RESEARCH ARTICLE

Yeast and yeast-like diversity in the southernmost glacier of Europe (Calderone Glacier, Apennines, Italy)

Eva Branda[1], Benedetta Turchetti[1], Guglielmina Diolaiuti[2], Massimo Pecci[3], Claudio Smiraglia[2] & Pietro Buzzini[1]

[1]Department of Applied Biology and Industrial Yeasts Collection DBVPG, University of Perugia, Italy; [2]Department of Health Sciences 'Ardito Desio', University of Milan, Italy; and [3]Italian Mountain Institute (EIM), Rome, Italy

Abstract

The present study reports the characterization of psychrophilic yeast and yeast-like diversity in cold habitats (superficial and deep sediments, ice cores and meltwaters) of the Calderone Glacier (Italy), which is the southernmost glacier in Europe. After incubation at 4 and 20 °C, sediments contained about 10^2–10^3 CFU of yeasts g^{-1}. The number of viable yeast cells in ice and meltwaters was several orders of magnitude lower. The concomitant presence of viable bacteria and filamentous fungi has also been observed. In all, 257 yeast strains were isolated and identified by 26S rRNA gene D1/D2 and internal transcribed spacers (1 and 2) sequencing as belonging to 28 ascomycetous and basidiomycetous species of 11 genera (*Candida*, *Cystofilobasidium*, *Cryptococcus*, *Dioszegia*, *Erythrobasidium*, *Guehomyces*, *Mastigobasidium*, *Mrakia*, *Mrakiella*, *Rhodotorula* and *Sporobolomyces*). Among them, the species *Cryptococcus gastricus* accounted for almost 40% of the total isolates. In addition, 12 strains were identified as belonging to the yeast-like species *Aureobasidium pullulans* and *Exophiala dermatitidis*, whereas 15 strains, presumably belonging to new species, yet to be described, were also isolated. Results herein reported indicate that the Calderone Glacier, although currently considered a vanishing ice body due to the ongoing global-warming phenomenon, still harbors viable psychrophilic yeast populations. Differences of yeast and yeast-like diversity between the glacier under study and other worldwide cold habitats are also discussed.

Introduction

Although microorganisms occurring in permanently cold ecosystems (which represent one of the largest biospheres on the Earth) have long been studied exclusively for their ability to survive under such extreme conditions (Abyzov, 1993; Ma *et al.*, 1999, 2000; Christner *et al.*, 2000; Poglazova *et al.*, 2001), more recent studies have provided evidence that such habitats (deep oceans, Arctic and Antarctic regions, mountain glaciers, etc.) can be colonized by both obligate and facultative psychrophilic microorganisms (Skidmore *et al.*, 2000, 2005; Deming, 2002; Bhatia *et al.*, 2006). In this sense, such ecosystems represent one of the last unexplored frontiers of ecology, and psychrophilic microbial populations sharing such habitats constitute an important part of cold-adapted biodiversity and play an essential role as

nutrient cyclers and organic matter mineralizers (Deming, 2002; Foght *et al.*, 2004).

There have been a number of studies on the microbiological composition of such psychrophilic populations in recent years. Viable bacterial communities have been observed beneath glaciers in the northern (Sharp *et al.*, 1999; Skidmore *et al.*, 2000) and southern hemisphere (Foght *et al.*, 2004). With reference to eukaryotic microorganisms, de García *et al.* (2007) described the occurrence of viable yeasts in meltwaters running off glaciers of northwest Patagonia, and Butinar *et al.* (2007) isolated culturable yeasts from basal ice layers of high arctic glaciers of the Svalbard Islands. Likewise, Margesin *et al.* (2002, 2007a) and Turchetti *et al.* (2008) described the occurrence of yeasts in some Alpine glacier habitats (e.g. cryoconites, ice cores, sediments and meltwaters). However, despite the profusion

of results, studies on microbial eukaryotic communities harboring in cold habitats of Mediterranean area are so far lacking.

It is well known that worldwide glaciers are strongly retreating due to ongoing climate change. In this context, the Mediterranean region represents a particularly delicate area, where glaciers of limited size are placed in mountain chains of relatively low altitude (the Pyrenees, Atlas Mountains, Maritime Alps, Apennines) (Messerli, 1980). With the disappearance of the Corral de la Veleta Glacier (Sierra Nevada, Spain) in 1913, the Calderone Glacier (Apennines, Italy) became the southernmost one of Europe. If the present trend continues, this glacier might soon share the fate of Corral de la Veleta (Pecci *et al.*, 2008). Accordingly, the study of psychrophilic microbial populations sharing such vanishing cold habitat is of increasing scientific interest.

The aim of the present investigation was to isolate and characterize yeast and yeast-like organisms from cold habitats of the Calderone Glacier (Apennines, Italy).

Materials and methods

Characteristics of the Calderone Glacier

The Calderone Glacier (altitude 2630–2830 m a.s.l.) is located in the Gran Sasso d'Italia group (Apennines, Italy) and is situated just beneath the top of Corno Grande (2912 m a.s.l.), the highest peak in the Apennine mountain range (Fig. 1). After the Last Glacial Maximum (about 18 000 years ago) it represents the residual presence of the past glaciarized area in the Apennines. Owing to its southernmost geographical placement (42°28′15″N), Calderone Glacier represents a *unicum* among European glaciers: its ice
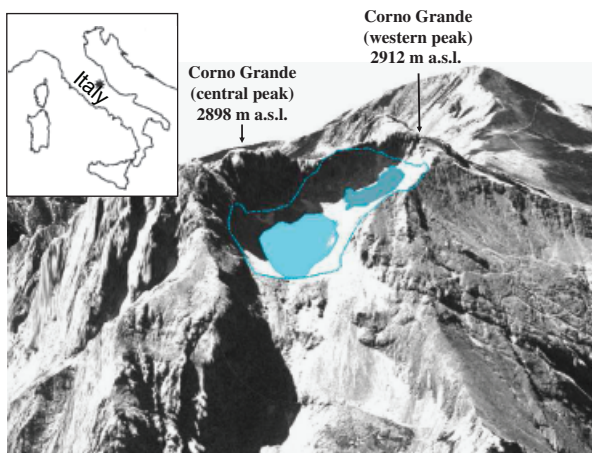

Fig. 1. Aerial map of the Calderone Glacier. Light-blue dotted line indicates the surface extension of the ice body in the last years of 19th century, whereas the two light-blue areas indicate the present extension of the two ice aprons.

mass survives below the Snow Line Altitude (which is found at this southern latitude at about 3100 m a.s.l.) due to its north-facing aspect, the shading effect provided by the mountains surrounding the glacier and the insulating action of the supraglacial debris cover, which have so far reduced the rates and magnitude of the ice melting (Pecci *et al.*, 2008). Notwithstanding these peculiar geographic settings and geomorphological features, a retreating trend has been observed in the last two centuries. In 1794, the Calderone Glacier volume was estimated at $> 4 \times 10^6 \, \mathrm{m}^{-3}$. By 1916, the glacier's volume was estimated at $3 \times 10^6 \, \mathrm{m}^{-3}$, and by 1990, at about $361 \times 10^3 \, \mathrm{m}^{-3}$ (D'Alessandro *et al.*, 2001).

At present, Calderone covers about 35 000 m². Although classified as a glacier because of the lack of any evident dynamic morphology (e.g. crevasses) and the subdivision of the ice body into two aprons, the Calderone Glacier can be actually considered a debris-covered *glacieret* [i.e. a small ice mass of indefinite shape, on a protected slope that originated from snow drifting and/or avalanching and exhibiting no marked ice flow pattern, *sensu* IUGG (CCS)-UNEP-UNESCO, 2005] exhibiting a barely retreating trend. The lower (and wider) ice apron is depressed and completely covered by white limestone rock debris, whereas the upper apron is characterized by the presence of seasonal snow (Pecci *et al.*, 2008). Accordingly, the reduced dimension and the general conditions of the glacier make it an interesting glaciological, geomorphological and environmental witness to global change (Gellatly *et al.*, 1994; D'Orefice *et al.*, 2000; D'Alessandro *et al.*, 2001, 2003; Pecci, 2001).

