RESEARCH ARTICLE

Cryptococcus species (Tremellales) from glacial biomes in the southern (Patagonia) and northern (Svalbard) hemispheres

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Abstract

Cryptococcus species (Basidiomycota) were isolated as the predominant yeast from glacial biomes of both Patagonia (Argentina) and the Svalbard archipelago (Norway). For a selected group of *Cryptococcus* belonging to Tremellales, assimilative profile, production of extracellular hydrolytic enzymes and ribosomal DNA internal transcribed spacer and large subunit (D1/D2) sequences were analysed. *Cryptococcus victoriae*, which was originally described from Antarctica, was the most frequently found species at both locations. High variability within the species was observed and described at the genotypic and phenotypic levels, two newly described species were found in both Patagonia and Svalbard: *Cryptococcus fonsecae* and *Cryptococcus psychrotolerans*. Two other new species were found only in Patagonia: *Cryptococcus frias* and *Cryptococcus tronadorensis*. Three additional new taxa were found, but they are not named as they were only represented by single isolates.

Introduction

Ice in nature has long been considered as only containing microorganisms that have been randomly deposited on its surface. However, it is now known that different types of ice provide biomes that can support active microbial growth and reproduction (Gostincar *et al.*, 2010). Initially, the microbial presence was investigated only at the prokaryotic level. However, recent studies have shown that fungi, as primarily as basidiomycetous yeast, also represent an important part of glacial microbial communities in both polar and mountainous glacial environments around the world (Margesin *et al.*, 2003; Bergauer *et al.*, 2005; Buzzini *et al.*, 2005; Butinar *et al.*, 2007; de Garcia *et al.*, 2007; Turchetti *et al.*, 2008, 2011; Branda *et al.*, 2010). Basidiomycetous yeast also predominates in permafrost soils of the Arctic and Antarctica (Vishniac, 2006). Species of the genus *Cryptococcus* (Connell *et al.*, 2008; Turchetti *et al.*, 2011) are among the most frequently isolated from such environments.

Cryptococcus species are distributed into four orders: *Tremellales*, *Trichosporonales*, *Filobasidiales* and *Cystofilobasidiales* (Kurtzman *et al.*, 2011). Species isolated from cold environments belong to all these orders. This is also the case for the *Cryptococcus sensu stricto* group (*Tremellales*), which includes the type species and also the most medically important species, *Cryptococcus neoformans* (Cooper, 2011). Agricultural importance has also been shown for this group. For example, *Cryptococcus laurentii* has been reported to inhibit growth of phytopathogenic fungi (Robiglio *et al.*, 2011), and it can be used together with *Cryptococcus albidus* for control of postharvest diseases in stored fruit (Abadias *et al.*, 2003; Qin *et al.*, 2004; Schisler *et al.*, 2011). *Cryptococcus* species can also be used for degradation of phenolic and polycyclic aromatic hydrocarbons (Johnson & Echavarri-Erasun, 2011). Consequently, the interest in these microorganisms is not limited to their biodiversity and ecological role, but includes potential industrial uses of economic value (Margesin *et al.*, 2007; Shivaji & Prasad, 2009).

Identification and phylogenetic placement of the basidiomycetous yeast can be difficult because of intraspecific variability (Fell *et al.*, 2000; Scorzetti *et al.*, 2002; Fonseca *et al.*, 2011). Particularly within *Tremellales*, *Cryptococcus* is highly heterogeneous and includes numerous species that are yet to be described (Fell *et al.*, 2000; Sampaio *et al.*, 2002; Scorzetti *et al.*, 2002; Inácio *et al.*, 2005; de Garcia *et al.*, 2010).

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The aim of the present study was to present and identify the predominant groups of basidiomycetous *Cryptococcus* yeast species, obtained in two independent studies in Norway and Argentina. The focus was on the species from the order *Tremellales* isolated from rarely explored glacial biomes in the northern and southern hemispheres, and to compare their biodiversity. *Cryptococcus* species predominated over the other yeast in both locations. Comparisons of physiology and phylogeny of isolates has revealed unexpected diversity, which has resulted in the description of new varieties and species, some of them found in both geographically distant locations.

Materials and methods

Description of sampling sites and isolation methods

Sampling in Patagonia (Argentina) was performed in February and March in 2004, 2008 and 2010. The samples were taken from: (1) meltwater from the Rio Manso, Castaño Overo and Frias glaciers of Mount Tronador (71°50′ W, 41°11′S), in the Nahuel Huapi National Park; (2) ice from the Perito Moreno glacier (73°51′W, 49°15′S) in the 'Los Glaciares' National Park; and (3) sea water from the meridian of Cape Horn, Argentinian Sea (66°34′W, 57° 25′S). Sampling in Svalbard (Norway) was performed in May 2001, August 2003 and July 2008. The samples were taken in Kongsfjorden, on the western coast of Spitsbergen (79°N, 12°E), from: (1) glacial meltwater; (2) superficial sea water; and (3) subglacial ice. The surface of the ice samples was melted and discarded, and the remaining ice was superficially rinsed with sterile distilled water and melted. The resulting water was filtered through Millipore membrane filters (0.45 μm pore diameter). The yeast from Patagonia were isolated after filtration and incubation at 10 °C, as described by de Garcia *et al.* (2007); those from Svalbard were isolated following the protocol of Gunde-Cimerman *et al.* (2003), and were incubated at 25 °C. The precise origins of all of the strains studied here are listed in Table 1.

Yeast characterisation and identification

Yeast characterisation and identification to the genus level was performed based on morphological characteristics, coupled with standard physiological tests (assimilation of carbon and nitrogen compound were performed in solid media, glucose fermentation was carry out in stationary liquid media), as described by Kurtzman *et al.* (2011). Mating experiments were performed on glucose yeast-extract agar (GY agar: 0.2% glucose, 0.1% yeast extract, 2% agar; Kurtzman *et al.*, 2011), with cultures incubated for 2 months at 18 °C and checked microscopically once per week.

Cell size and morphology were determined under differential interference contrast microscopy using an Olympus BX51 microscope with an attached DP12 camera and Cell^{B} Imaging Software. The cultures used here 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; Kurtzman *et al.*, 2011), incubated at 18 °C. One percent poly-l-lysine was used to attach the cells to the slides (Mazia *et al.*, 1975). The presence of capsules was observed after negative staining of cultures grown on YM agar incubated at 5 and 18 °C, using Indian ink. At least 50 cells were measured, and the mean values were calculated. The cell sizes of the type strains and of our isolates were compared using Students' *t*-tests.

The protocols for DNA extraction and the PCR conditions followed were as described by Libkind *et al.* (2003). For DNA sequence analysis, internal transcribed spacer (ITS) ribosomal (r)DNA was amplified using the ITS1 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers (White *et al.*, 1990). The D1/D2 domains of the large subunit of rDNA (LSU rDNA) were amplified using the NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAGACG G-3′) primers (Boekhout *et al.*, 1995).

Sequencing was performed by Macrogen Sequencing Service (Korea). BigDye terminator cycle sequencing kits were used in sequence reactions (Applied Biosystems, Foster City, CA). The sequences were obtained using an ABI Prism 3700 PCR machine (Applied Biosystems). The sequences downloaded from GenBank are indicated in the gene trees by their GenBank accession numbers; newly generated sequences are indicated by their strain numbers (see also Table 1).

PCR fingerprinting and mini/microsatellite-primed PCR (MSP-PCR) with the M13 primer were performed on all of the *Cryptococcus victoriae* isolates included in this study, according to Libkind *et al.* (2003).

