RESEARCH PAPER

Ancient fungi in Antarctic permafrost environments

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Abstract

Filamentous fungi in 36 samples of Antarctic permafrost sediments were studied. The samples collected during the Russian Antarctic expedition of 2007–2009 within the framework of the Antarctic Permafrost Age Project (ANTPAGE) were recovered from different depths in ice-free oases located along the perimeter of the continent. Fungal diversity was determined by conventional microbiological techniques combined with a culture-independent method based on the analysis of internal transcribed spacer (ITS2) sequences in total DNA of the samples. The study revealed a rather low fungal population density in permafrost, although the diversity found was appreciable, representing more than 26 genera. Comparison of the data obtained by different techniques showed that the culture-independent method enabled the detection of ascomycetous and basidiomycetous fungi not found by culturing. The molecular method failed to detect members of the genera *Penicillium* and *Cladosporium* that possess small-sized spores known to have a high resistance to environmental changes.

Introduction

Microorganisms in permanently frozen natural environments form specific communities that survive prolonged exposure to subzero temperatures in habitats with low water activity and low rates of nutrient and metabolite transfer. Studies by culture-dependent and culture-independent methods have revealed considerable abundance and diversity of eukaryotic organisms in low-temperature environments, including Antarctic active layer (ground surface; Malosso *et al.*, 2006; Ruisi *et al.*, 2007). Works on fungi in Antarctic permafrost (permanently frozen subsurface ground) studied by conventional microbiological techniques are, however, very few (Gilichinsky *et al.*, 2007; Onofri *et al.*, 2010). To the authors' knowledge, the data on fungal diversity in permafrost in general obtained by culture-independent methods are limited to the report by Lydolph *et al.* (2005) on Siberian Arctic samples. The list of taxa recovered and reported by Lydolph *et al.* (2005) and described by Ozerskaya *et al.* (2009) (the latter obtained by culturing) yield an overlap of no more than 40%.

As each approach has its limitations, a combination of culture-dependent and culture-independent methods appears to be more appropriate for studies of fungal diversity in permafrost environments. Culture-dependent techniques usually fail to detect some fungi owing to inappropriate pretreatments of samples (e.g. thawing regimes), occurrence of fungal spores not germinating on common nutrient media and particular organisms requiring specific culture conditions (media, moisture, exposure to light, temperature, level of oxygen etc.) closely associated with plants (Kochkina *et al.*, 2001). In turn, the culture-independent analysis using total DNA may underestimate the fungal diversity owing to the presence of lysis-resistant spores (e.g. in anamorphic ascomycetes) resulting in a limited amount of DNA available for studies (Prosser, 2002; Nikolcheva *et al.*, 2003; Nikolcheva & Bärlocher, 2005).

This article provides the results of a study of the fungal diversity in 36 Antarctic subsurface permafrost samples by conventional microbiological techniques along with assays of total DNA in nine samples. We have not set

specific targets of exact and indubitable identification of all species by their DNAs, as this requires a multilocus phylogenetic research. Our task was to make a tentative rough draft of fungal species diversity pattern.

Materials and methods

Samples collection

The permafrost sediments were sampled during the Russian Antarctic expedition (2007–2009) within the framework of the Antarctic Permafrost Age Project (ANTPAGE). The 36 samples analyzed in this study (Table 1) were recovered from different depth of the cores of 13 boreholes located at four stations (Bellingshausen, Novolazarevskaya, Progress, Russkaya), as well as in Banger Oasis and the Beacon Valley (Dry Valley's region) situated in ice-free oases of various geography and climate (Fig. 1).