Sample collection

Samples were collected in the summer of three consecutive years (2006–2008). For all sampling procedures, clean hand tools were surface-sterilized using 70% ethanol immediately before use and between each sample. Glass sample containers were previously sterilized in the laboratory by autoclaving them at 121 °C for 15 min.

Due to the high amount of rock debris covering the glacier surface, and in particular in the lower ice apron, the glacier bed beneath the snout area was inaccessible. Accordingly, meltwaters and ice cores were sampled in small holes in the debris-covering layer, from which it was possible to reach the ice mass. Aliquots (about 500 mL) of meltwaters were collected using sterile glass bottles. The bottles were immersed in the melt stream while still sealed and then opened while still under the water. Some liters of water were collected each year. Samples were stored at 4 °C until analysis, which was carried out within 72–96 h.

Ice cores were collected after removing and discarding about 5–10 cm of ice surface and were placed into bags containing dry ice (to prevent melting). A few kilograms of ice per year was collected. In the laboratory they were

refrigerated within 24–30 h at −18 °C. Ice cores never reached temperatures above 0 °C.

Superficial sediments (i.e. sediments occurring between coarse superficial debris and the top ice surface of the glacier, generally characterized by a grain size from small pebbles to sand) were sampled. After removing and discarding about 5–10 cm of surface sediment, the underlying layers were aseptically collected and placed into sterile glass containers. A few kilograms of sediments per year were collected from an average of five to six different sampling sites. As they were found unfrozen *in situ* when collected, they were stored at 4 °C until analysis (carried out within 72–96 h).

In addition, fine sediments (grain size from silt to clay) were also collected from the glacier surface in two different geomorphological conditions: (1) small dirt cones and (2) elongated little debris septa. Dirt cones originate from the melt out of either endoglacial (passively transported) or subglacial (actively transported) debris bands. Both phenomena are due to differential ablation (i.e. a process of losing mass at varying rates and magnitude due to surface glacier conditions), which produces such upstanding morphologies on the glacier surface (Fig. 2). Even though dirt cones are composed predominantly of ice and debris, they form a covering layer that causes reduced melting rates, thus supporting the rise of the cone (Benn & Evans, 1998). Small dirt cones can also be the result of deep-piping sediments (a phenomenon sometime occurring in glaciers)

Fig. 2. Schematic representation of the formation [time from (a) to (c)] of a dirt cone originating from an endoglacial debris band (modified from Benn & Evans, 1998).

originating from an internal high water pressure, which determines a local emission (squeezing-out) of a mix of water and fine deep sediments. When the water drains off (after the exhaustion of the internal pressure), some pseudo-circle-shaped material is visible on the glacier surface. In contrast, the elongated small debris septa probably represent either the release of a continuous debris-rich band (up-thrusting of subglacial debris along shear planes) or ice protected by a former bedload of an endoglacial stream (Benn & Evans, 1998).

Mainly because of their fine grain size distribution, both small dirt cones and elongated small debris septa appear to be inherited from the endoglacial or subglacial zone of the glacier. Accordingly, due to the present inaccessibility of the Calderone Glacier bed, and although microbial contamination could obviously be possible within and during the up-thrusting flow from deep zones to glacier surface, samples collected from small dirt cones and debris septa were labeled as deep sediments and considered representative of the environmental conditions occurring inside and beneath the glacier.

Deep sediments were collected aseptically by following the same procedure carried out for superficial ones. A few kilograms of sediments per year were collected from several small dirt cones and debris septa. They were stored at 4 °C until analysis (carried out within 72–96 h).

Physical, chemical and microbiological analyses

Due to the fact that sampling was carried out aseptically, both deep and superficial sediments and melting waters were processed in the laboratory without decontamination procedures. In contrast, ice cores were surface-decontaminated in the laboratory accordingly to Rogers *et al.* (2004) to exclude the presence of external microorganisms on the sample surfaces introduced during drilling procedures.

Ice, sediments and meltwaters were analyzed for dry weight (DW), pH, total organic carbon (TOC), total nitrogen (TN) and phosphorus (TP) using standard methods (Hunt & Wilson, 1986; Mudroch *et al.*, 1996).

Viable counts of yeasts and filamentous fungi were carried out accordingly to Turchetti *et al.* (2008) using Rose Bengal agar (RB)+tetracycline. Plate count agar was also used to enumerate bacteria: bacterial colonies were selectively counted after macroscopic and microscopic differentiation from yeast and fungal colonies growing on the same medium. All media were from Difco.

Melt water and ice (after melting) were filtered through 0.22-μm filters (Millipore), whereas sterile 0.1% sodium pyrophosphate was used for serial dilution of both deep and superficial sediments. Both serial dilutions and 0.22-μm filters were plated onto Petri dishes containing the above media and then incubated at two different temperatures

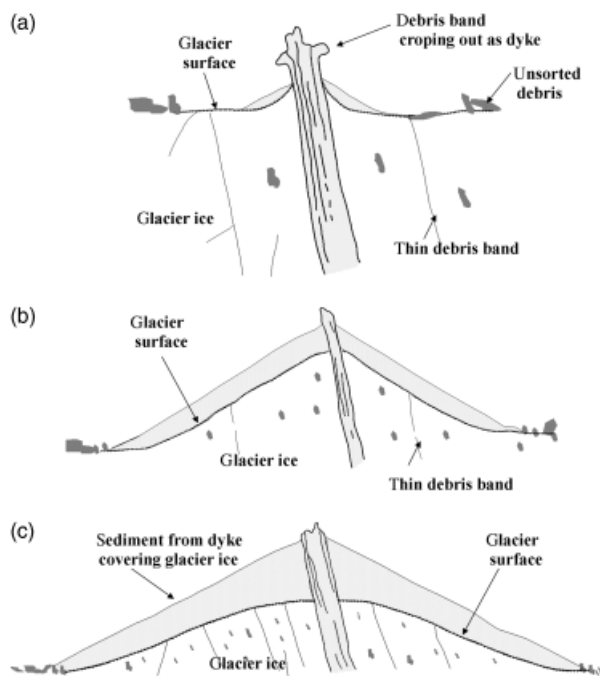(4 and 20 °C for 12 or 3 weeks, respectively). All chemical and microbiological analyses were carried out in triplicate and statistical evaluation of average values was carried out using ANOVA.

Yeast isolation

Yeast colonies grown on Petri dishes were periodically checked. To give a representative picture of the diversity of culturable yeast, colonies were selectively picked for isolation on the basis of both their morphology (paying attention to isolating all types occurring at the two different incubation temperatures) and frequency. After this initial isolation, colonies were transferred first to RB (without tetracycline) and secondly to malt extract agar (Difco)+a mixture of penicillin and streptomycin (100 IU mL^{-1}) for purification. All isolates are deposited in the Industrial Yeasts Collection DBVPG of the University of Perugia (Italy) (http://www.agr.unipg.it/dbvpg).

Preliminary phenotypic clustering of yeasts

The isolates were preliminarily typed using a few conventional phenotypic tests: macroscopic and microscopic morphology, Diazonium Blue B (DBB) assay (Yarrow, 1998), and growth at different temperatures (4, 10, 15, 20, 25, 30 and 35 °C) in YEPG (yeast extract 10 g L^{-1}, peptone 10 g L^{-1}, glucose 20 g L^{-1}, pH 6.5). Physiological tests were carried out in duplicate: no discrepant results were observed between repeated experiments.