Phylogenetic analysis

The ITS and LSU (D1/D2) rDNA sequences were automatically aligned using ClustalX, and the alignments were adjusted manually using Molecular Evolutionary Genetics Analysis software, version 5 (MEGA5; Tamura *et al.*, 2011). To estimate the phylogenetic relationships on the basis of the LSU rDNA (D1/D2 domains) and ITS sequences, neighbour-joining analysis (K2P) was performed using MEGA5 (Tamura *et al.*, 2011). To test the reproducibility of the results, the Bayesian Markov chain Monte-Carlo

Table 1. Strains of the genus *Cryptococcus* studied, and their origin and the GenBank accession number of their sequences

Species	Strain numbers	Substrate	Locality, Country	Isolated by	GenBank accession numbers	
					D1/D2	ITS
C. carnescens	**EXF-1549**	Subglacial ice	Austre Lovénbreen Glacier, Svalbard, Norways	N. Gunde-Cimerman	JN193440	–
	EXF-1551				JN193441	JN193461
	EXF-1591				JN193442	JN193462
	EXF-1621				JN193443	JN193463
C. foliicola	**CRUB 1267**	Glacial meltwater	Rio Manso (Garganta del Diablo waterfall), Río Negro, Argentina	V. de Garcia	GU560001	GU997160
C. frias sp nov.	**CRUB 1303**		Frias river, Río Negro, Argentina		GU560005	GU997163
	CRUB 1250				GU560004	GU997162
C. fonsecae sp. nov	**EXF-3792**	Hypersaline saltern water	Sečovlje, Slovenia	N. Gunde-Cimerman	JN193446	JN193466
	EXF-4087	Subglacial ice with gypsum inclusions	Austre Lovénbreen Glacier, Norway		JN193447	JN193468
	CRUB 1765	Sea water	Cape Horn Meridian Argentinian Sea	V. de Garcia	JN193448	JN193467
	CRUB 1766				JN193451	–
	CRUB 1767				JN193449	–
	CRUB 1768				JN193450	–
	SJ008	–	**San Juan Islands, Vancouver**	Fraser *et al.*	AY953961	–
	A57	Sea water	Portugal	M. Gadanho & J.P. Sampaio	AF485974	–
C. psychrotolerans sp. nov	**EXF-1583**	Basal ice	Conwaybreen glacier, Svalbard, Norway	N. Gunde-Cimerman	DQ644575	JN193464
	EXF-1528	Subglacial ice	Austre Lovénbreen Glaciers, Svalbard, Norway		JN193444	JN193465
	CRUB 1769	Sea water	Cape Horn Meridian Argentinian Sea	V. de Garcia	JN193445	–
	CBS 6578	Sea water	–	J.W. Fell	AB035053	AB035049
C. aff. tephrensis	CBS 9799	Arctic dwarf shrub *Dryas octopetala*	Norway	J. Pryce Miller	CBS database	CBS database
	CBS 9023	Flowering plant of *Pulmonaria stiriaca*	Hessen, Marburg, Germany	M. Herzberg	AF406896	–
	EXF-3999	Stream water	Kongsvegen, Svalbard, Norway	N. Gunde-Cimerman	GU586202	GU997158
	EXF-3749	Stream water			GU586203	GU997157
	EXF-3875				GU997140	GU997161
	EXF-6553	Surface ice			DQ640490	–

Table 1. Continued

Species	Strain numbers	Substrate	Locality, Country	Isolated by	GenBank accession numbers	
					D1/D2	ITS
C. tronadorensis sp. nov.	**CRUB 1258**	Glacial meltwater	Rio Manso (Garganta del Diablo waterfall), Rio negro, Argentina	V. de Garcia	GU560002	GU997164
	CRUB 1299				GU560003	GU997165
C. victoriae type group	CBS 8685^T	Soil	Antarctica	Montes *et al.*	AF363647	AF444469
	CBS 8920			S. Thomas-Hall	AY040650	AY040656
	CBS 8915				AY040652	AY040655
	CBS 9267	Aggregated grey-brown silty soil with coarse organic remains	Alaska, Nome	H.S. Vishniac	CBS database	CBS database
	CBS 9565					
	CRUB 1399	Acidic river	Rio Agrio-Lake Caviahue	G. Russo	EF585176	–
	DBVPG 4835	Glacial water	Italian Alps	E. Branda	EU287884	–
	DBVPG 4830				EU287882	–
	TP-Snow-Y33	Glacier surface snow	Tibetan Plateau China	S. Shao	JN400774	JN400815
	ESAB12	Food	Portugal: Tras-os-Montes	R. Calhelha	AJ749830	–
	I1-4	Processed meat products	Denmark	Nielsen *et al.*	EU194455	–
	CBS 9000	Flowering plant of *Helleborus foetidus*	Germany	M. Herzberg	AF406899	–
	CBS 6550	Forced rhubarb	UK	R. Buhagiar	AF444711	AF444447
	KCTC 17059	Rhizosphere soil of *Platycodon grandiflorum*	Korea	S.G. Hong & K.S. Bae	AF459674	–
	AY-99	*Deschampsia cespitosa* green leaves	Russia	A. Yurkov	FN357207	–
	AY-92	*Equisetum sylvaticum* green leaves			FN357206	–
	CRUB 1262	Glacial meltwater	Rio Manso (Garganta del diablo waterfall), Río Negro, Argentina	V. de Garcia	GU559996	GU997143
	CRUB 1254				GU559995	GU997147
	CRUB 1260		Frias river, Río Negro, Argentina		GU559997	GU997145
	CRUB 1264				GU559994	GU997148
	CRUB 1263				GU559998	GU997149
	CRUB 1266				GU559999	GU997150

Table 1. Continued

Species	Strain numbers	Substrate	Locality, Country	Isolated by	GenBank accession numbers D1/D2	GenBank accession numbers ITS
	EXF-3832	Subglacial ice	Kongsfjorden, Svalbard, Norway	N. Gunde-Cimerman	GU586196	GU997144
	EXF-1582	Darkly pigmented ice	Austre Brøggerbreen glacier, Kongsvegen, Norway		JN193432	JN193452
	EXF-1588	Sea water	Svalbard, Norway		JN193433	JN193453
	EXF-1623	Subglacial ice	Austre Lovénbreen Glacier, Svalbard, Norway		JN193434	JN193454
	EXF-3831	Melt water	Kongsfjorden, Svalbard,		GU586193	GU997141
	EXF-3748		Norway		GU586192	GU997142
	EXF-3721				GU586200	GU997154
	EXF-4012	Sea water			GU586195	GU997146
	EXF-3912				GU586194	GU997152
	EXF-4085				GU586198	GU997159
	EXF-3827	Subglacial ice with gypsum inclusions	Austre Lovénbreen Glacier, Norway		JN193435	JN193455
	EXF-6542	Subglacial ice	Kongsvegen Glacier, Svalbard, Norway		JN193436	JN193456
	EXF-6550		Austre Lovénbreen		JN193437	JN193457
	EXF-6549		Glacier, Svalbard,			JN193460
	EXF-6535		Norway		JN193438	JN193458
	EXF- 6534	Columnar ice crystals at the outflow of glacier melt water	Kongsfjorden, Svalbard, Norway		JN193439	JN193459
	EXF-4020	Sea water			GU586201	GU997156
	EXF-3923				GU586204	GU997155
	EXF-4011	Stream water			GU586197	GU997153
C. victoriae non-type group	**CRUB 1757**	Ice	Perito Moreno, Santa Cruz, Argentina	V. de Garcia	GU560000	GU997151
	CBS 8908	Soil	Antarctica	S. Thomas-Hall	AY040653	AY040654
	CBS 8884	Seawater (reef)	Bahamas	A. Statzell-Tallman	AF444741	AF444645
	VTT C-04542	Industrial malting ecosystem	Finland	Laitila *et al.*	DQ377664	–
	LU9-1	Grape	Denmark	Lederer *et al.*	HM146911	–
	A114	Sea water	Portugal	M. Gadanho & J. P. Sampaio	AF485971	–
Cryptococcus sp. 1	**EXF-1596**	Basal ice	Conwaybreen glacier, Svalbard, Norway	N. Gunde-Cimerman	DQ644575	–
Cryptococcus. sp. 2	**EXF-3926**	Subglacial ice	Kongsvegen, Svalbard,Norway		GU586199	GU997166

Numbers in bold are isolates from this study.

