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ORIGINAL PAPER

Cryptococcus vaughanmartiniae sp. nov. and *Cryptococcus onofrii* sp. nov.: two new species isolated from worldwide cold environments

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Abstract Twenty yeast strains, representing a selection from a wider group of more than 60 isolates were isolated from cold environments worldwide (Antarctica, Iceland, Russia, USA, Italian and French Alps, Apennines). The strains were grouped based on their common morphological and physiological characteristics. A phylogeny based on D1/D2 ribosomal DNA sequences placed them in an intermediate position between *Cryptococcus saitoi* and *Cryptococcus friedmannii*; the ITS1 and ITS2 rDNA phylogeny demonstrated that these strains belong to two related but hitherto unknown species within the order Filobasidiales, *albidus* clade. These two novel species are described with the names *Cryptococcus vaughanmartiniae* (type strain DBVPG 4736^T) and *Cryptococcus onofrii* (type strain DBVPG 5303^T).

Keywords Novel *Cryptococcus* species · *Cryptococcus vaughanmartiniae* sp. nov · *Cryptococcus onofrii* sp. nov. · Psychrophilic yeasts · Antarctica · Iceland · Russia · Alps · Apennines · Cold-adapted biodiversity

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Introduction

Since the 1960s, cold environments have proven to be a reservoir for diverse metabolically active cold-adapted microbial communities (Abyzov 1993; Skidmore et al. 2000; Deming 2002). Both psychrophilic and psychrotolerant bacteria have been previously studied with eukaryotic populations being more recently investigated (Shivaji and Prasad 2009; Margesin and Miteva 2011; Buzzini et al. 2012; Buzzini and Margesin 2014). Viable yeast populations were isolated from worldwide cold regions (Arctic and Antarctic areas, South American, European and Himalayan glaciers, as well as the deep sea) and from different types of substrates (permafrost soil, rocks and debris, ice cores and melt waters, cryoconite holes, sea water, glacial sediments, snow, saline lakes) (Brunati et al. 2009; Shivaji and Prasad 2009; Margesin and Miteva 2011; Buzzini et al. 2012; Connell et al. 2014; de García et al. 2014; Nagano et al. 2014; Selbmann et al. 2014; Turchetti et al. 2014; Zalar and Gunde-Cimerman 2014). In addition, the ecology of yeast populations sharing cold habitats has recently been investigated (Turchetti et al. 2013, 2014).

Basidiomycota represent hitherto over 80 % of psychrophilic and psychrotolerant yeasts isolated from worldwide cold environments and among them about one-third belong to the genus *Cryptococcus* (Buzzini et al. 2012). The well-known ability of *Cryptococcus* to produce an extracellular polysaccharide capsule (Vishniac 2006a; Selbmann et al. 2014) and its superior ability to assimilate carbon and nitrogen sources (Connel et al. 2008) are among the features that facilitate its apparent dominance in culturable yeast populations within cold ecosystems.

Cryptococcus is a polyphyletic genus and *Cryptococcus* species, including the ones isolated from cold habitats, currently pool in all the five major lineages within the class

Tremellomycetes, where they are sometimes grouped together with species of other anamorphic (e.g., *Bullera*) or teleomorphic (e.g., *Tremella*) genera (Fonseca et al. 2011).

In recent years, a number of new *Cryptococcus* species isolated from cold environments have been described including *Cryptococcus spencermartinsiae* García, Brizzio, Boekhout, Theelen, Libkind & van Broock, *Cryptococcus tronadorensis* de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock and *Cryptococcus frias* de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock from glacial melt waters of Argentinean glaciers (de Garcia et al. 2010, 2012); *Cryptococcus stepposus* Golubev & J.P. Samp. from a Russian grassland natural reserve (Prioksko-terrasny) (Golubev et al. 2006); *Cryptococcus fonsecae* de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock and *Cryptococcus psychrotolerans* de Garcia, Zalar, Brizzio, Gunde-Cimerman & van Broock from ice of Norwegian glaciers and sea water of Cape Horn Meridian (Argentina) (de García et al. 2012) and *Cryptococcus fildesensis* Zhang & Yu from moss sampled in maritime Antarctica (Zhang et al. 2014).

Relatively recently, some scientific expeditions aimed at studying cold-adapted yeast populations in cold environments worldwide (ice, snow, soil and melting water from Antarctica, Iceland, Russia, USA, Italian and French Alps, Apennines) have been conducted (Vishniac 2006b; Turchetti et al. 2008, 2013; Branda et al. 2010; Arenz and Blanchette 2011; Arenz et al. 2011). These efforts led to the isolation of a few hundred new yeast cultures. Among these strains isolated from different geographical regions, a few exhibited similar physiological profiles and sequences of the D1/D2 domains of the large subunit (LSU) and internal transcribed spacer (ITS) region of the rRNA gene. Analysis of the ITS presented an 9-bp long insertion and showed consistent differences when compared with the type strains of closely related species. Phylogenetic analysis confirmed the placement of the strains in two separate clusters. Consequently each cluster represent an undescribed *Cryptococcus* species. In this paper, *Cryptococcus vaughanmartiniae* and *Cryptococcus onofrii* have been described.

