

RESEARCH ARTICLE

Unique genomic traits for cold adaptation in *Naganishia vishniacii*, a polyextremophile yeast isolated from Antarctica

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One sentence summary: Through a comparative genomic approach, this study reveals a series of characteristics possibly involved in the evolution of *N. vishniacii* in the context of its extreme environment and its tolerance of extremely cold conditions.

Editor: Prof. Isak Pretorius

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ABSTRACT

Cold environments impose challenges to organisms. Polyextremophile microorganisms can survive in these conditions thanks to an array of counteracting mechanisms. *Naganishia vishniacii*, a yeast species hitherto only isolated from McMurdo Dry Valleys, Antarctica, is an example of a polyextremophile. Here we present the first draft genomic sequence of *N. vishniacii*. Using comparative genomics, we unraveled unique characteristics of cold associated adaptations. 336 putative genes (total: 6183) encoding solute transfers and chaperones, among others, were absent in sister species. Among genes shared by *N. vishniacii* and its closest related species we found orthologs encompassing possible evidence of positive selection ($dN/dS > 1$). Genes associated with photoprotection were found in agreement with high solar irradiation exposure. Also genes coding for desaturases and genomic features associated with cold tolerance (i.e. trehalose synthesis and lipid metabolism) were explored. Finally, biases in amino acid usage (namely an enrichment of glutamine and a trend in proline reduction) were observed, possibly conferring increased protein flexibility. To the best of our knowledge, such a combination of mechanisms for cold tolerance has not been previously reported in fungi, making *N. vishniacii* a unique model for the study of the genetic basis and evolution of cold adaptation strategies.

Received: 18 June 2020; Accepted: 15 October 2020

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Keywords: genomics; extremophile; cold adaptation; yeast; *Naganishia*; Antarctica

INTRODUCTION

A large proportion of the Earth's surface area is covered by permanently low temperature environments. Polar regions, Antarctica and areas within the Arctic circle, constitute 20% of the world's land surface area with less than 0.5% seasonally ice- or snow-free. Nevertheless several ecosystems are represented, with a native biota that has been adapted to the extreme conditions of these environments (Cowan et al. 2007; Convey and Peck 2019). The combination of low temperature, low liquid water availability, periodic freeze-thaw cycles, increased osmotic and oxidative stresses and high solar irradiation, *inter alia*, make these regions extremely inhospitable to most forms of life. However, such places have been successfully colonized by psychrophilic and psychrotolerant organisms. Current knowledge of microbial biodiversity has shown that cold habitats harbor a wide diversity of adapted prokaryotic and eukaryotic microbial life, including archaea, bacteria, cyanobacteria, protists, microalgae, virus, filamentous fungi and yeasts (Margesin and Miteva 2011; Connell et al. 2014; Zalar and Gunde-Cimerman 2014; Margesin and Collins 2019).

To successfully overcome the negative effects of low temperatures, organisms harbor a complex range of structural and functional adaptations. Changes in membrane fluidity by restructuring lipid composition thus counteracting membrane rigidifying, production of pigments and metabolites that act as reactive species scavengers and photoprotective compounds, synthesis of anti freezing proteins enabling survival to freeze-thaw cycles and accumulation of osmolytes that avoid freezing injury, have been reported as common mechanisms in a wide variety of cold tolerant organisms from vertebrates to microorganisms (Clark and Worland 2008; Singh et al. 2017; Collins and Margesin 2019). However, many of these responses and mechanisms are not fully characterized in cold tolerant yeasts. Understanding these mechanisms has been a challenge and a matter of interest, not only because of their biotechnological potential, but also because of their importance in biogeochemical nutrient cycling, ecological role and also to gain an understanding of the origins of life on our planet (Hébraud and Potier 1999; Albanese et al. 2020).

Yeasts are ubiquitous eukaryotic organisms belonging to the fungi and even though they are considered less tough than prokaryotic microorganisms, a great diversity of them are able to overcome a plethora of extreme conditions (Buzzini and Margesin 2014). *Naganishia vishniacii*, a basidiomycetous yeast (class Tremellomycetes, order Filobasidiales), was first isolated from the Beacon supergroup sandstone, Antarctica, and its discovery in the Ross Desert provided the first indication that indigenous microorganisms do inhabit the soils of the unglaciated high valleys (Vishniac and Hempfling 1979a). This species is apparently unique to the McMurdo Dry Valleys and even though its autochthony cannot be inferred based on the impossibility of finding it in similar soils, its ability to grow at 4°C and even at −3°C, but not at or above 21°C, and the fact that many other species found in these regions have also been isolated from other places, makes *N. vishniacii* an interesting candidate for indigenous microbiota. Little is known about cold-adapted yeast inhabiting these environments (Alcaíno, Cifuentes and Baeza 2015). The advent of 'omics' technologies with whole genome sequences of microbes inhabiting cold places are helping us understand their particularities and genes for cold adaptation,

cold shock proteins, pigments biosynthesis pathways, chaperones and DNA repair systems (Yadav et al. 2018). In this sense, the more sequenced genomes that become available, the more we will be able to increase and deepen our knowledge.

In this work, we report the genome of *N. vishniacii*. Using comparative genomics with model species and with phylogenetically closely related genomes, we were able to identify genome-wide characteristics of cold adaptation. Particularly, characterization of unique genes linked with protein stability, trends in amino acid composition and identification of specific pathways already associated with cold tolerance in other organisms (photoprotection, trehalose synthesis and lipid metabolism) were achieved. All these traits constitute valuable targets for further functional studies that may account for cold-adaptation in *N. vishniacii*.

MATERIALS AND METHODS

Isolate source

Naganishia vishniacii (ANT03-052; CBS 10616) was isolated from a soil sample collected in the Labyrinths, Wright Valley, South Victoria Land, Antarctica (160.702E, −77.562S). The soil was collected aseptically during the 2003–2004 Antarctic field campaign and isolates were cultured and identified as previously described (Connell et al. 2008).

Genome sequencing

The *N. vishniacii* genome was sequenced using two Illumina libraries, Regular Fragments (300bp insert-size) and Long Mate Pairs (LMP, 4kbp). For the Fragment library, 100 ng of DNA was sheared to 300 bp using the Covaris LE220 (Covaris, Woburn, MA, USA) and size selected using SPRI beads (Beckman Coulter, Indianapolis, IN, USA). The fragments were treated with end-repair, A-tailing and ligation of Illumina compatible adapters (IDT, Inc, Coralville, IA, USA) using the KAPA-Illumina library creation kit (KAPA Biosystems, Wilmington, MA, USA).

