RESEARCH ARTICLE

Yeasts from glacial ice of Patagonian Andes, Argentina

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Abstract

Glacial ice and snow are known habitats for cold-adapted microorganisms. Research on cold-adapted yeast biodiversity from Perito Moreno and Mount Tronador glaciers (Patagonia, Argentina), and production of extracellular enzymatic activity at low temperatures (5 and 18 °C), was performed and described in this study. Ninety percent (90%) of the isolates were basidiomycetous; 16 genera and 29 species were identified. Twenty-five percent (25%) of total isolates corresponded to psychrophilic yeasts, whereas 75% were psychrotolerant yeasts. Eighty-five percent (85%) of all isolates had at least one enzymatic activity. Multiple correspondence analysis and cluster classification revealed a relationship between certain genera and some enzymatic activities. Cold-adapted yeast isolates were able to hydrolyze natural compounds (casein, lipids, starch, pectin, and carboxymethylcellulose) at low temperatures, suggesting a significant ecological role of these organisms as organic matter decomposers and nutrient cyclers. These yeasts are especially relevant for metabolic and ecological studies, as well as for yeast-based biotechnological process at low temperatures.

Introduction

Ice is considered a life-preserving medium that can entrap randomly deposited microorganisms that may remain viable for a long time (Gunde-Cimerman *et al.*, 2003). Recent studies have shown that different types of ice (i.e. snow, sea ice, glacial ice) can be inhabited by psychrotolerant and psychrophile microorganisms (Margesin *et al.*, 2005; Butinar *et al.*, 2007; Turchetti *et al.*, 2008). These microorganisms can use complex biopolymers as energy sources, by synthesizing extracellular enzymes active at low temperatures (Margesin *et al.*, 2007a; Frisvad, 2008). Psychrophilic yeasts play an essential role in nutrient cycling and biomass production processes in cold ecosystems (Margesin *et al.*, 2007a). Studying microbial diversity in extreme environments, such as ice environments, is interesting because these micororganisms exhibit metabolic adaptations to extreme conditions and may provide insight into potential biotechnological applications. Furthermore, psychrophilic and psychrotolerant microorganisms found in these environments may be used as bio-indicators in studies monitoring global warming (Gunde-Cimerman, 2006; Kuhn, 2008).

Currently, most glaciers of the world are retreating rapidly as a result of global warming (Delgado Granados *et al.*, 2007). Glaciers of Mount Tronador Patagonia, Argentina, are a clear example, having experienced constant retreat during the past 100 years (Villarosa *et al.*, 2008). Perito Moreno glacier is one of the few glaciers that are in a stable situation, that is, it is neither advancing nor retreating. The study of psychrotrophic microbial populations in this vanishing or barely stable cold habitat is of increasing scientific interest (Branda *et al.*, 2010).

Glacier/climate interactions in Patagonian region are of relevance to understand the global climate change pattern. In addition, icefields and periglacial areas hold valuable information for Quaternary paleoenvironments (Stuefer *et al.*, 2007). There is also a dense forest cover, associated with recent glacial deposits. Lakes, rivers, and peat-lands are the main landforms that create conditions for the study of past and present environmental variations in the region (Villarosa *et al.*, 2008). Occurrence of cold-adapted yeasts in glacial environments in Patagonia was first studied from meltwater rivers of Mount Tronador (de Garcia *et al.*, 2007). Occurrence and diversity of yeast in glacier ice in Patagonia have not been studied before.

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The aim of this investigation was to assess the occurrence and biodiversity of cold-adapted yeasts in ice samples from Frias glacier (Mount Tronador) and Perito Moreno glacier (Patagonian icefields), and study their extracellular enzymatic activities.

Materials and methods

Area description and sampling

Ice samples were aseptically collected, in summer, from: (i) Frias glacier, Mount Tronador (71°50′W, 41°11′S) in February 2007; (ii) Perito Moreno glacier (73°51′W, 49°15′S) in March 2008.

Air temperature and GPS positioning were recorded *in situ*. Ice samples were melted aseptically at room temperature and then filtered through Millipore® membrane filters (pore size 0.45 μm, diameter 47 mm).

Yeast isolation and quantitative analysis

Volumes of 100 and 150 mL of melted ice were filtered through Millipore® nitrocellulose membranes, using a sterilized Nalgene® device. Filters were placed on MYP agar (malt extract 7 g L^{-1}; yeast extract 0.5 g L^{-1}; soytone 2.5 g L^{-1}; agar 15 g L^{-1}; pH 4) containing chloramphenicol 100 mg L^{-1} and in agar media with different substrates (proteins, lipids, starch, carboxy-methylcellulose, or pectin; see Extracellular Enzymatic Activity description below); Petri dishes were incubated at 10 °C for 1 week and at 5 °C for up to 1 month. Plates were periodically examined, and all emerging yeast colonies were transferred to MYP agar plates without antibiotics and purified. The isolates were stored at −80 °C and included in the CRUB Yeast Collection (CRUB: Yeast Collection of Centro Regional Universitario Bariloche). Yeast colony-forming units (CFU) were registered for quantitative analysis of yeast occurrence. Yeast cell counts from three replicates were used to calculate mean values and SDs.

