Polar Biol (2012) 35:749–757
DOI 10.1007/s00300-011-1119-6

ORIGINAL PAPER

Searching for eukaryotic life preserved in Antarctic permafrost

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Received: 7 July 2011 / Revised: 6 October 2011 / Accepted: 12 October 2011 / Published online: 11 November 2011

Abstract Fungi and yeasts isolated in pure culture from Antarctic permafrost collected at different depths in the McMurdo Dry Valleys were identified with cultural, physiological and molecular methods. Fungi belonged to the genera *Penicillium*, *Eurotium*, *Cladosporium*, *Alternaria*, *Engyodonthium*, *Aureobasidium*, *Cordyceps*, *Rhizopus* and yeasts to the genera *Cryptococcus* and *Sporidiobolus*. All the strains can be defined as mesophilic psychrotolerant. The molecular analyses revealed that these fungal genotypes do not deviate from the global gene pool of fungi commonly spreading worldwide at present, but possible ancestral strains have been found on the base of metabolic profiles.

Keywords Antarctica · Fungi · Yeasts · Metabolic profiles · UV

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Introduction

Permafrost is defined as a soil remaining at 0°C or below throughout two or more consecutive years (Muller 1947) while the active layer can be defined as the top layer of ground subject to annual thawing and freezing in areas underlain by permafrost (ACGR 1988). Heterogeneous permafrost ecosystems are present in nature, depending on climatic conditions. Mainly present in polar areas, permafrost occurs in all ice-free areas of Continental Antarctica (Bockheim et al. 2008). The McMurdo Dry Valleys, from which drilled samples originate, are the largest ice-free areas in Antarctica; the thickness of the active layer decreases from coastal areas to the polar plateau (Bockheim and Hall 2002) and in the high-elevation parts, and where the day-average surface temperature never exceeds the freezing point of water, the active layer may not form at all (Kreslavsky et al. 2008). The active layer in Victoria Valley has been monitored since 1999 ranging between 18 and 22 cm; mean annual air temperature was −20°C with large daily fluctuations during the winter (Adlam et al. 2010). A number of different viable microorganisms has been recorded from permafrost habitats. The microbial ecology and biodiversity of permafrost have been extensively reviewed (Wagner 2008). Rather wide microbial populations were recorded in Arctic permafrost samples, instead data on microorganisms preserved in subsurface Antarctic permafrost are scant (Ozerskaya et al. 2009). Viable microfungi and bacteria were reported from Antarctic permafrost sediments of different ages using cultural and molecular techniques (Vorobyova et al. 1997; Kochkina et al. 2001; Gilichinsky et al. 2007). Molecular studies have recently improved our knowledge on microbial diversity in permafrost from different Arctic and Antarctic locations, and a rather greater bacterial and yeast

diversity and abundance were found in permafrost soils, compared to filamentous fungi.

A community of predominantly psychrotrophic microorganisms, described as "a community of survivors" (Friedmann 1994), was reported as inhabiting the perennially frozen terrestrial permafrost. Recent data suggest considering the permafrost environment as an active microbial ecosystem rather than a frozen habitat with microbial survivors. Life maintenance of a psychrotolerant anaerobic bacterial community would be assured by an extremely low amount of biologically available liquid water, present as thin films firmly bound to soil particles (Rivkina et al. 2000). The stability of the environmental parameters below the superficial layer makes this environment less extreme than one can suppose and it may be ideal for the long-term preservation of microorganisms and biomolecules (Willerslev et al. 2004).

Permafrost seems to be also present on other planets of the Solar System, such as Mars. Water ice is assumed to be present on the Martian subsurface, in the large region surrounding the South Pole, where it may have remained entrapped when liquid water disappeared (Brack 2007). This evidence induces new interest on the possibility to investigate permafrost as possible reservoir of prokaryotic and eukaryotic spores outside the Earth, and Antarctic permafrost is considered the closest Mars analog model (Gilichinsky et al. 2007).

This contribution concerns the detection of fungal and yeast diversity in permafrost samples at different depths from the Upper Victoria Lake in the McMurdo Dry Valleys (Antarctica) studied by cultural, physiological and molecular methods.