For the LMP library, 5 µg of DNA was sheared using the Covaris g-TUBE (Covaris) and gel size selected for 4kb. The sheared DNA was treated with end repair and ligated with biotinylated adapters containing loxP. The adapter ligated DNA fragments were circularized via recombination by a Cre excision reaction (NEB). The circularized DNA templates were then randomly sheared using the Covaris LE220 (Covaris). The sheared fragments were treated with end repair and A-tailing using the KAPA-Illumina library creation kit (KAPA Biosystems) followed by immobilization of mate pair fragments on streptavidin beads (Invitrogen, Carlsbad, CA, USA). Illumina compatible adapters (IDT, Inc) were ligated to the mate pair fragments and eight cycles of PCR were used to enrich for the final library (KAPA Biosystems).

All libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument, multiplexed with other libraries and prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v3 and Illumina's cBot instrument to generate clustered flowcells for sequencing. Sequencing of the flowcells was performed on the Illumina HiSeq2000 sequencer using a TruSeq SBS

sequencing kit, v3, following a 2×150 bp (2×100 bp for LMP) indexed run recipes.

Genome assembly, gene predictions and annotations

Genomic reads from both Fragment and LMP libraries were initially assembled using AllPathsLG release version R41043, (Gnerre et al. 2011). Genome assembly was made available via the JGI fungal portal MycoCosm (mycocosm.jgi.doe.gov /Nagvi; Grigoriev et al. 2014). Reads were also assembled using SPAdes v3.6.0 (Bankevich et al. 2012) including adapter removal, trimming, quality filtering and error correction. Both assemblies were merged with the program NGAP4 (Nizovoy et al. 2018). Briefly, regions flanking undefined nucleotides in the initial AllPaths assembly were recovered and scanned for in SPAdes versions in order to correct nucleotide identities with those included in flanked fragments when possible. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JABEVT000000000. The version described in this paper is version JABEVT010000000.

Ab initio gene prediction with GeneMark-ES v2.3e (Ter-Hovhannisyan et al. 2008) was self-trained on the genome scaffolds (parameters: `-min.contig 8000 -max.nnn 1000`). These predictions as well as genome scaffolds were used to feed the Funannotate Pipeline (Palmer 2016). By means of this pipeline, Pfam domains, Basidiomycota BUSCO groups and functional categories were also annotated when possible. For genome comparisons, predictions and annotations were also performed for most closely related genomes available in NCBI portal, pertaining to the Filobasidiales order, namely *Naganishia albida* Y-1402 and NT2002 (DDBJ/EMBL/GenBank accession numbers PRJNA291988 and PRJNA298369; Vajpeyi and Chandran 2016; Yong et al. 2016), *Naganishia adeliensis* IF1SW-F1 (PRJNA623412; Bijlani et al. 2020), *Filobasidium wieringae* UCDFST 05-544 (PRJDB3683), *Solicozyma phenolicus* JCM11743 (PRJDB3680) and *Solicozyma terricola* JCM24523 (PRJNA327103). Strains selection of available assemblies was based on phylogenetic closeness to *N. vishniacii*, prioritizing already published genomes (for species with no publication available, assemblies were included upon permission of authors). Genomic sequences of *Cryptococcus neoformans* JEC21 (FungiDB-42) (Stajich et al. 2012) were processed equally in order to compare results with the model yeasts species.

Phylogenetic tree reconstruction

Set of single copy shared genes among Filobasidiales species and *Cryptococcus neoformans* JEC21 as determined by eggNOG-Mapper v4.5.1 were aligned by means of MUSCLE with default parameters (Edgar 2004) and converted to codon alignment with PAL2NAL (Suyama, Torrents and Bork 2006). Alignments were concatenated, gaps eliminated and the resulting length was 3082 974 columns. Rooted phylogenetic trees were constructed using RAxML v. 8.2.12 (Stamatakis 2014) with the PROTGAMMALG4M model of amino acid substitutions. Branch support values were determined using 1000 rapid bootstrap replicates. Evolutionary model was selected using ModelTest-NG with default parameters (Darriba et al. 2020). RAxML and ModelTest-NG were run on CIPRES science gateway (Miller, Pfeiffer and Schwartz 2010). The level of genomic sequence divergence between closely related species was estimated using de Kr value, an alignment-free pairwise distance measure calculated with Genome Tools (Gremme, Steinbiss and Kurtz 2013).

Orthologous genes and functional assignment

To find specific genes that may be involved in cold tolerance, species genes were categorized as *shared* or *unique* by means of fast orthology assignment using precomputed clusters. Clustering was performed with eggNOG v4.5.1 (Huerta-Cepas et al. 2017), employing the fungal database. This process enabled functional assignment of groups of genes.

Predicted CDS of *N. vishniacii* not matching with eggNOG database were classified into *shared* or *unique* genes by performing tblastn (e-value < $1e-05$, identity $\geq 50\%$) against the six Filobasidiales genomes. Predictions that did not group with any of those in the rest of the species were considered unique to *N. vishniacii*. Putative functions were inferred by homology search against non-redundant NCBI databases.

dN/dS ratio analysis

Ratio of non-synonymous (dN) and synonymous (dS) substitutions were computed for all the genes common to *N. vishniacii* and both *N. albida* strains. Analysis between single copy orthologs was performed by means of KaKs Calculator Version 2.0 (Wang et al. 2010). Predicted common proteins were aligned with MUSCLE with default parameters (Edgar 2004) and converted into gap-free codon alignments with PAL2NAL (Suyama, Torrents and Bork 2006). Derived *.axt files were split into 102 nucleotide-length chunks and analysed by a sliding window strategy (24 nucleotide steps) using YN model (Yang and Nielsen 2000). Analysis was also run for the unsplit alignments (whole sequences). All predictions with positive regions (dN/dS ratio > 1, e-value < $1e-02$) were subsequently compared between *N. vishniacii* and *F. wieringae*. Genes with positive regions for both pairs of species were evaluated with JCoDa (Steinway et al. 2010). Signals of positive selection were considered only if dN/dS > 1 was shared for the same region on the sequence among *N. vishniacii* vs. any or both *N. albida* strains and *N. vishniacii* vs. *F. wieringae* but not *N. albida* strains vs. *F. wieringae*.