CRUB, *Regional University Center* of *Bariloche* (Centro Regional Universitario Bariloche); EXF, Culture Collection of Extremophilic Fungi, Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia; CBS, Centraalbureau voor Schimmelcultures; DBVPG, Industrial Yeasts Collection of Dipartimento di Biologia Vegetale, Università di Perugia, Italy; KCTC, Korean Collection for Type Cultures; VTT, Technical Research Centre of Finland.

method of phylogenetic inference was applied, as implemented in the MRBAYES programme (Ronquist & Huelsenbeck, 2003). Parsimony networks were constructed from the aligned sequences with the TCS 1.21 programme (Clement *et al.*, 2000), with gapped positions excluded from the analysis.

Extracellular enzymatic activity

The strains were tested for their ability to degrade starch, protein (casein), pectin, carboxymethyl cellulose and fatty acids, according to procedures described by Brizzio *et al.* (2007). Calibrated suspensions of 1.0×10^6 cells mL^{-1} grown for 24–48 h were inoculated onto the surface of agar plates using a multipoint inoculation device (Brizzio *et al.*, 2007). The plates contained the substrates for above-mentioned activities, and they were incubated at 5 and 20 °C. The enzymatic activities were analysed after 5 days in the samples incubated at 20 °C, and after 21 days in those incubated at 5 °C. The enzymatic activities for the specific substrates were evaluated as described by Brizzio *et al.* (2007).

Results

According to the LSU rDNA analysis, psychrotolerant yeast (all the strains isolated grew at 5 °C and up to 25 °C) from the two sampling locations were grouped within three of six clades of *Tremellales*: *Bulleromyces*, *Victoriae* and *Kwoniella* (Figs 1 and 2). The results presented are also supported by Bayesian analysis (data not shown).

Cryptococcus victoriae and related species

The most frequent species isolated from these cold glacial environments of both Patagonia and Svalbard was *C. victoriae*, of which 27 strains were isolated and identified. The cell size of the *C. victoriae* type strain CBS 8586 described by Montes *et al.* (1999) was 3 × 2 μm, whereas Thomas-Hall *et al.* (2002) reported slightly larger cells of variable sizes, from 3 to 5 × 2 to 3 μm. Our measurements were in agreement with Thomas-Hall *et al.* (2002), with a median of 5 × 3 μm. The cell sizes of the related strains in the present study were variable, from 5 to 6 × 3 to 4 μm, and no statistical differences were found between the values obtained for the strains studied, or for the type strain. Small capsules were observed in all the strains studied, although there were no differences in their sizes when the cells were grown at low temperature (5 °C) (Fig. 3a–$a_{(i)}$ and b–$b_{(i)}$). No sexual reproduction was observed in the mating experiments.

The physiological tests showed variations in the assimilation of sorbitol, glucosamine, soluble starch, citrate, nitrate and creatinine, in comparison with the type strain CBS 8586 (see Table 2), as also observed for the MSP-PCR fingerprinting analysis (data not shown) and the sequence analysis.

Analyses of the LSU and ITS rDNA sequences revealed polymorphism in strains clustering together with *C. victoriae* type strain CBS 8586, which suggested a species complex (Fig. 4). The above-mentioned parsimony network analysis based on concatenated sequences showed that 16 out of 27 strains studied were identical to *C. victoriae* and were considered as an ancestral group, seven strains showed one nucleotide substitution in comparison to the type strain, and the remaining eight strains showed from 3 to 5 nucleotide substitutions. Based on these data, two groups were identified *C. victoriae* type group (identical to type strain) and *C. victoriae* nontype group, which showed more than one nucleotide difference in comparison to the type strain (Fig. 4).

The strains described by Thomas-Hall *et al.* (2002) from Lichen Valley (Antarctica) (CBS 8915, CBS 8920, CBS 8908) and 18 strains isolated from different regions around the world (Fig. 1) were conspecific with the *C. victoriae* type group reported here. Six strains showed one nucleotide difference in comparison to the type strain whereas five strains showed two or three differences; thus we include them in the *C. victoriae* nontype group, with this also supported by parsimony network analysis (Fig. 4).

The data for the six extracellular enzymatic activities tested showed that *C. victoriae* and related strains can produce cellulases and esterases at both of the temperatures tested (5 and 20 °C; Table 4).

Among the strains studied from the subglacial ice of the Austre Lovénbreen glacier (Svalbard), four strains of *Cryptococcus carnescens* were isolated that are identical to the type strain CBS 973. One *Cryptococcus* strain was isolated from glacial meltwater from Patagonia (CRUB 1267) that clustered together with the type strain of *Cryptococcus foliicola* and is related to the at present nonaccommodated *Cryptococcus* CBS 2339, which was isolated from human, and to *Cryptococcus heimaeyensis* (Sugita *et al.*, 2000; Takashima *et al.*, 2003).

Four strains that are closely related to *Cryptococcus tephrensis* were isolated from the stream water from Kongsfjorden (Svalbard). In the ITS region, two of these strains (EXF-3999, EXF-3875) showed six nucleotide differences to the type strain; these are related to CBS 9799, which was isolated from the dwarf shrub *Dryas octopetala* in the High Arctic, Svalbard, Norway, and to CBS 9023, which was isolated from the flowering plant *Pulmonaria stiriaca* in Germany (Fig. 1); these are currently identified as *C. victoriae*.

An undescribed species related to *C. tephrensis* was recognised based on three isolates in the present study.