Materials and methods

Sample collection

Core samples were collected on November 2003 in the Dry Valleys (Antarctica), close to the Easter side of the Upper Victoria Lake (77°20′03″S e 161°37′23″E), and transported and stored at −20°C at the University of Milano Bicocca (Department of Environmental Science). Permafrost temperature monitoring started in the closest borehole in Wright Valley only on 2007; mean annual permafrost temperature (MAPT, determined at the permafrost table) was −18.8°C in 2008, with daily fluctuations lower than 1°C/day already at 1 m depth until less 0.5°C/year at 17 m depth (Guglielmin et al. 2011), and the maximum thaw depth do not exceed 1 m. The temperature profile of the core samples was measured with a thermistor's chain (accuracy higher than 0.1°C) some days after the drilling and revealed temperatures ranging between −12.4 (1 m depth) and −25°C (5 m depth) with a mean value of −22.7°C.

The core may be defined as a ice-cemented permafrost with the surface greater than 70 cm (Bockheim et al. 2008). It was characterised by frozen sediments quite homogeneous for all the depths, composed by sand and siltysand with rare thin (2–3 cm) layers of gravel. The sediments were dry-frozen in the first 85 cm, while below the ice content was generally low (5–10% in volume) composed mainly by pore ice and rare ice lenses or sub-horizontal layers (1–3 cm of thickness). Three sections, corresponding to 233, 316 and 335 cm depths, were cut using a diamond saw; each section was divided in two parts to be processed for chemical and microbiological analyses respectively. The temperature measured in the borehole at the depth of the samples here considered ranged between −16 and −20.1°C.

Isolation

The cultivable microbial component of the core samples was isolated at the Department of Applied Biology (University of Perugia). A laboratory decontamination protocol (Rogers et al. 2004) was used in order to exclude the presence of external microorganisms on the core sample surfaces introduced during drilling procedures. Surface-sterilized micro-cores were melted, and 1 mL spread in Petri dishes (9 cm Ø) containing Rose Bengal (RB) agar + tetracycline; Petri dishes were incubated at 4 or 20°C for 12 or 3 weeks respectively, and the presence of colonies on the plates was periodically checked. Cultures were purified transferring colonies in Petri dishes containing RB agar + tetracycline. The isolates were preserved both as frozen (−80°C) and freeze-dried cultures. All fungal isolates are preserved in the CCFEE (Culture Collection of Fungi From Extreme Environments) at the University of Viterbo, whereas yeast strains are deposited in the Industrial Yeasts Collection DBVPG at the University of Perugia (http://www.agr.unipg.it/dbvpg).

Freeze-dried samples of the isolates were revitalized on Potato Dextrose Agar (PDA), a general raw medium for filamentous fungi, at 4 and 20°C, according with the isolating temperature. Fungal strains isolated from different permafrost samples were first grouped on the base of morphological characteristics observed in culture; few strains for each group were selected for further morphological, metabolic and molecular studies. All the yeast isolates were studied by a polyphasic approach combining conventional and molecular approaches.

Temperature preferences for filamentous fungi were tested by inoculating strains in Petri dishes containing suitable media, PDA for filamentous fungi (*Cladosporium*, *Alternaria*, *Engyodontium*, *Cordyceps*, and *Rhizopus*) and

Czapek Dox Agar (CzA) for *Penicillium* and *Aspergillus*, and incubating at 0 ± 1, 5 ± 1, 10 ± 1, 15 ± 1, 20 ± 1, 25 ± 1, 30 ± 1, and 35 ± 1°C. *A. pullulans* var. *pullulans* and yeast growth at different temperatures (within 4°–37°C) was checked in test tubes containing yeast extract-peptone-glucose (YEPG), specific for yeasts. All above tests were performed in triplicate.

Molecular analysis of filamentous fungi

Fungal genomic DNAs were extracted using Nucleospin Plant kit (Macherey–Nagel, Düren, Germany) following the protocol optimised for fungi.