Survey of genes involved in cold tolerance

We used tblastn and blastp to identify genes already known to be involved in pathways normally related to cold tolerance. A survey was made on Filobasidiales genomes and derived proteomes. Antifreeze proteins (AFPs) were screened using sequences obtained from NCBI database by using 'antifreeze' as keyword. Fungal (166) and bacterial (3832) query sequences were used for blastp suite search (e-val < $1e-5$) (available as Supplementary material: *.antifreeze.faa). Genes involved in the synthesis of photoprotection responses were identified in the predicted proteins using blastp (e-val < $1e-10$). Queries used are listed: genes involved in carotenoid synthesis: isopentenyl diphosphate isomerase (acc: BAA33979.1), farnesyltransferase (acc: AAY33922.1), farnesyl pyrophosphatase synthetase (acc: AHW57996.1), phytoene dehydrogenase (G01847.P), phytoene-beta carotene synthase (acc: AAO47570.1), NADP-cytochrome P450 reductase (acc: ACI43098.1), beta-carotene 15,15-monooxygenase (G04735.P). Genes involved in mycosporine synthesis: 2-epi-5-epi-valiolone synthase (G05217.P), catechol o-methyltransferase (G05218.P) and carbamoylphosphate synthase (G05219.P) previously characterized in *Phaffia rhodozyma* (Bellora et al. 2016). Backbone enzymes involved in the formation and degradation of trehalose, namely

trehalose-6-phosphate-synthase (acc: XP.02 554 8824.1), trehalose 6-phosphate phosphatase (acc: NP.594 975) and trehalases (acc: NP.0 09555.1, NP.01 0284.1 and NP.01 5351.1) were prospected in proteomes using fungal homologues queries. Identity was confirmed by reciprocal-blastp (e-values $\leq 2e-48$), specific Pfam domains presence (PF02358, PF0982/PF07492, PF1204) and grouping into common clusters of orthologous genes. Fatty acid desaturases genes were screened in all predicted proteomes by searching specific Pfam domains (PF00487 and PF08557). Identity of putative targets was confirmed by reciprocal blastp (e-values $\leq 4e-27$). Also differences in copy numbers of enzymes involved in lipid metabolism determined by eggNOG clustering were analyzed among Filobasidiales species.

Analysis of Amino Acids usage

The set of 248 core eukaryotic genes (CEGs; Parra et al. 2008) was scanned with HMMER v 3.1b1 (Eddy 2008) in all proteomes from Filobasidiales species as well as in *C. neoformans*. Screening was accomplished by applying the specific models with a threshold defined by Parra et al. (2008). Sets of common CEGs among each of the Filobasidiales species and *C. neoformans* (among 239 and 243) were pairwise aligned using MUSCLE (Edgar 2004) with default parameters and converted to codon alignment with PAL2NAL (Suyama, Torrents and Bork 2006). Translated alignments were concatenated and gaps eliminated. Concatenated sequences of each species, as well as those resulting from pairwise alignment of 2130 genes common to all, as determined by eggNOG-Mapper v4.5.1, were analyzed in order to study if there was an amino acid composition bias. Comparisons of amino acid composition were made over both sets of resulting sequences using Composition Profiler with default parameters (Vacic et al. 2007) and the analysis was referred to *C. neoformans*.

Experimental confirmation of carotenoids and mycosporines production in *N. vishniacii*

Yeasts were cultured in modified MMS agar (Moliné et al. 2011) under three different treatments: (a) radiation, (b) oxidative stress and (c) low temperature. Plates were inoculated with 100 μ L of a 10^6 cell suspension with a Drigalski-spatula until dry and cultured: (a) at 19°C under photosynthetically active irradiation in an environmental test chamber with light provided by 10 white light fluorescent tubes (Sanyo, 40 W); (b) at 19°C adding 1 mL of H₂O₂ 0.01 mM and (c) at 0.5°C. Carotenoid extraction was performed as described by Sedmak (Sedmak, Weerasinghe and Jolly 1990), with modifications proposed by Moliné et al. (2009). Samples were suspended in n-hexane and the absorbance was measured at 473 nm in a UV-visible spectrophotometer (Shimadzu UV1800). Mycosporines extraction was performed as described before (Moliné et al. 2011) and absorbance was measured at 310 nm.

RESULTS

Genome assembly, annotations and phylogenetic reconstruction

The 19.7-Mbp genome of *N. vishniacii* (ANT03-052; CBS 10616) was assembled into 38 scaffolds, ranging from 2.1 kb to 1.8 Mb, with a 146.7-fold depth of coverage and a very low number of undefined nucleotides (25 706 Ns, 0.13%). It was 90.9% complete using the Basidiomycota BUSCOs and 99.2% complete

based on CEGs genes. Gene predictions yielded 6183 putative coding genes accounting for 53.4% of the genome sequence. From these models 4956 putative genes (80.3%) were assigned to 23 different eggNOG functional categories and classified into 4609 orthogroups. 2613 (42.3%) gene predictions were annotated with a putative function. G + C content among this species was considerably higher than the model species *Cryptococcus neoformans* JEC21 as shown in Fig. 1.

The phylogenetic tree for the eight species constructed based on common single copy orthologs clusters (described below) grouped species by genera (Fig. 1).

Orthologous genes and functional assignment

Orthology inference analysis of the 4609 different groups revealed that among them, 4593 (~99.7%) were shared between *N. vishniacii* and one or more of the related species. 2926 orthogroups were common to the seven Filobasidiales species, whereas 16 groups were only found in the *N. vishniacii* genome. Almost 18% of predicted genes in *N. vishniacii* ($n = 1073$) did not match any orthogroup of the eggNOG database. Tblastn of derived proteins performed against the rest of the species genomes for this set of genes yielded 753 (70%) predictions with positive matches in any or several species. For the rest of the 320 predictions without significant matches and no orthology assignment, the search against NCBI non redundant database resulted in 297 of these annotations (almost 93%) not matching any deposited query, while 23 (~7%) were linked to hypothetical proteins or partially matched with a deposited sequence and six of them (~2%) were linked with putative functions. Complete list is available as Table S1 (Supporting Information; Fig. 2).

Among 16 orthogroups found only in the *N. vishniacii* genome, the eggNOG functional categories most represented included unknown function (43%), signal transduction mechanisms (13%), secondary metabolites biosynthesis transport and catabolism (13%) and transcription (13%). Also carbohydrate transport and metabolism and RNA processing and modification were present. Functional description resulted, among others, in putative transferases (Nv.005274), solute transporters (NV.002329) and chaperones (Nv.005012). A complete list is available as Supplementary material (Table S2, Supporting Information).

