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TAXONOMIC DESCRIPTION

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Description of *Dioszegia patagonica* sp. nov., a novel carotenogenic yeast isolated from cold environments

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Abstract

During a survey of carotenogenic yeasts from cold and oligotrophic environments in Patagonia, several yeasts of the genus *Dioszegia* (Tremellales, Agaricomycotina) were detected, including three strains that could not be assigned to any known taxa. Analyses of internal transcribed spacer and D1/D2 regions of the large subunit rRNA gene showed these strains are conspecific with several other strains found in the Italian Alps and in Antarctica soil. Phylogenetic analyses showed that 19 of these strains represent a novel yeast species of the genus *Dioszegia*. The name *Dioszegia patagonica* sp. nov. is proposed to accommodate these strains and CRUB 1147^T (UFMG 195^T=CBMAI 1564^T=DBVPG 10618^T=CBS 14901^T; MycoBank MB 819782) was designated as the type strain. This *Dioszegia* species accumulates biotechnologically valuable compounds such as carotenoid pigments and mycosporines.

The genus *Dioszegia* constitutes a monophyletic group within the Tremellales (Tremellomycetes, Agaricomycotina) [1], which currently includes 17 species [2–10]. Recently, the phylogeny of the tremellomycetous yeasts and related dimorphic and filamentous Tremellomycetes was reconstructed by analysing sequences from seven genes [1]. *Dioszegia* were included in the novel proposed family *Bulleribasidiaceae* within the order Tremellales. The yeasts included in the genus *Dioszegia* are generally characterized by the salmon, pink or red colour of their colonies, which is a result of the intracellular accumulation of carotenoid pigments [11, 12]. All species characterized up to date are non-fermentative, may or may not form ballistoconidia and there is no evidence of a sexual stage. Regarding the ecology, most species of the genus *Dioszegia* have been isolated from plant leaves, roots or soil [13]. Previous reports showed that cold aquatic environments harbour an interesting diversity of carotenogenic and cold-adapted yeasts [14, 15], including several novel species [16–18]. In the present work a new species of *Dioszegia*, named *Dioszegia patagonica* sp. nov., isolated from cold water, soil, ice and glacial sediments from South America, Antarctica and Europe (Italy) is described.

The origin of the 20 strains studied here is shown in Table 1. The morphological and physiological characterization was done according to Kurtzman *et al.* [19]. Mycosporine and lipid content, and UVB survival were tested previously for strain CRUB 1147^T [15, 20]. The carotenoid content of the type strain was assessed spectrophotometrically in this study as previously described [21].

Identification of the yeast isolates was based on internal transcribed spacer (ITS) and large subunit (LSU) rRNA gene sequencing. For DNA sequence analysis, DNA extraction, PCR amplification, purification and cycle sequencing, the protocol establised in Sampaio *et al.* [22] was followed. For the 26S rRNA gene D1/D2 domain sequencing, forward primer NL1 (5-GCATATCAATAAGCGGAGGAAAAG-3) and reverse primer NL4 (5-GGTCCGTGTTTCAAGACGG-3) were employed. The ITS region was sequenced using the forward primer ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and the reverse primer ITS4 (5-TCCTCCGCTTATTGATATGC-3). Sequences were aligned by using the CLUSTAL algorithm and manually corrected. The phylogenetic analysis was carried out using the neighbour-joining method [23] in MEGA 6 [24]. The evolutionary distances

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Keywords: *patagonia*; cold environments; red yeast; psychrophile.

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are EF595753, KY438212, KY438211, MF100849, MF100848, KC433801, KC433854 (26SrDNA D1/D2) and KY449061, KY438210, KY438209, MF100847, MF100846, KC455892, KC455907 (complete ITS region and 5.8S rDNA).

Two supplementary tables are available with the online Supplementary Material.