The amplifications of the ITS rDNA portion for fungi were performed using BioMix (BioLine GmbH, Luckenwalde, Germany) employing primers ITS1, ITS4 (White et al. 1990), ITS5, and ITS4a (Larena et al. 1999). The amplifications were carried out using MiniCycler™ (MJ Research, Waltham, Massachusetts, USA) equipped with a heated lid. The program for amplification was as follows: a first denaturation step at 95°C for 5 min was followed by denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 32 s. The last three steps were repeated 35 times, with a last extension at 72°C for 5 min.

The PCR products were visualized on agarose gel electrophoresis and quantified by comparison with the Ladder GeneRuler™ 1 kb DNA (Fermentas). The products were purified using Nucleospin Extract kit (Macherey–Nagel, Düren, Germany). Sequencing reactions were performed by Macrogen Inc. (Seoul, Korea) (http://www.macrogen.com) according to the dideoxynucleotide method (Sanger et al. 1977). Sequence assembly was done using the software Chromas (version 1.45 1996–1998, Conor McCarthy School of Health Science, Griffith University, Southport, Queensland, Australia). Sequences with high similarity available in the NCBI Genbank were identified using BLASTn search (Altschul et al. 1997; http://www.ncbi.nlm.nih.gov/BLAST). The comparisons were based on fragments with lengths ranging from 530 to 590 base pairs.

Metabolic profiles of filamentous fungi

The Penicillia were grown on Czapek Yeast Agar (CYA) and Yeast extract-sucrose (YES) for optimal secondary metabolite production. Secondary metabolites were extracted after incubation at 25°C for 7 days. Five plugs of each agar medium were taken and pooled together into same vial for extraction with 0.75 mL of a mixture of ethyl acetate/dichloromethane/methanol (3:2:1) (v/v/v) with 1% (v/v) formic acid. The extraction liquid was evaporated and the dry extracts was redissolved in methanol, filtered and analyzed by HPLC using alkylphenone retention indices and diode array UV–VIS detection as described by Frisvad and Thrane (1987), with minor modifications as described by Smedsgaard (1997). The column used was a 50 × 2 mm Luna C-18 (II) reversed phase column (Phenomenex, CA, USA) fitted with a 2 × 2 mm guard column.

Molecular analysis of *A. pullulans* var. *pullulans* and yeasts

Total genomic DNA extraction was done by NucleoSpin® Tissue DNA extraction kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) using the yeast protocol with some modifications. Disruption of the cell wall was achieved by suspending three loopfuls of 48 h cultures in YEPG agar in 300 µL of sterile water and 200 µL (calculated as equivalent volume) of glass beads (diameter = 425–600 µm) were added. After vortexing for 2 min, the tubes were incubated for 1 h at 65°C, after which samples were vortexed again for 1 min. The suspension was then handled according to the protocol (Turchetti et al. 2008).

Yeasts isolated were subjected to sequencing of the D1/D2 domain of 26S rRNA gene. DNA was first amplified by using the primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G) and LR6 (5′-CGC CAG TTC TGC TTA CC) (Sigma Aldrich). A 600-650 bp region was sequenced by the forward primer NL1 (5′-5′-GCA TAT CAA TAA GCG GAG GAA AAG) and the reverse primer NL4 (5′-GGT CCG TGTTTC AAG ACG G) (Sigma Aldrich). ITS rDNA was sequenced for strains exhibiting ambiguous results in D1/D2. ITS sequences were obtained using the primers RLR3R (5′-GGT CCG TGT TTC AAG AC) and V9 (5′-TGC GTT GAT TAC GTC CCT GC) (Sigma Aldrich). A 600–650 bp region was sequenced by the forward ITS 1 (5′-TCC GTA GGT GAA CCT GCG G) and the reverse ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC) primers (Sigma Aldrich).

Sequences were obtained using an Applied Biosystems DNA Sequencer, mod. ABI3730XL (Applied Biosystems, USA) using standard protocols. Strains were identified by comparing the sequences obtained with the GenBank database (BLASTN freeware from http://www.ncbi.nlm.nih.gov/BLAST).