Methods of sampling, storage, transportation and control, as well as the specialized tests, were chosen to ensure that the microorganisms found in all probability were indigenous to the sample and not contaminants. The cores (diameter 5–10 cm and length 15–30 cm) were collected using a dry drilling technique developed specifically for microbiological studies of permafrost (Gilichinsky *et al.*, 1989, 2007). The dry drilling and sampling prevent down-hole contamination caused by drilling fluids. The sampling is achieved by dry shaving of the core back to native ice-cemented sediment. Possible contamination during the drilling was monitored by several tests. Previous studies have employed fluidless drilling techniques combined with an exogenous bacterial tracer such as a pure culture of *Serratia marcescens*. In tests using the isolation techniques, *S. marcescens* bacteria were found only on the surface of the frozen sample, never inside the frozen cores (Gilichinsky *et al.*, 2007).

After removal from the corer, the core surfaces were cleaned by shaving with a sterile knife, followed by splitting into *c.* 5-cm-long segments and placing them into sterile aluminum boxes or plastic bags. The samples were stored and transported frozen and then maintained in the laboratory at − 20 °C until analyses.

The indigenous origin of microorganisms is ensured by the nature of investigated specimens proper, that is, the permafrost sediments from the closed environmental system (Gilichinsky *et al.*, 1989, 2007; Shi *et al.*, 1997; Steven *et al.*, 2006).

Isolation of fungi

It was shown that the thawing of samples at a high temperature (45–52 °C) considerably increased the yield of viable cells of some eukaryotes as compared with their thawing at 20 °C (Lozina-Lozinskii, 1966). In our experiments, the thawing of samples at 35 and 52 °C led to the recovery of the fungal genera that could not be recovered at lower temperatures (Kochkina *et al.*, 2001).Therefore, the samples were treated in three ways. To isolate fungi, 0.5-g portions of a core sample were placed in test tubes with 5 mL of sterile water heated to 20, 35, and 52 °C for 1 min and then were shaken (3000 rev min^{-1}) at room temperature for 10 min. The suspension was spread (in triplicate for each variant of isolation) onto plates containing the Czapek's agar with 2% w/v sucrose (Cz) and Malt extract agar (MEA) both supplemented with lactic acid (0.4 vol %). The plates were incubated at 25 and 4 °C over a month for isolating mesophilic and psychrotolerant organisms, respectively.

In parallel, accumulative method to isolate fungi was used. The permafrost samples were filled with liquid wort (3.5 °B) and incubated at 4 °C for 60 days with a periodic visual inspection to pinch out colonies of slow-growing fungi. All distinguishable fungal colonies were isolated on solid media and identified.

Tap water used for preparing sample suspensions was tested for sterility by inoculation into appropriate media. To control the sterility of air in the microbiological box, open Petri dishes with solid media (MEA and Cz) were exposed to the air for 10–15 min and then were incubated at 4 and 25 °C.

Identification of fungi

The isolated fungi were identified on the genus/species level on the basis of cultural, morphological, physiological, and biochemical characteristics using suitable media according to the relevant current manuals (Samson & Frisvad, 2004; Crous *et al.*, 2007; Jaklitsch, 2009; etc.).

DNA extraction and amplification

Total genomic DNA was extracted from nine permafrost samples (0.62–0.97 g each; Table 1), which were pre-sonicated to facilitate desorption of fungal cells from the sediment particles and increasing the DNA yields. DNA was isolated using UltraClean soil DNA isolation kits (Mo Bio Laboratories, Inc., Carlsbad, CA), according to the manufacturer's instructions.

The resulting genomic DNA was used as a PCR template to amplify ITS2 gene region using the most 'universal' of 'panfungal' primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.*, 1990). The reaction mixture for PCR had the following composition: Tris-HCl, 50 mM (pH 9.0); KCl, 50 mM; $MgCl_2$, 1.5 mM; Triton X-100, 0.1%; glycerol,

