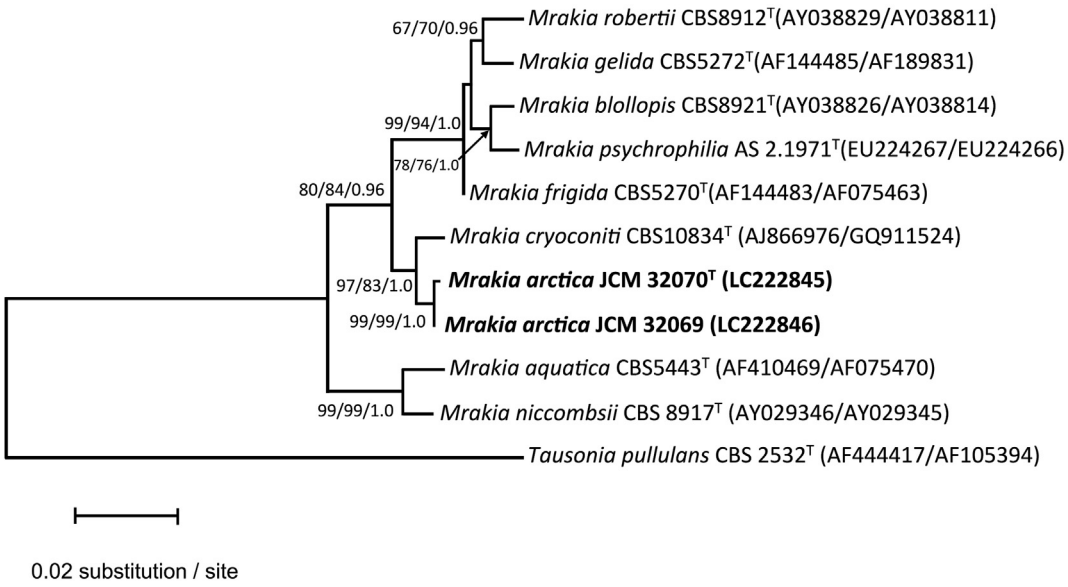

Fig. 1. Phylogenetic tree based on the ITS region-LSUD1/D2 domain sequences. Maximum likelihood analysis of the ITS region-LSU D1/D2 domain sequences of *Mrakia arctica* and closely related species. *Mrakia arctica* strains investigated in this study are highlighted in bold font. *Tausonia pullulans* CBS 2532 was designated as the outgroup. The tree backbone was constructed by maximum likelihood analysis with MEGA7. Bootstrap percentages of maximum likelihood and maximum parsimony analyses over 50% from 1000 bootstrap replicates and posterior probabilities of Bayesian inference above 0.9 are shown from left on the branches. The scale bar represents 0.02 substitutions per nucleotide position.

Table 1
Number of nucleotide substitutions in the LSU D1/D2 domain sequence and the ITS region sequences among the type strains of species in the genus *Mrakia*. Right upper triangle shows the number of nucleotide substitutions (nt) in LSU D1/D2 domain sequence. Left lower triangle indicates the number of nucleotide substitutions (nt) and the sequence similarity (%, in parentheses) between pairs of species in the ITS region sequences.

Species	*M. aquatica*	*M. arctica*	*M. blollopis*	*M. cryoconiti*	*M. frigida*	*M. gelida*	*M. niccombsii*	*M. psychrophila*	*M. robertii*
M. aquatica	–	16	19	16	18	18	10	18	19
M. arctica	52 (91.7)	–	10	7	7	7	13	10	8
M. blollopis	53 (91.8)	35 (94.4)	–	11	3	3	15	2	4
M. cryoconiti	49 (92.0)	16 (97.4)	34 (94.4)	–	10	10	14	11	11
M. frigida	50 (92.3)	30 (95.2)	11 (98.3)	31 (94.9)	–	0	14	3	1
M. gelida	53 (91.8)	38 (93.9)	15 (97.7)	37 (93.9)	16 (97.5)	–	14	3	1
M. niccombsii	8 (98.8)	52 (91.7)	54 (91.7)	50 (91.8)	52 (92.0)	52 (92.0)	–	13	18
M. psychrophila	53 (91.3)	30 (94.9)	8 (98.7)	31 (94.6)	11 (98.2)	8 (97.0)	55 (91.0)	–	4
M. robertii	52 (92.0)	40 (93.5)	17 (97.3)	37 (93.9)	17 (97.4)	18 (97.2)	53 (91.8)	17 (97.2)	–