Nutritional profiles of *A. pullulans* var. *pullulans* and yeasts

The isolates were analyzed for their assimilation of different carbon and nitrogen sources by using the standard protocols (Yarrow 1998).

Results

Eighty-five fungal strains have been isolated. The isolates belonging to the same species were grouped according with

the sample depth and incubation temperature and few strains for each group were selected for molecular investigations (Table 1). Table 2 shows the highest similarity percentages with sequences in the public domain.

The blast analysis carried out on strains 28 and 65 gave 100% similarity with both *A. alternata* (Fr.) Keissl. and *A. tenuissima* (Nees) Wiltshire sequences deposited in NCBI Genbank; spore morphology and dimensions suggest a closer relationship with *A. alternata*, but the genus itself shows a wide morphological variability and ITS genes are not suitable to discern closely related species (Serdani et al. 2002). Phylogenetic analyses based on ITS and SSU rDNA sequences, performed with the neighbour joining and maximum parsimony methods, also placed *A. tenuissima* and *A. alternata* in a species-clade supported by 100% bootstrap value (Pryor and Gilbertson 2000). Strain 14 showed 100% similarity with deposited sequences of *Aureobasidium pullulans* var. *pullulans* (De Bary) G. Arnaud strains in the CBS collection. Strain 20 showed 100% similarity in ITS with *Eurotium amstelodami* L. Mangin, teleomorph of *A. vitis* Novobr. Among 17 isolates belonging to *Cladosporium*, strains 27, 37, 58 and 88 showed 99–100% similarities with deposited sequences of *C. cladosporioides* (Fresen.) G. A. de Vries, and strains 64, 32 and 81, 99–100% similarities with deposited sequences of *C. herbarum* (Pers.: Fr.) Link, the teleomorph *Davidiella tassiana* (De Not.) Crous and U. Braun and *C. macrocarpum* Preuss (syn. of *C. herbarum* var. *macrocarpum*). On the base of ITS similarities sample 50 belongs to *Engyodontium album* (Limber) de Hoog and sample 59 to *Rhizopus oryzae* Went & Prins. Geerl. Samples 63 and 75 can be assigned with certainty to *Cordyceps bassiana* Z. Z. Li et al. and the phylogenetic analysis confirm the classification, with a well supported cluster (data not shown).

Penicillia represent the widest group and have been determined on the base of strains morphology, physiology and by molecular tools. Strain n. 79 showed 100% similarity with the species *Penicillium expansum* Link and strains ns 15 and 36 with 99 and 100% similarity belong to *Penicillium glabrum* (Wehmer) Westling. Strains 45, 47, 55, and 87 remained undetermined; they were 100% similar to more strains as a *P. commune* Thom (AF455544.1) strain isolated from nasal mucus (Buzina et al. 2003) and an undetermined lead resistant *Penicillium* strain Psf-2 (EF660439.1) isolated from the deep sea sediment of the Pacific Ocean (Sun and Shao 2007). Many of the analysed strains, showing high ITS similarities in GenBank with different *Penicillium* species, have been identified as *P. palitans* Westling and *P. chrysogenum* Thom on the base of morpho-physiological characteristics rather than molecular results. In particular 10 strains, representative of 39 isolates, belonged to *P. palitans* and eight strains, representative of 10 isolates belonged to *P. chrysogenum*; their metabolic profiles are reported in Table 3.

Table 1 Isolation number of the fungi grown from each permafrost sample (sample 1 = 233 cm, sample 2 = 316 cm, sample 3 = 335 cm) and incubation temperature

