Antonie van Leeuwenhoek (2007) 92:43–51
DOI 10.1007/s10482-006-9133-3

ORIGINAL PAPER

Penicillium svalbardense, a new species from Arctic glacial ice

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Received: 10 July 2006 / Accepted: 20 November 2006 / Published online: 25 January 2007

Abstract During investigations of mycobiota in the coastal Arctic polythermal glaciers, different species of the ubiquitous genus *Penicillium* were isolated from the extreme subglacial environment. A group of *Penicillium* strains was obtained that did not belong to any known *Penicillium* species. This species was isolated in high numbers from the Kongsvegen subglacial ice and was not detected in the surrounding environment. A detailed analysis of secondary metabolite profiles, physiological and morphological characteristics, and partial β-tubulin gene sequences showed that the proposed new species *Penicillium svalbardense* is closely related but not identical to *Penicillium piscarium* and *Penicillium simplicissimum*. It differs in the production of secondary metabolites and in the morphological features of conidia and penicilli, and it is therefore described as a new species.

Keywords Arctic · Partial β-tubulin gene sequences · *Penicillium svalbardense* sp. nov. · Secondary metabolites · Subglacial ice

Abbreviations

CFU	colony forming units
DIC	differential interference contrast
HPLC	high-performance liquid chromatography
PCR	polymerase chain reaction
ITS	internal transcribed spacer

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Introduction

The ubiquitous genus *Penicillium* contains mainly food, soil and airborne species (Pitt et al. 2000). This genus shows tolerance to cold environments, as demonstrated by the many species that grow on refrigerated food (Pitt and Hocking 1999) and that have been isolated from high mountain soils (Domsch et al. 1980; Petrovič et al. 2000). *Penicillium* spp. also inhabit extremely cold, polar areas of the world. They have been isolated from Arctic and Antarctic soils, permafrost, snow, sea ice and sea water (Vishniac 1993; McRae et al.

1999; Gunde-Cimerman et al. 2003; Frisvad 2004; Ivanushkina et al. 2005; Frisvad et al. 2006), and from glacial ice cores up to 38,600 years old (Abyzov 1993; Ma et al. 1999, 2000). Recent studies have revealed a new habitat for microorganisms within the Arctic polythermal glaciers. These are represented by bacteria (Foght et al. 2004) and fungi, primarily yeasts and the genus *Penicillium* (Sonjak et al. 2006). Among the *Penicillium* spp. detected, a group of biverticillate *Penicillium* strains was obtained that did not belong to any known *Penicillium* species. These strains were isolated exclusively from this extreme environment and were not detected in the neighbouring coastal areas (Sonjak et al. 2006). Here, we present the description of this new biverticillate *Penicillium* species, named *Penicillium svalbardense*, using morphological, physiological and molecular criteria.

Materials and methods

Site and sampling

In June and August 2001, fieldwork was undertaken in an area of Kongsfjorden, one of the largest fjords in west Spitsbergen, Svalbard (79°N, 12°E), Norway. Sediment-rich subglacial ice and the overlying clear glacial ice of the Kongsvegen polythermal glacier were sampled at the glacier margins where it was exposed. Samples were collected in sterile polypropylene bags using surface sterilized tools and transported to the laboratory, where they were processed as previously described (Sonjak et al. 2006). Aseptically melted inner layers of the glacial ice mass were filtered in aliquots of 100 ml. The membrane filters used (0.45 μm; Millipore) were placed on solid agar media. Additionally, mineral ice inclusions were collected aseptically, and 1 g of this sediment was spread directly over agar plates. Up to ten replicates of enumeration and different selective media were used (Sonjak et al. 2006). All of the plates were incubated for up to 14 weeks at 10°C and 24°C. After incubation, the colony forming units (CFU) were counted, with the mean CFUs calculated and expressed as CFU l^{-1} for the melt-water and CFU g^{-1} for the direct spreading of the sediments.