Yeast characterization and identification

Yeast characterization and identification up to genus level (morphological features and physiological tests) were performed according to standard methods, as described by Kurtzman *et al.* (2011).

In a previous study on yeasts in glacial meltwater (de Garcia *et al.*, 2007), 38 strains remained unidentified. These 38 strains were included in this study for identification, phylogenetic analyses, extracellular enzymatic activity and were also included in the discussions of yeast diversity.

PCR fingerprinting analysis was performed using mini/microsatellite-primed PCR technique (MSP-PCR). Protocols for DNA extraction, PCR, and electrophoresis conditions were those described by Libkind *et al.* (2003), and primer M13 was used. For DNA sequence analysis, D1/D2 domains of the large subunit of ribosomal DNA (LSU rDNA) was studied, primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAGACG G-3′) were employed, and internal transcribed spacer (ITS) region was sequenced using the forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGAT ATGC-3′).

Sequencing was performed by the Sequencing Service Macrogen (Korea). BigDye terminator cycle sequencing kits were used in sequence reactions (Applied Biosystems, Foster City, CA). Sequences were obtained with an ABI Prism 3700 (Applied Biosystems). Sequences downloaded from GenBank are indicated in the gene trees by their GenBank accession number; newly generated sequences are indicated by their strain number (CRUB) and GenBank accession number (Table S1, Supporting information).

Phylogenetic analyses

Sequences were automatically aligned using CLUSTALX, and alignments were adjusted manually using MEGA5 (Tamura *et al.*, 2011). To estimate phylogenetic relationships on the basis of LSU rDNA (D1/D2 domains), neighbor-joining analysis (K2P) was performed using MEGA software, version 5 (Tamura *et al.*, 2011).

Extracellular enzymatic activity

The isolated strains were tested for their ability to degrade starch, protein (casein), pectin, carboxymethylcellulose, and Tween-80 according to the procedures described by Brizzio *et al.* (2007). Calibrated suspensions of 10^6 cells mL^{-1}, grown for 24–48 h, were surface inoculated on agar plates using a multipoint inoculation device. Plates containing each substrate were incubated at 5 and 18 °C. Enzymatic activity was recorded after 5 days in samples incubated at 18 °C and after 21 days in those incubated at 5 °C.

Statistical analyses

Yeast diversity in each glacier was studied using the Shannon–Weaver (H) index with Hutcheson's t-test ($a = 0.05$) as described in Moreno (2001). Similarities between communities were studied with Jaccard index (J) according to Chao *et al.* (2005), using the occurrence frequency of each isolated species.

Enzyme production at both temperatures was compared using Mann–Whitney–Wilcoxon nonparametric test for two independent samples, and the analysis was carried out using SIGMASTAT V2.03 program. Analysis of multiple correspondences and hierarchical classification were carried out to evaluate the results of semiquantitative extracellular enzymatic activity. STATISTICA 6.0 software package was used.

Results and discussion

Yeast occurrence and quantitative analyses

Average yeast counts for each sampling site are shown in Table 1. Yeast counts between sampling sites were not significantly different ($P > 0.117$), nor were counts for different selective media within each sampling site ($P > 0.117$). No yeasts colonies were observed in selective media with casein and Tween 80 substrates (Table 1). Yeast counts were similar to those obtained for meltwater rivers of Mount Tronador (de Garcia *et al.*, 2007) and in other aquatic environments of Patagonia Argentina ($1–2 \times 10^3 \pm 1–4 \times 10^2$ CFU L^{-1}; Libkind *et al.*, 2003; Brandao *et al.*, 2011). Studies in similar environments of the Italian Alps also registered similar values of yeast counts, 1×10^1 and 4×10^3 CFU L^{-1} (Buzzini *et al.*, 2005; Turchetti *et al.*, 2008; Branda *et al.*, 2010). These values are relatively low when compared with coastal or polluted aquatic systems (Nagahama, 2006), because of the oligotrophic nature of the ice samples (Hagler & Ahearn, 1987; Foght *et al.*, 2004).

A total of 153 isolates were classified. Of these, 115 corresponded to the ice samples of the Frias and Perito Moreno glaciers. These were assigned to 16 genera and 29 species (Table 2). The remaining 38 corresponded to the unidentified isolates from a previous study on yeasts in meltwaters of Mount Tronador (de Garcia *et al.*, 2007). These were classified into five genera and 16 different species (Table 2). Results in this section will refer to all 153 isolates. Ninety percent of the total yeast strains studied (153 isolates) belonged to the phylum *Basidiomycetes*.

All isolates were adapted to living at cold temperatures, 75% were psychrotolerant (growth at 5–25 °C), while the remaining 25% were psychrophilic (growth at 5–15 °C). The occurrence of psychrophilic yeasts in cold environments reported here was similar to that found in Alpine glaciers (17%) by Turchetti *et al.* (2008) and Branda *et al.* (2010), and in Arctic glaciers (30%) by Pathan *et al.* (2010).