Table 1. The viable mycelial fungi in Antarctic permafrost sediments

No	Location	Bore-hole	Latitude	Longitude	Alti-tude (m)	Age (years)	Mean annual temperature (°C)	Depth (cm)	Max.number (CFU g^{-1})	Fungal taxa
1	Bellingshausen Station	A11/08	62°11′48″S	58°7′56″W	15	7485 ± 40	− 1.0	320–330	18.1	*Aspergillus sydowii*, *Chrysosporium europae*, *Penicillium chrysogenum*
2								410–420	0	
3								500–520	12.9	*Lecanicillium muscarium*, *Penicillium brevicompactum*, *P. chrysogenum*, *P. variabile*, *Trichoderma harzianum*
4								530–550	0	
5								585–600	0	
6								615–625	21.8	*Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *P. variabile*, *P. waksmanii*
7								680–700	0	
8								850–860	6.5	*Trichoderma atroviride*
9								965–975	53.1	*Penicillium chrysogenum*, *P. olsonii*
10	Progress Station	A1/07	69°24′04″S	76°23′33″W	56	No date	− 8.5	70–80	0	
11								90–100	1.9	Mycelia sterilia (dark)
12								190–200	0	
13		A2/07	69°24′14″S	76°20′36″W	96			100–110	0	
14								230–240	4.4	*Cryomyces* sp., *Penicillium chrysogenum*, Coelomycetes Incertae sedis*
15		A3/07	69°24′0″S	76°20′46″W	98			110–130	0	
16								160–170	0	*Aureobasidium pullulans* var.*pullulans**
17								180–190	0	
18	Banger Oasis	A5/08	66°16′31″S	100°4′36″E	7	23 705 ± 110	− 7.8	100–105	8.1	*Penicillium chrysogenum*
19								115–125	11.4	*Cladosporium herbarum*, *Phoma eupyrena*, *Cladosporium antarcticum*, *Chaetophoma* sp., Coelomycetes Incertae sedis
20								145–150	0	
21								175–185	8.6	*Aspergillus sydowii*
22								320–325	5.9	*Cladosporium cladosporioides*
23	Russkaya Station	A6/08	74°45′47″S	136°47′53″W	64		− 10.8	8–15	2.7	Mycelia sterilia (dark)
24								15–20	2.2	*Cladosporium herbarum*
25								20–26	5.2	*Cladosporium cladosporioides*
26								26–33	0	
27								65–70	0	
28		A8/08	74°45′48″S	136°47′47″	76			40–70	0	
29								115–120	91.1	*Gliocladium* sp., *Aureobasidium pullulans* var. *pullulans*, Ascomycota Incertae sedis, *Exophiala* sp., *Ascochyta* sp.
30								130–140	8.0	Ascomycota Incertae sedis

Table 1. Continued

No	Location	Bore-hole	Latitude	Longitude	Alti-tude (m)	Age (years)	Mean annual temperature (°C)	Depth (cm)	Max.number (CFU g^{-1})	Fungal taxa
31	Novolazarev-	1/09	70°45′45″S	11°47′27″E		> 50 000	− 8.3	80–95	0	
32	skaya	2/09						95–105	0	
33	Station	3/9						60–70	17.1	*Penicillium expansum*, Mycelia sterilia (dark)
34		4/9						85–100	0	*Cladosporium herbarum**
35	Beacon	10/99	77°48′S	160°42′E	1000	> 3 000 000	− 22.5	390–450	0	
36	Valley	11/99						570–600	0	

*Species were isolated using the accumulative method.

10%; cresol red (Na), 0.05 mM; dNTP mix, 0.2 mM of each nucleotide (Promega); primers, 0.05 μM each (Syntol); Taq polymerase with inhibitory monoclonal antibodies, 25 U mL^{-1} (Syntol). The volume of the reaction mixture was equal to 20 μL, of which up to 20% accounted for the solution of template DNA. PCR program: primary template melting, 7 min at 95 °C; 35 cycles of 30 s at 95 °C (melting), 45 s at 54 °C (primer annealing), and 60 s at 72 °C (elongation); 10 min at 72 °C (final elongation). The thermal cycler used in these experiments was a BioRad MyCycler 580BR 2261. The reaction products were separated electrophoretically in 2% agarose gels, stained with ethidium bromide, and visualized and documented on a BioRad Molecular Imager Gel Doc XR System (p/n 170–8170).