In order to explore possible gene redundancy in *N. vishniacii*, we compared orthogroups copy number. Analysis revealed that 2366 shared groups (~81%) encompassed one copy candidate by species, 19 (~1%), two copies and 1, three copies, while 540 groups (18%) showed differences in copy numbers among species. When comparing the copy numbers between *N. vishniacii* and *N. albida* strains we found that in 12 orthogroups *N. vishniacii* gene redundancy was higher than that in both *N. albida* strains. (Fig. 3). Genes involved in RNA processing and modification, amino acids and solute transport and metabolism and energy production were more abundant in *N. vishniacii* regarding both *N. albida* strains. For groups ENOG410PJ40, ENOG410PFJG, ENOG410PH2P, ENOG410PFKH and ENOG410PF97 (green highlighted), even though *N. vishniacii* surpassed *N. albida* copy numbers, it showed equal or even lower number of genes than those in the rest of the Filobasidiales species belonging to non *Naganishia* genera.

dN/dS analysis

Distribution of dN/dS mean values for the 3942/3991 single copy common genes between *N. vishniacii* and *N. albida* strains

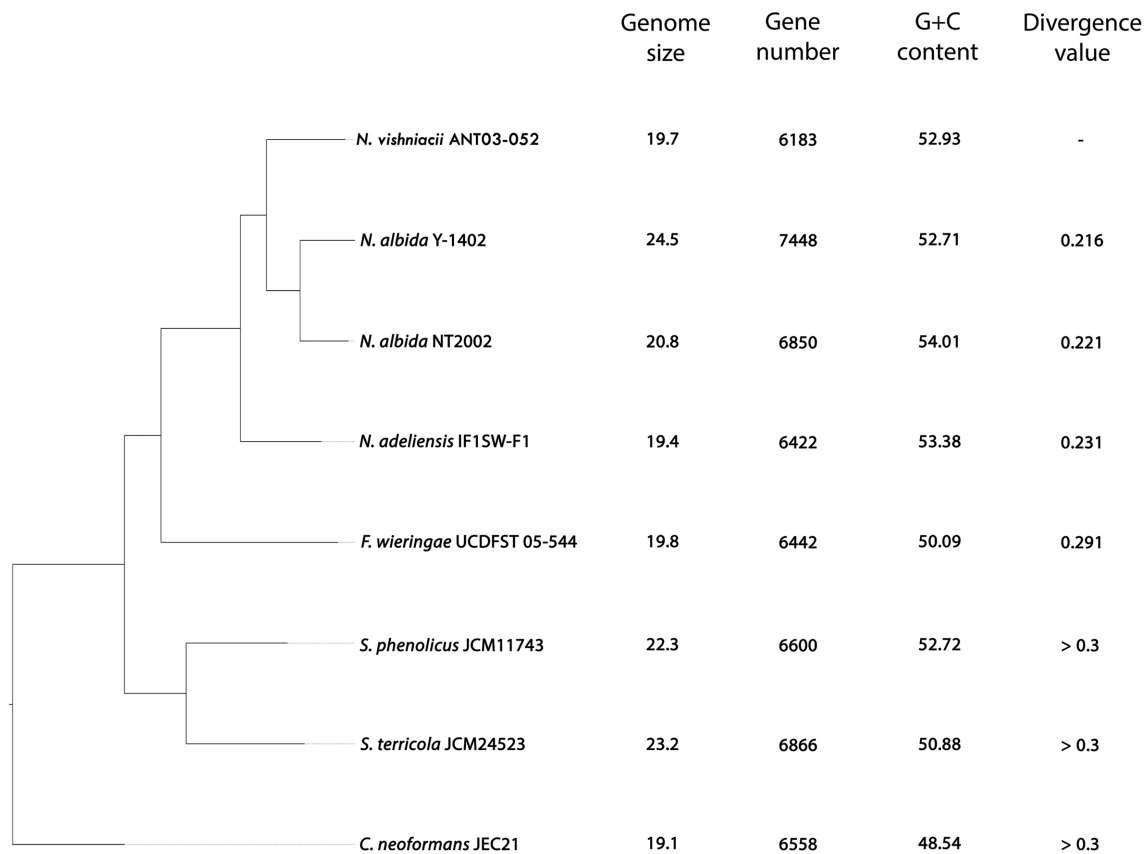


Figure 1. Phylogenetic tree of the organisms used in this study, constructed from 2130 single copy ortholog protein sequences using RAxML v. 8.2.12 with the PROTGAM-MALG4M model of amino acid substitution. All nodes showed 100% bootstrap support.

(Y-1402/NT2002 respectively) are shown in Fig. 4A. The dN/dS ranged from 0 to 0.526/0.590 although the 98% of the genes had $dN/dS < 0.2$ (81/79% with $dN/dS < 0.1$, respectively). Even though no gene showed dN/dS mean value > 1 , by means of a sliding windows strategy we could detect that 65/77 annotations (1.9/1.6% respectively) overcame $dN/dS > 1$ in any region between *N. vishniacii* and *N. albida* strains and 49/23 of those also showed a $dN/dS > 1$ between *N. vishniacii* vs. *F. wieringae* pairwise comparisons. One by one comparison of these annotations among the three species using JCoDA resulted in 31/13 (0.79/0.30%) orthologs encompassing possible evidence of positive selection, with $dN/dS > 1$ in shared regions between *N. vishniacii* vs. *N. albida* (YN-102/NT2002 strains) or *F. wieringae*, but not between *F. wieringae* vs. *N. albida* (numbers shown in brackets in Fig. 4A). Among them, nine showed evidence of positive selection in both *N. albida* strains. Orthogroups were classified by means of eggNOG functional categories (Fig. 4B). Function of most of the predicted genes (42.9%) could not be inferred (grouped as Function unknown). Post-translational modification, protein turnover and chaperones (20%), RNA processing and modification (8.6%) and Replication, recombination and repair (8.6%) were the most represented categories. A total of ten of these 35 genes also matched BUSCO database (EOG nomenclature in Fig. 4B).

Survey of genes involved in cold tolerance

A survey of specific genes related to cold adaptations demonstrated that *N. vishniacii* possesses genes involved in photoprotection and antioxidant responses as well as in the synthe-

sis of trehalose. Furthermore, we were able to identify genes involved in lipid transport and metabolism in *N. vishniacii*. Homology search of antifreeze proteins yielded positive results in *N. adeliensis* genome only. This species possesses a gene with a high homology ($< 1e-11$) to a characterized AFP glycoprotein from *Rhodotorula toruloides*. For the rest of the Filobasidiales species, we could not find any homology evidence employing the 3998 AFPs queried. Regarding genes involved in photoprotection and antioxidant responses, a cluster of three genes previously shown to be involved in mycosporine-glutaminol-glucoside (MGG) synthesis (Bellora et al. 2016), consisting of DHQS homolog (2-epi-5-epi-valiolone synthase, EEVS-like; Nv.000059), O-methyltransferase (O-MT; Nv.000060) and an ATP-grasp (Nv.000058) was found in all the *Naganishia* genomes as well as in *F. wieringae* and *S. terricola*. Queried sequences were not found in *S. phenolicus* nor in the outgroup species. MGG biosynthesis was experimentally confirmed for all positive species: *F. wieringae* (Muñoz 2010, Brandão et al. 2011), *S. terricola* (Libkind et al. 2009) and *Naganishia* spp. (Guzmán 2016 and in all three conditions tested in this work). The complete set of genes involved in the synthesis of carotenoids were characterized in *N. vishniacii* genome (Nv.003146: isopentenyl diphosphate isomerase; Nv.004897: farnesyltransferase; Nv.000648: farnesyl pyrophosphatase synthetase; Nv.002341: phytoene dehydrogenase; Nv.005201: phytoene-beta carotene synthase; Nv.004862: NADP-cytochrome P450 reductase; Nv.000692: beta-carotene 15,15-monooxygenase) as well as in both *N. albida* strains, *N. adeliensis* and *F. wieringae*, but not in the other studied species (Table S3, Supporting Information). *Naganishia vishniacii* colonies are creamy to light pink colored when cultured under different