Species diversity values, measured with the Shannon–Weaver index, were higher in the ice from Frias glacier and meltwaters from Mount Tronador ($H = 2.23$ and $H = 2.52$, respectively) than in ice from Perito Moreno ($H = 1.72$). There were no significant differences for Shannon–Weaver diversity indices among all compared communities ($P > 0.05$). Jaccard index for community similarity analysis showed that Frias and meltwater yeast communities and Perito Moreno and meltwater yeast communities were the most similar ($J = 0.15$ and $J = 0.13$, respectively); however, the index values were low for all the compared communities, and no similarity was found when yeasts communities of Perito Moreno and Frias were compared.

A relatively higher richness index of taxa among ice and meltwater samples was observed, compared to the values reported for soil samples in Patagonian forest by Mestre *et al.* (2011). In addition, Brandao *et al.* (2011) mentioned similar values in water samples from Nahuel Huapi Lake (Patagonia, Argentina; coast sites $H = 2.2$ and pelagic sites $H = 2.8$).

Yeast identification, diversity, and ecology

Basidiomycetous yeasts were the predominant group in ice from Patagonian glaciers, belonging mostly to subphylum *Agaromycotina*, particularly to *Tremellales* (55 isolates) and *Cystofilobasidialles* orders (27 isolates). Similar results from different cold environments of the world

Table 1. Location of sampling sites and viable yeast counts

Sampling sites	Frías Glacier (Mount Tronador)	Perito Moreno Glacier
Location	41°08′43″S, 71°49′01″W	49°S, 73°W
pH of the sample	6.5	6.4
Yeast viable counts in general and selective media* Media ± SD (CFU L^{-1})		
MYP	$3.3 \times 10^3 \pm 9.6 \times 10^2$	$3.3 \times 10^3 \pm 2.1 \times 10^3$
YNB + Pectin pH 5	$2.5 \times 10^3 \pm 8.0 \times 10^2$	$5.3 \times 10^3 \pm 3.7 \times 10^3$
YNB + Pectin pH 7	$2.1 \times 10^3 \pm 6.8 \times 10^2$	$3.6 \times 10^3 \pm 1.0 \times 10^3$
YNB + CMC	0	$4.0 \times 10^3 \pm 2.8 \times 10^3$

*No yeasts colonies were observed in selective media with casein and Tween 80 substrates.

Table 2. Yeast species isolated from glacial meltwater and ice from Patagonia Argentina, number of isolates and origin

Species	Total number of isolates	Origin of strains		
		Meltwaters	Ice	
		Mount Tronador	Frias Glacier	Perito Moreno Glacier
Dioszegia crocea	24 (15.6)	21	3	–
Psychrophilic strain sp. 1*	19 (12.5)	–	–	19
Sporobolomyces ruberrimus	15 (9.8)	–	15	–
Dioszegia fristingensis	12	1	11	–
Udeniomyces pannonicus	8	–	8	–
Cryptococcus victoriae	7	6	–	1
Udeniomyces pyricola	5	–	5	–
Mrakiella aquatica	4	–	4	–
Rhodotorula sp. 1*	3	3	–	–
Rhodotorula mucilaginosa	3	–	–	3
Dioszegia butyracea	3	–	3	–
Cr. spencermartinsiae†	3	3	–	–
Udeniomyces megalosporus	3	–	–	3
Mrakia robertii	3	–	3	–
Aureobasidium pullulans	3	–	3	–
Candida sp. 1*	3	3	–	–
Cryptococcus sp. 1*	2	2	–	–
Cryptocccus sp. 2*	2	2	–	–
Holtermanniella festucosa	2	–	–	2
Candida mesenterica	2	1	–	1
Mastigobasidium intermedium	1	–	1	–
Rhodotorula sp. 2 CRUB 1756*	1	–	–	1
Cryptococcus sp. 3 CRUB 1267*	1	1	–	–
Holtermanniella sp. 1 CRUB 1256*	1	–	–	1
Udeniomyces sp. 1 CRUB 1695*	1	–	1	–
Mrakia sp. 1 CRUB 1707*	1	–	1	–
Mrakia sp. 2 CRUB 1706*	1	–	1	–
Guehomyces pullulans	1	–	–	1
Bensingtonia yamatoana	1	–	1	–
Cryptococcus sp. 4 CRUB 1245*	1	1	–	–
Cryptococcus terrícola	1	–	1	–
Cryptococcus wieringae	1	1	–	–
Ascomycota sp. 1 CRUB 1755*	1	–	–	1
Phaeococcomyces sp. 1 CRUB 1760*	1	–	–	1
Wickerhamomyces patagonicus†	1	1	–	–
Debaryomyces hansenii	1	–	1	–
Candida sp. 2 CRUB 1719*	1	–	–	1
Candida sp. 3 CRUB 1220*	1	1	–	–
Candida sp. 4 CRUB 1295*	1	1	–	–
Candida maritima	1	1	–	–
Total	153	38‡	80	35

Occurrence frequencies percentage are shown in parentheses.

*Possible new species.

†New species recently described.

‡These were sampled in a previous study on yeasts in glacial meltwater (de Garcia *et al.*, 2007), but had remained unidentified in that study.

