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TAXONOMIC DESCRIPTION
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Novel yeast taxa from the cold: description of *Cryolevonia giraudoae* sp. nov. and *Camptobasidium gelus* sp. nov.

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Abstract

Twenty-one psychrophilic yeast isolates related to the *Camptobasidiaceae* family in the *Microbotryomycetes* class were obtained from ice collected from cold environments worldwide. A new psychrophilic species from the recently described genus *Cryolevonia*, *Cryolevania giraudoae* is proposed to accommodate 18 isolates from Patagonia (Argentina) and Antarctica (holotype CRUB 2086^T). In addition, a new psychrophilic species in the genus *Camptobasidium* is described as *Camptobasidium gelus* sp. nov. (holotype CBS 8941^T), based on three isolates from glacial ice in the Russel glacier (Greenland ice sheet) and Antarctica. The strict psychrophilic profile is the salient feature of both novel species.

Cold ecosystems constitute one of the largest biospheres on Earth, although prokaryotic and eukaryotic organisms in these extreme habitats are still poorly understood. Research on the biodiversity of cold-adapted yeasts in cold ecosystems is essential to establish yeast species richness, to decrypt the molecular basis of their adaptation to cold and to examine new strains for the production of useful compounds [1].

Cold adapted yeasts belong to multiple taxonomic groups among the Ascomycota and the Basidiomycota. The class Microbotryomycetes (phylum Basidiomycota, subphylum Pucciniomycotina) is particularly enriched in several psychrotolerant and psychrophilic yeasts lineages, mostly contained in the families Kriegerieaceae, Camptobasideaceae and Leucosporidiaceae, particularly in the genera *Phenoliferia*, *Leucosporidium*, *Glaciozyma*, *Camptobasidium* and *Cryolevonia* [2–6]. These interesting psychrophilic taxa are monotypic, or contain very few known species [2], hence robust taxonomic analyses are difficult to achieve. An example is the Camptobasidiaceae family, which was recently considered as '*incertae sedis*' in the class Microbotryomycetes [7], including the genera *Camptobasidium* and *Glaciozyma;* and more recently *Cryolevonia*. This new genus contains only one species, *Cryolevonia schafbergensis*, isolated in an ancient permafrost layer in the Alps and in melted sea ice in Bafn Island in Canada. The genus *Camptobasidium* comprises a single species, *Camptobasidium hydrophilum*, a slow-growing psychrophilic fungus without a yeast stage [2], whereas *Glaciozyma* contains four psychrophilic yeast species: *G. antarctica, G. martinii, G. watsonii* and *G. litoralis* [4, 8]. In recent years, these four species, together with other psychrophilic yeasts are being extensively studied as a source of cold active proteins and enzymes of high biotechnological value [9–11]. Also, the strategies evolved by these taxa to cope with cold conditions are a matter of substantial investigation nowadays [8]. Hence, the description of novel psychrophilic Microbotryomycetes is important for improving the group taxonomy, and also for ecological and biotechnological fundamental and applied research.

During different surveys in cold environments from different cold regions of the world, 21 psychrophilic yeast isolates were obtained mainly from the southern hemisphere. Several isolates from Argentina and Antarctica were found to represent a novel species within Cryolevonia, here described as *Cryolevonia giraudoae* CRUB 2086^T. Also, the new species *Camptobasidium gelus* sp. nov. CBS 8941^T is proposed to accommodate three yeast strains obtained from ice from the Russel glacier (Greenland ice sheet) and Antarctica.

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Keywords: Basidiomycota; Microbotryomycetes; psychrophilic yeasts; taxonomy.
Abbreviations: CBS, CBS-KNAW culture collection; CRUB, Culture Collection of Yeasts from Centro Regional Universitario Bariloche; YNB, Yeasts Nitrogen base.
The MycoBank numbers are MB 835167, MB 830185. The GenBank accession numbers for the sequences of the ITS region and the D1/D2 domains of the large subunit rRNA gene determined in this study are: MN622687 to MN622704, MN626542 and MN626543; MN626546 to MN626561, MN626544 and MN626545.
Four supplementary figures and one table are available with the online version of this article.