Table 1. List of studied strains

Strain code*	Locality	Substrate	Reference
CRUB 1147^T=CBS 14901^T= UFMG-CM-Y195^T= CBMAI 1564^T= DBVPG 10618^T	Toncek lake, Parque Nacional NH†, Río Negro, Argentina	Water	[15]
CRUB 1909	Nahuel Huapi lake, Parque Nacional NH, Argentina	Water	[43]
CRUB 1910	Nahuel Huapi lake, Parque Nacional NH, Argentina	Water	[43]
UFMG-CM-Y6157 (=ANT99)	Antarctica	Rhizosphere of *Deschampsia antarctica* Desv. (Poaceae)	[41]
DBVPG 5452	Helbronner peak‡, Mont Blanc, Italy	Snow with superficial sediment	[44]
DBVPG 5526	Helbronner peak, Mont Blanc, Italy	Snow with superficial sediment	[44]
DBVPG 5619	Helbronner peak, Mont Blanc, Italy	Snow with superficial sediment	[44]
DBVPG 5716	Helbronner peak, Mont Blanc, Italy	Snow with superficial sediment	[44]
DBVPG 5739	Helbronner peak, Mont Blanc, Italy	Snow	[44]
DBVPG 5932	Miage glacier§, Mont Blanc, Italy	Glacial melting water	[44]
DBVPG 10224	Miage glacier, Mont Blanc, Italy	Sediment	
DBVPG 10252	Miage glacier, Mont Blanc, Italy	Sediment	
DBVPG 10471	Miage glacier, Mont Blanc, Italy	Sediment	
DBVPG 10487	Miage glacier, Mont Blanc, Italy	Sediment	
DBVPG 10506	Miage glacier, Mont Blanc, Italy	Sediment	
DBVPG 10510	Miage glacier, Mont Blanc, Italy	Sediment	
DBVPG 5456	Helbronner peak, Mont Blanc, Italy	Snow with superficial sediment	
DBVPG 5478	Helbronner peak, Mont Blanc, Italy	Snow with superficial sediment	
DBVPG 10049	Miage glacier, Mont Blanc, Italy	Ice	
TP-Snow-Y51	Tibetan Plateu, China	Glacier surface snow	Shao, S. and Ma, X. (unpublished)

*CBS, CBS Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; UFMG, Culture Collection of the Universidade Federal de Minas Gerais; CBMAI, Brazilian Collection of Environmental and Industrial Microorganisms; DBVPG, Industrial Yeasts Collection of the University of Perugia, Italy; CRUB, Centro Regional Universitario Bariloche, Argentina.

†Parque Nacional Nahuel Huapi (Nahuel Huapi National Park).

‡Helbronner peak, Glacier du Geant (3430 m a.s.l.).

§Miage glacier (from 1720 to 2400 m a.s.l.).

were computed by using the Kimura two-parameter method [25] and bootstrap analysis was inferred from 1000 replicates [26]. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Network analysis was performed using the median-joining method [27] with NETWORK 5.0.0.1 software with the same multiple alignment used for phylogenetic analysis without gaps. Percent similarities were computed from pairwise comparisons of sequences as the percentage identity of the overlap between the two sequences, by using the BLAST algorithm [28], for which only substitutions were considered.

PHYLOGENETIC PLACEMENT AND PHYSIOLOGICAL CHARACTERISTICS

During a number of surveys, 19 strains of a putative novel species of *Dioszegia* were isolated (Table 1). These included three isolates recovered from water samples of ultraoligotrophic mountain lakes in Patagonia, one isolate from the rhizosphere of *Deschampsia antarctica* in Antarctica and several isolates from snow, ice, glacial melt water and glacial sediments of Mont Blanc, Italy. In addition, closely related ITS and/or D1D2 sequences from public databases were retrieved and analysed. Three strains in which both rRNA gene regions were available were kept for analysis: CBS 5124 (GenBank AF272664/AF314231), CBS 6953 (AF272668/AF314240) and TP-Snow-Y51 (JN400821/JN400792). Other strains without a complete set of available sequences and related metagenomics sequence data are discussed later in the manuscript. Strains CBS 5124 and CBS 6953 were formerly misclassified as *D. hungarica* [29], although later this was revised based on rRNA gene sequence analyses [9, 30, 31]. Both strains, like in previous works [9], were excluded from the final dataset due to the low quality of their available sequences. Re-sequencing of both strains would be necessary for their adequate taxonomic classification.