(Antarctica, Alpine glaciers, Alaska, and Arctic) have been reported (Thomas-Hall *et al.*, 2002, 2010; Bergauer *et al.*, 2005; Margesin *et al.*, 2005, 2007b; Butinar *et al.*, 2007; Connell *et al.*, 2008, 2010; Turchetti *et al.*, 2008; Shivaji & Prasad, 2009; Branda *et al.*, 2010; Uetake *et al.*, 2011; Vaz *et al.*, 2011) and also from other aquatic environments of Patagonia (Libkind *et al.*, 2003; de Garcia *et al.*, 2007; Brandao *et al.*, 2011).

Connell *et al.* (2008) mentioned that in Antarctic soils (South Victoria Land), 90% of yeast isolates were basidiomycetous, 43% of these corresponded possible new species. In this study, 40% (11 species) of basidiomycetous

isolates were also possible new species. Several authors have suggested that the predominance of basidiomycetous yeasts in these extreme environments is because they are more nutritionally versatile and more tolerant to extreme environmental conditions than ascomycetous yeasts (Sampaio, 2004; Brandao *et al.*, 2011).

The species most frequently recovered from Perito Moreno glacier ice was a yeast identified as psychrophilic yeasts sp. 1 (19 isolates), followed by and *Sporobolomyces ruberrimus* (15 isolates) identified by MSP-PCR fingerprinting (data not shown) isolated from ice from Frias glacier. While *Dioszegia* species were most frequently recovered (*D. crocea* 24 and *D. fristingensis* 12 isolates) from Mount Tronador Glaciers (ice and meltwaters). Species belonging to the *Dioszegia* genus are frequently found associated with plants and terrestrial substrates (Inácio *et al.*, 2005), while *D. crocea* species more commonly related to cold environments.

Some cosmopolitan species, such as *Rhodotorula mucilaginosa* (three strains from Perito Moreno glacier), *Cryptococcus victoriae* (six strains from meltwaters of Mount Tronador and one strain from Perito Moreno Glacier), and *Aureobasidium pullulans* (three strains from Frias glacier) were isolated.

Psychrophilic yeasts sp. 1 was shown to be identical in D1/D2 sequence to yeasts isolated in Alaska (*Basidiomycota* sp. GU 74), and considering ITS region, the closest related strain was an Antarctic yeast (CBS 8941), with 94% similarity in BLAST result. This species is closely related to *Camptobasidium hydrophilum* and together with the unidentified species from Alaska and Antarctica (GU 54 and CBS 8941) formed a new clade within the class (Fig. 1). The presence of teliospores without mating was observed in some isolates of this species.

Strains related to the genus *Rhodotorula* were found (*Rhodotorula* sp. 1 and *Rhodotorula* sp. 2 CRUB 1756). Species *Rhodotorula* sp. 1 was related to *Rh. glacialis* and had four nucleotide differences in the D1/D2 region. Further analyses are needed to determine whether these strains represent new species. *Rhodotorula* sp. 2 CRUB 1756 had 15 nucleotide differences in D1/D2 region and 11 in ITS region, with the closest species *Rhodotorula laringys*^{T}, having a basal position in *Rh. laringys* clade.

Three potential new *Cryptococcus* species, related to *Tremellales* order, were identified, *Cryptococcus* sp. 1 (related to *Cr. foliicola*), *Cryptococcus* sp. 2 (related to *Cr. laurentii* clade), and *Cryptococcus* sp. 3 (related to *Kwoniella* clade formal *Cr. heaveanensis* clade). Formal description of these species is in progress. Species *Cr. spencermartinsiae* sp. nov. has been recently described (de Garcia *et al.*, 2010a).

Species of genera *Udeniomyces* and *Guehomyces* were identified (*U. pannonicus, U. pyricola, U. megalosporus*, *Udeniomyces* sp. 1 CRUB 1697 and *G. pullulans*); *Udeniomyces* sp. 1 CRUB 1697 had 11 nucleotide differences in the D1/D2 region and 16 in the ITS region with the closest species being *U. pseudopyricola*^{T}.

Hortemanniella festucosa (*Cryptococcus festucosus*) of the recently described order *Holtermanniales* (Wuczkowski *et al.*, 2010) was also isolated.

Udeniomyces pannonicus, G. pullulans and *H. festucosa*, species are psychrophilic or psychrotolerant and have been isolated from different cold terrestrial regions of the world (Fell & Guého-Kellermann, 2011; Takashima & Nakase, 2011). Wuczkowski *et al.* (2010) concluded that *Holtermanniella* species possess significant amounts of polyunsaturated fatty acids, a fatty acid composition typical of yeasts adapted to cold environments.

Regarding the *Filobasidialles* order, *Cryptococcus terricola*, *Cr. wieringae* and a possible new species *Cryptococcus* sp. 4 CRUB 1245 (13 nucleotide difference in D1/D2 region and 9 in ITS region with the closest species *Cryptococcus arrabidensis* CBS 8678^T) were identified.

Representatives of the psychrophilic genus *Mrakia* and the anamorphic-related genus *Mrakiella* were isolated in this survey: three strains of *Mrakia robertii*, two possible new species (*Mrakia* sp. 1 CRUB 1706 and *Mrakia* sp. 2 CRUB 1707), and four strains of *Mrakiella aquatica*.