Materials and methods

Yeasts isolation

The 20 yeast strains analysed in this study (Table 1) are a representative selection from a wider group of more than 60 isolates sharing some common morphological, physiological and genetic features. The selection was made in order to have a broad variety of isolates from different substrates and geographical origins (Table 1).

Ten strains were isolated from superficial debris, ice, snow and melting water collected during sampling campaigns carried out from 2004 to 2010 in Italian and French Alpine glaciers (Turchetti et al. 2008, 2013). One isolate was obtained from deep-piping sediments of Calderone glacier, Apennines, Italy (Branda et al. 2010). Three strains were isolated from soil and moss collected from Kay Island, Victoria Land, Antarctica as well as from soil at Hope Bay and Anvers Island in the Antarctic Peninsula; they were part of two distinct investigations focused on studying eukaryotic microbial diversity of some Antarctic habitats (Selbmann et al. 2014; Arenz and Blanchette 2011). The remaining 6 strains were isolated from soil of different geographical areas; in particular the strains selected for this study were isolated from cold habitats of Alaska and Colorado in the United States as well as Russia and Iceland (Vishniac 2006b).

All cultures are preserved in the Industrial Yeasts Collection DBVPG of the University of Perugia (http://www.dbvpg.unipg.it), Italy, as lyophilized (4 °C) and long-term cryopreserved (−80 °C) cultures. Working cultures have been maintained on YEPG (Yeast Extract 10 %, Peptone 10 %, Glucose 20 %, agar 2 %) medium.

Physiological tests and morphology

Physiological and biochemical tests were performed according to the protocols described by Kurtzman et al. (2011) using the following media: Malt Extract broth (ME, Oxoid), Malt Extract Agar (MEA, Oxoid), Potato Dextrose Agar (PDA, Oxoid), and Corn Meal Agar (CMA, Difco). All tests were done in duplicate at 25 °C and results were recorded at 2 and 4 weeks after inoculation. No discrepant results were obtained in duplicate experiments. Images of colony morphology were taken using a stereo-microscope and were photographed directly with a Nikon zoom digital camera, COOLPIX950. Standard light microscopy and scanning electron microscopy (SEM) were employed using a Nikon Microphot-FX microscope and a Philips Electron Optics-FEI Company, model XL30, respectively. A BAL-TEC apparatus CPD 030 (BalTec, Pfäffikon, CH), using liquid CO_2 for critical-point drying, was used for sample fixation. The ability to secrete extracellular hydrolytic enzymes (amylase, protease, lipase and esterase) was evaluated at 4 and 25 °C according to previously described procedures (Buzzini and Martini 2002; Brizzio et al. 2007; de García et al. 2007).

Phylogenetic analysis

The D1/D2 domains of the large subunit (LSU) ribosomal gene region and the Internal Transcribed Spacer region (ITS1 and 2) including the 5.8S rRNA gene were amplified

Table 1 List of the yeast strains of *Cr. vaughanmartiniae* and *Cr. onofrii* considered in the present study: strain accession numbers, substrate and locality of isolation, GenBank accession numbers of the D1/D2 rRNA gene and those of the ITS rDNA sequences, http://www.ncbi.nlm.nih.gov

Species	Strain numbers		Isolation source	Locality	GenBank accession numbers	
					LSU	ITS1 and 2
Cryptococcus vaughanmartiniae GROUP A	DBVPG 4736^T	CBS 13731^T	Superficial sediments	Sforzellina glacier, Ortles-Cevedale complex, Alps, Italy	KF861779	KF861792
	DBVPG 4804		Ice cores	Sforzellina glacier, Ortles-Cevedale complex, Alps, Italy	EF643744	KF861793
	DBVPG 5146		Deep-piping sediments	Calderone glacier, Apennines, Italy	GQ911503	KF861794
	DBVPG 5279		Superficial sediments	Miage glacier, Mount Blanc, Alps, Italy	KF861780	KF861795
	DBVPG 5325		Superficial sediments	Miage glacier, Mount Blanc, Alps, Italy	KC433840	KC455904
	DBVPG 5412		Ice cores	Miage glacier, Mount Blanc, Alps, Italy	KF861781	KF861796
	DBVPG 5506		Superficial sediments	Miage glacier, Mount Blanc, Alps, Italy	KF861782	KF861797
	DBVPG 5721		Snow	glacier du Geant, Mount Blanc, Alps, France	KC433783	KC455888
	DBVPG 5728		Snow	glacier du Geant, Mount Blanc, Alps, France	KF861783	KF861798
	DBVPG 5862	CCFEE 5425	Soil under moss	Kay Island, Victoria Land, Antarctica	KF861785	KF861800
	DBVPG 7758	CBS 9231	Soil	Ring Road to a branch of Vatnajokull, Iceland	KF861786	KF861801
	DBVPG 7765		Fine sandy soil	Gothafoss, Iceland	KF861787	KF861802
	DBVPG 7818	CBS 9271	Soil	Nome, Alaska, USA	KF861788	KF861803
	DBVPG 7855	CBS 9295	Soil	Providenya, Russian Far East, Russia	KF861789	KF861804
	DBVPG 10101		Soil	Anvers Island, Antarctic Peninsula	KM077483	HM589283
	DBVPG 10102		Soil	Hope Bay, Antarctic Peninsula	KM077484	FJ236008
Cryptococcus onofrii GROUP B	DBVPG 5303^T	CBS 13732^T	Melting water	Miage glacier, Mount Blanc, Alps, Italy	KC433831	KC455900
	DBVPG 5737		Snow	Glacier du Geant, Mount Blanc, Alps, France	KF861784	KF861799
	DBVPG 7957	CBS 9622	High mountain soil	Krumholtz, Colorado, USA	KF861790	KF861805
	DBVPG 7967	CBS 9632	High mountain soil	Horse Shoe Ridge, Colorado, USA	KF861791	KF861806