Strains examined

Strains of the proposed new *Penicillium* species are maintained in the EX-F culture collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia and in the IBT culture collection of The Centre for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark. The strains included in the analysis are listed in Table 1. Additional strains of related species, *Penicillium simplicissimum*, *Penicillium piscarium* and *Penicillium cremeogriseum*, were obtained from the IBT collection and the CBS culture collection of the Centraalbureau voor Schimmelcultures of the Royal Netherlands Academy of Arts and Sciences, Utrecht, The Netherlands (Table 1).

Morphology, physiology and extracellular enzymatic activity

For the determination of the morphological characteristics, the isolates were inoculated as three-point cultures on Czapek yeast autolysate agar (CYA), CYA with 5% NaCl (CYAS), malt extract agar (MEA), yeast extract sucrose agar (YES) and creatine sucrose agar (CREA), and grown for seven days at 25°C (CYA also at 15°C) in the dark (Frisvad and Samson 2004). For the determination of micro-morphological characteristics, microscope slides were prepared from MEA medium. Water solution of 60% (v/v) lactic acid without a colour dye was used as the mounting medium. The slides were examined under oil immersion with a BX51 microscope (Olympus, Japan) by differential interference contrast (DIC), at up to 1000× magnification. Digital micrographs were taken with DP12 digital camera and analysed using the DPSOFT 3.2 application software (Olympus, Japan). For the selected strains (Table 1), the following extracellular enzymatic activities were tested: fatty acid esterase (Tween 80 medium), protease (casein hydrolysis medium, gelatine hydrolysis medium), amylase (starch agar), β-glucosidase (aesculin

Table 1 List of seven *P. svalbardense*, two *P. simplicissimum*, three *P. piscarium*, and two *P. cremeogriseum* isolates examined

Taxon name	Collection numbers	Source	GenBank Number
P. svalbardense	EX-F 1097, IBT 23851[a]	Glacial ice, Svalbard	
P. svalbardense	EX-F 1174, IBT 24266[b]	Glacial ice[c], Svalbard	
P. svalbardense	EX-F 1186, IBT 24243[b]	Glacial ice[c], Svalbard	
P. svalbardense	EX-F 1227, IBT 24101[a]	Glacial ice[c], Svalbard	DQ834933
P. svalbardense	EX-F 1233, IBT 23849[a]	Glacial ice[c], Svalbard	
P. svalbardense	EX-F 1307[T], IBT 23856[a]	Glacial ice, Svalbard	DQ486644
P. svalbardense	EX-F 1319, IBT 23855[a]	Glacial ice, Svalbard	DQ486643
P. simplicissimum	CBS 372.48[T], IBT 4405, EX-F 2691	Flannel bag, South Africa	DQ486650
P. simplicissimum	IBT 15303, EX-F 2692	Cereal feed, Norway	DQ834935
P. piscarium	IBT 21815, CCF 2003, EX-F 2639	Soil, Czech Republic	DQ486648
P. piscarium	IBT 21002, EX-F 2640	Air, Czech Republic	DQ486649
P. piscarium	IBT 12452, EX-F 2423, NRRL 1075, CBS 362.48[T]	Cod-liver oil emulsion, Germany	DQ834934
P. cremeogriseum	CBS 223.66[nT], IBT 13212, EX-F 2767	Forest soil, Ukraine	DQ834936
P. cremeogriseum	IBT 15467, EX-F 2641	Agricultural soil, Australia	DQ486651

[a] Strains selected for secondary metabolite analysis

[b] Strains selected for testing of enzymatic activities

[c] Glacial ice with sediment

[T] Strain derived from the type

[nT] Strain derived from the neotype

agar) and urease (urease test agar) (Paterson and Bridge 1994).