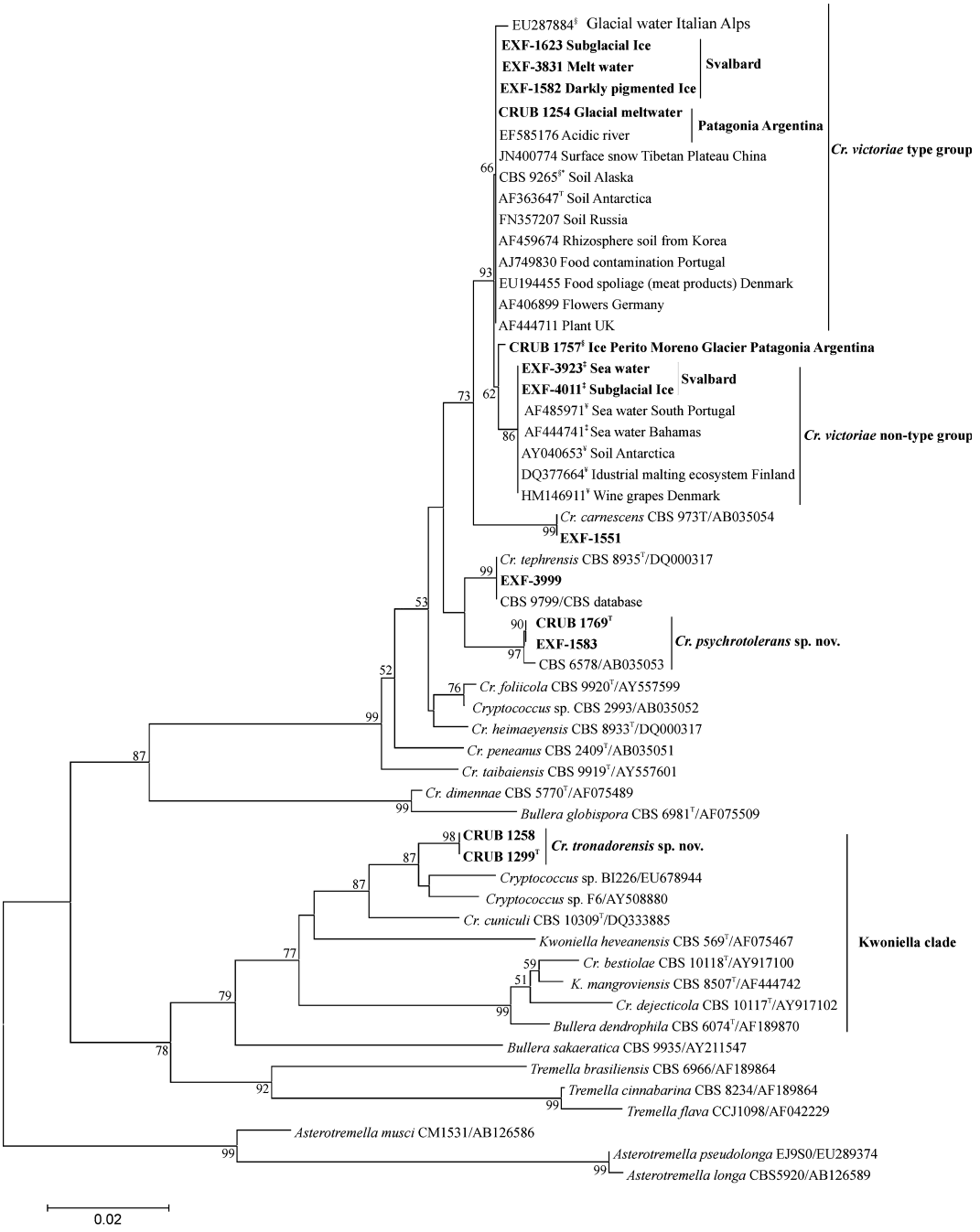

Fig. 1. Phylogenetic placement of the species in *Victoriae* and *Knowiella* clades (*Tremellales*) obtained by neighbour joining (distance K2P method) of the LSU rDNA gene D1/D2 domains. Bar, substitutions accumulated every 100 nucleotides. Nucleotide differences (nd) of *Cryptococcus victoriae* strains with type strain are represented as §: 1 nd; ¥: 2 nd and ‡: 3 nd. *The sequence was obtained in CBS databank, no GeneBank number was available. Strain numbers in bold represent isolates from the present study. Bootstrap values higher than 50% are shown (1000 replicates). [T]Type strain. *Asterotremella music*, *Asterotremella pseudolonga* and *Asterotremella longa* were designated as the outgroup species for this analysis.

One strain originated from the water of the Austral Argentinean Sea (CRUB 1769), and two from the ice of the Svalbard glacier in Norway (EXF-1528, EXF-1583). The strain CBS 6578 was isolated from sea water in the Pacific Ocean (Fell & Jones, 1976), it was originally identified as *C. laurentii*, and is currently identified as

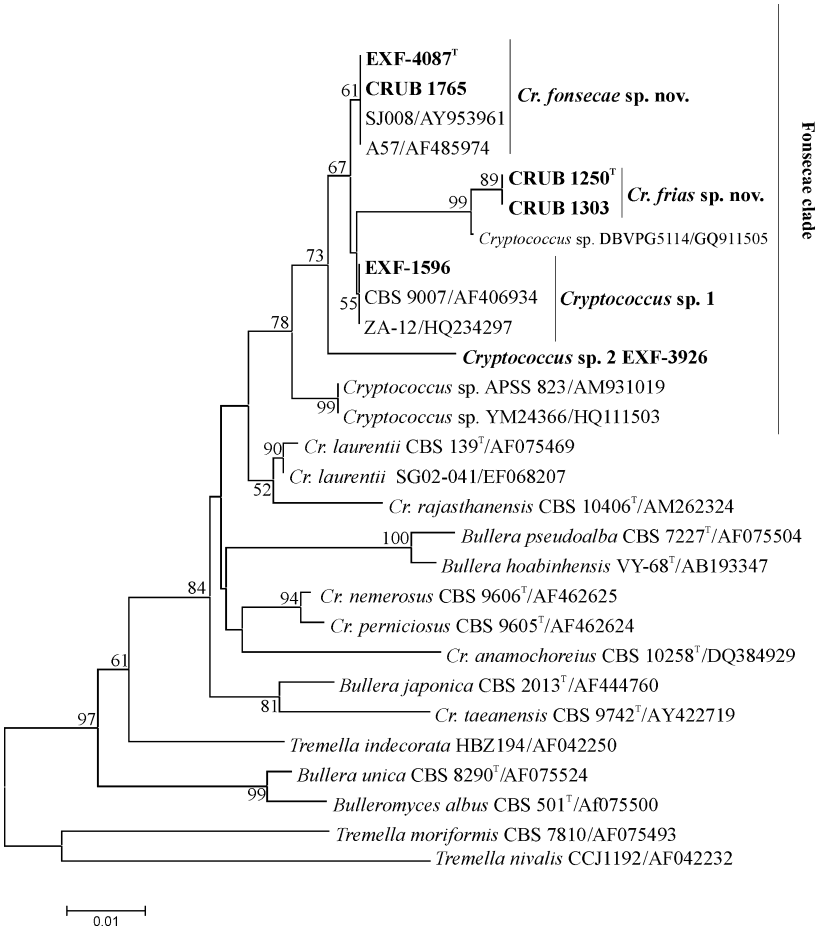

Fig. 2. Phylogenetic placement of species in *Bulleromyces* clade (*Tremellales*) obtained by neighbour joining (distance K2P method) of the LSU rDNA gene D1/D2 domains. Bar, substitutions accumulated every 100 nucleotides. Strain numbers in bold represent isolates from the present study. Bootstrap values higher than 50% are shown (1000 replicates). ^{T}Type strain. *Tremella moriformis* and *Tremella nivalis* were designated as the outgroup species for this analysis.

C. tephrensis (Takashima *et al.*, 2003); according to results of this study it is related to the newly proposed species *Cryptococcus psychrotolerans*.

Within this group, there were 7 (1.5%) nucleotide differences in the LSU region and 28 (6.9%) nucleotide differences in the ITS region, in comparison with the sequences of *C. tephrensis* CBS 8935^{T}; this justifies the decision to describe a new species, named here as *C. psychrotolerans*. In addition, this species differs from the closely related *C. tephrensis* by the absence of cellulolytic activity (Table 4).

Kwoniella clade

In the second group of *Cryptococcus* (*Tremellales*), strains related to *Cryptococcus heveanensis* (*Kwoniella heveanensis*) were also isolated, and they are recognised and proposed here as a new species, named *Cryptococcus tronadorensis*. These isolates originated from the glacial meltwater from Patagonia Argentina (Mount Tronador). Compared with the closest sequences, their LSU sequences showed six nucleotide differences to *Cryptococcus* sp. F6 (Wang & Yang, genebank), and 14 to *Cryptococcus* sp. BI226 (Landell *et al.*, GeneBank). The ITS sequences were not available in genebank for comparison.

Sexual genus *Kwoniella*, and species *Kwoniella mangroviensis* closely related to *C. heveanensis* was discovered by Statzell-Tallman *et al.* (2008); recently Metin *et al.* (2010) described sexual state of this species (*K. heveanensis*). *Cryptococcus tronadorensis*, belongs to *Kwoniella* clade (Fig. 1), however sexual state was not observed, to perform a deeper analysis in this regard, higher number of isolates are needed to define if this species belongs to this clade or a new one.