When compared by multiple alignment of the LSU rRNA gene D1/D2 and ITS regions, low nucleotide heterogeneity for most of the 20 analysed strains was found. A main group

of 15 strains either shared identical sequences to CRUB 1147^T (CRUB 1909, CRUB 1910, DBVPG 5526, DBVPG 5716, DBVPG 5932, DBVPG 10252, DVBPG 10487, DVBPG 10506 and ANT99) or differed in a single but variable position (DBVPG 5739, DBVPG 10224, DBVPG 5452, DBVPG 5619, DVBPG 10510 and DBVPG 10471) (Fig. 1). Four remaining strains were more divergent, constituting distinct phylotypes: DBVPG 5456 and DBVPG 5478 (identical sequences) with five substitutions at the D1D2 region and no substitutions at the ITS region; TP-Snow-Y51 with six substitutions at D1D2 region and one substitution at the ITS region; and DBVPG 10049 with nine substitutions at the D1D2 region and five substitutions at the ITS region. Of note were the atypical higher genetic polymorphisms found in the D1D2 region in comparison to the ITS region for these strains (Table S1, available with the online Supplementary Material), which adds to a small list of similar exceptions among yeasts [16, 32, 33] and increases the difficulty of taxonomic circumscription at the species level.

Phylogenetic analysis together with hitherto recognized *Dioszegia* species and representative *Nielozyma* taxa (Tremellales), confirmed that all the isolates were placed within the genus *Dioszegia* though forming a separate sub-clade (Fig. 1). To better circumscribe the novel taxon, a network analysis was performed [27] and also percent similarities for ITS and D1D2 regions were computed (Fig. 2 and Table S1). As shown in Fig. 2, most of the strains grouped in a single central node (no substitutions), represented by CRUB 1147^T. A few other strains were connected to this node with several substitutions (circled in Fig. 2), all with similarities above 99 % for both ITS and D1D2 regions