Yeasts belonging to phylum *Ascomycota* were less abundant, being approximately 10% of total isolates. Species *Candida maritima*, *Debaryomyces hansenii*, *Candida mesenterica*, and *A. pullulans* were identified, and the new species *Wickerhamomyces patagonicus* was recently described (de Garcia *et al.*, 2010b).

From 11 identified species, seven (63%) failed to match the already-described species, and most probably represent new ones. Four species were related to *Candida* in *Saccharomycetales* order, one strain was related to the species *Hyalodendriella betulae* (*Helotiales*), and one to the melanin-producing species *Phaecoccomyces nigricans* (*Chetothyriales*; Fig. 2).

As was mentioned, isolates of species *Rhodotorula* sp. 1 related to *Rh. glacialis* were identified. Gadanho & Sampaio (2009) proposed the 'ecoclade' concept, which refers to species that are phylogenetically related and show metabolic adaptations associated with physicochemical conditions present in the environment from which they were recovered. These authors suggest the existence of two 'ecoclades', acidic and psychrophilic. The psychrophilic ecoclade is defined by species from glacial environments from the Alps and mountainous areas from Himalaya. *Rhodotorula* sp. 1, isolated from glacier meltwater from Mount Tronador belongs to this clade, supporting this ecoclade proposal.

All *Mrakiella* strains isolated here produced mycelium and teliospores in culture medium without nitrogen (Yeasts Carbon Base). Teliospores were placed in distilled

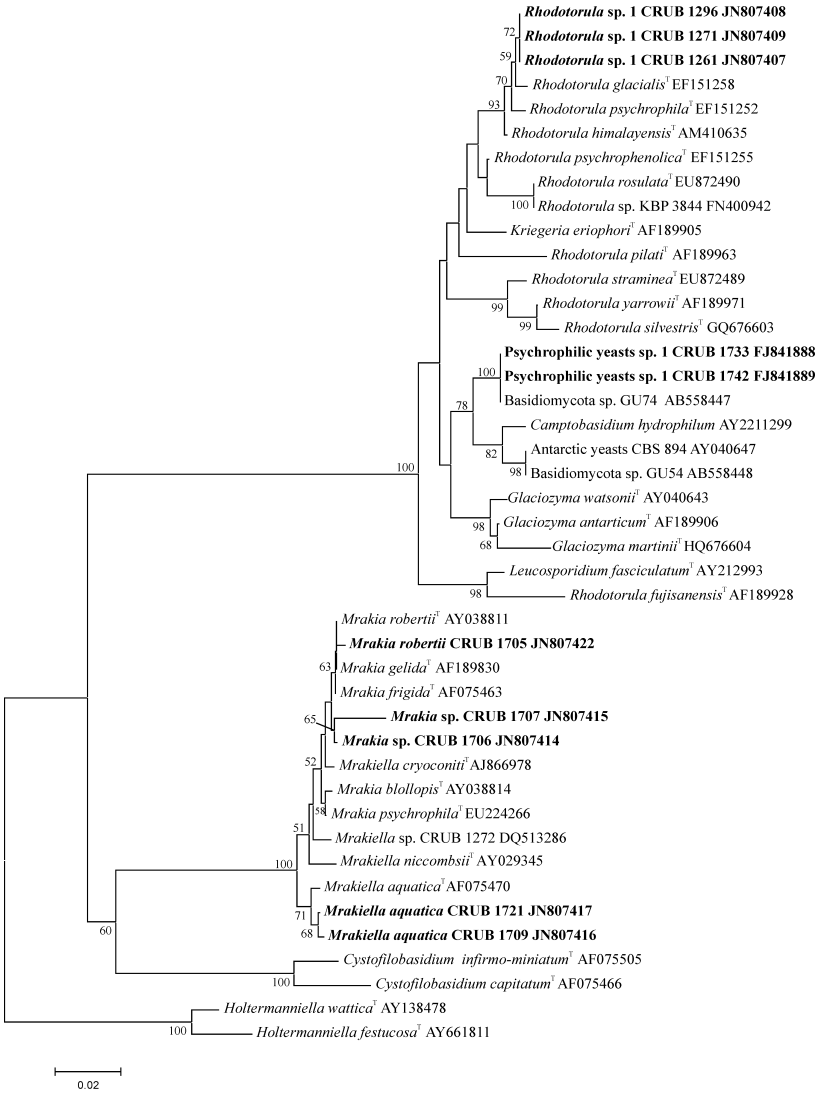

Fig. 1. Phylogenetic placement of psychrophilic basidiomycetous Patagonian yeasts species obtained by neighbor-joining (distance K2P method) of the LSU rRNA gene D1/D2 domains. Names in bold type are strains described in this work. Bar, substitutions accumulated every 100 nucleotides. Bootstrap values higher than 50% are shown (1000 replicates). ^{T}Type strain.

water and incubated at 5 °C for up to 1 year; after this period, agar blocks containing teliospores were placed in agar-water and, after 2 months, germinating teliospores were observed in two strains (*Mrakiella* sp. CRUB 1272 and *M. aquatica* CRUB 1209). Hyphae and teliospores developed directly from a single cell, without mating, and clamp connections were not observed. Germinating teliospores of strain CRUB 1272 produced three to five single-celled structures (Fig. 3), a septate structure was observed (Fig. 3c).