Secondary metabolite analysis

Agar plugs (6 mm in diameter) were cut out of seven-day-old cultures growing on CYA and YES media. According to the method of Smedsgaard (1997), the cultures were extracted ultrasonically for 60 min with 500 µl of the solvent mixture of methanol/dichloromethane/ethyl acetate (3:2:1) containing 0.5% (v/v) formic acid. The organic solvent was transferred to a clean vial and evaporated by vacuum centrifugation. The residues were re-dissolved in 500 µl methanol, filtered through 0.45 µm Minisart filters, and analysed by high-performance liquid chromatograph (HPLC) (A1100; Agilent, Germany) with diode array detection at 210 nm, with 5 µl injections (Frisvad and Thrane 1987, 1993; Smedsgaard 1997). Separation was obtained with a 2×100 mm Luna2 OOD-4251-BO-C_{18} column (Phenomenex, Germany). The elution gradient was initially linear, from 85% water/15% acetonitrile to 100% acetonitrile over 20 min; 100% acetonitrile was then maintained for 5 min. A flow-rate of 0.4 ml min^{-1} was used. Both eluents contained 0.005% (v/v) trifluoroacetic acid. An alkylphenone analytical standard was used to define the retention time and an index was calculated for each peak detected. The secondary metabolites were identified by comparison with standard and by their characteristic UV spectra.

DNA isolation, amplification and sequence analysis

For selected isolates (Table 1), DNA was extracted following mechanical lysis (Gerrits van den Ende and de Hoog 1999), from ~200 mg of four-day-old cultures grown on complete yeast extract medium (CYM; Raper et al. 1972), at 25°C in the dark. Amplification of the partial β-tubulin gene was carried out as described by O'Donnell and Cigelnik (1997) using the T1 and T22 primers. PCR was performed in a 50 µl reaction mixture with ~10 ng of genomic DNA (GeneAmp PCR system 2400; Perkin Elmer, USA). The PCR products were separated electrophoretically on 1% agarose gels, and the expected bands were excised and purified

with the DNA Gel Extraction Kit (Promega, USA), following the manufacturer protocol. The PCR fragments were sequenced with the T1 and T22 primers using a BigDye™ terminator Ready Reaction Cycle Sequencing Kit on an ABI 3730xl DNA Analyser (Applied Biosystems, USA), as provided by the Macrogen Company (Korea). The partial β-tubulin gene sequences were aligned using CLUSTAL W (Thompson et al. 1994). For the phylogenetic analysis, the neighbor-joining (NJ) method (Saitou and Nei 1987) was used. The data were first analysed using the Tamura-Nei parameter distance calculation model (Tamura and Nei 1993), which was then used to construct the NJ tree using MEGA3.1 software (Kumar et al. 2004). To determine the support for each clade, bootstrap analysis was performed with 1000 replications. All of the sequences generated in this study have been deposited at GenBank; their accession numbers are given in Table 1.

Results and discussion

Polythermal glaciers are characterized by areas of massive surface ablation (80–100 m) that are drained by a stable, open-channel system with ice temperatures below zero and with cold (subfreezing) ice at the surface, margins and terminus of the glacier. The glaciers are exposed to rapid movements (Ekström et al. 2003; Fahnestock 2003), and consequently to frictional and geothermal melting of the ice at their base. Together with groundwater and seasonal supraglacial water, the water arising at the base contributes to the subglacial waters, which interact with rocks and sediments beneath the glacial ice (Foght et al. 2004). These processes create subglacial environments that have until recently been considered abiotic. However, a very high number of *Penicillium* species, at up to 13,000 CFU l^{-1}, was recently isolated from the subglacial ice of this extreme environment (Gunde-Cimerman et al. 2003).

One of the *Penicillium* species isolated almost exclusively from the Kongsvegen glacier (Gunde-Cimerman et al. 2003; Sonjak et al. 2006) has characteristics that are different from known species and was therefore acknowledged as a new species; this was named *P. svalbardense*. It was isolated from subglacial ice in total counts of 200 CFU l^{-1}, with the highest proportions obtained on a medium used for the detection of moderate xerophiles, dichloran 18% glycerol agar (DG18; Hocking and Pitt 1980), at 10°C, followed by malt extract agar with 5% NaCl (MEA5NaCl; Gunde-Cimerman et al. 2003) and malt extract agar with 15% NaCl (MEA15NaCl; Gunde-Cimerman et al. 2003), at 24°C. It was isolated from clear glacial ice at lower total counts, of up to 43 CFU l^{-1}, again with the highest proportion obtained on DG18 at 10°C. The species was also isolated from Kongsvegen subglacial mineral ice inclusions, with the highest number (10 CFU g^{-1}) obtained on MEA5NaCl at 10°C (Table 2).