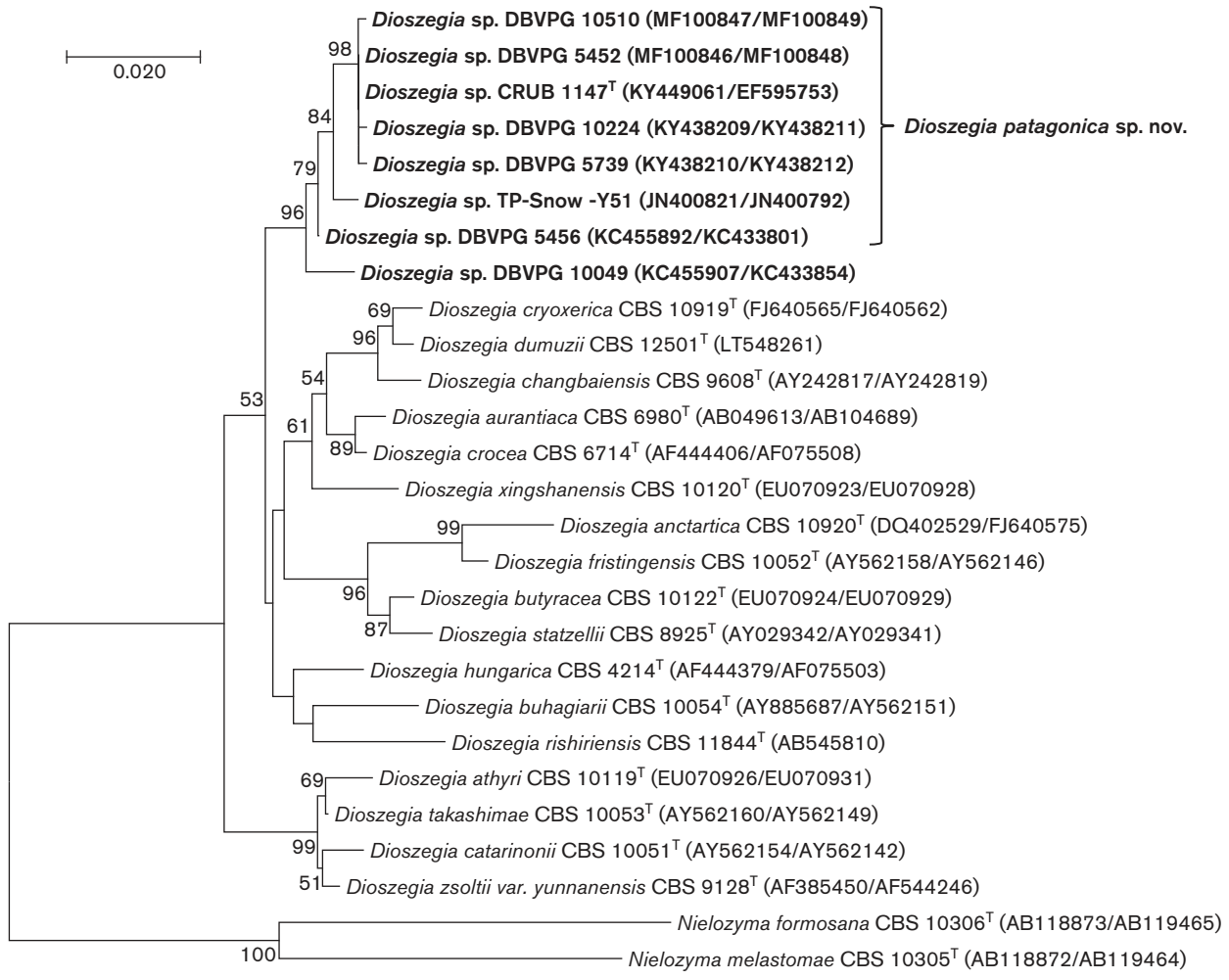

Fig. 1. Evolutionary relationships of taxa. Phylogenetic placement of *Dioszegia patagonica* sp. nov. obtained by neighbour-joining analyses of the ITS and D1/D2 domains of the large subunit of the rRNA gene. The evolutionary distances were computed using the Kimura two-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 904 positions in the final dataset. Bar, number of substitutions accumulated every 100 nucleotides. Bootstrap values higher than 50 % are shown (1000 replicates). GenBank accession numbers are given in parentheses (ITS/D1D2).