Is not clear whether teliospores found here in *Mrakiella* species were from asexual or sexual origin. Generally, germination of sexual teliospores in *Mrakia* species is not common and in some species has rarely been observed (*Mrakia frigida* and *M. gelida*; Fell, 2011). Further studies will be necessary to determine the sexual state of *Mrakiella* strains.

The presence of teliospores has been observed in almost all psychrophilic species described (*Mrakia* and *Glaciozyma*; Thomas-Hall *et al.*, 2010; Fell *et al.*, 2011; Turchetti *et al.*, 2011). Production of this structure can enhance survival in a diverse array of harsh environmental conditions, including cold habitats.

The presence of these extremophilic microorganisms in geographically distant regions could be the result of ecological fitting and genetic adaptation that allowed them to increase and improve their survival in these specific environments (Margesin *et al.*, 2007b; Rossi *et al.*, 2009).

In summary, and given the hypothesis that microorganisms in extreme environments could have differential evolutionary ratios compared with those in temperate environments (Skidmore *et al.*, 2000; Rosenberg & Hastings, 2003, 2004), studying and understanding the evolution of extremophiles will increase the basic knowledge of

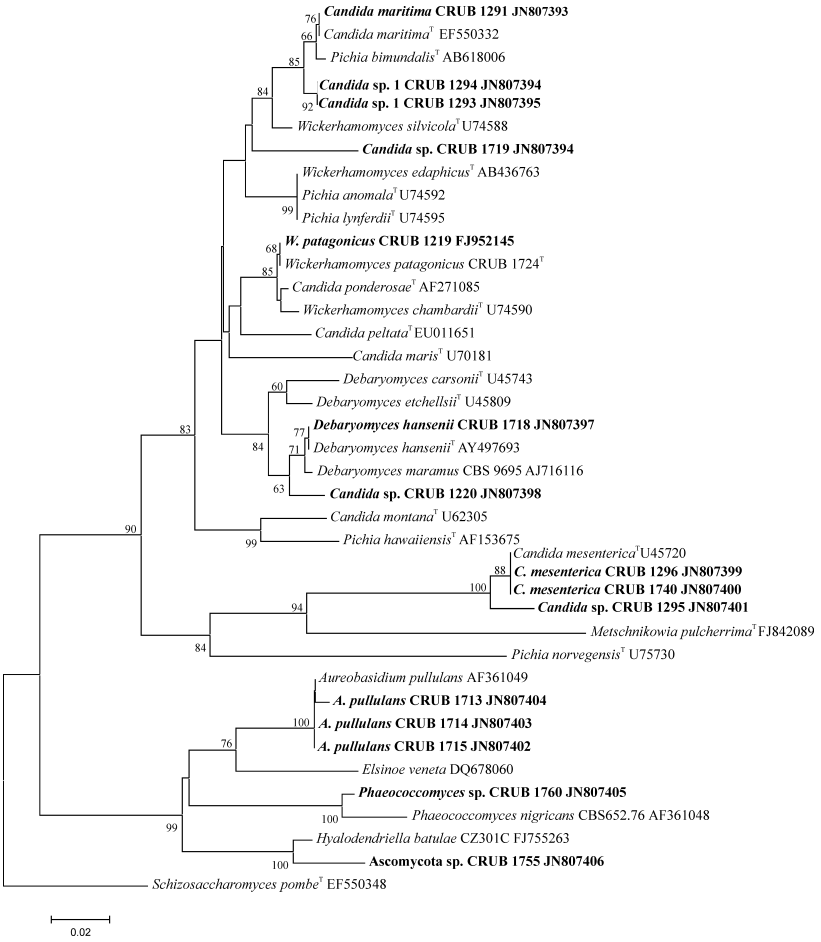

Fig. 2. Phylogenetic placement of Ascomycetous Patagonian yeasts species obtained by neighbor-joining (distance K2P method) of the LSU rRNA gene D1/D2 domains. Names in bold type are strains described in this work. Bar, substitutions accumulated every 100 nucleotides. Bootstrap values higher than 50% are shown (1000 replicates). ^{T}Type strain.

evolutionary processes, allowing a better evaluation of potential ecological consequences of environmental changes and possible effects on human health (Gostincar *et al.*, 2011).

Enzymatic analyses

Several selective media containing different substrates were included in the isolation step, attempting to improve the recovery of yeast strains with the ability to metabolize these different substrates. Results were not significantly different to those obtained with MYP agar, indicating that yeast diversity in the samples was adequately reflected by culturing in this generic medium.

Extracellular activity of selected yeast strains was previously reported (de Garcia *et al.*, 2007). However, in that study, the evaluation was not complete, as it included only some strains for the enzymatic analyses. Those strains were therefore included in this work, for a complete analysis of enzymatic production (from ice and meltwater samples) at 5 and 18 °C. Thus, a total of 212 strains were assessed (115 strains from ice and 97 from meltwaters), and the results are shown in Fig. 4.