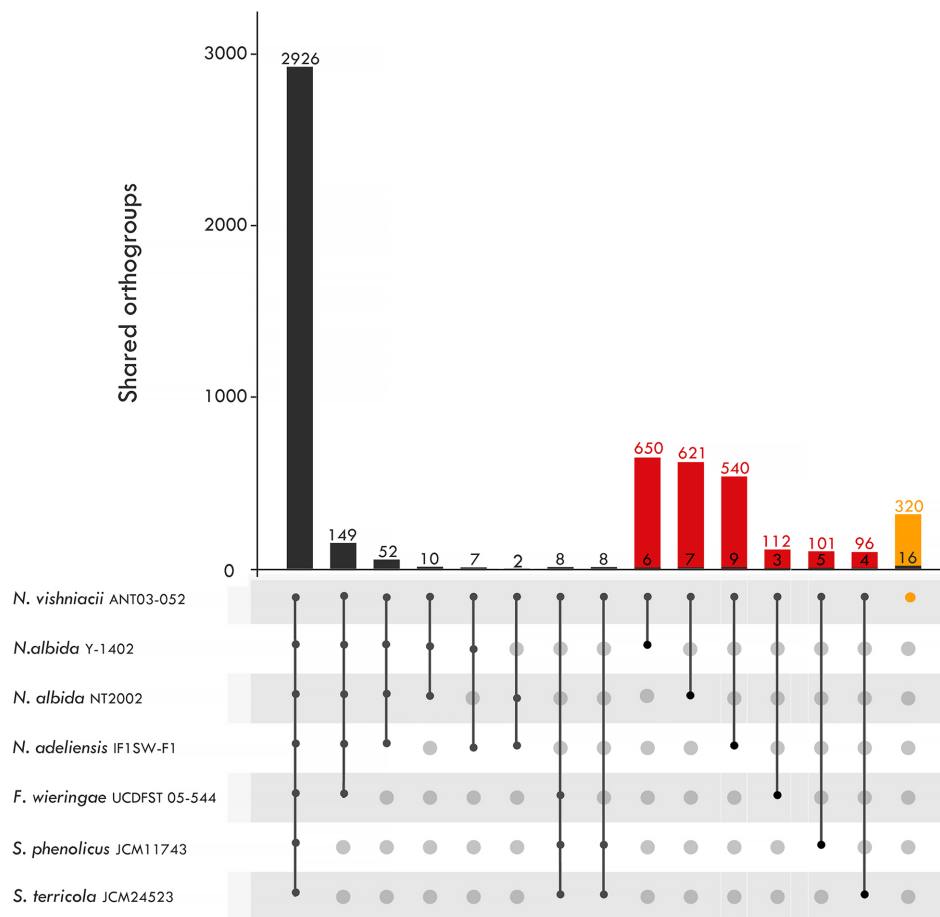


Figure 2. Orthology analysis of seven Filobasidiales species. Black bars represent the number of genes present in shared orthogroups. Filled dark dots denote presence and empty dots (light gray) indicate absence of orthogroups in each species. Only main intersections/overlap relative to *N. vishniacii* are shown. Predicted proteins pertaining to *N. vishniacii* that did not cluster in orthogroups but with positive tblastn hits against the other genomes are represented by red bars above each pairwise intersection. Yellow vertical bar represents genes in *N. vishniacii* not clustered by eggNOG and not matching the rest of the Filobasidiales genomes used in this study. Plot visualized with UpSetR (Conway, Lex and Gehlenborg 2017).

conditions, however experimental confirmation of carotenoid production was negative in all tested conditions used in this work.

Genes involved in trehalose synthesis were identified in all of the eight species in this work. Backbone enzymes involved in the formation (TPS1 and TPS2) and degradation (NTH1/2, ATH1) of these two glucose molecules disaccharide were screened in the proteomes. One putative copy of TPS1 and TPS2 enzymes were found in all Filobasidiales species (TPS1 was found in consecutive predictions on scaffold 2: Nv_002644 and Nv_002645 and TPS2 was found on scaffold 17: Nv_002256). Trehalase gene NTH1 (Nv_002469) was found as a single copy in all the genomes (Table S4, Supporting Information). Regarding lipid metabolism genes, $\Delta 4$ (Nv_004586), $\Delta 8$ (Nv_004525) and $\Delta 12$ (Nv_000815) desaturases were found in all the genomes from Filobasidiales species by looking for specific protein domains and the presence of $\Delta 9$ (Nv_003331) desaturase was confirmed in all analyzed genomes except for *S. terricola*. Orthology clustering of enzymes involved in lipid metabolism yielded between 119 and 136 groups composed of unidentified hypothetical proteins in the seven Filobasidiales species, with 78 groups common to all. In addition, 49 groups were annotated with associated functions, with 37 groups shared among all the species. Even though the copy numbers of enzymes pertaining to each group were similar among species, an enzyme related to putative enoyl-CoA-hydratase was

not found in any of the *Naganishia* species and sterol 3-beta-glucosyltransferase was not found in *Naganishia* or in *F. wieringae* genomes (Table S5, Supporting Information).

Analysis of amino acids usage

Differences in amino acid usage was computed for the common-to-all set of single copy orthologous genes (ScCOg) among the seven Filobasidiales species and differences in usage were compared with *C. neoformans*, and also between *N. vishniacii* and the rest of the *Naganishia* species. Enrichment in six amino acid usage (Ala, Arg, Asp, Gln, Met and Thr), and depletion in other five amino acids (Glu, Lys, Pro, Ser and Trp) was observed in all the *Naganishia* species compared with *C. neoformans* ($p < 0.001250$; Fig. 5).

Comparison among *Naganishia* species showed a lower frequency of Ala usage in *N. vishniacii* with the other studied species belonging to the genera. A depletion in Pro and an enrichment in Ser was also confirmed when comparison was made against both *N. albidia* strains.