DBVPG Industrial Yeast Collection, University of Perugia, Italy; CBS Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CCFEE Culture Collection of Fungi from Extreme Environments, University of Tuscia, Viterbo, Italy; T type strain

using standard primers (NL1 and RLR3R for LSU; ITS1 and ITS2 for ITS regions) (Thomas-Hall et al. 2010). For amplification, the T personal Combi Thermal Cycler (Biometras Gmbh, Goettingen, Germany) was employed, using amplification protocols reported by Selbmann et al. (2014). Sequences were obtained with an ABI PRISM 3730XL Capillary Sequencer using the standard protocol recommended by the manufacturer. ITS and D1/D2 sequences were blasted in the NCBI database. The most similar ITS and D1/D2 sequences were exported and aligned iteratively with MUSCLE (Thompson et al. 1997). Phylogenetic analysis was performed using Molecular

Evolutionary Genetics Analysis (MEGA) Software Version 4.0 (Tamura et al. 2007) using maximum parsimony analysis (D1/D2 phylogenetic analysis not shown). Bootstrap analysis (1000 replicates) was performed using a parsimonious heuristic search with random addition of sequences (100 replicates).

Results

Twenty yeast strains isolated during different sampling campaigns carried out in many cold environments (Table 1) were selected for their common morphological, physiological and genetic features. The cell shape was invariably globular with polar budding and extracellular starch-like polysaccharides (Figs. 1c, d, 2c, d). Sexual reproduction was not observed. All the strains synthesized extracellular starch-like polysaccharides and gave positive results to starch, Diazonum Blue B (DBB) and urea tests (Table 2).

From a molecular point of view, all the strains showed 100 or 99 % similarity in D1/D2 sequences (with only 1 bp of difference) with *Cryptococcus friedmannii* CBS 1975^T and *Cryptococcus saitoi* CBS 7160^T, respectively. Both species are members of the *albidus* clade of the order Filobasidiales (Fonseca et al. 2011). Even if *Cr. friedmannii* and *Cr. saitoi* showed a significant physiological divergence, they differ by only one nucleotide substitution in D1/D2 (the fifteenth nucleotide subsequent to the sequence of the primer NL1) and by five in ITS sequences (Fonseca et al. 2011). In the last few years, several strains of these two species were identified solely considering differences in D1/D2 sequences or even D2 only, leading sometimes to an ambiguous identification; accordingly, some strains were tentatively reported as *Cr. friedmannii/saitoi* (Vishniac 2006b; Fonseca et al. 2011).

The alignment of the polymorphic ITS1 and ITS2 regions of the species belonging to the *albidus* clade (Figs. 3, 4), showed that *Cr. friedmannii* and *Cr. saitoi* contain a 9-bp long insertion in the first part of the sequence (from the 122th nucleotide to the 130th after the primer NL1) that is absent in any other closely related species (Fig. 4). Surprisingly, not all the strains herein studied and selected for their similarity in D1/D2 to *Cr. friedmannii* and *Cr. saitoi* showed the same insertion. In the ITS phylogenetic tree, the strains studied were divided in 2 groups (Fig. 3).

The first (Group A) contained 16 strains with 100 and 99 % similarity in D1/D2 with *Cr. friedmannii* and *Cr. saitoi* but 96 and 97 % in ITS, respectively (Table 1). In particular these strains did not show the 9-bp long insertion described above but they exhibited a deletion of three nucleotides in the same site (Fig. 4) and an additional three and six mismatches when compared with the type strains of *Cr. friedmannii* and *Cr. saitoi* respectively. All 16 strains belonging to Group A (Table 1) had the same ITS sequences with the sole exception of strain DBVPG 5146 where the deletion was one nucleotide longer (108th nucleotide) with an extra mismatch (Fig. 4).