Bulleromyces clade

A new clade was discovered that is composed of four so-far-undescribed taxa related to *C. laurentii* (Fig. 2). Two of these are described in the present study as *Cryptococcus frias* and *Cryptococcus fonsecae*. One of the remaining potentially new species is represented by a single isolate (EXF-3926) from subglacial ice from Kongsfjorden glacier (Norway), and the second is represented by four

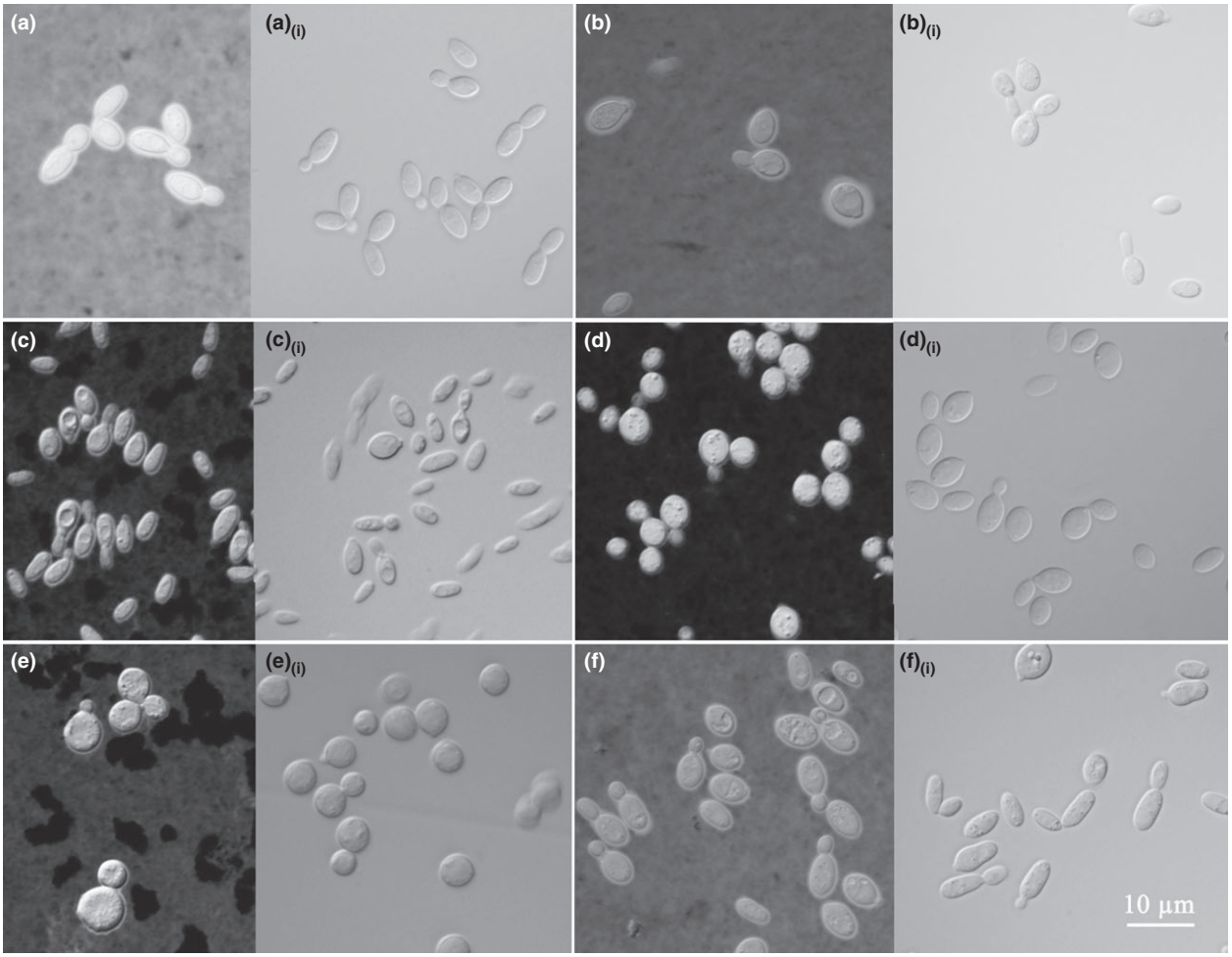

Fig. 3. Micromorphology of vegetative cells after 3 days of growth on yeast extract-malt extract agar incubated at 18 °C. Column 1 and 3: cells and capsules negative stained with Indian ink; column 2 and 4: budding cells (Nomarski optics). Bar, 10 μm, valid for all pictures. (a, $a_{(i)}$) *Cryptococcus victoriae*, CBS 8685^T; (b, $b_{(i)}$) *C. victoriae* nontype group, EXF-4020; (c, $c_{(i)}$) *Cryptococcus psychrotolerans* sp. nov., CRUB 1769^T; (d, $d_{(i)}$) *Cryptococcus tronadorensis* sp. nov., CRUB 1299^T; (e, $e_{(i)}$) *Cryptococcus fonsecae* sp. nov., EXF-4087^T; (f, $f_{(i)}$) *Cryptococcus frias* sp nov., CRUB 1250^T.

isolates: Two endophytic yeasts from *Populus euphractica* (China), one isolate from the flowers of *Helleborus foetidus* (Germany) and one from the Svalbard glacial environment (EXF-1596) (Fig. 2).

The newly proposed species *C. frias* is described based on two isolates from the glacial environments of Patagonia, Argentina. The most closely related strain was isolated from the glacial environments of the Italian Alps, with five nucleotide differences in the LSU. This species shows 7 and 12 differences in the LSU to *C. fonsecae* and *Cryptococcus* sp. 1, respectively (Fig. 2).

Cryptococcus fonsecae sp. nov. is described based on eight isolates: four from the Austral Sea (Patagonia), one from subglacial ice with gypsum inclusions (Svalbard) and a single strain from geographically diverse sea-water habitats: hypersaline saltern water on the Mediterranean coast in Slovenia (the present study), sea water in south Portugal (Gadanho *et al.*, 2003) and the San Juan Islands, Vancouver Island (Fraser *et al.*, 2006).

Table 3 summarises the differential phenotypic characteristics selected (assimilation tests) for the proposed new species and the related species described.

All the *Cryptococcus* strains isolated from cold environments showed the production of extracellular enzymes at low (5 °C) and moderate (20 °C) temperatures. Extracellular esterase and cellulase activities were more frequent (Table 4). Similar assimilation patterns were seen within each species, and some differences were seen among the different species. The most active species were *C. tronadorensis* sp. nov., *C. fonsecae* sp. nov. and *Cryptococcus*

Table 2. Assimilation profiles of the *Cryptococcus victoriae* strains

Species	Sor	Glm	Ino	Suc	Sta	Cit	Nit	Cre	SF
C. victoriae type group									
1									
CBS 8586^T	–	w	+	+	–	w	+*	+*	+
CRUB 1262	+	+	+	+	–	+	–	+	+
CRUB 1254	+	+	+	+	–	+	–	+	+
CRUB 1266	–	+	+	+	–	+	–	+	+
CRUB 1264	–	w	+	+	–	+	–	+	+
EXF-3832	w	+	–	w	–	+	–	+	+
EXF-3721	–	+	+	+	–	+	–	+	+
EXF-4085	–	+	+	+	–	+	–	+	+
EXF-3831	–	+	+	+	–	+	–	+	+
EXF-3748	–	+	+	+	–	+	–	+	+
2									
CBS 8915	v	+	w	+/s	+	w	–	–	+/w
CBS 8920	v	w	–	+/s	+	+	–	–	+/w
CRUB 1263	–	+	+	+	–	w	–	–	+
CRUB 1260	–	+	+	+	–	+	–	s	+
EXF-3912	+	w	+	+	–	+	–	s	+
CRUB 1757	+	w	+	+	–	+	–	–	+
C. victoriae non-type group									
EXF-4012	–	+	+	+	–	+	–	s	+
EXF-4020	–	+	+	+	–	+	–	–	+
EXF-4011	–	+	+	+	–	+	–	w	+
CBS 8908	v	w	w	+/s	+	+	–	–	+/w
EXF-3923	w	+	+	+	–	–	–	+	+

1 – Group of strains with no LSU (D1/D2 domain) rDNA nucleotide differences in comparison to the type strain.

2 – Group of strains with one nucleotide difference in comparison to the type strain.

Growth tests: C-sources: Sor, L-sorbose; Glm, glucosamine; Ino, *myo*-inositol; Suc, succinate; Sta, soluble starch; Cit, citrate; N-sources: Nit, nitrate; Cre, creatinine; SF: starch formation. –, negative; +, positive; s, slow; v, variable; w, weak.