Table 1. List of *Cryolevonia giraudoae* sp. nov. and *Camptobasidium gelus* sp. nov. isolates

Species	Strains code	GenBank accession number (ITS/D1D2)	Isolation substrate	Locality	Reference
Cryolevonia giraudoae	CRUB 2086^T	MN622687/MN626546	Glacial Ice	Castaño Overa Glacier Mount Tronador, Patagonia Argentina	This study
	CRUB 2089	MN622688/MN626547			
	CRUB 2090	MN622689/MN626548			
	CRUB 2091	MN622690/MN626549			
	CRUB 2092	MN622691/MN626550			
	CRUB 2093	MN622692/MN626551			
	CRUB 2094	MN622693/MN626552			
	CRUB 2095	MN622694/MN626553			
	CRUB 2096	MN622695/MN626554			
	CRUB 2097	MN622696/MN626555			
	CRUB 2098	MN622697/MN626556			
	CRUB 2068	MN622698/MN626557	Marine ice	Bellingshausen sea, Antarctic Peninsula	This study
	CRUB 2069	MN622700/MN626559			
	CRUB 2100	MN622699/MN626558			
	CRUB 1733	MN622701/FJ841888	Glacial Ice	Perito Moreno Glacier, Patagonia Argentina	[12]
	CRUB 1737	MN622702/FJ841890			
	CRUB 1741	MN622703/MN626560			
	CRUB 1745	MN622704/MN626561			
Camptobasidium gelus	09 GL-9	MN626542/MN626544	Glacial Ice	Russel Glacier, Greenland Ice sheet	This study
	09 GL-23	MN626543/MN626545			
	CBS 8941^T	AY040665/AY040647	Ice	Vestfold Hills area of Davis base, Antarctica	[14]

DESCRIPTION OF SAMPLING SITES AND ISOLATION METHODS

Ice samples were obtained from two locations in Patagonia and one in Antarctica: (i) Castaño Overa glacier at Mount Tronador (71°50′W, 41°11′S) in summer of 2014; and (ii) Perito Moreno glacier (73°51′W, 49°15′S) in the summer of 2008 [11]; and Bellingshausen sea (61°00′W, 60°51′S) in the summer of 2012. The surface of the ice samples was melted and discarded, and the remaining ice was superficially rinsed with sterile distilled water and melted in aseptic conditions. The resulting water was filtered through sterile nitrocellulose membrane filters (0.45 µm pore diameter). Yeasts from Patagonia and Antarctica were isolated after filtration and incubation at 5 °C in Petri dishes MYP agar as described by de Garcia [12].

Ice samples from the Russel glacier in Greenland ice sheet were collected in the spring of 2009, and yeasts were isolated as described by Uetake [13].

Strain CBS 8941 was isolated from benthic mat positioned on an ice top at Chelnok Lake (68° 38′44′ S, 78° 20′03′ E), in the Vestfold Hills area of Base Davis, Antarctica by Thomas-Hall in 1998 [14]. The precise origin of all isolates is listed in Table 1.

Yeast characterization was performed based on morphological traits, coupled with standard physiological tests (assimilation of carbon and nitrogen compounds were performed in solid media, glucose fermentation was carried out in stationary liquid media), as described by Kurtzman [15]. For mating experiments 48 h cultures were paired (all combinations of strains were tested) or maintained singly on glucose yeast-extract agar (GY agar: 0.2% glucose, 0.1% yeast extract, 2% agar [15]), incubated for 2 months at 10 °C and observed microscopically once per week.