Eighty-five percent (85%) of the 212 yeasts strains were able to produce at least one enzymatic activity, and 18% produced five different enzymatic activities. Differences in qualitative (number of positive strains) and semiquantitative (intensity of degrading activity) expression of extracellular enzymatic activities at 5 and 18 °C were investigated. Higher activity at 5 °C was observed for all extracellular activities analyzed (both in number of positive strains and in intensity), and statistically significant differences were found for proteolysis ($P \leq 0.001$), hydrolysis of carboxymethyl-cellulose (cellulose activity; $P = 0.025$), and hydrolysis of Tween-80 ($P = <0.001$; Fig. 4).

Turchetti *et al.* (2008) reported similar results for yeasts isolated from Alpine glaciers, indicating that 73 selected isolates had enzymatic activity at low temperatures (4 °C). However, Pathan *et al.* (2010) observed higher degradation at 22 than at 8 °C in psychrotolerant yeasts from Arctic glacier meltwaters, but in this case, the authors used an incubation period of only 10 days at 8 °C, when, in general, 20 days are needed at

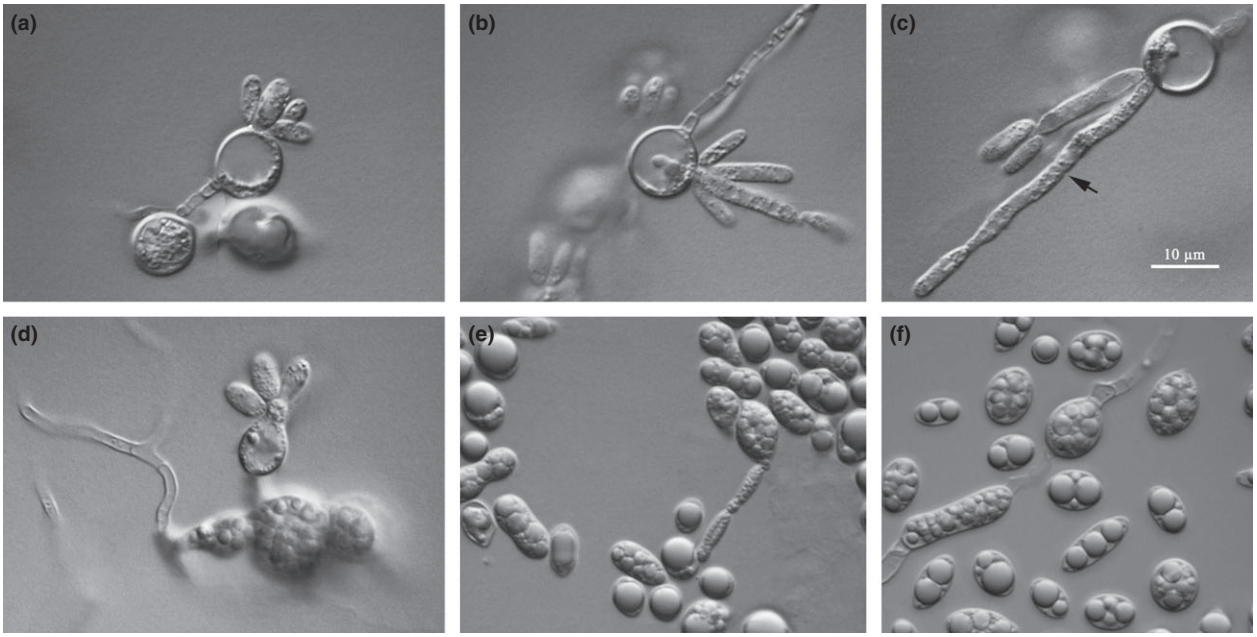

Fig. 3. Phase-contrast micrograph of teliospores of *Mrakiella* strains isolated from Patagonian glaciers. *Mrakiella* sp. CRUB 1272 (a–c), after 7 weeks in YCB agar media at 10 °C followed by 1 year in distilled water, (a, b) Teliospores germinated, (c) Teliospores germinated, arrow is showing septated structure. (d) *Mrakiella aquatica* CRUB 1709, germinated teliospores. (e, f) Teliospores of *M. aquatica* CRUB 1708 and CRUB 1721, respectively. On 2% agar after 2 months. Bar = 10 μm.

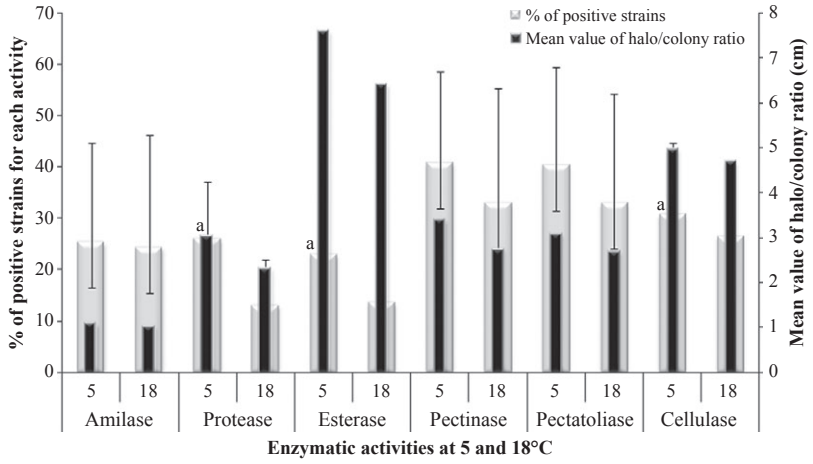

Fig. 4. Extracellular enzymatic activity of yeast strains from ice and meltwater rivers from Perito Moreno glacier and Mount Tronador glaciers. Bars indicate the SD of halo/colony mean values. *N* indicates the number of positive strains (of 212 strains) for the corresponding enzymatic activity. **a,** Significantly different activity at 5 and 18 °C ($P = 0.001$).