To date, the genus *Penicillium* comprises approximately 225 described species that show a

Table 2 CFU numbers of *P. svalbardense* obtained on different media and at different temperatures. From Kongsvegen clear basal glacial ice (Kc) and subglacial ice (Ks) *P. svalbardense* was isolated using filtration method, whereas from Kongsvegen mineral ice inclusions it was isolated by direct spreading of the sediment (Km)

Medium	T (°C)	CFU l^{-1}		CFU g^{-1} Km
		Kc	Ks	
DRBC	10			
	24	1	13	
DG18	10	26	57	
	24			
MY20G	10			1
	24	1	3	2
MY35G	10			1
	24		16	
MY50G	10			
	24			
MY10-12	10	1		
	24			
MEA5NaCl	10	10		10
	24	2	54	
MEA10NaCl	10		3	
	24	2	14	1
MEA15NaCl	10			
	24		40	
Total		43	200	15

DRBC, dichloran rose bengal chloramphenicol agar; DG18, dichloran 18% glycerol agar; MY20G, MY35G, MY50G, malt yeast X% glucose agar, $X = 20, 35, 50$; MY10–12, malt yeast 10% glucose and 12% NaCl agar; MEA5NaCl, MEA10NaCl, MEA15NaCl, malt extract agar with X% NaCl, $X = 5, 10, 15$ (Gunde-Cimerman et al., 2003)

high diversity of morphology and secondary metabolites (Pitt et al. 2000). A combination of micromorphological, macromorphological and physiological characters is therefore needed to achieve satisfactory identification and classification (Frisvad and Samson 2004).

The proposed new species of *P. svalbardense* is characterized by its biverticillate penicilli, rough-walled stipes, globose to subglobose, smooth to slightly rough-walled conidia, good growth on CYA at 25°C and 30°C, and moderate to good growth on CREA. The morphology of their penicilli resembles most those of *P. simplicissimum* and *P. piscarium* from the subgenus *Furcatum*; however, there are differences in size, ornamentation and shape of the conidia (Table 3), which are among the most stable morphological characters in the genus *Penicillium* (Frisvad and Samson 2004).

Since *Penicillium* species produce several mycotoxins and other secondary metabolites that are species specific and usually very consistently expressed, chemotaxonomic studies are often used for their identification and classification (Frisvad et al. 1998). Thus, secondary metabolite production has been used extensively to distinguish between *Penicillium* species in the subgenus *Furcatum* (Frisvad and Filtenborg 1990). On the basis of secondary metabolites, *P. svalbardense* is phenotypically most closely related to *P. piscarium*, since they both produce indole diterpenes. However, *P. piscarium* does not produce xanthoepocin, which is found in *P. svalbardense* and was reported to be produced by *P. simplicissimum* (Igarashi et al. 2000). Both *P. piscarium* and *P. simplicissimum* also produce additional secondary metabolites that are not found in *P. svalbardense* (Tuthill et al. 2001) (Table 3).