Molecular identification of yeasts: DNA extraction

Total genomic DNA extraction was performed using a 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.

Molecular identification of yeasts: sequencing of the 26S rRNA gene D1/D2 and internal transcribed spacer (ITS) region

All isolated strains were subjected to sequencing of the D1/D2 domain of 26S rRNA gene. DNA was first amplified using the primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and LR6 (5′-CGC CAG TTC TGC TTA CC-3′) (MWG Biotech). A 600–650-bp region was sequenced by the forward primer NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and the reverse primer NL4 (5′-GGT CCG TGTTTC AAG ACG G-3′) (MWG Biotech). Strains exhibiting ambiguous results of D1/D2 sequences underwent sequencing of the ITS 1 & 2 region. ITS sequences were obtained using the primers RLR3R (5′-GGT CCG TGT TTC AAG AC-3′) and V9 (5′-TGC GTT GAT TAC GTC CCT GC-3′) (MWG Biotech). A 600–650-bp region was sequenced by the forward primer ITS 1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and the reverse primer ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (MWG Biotech).

Sequences were obtained by an Applied Biosystems DNA Sequencer, model ABI3730XL (Applied Biosystems) using standard protocols. Alignments were made using VECTOR NTI SUITE 8 CONTIG EXPRESS (Informax, Invitrogen). In both cases, strains were identified by comparing the sequences obtained with the GenBank database (BLASTN freeware from http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic analysis was performed using molecular evolutionary genetics analysis (MEGA) software 6 version 4.1 (Tamura et al., 2007) using neighbor-joining analysis. Bootstrap analysis (1000 replicates) was performed using a full heuristic search.

Results

Physical and chemical characteristics of sampling habitats

The pH of sediments, ice cores and meltwaters was within a subalkaline range (7.9–8.2) with no significant ($P > 0.01$) differences between deep and superficial origin (Table 1). A

Table 1. Average values of pH, DW%, TOC, TN and TP of (superficial and deep) sediments, ice cores and meltwaters collected in the Calderone Glacier (Italy) during 2006–2008

| | Superficial sediments | Deep sediments | | Ice cores | Meltwaters |
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD
DW (%)	83.1 ± 7.1	82.8 ± 3.1	DW (%)	0.46 ± 0.47	0.04 ± 0.06
pH	8.2 ± 0.6	8.3 ± 0.3	pH	7.9 ± 0.3	8.0 ± 0.5
TOC, g kg^{-1} DW	2.13 ± 0.44	3.19 ± 1.78	TOC, mg L^{-1} DW	0.46 ± 0.34	0.30 ± 0.41
TN, g kg^{-1} DW	3.28 ± 0.34	3.26 ± 0.23	TN, mg L^{-1} DW	0.54 ± 0.16	0.13 ± 0.06
TP, mg kg^{-1} DW	731.4 ± 22.9	960.8 ± 143.2	TP, mg L^{-1} DW	57.3 ± 27.6	44.4 ± 7.8

DW as high as 80% characterized both sediments, whereas ice cores exhibited a DW about 10 times higher than that observed in meltwaters. At the same time, TOC, TN and TP of both deep and superficial sediments were about from 4 to 25 times higher than those observed in ice and meltwaters (Table 1). No significant ($P > 0.01$) differences between data of the 3 years of sampling were observed.

Yeast occurrence in sampling habitats

The average number of culturable yeasts in superficial and deep sediments ranged from 10^2 to 10^3 CFU g^{-1} DW, respectively, with no significant ($P > 0.01$) differences between the counts carried out at 4 and 20 °C (Table 2). On the contrary, the number of yeast cells observed in ice cores and meltwaters was several orders of magnitude lower, with significantly ($P < 0.01$) higher values at 4 °C (Table 2).

The concomitant occurrence of both bacteria and filamentous fungi was observed. There were 10^2–10^3 CFU g^{-1} DW culturable filamentous fungi in both deep and superficial sediments. In contrast, ice cores and meltwaters contained from 10 to 10^2 CFU L^{-1}, respectively, with no significant ($P > 0.01$) differences between 4 and 20 °C (Table 2). On the whole, the enumeration of culturable bacteria gave results several orders of magnitude higher than those observed for yeasts and filamentous fungi: in both sediments a number of viable bacterial cells of 10^5 g^{-1} DW were observed, whereas ice cores and meltwaters contained from 10 to 10^4 CFU L^{-1}, respectively. In this case as well, no significant ($P > 0.01$) differences between the enumeration carried out at 4 and 20 °C were observed (Table 2).

Viable microbial enumerations exhibited no significant ($P > 0.01$) differences among data of the 3 years of sampling.

Yeast diversity in sampling habitats

In all, 284 yeasts were isolated. They were identified by D1/D2 domain of the 26S rRNA gene sequencing. In several cases, identification was confirmed by ITS sequencing. As suggested by Fell *et al.* (2000), strains that differed from the closest related type strain by two or fewer nucleotides in the D1/D2 domain were considered to be the same species. Selected sequences obtained in this study were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) and their accession numbers are shown in Table 3. The D1/D2 domain of the 26S rRNA gene and ITS region sequence analysis of 257 yeast strains allowed them to be assigned to 28 species (belonging to both ascomycetous and basidiomycetous genera): *Candida santamariae*, *Cryptococcus adeliensis*, *Cryptococcus aerius*, *Cryptococcus albidosimilis*, *Cryptococcus dimennae*, *Cryptococcus festucosus*, *Cryptococcus gastricus*, *Cryptococcus macerans*, *Cryptococcus oeirensis*, *Cryptococcus saitoi*, *Cryptococcus stepposus*, *Cryptococcus tephrensis*, *Cryptococcus victoriae*, *Cryptococcus watticus*, *Cryptococcus wieringae*, *Cystofilobasidium capitatum*, *Dioszegia crocea*, *Erythrobasidium hasegawianum*, *Guehomyces pullulans*, *Mastigobasidium intermedium*, *Mrakia blollopis*, *Mrakia gelida*, *Mrakiella aquatica*, *Mrakiella cryoconiti*, *Rhodotorula colostri*, *Rhodotorula laryngis*, *Rhodotorula psychrophenolica* and *Sporobolomyces roseus*. In the same way, 12 strains were identified as belonging to two yeast-like species: *Aureobasidium pullulans* and *Exophiala dermatitidis* (Table 3).

In a handful of species (*A. pullulans*, *C. macerans*, *C. victoriae*, *M. gelida*, *M. aquatica*, *R. laryngis* and *G. pullulans*) heterogeneous sequences were observed, whereas all other species exhibited a close 26S rRNA gene D1/D2 and ITS sequence homology (Table 3).

Based on their D1/D2 domain of the 26S rRNA gene and ITS sequences, seven yeast strains were characterized by an homology $\leq 98\%$ with two sequences (deposited in GenBank database as DQ377668 and FM246504, respectively) belonging to strains so far unidentified and labeled as *Cryptococcus* sp.: accordingly, the seven strains isolated in the present study were preliminarily identified as *Cryptococcus* sp. 1. Likewise, the D1/D2 and ITS sequences of two strains exhibited a low homology with those of the taxonomically closest species *Cryptococcus laurentii* (98% homology with the D1/D2 sequence DQ538362 and 90% with ITS sequence AF410468). Accordingly, both studied strains were preliminarily identified as *Cryptococcus* sp. 2.