Species	Sample 1		Sample 2		Sample 3	
	20°C	4°C	20°C	4°C	20°C	4°C
Alternaria alternata			**65**		**28**	
Aureobasidium pullulans var. *pullulans*					**14**	
Cladosporium cladosporioides	**37**		56, **58**, 69	**88**, 94	**27**	
Cladosporium herbarum			57, 61, 62, **64,** 70, 77, 80	**81**, 92		**32**
Cordyceps bassiana			**63**, **75**			
Cryptococcus stepposus					**25**, **26**	
Eurotium amstelodami					**20**	
Engyodontium album	**50**					
Penicillium chrysogenum	**41**, **43**		**60,** 67, **71**, 78	**86**	**9**, **13**, **24**	
Penicillium expansum			**79**			
Penicillium glabrum					**15**, 18	**36**
Penicillium palitans	38, 40, 42, 44, **49**	**46**, 51, 52, 76, **96**	33, 66, 72, 73, **74**, 97	82, **83**, 84, 85, 89, 90, 91, 93	1, 2, 5, 8, 10, **11**, **19**, 21, 22, **23**, 48	**29**, 31, 34, **95**
Penicillium sp.		**45**		**87**	**47**	**55**
Rhizopus oryzae			**59**			
Sporidiobolus metaroseus	**54**					

Isolates selected for molecular studies are reported in bold

Table 2 Species identification on the base of highest similarity percentages with sequences in the NCBI GenBank

Species	Nearest neighbour (accession number)	Similarity (%)
Alternaria alternata	*A. alternata* AF455539.1, AF455516.1, *A. tenuissima* AY154712.1	100
Aureobasidium pullulans var. *pullulans*	*A. pullulans* var. *pullulans* CBS 584.75^{T} (DQ321374), CBS 100524 (FJ150952), CBS 701.76 (FJ150951), CBS 146.30 (FJ150916)	100
Cladosporium cladosporioides	*C. cladosporioides* AY463365.1, AY361966.1, AY463363.1, AJ876482.1	99–100
Cladosporium herbarum	*C. herbarum* AF455517.1, AF455479.1, *Davidiella tassiana* AY463366.1, AY361993.1, *C. macrocarpum* EF679379.1, EF679380.1	99–100
Cordyceps bassiana	*Cordyceps bassiana* AJ564898.1, AJ564897.1, AJ564889.1, AY532052.1, AY532051.1	99–100
Cryptococcus stepposus	*Cr. stepposus* PTZ139^{T} (DQ222455), *Cryptococcus* sp. CBS 10179 (EU002867), CBS 10180 (EU002868)	99–100
Eurotium amstelodami	*E. amstelodami* AF455520.1, AF455536.1, AJ876482.1,	100
Engyodontium album	*E.album* DQ649066.1, AB106650.1	100
Penicillium chrysogenum	*P. aurantiogriseum AY280953.1*, *AY280956.1*, *P. brevicompactum DQ682592.1*, *AY373897.1*, *AF521657.1*, *P. camemberti AJ004817.1*, *P. chrysogenum* AY373902.1, AY213670.1, *P. citrinum* DQ681331.1, *P. commune* AF455544.1, AY373905.1, AY213672.1, AF455436.1, *P. dipodomyicola* DQ339570.1, *P. glandicola* DQ339565.1, *P. granulatum* DQ681334.1, *P. griseofulvum* DQ339557.1, *P. lanosum* DQ681336.1, *P. vinaceum* DQ681340.1, *Penicillium* sp. AF177735.1, EF660439.1, EF105368.1, AJ279476.1,	99–100
Penicillium expansum	*P. expansum* DQ339562.1, DQ339558.1, DQ339556.1	100
Penicillium glabrum	*P. glabrum* DQ682590.1, DQ681321.1	99–100
Penicillium palitans	*P. commune* AY373905.1, AY213672.1,AF236103.1, AF455471.1, AJ004813.1, *P. aurantiogriseum* AY280956.1, AY280953.1, *P. camemberti* AJ004814.1, *Penicillium* sp. DQ8101185.1, EF660439, EF660439.1, *P. vinaceum DQ681340.1*, *P. citrinum DQ681331.1P. granulatum* DQ681334.1	99–100
Penicillium sp.	*Penicillium* sp. EF660439.1, EF105368.1, *P. commune* AF455544.1, AF455436.1	100
Rhizopus oryzae	*R. oryzae* AB279763.1, AB097333.1, AB097332.1, AB2097331.1	99–100
Sporidiobolus metaroseus	*Sp. metaroseus* CBS 7683^{T} (EU003461), CBS 993 (EU003467), *S. roseus* CBS 2841 (AY070016)	99–100