M. frigida and *M. gelida*, respectively. *M. frigida* was shown to differ by 1 nucleotide from *M. robertii*, and *M blollopis* exhibited 2 and 4 nucleotide variations compared with *M. psychrophila* and *M. robertii*, respectively (Table 1). Furthermore, JCM 32070^T exhibited 2 nucleotide substitution compared with JCM 32069 in the LSU D1/D2 domain sequence. With respect to the nucleotide similarities and substitutions in the ITS region sequences, *M. arctica* demonstrated 16 and 30 nucleotide substitutions, and 97.4% and 95.2% sequence similarity with *M. cryoconiti* and *M. frigida*, respectively, and 38 nucleotide changes and 93.9% sequences similarity with *M. gelida*. In addition, *M. blollopis* showed 8 nucleotide differences and 98.7% similarity with *M. psychrophila*, and *M. gelida* had 8 nucleotide substitutions and 97.0% sequence similarity compared with the ITS region sequence of *M. psychrophila* in the ITS region sequence (Table 1). Moreover, JCM 32070^T showed 100% similarity with JCM 32069 in the ITS region sequence. Therefore, considering the results of the phylogenetic analysis of the LSU D1/D2 domain, and nucleotide substitutions of the LSU D1/D2 rDNA and ITS region sequences, JCM 32070^T and JCM 32069 should be considered as representing novel species.

The genus *Mrakia* is characterized by the following features: yeast cells are ovoid to elongate; nitrate is assimilated; starch-like compounds are produced; the maximum growth temperature is below 25 °C; and diazonium blue B and urease reactions are positive (Fell 2011; Fell and Margesin 2011). Our isolate from the Arctic ice island exhibited these characteristics, confirming that it belongs to the genus *Mrakia*. Cultures of *M. arctica* could ferment glucose and could grow in vitamin-free conditions, but were not able to utilize D-ribose, D-xylose, or lactose. In contrast, *M. cryoconiti* showed a lack of fermentation ability on glucose, could not grow in vitamin-free conditions and was able to assimilate D-ribose, D-xylose, and lactose (Table 2). A comparison of physiological characteristics of *M. arctica* and *M. frigida* showed that *M. frigida* could not assimilate maltose and was able to utilize D-ribose and D-xylose, and could not grow in vitamin-free media, whereas *M. arctica* showed the opposite physiological characteristics. *Mrakia arctica* and *M. gelida* showed similar carbon assimilation patterns, but *M. gelida* could assimilate D-xylose (Table 2). The optimum growth temperature of *M. arctica* was 15 °C and the maximum growth temperature tolerated by this new species was 20 °C. *Mrakia arctica* cells grow well on PDA at 15 °C, but did not grow efficiently on YM agar, 5% MA, or CMA. The cell size of *M. arctica* was 8–10 μm × 5–7 μm, and the cell shape was ovoid to elongated on CMA after 10 d incubation at 15 °C (Fig. 2). After 8 wk on YM, 5% MA,

Table 2
Comparison of assimilation and growth characteristics among species of the genus *Mrakia*.

Species	D-arabinose	Maltose	D-ribose	D-xylose	Lactose	Inulin	Citrate	Vitamin-free	Glucose fermentation
M. aquatica	−	+	−	+	+	−	s	−	+
M. arctica	+	+	−	−	−	−	−	+	+
M. blollopis	w/−	+	w/+	w/+	w	−	w/+	w	+
M. cryconiti	+	+	+	+	+	v	−	−	−
M. frigida	+	−	−	+	v	−	v	−	+
M. gelida	v	+	v	+	−	−	v	−	+
M.niccombsii	w	+	w/+	+	w	w	w	−	+
M. psychrophila	+	+	+	+	+	+	−	+	+
M. robertii	w/−	+	w/−	w/+	−	w	w/+	w/+	+

Main assimilation and growth test results for the characteristics of *M. arctica* and related species are shown. Physiological data of related species were taken from Fell and Margesin (2011), Fell (2011), Thomas-Hall et al. (2010), Xin and Zhou (2007) and this study. +, positive; w, weak; s, slow; -, negative; v, variable. The characteristics of the new species *Mrakia arctica* are highlighted in bold.

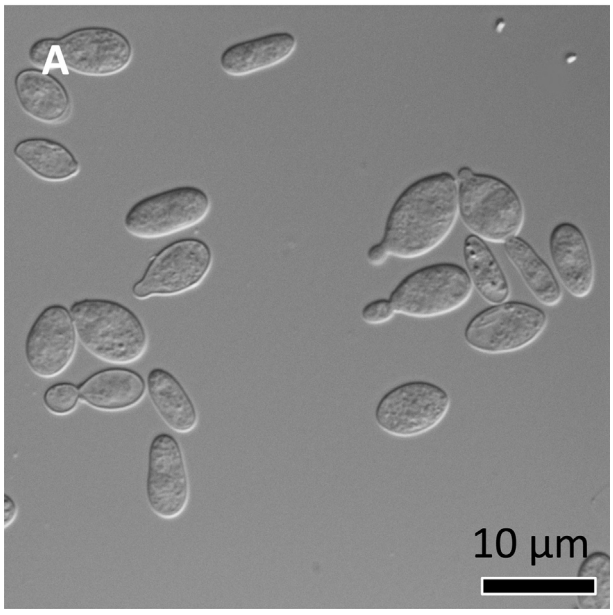

Fig. 2. Morphology of *Mrakia arctica*. Photomicrograph of *Mrakia arctica* grown on CMA for 10 d at 15 °C. *Bar*: 10 µm.

and CMA at 15 °C, no pseudohyphae or true hyphae were formed. *M. arctica* cell division was by polar budding. None of *M. arctica* strains exhibited sexual activity, or produced ballistoconidia.