Molecular phylogenetic analyses based on ITS (Peterson 2000; Tuthill 2001) and recently particularly of the partial β-tubulin gene sequences (Seifert and Louis-Seize, 2000; Samson et al. 2004; Frisvad et al. 2006) have been used for *Penicillium* species delimitation. Skouboe et al. (1996) showed that ribosomal internal transcribed spacer (ITS) sequences were to invariant to provide sufficiently resolved phylogram. Therefore, in the case of *P. svalbardense*, partial β-tubulin gene sequencing was performed. We used T1 and T22 primers to amplify about 1500 bp of the β-tubulin gene. The smaller portion of about 700 bp that included the bt2a–bt2b region (Glass and Donaldson 1995) was sequenced. The sequences obtained were compared to the available sequences of the National Center for Biotechnology Informatic (NCBI) by using blast program BLAST-n (Altschul et al. 1990, 1997). Due to the lack of the *Penicillium* β-tubulin sequences in gene banks no close matches were obtained. The phylogenetic relationships of the partial β-tubulin gene sequences of ten strains belonging to different species from the subgenus *Furcatum* and additionally of the *P. janthinellum* strain used as an outgroup were inferred from NJ analysis. The tree produced is shown in Fig. 1. The *P. svalbardense* strains included in the analysis were grouped into a cluster with a 100% bootstrap value. The alignment of these strains showed no differences in base composition. *P. svalbardense* is phylogenetically most closely related to *P. piscarium*. The tree topology

Table 3 Comparison of some of the diagnostic features between *P. svalbardense* and related species (from Tuthill et al. 2001)

	P. svalbardense	*P. simplicissimum*	*P. piscarium*
Conidia wall	Smooth to slightly rough	Smooth	Echinulate
Conidia shape	Globose to subglobose	Subglobose to elipsoidal	Globose
Conidia size (μm)	2.7–3.4 in diam.	3.4–4.2 × 2.7–3.4	3–3.8 in diam.
Stipe wall	Rough	Rough	Rough
Secondary metabolites	Xanthoepocin, metabolite with penitremone chromophore	Paraherquamides, austalides	Janthitrems, pulvilloric acid, sometimes anthraquinone

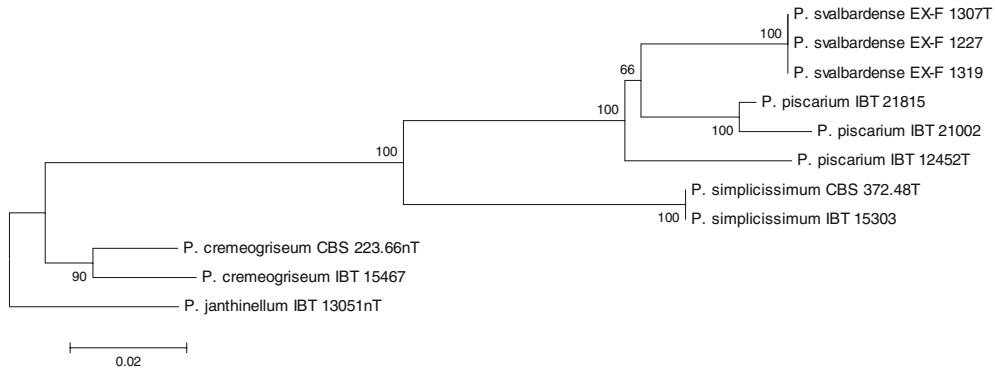

Fig. 1 Phylogenetic tree inferred from neighbor-joining analysis of the partial β-tubulin gene sequences. The numbers at the nodes represent the bootstrap values of >60% (out of 1000 bootstrap replications). The number of nucleotide changes between taxa is represented by the branch length

indicates on the intra-specific variation of β-tubulin sequences in single species *P. piscarium* and therefore on possible existence of two races, one being *P. svalbardense* and the other the two *P. piscarium* strains IBT 21815 and IBT 21002. However, the genetic dissimilarity between *P. svalbardense* and the closest *P. piscarium* strain, and particularly additional differences in morphology and secondary metabolite profiles clearly point to a new species.

Penicillium svalbardense Frisvad, Sonjak & Gunde-Cimerman, sp. nov.

Coloniae in agaro CYA 29–36 mm diam. post 7 dies 25°C, 30°C 40–46 mm, planae. Conidiophora mononematosa, biverticillata; stipites 250–500 μm longi, 2.5–3.5(–4) μm lati, asperulati; metulae leves, 13–25 × 2.5–3.5 μm; phialides lageniformes, collulo brevi praeditae, 9–13 × 2.3–3.5 μm. Conidia globosa vel subglobosa, leves vel asperulata, 2.7–3.4 μm diam, acervata caeruleo-grisea. Metabolitum: xanthoepocinum.