All isolates were tested for their ability to grow at 0, 4, 10, 13 and 18 °C on YNB glucose liquid media. The 96 well microplates were inoculated with a calibrated suspension (1×10^6 cells

ml^{-1}) of exponentially growing yeast cells, and incubated at different temperatures. Experiments were performed in triplicates. Growth kinetics were measured photometrically at 640 nm at 24 h intervals (Elisa microplate reader Mindray MR-96A Spectrophotometer) and dry biomass was determined after 210 h of growth [16].

Cell size and morphology were determined under differential interference contrast microscopy using an Olympus BX51 microscope with an attached DP12 camera and CellB Imaging Software. Cultures were grown on yeast-extract, malt-extract, peptone-glucose agar (YM agar: 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar [15]), incubated at 10 °C.

DNA extraction and PCR amplification conditions were those described by Libkind [17]. For DNA sequence analysis, internal transcribed spacer (ITS) ribosomal rDNA was amplified using ITS1 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers [14]. D1/D2 domains of rDNA large subunit (LSU rDNA) were amplified using NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAGACG G-3′) primers [18, 19]. Sequencing was performed by Macrogen Sequencing Service (Korea). The sequences downloaded from GenBank are indicated in the phylogenetic tree by their accession numbers.

Multiple sequence alignment using ITS and D1/D2 domains of the LSU rRNA sequences was performed using MAFFT version 5 [20] via the CIPRES Science Gateway [21]. Estimation of the phylogenetic relationships, on the basis of the ITS and D1/D2 domains of the LSU rRNA sequences, was done with neighbour-joining (NJ) method using Kimura two-parameter (K2P) model to estimate the evolutionary distances (Fig. 1), and by maximum-likelihood (ML) using a General Time Reversible Model (GTR) of substitution in MEGA 5 [22, 23] (Fig. S1, available in the online version of this article). Pairwise similarities were computed after pairwise alignments using Clustal W [24]. All ITS and D1/D2 LSU rRNA sequences closely related to our 21 strains, were retrieved until November of 2019 (Table S1)

SPECIES DELINEATION AND PHYLOGENETIC PLACEMENT

In different sampling campaigns of cold environments, 21 yeast isolates related to the Microbotryomycetes class were obtained and analysed. These isolates were obtained from: Castaño Overa glacier ice from Mount Tronador, Nahuel Huapi National Park, Argentina (11 isolates); Perito Moreno Glacier ice, Los Glaciares National Park, Argentina (four isolates); marine ice from Bellingshausen sea (three isolates); ice top at Chelnok Lake in Antarctica (one isolate); and from Russel glacier in the Greenland ice sheet (two isolates) (Table 1).

Comparison by multiple alignment and phylogenetic analysis using the ITS and D1/D2 LSU rRNA sequences of our 21 isolates, and available related sequences from Microbotryomycetes species, confirmed their placement within the Camptobasidiaceae family. Eighteen of these 21 isolates grouped together with the recently described species *Cryolevonia schafbergensis* [6] which is phylogenetically located near to the genera *Glaciozyma* and *Camptobasidium* (clade one). The remaining three isolates were closely related to *C. hydrophilum* (clade two) (Figs 1 and S1). The isolates within clade one showed sequences with no more than one nucleotide difference suggesting their conspecificity. A group of 10 isolates shared identical sequences both at ITS and D1/D2 LSU rRNA sequences (CRUB 2086^T, CRUB 2089, CRUB 2090, CRUB 2091, CRUB 2093, CRUB 2095, CRUB 2096, CRUB 2097, CRUB 2098, CRUB 1745, CRUB 1733). Strain CRUB 2068 differed in a single position at the ITS region from all other isolates; whereas CRUB 2100, CRUB 2069, CRUB 1737 and CRUB 1741 shared one difference at a single position. Isolates CRUB 2092 and CRUB 2094 each one had one nucleotide difference at D1/D2 region from all other isolates.

Additionally, pairwise nucleotide similarities were computed at the ITS and D1/D2 LSU rRNA sequences for strain CRUB 2086^T and *Cryolevonia schafbergensis* PYCC 8347^T, showing a similarity of 97.9% at the ITS region (11 differences) and 98.7% at the D1/D2 region (six differences), indicating that these are closely related species. For other Microbotryomycetes species, all identities were below 90 and 97% for the ITS and D1/D2 regions, respectively.