In our tests of the extracellular enzyme secretion of lipase, cellulase and protease enzymes over the range −3 to 20 °C, the optimum temperature for lipase secretion by *M. arctica* was −3 °C, with evidence of strong lipase activity, even at 20 °C (Table 3). We have previously reported that lipase activity of *Mrakia* from Skarvsnes ice-free area was higher at 4 °C than at 15 °C (Tsuji et al. 2013b, 2014, 2015b). *Mrakia arctica* exhibited strong cellulase activity between −3 and 15 °C, and this enzyme secretion was most pronounced at 4 °C. *Mrakia arctica* showed weak secretion of this enzyme at −3 °C, but strong secretion of this enzyme between 4 and 20 °C (Table 3). The best protein degradation activity was 10 °C. This contrasts with protease studies on *Mrakia* elsewhere: Singh et al. (2016) reported that *M. blollopis*, isolated from the Norwegian Arctic, showed weak protease activity, and our previous whole genome analysis of an Antarctic strain of *M. blollopis* strain showed that it lacked the protease K gene (Tsuji et al. 2015a).

Table 3
The extracellular enzyme secretion ability of *Mrakia arctica*.

	Lipase	Cellulase	Protease
−3 °C	6.15 ± 0.68	5.34 ± 0.78	0.75 ± 0.12
4 °C	5.98 ± 1.13	6.35 ± 0.57	2.08 ± 0.27
10 °C	5.83 ± 0.24	5.58 ± 0.07	3.12 ± 0.15
15 °C	5.32 ± 0.84	5.33 ± 0.42	2.66 ± 0.08
20 °C	3.92 ± 0.12	N	2.42 ± 0.18

The values represent the difference between the diameters of the zone of clearance and the colony, expressed as a proportion of the colony size (means ± SD for triplicates).
N; no activity.

The extracellular enzyme tests indicate that *M. arctica* could decompose a variety of organic materials over a wide range of temperatures. Previous metagenomic analysis of microbial mats in these High Arctic ice island and ice shelf environments has shown a high capacity for decomposition, nutrient recycling and scavenging (Varin et al. 2010) and *M. arctica* is likely to be an active contributor to these processes.

Taxonomy

Mrakia arctica M. Tsuji, sp. nov. Fig. 2.
MycoBank no.: MB 821502.

Etymology: "*arctica*" referring to the origin of this species.

Type: Canada, Ellesmere Island (lat. 82°50′N, long. 73°40′W), 18 Jul 2016, isolated from an ice island microbial mat in Disraeli Fjord (holotype: strain JCM 32070^T preserved in a metabolically inactive state at Japan Collection of Microorganisms, Riken, Japan; ex-type culture: HUT7420^T deposited at the HUT Culture Collection, Hiroshima University, Japan; paratype: JCM 32069, HUT7419); ITS and LSU D1/D2 domain: LC222845 (JCM 32070^T), LC222846 (JCM 32069).

Yeast cells after 10 d on YM agar ovoid to elongated, 8–10 µm × 5–7 µm, proliferating by polar budding. Sexual activity is not observed. Pseudohyphae and true hyphae are not formed. Streak culture after one wk on 5% malt extract agar at 15 °C: colonies are a yellowish cream color, round convex and smooth with entire margin.

Glucose and sucrose are fermented. Assimilation of carbon compounds: D-arabinose, L-arabinose, cellobiose, D-galactose, D-glucose, maltose, melibiose, melezitose, raffinose, L-sorbose, sucrose, trehalose D-glucitol glycerol, ethanol (weak), erythritol, D-mannitol, ribitol, D-xylitol starch, salicin, succinate, D-gluconate, D-glucuronate, D-glucosamine, *N*-acetyl-D-glucosamine, potassium nitrate, and sodium nitrate. Inulin, D-xylose, lactose, methyl-α-D-glucoside, L-rhamnose, D-ribose, methanol, galactictol, *myo*-inositol, DL-lactate and citrate are not assimilated. Growth on 50% (w/v) glucose medium. No growth occur on 5% glucose medium with 10% NaCl (w/v), 0.01% cycloheximide. DBB and urease reactions are positive. Amino acids and vitamins are not required for

growth. Maximum temperature for growth is 20 °C, and optimal growth is at 15 °C. Has ability to grow at −3 °C on PDA. Lipase enzymes are secreted ranging from −3 to 20 °C, cellulase enzymes are active from −3 to 15 °C, and protease activity is present at −3 to 20 °C.

Habitat: Ice island melt-pool, Disraeli Fjords, northern Ellesmere Island, High Arctic Canada.

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