Cloning

Molecular cloning of the amplicon library, based on blue/white selection, was performed by ligation into pGEM®-T Vector System (Promega), using competent *Escherichia coli* strain JM109 cells (Promega), as instructed by the manufacturer. To verify the presence of the target nucleotide insert in the colony cells, the amplification was run with the ITS3/ITS4 primer pair (the composition of the mixture and the parameters of the reaction were similar to those described above); a fragment of an individual *E. coli* colony was used as a template. We expected to receive no < 50 clones at the blue-white screening stage. However, the number of isolated clones ranged from 30 to 70 per sample.

Sequence analyses

Direct sequencing of DNA fragments was performed by the method of fluorescent dye-labeled terminators on a Beckman Coulter CEQ 8000 Genetic Analysis System according to the manufacturer's protocol; the reaction was prepared using a Beckman Coulter Quick Start Kit and ITS3 as a primer (4 pmol per reaction). The sequences obtained were aligned and edited using CEQ 8000 Software package (Beckman Coulter). Differing sequences were taken to represent operational taxonomic units (OTUs) and identified taxonomically by checking against the International Nucleotide Sequence Database (www.insdc.org) using BLASTN software (Blastn and megablast algorithms at a threshold of 10); www.ncbi.nlm.nih.gov/books/NBK21097/ to exercise caution in interpreting the results, sequences of low in credibility were eliminated from the comparison database.

To control for the occurrence of possibly chimeric sequences, all sequenced clones were checked using UCHIME (Edgar *et al.*, 2011). Of 124 sequences, 108

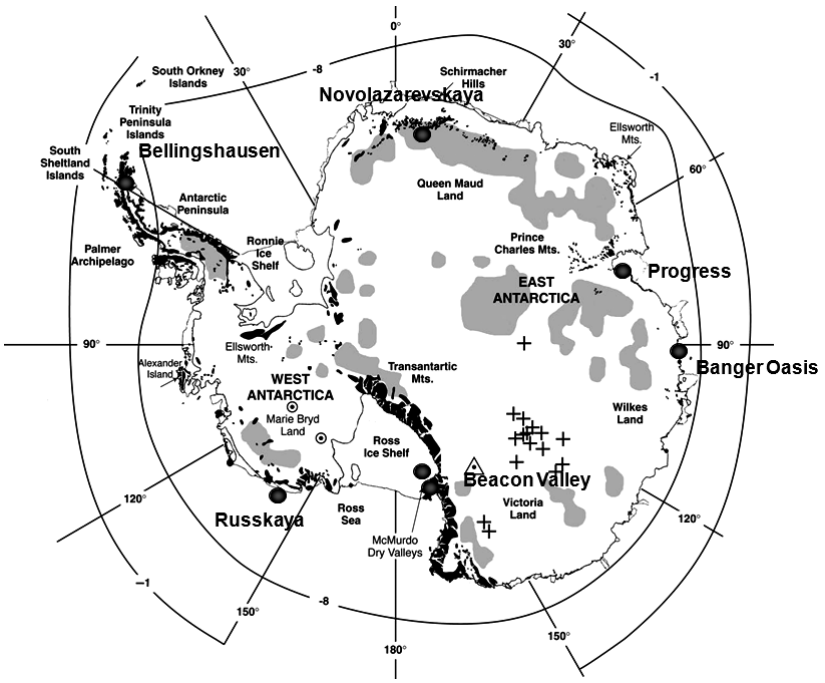

Fig. 1. Antarctic map. Locations of the sites where cores were recovered.

(87, 1%) were non-chimeric. The non-chimeric sequences were submitted to GenBank and given accession numbers HM588009–HM588115, HM622757.