Both yeast strains 25 and 26 exhibited an identity from 99 to 100% in D1/D2 with few unidentified strains of *Cryptococcus* and with the type strain (PTZ139) of the species *Cryptococcus stepposus* Golubev & Sampaio (Golubev et al. 2006). The analysis of ITS sequence confirmed the 100% homology with *Cr. stepposus* PTZ139. Finally, D1/D2 sequence of the strain 54 exhibited from 99 to 100% similarity with those obtained from the species *Sporidiobolus metaroseus* Sanpaio & Valerio and *Sporobolomyces roseus* Kluyver and C. B. Niel, the last recently reclassified as synonym of *Sp. metaroseus* (Valerio et al. 2008). The metabolic profiles of the dimorphic fungus *A. pullulans* var. *pullulans* and yeasts are reported in Table 4.

All fungal strains studied showed optimum growth temperatures in the range 20–30°C. In particular 14 strains showed optimal growth temperature at 20°C, 15 strains at 25°C, and 9 strains at 30°C. With the only exception of *E. album*, all the strains tested showed a minimal growth also at 0°C after 14 days of incubation. *A. pullulans* var. *pullulans* and yeasts grew within a wide range of temperature (from 4 to 25°C, data not shown).

Discussion

This paper represents a contribution to the knowledge of the cultivable fungal and yeast component of Antarctic permafrost. Two incubation temperatures (4 and 20°C) were used to get a number as large as possible of isolates (Kochkina et al. 2001), obtaining eighty-five fungal isolates. The most frequently recorded fungal species are *P. palitans* and *P. chrysogenum*, with 39 and 10 isolates respectively. All the strains found, with the only exception of *E. amstelodami* and *Cr. stepposus*, have been already recorded in Antarctic ecosystems as well as in other cold habitats (Onofri et al. 2007). Among them, *C. cladosporioides* and *C. herbarum* have already been reported as the most frequently strains isolated from the Dry Valleys permafrost samples (Gilichinky et al. 2007), and as the most widespread fungal species in Antarctica (Onofri et al. 2007). *C. herbarum*, as well as *A. alternata*, are also among the fungal strains isolated from cryopegs (Siberia) (Ozerskaya et al. 2004), and have been recorded as components of an halotolerant and psychrophylic community isolated from cryopegs in Arctic (Gilichinsky et al. 2005)

Table 3 Metabolic profiles of *Penicillium palitans* and *P. chrysogenum* strains

	Penicillium palitans strains	*Penicillium chrysogenum* strains
Andrastin X	19, 23	–
Asperenone X	11, 19, 95, 23 (trace), 29, 49, 74, 83, 96 (trace)	–
Cyclopenin	19	–
Cyclopenol	11, 19	–
Cyclopiazonic acid	11, 19, 95, 23, 29, 46, 49, 74, 83, 96	–
Dehydrocyclopeptin	19	–
Frequentin	95	–
"FOM"	23, 29, 46, 49, 74, 83, 96	–
"HUJI"	23, 29, 46, 49, 74, 83, 96	–
Palitantin	95	–
Viridicatin	19	–
Viridicatol	11, 19	–
Antraquinones		86, 24, 41, 60
Andrastin A		9, 13, 41, 60
Chrysogine		24, 9, 43
Lumpidin X		13, 86, 24, 9, 41, 60
Meleagrin		13, 24, 9, 43, 41, 60
Roquefortine C		9
Secalonic acid D		13, 86
Secalonic acid F		13, 86
Sorbicillins		9, 43
"STERO"		41, 60
"SULO"		41, 60

Table 4 Assimilation of different carbon and nitrogen sources for *A. pullulans* var. *pullulans* and yeast strains