On CYA at 25°C after 7 days (Fig. 2a): colonies 29–36 mm in diameter, low, plane, with few light radial and annular zones, mononematous; conidiogenesis moderate to good, bluish-grey; mycelium white at margins; creamy-rose-coloured exudate, occasionally present; reverse light brown in centre.

On MEA at 25°C after 7 days (Fig. 2b): colonies 35–39 mm in diameter, low, plane, mononematous; conidiogenesis good, bluish-grey; mycelium white at margins; no exudate and soluble pigments; reverse uncoloured.

On YES at 25°C after 7 days (Fig. 2c): colonies 37–39 mm in diameter, low, umbonate in centre, with radial and annular zones; conidiogenesis weak to moderate, bluish green to brown; mycelium white; no exudate and soluble pigments; reverse yellow brown.

On CREA at 25°C after 7 days: colonies 22–25 mm in diameter, good growth, no acid or low acid production.

On CYA at 30°C after 7 days: colonies 40–46 mm in diameter.

On CYA at 15°C after 7 days: colonies 18–25 mm in diameter.

On CYAS at 25°C after 7 days: colonies 5–8 mm in diameter.

Extracellular enzymatic activities: positive fatty acid esterase, β-glucosidase and urease activities.

Conidiophores (Figs. 2d–h, 3) biverticillate, stipes 250–500 μm × 2.5–3.5 (–4) μm, rough-walled; metulae smooth-walled, 13–25 μm × 2.5–3.5 μm; phialides flask-shaped with a short neck 9–13 μm × 2.3–3.5 μm; conidia (Figs. 2i, 3) globose to subglobose, smooth to slightly rough-walled, 2.7–3.4 μm in diameter.

Etymology: the species is named after its origin, Svalbard archipelago.