In contrast, when comparison was made over CEG dataset significant differences were found in one amino acid only, with an enrichment of Gln (p -value = 0.001250) for *N. vishniacii* and *N. adeliensis* species with respect to *C. neoformans*, but no significant differences in amino acid composition were observed between

Orthogroup	<i>N. vishniacii</i> ID	COG functional category	Functional description	#Nv	#NaY	#NaN	#Nad	#roF
ENOG410PGGB	Nv_000525 Nv_001541 Nv_003488	RNA processing & modification	Dicer-like endonuclease	3	2	2	1	2
ENOG410PGKM	Nv_002463 Nv_003321 Nv_003922	AA transport & metab. post-translational modif. prot. turnover & chaperones	Aminopeptidase	4	2	1	1	1-2
ENOG410PMCP	Nv_003280 Nv_003328 Nv_004462 Nv_005845 Nv_005846	Energy production & conversion	Oxidoreductase	5	4	3	3	2-4
ENOG410PII4	Nv_000220 Nv_003422 Nv_005291 Nv_005418	Carbohydrate transport & metabolism	alpha-1-6- mannosyltransferase	4	2	1	2	1-3
ENOG410PNF7	Nv_003563 Nv_004049 Nv_004279		Glycosyl hydrolase family 71	3	1	1	3	1-2
ENOG410PNRT	Nv_002066 Nv_002068 Nv_002408 Nv_004073	Function unknown	SUR7/Pall family	4	3	3	3	1-2
ENOG410PWG1	Nv_001317 Nv_001333 Nv_003778		NA	3	2	2	5	2
ENOG410PJ40	Nv_001387 Nv_001586 Nv_003277	AA transport & metab. post-translational modif. prot. turnover & chaperones	Amidohydrolase	3	2	1	2	1-4
ENOG410PFJG	Nv_000658 Nv_003680 Nv_003681		MFS transporter	3	1	2	2	1-3
ENOG410PH2P	Nv_000862 Nv_001298 Nv_005799			3	2	2	2	2-3
ENOG410PFKH	Nv_000275 Nv_000284 Nv_002412 Nv_005830	Carbohydrate transport & metabolism	Quinate permease	4	3	3	3	3-7
ENOG410PF97	Nv_001371 Nv_002166 Nv_002181 Nv_002182 Nv_005515		Maltose permease, trehalose transport- related protein	5	3	1	4	5-16

Figure 3. Orthogroups in which *N. vishniacii* copy numbers were higher than *N. albida*. Copy numbers in *N. vishniacii* (#Nv), *N. albida* Y-1402 (#NaY), *N. albida* NT2002 (#NaN) and *N. adeliensis* (#Nad) and observed range in the rest of the Filobasidiales species (#roF) are shown. Green highlighted: groups in which the rest of the Filobasidiales showed higher or equal copy numbers than *N. vishniacii*.

both *Naganishia*. Similar magnitude of fold change was observed for both gene sets (SccOg and CEGs) for that amino acid. A complete list with compositional changes for both sets of genes is available as Table S6 (Supporting Information).

DISCUSSION

Genome assembly, gene predictions and annotations

Using a combinatorial approach as described in the methods, we were able to generate a high quality genome assembly for *N. vishniacii* (ANT03-052; CBS 10616) with a low number of scaffolds (38), a very low number of Ns (0.13%) and a high number of identified complete BUSCO (90.9%) and CEGs genes (99.2%). The genome size of *N. vishniacii* was smaller than those of *N. albida*

strains but similar to *F. wieringae* and *N. adeliensis*. The predicted gene number was also considerably lower than that observed for all the other species in this study. Gene loss is a pervasive phenomenon in all organisms with positive effects (Albalat and Cañestro 2016) given that in free living microorganisms gene loss can be beneficial when they are inhabiting oligotrophic or extreme environments (García-Fernández, de Marsac and Diez 2004; Ullrich et al. 2016; Zhang et al. 2017) or are in complex microbial communities, particularly in homogeneous environments (Morris, Lenski and Zinser 2012; Mas et al. 2016). Although the impact of gene loss in yeast fitness is still a matter of debate (Thatcher, Shaw and Dickinson 1998; Sliwa and Korona 2005; Lang, Murray and Botstein 2009), the considerably low number of genes in *N. vishniacii* with respect to the other related species suggest that this can be an adaptive feature to an environment

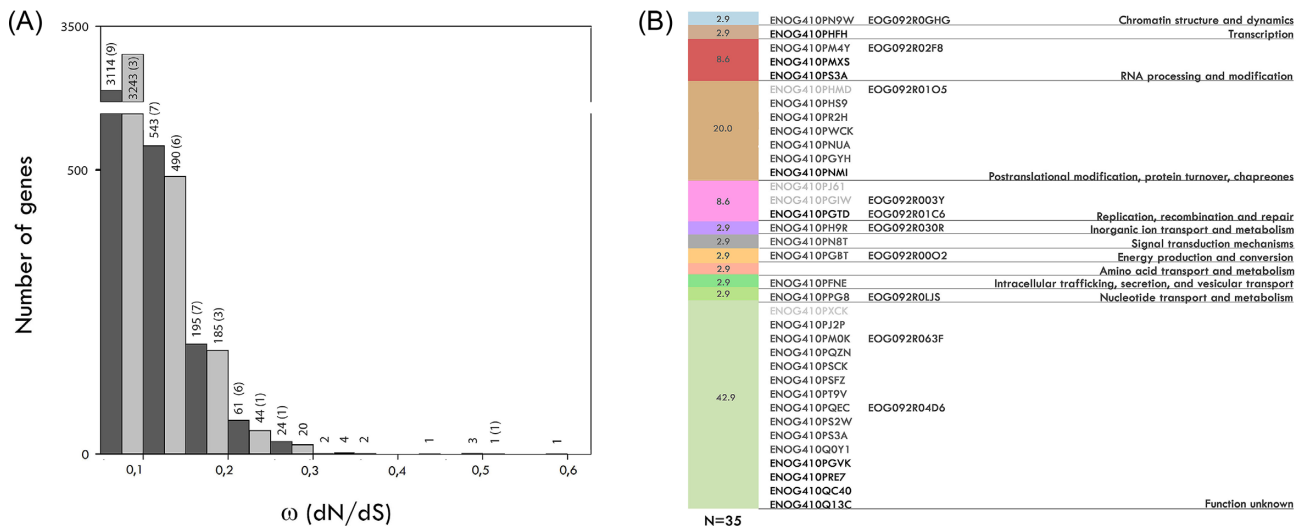


Figure 4. dN/dS analysis for single copy shared genes. **(A)** Distribution of dN/dS mean values for 3942/3991 orthogroups shared among *N. vishniacii* and *N. albida* Y-1402 (dark bars) and NT2002 (light bars) strains. Shown in brackets: numbers of genes with dN/dS > 1 considered under positive selection in any region (n = 35). **(B)** eggNOG functional categories of genes under positive selection. Groups with positive signal among *N. vishniacii* and both strains (bold), Y-1402 (dark color) only and NT2 (light color) only. Genes belonging to the BUSCO database are shown (EOG).