Carbon and nitrogen sources	*A. pullulans* var. *pullulans* strain	*Cr. stepposus* strains		*Sp. metaroseus* strain
	14	25	26	54
D-glucose	×	×	×	×
D-galactose	×	w	×	
L-sorbose	×		×	w
D-glucosamine		×	×	
D-ribose	×	×	w	×
D-xylose	×	×	x	
L-arabinose	×	w	×	
L-rhamnose	×	×	×	
Sucrose	×	×	×	×
Maltose	×	×	×	×
α-trehalose	×	×	×	×
Methyl-α-D-glucoside		×	×	
Cellobiose	×	×	×	×
Melibiose	×	×	w	
Lactose		×	×	
Raffinose	×	×	×	×
Melizitose	×	×	×	×
Glycerol	w	×	×	×
Erythritol	×		w	
D-glucitol	×	×	×	×
D-mannitol	×	×	×	×
Myo-inositol	×	×	×	
D-gluconate	×	×	w	×
2-keto-D-gluconate	×	×	w	
D-glucuronate		×	×	
DL-lactate		×		w
Nitrate	×	w	×	w

w = weak growth

and Russian permafrost (Ozerskaya et al. 2004). *Penicillium* was also among the most frequently genus recorded in ice samples with higher quantities of sediments in Arctic ice (Gunde-Cimerman et al. 2003; Sonjak et al. 2006). It is therefore confirmed here, that there are similar species in the poles and other cold environments (Gilichinsky et al. 2007). However, psychrotolerant species that hardly grow at 25°C, such as *P. jamesonlandense* Frisvad & Overy (Frisvad et al. 2006), have not yet been found on Antarctica. All species found so far on Antarctica grow and sporulate well at 25°C.

A. pullulans var. *pullulans* is a widespread oligotrophic dimorphic fungus. This species has been previously isolated from natural or man-associated environments with fluctuating water activities (Andrews et al. 1994; Samson et al. 2004). It was also found in osmotically much stressed environments, e.g. hypersaline waters (Gunde-Cimerman et al. 2000), rocks and monuments (Urzì et al. 1999). Due to the production of large quantities of yeast-like propagules, this fungus disperses globally, although it has only rarely been reported in cold environments so far (Vishniac 1993; Lydolph et al. 2005; Branda et al. 2010). Likewise, *Sp. metaroseus* is one of the most widespread phylloplane-associated yeast (Fonseca and Inácio 2006), which has been isolated from other substrates, such as air, seawater and freshwater (Hirst 1953; Gadanho et al. 2003; Libkind et al. 2003). In recent years, this species has been isolated from Siberian, Alpine and Antarctic habitats (Poliakova et al. 2001; Bergauer et al. 2005; Vishniac 2006; Turchetti et al. 2008). On the contrary, the species *Cr. stepposus* was previously isolated from steppe area of the Prioksko-Terrasny biosphere reserve (Russia) (Golubev et al. 2006) and more recently from glaciers of south Europe (Branda et al.

2010). Moreover, this species was found as predominant in meltwaters draining from some Patagonian glaciers (D. Libkind, personal communication 2009). This is the first study reporting its isolation in Antarctica.

The highest number of isolates was obtained from sample 2, collected at an intermediate depth; no significant differences with respect the number of isolates were observed at the two temperatures used for incubation even if low-temperature cultivation was reported to be quite effective for recovery of bacteria (Vishnivetskaya et al. 2000).

All strains of *Penicillium chrysogenum sensu lato* found in this study could be placed in the well known species rather than its sister species *P. rubens* Biourge (Houbraken et al. 2011), as they produced lumpidin X and or secalonic acids D & F (Table 3). Interestingly some of the strains (no. 41 and 60) accumulating monodictyphenone, a precursor to secalonic acid A and D, but not secalonic acid D & F, and these two strains may be assumed as putatively "ancestral" of *P. chrysogenum*; this may fit with the estimated age of 15–20 Kyears of the examined permafrost samples. The remaining secondary metabolites produced by *P. chrysogenum* have been found in that species earlier (Houbraken et al. 2011). *Penicillium chrysogenum* is common in indoor air worldwide (Frisvad and Samson 2004; Scott et al. 2004), and the type culture was found on refrigerated cheese. *Penicillium palitans* was another dominant species, and strains from Antarctica produced cyclopiazonic acid, palitantin, cyclopenins, and an asperenone, metabolites that are also found in other strains of *P. palitans* from refrigerated cheese (Frisvad and Samson 2004). Secondary metabolite production in *Penicillium* fungi isolated from ancient Russian permafrost deposits were also helpful in species identification (Zhelifonova et al. 2009) and new species from ice in Greenland (Frisvad et al. 2006).