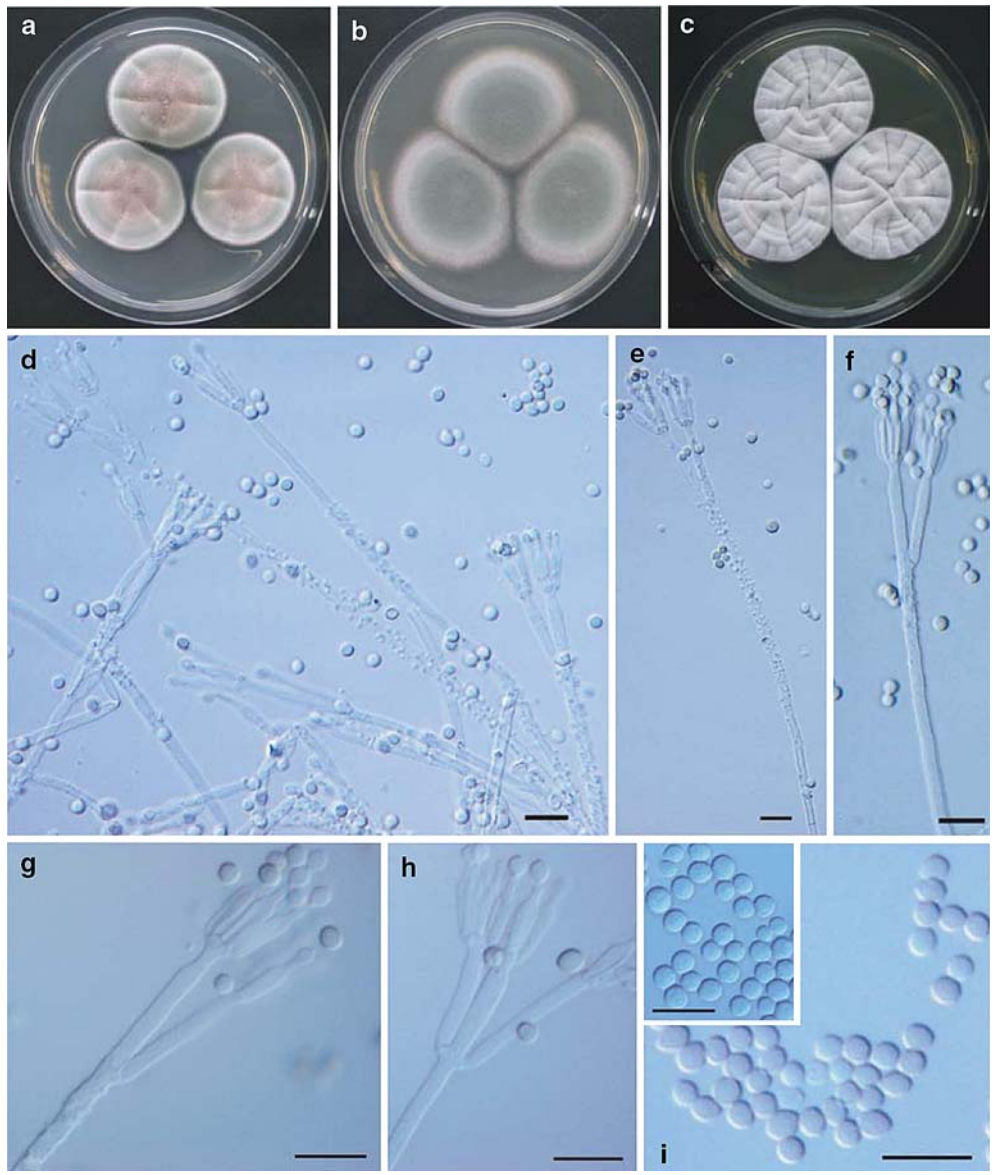

Fig. 2 Morphological features of *P. svalbardense*. (**a**–**c**) *P. svalbardense* colonies grown for seven days on CYA, MEA and YES, respectively. (**d**–**i**) DIC micrographs of *P. svalbardense* conidiophores (**d**–**h**) and conidia (**i**). Scale bars, 10 μm

GenBank accession numbers of partial β-tubulin gene sequences

EX-F 1227—DQ834933, EX-F 1307—DQ486644, EX-F 1319—DQ486643, CBS 372.48—DQ486650, IBT 13051—EF123659; IBT 15303—DQ834935, IBT 21815—DQ486648, IBT 21002—DQ486649, IBT 12452—DQ834934, CBS 223.66—DQ834936, IBT 15467—DQ486651.

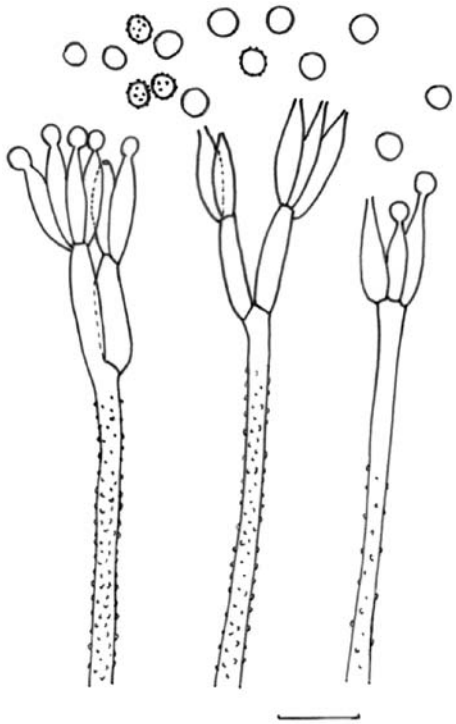

Fig. 3 Illustration of *P. svalbardense* micromorphological features: conidiophores and conidia. Scale bar, 10 μm

Acknowledgements We thank Polona Zalar for the illustration of the *P. svalbardense* micromorphological features and Dr. Walter Gams for the latin diagnosis.

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