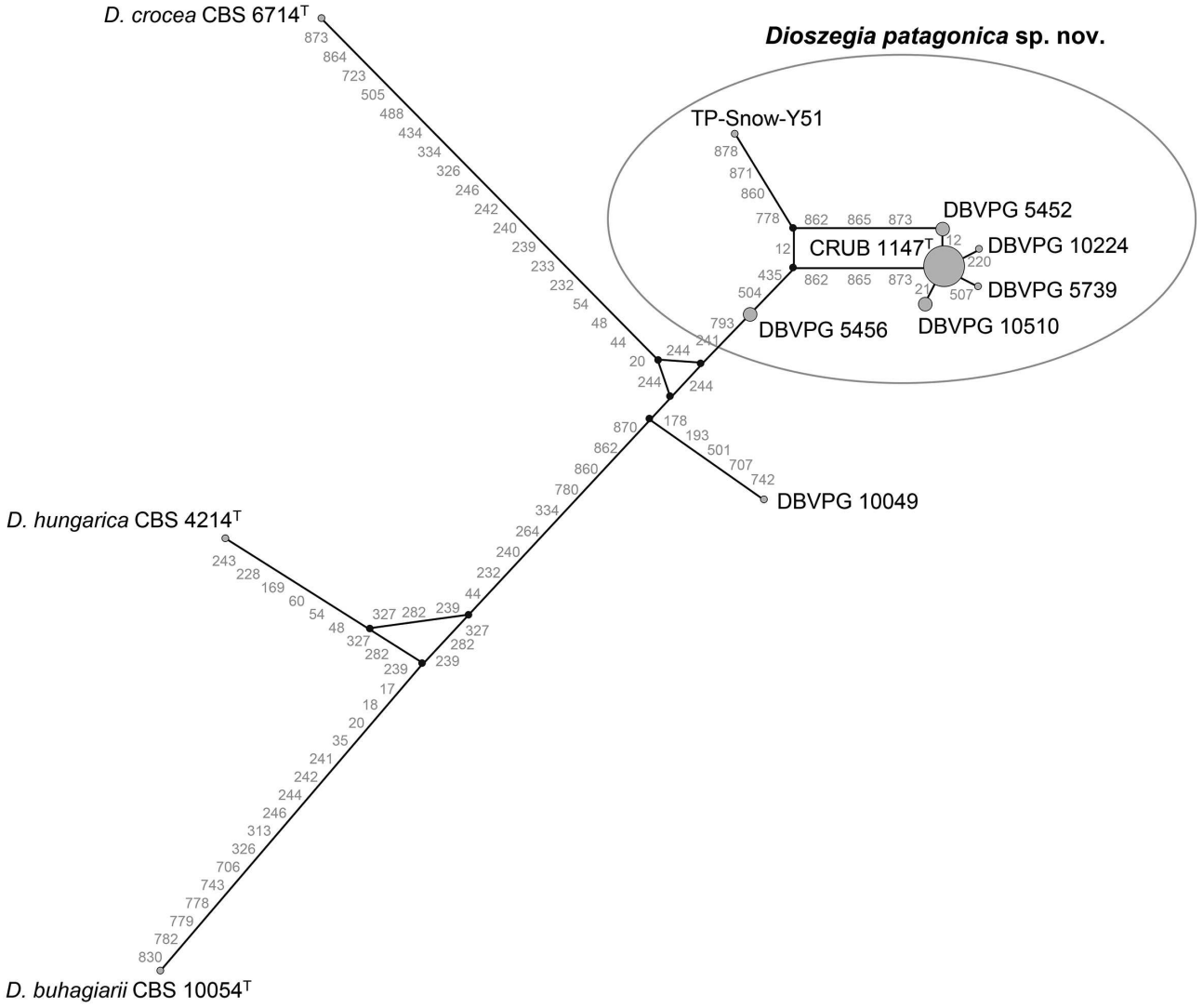

Fig. 2. Network analysis of intraspecific variation in *Dioszegia patagonica*. Network displaying the sequence variation of 21 sequences corresponding to different strains of *Dioszegia* species, including ITS and D1D2 regions (without gaps). Grey circles (nodes) represent sequence types (GenBank numbers are included in Fig. 1). Black nodes are median vectors (unsampled sequences). Node sizes are proportional to the frequency of sequences representing each type. Mutations are referred to by the nucleotide positions in the alignment (numbers). Nodes DBVPG 5456, CRUB1147^T, DBVPG 5452 and DBVPG 10510 represent strains DBVPG 5456/DBVPG 5478, CRUB 1147^T/DBVPG 10252/DBVPG 5526/DBVPG 5716/DBVPG 5932/ANT99/CRUB 1909/CRUB 1910, DBVPG 5452/DBVPG 5619 and DBVPG 10510/DBVPG 10471 respectively. Sequences corresponding to strains DBVPG 10506 and DBVPG 10487 are identical to CRUB 1147^T but were excluded from the analysis since their sequences were a few nucleotides shorter at the ITS region.

when compared to CRUB1147^T. Strain DBVPG 10049 was more distantly located in the network analysis, and showed percent similarities below 99 % for both ITS and D1D2 regions (Table S1). Based on these results we conclude that the group of isolates under study (excluding DBVPG 10049) can be accommodated in a novel species, for which the name *Dioszegia patagonica* sp. nov. is proposed, and for which CRUB 1147^T is selected as the type strain. Further analyses are needed to determine the taxonomic classification of DBVPG 10049, as well as additional closely related strains detected in public databases with single rDNA gene markers. Isolates with sequences in public databases that were not included in the analysis due to absence of ITS information deserve further attention; since their D1D2 sequences are highly identical to either CRUB1147^T or DBVPG 5456 or TP-SnowY51 phylotypes (Table S2). A correlation of the different phylotypes with distinct geographic location or types of substrates was not found, instead in some cases isolates of different phylotypes were recovered from the same substrate or sampling area. This was the case for DBVPG 5456 and DBVPG 5478, both sampled from snow with superficial sediment at the Helbronner peak,

Mont Blanc; together with other strains from lineage CRUB 1147^T (Table 1).

In terms of physiological characteristics, high variability was observed among *D. patagonica* strains (assimilation of 19 compounds had variable results). The more genetically divergent strains, DBVPG 5456 and DBVPG 5478, did not differ physiologically with the core group of CRUB 1147 ^{T}; with the only exception being ribitol assimilation, which was negative for these two strains and positive or weak for all the others. The more salient traits of *D. patagonica* sp. nov. in comparison to other *Dioszegia* species are presented in Table 2, and the former can be distinguished by its lack of ability to assimilate melibiose, shared only with *D. hungarica*, *D. buhagiarii* and *D. rishiriensis*.