Table 2. Enumeration of yeasts, filamentous fungi and bacteria in (superficial and deep) sediments, ice cores and meltwaters collected in the Calderone Glacier (Italy) during 2006–2008

	Superficial sediments (CFU g^{-1} DW)	Deep sediments (CFU g^{-1} DW)	Ice cores (CFU L^{-1})	Meltwaters (CFU L^{-1})
Enumeration at 4 °C				
Yeasts	$(2.1 \pm 2.3) \times 10^3$	$(2.0 \pm 0.7) \times 10^2$	$(6.1 \pm 6.7) \times 10$	$(2.0 \pm 2.5) \times 10^2$
Filamentous fungi	$(5.2 \pm 7.0) \times 10^4$	$(5.9 \pm 3.0) \times 10^3$	$0.7 \pm 1.4 \times 10$	$(4.0 \pm 4.1) \times 10^2$
Bacteria	$(7.6 \pm 12.7) \times 10^5$	$(1.7 \pm 2.1) \times 10^5$	$(1.1 \pm 3.3) \times 10$	$(9.6 \pm 7.1) \times 10^3$
Enumeration at 20 °C				
Yeasts	$(2.1 \pm 2.5) \times 10^3$	$(1.2 \pm 0.7) \times 10^2$	$< 1 \times 10$	$(3.5 \pm 7.2) \times 10$
Filamentous fungi	$(5.8 \pm 7.7) \times 10^4$	$(5.8 \pm 2.9) \times 10^3$	$(3.6 \pm 4.7) \times 10$	$(3.2 \pm 2.2) \times 10^2$
Bacteria	$(6.8 \pm 10.1) \times 10^5$	$(1.3 \pm 0.8) \times 10^5$	$(2 \pm 4) \times 10$	$(1.0 \pm 0.6) \times 10^4$

Table 3. Identification of yeast isolated from the Calderone Glacier on the basis of their D1/D2 domain of the 26S rRNA gene and ITS (1 and 2) sequences

Species	Strains (DBVPG accession number)	GenBank accession no. D1/D2 26S rRNA gene	GenBank accession no. ITS 1 and 2
Ascomycetous yeasts			
Candida santamariae	5182	GQ911489	
Basidiomycetous yeasts			
Cryptococcus adeliensis	4819, 5081, 5187, 5193, 5195, 5149, 5152	EU287877	
Cryptococcus aerius	5150	GQ911490	GQ911534
Cryptococcus albidosimilis	5184	GQ911491	
Cryptococcus dimennae	4996	GQ911492	
Cryptococcus festucosus	5005	GQ911493	
Cryptococcus gastricus	4816, 4818, 4820, 4821, 4823–4825, 4827, 4829, 4831–4834, 4838–4840, 4842, 4843, 5016, 5023, 5032–5034, 5036–5038, 5054, 5058–5069, 5072, 5073, 5078, 5080, 5082, 086, 5090–5094, 5096, 5100–5102, 5104, 5107, 5109, 5110, 5112, 5113, 5122, 5123, 5125–5130, 5133–5141, 5143, 5145, 5147, 5148, 5157–5167, 5181, 5183, 5185, 5186, 5188–5190, 5192, 5194, 5196–5199, 5201, 5203–5206, 5208–5211, 5217	EU287878 EU287883 EU287887 EU287889 GQ911494 GQ911495 GQ911496 GQ911497	
Cryptococcus macerans	4971, 4975, 4976, 4980–4982; 4984, 4989, 4993, 4998, 5001, 5009, 5027, 5031	GQ911498 GQ911499 GQ911500	
Cryptococcus oeirensis	5097, 5115	GQ911501 GQ911502	
Cryptococcus saitoi	5146	GQ911503	
Cryptococcus sp. 1	5011, 5012, 5021, 5025, 5026, 5117, 5118	GQ911504	GQ911535
Cryptococcus sp. 2	5114, 5116	GQ911505	
Cryptococcus sp. 3	5014, 5019, 5024, 5119, 5213	GQ911506	GQ911536
Cryptococcus stepposus	4988	GQ911507	GQ911537
Cryptococcus tephrensis	5111	GQ911508	GQ911538
Cryptococcus victoriae	4826, 4830, 4835, 4965, 4967, 4968, 4973, 4978, 4986, 5013, 5015, 5017, 5055–5057, 5132, 5207, 5212	EU287880 EU287882 EU287884 GQ911509	
Cryptococcus watticus	4837, 5079	EU287886	
Cryptococcus wieringae	5085, 5089, 5095, 5099, 5153–5156	GQ911510	
Cystofilobasidium capitatum	4845, 4985, 4987, 4991, 4997, 5002–5004, 5008, 5214	EU287890 GQ911511	
Dioszegia crocea	5030	GQ911512	GQ911539
Erythrobasidium hasegawianum	5083	GQ911513	GQ911540
Guehomyces pullulans	4822, 4836, 4969, 4970, 4972, 5007, 5105, 5108, 5170–5172, 5215	EU287879 EU287885 GQ911515	
Leucosporidium sp.	4841	EU287888	
Mastigobasidium intermedium	5216	GQ911516	
Mrakia blollopis	4974	GQ911517	GQ911542
Mrakia gelida	4844, 4977, 4983, 4995, 5106, 5200, 5202, 5218– 5220	GQ911518 GQ911519 GQ911520 GQ911521	GQ911543 GQ911544 GQ911545 GQ911546
Mrakiella aquatica	4979, 4990, 4994, 4999, 5000	GQ911522 GQ911523	GQ911547 GQ911548
Mrakiella cryoconiti	5179, 5180	GQ911524	GQ911549
Rhodotorula colostri	5006	GQ911527	
Rhodotorula laryngis	5035, 5084, 5088, 5098, 5151	GQ911525 GQ911526	GQ911550
Rhodotorula psychrophenolica	4817, 5039–5053, 5070, 5071, 5074–5076, 5087, 5174–5178, 5191	EU287876 GQ911528 GQ911529 GQ911530	
Sporobolomyces roseus	5010, 5018, 5020, 5022, 5029, 5120	GQ911531	
Yeast-like organisms			
Aureobasidium pullulans	4828, 4992, 5028, 5077, 5103, 5121, 5131, 5168, 5173	EU287881 GQ911487 GQ911488	GQ911532 GQ911533
Exophiala dermatitidis	5124, 5142, 5144	GQ911514	GQ911541

The D1/D2 domain and ITS sequences of five strains isolated in the present study exhibited 99% homology with those belonging to the type strains of two species: *C. oeirensis* (deposited as AF181519 and AF444349, respectively) and *C. magnus* (AF181851 and AF190008). Because of this uncertain condition, which makes it impossible to assign an unambiguous taxonomic designation, all five strains were preliminarily labeled as *Cryptococcus* sp. 3.

Finally, one additional yeast strain was characterized by a 26S rRNA gene D1/D2 domain and ITS sequences exhibiting a homology ≥ 99% with those (deposited as AY040646 and AY040664, respectively) obtained by a handful of strains so far unidentified and labeled as 'Antarctic yeasts'. The D1/D2 domain sequence of the yeast under study differed substantially (2%, corresponding to seven substitutions) from that of the closest known species *Leucosporidium antarcticum* (AF189906, corresponding to the strain CBS 5942). Accordingly, this strain was preliminarily identified as *Leucosporidium* sp.

Based on the above results, all the above 15 strains presumably belong to four new species, which remain to be described: their formal taxonomic description is in progress.

In agreement with the subdivision of basidiomycetous yeasts in the three Agaricomycotina, Pucciniomycotina and Ustilagomycotina subphyla (Bauer *et al.*, 2006), the phylogenic placement of yeasts isolated in the present study (obtained by neighbor-joining clustering of the D1/D2 domain of the 26S rRNA gene), is reported in Figs 3 and 4, respectively.

The frequency of the yeast and yeast-like species isolated from the different habitats of the Calderone Glacier is reported in Table 4. With the sole exception of one strain identified as *C. santamariae*, all isolated yeasts were basidiomycetes. Only a few yeast and yeast-like species were isolated from all sampled habitats (i.e. deep and superficial sediments, ice cores and meltwaters): *C. gastricus*, *C. victoriae*, *G. pullulans*, *M. gelida* and *A. pullulans*. The other species were observed more sporadically (Table 4).