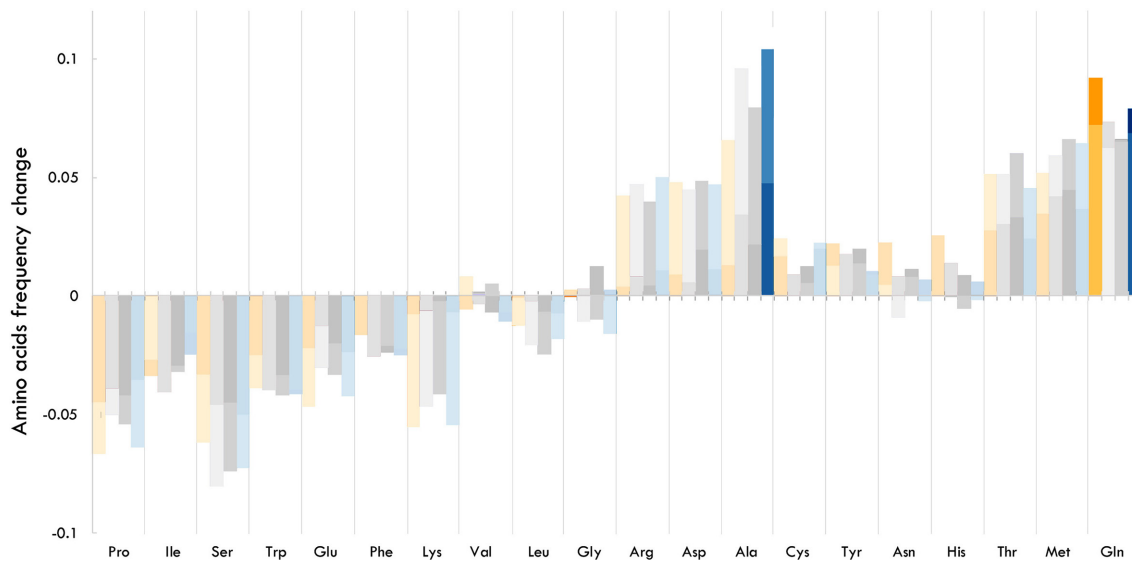


Figure 5. Amino acid frequency changes in *N. vishniacii* (orange), *N. albida* strains Y-1402 and NT2 (dark and light gray respectively) and *N. adeliensis* (blue) with respect to *C. neoformans*, tested in conserved CEGs genes (dark colors) and SccOg (light colors). Highlighted bars show amino acids with significant differences for both sets of genes.

with low resources and many stressing factors but relatively constant conditions such as low temperature, low water activity and high solar irradiation (Selbman et al. 2014).

Orthologous genes and functional assignment

Comparison between orthologous groups of genes among all the Filobasidiales genomes available to date as well as *C. neoformans* allowed us to identify that most of the genes present in *N. vishniacii* were also present in related species. Interestingly, orthology inference resulted in a set of 44 candidate genes corresponding to 12 orthogroups (Fig. 3) with increased copy numbers (gene redundancy) in *N. vishniacii* genome when compared with *N. albida* strains and, in most cases, also with the rest of the Filobasidiales species, and 16 genes (corresponding to 16 orthogroups) unique to *N. vishniacii*.

Gene redundancy is usually related to an increase in dosage of encoded genes (Schuster-Böckler, Conrad and Bateman 2010) and a mechanism of adaptation to stressful or novel environmental conditions (Kondrashov 2012). Among the functional groups with greater gene redundancy in *N. vishniacii*, the major facilitator superfamily (MFS) reported here has been previously proposed as part of an important mechanism for cold adaptation in the obligate psychrophilic yeast *Mrakia psychrophila* (Su et al. 2016), probably contributing to the accumulation of nutrients from the environment in order to give rise to all the other metabolic and rearrangement modifications needed to counteract cold imposed conditions. For other functional groups, the relationship with cold is not clear. Different organisms counteract shifts in temperature by overexpression of genes involved in modulating RNA processing in different ways (Phadtare, Inouye and Severinov 2004; Zhu, Dong and Zhu 2007) such as by the

production and accumulation of reserve carbohydrates as cold protectant metabolites (Schade et al. 2004), and by modification of membrane lipid composition to maintain an appropriate physical state and functionality (discussed below). Gene redundancy in Dicer-like endonucleases, oxidoreductases (enoyl reductases), and in trehalose transport proteins could be related to those general responses.

By means of our comparative approach we were also able to unravel 16 orthogroups that appeared to be present only in the *N. vishniacii* genome (with respect to the other six genomes used for comparison in this work). Most of them were associated with signal transduction mechanisms, secondary metabolites biosynthesis/transport/catabolism and transcription. Also, groups related to carbohydrate transport and metabolism, RNA processing and modification and cell wall/membrane biogenesis were present. Among them we found one gene containing the MFS domain (Nv.002329) that provides additional evidence for the role of this group in cold adaptation; one transferase (Nv.005274), a functional group that was previously suggested as having important role in cold tolerance of Antarctic bacteria for being involved in membrane biogenesis, carbohydrate, proteins or lipids modifications (Grzymalski et al. 2006) and one gene related to chaperones (Nv.005012), enzymes required for protein folding, important for protein conformation maintenance in the cold (Ferrer et al. 2003) and reported to be involved in a synergistic effect together with trehalose during yeast adaptation to near-freezing temperatures (Kandror et al. 2004).

Finally, for the 320 unique genes of *N. vishniacii*, not grouped by orthology clustering nor found in the other species in this work, the vast majority (297) did not yield significant matches in the GenBank non-redundant database. Similar numbers of genes without matches were observed in yeasts belonging to the Basidiomycetes and the Ascomycetes (Bellora et al. 2016; Levasseur et al. 2014; Blevins et al. 2019) and are usually categorized as putative orphan or lineage-specific genes. These are in some cases novel genes but they mostly have undetectable homology with other related sequences (Weisman, Murray and Eddy 2020) and are often related to ecological adaptations (Wilson et al. 2005). Further analyses are needed to understand if any of these predictions are related to the extreme habitat preference of *N. vishniacii*.

dN/dS analysis

Distribution of dN/dS mean values among *N. vishniacii* and *N. albida* strains' single copy shared genes revealed that most genes are under negative selection. However, selective pressure on genes generally occurs during some lapses of time on the evolution affecting only a few amino acids on specific regions of domains and dN/dS is usually lower than one in divergent species, moreover values lower or higher than one can occur under both negative and positive selection (Kryazhimskiy and Plotkin 2008; Spielman and Wilke 2015). For those reasons the averaged dN/dS ratio is tricky and rarely exceeds values greater than one (Gillespie 1994). By means of a sliding windows strategy we could find 35 genes with regions exceeding dN/dS > 1 thus encompassing possible evidence of positive selection. Since our search strategy was highly conservative and we only kept genes with selection signals present in *N. vishniacii* only, this set of 35 genes is highly significant in terms of evolutionary history.