*Results obtained from Montes *et al.* (1999).

sp. 2, where all the enzyme activities tested were seen at both of the temperatures tested (Table 4).

Discussion

Cryptocccus victoriae was first described by Montes *et al.* (1999) in association with soil from Southern Victoria Land in Antarctica. Soon after the publication of the original description, Thomas-Hall *et al.* (2002) described new isolates that differed morphologically and phylogenetically from the Antarctic type strain. The truly cosmopolitan distribution of this species in cold areas of the world became more apparent with successive isolations from extremely cold water-related environments, such as glacial ice from the Arctic (Butinar *et al.*, 2007) and from the Italian Alps (Turchetti *et al.*, 2008; Branda *et al.*, 2010). Its poly-extremotolerant character became further evident with its isolation from the acidic volcanic waters of the Río Agrio in Patagonia (Russo *et al.*, 2008). Although mainly found in cold terrestrial habitats, isolates of *C. victoriae* have also been obtained from a range of different habitats in temperate regions: soil and rhizosphere soil in Korea (Hong *et al.*, 2002), sea water in Portugal (Gadanho *et al.*, 2003), roots, rhizosphere and seeds of different plants in Germany and Austria (Renker *et al.*, 2004; Wuczkowski & Prillinger, 2004), the gut of the insect *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) in the USA (Woolfolk & Inglis, 2004), an industrial malting area and indoor air in Finland (Laitila *et al.*, 2006; Pitkäranta *et al.*, 2008) and a dry meat processing factory in Norway (Asefa *et al.*, 2009).

The presence of *C. victoriae* in aquatic environments outside the polar areas has been less frequently documented. In summary, *C. victoriae* is a species that inhabits very diverse environments and climatic zones, and that has the ability to adapt to a variety of environmental conditions. Thus, *C. victoriae* can be considered as a generalist species, which are typically characterised by the ability to tolerate a variety of stressful environments, but not the most extreme conditions.

These generalist species can adapt because of their so-called 'robust genotypes', which allow their persistence across varied environments without obligate adaptation to local conditions. This is achieved by structuring their

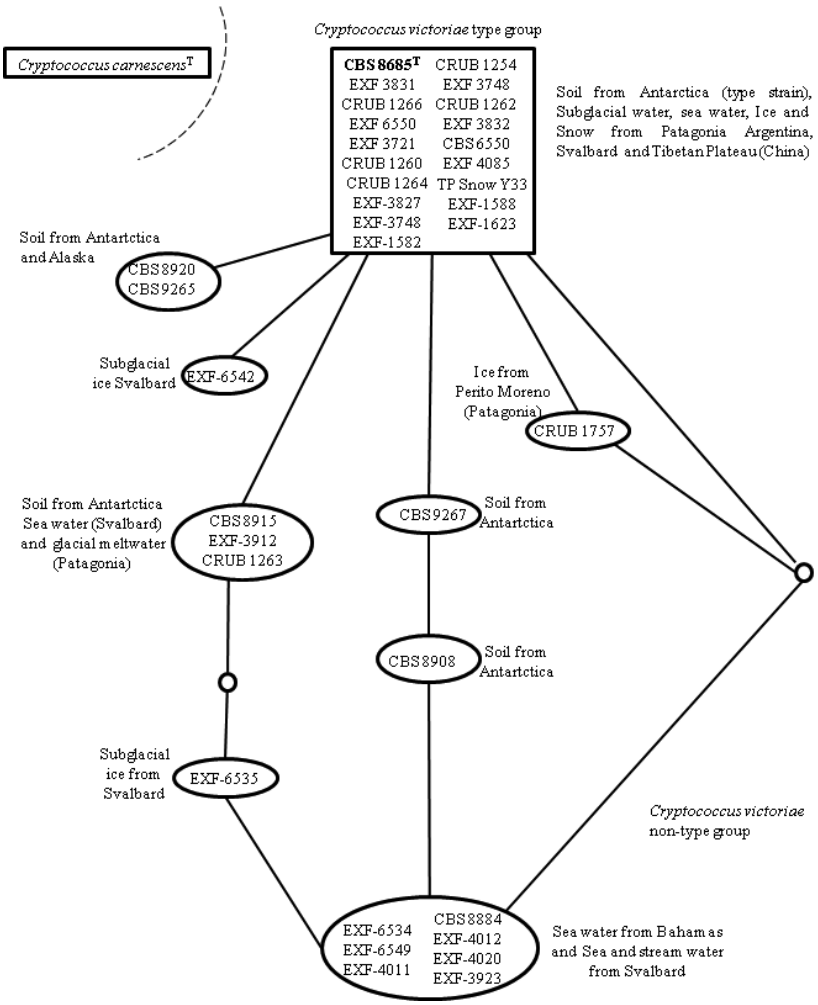

Fig. 4. Parsimony network analysis of the combined ITS and LSU rDNA gene D1/D2 domains of strains of *Cryptococcus victoriae* and its relatives. Each connecting line represents one substitution and each small circle represents a missing intermediate sequence. A rectangle identifies the sequence identified as ancestral by the analysis. The shaded area shows a subset of strains that differ from the type by three or fewer substitutions. The dashed line shows that *Cryptococcus carnescens*^{T} was excluded from the network.

populations into groups, and thus initiating their specialisation into specialist species (Gostincar *et al.*, 2010).

Parsimony network analysis provides a statistical, theory-laden approach to the delineation of phylogenetic yeast species from sequence data. This method aims to distinguish within sequence space, variations that can be regarded as polymorphisms (within species) from differences that are the consequences of speciation (Lachance *et al.*, 2010). The parsimony network analyses of the ITS, and the LSU rDNA sequences of the *C. victoriae* isolates occupying different ecological niches reinforces the concept that they are members of a single evolutionary lineage. The variability observed in the *C. victoriae* strains can be considered either an intrinsic characteristic of the species, or a possible initiation of speciation. The data presented in the present study support this statement, through showing the high plasticity of *C. victoriae* in terms of its physiology, morphology and molecular characteristics.

According to Fonseca *et al.* (2011), minor differences in the nucleotide sequences of LSU rDNA in comparison to the *C. victoriae* type strain do not suffice for the description of new species; therefore, other loci and phenotypic characteristics also have to be studied. Based on the dataset used in multilocus analysis in *C. neoformans* (*Tremellales*) (Findley *et al.*, 2009), analyses of the largest subunit of RNA polymerase II (*RPB1*), the second largest subunit of RNA polymerase II (*RPB2*) (degenerate primers were used), and elongation factor 1 alpha (*EF1*) were also planned for these *C. victoriae* isolates. However, no PCR products with published primers were obtained for the *C. victoriae* strains. In our experience, deeper analysis

Table 3. Selected phenotypic characteristics of the newly described *Cryptococcus* and related known species

Species	Mel	Sor	Eth	Ery	Glu	Glm	Sta	Man	Cre	Growth at 5 °C
C. cuniculi	–	s	+	–	nd	–	+	+	–	nd
C. *fonsecae* sp. nov.	+	–	–	–	–	w	–	–	+	+
C. *frias* sp. nov.	+	–	–	–	+	+	–	+	+	+
C. heveanensis	–	+	+	+	+	–	–	+	–	nd
C. laurentii	+	–/s	+/s	+	+	+	+	+	+	nd
C. nemorosus	+	+	+	+	+	+	+	+	–	nd
C. perniciosus	+	+	–	+	–	+	+	+	–	nd
C. *psychrotolerans* sp. nov.	+	–	–	+	+	w	–	+	+	+
C. tephrensis	+	+	–	+	+	+	+/s	+	–	nd
C. *tronadorensis* sp. nov.	w	+	+	–	+	w	–	+	–	+
***Cryptococcus* sp. 1 EXF-1596**	w	w	–	–	–	w	w	+	–	+
***Cryptococcus* sp. 2 EXF-3926**	+	w	–	–	–	–	–	–	–	+

Species in bold are isolates from this study.