Results and discussion

The fungi found in permafrost sediments and discussed in this study are very probably of indigenous nature. Nonindigenous microorganisms are unlikely to penetrate permafrost layers either from the surface or from other layers through possible vertical movement of water. The surface beneath the seasonally thawing (active) layer (Graham *et al.*, 2012) is the permafrost. This creates physical and biogeochemical barriers that restrict the influence of external factors and processes. The underlying permafrost horizons are firmly cemented, showing no signs of water-bearing cavities and water infiltration. Because of low temperature, the amount of unfrozen water that could exist under such conditions is so small that it is unlikely that translocation of cells can occur along water films, thereby making migration of microbes unlikely. The permafrost, therefore, very probable resist penetration by modern microorganisms into the frozen depths and significantly reduces the probability of contamination (Gilichinsky *et al.*, 1989, 2007; Shi *et al.*, 1997; Steven *et al.*, 2006).

Using cultural methods, the density of fungal population in permafrost was found to be low (Table 1). Nearly 50% of samples appeared not to contain culturable fungi under the experimental conditions used. The number of fungal colonies developed from the other sediment samples ranged from 2 to 91 CFUs (colony forming units) calculated per 1 g of air-dry sample. Most isolates were detected in the rock-rich glacial marine sediments at the Bellingshausen Station (the site that is also characterized by the highest mean annual air temperature, − 1 °C). Loose rocks are known to facilitate the solid-phase immobilization of cells and thereby may favor their preservation. Conversely, the gravel-sand sediments from the Progress Station were practically devoid of microscopic fungi. Meanwhile, a few fungal organisms (*Cryomyces* sp., *Aureobasidium pullulans* var. *pullulans* and *Cladosporium herbarum*) found in two samples (nos 14 and 16) from the Progress Station and one sample (no. 34) from the Novolazarevskaya Station were recovered using exclusively the isolation via accumulative culturing.

The observed distribution in quantity had a focal character and was rather not related to the depth or age of sediments, which is generally similar to that in Arctic permafrost (Kochkina *et al.*, 2001; Gilichinsky *et al.*, 2007; Ozerskaya *et al.*, 2008). However, the total number of fungal isolates detected in deep horizons of Antarctic sediments (Fig. 2) was considerably lower than in the Arctic, at least for specimens analyzed.

Throughout the entire period of Antarctic subsurface permafrost studies (1998–2011), we have isolated fungi of more than 26 genera along with unidentified strains with sterile mycelia. The data of this latest work and of our previous research into the diversity of fungi sampled in

dry valleys of Antarctica (Gilichinsky *et al.*, 2007) are summarized in Table 2.

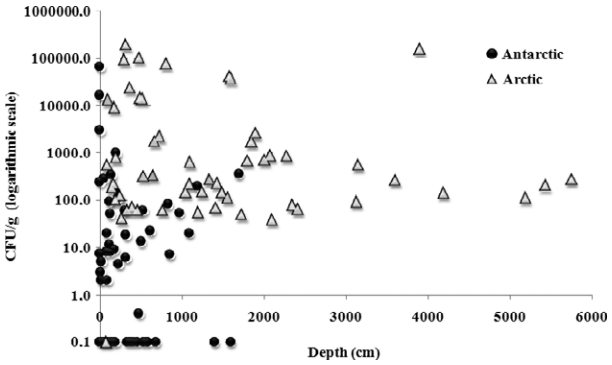

Fig. 2. Number of fungi in permafrost sediments.

Our previous assessment of the influence of the temperature regime of sample defrosting and heating on the taxonomic composition of micromycetes has shown that the exposures of the samples at 35 and 52 °C lead to the expansion the spectra of the taxa has revealed (Kochkina *et al.*, 2001). Notably, a strain with dark mycelium (identified subsequently as *Exophiala* sp.) and a representative of keratinolytic species *Chrysosporium europae* were isolated solely from the specimens heated at 52 °C. The exposure of samples at 20 °C resulted in the detection of maximal number of fungal taxa. The incubation temperature and the isolation method also influenced the taxonomic composition of the isolated micromycetes. In particular, quite a rare psychrotolerant fungus of the genus *Cryomyces* with a monilioid hyphal structure (not yet identified at the species level) was isolated at 4 °C through accumulative culturing of a sample in liquid medium.