The most frequently isolated genera were *Cryptococcus* (over 65% of total strains) and *Rhodotorula* (about 12%). Among them, the isolates belonging to the species *C. gastricus* constituted over 40% of all strains. Other frequently isolated species were *R. psychrophenolica* (10%), *C. victoriae* (6%), *C. macerans* (5%) and *G. pullulans* (4%). The sum of strains belonging to the two yeast-like species (*A. pullulans*+ *E. dermatitidis*) accounted for about 4% of the total, and the strains belonging to the species so far unidentified (*Cryptococcus* sp. 1, *Cryptococcus* sp. 2, *Cryptococcus* sp. 3 and *Leucosporidum* sp.) accounted for about 5% (Table 4).

Effect of temperature on yeast growth

All isolated yeast and yeast-like strains were able to grow at 4, 10 and 15 °C. A variable upper growth limit was observed for the strains belonging to the species: *C. gastricus*, *C. oeirensis*, *Cryptococcus* sp. 1, *Cryptococcus* sp. 3, *C. victoriae*, *M. gelida*, *M. cryoconiti*, *R. laryngis*, *R. psychrophenolica*, *S. roseus* and *A. pullulans* (Table 5). On the whole, only a handful of strains (belonging to the species *M. cryoconiti*, *M. gelida*, *R. psychrophenolica* and *Leucosporidium* sp., together representing about 2% of total isolates) could not grow at 20 °C. In contrast, 30 °C represented the upper limit of growth for all strains of the species *C. santamariae*, *C. adeliensis*, *C. aerius*, *C. albidosimilis* and *C. tephrensis*, whereas all isolates of *C. dimennae*, *C. festucosus*, *C. macerans*, *C. saitoi*, *Cryptococcus* sp. 2, *Cr. stepposus*, *C. watticus*, *C. wieringae*, *C. capitatum*, *D. crocea*, *E. hasegawianum*, *G. pullulans*, *M. intermedium*, *M. blollopis*, *M. aquatica* and *R. colostri* grew at 25 °C, but not at 30 °C. Only the strains of the species *E. dermatitidis* grew at 35 °C (Table 5).

Discussion

Mountain glaciers contain only a small part of the total ice mass existing on Earth: the retreat of such glaciers subsequent to ongoing climate change has been documented extensively since the 1980s (Meier, 1984; Benn & Evans, 1998; Nesje & Dahl, 2000; Paul *et al.*, 2004; Zemp *et al.*, 2006; Citterio *et al.*, 2007). In more recent years, a few studies developed analytical models to forecast the retreating trend of glaciers in the future. They predicted that a consistent loss (or even the complete extinction) of most of the ice masses will be observed by the end of this century in which case should the current climate trend continue, and concluded that small glaciers of southern Europe will be among the most reliable witnesses of global warming (Oerlemans, 1997; Zuo & Oerlemans, 1997; Benn & Evans, 1998; Nesje & Dahl, 2000; Zemp *et al.*, 2006). Accordingly, such southern glaciers can be considered important for studying climate and environmental changes occurring in the Mediterranean region.

In this context, studies on psychrophilic microorganisms sharing cold habitats of the Calderone Glacier acquire increasing scientific interest, mainly because such cold-adapted microbial populations (which can be considered in danger of disappearing) could give additional information about the persistence of cold conditions in such an environment.

Physical and chemical analysis of both deep and superficial sediments collected in the Calderone Glacier revealed concentrations of organic carbon, nitrogen and phosphorous considerably higher than those previously observed in other glaciers (Sharp *et al.*, 1999; Foght *et al.*, 2004; Turchetti *et al.*, 2008). In addition, the subalkaline range of pH observed in sediments, ice cores and meltwaters was apparently the immediate consequence of the limestone nature of debris lithology of mountains surrounding the glacier.
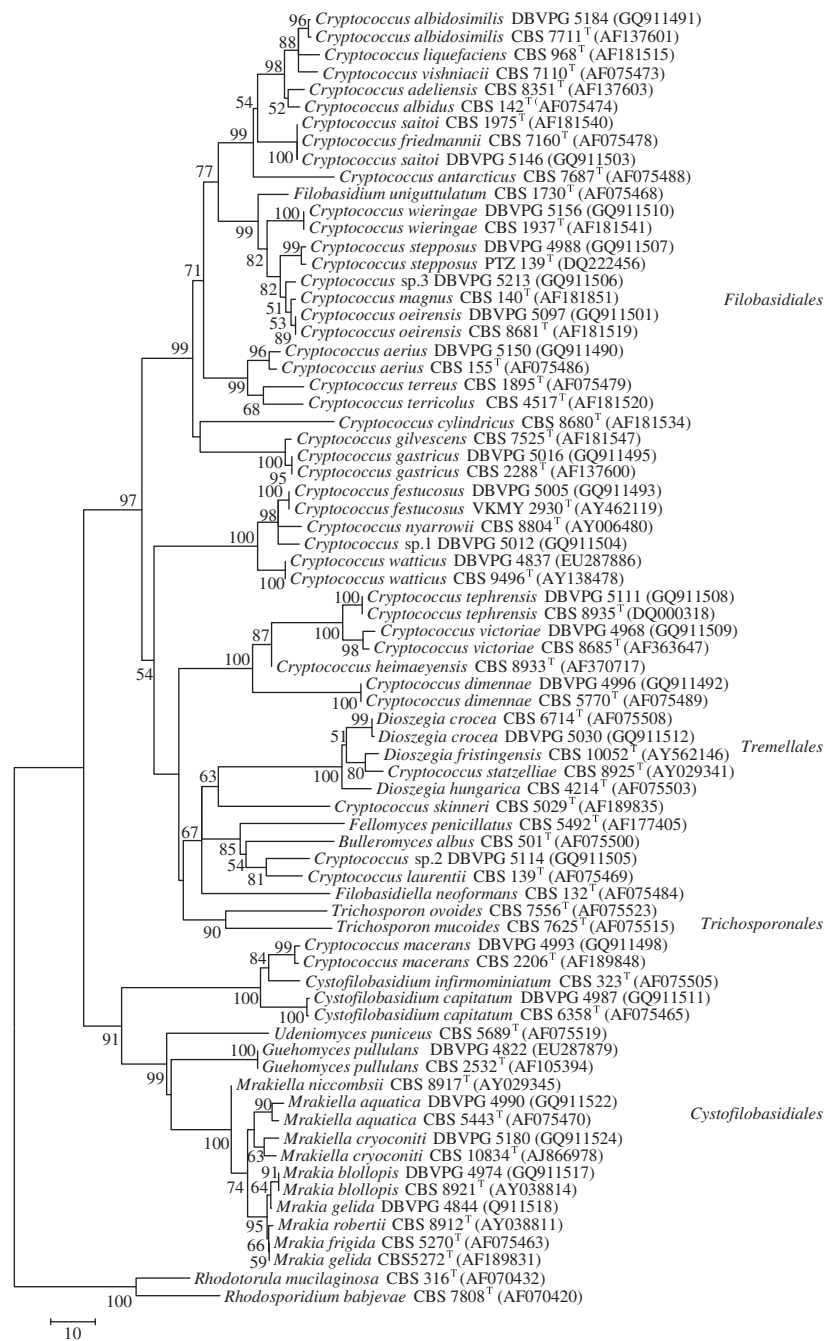
Fig. 3. Phylogenic placement within Agaricomycotina subphylum of the species identified in the present study (labeled as DBVPG) and of the species previously observed in cold habitats. The tree derived from neighbor-joining analysis of the 26S rRNA gene D1/D2 domain. Numbers on branches represent bootstrap percentages from 1000 replicates in a full heuristic search (values below 50% are not shown). *Rhodosporidium babjevae* CBS7808[T] and *Rhodotorula mucilaginosa* CBS316[T] were used as outgroups. GenBank accession numbers are indicated in parentheses. T, type strains.