this temperature for psychrophilic and psychrotolerant strains to achieve stationary growth phase, in which extracellular enzymes are produced.

Hydrolysis of Tween-80 (esterase activity) was the most common activity, being present in 146 isolates (69%). These results are in agreement with those reported by Brandao *et al.* (2011) for enzymatic activity of yeasts from an oligotrophic lake of Patagonia (71.8% of the total isolates) and by Margesin *et al.* (2005), Buzzini *et al.* (2005) and Turchetti *et al.* (2008) for yeasts isolates from Alpine glacial environments (89%, 46%, and 86% respectively). Hydrolysis of carboxymethyl-cellulose (96 isolates) was the second most frequent activity, followed by proteolysis (60 isolates) and pectinolysis (62 isolates), amylase activity was the least frequent of the activities (27 isolates).

An association between yeast genera and the ability to produce extracellular enzymes was found through multiple correspondence and hierarchical classification analysis. Six different classes were proposed:

Class 1: *Sporobolomyces* isolates produce amylase at 5 and 18 °C.
Class 2: *Leucosporidiella* and *Udeniomyces* isolates produce protease, CMC-cellulose, pectinase, and esterase at 5 and 18 °C.
Class 3: *Cryptococcus* isolates produce CMC-cellulase at 5 and 18 °C.
Class 4: *Dioszegia* isolates produce esterase at 5 and 18 °C.
Class 5: *Mrakia* and *Mrakiella* isolates produce protease, esterase, and pectinase at 5 °C.
Class 6: *Ascomycota* and *Rhodotorula* isolates were not associated with enzymatic activity tested in the essay conditions. It must be noted that even though, according to multiple correspondence and hierarchical classification analysis, ascomycetous yeasts were not associated with any enzymatic activity; *A. pullulans* isolate had more than two extracellular activities, which is in agreement with other reports for this species (Turk *et al.*, 2007; Zalar *et al.*, 2008). Buzzini *et al.* (2005) found similar results for ascomycetous yeast isolated from Alpine glacier.

In conclusion, results of the laboratory cultures carried out in this study support that the yeasts in these extreme (cold) habitats possess metabolic adaptation to low temperatures. Cold-adapted *Cryptococcus* isolates with the ability to produce more than one hydrolytic cold-active enzyme were obtained from Patagonian glaciers. Also *Mrakia* and *Mrakiella* isolates able to produce up to five different extracellular cold-active enzymes, and resistance structures (teliospores) were recovered and characterized. These microorganisms are heterotrophic, and their ability to degrade organic macromolecules through the secretion of extracellular hydrolytic cold-adapted enzymes suggest that, as proposed by Turchetti *et al.* (2008), they may have a significant ecological role in organic matter decomposition and nutrients in glacial environments. This role is also supported by the presence of organic carbon and organic and inorganic nitrogen in glacial meltwater and ice (Skidmore *et al.*, 2000; Foght *et al.*, 2004; Margesin *et al.*, 2007a).

The biotechnological (and industrial) relevance of cold enzymes from psychrophilic yeasts has been emphasized (Thomas-Hall *et al.*, 2010). Association observed here between certain taxa of basidiomycetous yeasts and extracellular enzymatic activities facilitates a directed search of genera of interest, both in culture collections and in the environment, to find strains with possible biotechnological applications.

Basidiomycetous yeasts are a diverse group of fungi with considerable industrial and medical importance and have undeniable potential for economic exploitation (Abadias *et al.*, 2003; Qin *et al.*, 2004; Schisler *et al.*, 2011). This study has contributed to the understanding of their biodiversity and ecological roles.

Final remarks

The Patagonian Andes possess unique physical and environmental characteristics. Glaciers present in this area have a high potential for glaciological and paleoclimatic studies, and also for microbiological surveys, as shown in this work. The relevance of studies on the effects of climatic and environmental changes on continental glaciers has been shown elsewhere (Villarosa *et al.*, 2008; Branda *et al.*, 2010). Microorganisms inhabiting these withdrawing glaciers may be released into soil, rivers, and oceans, possibly complementing or changing the existing microbial communities (Butinar *et al.*, 2007).

Glaciers of Patagonia Argentina offer unexplored environments and are true cold-adapted yeast reservoirs. Furthermore, these yeasts possess adaptations, such as cold-active enzymes, which may undeniably contribute to biotechnological research and application.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Yeast isolates GenBank accession number of 26S rRNA gene D1/D2.

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