When the physiological profile of the 18 isolates included in clade one was compared to other Microbotryomycetes, we observed that they differed from *Cryolevonia schafbergensis* in their positive assimilation of ribitol and xylitol as sole carbon sources and lysine as sole nitrogen source. They differed from *Glaciozyma* spp. in their positive assimilation of melezitose and glycerol as a sole carbon sources; and differed from *C. hydrophilum* CCM 8060^T in their ability to assimilate glycerol and lack of assimilation of lactose as sole carbon source, as well as the usage of nitrate and nitrite as sole nitrogen sources (Table 2). In addition, none of the isolates in clade one showed a filamentous state such as *C. hydrophilum* CCM 8060^T, which lacks a yeast-like state [2, 6, 7]. Isolates included in clade one differed from *C. gelus* sp. nov., in their positive assimilation of melezitose and negative assimilation of melibiose (Table 2).

Based on these results we propose the new species *Cryolevonia giraudoae* sp. nov. to include the 18 isolates from clade one.

Multiple alignment of ITS and D1D2 LSU rRNA sequences showed that the three isolates in clade two are identical, and have five nucleotide substitutions at the D1/D2 region (99% similarity) and 20 at ITS (96% identity) when compared to *C. hydrophilum* CCM 8060^T sequences. Physiological analysis showed that, in contrast to *C. hydrophilum*, these isolates did not display a filamentous state in any of the conditions tested. Also, in contrast to other taxa in the Microbotryomycetes class, these isolates do assimilate melibiose as a sole carbon source. Unlike *C. hydrophilum*, these strains cannot use lactose as sole carbon source but utilize glycerol, and can use nitrate and nitrite as sole nitrogen sources (Table 2). Based on the genetic and phenotypic