Table 2. Complete list of filamentous fungi isolated from Antarctic permafrost sediments

Taxa	Number of samples in which the taxa occurs	Taxa	Number of samples in which the taxa occurs
Arthrinium arundinis (Corda) Dyko et B. Sutton*	1	*P. chrysogenum* Thom	8
Ascochyta sp.	1	*P. citrinum* Thom†	2
Ascomycota Incertae sedis	2	*P. commune* Thom†	1
Aspergillus sydowii (Bainier et Sartory) Thom et Church	3	*P. expansum* Link	3
A. versicolor (Vuill.) Tirab.†	3	*P. funiculosum* Thom†	1
Aureobasidium pullulans (de Bary) G. Arnaud	2	*P. glabrum* (Wehmer) Westling†	1
Chaetophoma sp.	1	*P. granulatum* Bainier†	2
Chrysosporium europae Sigler, Guarro et Punsola	1	*P. jensenii* K. M. Zalessky†	1
C. xerophilum Pitt*	1	*P. implicatum* Biourge†	1
Cladosporium antarcticum K. Schub., Crous et U. Braun	1	*P. miczynskii* K. M. Zalessky†	1
C. cladosporioides (Fresen.) G. A. de Vries	4	*P. minioluteum* Dierckx†	7
C. herbarum (Pers.) Link	6	*P. olsonii* Bainier et Sartory	1
C. sphaerospermum Penz.	2	*P. purpurogenum* Stoll†	1
Coelomycetes Incertae sedis	2	*P. rugulosum* Thom*	1
Cryomyces sp.	1	*P. variabile* Sopp	4
Dipodascus aggregatus Francke-Grosm.	1	*P. viridicatum* (Westling) Frisvad et Filt.†	1
Eurotium herbariorum (F.H. Wigg.) Link	2	*P. waksmanii* K. M. Zalessky	1
Exophiala sp.	2	*Phoma eupyrena* Sacc.	1
Fusarium oxysporum Schltdl.†	1	*Ph. leveillei* Boerema et G. J. Bollen*	1
Geotrichum candidum Link*	1	*Pochonia suchlasporia* var. *catenata* (W. Gams et Dackman) Zare et W. Gams*	1
Gliocladium sp.	1	*Trichoderma atroviride* P. Karst.	1
Lecanicillium muscarium (Petch) Zare et W. Gams	1	*T. harzianum* Rifai	1
L. lecanii (Zimm.) Zare et W. Gams†	1	*Ulocladium botrytis* Preuss*	1
Mortierella sp.*	1	*Wardomyces anomalus* F. T. Brooks et Hansf.*	1
Oidiodendron sp.*	1		
Penicillium aurantiogriseum Dierckx*	2	Mycelia sterilia (dark)	5
P. brevicompactum Dierckx	1	Mycelia sterilia (white)‡	5

*Data of previous studies (Gilichinsky *et al.*, 2007).

†Unpublished data of the authors obtained in the study of different Antarctic permafrost samples in 2000–2010.

‡With using molecular-biological methods, several strains of Mycelia sterilia found to be similar to *Venturia* sp., *Thanatephorus cucumeris,* and *Bjerkandera adusta*.

The taxonomic diversity of the fungi found in each fungus-containing sample was insignificant, mostly 1–3 species, increasing up to 4–5 species in marine-related sediments from the Bellingshausen Station and Banger Oasis. The most frequently occurring micromycete genera in the tested samples were *Penicillium* (19 species) and *Cladosporium* (four species). These fungi usually dominate the fungal population both in Arctic and in Antarctic permafrost sediments (Gilichinsky *et al.*, 2007; Ozerskaya *et al.*, 2009), with *Penicillium chrysogenum* and *Penicillium minioluteum* being found most commonly. Onofri *et al.* (2010) also mentioned *P. chrysogenum* among the most frequently encountered fungi occurring in the deep horizons (233–335 cm) in the Beacon Valley, Antarctica. An isolate similar to *P. chrysogenum* was recently recovered from the ice of Lake Vostok in Antarctica (D'Elia *et al.*, 2009). The psychrotolerant fungi *Penicillium olsonii*, *Cladosporium sphaerospermum*, and *Trichoderma atroviride* revealed in permafrost were also isolated from arctic ice, salted marine sediments, saline soils, etc. (Gunde-Cimerman *et al.*, 2003; Zalar *et al.*, 2007; Hujslová *et al.*, 2010). The ability to grow at, or be tolerant to, low water activity is rather characteristic of these fungi.