Group B included 4 strains (Table 1) with 100 and 99 % similarity in D1/D2 with *Cr. friedmannii* and *Cr. saitoi* but 99 and 98 % in ITS respectively (corresponding to 5 and 8 substitutions). The strains of this group showed the 9-bp long insertion present also in *Cr. friedmannii* and *Cr. saitoi* even if one mismatch was present (Fig. 4).

Based on the above considerations, the species of the *albidus* clade could be divided into two sets: species with the insertion (*Cr. friedmannii*, *Cr. saitoi* and the four strains clustered in Group B) and species without insertion (all the other species in *albidus* clade and the 16 strains clustered in Group A). The phylogenetic analysis of ITS sequences confirmed the relation between *Cr. friedmannii* and *Cr. saitoi* and clearly split the strains herein studied into two groups (Group A and Group B): all of them are included inside a well-defined cluster falling within *albidus* clade, supported by high bootstrap values (Fig. 3).

Physiological characteristics of the studied strains are presented in Table 2. Strains belonging to Group A differed from strains of Group B in their abilities to assimilate ribose, lactose, raffinose, glycerol, ribitol, myo-inositol and glucono d-lactone; additionally, although all the strains exhibited the capability to synthesize extracellular starch-like polysaccharides, the production of capsule was observed only in strains of Group B.

Even if the strains of Group A and B demonstrated closer phylogenetic relation with *Cr. friedmannii* than *Cr. saitoi*, from a physiological viewpoint they both exhibited higher similarity with *Cr. saitoi*. For example, *Cr. friedmannii* did not assimilate galactose, ribose, arabinose, rhamnose, lactose, glycerol, xylitol, glucitol and mannitol while strains belonging to Group A and B showed variable results depending on the strain. On the contrary *Cr. saitoi* differed from Group A and B strains only in its ability to assimilate arabinose, citrate and malic acid (Table 2).

Strains of both Group A and B grew easily at 25 °C but not at 30 °C, as already observed for *Cr. saitoi* and *Cr. friedmannii* (Turchetti et al. 2008; Fonseca et al. 2011; Selbmann et al. 2014) and they showed a slower growth at 4 °C, demonstrating a psychrotolerant nature. Indeed, *Cr. friedmannii* was first described by Vishniac (1985) as unable to grow at 25 °C, but more recent studies reported that some strains belonging to the same species exhibited growth at 25 °C (Turchetti et al. 2008; Selbmann et al. 2014).

All studied strains were unable to grow under increased osmotic pressure (50 % glucose) or increased NaCl concentrations (5 % glucose + 10 % NaCl) (Table 2). The strains showed comparable abilities to secrete extracellular enzymes

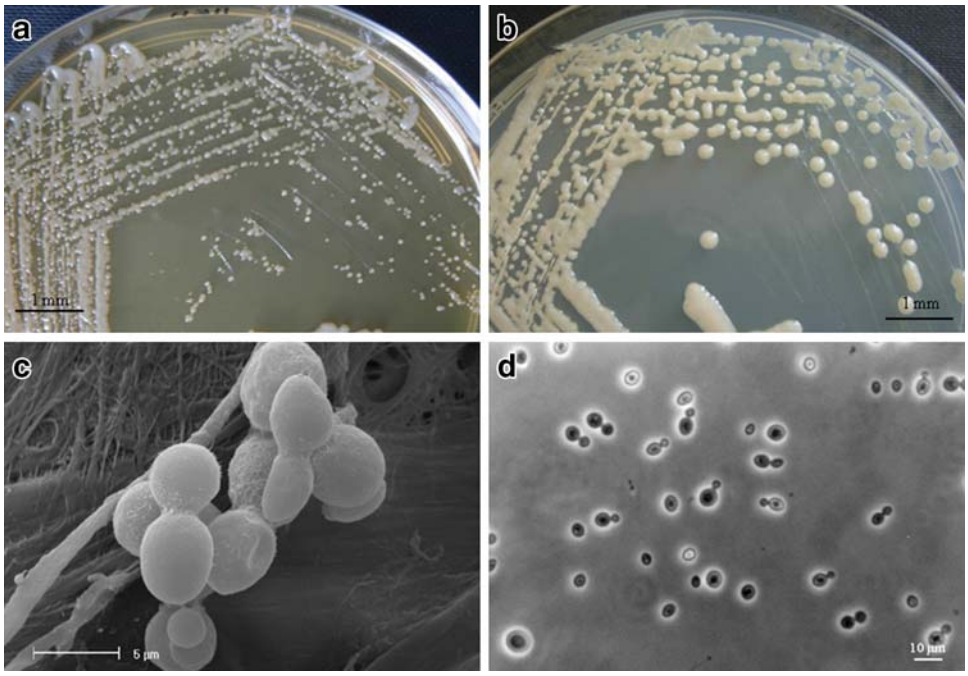

Fig. 1 *Cryptococcus vaughanmartiniae* (type strain DBVPG 4736). **a** Colonies on MEA after 3 days incubation at 25 °C: colonies circular, smooth, glistening, flat, with entire margins, *white coloured*. **b** Colonies on PDA after 5 days incubation at 25 °C: colonies wide, circular, with entire margins, *white-yellowish coloured*. **c** Scanning electron micrograph (SEM) of cells in ME broth at 25 °C after 3 days: cells globose to subglobose, with extracellular starch-like polysaccharides and polar budding occurring in parent-bud pairs and in short chains of 3 cells. **d** Polar budding cells after 3 days incubation on YEPG at 25 °C