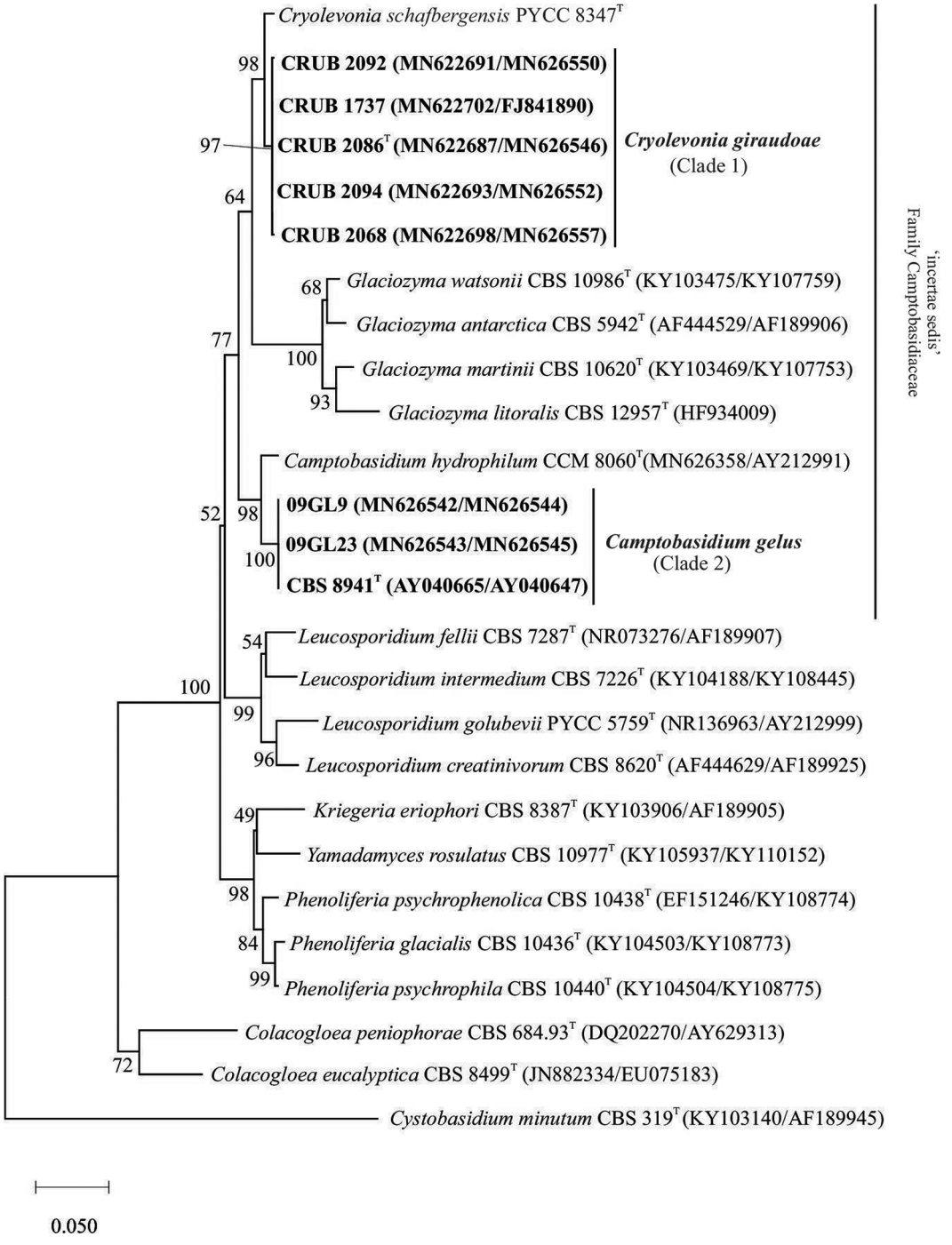

Fig. 1. Phylogenetic placement of *Cryolevonia giraudoae* sp. nov. and *Camptobasidium gelus* sp. nov. based on the ITS and D1/D2 domains of the LSU rRNA sequences. The tree was reconstructed using neighbour-joining analysis and Kimura's two-parameter distance model. Bootstrap values were determined from 1000 pseudoreplicates. Bar, 0.005 substitutions per site. T, Type strain. In bold are the strains studied in this report. GenBank accession numbers are given in parentheses (ITS, D1/D2).

differences, we propose the new species *Camptobasidium gelus* sp. nov. to include isolates from clade two.

A number of ITS and D1/D2 sequences were found in public databases, similar to those from the new taxa described here. From these, 67 belonged to cultured yeasts and 38 to uncultured fungi from cold environments worldwide (Table S1). Five environmental sequences (D1/D2), two from ice in Gulkana glacier, Alaska [13] and three from dark ice in the Greenland ice sheet [32], are identical to the D1/D2 region of *Cryolevonia giraudoae* CRUB 2086T (Figs S2 and S3). Thus,

Table 2. List of salient physiological differences of *Cryolevonia giraudoae sp. nov.* and *Camptobasidium gelus* sp. nov. compared to related *Microbotryomycetes*. 1, Sucrose; 2, Raffinose; 3, Lactose; 4, Trehalose; 5, Maltose; 6, Melezitose; 7, Melibiose; 8, Methyl-α-D-glucoside; 9, Soluble starch; 10, L-Arabinose; 11, D-Arabinose; 12, Glycerol; 13, myo-Inositol; 14, DL-Glucoside; 15, Nitrate; 16, Nitrite; 17, Lysine. –, Negative; +, positive; d, delayed; v, variable; W, weak; dw, delayed and weak; ND, not determined.