The presence of multiple types of sterile mycelium is another prominent characteristic of fungal organisms in permafrost sediments. Recent identification of five representatives of such strains on the basis of DNA sequence analysis of the ribosomal RNA gene cluster revealed these strains to be most close to ascomycetous *Venturia* sp. and two basidiomycetous species, *Thanatephorus cucumeris* (GenBank FJ609294) and *Bjerkandera adusta* (GenBank FJ609296; Ozerskaya SM., Kochkina GA, Ivanushkina NE & Vasilenko OV, unpublished data).

Throughout the study of permafrost samples by culture-independent techniques, we succeeded only in isolation of total fungal DNA from as little as three samples (nos 9, 10, and 20) out of nine. It produced 108 OTUs of ITS2 region, of which 100 OTUs were pertinent to the Kingdom Mycota, 2 to the Kingdom Metazoa, and 6 OTUs exhibited no relatedness to any sequences deposited so far in the GenBank database (Table 3). In all specimens analyzed, OTUs close to ascomycetous yeasts (*Candida albicans* and *Pichia kluyveri*) and basidiomycetous yeasts (*Cryptococcus pseudolongus*) were the most abundant. These yeast species are cosmopolitan, and the general abundance of yeast (including species of the genus *Cryptococcus*) has been reported in many publications on Antarctic microorganisms (Scorzetti *et al.*, 2000; Thomas-Hall *et al.*, 2002; Connell *et al.*, 2008; etc.).

Note that our data on the abundance of *C. albicans* is likely to be overestimated, as the DNA of this fungus may have competitive advantages over other DNAs with respect to amplification (Playford *et al.*, 2006).

In samples 9 and 10 from Progress Station and Bellingshausen Station, ITS2 sequences highly similar to that of the lichenized fungus *Lecania brialmontii* common to Antarctica (Kim *et al.*, 2006) were detected. Sample 10 also contained a sequence exhibiting a maximum similarity (86%) to that of the cosmopolitan lichen *Roccella gracilis*. One more sequence from this sample was very close (92%) to the sequences of the cosmopolitan fungus *Diaporthe helianthi*, a phytopathogen causing sunflower stem canker. The fungus from sample 9 had the ITS2 sequence identical to that of the ectomycorrhizal fungus *Cortinarius scaurus* f. *phaeophyllus*, which is likewise also widespread. Specific growth requirements do not favor the development of this fungus on nutrient media commonly used for isolation. In addition, we revealed three sequences referred to *Pezizomycotina* (samples 9 and 10). One exhibited a maximum sequence similarity (84%) to *Dwayaangam colodena*, a fungus found previously in Canada (Quebec) and known to realize its developmental cycle both in terrestrial and in water environments (Sokolski *et al.*, 2006). The second is related (90%) to *Imaia gigantea*, a truffle fungus reported to occur in Japan and North America (Appalachian Mountains; Kovacs *et al.*, 2008). The third sequence has 86% similarities with lichen *R. gracilis*. Yet another OTU, denoted Fungus Incertae sedis, exhibited a maximum similarity to a GenBank sequence (EU516679.1), its taxonomic position remains to be established. Finally, there were also six sequences (from samples nos 10 and 20) that we failed to assign to any taxonomic group owing to their quite low similarities to any sequence available in the GenBank.