Growth tests: C-sources: Mel, melibiose; Sor, L-sorbose; Eth, Ethanol; Ery, erythritol; Glu, glucitol; Glm, glucosamine; Sta, soluble starch; Man, mannitol; N-source: Cre, creatinine.

–, negative; +, positive; s, slow; w, weak; nd, not determined.

Table 4. Enzymatic profiles of the *Cryptococcus* species two different temperatures

	Esterase		Pectinase pH5		Pectinase pH7		Cellulase	
Species	5 °C	20 °C	5 °C	20 °C	5 °C	20 °C	5 °C	20 °C
C. victoriae type group	+	+/–	–	–	–	–	+/–	+/–
C. victoriae nontype group	+	+/–	–	–	–	–	+	+
C. aff. tephrensis	+	+	–	–	–	–	+	+
C. carnescens	+	+	–	–	–	–	+	+
C. foliiacea	+	+	–	–	–	–	–	+
C. psychrotolerans sp. nov	+	+	–	–	–	–	–	–
C. tronadorensis sp. nov.	+	+/–	+	+	+	+/–	+	+
C. fonsecae sp. nov	+	+	+	+	+/–	–	+	+
Cryptococcus sp. 1	+	+	–	–	–	–	+	+
Cryptococcus sp. 2	+	+	+	+	+	+	+	+
C. frias sp. nov.	+/–	+/–	–	–	–	–	+	+

Amylase and protease activities were negative for all the strains tested, at both temperatures. Values for 5 °C were obtained after 20 days of incubation, and values at 20 °C after 5 days of incubation.

–, negative; +, positive; w, weak.

and the construction of new primer sets are necessary for multilocus analysis of this species.

Cryptococcus tephrensis was initially isolated from soil in Iceland and described by Vishniac (2002), and later it was also isolated from soil in the Moscow region (Yurkov, 2006), and recently from glacial Alpine environments (Branda *et al.*, 2010). *Cryptococcus tephrensis* is closely related to *C. victoriae*, *C. foliicola* and *Cryptococcus peneanus* (Montes *et al.*, 1999; Takashima *et al.*, 2003). In the present study, four strains that deviate from *C. tephrensis* were isolated from the Arctic glacial environments. To date, all the reports of this species have originated exclusively from cold environments, indicating its psychrotolerant nature. Our data indicate that *C. tephrensis* and the related strains obtained in the present study (EXF-3749, EXF-3875, EXF-3999, EXF-6553) showed variability. To determine a potential complex of yet-unidentified species, additional studies of different molecular markers will be necessary, as well as additional information on their life cycle (e.g. sexual state); both these will be facilitated by the isolation of related species.

A new species *C. psychrotolerans* (Victoriae clade) that is related to *C. tephrensis* is described based on four isolates, two of which originated from glacier ice (EXF-1528, EXF-1583), and two from sea water (CRUB 1769, CBS 6578). Strain CBS 6578 was previously identified as *C. laurentii*, and it is included in the description of this new species. All the relevant information was obtained

from the online CBS page (http://www.cbs.knaw.nl/collections/BioloMICS.aspx) and from Takashima *et al.* (2003).

The description of the newly proposed species, *C. tronadorensis* (*Kwoniella* clade) from the glacial environments is based on the D1/D2 LSU rDNA sequences, which are closely related to other two strains: one isolated from Taiwan (*Cryptococcus* sp. F6; Wang and Yang, genebank, unpublished) and one of unknown provenance (*Cryptococcus* sp. BI226; Landell M, Ramos J, Leoncini O and Valente P GeneBank, unpublished data). Unfortunately, there is no information available on the origins of these strains; additional information and other isolates of this species are needed for a better understanding of its ecology and life cycle.

Based on the good bootstrap support of a new clade related to *C. laurentii* (*Bulleromyces* clade), the description of two new species isolated from the glacial and saline environments is performed. The new clade is composed of yet-undescribed taxa, the majority of which originate from cold and marine environments in the northern and southern hemispheres (*C. frias* sp. nov., *Cryptococcus* sp. DBVPG 5114, *C. fonsecae* sp. nov., *Cryptococcus* sp. 2 EXF-1569, *Cryptococcus* sp. 3 EXF-3926). Isolates phylogenetically related to *Cryptococcus* sp. 2, included in *C. laurentii* or *C. aff. laurentii*, were isolated from nectar of *H. foetidus* in Germany (CBS 9007; Herzberg *et al.*, 2002) and from *P. euphractica* along the Tarim River in China (ZA-12 and ZA-3, Abdurehim Z., GeneBank).

The state of the genus *Cryptococcus* represents probably one of the most complex taxonomic problems in yeast systematics (Wuczkowski *et al.*, 2011). New species descriptions within this taxonomic group are evidently needed, as exemplified by the new species related to *C. laurentii* described in the present study. As Shivaji & Prasad (2009) showed, *Cryptococcus* species have a ubiquitous presence in polar areas. Although *Cryptococcus* species have been reported in most yeast studies from Antarctica, the delineation of these species based on phenotypic characteristics might have been incorrect. Sequence analyses have resulted in the description of several additional new *Cryptococcus* species that were previously considered to be *C. laurentii.* Several reports have also indicated misidentification of *Cryptococcus flavescens* and *C. victoriae* (Fonseca *et al.*, 2011). As Fonseca *et al.* (2011) pointed out recently, in the last edition of 'The Yeasts', some species are not as common as previously considered and new ones can be identified, as included in the present study.

Cryptococcus strains isolated show heterotrophic metabolism and the ability to degrade organic macromolecules through the secretion of extracellular hydrolytic enzymes. This indicates high plasticity and their potential auxiliary role as biogeochemical nutrient recyclers in these environments. They have relatively broad amplitude of ecological tolerance, as they can survive acidic conditions, low temperatures and low nutrient concentrations. This 'phenotypic plasticity' is also characterised by the presence of a capsule, which confers stress tolerance in glacial biomes, as well as in the human body (Gostincar *et al.*, 2010).

The present study shows that not only extremely cold terrestrial environments but also extremely cold aquatic environments provide sources for new fungal taxa with distinct metabolic and potentially interesting biotechnological properties. Glacial biomes represent unique habitats and are generally unexplored reservoirs of unknown microbial species. The microbial biodiversity of these biomes is much higher than previously expected, at the level of both bacteria and eukaryotic species. As global warming is resulting in the melting of glaciers throughout the regions of the world, these cold ecosystems are in danger of disappearing (Thomas-Hall *et al.*, 2010). The isolation of these yeasts will allow the collection, discovery and description of new species before they are being released into the soil, rivers and oceans of the world.

Description of four new *Cryptococcus* species

These new species are anamorphic yeasts that are related to the subphylum *Agaromycotina*, class *Tremellomycetes*, order *Tremellales*, family *Tremalaceae*.

Cryptococcus psychrotolerans sp. nov. de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock.

Etymology: *C. psychrotolerans* (psy.chro.tol′er.ans. Gr. adj. *psychros* cold; L. pres. part. *tolerans* tolerating; N.L. part. adj. *psychrotolerans* cold-tolerating).

MycoBank: MB 800033

After 7 days on malt extract/yeast extract agar at 18 °C, the colonies are cream coloured, smooth and opaque, with an entire margin. The cells are ovoidal to ellipsoidal, 5.9–7.0 × 4.2–5.4 μm, and they multiply by multilateral budding. In Dalmau plates, after 2 weeks on cornmeal agar, pseudohyphae and true hyphae are not formed. No positive mating reactions are observed among the three strains isolated.