Taxon	Assimilation of																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
***Cryolevonia giraudoae* sp. nov.**	+	v	–	–	v	+	–	–	–	–	–	+	–	–	+	+	+
Cryolevonia schafbergensis	+	–	–	–	+	+	–	–	–	–	–	d	–	ND	+	+	–
***Camptobasidium gelus* sp. nov.**	+	+	–	–	w	–	+	–	–	–	–	+	–	–	+	+	d
Camptobasidium hydrophilum	+	+	+	–	w	+	–	w	–	w	w	–	–	–	–	–	ND
Glaciozyma martinii	+	–	dw	dw	v	–	–	–	–	w/-	–	v	dw	–	+	w	w
Glaciozyma antarctica	+	v	–	v	v	–	–	–	–	–	–	w/+	–	ND	+	+	ND
Glaciozyma watsonii	+	–	–	dw	v	dw	–	–	–	v	–	–	dw	–	w/+	w	–
Glaciozyma litorale	+	–	–	v	–	–	–	–	–	–	–	w	ND	ND	–	ND	ND
Kriegeria eriophori	+	–	–	+	+	+	–	+	ND	+	–	+	ND	ND	+	+	w
Phenolifera (glacialis clade)	+	+	–	–	–	+	–	–	ND	–	–	–	–	–	+	–	ND

it is very likely that this species is also present in glaciers and permanent ice in the northern hemisphere.

Regarding *C. gelus* sp. nov., we found isolates with identical or almost identical D1/D2 regions: one isolate from surface snow from a Tibetan plateau glacier, PR China (JQ768846); two from Gulkana glacier surface ice in Alaska [13], 15 from subglacial ice from Svalbard and 32 from the Greenland ice sheet. In addition, we found 13 similar D1/D2 sequences from uncultured fungi, obtained mostly from soil samples (Figs S2 and S3).

The data here presented from available environmental sequences collected worldwide suggests that the two yeast species described are distributed in many cold environments from the world (Alaska, Russia, Svalbard, China, Antarctica, Patagonia, Mexican glaciers, among others). Different surveys using either conventional isolation or metagenomics approaches show there is still a large yeast biodiversity to be uncovered in glacial environments [12, 25, 26].

Different topologies are obtained when single markers are used alone (ITS or D1/D2), compared to the phylogenetic tree built with both concatenated sequences (Figs 1, S1–S3). Nevertheless single marker analyses contain fewer informative sites and are not recommended for species or strain delimitations; and are only presented to discuss species ecology and to help delineate future description of related species. Further studies with additional markers (7) on cold adapted yeasts, particularly those from the Microbotryomycetes class, will certainly help to resolve phylogenetic ambiguities among these understudied groups.

Also, it will be important to expand the studies of the mechanisms that enable survival in cold temperatures, including the production of novel molecules with biotechnological applications, such as cold active enzymes and antifreeze proteins [9–11, 27]. The genetic diversity present in permanently cold habitats like glacial ice or permafrost needs to be urgently preserved, since most of these are endangered as a result of global warming [28, 29].

DESCRIPTION OF *CRYOLEVONIA GIRAUDOAE* DE GARCIA, TROCHINE & LIBKIND SP. NOV.

Etymology: gi.rau.do'ae. N.L. gen. n. *giraudoae* named in honour of the Argentinian researcher Dr. María Rosa Giraudo for her contributions to yeast systematics, ecology and biotechnology.

MycoBank accession number: MB 835167

After 10 days on malt extract/yeast extract agar at 10 °C, the colonies are white yellowish and after a few days they turn light pink, smooth and opaque, with friable texture, and undulated with lobulated margin (Fig. 2a–c). The cells are globose to elongated, and multiply by multilateral budding, budding end in a stalk (Fig. 3). After 3 weeks on GSA medium pseudohyphae may be formed. No positive mating reactions were observed among all *Cryolevonia giraudoae* isolates.