ECOLOGICAL AND PHENOTYPIC TRAITS

Patagonian isolates were obtained from independent water samples from lakes Toncek (CRUB 1147^T) and Nahuel Huapi (CRUB 1909 and CRUB 1910 strains), both oligotrophic water bodies from northwestern Patagonia at the Nahuel Huapi National Park. Both lakes are characterized by clear water, with high UV radiation penetration [15, 34, 35]. Another isolate was obtained from rhizosphere of *D. antarctica* Desv. (Poaceae) in Antarctica while the other 15 strains were found in snow collected at Helbronner peak, Glacier du Geant (3430 m a.s.l.), and melting water, ice and sediment of Miage glacier (from 1720 to 2400 m a. s.l.), both located in the Mont Blanc Massif, Italy. Isolation of strain TP-Snow-Y51 is reported from glacier surface snow at the Tibetan Plateau (China). Also, environmental sequences belonging to uncultured organisms very closely related to *Dioszegia patagonica* sp. nov. (based on ITS region, identity near 99 % or above), are available in public databases. They mostly originate from cold substrates such as Tibetan Plateau permafrost (KT265191), soil in Prince Patrick Island Canada (KC966099, KC966100), subalpine soil (KM504441), dust in Finland (FR682194, AM901780) and Alpine soil (KP714641, KP714701). Additionally, isolates with closely related D1D2 sequences also belong to cold substrates (Table S2), including water from high altitude clouds [36], and deserve further studies to assure their taxonomic position.

All of these habitats are characterized by the presence of low temperatures but other harsh conditions may be present including low water activity, characteristic of snow/ice, and also low nutrient availability and oxidative stress (e.g. due to high solar irradiation) [19]. In previous studies the ability of one strain of *Dioszegia patagonica* sp. nov. (CRUB 1147^T) to accumulate large quantities (47.8±0.4 mg g^{-1}) of the UV-B absorbing molecule mycosporine–glutaminol–glucoside (MGG) as a response to photostimulation was reported [37]. The accumulation of MGG in this strain was even higher than in strains of *D. hungarica* [31] and ever higher than in *Phaffia rhodozyma*, a yeast species that is heavily equipped for coping with UV-B radiation [15]. Later, strain

Table 2. List of salient physiological differences of *D. patagonica* compared to other *Dioszegia* species

1, Sorbose; 2, Lactose; 3, Melibiose; 4, Glicerol; 5, Galactitol; 6, Hexadecene. −, Negative; +, positive; d, delayed; v, variable; w, weak; dw, delayed and weak; ND, not determined.

Taxon	Assimilation of					
	1	2	3	4	5	6
D. patagonica sp. nov.*	−/d	−	−	+/d/w	+/d	−
D. aurantiaca	−	−	+	w	+	−
D. crocea	dw/−	−	+	d/dw	+	−
D. dumuzii	−	w	ND	w	ND	ND
D. cryoxerica	+	+	+	+	+	+
D. changbaiensis	−	−	+	−	+	−
D. xingshanensis	−	−	+	−	dw	−
D. statzelliae	−	−	w	−	+	−
D. butyracea	−	−	+	−	+	−
D. fristingensis	−	−	+	−	+	−
D. antarctica	v	+	+/w/d	+/d	+	v
D. hungarica	−	−	−	−/dw	+	−
D. buhagiarii	d	−	−	d/dw	+	−
D. rishiriensis	dw	−	−	−	+	−
D. athyri	−	−	+	−	w	−
D. takashimae	ND	+/d	+/d	−	+/d	−
D. zolstii var. *yunnanensis*	v	v	+	−	+/w	−
D. catarinonii	d/−	d/w/dw	+	d/dw	+/d	−

*Results from all strains included in Table 1 with exception of TP-Snow-Y51 for which no physiological data is available.

CRUB1147^T and two other *D. fristingensis* strains were demonstrated to be highly tolerant to UV-B radiation exposure [15], which is in agreement with their ability to synthesize UV-B protectants such us MGG. Based on these results, and the ability of other nine *Dioszegia* species to synthesize MGG (Libkind, unpublished) and the fact that this character typically behaves as a taxon-specific trait [38], we hypothesize that MGG production is a feature common to the genus *Dioszegia*.

The type strain of *D. patagonica* was found to synthesize an average of 116.80±20.38 µg g^{-1} of total carotenoid pigments, which is similar or less than values observed previously for other *Dioszegia* species [12, 39]. Very few studies deal with the carotenoid composition in *Dioszegia* species; and suggest plectaniaxanthin could be the major carotenoid in this clade, a xanthophyll not found in other yeast species [40]. Interestingly, Madhour *et al.* [12] showed that plectaniaxanthin synthesis in *Dioszegia takashimae* was increased under oxidative stress, which suggests a potential photoprotective role of this carotenoid in this yeast. Further analyses with a larger set of species are required in order to determine if the synthesis of MGG and plectaniaxanthin are taxonomical relevant traits in the genus *Dioszegia* and if they are responsible for the increased UV radiation tolerance in many of these yeasts [15].