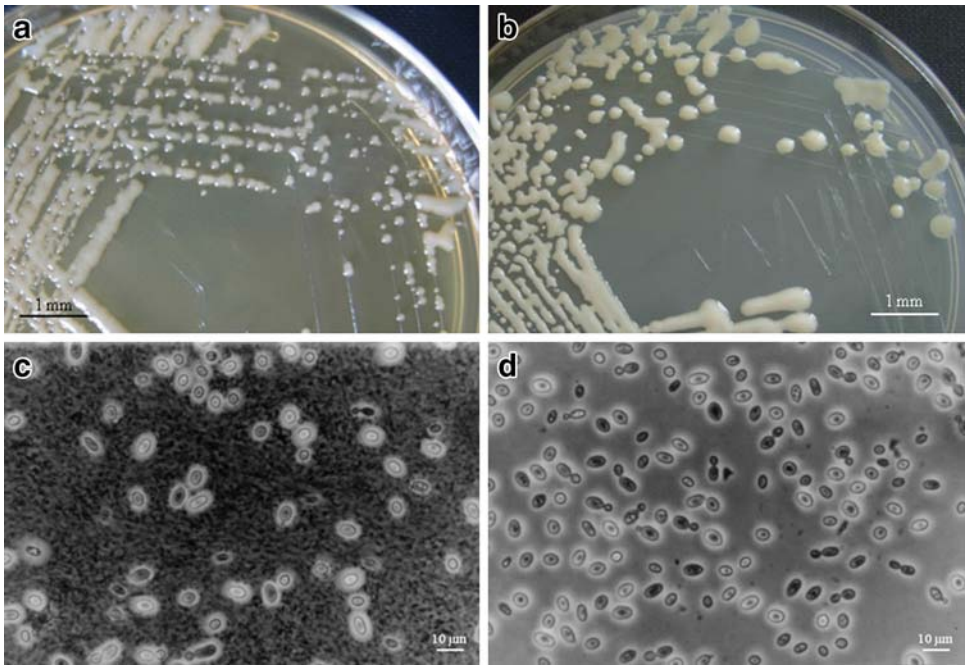

Fig. 2 *Cryptococcus onofrii* (type strain DBVPG 5303). **a** Colonies on MEA after 3 days incubation at 25 °C: colonies circular, smooth, glistering, flat, with entire margins, *white coloured*, with very mucoid texture close to being liquid. **b** Colonies on PDA after 5 days incubation at 25 °C: colonies wide, *white-yellowish coloured*, with dull surface and butyrous texture. **c** Cells on MEA after 10 days incubation at 25 °C after ink coloration, abundant capsules are visible. **d** Polar budding cells after 3 days incubation on YPD at 25 °C: cells are mainly ellipsoidal

at 4 °C as well as 25 °C: they hydrolyzed two lipophilic substrates (Tween 80 and tributyrin) while the ability to degrade starch and proteins was not apparently expressed.

When considering ITS1 and ITS2 sequences, the strains herein studied clustered into two different anamorphic clades that we initially named Group A and Group B. Based on the evidence provided in the present study, we here describe the 16 and 4 isolated strains belonging to Group A and B, respectively, as two novel yeast species for which the names *Cryptococcus vaughanmartiniae* (MycoBank No. MB809276) and *Cryptococcus onofrii* (MycoBank No. MB809277) are proposed.

Discussion

A large part of the Earth's environment (Arctic and Antarctic snow and ice caps, permafrost soils, high mountain

Table 2 Physiological characteristics of the species *Cr. vaughanmartiniae* and *Cr. onofrii*