Glucose is not fermented. Carbon source assimilated: glucose, raffinose (variable), D-galactose (variable), L-sorbose, maltose (variable), cellobiose (variable), melezitose, glycerol, D-glucitol (weak), ribitol, D-mannitol, xylitol (weak), salicin (weak), succinate (variable), arabutine (weak), and ethanol (variable). No growth occurs on lactose, trehalose, melibiose, methyl-α-D-glucoside, soluble starch, L-arabionose, D-arabinose, myo-inositol, DL-glucoside, D-glucosamine, D-ribose, D-xilose, L-rhamnose, sacarose, meso-erythritol, galactitol, glucono-δ-lactona, D-gluconate, D-galacturonate, citrate and methanol. Assimilation of nitrogen compounds: positive for nitrite, nitrate and L-lysine. No growth was observed on D-glucosamine. Growth is observed between −2 °C up to 13 °C, no growth is

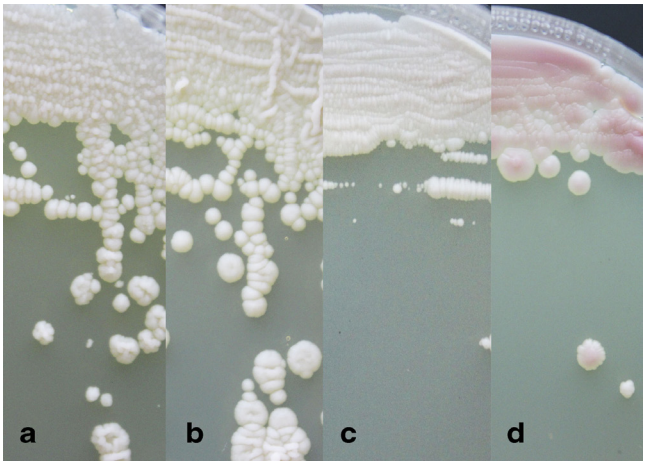

Fig. 2. Colonies of *Cryolevonia giraudoae* sp. nov. strains isolated from different locations and *Camptobasidium gelus* sp. nov. in malt extract/yeast extract agar at 10 °C after 10 days. (a) *Cryolevonia giraudoae* CRUB 2068^{T} isolated from ice from Castaño Overa glacier (Mount Tronador, Patagonia Argentina); (b) *Cryolevonia giraudoae* CRUB 2068 ^{T} isolated from marine ice in Bellingshausen sea (Antarctic peninsula); (c) *Cryolevonia giraudoae* CRUB 1737 isolated from ice from Perito Moreno glacier (Patagonia Argentina); (d) *Campatobasidium gelus* GL9 isolated from ice from Russel Glacier, Greenland.

observed above 15 °C. Growth on YM agar with 10% sodium chloride is absent. Starch-like compounds are not produced. Urease activity is positive.

Holotype: isolated from glacial ice in Castaño Overa glacier in Mount Tronador, deposited at the Centro Regional Universitario Bariloche (CRUB), Bariloche, Argentina as CRUB 2086^{T} and stored in a metabolically inactive form. Ex-holotype strain has been deposited at the CBS Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, as CBS xxx.

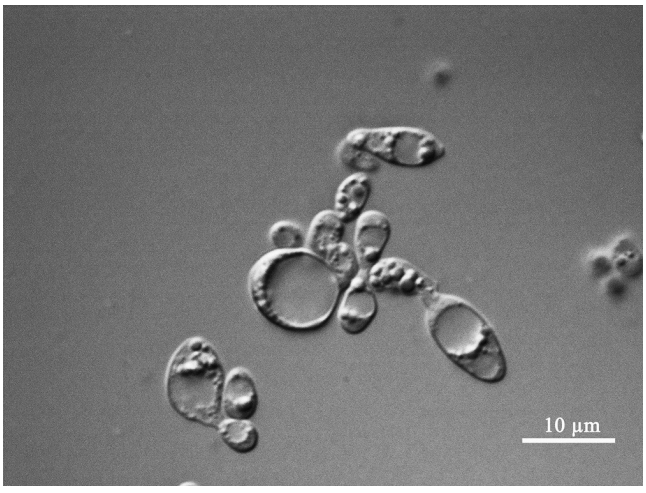

Fig. 3. Phase contrast micrograph of *Cryolevonia giraudoae* CRUB 2068^{T} sp. nov. on malt extract/yeast extract agar after 10 days at 10 °C. Bar=10 µm.