Tests on temperature preferences show a fungal and yeast community composed of mesophilic psychrotolerant (optimal growth temperatures above 20°C, minimal growth at 0°C after 14 days of incubation) strains, according to previous data on microorganisms isolated from Arctic and Antarctic permafrost soils. A predominantly psychrotolerant community of fungal microorganisms was reported for Arctic and Antarctic permafrost soils (Finegold 1996; Gilichinsky 2002; Rivkina et al. 2004; Gilichinsky et al. 2007); a large presence of psychroactive, in that they grow at −2.5°C (Vishnivetskaya et al. 2006), or psychrotolerant (Steven et al. 2007) bacteria was reported, able to produce lipids in permafrost at temperatures down to −20°C (Rivkina et al. 2000) and to metabolise even at temperatures below −16.5°C, at only 1–2% of liquid water (Rivkina et al. 2004); psychroactive bacteria with the lowest growth temperature varying from −1°C to −17°C were also reported from Alaskan soil and permafrost (Panikov and Sizova 2007). Even yeasts and filamentous fungi grow up to −17°C, while when cooled below −18°C they are not able to sustain metabolic activity continuously and after 3 weeks enter a state of reversible dormancy (Panikov and Sizova 2007). Considering the annual mean temperature of our samples ranging between −16 and −20.1°C, our isolates presumably survived in a resting state. A natural contamination of the sampled depths from the surface throughout permafrost layers might be excluded since a maximum thaw depth not exceeding 1 m is reported for the sampling site (Guglielmin et al. 2011); fungi isolated have most probably remained trapped in permafrost layers for an estimated age of up to 15–20 Kyears. Permafrost is believed to be a natural collector and storehouse of ancient microbes, possibly owing the survival of cells for hundreds of thousands to millions of years, and maybe full genome sequencing of some strains could prove this hypothesis. The stressing conditions may have selected few but resistant fungal species; this may justify the small number of species found. Fungal diversity recorded in permafrost samples here examined is similar to data previously reported but it deeply differs from pattern of actual colonizers of rock and soils of the McMurdo Dry Valleys (Vishniac 1993; Selbmann et al. 2005, 2008; Arenz and Blanchette 2011). This apparent contradiction could be explained considering that at present the air-born propagules are not able to resist few of environmental parameters, in particular UV exposition, when accidentally land on rock and soil surfaces; this stressor has dramatically improved in the last decades and is supposed to be the most important limiting ecological factor for soil mycobiota in continental Antarctica (Tosi et al. 2005). The actual environmental stressors fail to damage the permafrost inhabitants where they survive for geological times thanks to the stable low temperatures of the perennially frozen ground. Our isolates, all derived from more than 1 m depth, do not show deviating genotypes in the rDNA with respect to the actual microbial component, indicating that no significant evolutionary changes took place within the time of about 15–20 Kyears. This apparent absence of evolution may be a consequence of sampling that led to the isolation of only few haplotypes. In fact, the estimated age of 15–20 Kyears of the permafrost samples fits with the finding of some putative ancestral Penicillia strains accumulating metabolic precursors.

The presence of living microorganisms in terrestrial permafrost increases optimisms about the capacity of similar extraterrestrial planetary environments to preserve life. Researches on the diversity of permafrost microbial community and life strategies, might give new insights to evaluate the habitability of other Earth-like planets and to search and detect microbial life. Survival in extreme conditions, without real growth, is a phenomenon of utmost importance in astrobiology. The long term preservation of

viable eukaryotic cells in permafrost, as shown by cultural results, supports the possibility of transport through interplanetary flight.

Acknowledgments These studies were carried out within the framework of the Italian National Programme for Research in Antarctica (PNRA). Authors deeply thank Prof. Ann Vaughan-Martini for her precious scientific and technical skill in the course of the study.

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