On the whole, the average number of culturable psychrophilic yeasts found in deep sediments of the glacier under study was of the same order of magnitude as that previously observed in other glaciers (Butinar *et al.*, 2007; de García *et al.*, 2007; Turchetti *et al.*, 2008). Based on this similarity, we could postulate that, despite the dramatic and continuous loss of ice mass observed in the last decades, the bed of the Calderone Glacier still exhibits cold conditions harboring psychrophilic yeast populations. The concomitant occurrence of both bacteria and filamentous fungi actively growing at 4 °C, in close analogy with other observations (Foght *et al.*, 2004; Turchetti *et al.*, 2008), constitutes an indirect confirmation of the above hypothesis.

We observed that the superficial sediments of Calderone Glacier exhibited about 100 times more psychrophilic yeasts higher than observed in Alpine glaciers (Turchetti *et al.*,
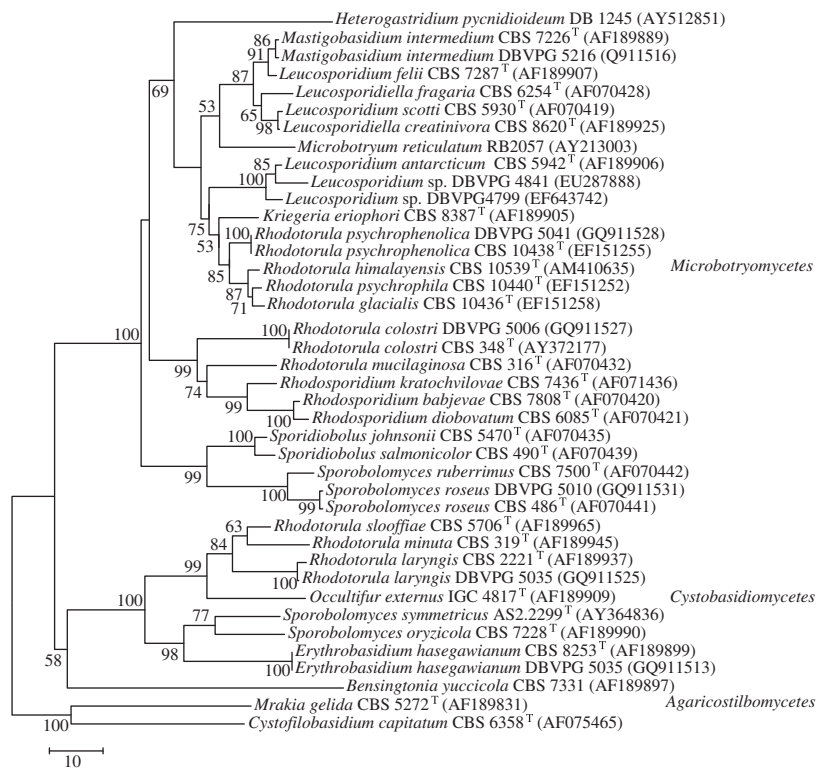
Fig. 4. Phylogenic placement within Pucciniomycotina subphylum of the species identified in the present study (labeled as DBVPG) and of the species previously observed in cold habitats. The tree derived from neighbor-joining analysis of the 26S rRNA gene D1/D2 domain. Numbers on branches represent bootstrap percentages from 1000 replicates in a full heuristic search (values below 50% are not shown).
Mrakia gelida CBS5272[T] and *Cystofilobasidium capitatum* CBS6358[T] were used as outgroups. GenBank accession numbers are indicated in parentheses. T, type strains.

2008). A few causes could apparently justify such a discrepancy. The first might be, as stated above, the lithological nature (white limestone) of rock debris constituting the superficial sediments of the Calderone Glacier. A recent study (Mihalcea *et al.*, 2008) reported that white limestone is characterized by a higher reflectivity value (about 0.30) than that observed in the largest part of siliceous debris covering Alpine glaciers (from about 0.15 to 0.09). This causes a lower absorption of incoming solar energy in summertime and, consequently, a considerably smaller seasonal increase of surface temperature of supraglacial sediments.

Secondly, the thickness of superficial sediments observed on the Calderone Glacier surface, which can be quantified from some decimeters up to 1 m in the lower (and wider) ice apron (Pecci *et al.*, 2008), was conspicuously greater than the sediments observed on the largest part of Alpine debris-free glaciers, which were absent or constituted thin layers up to a few decimeters thick.

Finally, the sediments sampled from the Calderone Glacier exhibited a higher (about from 10 to 100 times) organic carbon content than those sampled from Alpine glaciers (Turchetti *et al.*, 2008), thus constituting a richer substrate, allowing an active growth of microbial heterotrophic populations.

The low yeast counts observed in both ice cores and meltwaters are in agreement with other reports describing

the presence of a low number of viable yeast cells in lakes, lagoons and meltwaters from glaciers of the Patagonian Andes (Libkind *et al.*, 2003; de García *et al.*, 2007) and in ice cores and meltwaters draining beneath glaciers in the Italian Alps (Buzzini *et al.*, 2005; Turchetti *et al.*, 2008). As previously suggested, these results could be related to the oligotrophic nature of both ice and meltwater rivers running off from glaciers (Pedrozo *et al.*, 1993; Foght *et al.*, 2004; de García *et al.*, 2007).

Vishniac (1993) underlined early that, in contrast to temperate or tropical habitats, yeast populations sharing glacial environments contain an apparently restricted number of genera and species. The observation that almost all strains isolated from the glacier under study were basidiomycetous yeasts (in particular, species predominantly identified as belonging to the genus *Cryptococcus*) is in agreement with the yeast diversity recently found in Alpine, Arctic and Patagonian glaciers (Butinar *et al.*, 2007; de García *et al.*, 2007; Turchetti *et al.*, 2008). In contrast, the evidence that only a few isolates did not exhibit the ability to grow at 20 °C is in disagreement with data recently reported by Turchetti *et al.* (2008), who found that about 20% of yeasts found in Alpine glaciers were obligate psychrophiles. Based on this evidence, we might formulate a supplementary hypothesis: although the bed of the retreating glacier under study still exhibits cold conditions (as postulated above), cold-adapted yeast populations sharing this habitat

Table 4. Frequency of isolation of the yeast and yeast-like species from the different cold habitats of the Calderone Glacier

Species	Habitats			Total strains	% on total strains
	1	2	3		
Ascomycetous yeasts					
Candida santamariae	1			1	0.4
Basidiomycetous yeasts					
Cryptococcus adeliensis	4		3	7	2.7
Cryptococcus aerius			1	1	0.4
Cryptococcus albidosimilis	1			1	0.4
Cryptococcus dimennae		1		1	0.4
Cryptococcus festucosus		1		1	0.4
Cryptococcus gastricus	46	9	60	115	44.7
Cryptococcus macerans	2	12		14	5.5
Cryptococcus oeirensis		1	1	2	0.8
Cryptococcus saitoi			1	1	0.4
Cryptococcus sp. 1	7			7	2.7
Cryptococcus sp. 2		2		2	0.8
Cryptococcus sp. 3	4	1		5	1.9
Cryptococcus stepposus		1		1	0.4
Cryptococcus tephrensis			1	1	0.4
Cryptococcus victoriae	8	8	2	18	7.0
Cryptococcus watticus	1		1	2	0.8
Cryptococcus wieringae			8	8	3.1
Cystofilobasidium capitatum		10		10	3.9
Dioszegia crocea	1			1	0.4
Erythrobasidium hasegawianum			1	1	0.4
Guehomyces pullulans	3	5	4	12	4.7
Leucosporidum sp.	1			1	0.4
Mastigobasidium intermedium		1		1	0.4
Mrakia blollopis		1		1	0.4
Mrakia gelida	2	7	1	10	3.9
Mrakiella aquatica		5		5	1.9
Mrakiella cryoconiti	2			2	0.8
Rhodotorula colostri		1		1	0.4
Rhodotorula laryngis	1		4	5	1.9
Rhodotorula psychrophenolica	22		6	28	10.9
Sporobolomyces roseus	6			6	2.3
Yeast-like organisms					
Aureobasidium pullulans	3	1	5	9	3.5
Exophiala dermatitidis	1		2	3	1.2