Comparison of the data generated by culture-dependent vs. culture-independent studies showed just one sample (no. 9), in which the fungi were revealed by both techniques. The culturing enabled detecting the ascomycetous species *P. chrysogenum* and *P. olsonii* which produce small-sized spores and are widely distributed in low-temperature habitats (Kochkina *et al.*, 2001). Direct DNA analysis failed to detect these organisms but showed the presence of a fungus closely related to *C. scaurus* f. *phaeophyllus* which is likewise widespread. No fungi were detected by both techniques in the oldest sediments analyzed (samples 35 and 36; 4–6 m, 3 000 000 years), these are from Beacon Valley.

The fungal DNA not yet assigned to any taxonomic group, except *D. helianthi* mentioned before was revealed in samples nos 10 and 20, whereas no fungal colonies appeared at the culturing procedures. On the other hand, the analysis of genomic DNA from samples nos 1, 3, 6, and 18 failed to detect any fungi, whereas inoculation of specimens into culture media revealed a number of species

Table 3. Diversity of fungal OTU defined by molecular methods in Antarctic permafrost sediments

OTU types	Number of OTU in samples			NCBI match				Length, bp	Ecological characteristics of taxa
	9	10	20	Taxa	Homology, %	Coverage, %	*E*-value		
1	20	22	19	*Candida albicans* (C. P. Robin) Berkhout	81–100	72–100	$< 3e^{-39}$	163–339	Mammals and birds commensal and pathogenic fungus
2	11	17		*Cryptococcus pseudolongus* M. Takash., Sugita, Shinoda et Nakase	81–100	88–99	$< 3e^{-59}$	245–313	Dead tree, soil
3	2	1		*Pichia kluyveri* Bedford ex Kudryavtsev	98–99	100	$< 3e^{-97}$	207–259	Plant pathogen
4	1	1		*Lecania brialmontii*	94–98	98–100	$< 4e^{-122}$	250–326	Antarctic lichen
5			2	*Cyrtohymena citrina*	94–95	99–100	$< 8e^{-130}$	302–303	Prorozoa, infusorium
6		1		*Roccella gracilis* Bory	87	12	0.39	311	Lichen, coastal areas, grows on vertical rocks, trees and shrubs
7		1		*Diaporthe helianthi* Munt.-Cvetk., Mihaljč. et M. Petrov	92	23	$4e^{-23}$	335	Plant pathogen (sunflowers, etc.), common in Europe
8		1		Fungus Incertae sedis	86	72	$4e^{-42}$	221	
9		1		*Dwayaangam colodena* Sokolski et Bérubé	84	39	$7e^{-12}$	230	Aquatic fungus, mixed-wood forests
10		1		*Imaia gigantea* (S. Imai) Trappe et Kovács	89	22	$1e^{-12}$	292	Mycorrhizal fungi
11	1			*Cortinarius scaurus* (Fr.) Fr. f. *phaeophyllus* M. M. Moser	100	10	$2e^{-7}$	474	Forests of N. America, Europe, and New Zealand, plantation of S. America

characteristic of low-temperature ecotopes (*Aspergillus sydowii*, *C. sphaerospermum, Penicillium brevicompactum*, *P. chrysogenum*, *P. variabile*, *P. waksmanii* and *Trichoderma harzianum*). This finding is consistent with the previously reported data on Arctic permafrost concerned with extremotolerant fungi forming small-size spores (Lydolph *et al.*, 2005; Ozerskaya *et al.*, 2009). This finding may be related to the difficulties of isolating the DNA of these fungi.

This is the first report of a comprehensive study of filamentous fungi in Antarctic subsurface permafrost sediments by applying culture-dependent techniques along with culture-independent methods. The numbers of fungal isolates, their microfocal profile distribution, and their taxonomic composition were determined. The study revealed a low density of fungal population in Antarctica, which contrasted with the appreciable taxonomic diversity of fungi identified. Of particular interest is the observation concerning the genera *Penicillium*, *Cladosporium*, etc. These are commonly found in permafrost by conventional microbiological techniques, but fail to be detected by the molecular methods used. Further studies will develop adequate molecular techniques to enable revealing these and other fungal groups that have thus far escaped detection, including efficient methods of extracting total genomic DNA and designing appropriate primers.

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