	Cryptococcus vaughanmartiniae[a]	*Cryptococcus onofrii*[a]	*Cryptococcus saitoi*[b]	*Cryptococcus friedmannii*[b]
Assimilation of carbon sources				
D-Glucose	+	+	+	+
D-Galactose	+	+	−/w	−
L-sorbose	v	v	−	−
D-Ribose	+	v	v	−
D-Xylose	+	+	+	+
L-Arabinose	+	+	+	+
D-Arabinose	+	+	−	−
L-Rhamnose	+	+	+	−
Sucrose	+	+	+	s
Maltose	+	+	+	+
α,α trehalose	+	+	+	+
Methyl α-glucoside	+	+	+	s
Cellobiose	+	+	+	+
Salicin	+	+	+	+
Arbutin	+	+	n	n
Melibiose	−	−	−	−
Lactose	v	+	v	−
Raffinose	−/w	+	+/w	−
Melezitose	+	+	+	+
Glycerol	v	+	−/s	−
Meso erythritol	−	−	−	−
Ribitol	−	v	−	−
Xylitol	+	+	−/s	−
D-glucitol	+	+	+	−
Mannitol	+	+	+	−
Galactitol	−	−	−	−
Myo-inositol	v	+	+	−
Glucono D-lactone	−	v	n	n
D-Gluconate	+	+	+	s
D-Glucuronate	+	+	+	+
D-Galacturonate	−	−	n	n
DL-Lactate	−	−	−	−
Succinate	v	v	+	−
Citrate	−	−	+	s
Methanol	−	−	−	−
Ethanol	+	+	+	−
L-Malic acid	−	−	+	s
Hexadecane	−	−	n	n
N-acetyl D-glucosamine	−	−	n	n
Ethyl acetate	+	+	n	n
Assimilation of nitrogen sources				
Nitrate	+	+	+	+
Ethylamine	+	+	+	−
L-Lysine	+	+	+	+
Cadaverine	+	+	+	+
Fermentation of				
Glucose	−	−	−	−

Table 2 continued

	Cryptococcus vaughanmartiniae[a]	*Cryptococcus onofrii*[a]	*Cryptococcus saitoi*[b]	*Cryptococcus friedmannii*[b]
Growth on				
0.01 % cycloheximide	−	−	−	−
50 % glucose	−	−	n	n
5 % glu + 10 % NaCl	−	−	n	n
Other tests				
Starch production	+	+	+	+
Urea test	+	+	+	+
DBB reaction	+	+	+	+
Growth at				
4 °C	+	+	n	n
10 °C	+	+	n	n
15 °C	+	+	n	n
20 °C	+	+	n	+
25 °C	+	+	+	+[c]
30 °C	−	−	+	−[c]
35 °C	n	n	−	n

[a] Data from the present study

[b] Data obtained from Fonseca et al. (2011)

[c] Data obtained from Turchetti et al. (2008) and Selbmann et al. (2014)

glaciers, sea ice, deep oceans, etc.) is characterized by average temperatures below 5 °C (Buzzini and Margesin 2014). Current literature reports that cold habitats regularly harbour assorted cold-adapted microbial communities (including yeasts), whose biodiversity is still not fully investigated: it has been estimated that 15–40 % of total culturable yeasts isolated from these ecosystems may belong to novel species (Butinar et al. 2007; de García et al. 2007; Connel et al. 2008; Turchetti et al. 2008; Branda et al. 2010; Brandão et al. 2011; Butinar et al. 2011, Selbmann et al. 2014). All strains considered in this study were isolated from environments located in different areas of the globe, but all characterized by low average temperature. Strains of both *Cr. vaughanmartiniae* and *Cr. onofrii* herein studied (Table 1) exhibited the status of psychrotolerant organisms because of their ability to grow and express enzymatic activities both at 4 and 25 °C. This behaviour is not particularly surprising because their native habitats are currently characterized by seasonal (or even in some cases daily) thermal variations. The psychrotolerance exhibited by both species may be regarded as a potential competitive advantage towards obligate psychrophilic yeasts and could justify their apparent cosmopolitan geographical distribution (Buzzini and Margesin 2014). Accordingly, although all strains presented in this study have been isolated from cold habitats, their ability to grow at 25 °C does not exclude their possible future isolation from moderately cold (or even temperate) ecosystems. The ability of *Cr. vaughanmartiniae* and *Cr. onofrii* to assimilate a wide range of carbon and nitrogen sources (Table 2) may corroborate this hypothesis: in fact, this may represent an advantage for their adaptation to mesophilic ecosystems, where competition among microorganisms could be significantly higher than that supposedly observed in cold environments (Gostinčar et al. 2010). In particular, their aptitude to assimilate some compounds common in some natural habitats (e.g. mono- and oligosaccharides, namely xylose and cellobiose; glycosylated hydroxyl-phenols, like salicin and arbutin; nitrogen compounds, namely nitrates) could suggest that the ability of such yeasts to colonize a variety of ecosystems cannot be a priori excluded. On the other hand, their inability to survive under high osmotic pressure or halophilic conditions could represent a limitation in colonizing some extreme niches.

It has been suggested that extreme conditions (e.g. low temperatures) may lead to an increase of selective pressure for inhabiting stressful environments. In such conditions, generalist species can be considered to have a reservoir of characteristics useful for the evolution of extremophiles (Gostinčar et al. 2010). Microbial populations colonizing ecologically extreme environments can be subjected to multiple stresses (e.g. low temperature, low water activity, oligotrophy, etc.) that can select for increased mutation rates, which may be considered a fundamental trend promoting microbial adaptation to new conditions (Heidenreich et al. 2003; Stoycheva et al. 2007; Stamenova et al. 2008). Genetic variations occurring in microbial genomes can remain sometimes cryptic and not detectable by regular