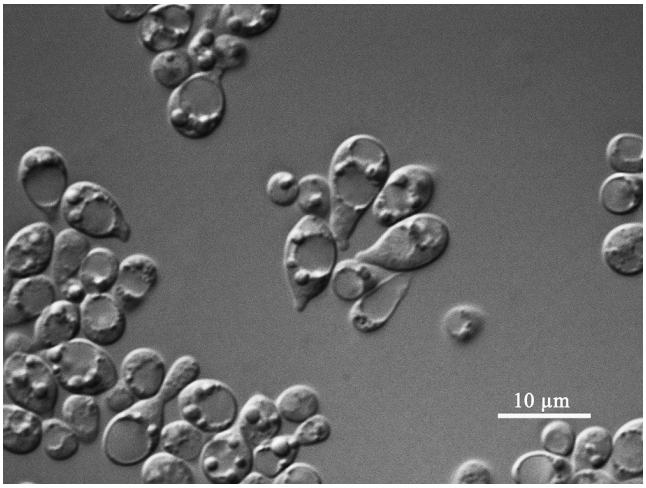

Fig. 4. Phase contrast micrograph of *Camptobasidium gelus* CBS 8941^{T} sp. nov. on malt extract/yeast extract agar after 10 days at 10 °C. Bar=10 µm.

DESCRIPTION OF *CAMPTOBASIDIUM GELUS* DE GARCIA, TROCHINE, UETAKE, & LIBKIND SP. NOV.

Etymology: (ge'lus. L. gen. n. *gelus*, of ice) *Camptobasidium gelus*, the species name refers to the substrate (ice) from which the isolates were obtained.

MycoBank: MB 830185

After 10 days on malt extract/yeast extract agar at 10 °C, the colonies are light purple to light pink, smooth and opaque, with friable texture, and undulated with lobulated margin (Fig. 2d). The cells are ovoidal to ellipsoidal, and multiply by multilateral budding. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae and true hyphae are not formed (Fig. 4). No positive mating reactions were observed.

Glucose is not fermented. Assimilation of carbon source occurs in: glucose, raffinose, D-galactose (variable), L-sorbose, sacarose, maltose (weak), cellobiose (weak), melibiose, glycerol, D-glucitol, ribitol, D-mannitol, xylitol, salicin (weak), arbutin (weak), succinate (variable), and methanol (weak). No growth occurs on lactose, trehalose, methyl-α-D-glucoside, soluble starch, L-arabionose, D-arabinose, myo-inositol, melezitose, DL-glucoside, D-glucosamine, D-ribose, D-xilose, L-rhamnose, meso-erythritol, galactitol, glucono-δ-lactona, D-gluconate, D-galacturonate, citrate and ethanol. Assimilation of nitrogen compounds: positive for nitrite, nitrate and L-lysine (slow). No growth was observed on D-glucosamine. Maximum growth is observed between −2 °C up to 13 °C, no growth is observed above 18 °C. Growth on YM agar with 10% sodium chloride is absent. Starch-like compounds are not produced. Urease activity is positive.

Holotype: isolated from ice in the Vestford Hills, Base Davis, Antarctica, deposited at the CBS Westerdijk Fungal

Biodiversity Institute, Utrecht, The Netherlands, as CBS 8941^T, and stored in a metabolically inactive form.

PROTOLOGUE

The MycoBank numbers are MB 835167, MB 830185

The GenBank accession numbers determined in this study for the sequences of the ITS region are MN622687 to MN622704, MN626542 and MN626543 and the D1/D2 domains of the large subunit rRNA gene are MN626546 to MN626561, MN626544 and MN626545

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Conflicts of interest
The authors declare that there are no conflicts of interest

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