Glucose is not fermented. Glucose, D-galactose, D-glucosamine (weak), D-xylose, L-arabinose, D-arabinose, L-rhamnose, maltose, trehalose, cellobiose, arbutin, salicin, melibiose, lactose, raffinose, melezitose, inulin, meso-erytritol, xylitol, D-glucitol, D-mannitol, myo-inositol, glucono-δ-lactose, D-gluconate, D-glucuronate, D-galacturonate, succinate and lactate (weak) are assimilated. No growth occurs on L-sorbose, D-ribose, soluble starch, glycerol, ribitol, galactitol, citrate, methanol, ethanol, hexadecane and isopropanol. Assimilation of nitrogen compounds: posi-

tive for nitrite, l-lysine and creatinine. No growth is observed on nitrate. Growth in vitamin-free medium is positive. Growth in amino-acid-free medium is positive. Growth observed at 5–25 °C; no growth at 30 °C. No growth on YM agar with 10% sodium chloride. No growth observed in 50% glucose/yeast extract (0.5%). No growth in 100 μg mL^{-1} cycloheximide. Urease activity is positive. Diazonium Blue B reaction is positive.

Holotype: The type strain of *C. psychrotolerans* sp. nov. is CRUB 1769, which was recovered from sea water at the Cape Horn Meridian in the Argentinian Sea. The strain has been deposited in the Culture Collection of Extremophilic Fungi (EX), Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia, as EXF-7039^T, and in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as CBS XX.

Cryptococcus tronadorensis sp. nov. de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock.

Etymology: *C. tronadorensis* (N.L. masc. adj., *tronadorensis*, referring to Mount Tronador, the name of the mountain with the glacial meltwaters from which the strains of this species originated).

MycoBank: MB 800034

After 7 days on malt extract/yeast extract agar at 18 °C the colonies are cream coloured, smooth and opaque, with an entire margin. The cells are subglobose to ellipsoid, 6.2–4.7 × 3.4–3.1 μm, with a capsule, and they multiply by multilateral budding. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae and true hyphae are not formed. No positive mating reactions are observed among the two strains of *C. tronadorensis*.

Glucose is not fermented. Glucose, L-sorbose, sucrose, D-galactose, D-glucosamine (weak), D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, glucono-δ-lactose, salicin, maltose, trehalose, cellobiose, arbutin, melibiose (weak), lactose, raffinose (weak), melezitose, ribitol, glycerol (slow), xylitol, D-glucitol, D-mannitol, galactitol (weak), myo-inositol, D-glucuronate, succinate, ethanol are assimilated. No growth occurs on meso-erythritol, soluble starch, citrate, methanol, hexadecane and isopropanol. Assimilation of nitrogen compounds: positive for l-lysine, D-glucosamine and cadaverine. No growth is observed on creatine, creatinine, nitrite and nitrate. Growth in vitamin-free medium is positive. Growth in amino-acid-free medium is positive. Growth observed at 5 °C and is weak at 25 °C; no growth at 30 °C. Growth on YM agar with 10% sodium chloride is absent. Growth in 50% glucose/yeast extract (0.5%) is negative. Starch-like compounds are produced. In 100 μg mL^{-1} cycloheximide growth is absent. Urease activity is positive. Diazonium Blue B reaction is positive.

Holotype: the type strain of *C. tronadorensis* sp. nov. is CRUB 1299^T, which was isolated from the Rio Manso (Garganta del Diablo waterfall), which originates from the Manso glacier of Mount Tronador, Nahuel Huapi National Park, San Carlos de Bariloche, Río Negro, Argentina. The strain has been deposited in the Culture Collection of Extremophilic Fungi (EX), Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia, as strain EXF-6801^T, and in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as CBS XX.

Cryptococcus fonsecae sp. nov. de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock.

Etymology: *C. fonsecae* (fon.se′cae. L. gen. sing. m. adj. *fonsecae* of Fonseca, in honour of the Portuguese yeast researcher Alvaro Fonseca for his contributions to yeast systematics and ecology).

MycoBank: MB 800035

After 7 days on malt extract/ yeast extract agar at 18 °C, the colonies are light pink, smooth and opaque, with an entire margin. The cells are mainly globose, 5.7–4.9 × 5.6–4.5 μm, with capsule, and they multiply by multilateral budding. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae and true hyphae are not formed. No positive mating reactions are observed among the strains of *C. fonsecae.*

Glucose is not fermented. Glucose, sucrose, D-galactose, D-glucosamine (weak), D-xylose, L-rhamnose (weak), D-mannitol (slow), myo-inositol, salicin (weak), arbutin (weak), lactose, maltose, melibiose (weak), L-arabinose, trehalose, cellobiose, raffinose, melezitose, glycerol (slow), D-glucuronate (weak), xylitol are assimilated. No growth occurs on L-sorbose, D-ribose, D-arabinose, meso-erythritol, ribitol, D-glucitol, galactitol, glucono-δ-lactose, galacturonate, succinate, citrate, ethanol, soluble starch, methanol, hexadecane and isopropanol. Assimilation of nitrogen compounds: positive for nitrite, l-lysine, D-glucosamine, creatine (weak) and cadaverine. No growth is observed on nitrate and creatinine. Growth in vitamin-free medium is positive. Growth in amino-acid-free medium is positive. Growth observed at 5 °C, and is weak at 25 °C; no growth at 30 °C. Growth on YM agar with 10% sodium chloride is absent. Growth in 50% glucose/yeast extract (0.5%) is negative. Starch-like compounds are produced. In 100 μg mL^{-1} cycloheximide, growth is absent. Urease activity is positive. Diazonium Blue B reaction is positive.

Holotype: the type strain of *C. fonsecae* sp. nov. is EXF-4087^T, which was isolated from subglacial ice from Svalbard, from the Austre Lovénbreen glacier in Kongsfjorden, on the western coast of Spitsbergen.

Cryptococcus frias sp. nov. de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock.

Etymology: *C. frias* (N.L. masc. adj., *frias*, referring to Frias glacier, name of the glacier where the meltwater originated, as the source of this species).

MycoBank: MB 800036

After 7 days on malt extract/yeast extract agar at 18 °C, the colonies are yellow, smooth and opaque, with an entire margin. The cells are ovoidal to ellipsoidal, 6.9–5.6 × 3.8–3.4 μm, with a capsule, and they multiply by multilateral budding. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae and true hyphae are not formed. No positive mating reactions are observed among the two strains of *C. tronadorensis*.

Glucose is not fermented. Glucose, sucrose, D-galactose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, glucono-δ-lactose (weak), salicin (weak), maltose, trehalose, cellobiose, arbutin, melibiose, lactose, raffinose, melezitose, ribitol, glycerol (slow), D-glucuronate (weak), xylitol, D-glucitol, D-mannitol, galactitol (weak), myo-inositol, succinate are assimilated. No growth occurs on L-sorbose, meso-erythritol, soluble starch, citrate, ethanol, methanol, hexadecane and isopropanol. Assimilation of nitrogen compounds: positive for l-lysine, D-glucosamine, creatinine, creatine (weak) and cadaverine. No growth is observed on nitrite and nitrate. Growth in vitamin-free medium is positive. Growth in amino-acid-free medium is positive. Growth observed at 5 °C and is weak at 25 °C; no growth at 30 °C. Growth on YM agar with 10% sodium chloride is absent. Growth in 50% glucose/yeast extract (0.5%) is negative. Starch-like compounds are produced. In 100 μg mL^{-1} cycloheximide growth is absent. Urease activity is positive. Diazonium Blue B reaction is positive.

Holotype: The type strain of *C. frias* sp. nov. is CRUB 1250^T, which was isolated from the Frias River meltwaters that originate from the Frias glacier of Mount Tronador, Nahuel Huapi National Park, San Carlos de Bariloche, Río Negro, Argentina. The strain has been deposited in the Culture Collection of Extremophilic Fungi (EX), Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia as EXF-5992^T, and in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as CBS XX.

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Supporting Information

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

Fig. S1. Phylogenetic placement of species in *Victoriae* clade (*Tremellales*) obtained by neighbour joining (distance K2P method) of the LSU and ITS rDNA gene.

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