The lipid content of *D. patagonica* CRUB 1147^T was also analysed and an enrichment in polyunsaturated fatty acids (PUFAs, 42.7–57.3 % cell dry weight) typical of cold adapted yeasts was observed [20]. Other evidence of the cold adapted nature of *D. patagonica* is the synthesis of extracellular enzymes at 4 °C, including amylase, esterase, pectinase, cellulase and lipase, detected for several isolates from Patagonia and Antarctica [41, 42]. In summary, the data suggest adaptation of *Dioszegia patagonica* sp. nov. to several extreme conditions including low temperatures and high UV-radiation, although further studies would be necessary to dissect the specific genes/metabolites responsible for each of these adaptations.

DESCRIPTION OF *DIOSZEGIA PATAGONICA* TROCHINE,TURCHETTI, VAZ, BRANDAO, ROSA, BUZZINI, ROSA AND LIBKIND SP. NOV.

Dioszegia patagonica (pa.ta.go′ni.ca. N.L. fem adj. patagonica of Patagonia, referring to the name of the region where a number of isolates were found).

Belongs to the subphylum *Agaricomycotina*, class *Tremellomycetes*, order Tremellales. Yeast cells after 5 days at 20 °C on yeast–malt (YM) agar are ovoid to elongated, measuring 11 µm in average length and 3.4 µm in average width (Fig. 3). Budding is polar. Hyphae or pseudohyphae are not formed. On YM agar, colonies appear convex, circular, with an entire margin, are orange, semi-glistening and pasty in texture. Glucose is not fermented. Sexual reproduction is not observed. Physiological and biochemical discriminating characters are depicted in Table 2; phylogenetic placement

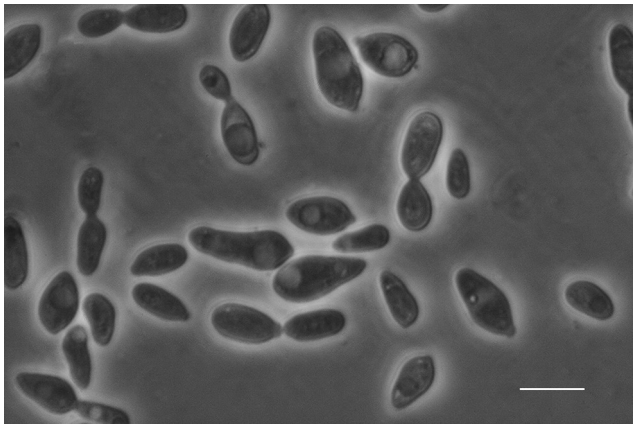

Fig. 3. Phase-contrast micrograph of strain CRUB 1147^T on YM agar after 3 days at 20 °C. Budding cells are visible. Bar, 10 µm.

is presented in Fig. 1. The substrates of origin of 20 strains are shown in Table 1. No physiological data is available for strain TP-Snow-Y51. *D. patagonica* assimilates D-glucose, D-xylose, trehalose, glicerol (some strains delayed), galactitol (some strains delayed), D-mannitol (some strains delayed) and D-gluconate (some strains delayed). Variable results among strains were obtained for assimilation of galactose, D-ribose, ribitol (two strains negative), L-arabinose, D-arabinose, L-rhamnose (one strain negative), sucrose (one strain negative), maltose, cellobiose, salicin, raffinose, melezitose, meso-erythritol, xylitol, D-glucitol, *myo*-inositol, DL-lactate, succinate and ethanol. It does not assimilate lactose, melibiose, methanol or n-hexadecane. It assimilates L-lysine hydrochloride but does not assimilate potassium nitrate. It grows at 25 °C (except for ANT99 strain) but not at 30 °C.

Dioszegia patagonica type strain CRUB1147^T was isolated from water of the Toncek lake, Parque Nacional Nahuel Huapi, Argentina. The type strain has been deposited at the CBS Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands as CBS 14901^T and is stored in a metabolically inactive form in accordance with the Code. It was also deposited at the Culture Collection of the Universidade Federal de Minas Gerais (UFMG), at the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), at the Industrial Yeasts Collection (DBVPG) of the University of Perugia, Italy, and at the Centro Regional Universitario Bariloche (CRUB), as UFMG-CM-Y195^T, CBMAI 1564^T, DBVPG 10618^T and CRUB 1147^T, respectively.

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

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