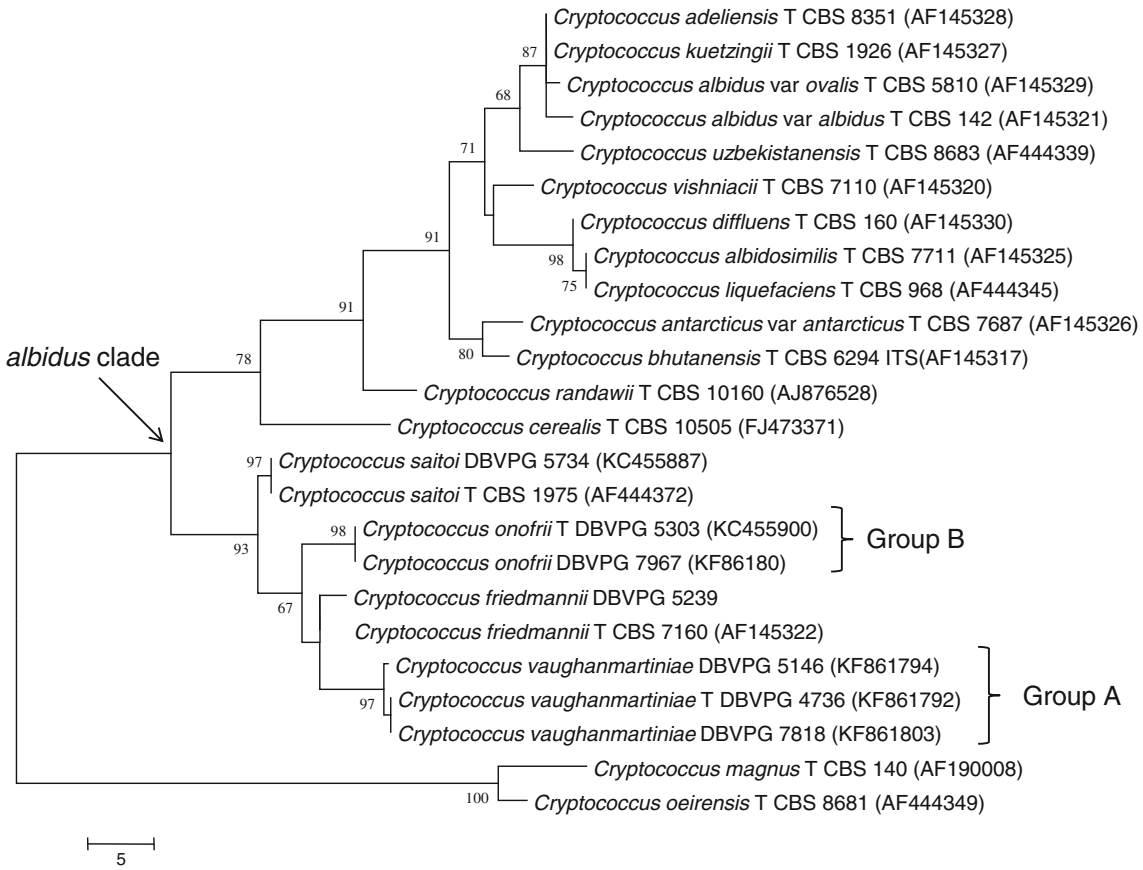

Fig. 3 ITS phylogenetic tree of *Cr. vaughanmartiniae*, *Cr. onofrii* and related taxa of *albidus* clade (showed only in part), class Tremellomycetes, order Filobasidiales. The tree was rooted with *Cr. magnus* and *Cr. oeirensis* type strains. Bootstrap percentages from 1000 replications are shown on the branches (values below 50 % are not shown). GenBank accession numbers of the sequences are indicated after strain numbers

Fig. 4 Partial alignment of ITS sequences of strains considered for the phylogenetic analysis of Fig. 1. The fragment considered shows the insertion and the gaps characterizing *Cr. vaughanmartiniae* and *Cr. onofrii* inside *albidus* clade

sequence analysis of typical marker genes (Schlichting 2008). Strains belonging to *Cr. saitoi*, *Cr. friedmannii* and *Cr. onofrii* showed a 9-bp long insertion and a gap of 4 bp in the polymorphic ITS1 and ITS2 regions of rDNA (including the 5.8S rRNA gene) if compared with the most phylogenetically closest species (Fig. 4). On the other hand, *Cr. vaughanmartiniae* strains exhibit the same gap, but not the 9-bp long insertion (Fig. 4). Based on these observations and in close conformity with the above reported consideration, we can speculate that such variations observed in a well-known genetic region (ITS1 and 2) suggests the presence of additional DNA mutations in taxonomically related species as a consequence of adaptation to low temperatures. The possible physiological and ecological significance of such genetic variations observed in *Cr. vaughanmartiniae* and *Cr. onofrii* still remains an open question. The description of future additional novel *Cryptococcus* species that are related to *Cr. vaughanmartiniae* and *Cr. onofrii* could give additional insights on the significance of such molecular differences, mainly in terms of elucidation of specific physiological and ecological features.