Table 5. Growth temperatures of yeast species isolated from the Calderone Glacier

Species	Growth at (°C)						
	4	10	15	20	25	30	35
Ascomycetous yeasts							
Candida santamariae	+	+	+	+	+	+	−
Basidiomycetous yeasts							
Cryptococcus adeliensis	+	+	+	+	+	+	−
Cryptococcus aerius	+	+	+	+	+	+	−
Cryptococcus albidosimilis	+	+	+	+	+	+	−
Cryptococcus dimennae	+	+	+	+	+	−	−
Cryptococcus festucosus	+	+	+	+	+	−	−
Cryptococcus gastricus	+	+	+	+	+	v*	−
Cryptococcus macerans	+	+	+	+	+[†]	−	−
Cryptococcus oeirensis	+	+	+	+	+	v*	−
Cryptococcus saitoi	+	+	+	+	+	−	−
Cryptococcus sp. 1	+	+	+	+	+	v	−
Cryptococcus sp. 2	+	+	+	+	+	−	−
Cryptococcus sp. 3	+	+	+	+	+	v[‡]	−
Cryptococcus stepposus	+	+	+	+	+	−	−
Cryptococcus tephrensis	+	+	+	+	+	+	−
Cryptococcus victoriae	+	+	+	+	+	v	−
Cryptococcus watticus	+	+	+	+	+	−	−
Cryptococcus wieringae	+	+	+	+	+	−	−
Cystofilobasidium capitatum	+	+	+	+	+	−	−
Dioszegia crocea	+	+	+	+	+	−	−
Erythrobasidium hasegawianum	+	+	+	+	+	−	−
Guehomyces pullulans	+	+	+	+	+	−	−
Leucosporidium sp.	+	+	+	−	−	−	−
Mastigobasidium intermedium	+	+	+	+	+	−	−
Mrakia blollopis	+	+	+	w[§]	−	−	−
Mrakia gelida	+	+	+	w[†]	−	−	−
Mrakiella aquatica	+	+	+	+	w[§]	−	−
Mrakiella cryoconiti	+	+	+	v[‡]	−	−	−
Rhodotorula colostri	+	+	+	+	+	−	−
Rhodotorula laryngis	+	+	+	+	+	v[†]	−
Rhodotorula psychrophenolica	+	+	+	v	−	−	−
Sporobolomyces roseus	+	+	+	+	+	v[†]	−
Yeast-like organisms							
Aureobasidium pullulans	+	+	+	+	+	v[†]	−
Exophiala dermatitidis	+	+	+	+	+	+	+

v, variable growth; w, weak growth.
*Growth was observed in only one strain.
[†]Growth sometimes weak (or very weak).
[‡]Weak growth was observed in one strain.
[§]A very weak growth was observed.

have probably evolved to replace obligate psychrophilic species with facultative ones. Conventionally, obligate psychrophiles have a maximum temperature for growth below 20 °C, with an optimum temperature at 10 °C, and a minimum at 0 °C or subzero temperatures. Their existence under such cold conditions is the result of adaptation (observed over a time scale of several generations) by evolutionary selection of the gene alleles that increase their fitness for survival in a specific environmental niche (Morgan-Kiss *et al.*, 2006). Alternatively, facultative psychrophiles can be regarded as mesophilic ones that evolve to tolerate cold. Their optimum temperatures are at about 20 °C and they are capable of growth around 0 °C (Cavicchioli &

Tortsen, 2000; Price & Sowers, 2004; Raspor & Zupan, 2006; Margesin *et al.*, 2007b; Rossi *et al.*, 2009). In this case, the prolonged exposure to suboptimal temperature leads to acclimation, which implicates regulatory mechanisms resulting in the full adjustment of the genomic expression and the physiological state during growth under cold conditions (Morgan-Kiss *et al.*, 2006).

The yeast species isolated in this study are mainly placed in the Agaricomycotina subphylum and in particularly in

the *Filobasidiales* and *Cystofilobasidiales*. This is in accordance with recent papers reporting yeast strains isolated from cold habitats (Scorzetti *et al.*, 2000; Thomas-Hall *et al.*, 2002, 2010; Golubev *et al.*, 2006; Butinar *et al.*, 2007; de García *et al.*, 2007; Margesin *et al.*, 2007a; Xin & Zhou, 2007; Connell *et al.*, 2008; Margesin & Fell, 2008; Shivaji *et al.*, 2008; Turchetti *et al.*, 2008). However, some questions still remain about the biodiversity of psychrophilic yeasts in glacial habitats. Among them, the existence of microbial species that cannot be cultured under laboratory conditions is undoubtedly the most relevant. As a consequence, the results of this study might represent at best a partial picture of the yeast and the yeast-like diversity occurring in studied glacial ecosystems.

Yeast diversity found in the Calderone Glacier has been compared with that previously observed worldwide in cold habitats both associate or not with glaciers (Table 6). Butinar *et al.* (2007) found that *Cryptococcus liquefaciens* represented over 90% of isolates from glaciers of the Svalbard Islands, whereas Turchetti *et al.* (2008) reported that over 50% of strains from Alpine glaciers belonged to the species *Cryptococcus gilvescens*. Interestingly, none of these

Table 6. Comparison of yeasts isolated from glacial meltwater rivers from cold habitats of the Calderone Glacier with those isolated from other cold environments worldwide

Species	Calderone Glacier	Alpine glaciers*	Patagonian habitats†	Arctic habitats‡	Antarctic habitats§	Other cold habitats¶
Ascomycetous yeasts						
Candida famata			+			
Candida intermedia					+	
Candida psychrophila					+	
Candida santamariae	+					
Clavispora lusitaniae					+	
Debaryomyces hansenii				+	+	+
Dipodascus australiensis					+	
Pichia guilliermondii				+		
Wickerhamomyces patagonicus			+			
Basidiomycetous yeasts						
Bulleromyces albus				+	+	+
Cryptococcus adeliensis	+		+	+	+	
Cryptococcus aerius	+					+
Cryptococcus albidosimilis	+			+	+	
Cryptococcus albidus				+	+	
Cryptococcus antarcticus					+	
Cryptococcus carnescens				+	+	+
Cryptococcus cylindricus			+			+
Cryptococcus dimennae	+					+
Cryptococcus festucosus	+		+			+
Cryptococcus foliicola					+	
Cryptococcus friedmannii					+	
Cryptococcus gilvescens		+		+		+
Cryptococcus gastricus	+					+
Cryptococcus heimaeyensis				+		
Cryptococcus hungaricus					+	
Cryptococcus laurentii			+	+	+	+
Cryptococcus liquefaciens				+		
Cryptococcus macerans	+		+	+	+	+
Cryptococcus magnus				+		+
Cryptococcus nyarrowii					+	
Cryptococcus oeirensis	+			+		+
Cryptococcus saitoi	+	+		+	+	
Cryptococcus skinnerii				+	+	
Cryptococcus spencermartinsiae			+			
Cryptococcus statzelliae					+	
Cryptococcus stepposus	+		+			+
Cryptococcus tephrensis	+			+		+
Cryptococcus terricolus		+				+
Cryptococcus victoriae	+			+	+	+
Cryptococcus vishniacii					+	
Cryptococcus watticus	+				+	
Cryptococcus wieringae	+				+	+
Cystofilobasidium capitatum	+		+	+		+
Cystofilobasidium infirmominiatum			+			+

Table 6. Continued.