Taxonomic considerations

Cryptococcus vaughanmartiniae Turchetti, Blanchette and Arenz sp. nov

Cryptococcus vaughanmartiniae (vau.ghan'mar.ti.niae N.L. gen. sing. fem. n. referring to Ann Vaughan-Martini, a yeast biologist at the University of Perugia, Italy, past curator and co-founder of the DBVPG Collection, in whose honour the species is named).

Novel yeast species belonging to phylum Basidiomycota, class Tremellomycetes, order Filobasidiales.

The type strain of *Cryptococcus vaughanmartiniae* has been deposited at the Industrial Yeasts Collection DBVPG (Department of Agricultural, Food and Environmental Science) University of Perugia, Italy, and at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, under the codes DBVPG 4736^{T} and CBS 13731^{T}, respectively.

MycoBank No: MB809276.

Gene sequence accession numbers of the type strain: D1/D2 LSU rRNA = KF861779, ITS = KF861792.

The sixteen strains reported in the present paper were all isolated from cold habitats located in Italian Alps and Apennines, Antarctica, Iceland, Alaska and Russia (Table 1).

The physiological and biochemical characteristics of *Cr. vaughanmartiniae* are indicated in Table 2 and its phylogenetic placement is presented in Fig. 3.

Morphology

Cr. vaughanmartiniae strains at 25 °C showed standard growth on all the media tested, with smaller colonies on CMA. After 3 days colonies on MEA were circular, smooth and glistering, flat with entire margins, white coloured with mucoid texture (Fig. 1). Growth on PDA was more abundant and white-yellowish coloured.

After 2 weeks at 25 °C on MEA and PDA, colonies reached 4–5 mm in diameter, showed a raised profile in the central part and slightly irregular margins. Some strains (DBVPG 4736, 5279, 5325, 5728, 5721, 5146) showed less mucoid texture and dull surface, other strains maintained a more fluid consistency. On CMA, colonies were always small (1 mm diam), flat, white and glistering.

Microscopy

Growth in ME broth at 25 °C after 3 days resulted in the formation of mucoid sediment. After 7 days, a light superficial ring was present in all the strains considered. In YEPG and ME after 4 weeks at 25 °C, compact and wrinkled superficial pellicle was developed by DBVPG 5506, 7765, 7855 and 5721. After 3 days on MEA, cells were globose to subglobose, measuring 0.5–1 × 1–3 μm. Wider cells occurred on PDA, measuring 1–1.5 × 1–4 μm. Budding was polar and the cells occurred singly, in parent-bud pairs and in a few cases also in short chains of 3 cells (Fig. 1) showing globose shape. No hyphae and pseudohyphae were observed on any media.

Cryptococcus onofrii Turchetti, Selbmann & Zucconi sp. nov

Cryptococcus onofrii (o.no'fri.i, N.L. gen. sing. masc. n. referring to Silvano Onofri, a mycologist at the University of Tuscia, Viterbo, Italy, in whose honour the species is named).

Novel yeast species belonging to the phylum Basidiomycota, class Tremellomycetes, order Filobasidiales.

The type strain of *Cryptococcus onofrii* has been deposited at the Industrial Yeasts Collection DBVPG (Department of Agricultural, Food and Environmental Science) University of Perugia, Italy, and at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, under the codes DBVPG 5303^{T} and CBS 13732^{T}, respectively.

MycoBank No: MB809277.

Gene sequence accession numbers of the type strain: D1/D2 LSU rRNA = KC4338319, ITS = KC455900.

Four strains were reported in the present paper all isolated from cold habitats located in Alps (Mount Blanc) and Colorado (USA) (Table 1).

The physiological and biochemical characteristics of *Cr. onofrii* are indicated in Table 2 and its phylogenetic placement is showed in Fig. 3.

Morphology

Cr. onofrii strains at 25 °C showed standard growth on all the media tested, with smaller colonies on CMA. After 3 days, colonies on MEA were circular, smooth and glistering, flat with entire margins, white coloured with very mucoid texture close to being liquid (Fig. 2). Growth on PDA was more abundant and after 7 days DBVPG 7957 showed a dull surface, butyrous texture and white-yellowish colour.

After 2 weeks at 25 °C on MEA and PDA, colonies reached 7 mm in diameter and showed raised profile in the central part and slightly undulated margins. On CMA, the colonies were always smaller and flat, very liquid and glistening.

Microscopy

Growth in ME broth at 25 °C after 3 days resulted in the formation of mucoid sediment and superficial ring. After 7 days, a superficial ring was present in all the strains grown in ME and YEPG.

After 3 days on MEA cells were ellipsoidal, measuring 1–2 × 2–3 μm. On PDA cylindrical cells occurred too. Budding was polar on a narrow base and the cells occur singly or in parent-bud pairs. After 7 days on MEA a high percentage of globose cells occurred and multilateral budding was observed. No hyphae and pseudohyphae were observed in any media. After 7 days on MEA, abundant capsules were observed in all the strains considered (Fig. 2).

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