Species	Calderone Glacier	Alpine glaciers*	Patagonian habitats†	Arctic habitats‡	Antarctic habitats§	Other cold habitats¶
Dioszegia antarctica					+	
Dioszegia crocea	+		+			+
Dioszegia cryoxerica					+	
Dioszegia fristingensis			+			
Dioszegia hungarica			+		+	+
Erythrobasidium hasegawianum	+					+
Filobasidium uniguttulatum				+		
Guehomyces pullulans	+				+	+
Leucosporidiellla creatinovora		+	+			+
Leucosporidiella fragaria			+	+		
Leucosporidium antarcticum					+	
Leucosporidium scottii					+	
Malassezia restricta					+	
Mastigobasidium intermedium	+				+	+
Mrakia blollopis	+				+	
Mrakia frigida		+	+		+	+
Mrakia gelida	+	+			+	
Mrakia robertii		+			+	
Mrakiella aquatica	+	+			+	
Mrakiella cryoconiti	+	+		+		
Mrakiella niccombsii					+	
Rhodosporidium diobovatum				+		
Rhodosporidium kratochvilovae			+		+	
Rhodotorula babjevae			+			+
Rhodotorula bacarum		+				+
Rhodotorula colostrii	+		+		+	+
Rhodotorula glacialis		+				
Rhodotorula himalayensis						+
Rhodotorula laryngis	+	+	+	+	+	+
Rhodotorula minuta			+	+	+	+
Rhodotorula mucilaginosa			+	+	+	+
Rhodotorula pinicola			+			+
Rhodotorula psychrophenolica	+	+				
Rhodotorula psychrophila		+			+	
Rhodotorula slooffiae			+			
Sporobolomyces roseus	+	+	+		+	+
Sporobolomyces ruberrimus			+			
Sporidiobolus salmonicolor			+		+	
Sporidiobolus symmetricus					+	
Trichosporon mucoides				+		
Trichosporon middelhovenii					+	
Trichosporon shinodae					+	
Udeiomyces pannonicus			+			
Yeast-like organisms						
Aureobasidium pullulans	+	+		+	+	+
Cladophialophora minutissima					+	
Endocarpum pallidulum					+	
Exophiala dermatitidis	+				+	
Exophiala spinifera					+	
Staurothele marmoreum					+	
Staurothele frustulenta					+	
Verrucaria marmorea					+	

*Margesin *et al.* (2002, 2007a), Margesin (2007), Turchetti *et al.* (2008), Margesin & Fell (2008), Thomas-Hall *et al.* (2010).
†Libkind *et al.* (2003), de García *et al.* (2007, 2009, 2010), D. Libkind, pers. commun. (2009).
‡Vishniac (2002), Birgisson *et al.* (2003), Gilichinsky *et al.* (2005), Butinar *et al.* (2007), Turk *et al.* (2007), Margesin & Fell (2008), Zalar *et al.* (2008).
§Vishniac & Kurtzman (1992), Vishniac (1996, 2006), Deegenaars & Watson (1998), Montes *et al.* (1999), Scorzetti *et al.* (2000), Pavlova *et al.* (2002), Thomas-Hall *et al.* (2002, 2010), Thomas-Hall & Watson (2002), Guffogg *et al.* (2004), Arenz *et al.* (2006), Xin & Zhou (2007), Connell *et al.* (2008), Bridge & Newsham (2009), Connell *et al.* (2009).
¶Fell & Phaff (1967), Spencer & Spencer (1997), Fell & Statzell-Tallman (1998), Sláviková & Vadkertiová (2000), Poliakova *et al.* (2001), Zhao *et al.* (2002), Golubev *et al.* (2003, 2004, 2006), Nakagawa *et al.* (2004, 2005), Wuczkowski & Prillinger (2004), Bergauer *et al.* (2005), Inácio *et al.* (2005), Wuczkowski *et al.* (2005), Nagahama (2006), Nakagawa *et al.* (2006), Sperstad *et al.* (2006), Kachalkin *et al.* (2008), Shivaji *et al.* (2008).

two species was isolated from the Calderone Glacier. In addition, the species *Cr. stepposus*, isolated early on from the steppe area of the Priokso-Terrasny biosphere reserve (Russia) (Golubev *et al.*, 2006), was predominant among yeast strains found in meltwaters from three Patagonian glaciers (D. Libkind, pers. commun., 2009). Only one strain of this species has so far been isolated from the glacier under study.

With the sole exception of *C. santamariae*, all species isolated from the Calderone Glacier have been observed previously in other cold habitats. In particular, 21 yeast and yeast-like species were previously isolated from at least two habitats of glaciers worldwide, Antarctica and other cold environments (Table 6): *A. pullulans*, *C. adeliensis*, *C. albidosimilis*, *C. festucosus*, *C. macerans*, *C. oeirensis*, *C. saitoi*, *C. stepposus*, *C. tephrensis*, *C. victoriae*, *C. wieringae*, *C. capitatum*, *D. crocea*, *M. intermedium*, *M. gelida*, *M. aquatica*, *M. cryoconiti*, *R. colostri*, *R. laryngis*, *R. psychrophenolica* and *S. roseus* (Vishniac & Kurtzman, 1992; Deegenaars & Watson, 1998; Montes *et al.*, 1999; Scorzetti *et al.*, 2000; Sláviková & Vadkertiová, 2000; Thomas-Hall *et al.*, 2002; Vishniac, 2002; Birgisson *et al.*, 2003; Libkind *et al.*, 2003; Golubev *et al.*, 2004, 2006; Nakagawa *et al.*, 2004; Wuczkowski & Prillinger, 2004; Bergauer *et al.*, 2005; Gilichinsky *et al.*, 2005; Inácio *et al.*, 2005; Arenz *et al.*, 2006; Butinar *et al.*, 2007; de García *et al.*, 2007; Margesin *et al.*, 2007a; Connell *et al.*, 2008; Margesin & Fell, 2008; Turchetti *et al.*, 2008; D. Libkind, pers. commun., 2009). Among them, the most cosmopolitan species was *R. laryngis*, which was previously observed from all studied cold environments (Nagahama, 2006; Butinar *et al.*, 2007; de García *et al.*, 2007; Connell *et al.*, 2008; Turchetti *et al.*, 2008).

In contrast, two more specialized species (*C. watticus* and *M. blollopis*) have so far been isolated only from Antarctica (Vishniac, 1996; Guffogg *et al.*, 2004; Thomas-Hall *et al.*, 2010), and *R. psychrophenolica* only from Alpine glaciers (Table 6) (Margesin *et al.*, 2007a; Turchetti *et al.*, 2008). This is the first study reporting their isolation outside such environmental niches.

A few species (*C. dimennae*, *C. gastricus* and *E. hasegawianum*) were hitherto previously isolated from other cold habitats not associated with glaciers (Table 6). *Cryptococcus dimennae* and *E. hasegawianum* were first isolated from pasture plants and aquatic environments, respectively (Fell & Phaff, 1967; Nagahama, 2006). On the other hand, *C. gastricus*, which represented the predominant yeast species occurring in the cold habitats of the Calderone Glacier, was isolated early from New Zealand soils (Fell & Statzell-Tallman, 1998) and, more recently, from the Roopkund Lake of the Himalayan mountains (India) (Shivaji *et al.*, 2008). This is the first study reporting its isolation as predominant species in a cold habitat (Calderone Glacier) outside Asia and Oceania.

Acknowledgements

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