Among them, Almost 40% of the genes were functionally related with translational modification, protein turnover and chaperones, RNA processing and modification and replication, recombination and repair, as the main represented groups. In

this sense, it is important to highlight that all these genes are involved in genome expression and protein folding and, as mentioned before, the role of these functional categories in cold tolerance has already been reported for psychrotolerant organisms (Phadtare, Inouye and Severinov 2004). In total, two genes are remarkably important considering the environment from which *N. vishniacii* was isolated. The gene Nv.005203 (ENOG410PSCK) showed the highest mean value for those whose function could be inferred. HMMER search of Nv.005203 reveals that this gene is related to the Uniprot target A0A1E3HK01 of *C. depauperatus*, and Q5KHT7 of *C. neoformans*, both related with the function of sensing extracellular osmolarity to initiate a change in cell activity, and to span the membrane of the cell. *N. vishniacii* was described not only as a psychrophilic yeast, but also as a xerotolerant species (Vishniac 2006). Considering that the McMurdo Dry Valleys are the most extreme cold desserts known (Vishniac and Hempfling, 1979b) and lack of water of its xeric soils is one of the main factors limiting microbial growth there (Klingler and Vishniac 1988), this selection pressure on Nv.005203 gene is most probably related with the need to survive in environments with low water activity. One of the most studied responses in xerophilic fungi to survive and grow at reduced water availability is the intracellular accumulation of compatible solutes like glycerol, to maintain balance between water availability inside and outside the cell (Vinnere and Leong 2001). The membrane osmosensor Nv.005203 found in this analysis is highly similar to the Sho1 gene described for *S. cerevisiae* and *Aspergillus* spp., involved in the synthesis and accumulation of glycerol (Salazar et al. 2009). In *S. cerevisiae* this protein is part of a transduction cascade that, upon different stresses (including cold, hyperosmotic and oxidative stresses), trigger transcriptional responses (Rodríguez Peña et al. 2010). Also remarkable was the positive signal in gene Nv.003572 (ENOG410PGIW). In this case, HMMER search yielded a putative serine threonine protein kinase related to A0A1E3IMU4 Uniprot target, from *C. depauperatus*. This protein is a DNA damage response kinase that belongs to the phosphatidylinositol 3-kinase-like (PIKK) family. Members of this family are activated upon DNA damage further amplifying the signal by activating downstream kinases (Durocher and Jackson 2001). In cells with damaged DNA due to genotoxic stresses such as ionizing radiation, ultraviolet light or DNA replication stalling (Kozhina, Kozhin and Korolev 2011), propagation of the signal results in cell-cycle arrest and stimulation of DNA repair (Bøe et al. 2018). Selection of this gene, thus, may be related to the high incidence of solar radiation characteristic of this harsh environment (Cary et al. 2010) as will be discussed below.

The function of the remaining genes with strong signals of positive selection could not be inferred, and were annotated as hypothetical proteins. Functional relevance of these predictions in the response of *N. vishniacii* to its habitat's extreme conditions should be the target for further studies.

Survey of genes involved in cold tolerance

Genome mining in the genome of *N. vishniacii* for genes previously known to be related to cold tolerance in yeasts and other organisms suggested the presence of most of these traits but surprisingly the lack of others, for example antifreeze-proteins (AFPs). AFPs have been identified in various psychrophilic organisms (Ewart et al. 1999), including yeasts such as basidiomycetous species of the genera *Glaciozyma*, *Leucosporidium* and *Rhodotorula* (Kim et al. 2014). Although AFPs are thought to prevent cell damage from recrystallization of extracellular ice

(Lee et al. 2010; Hashim et al. 2014), after a deep search of available yeast and bacterial AFPs, we were not able to find similar sequences in *N. vishniacii*. Because this species lives in Antarctic soils, a strategy to avoid the formation of ice crystals was expected. However, it is important to highlight that AFPs possess a large, relatively flat and hydrophobic plane on the protein surface and commonly have repeating motifs (Wang et al. 2017), and since this characteristic is common to several proteins, AFPs evolved several times in different organisms from different peptides (Cheng 1998). For those reasons, we cannot eliminate the possibility of other still unknown AFP in *N. vishniacii*, different from those already described in other organisms.

We were able to detect genes involved in the synthesis of antioxidant and UV sunscreen molecules (carotenoid pigments and mycosporines). Production of carotenoids and UV-screening compounds is a wide-spread trait present in several yeasts species (Libkind et al. 2011; Moliné et al. 2014) and both compounds play an important role in photoprotection and possess antioxidant properties (Moliné et al. 2009; 2011). In cold rock-associated ecosystems, such as the ones of the McMurdo Dry Valleys, high solar irradiation is a limiting stress factor for life (Onofri et al. 2007). In these environments yeasts species from the orders Filobasidiales and Tremellales are usually isolated (Selbmann et al. 2014). The presence of photoprotective compounds in yeasts from such environments was expected. All known genes responsible for the synthesis of both carotenoid pigments and mycosporines (particularly MGG) were unambiguously found in *N. vishniacii*, as well as in the rest of the *Naganishia* species and *F. wieriingae* but not in the other species in this study. The presence of genes involved in the production of carotenoids and MGG make sense considering that Antarctica is a region of high UV radiation incidence as a result of the long solar radiation periods in summer and the ozone reduction (Madronich et al. 1998; McKenzie et al. 2011) and it has been reported that *Naganishia* species are among the more resistant organisms to UV radiation (Schmidt et al. 2017). However, we could not experimentally confirm carotenoid synthesis in *N. vishniacii* even under photostimulation and oxidative conditions. Moreover, the three *Naganishia* species and *F. wieriingae* usually grow in culture as white to creamy colored colonies (Viviani and Tortorano 2009; Kurtzman, Fell and Boekhout 2011). It is still not clear whether the lack of carotenoids in culture colonies of *N. vishniacii* is due to a failure to meet the necessary conditions to stimulate their synthesis or a possible inhibition at the level of gene expression. On the contrary, the accumulation of MGG was experimentally confirmed for *N. vishniacii*, suggesting that this molecule is enough to cope with extreme UV radiation conditions in Antarctic environments.

The presence of the primary enzymes involved in the synthesis and degradation of trehalose were also confirmed in all the Filobasidiales genomes. Trehalose is a well-known protective metabolite against harsh environmental conditions such as freezing temperatures (Wiemken 1990) that helps to maintain membrane integrity and function (Rudolph, Crowe and Crowe 1986). In yeasts, increasing intracellular trehalose accumulation is sufficient to confer high tolerance to desiccation (Tapia et al. 2015). The presence of trehalose genes together with the expanded trehalose transport proteins described above, suggests their capability to synthesize this important compound that probably helps to withstand cold conditions and low water availability of the McMurdo Dry Valleys.

In the same way, the presence of a complete set of desaturases genes in *N. vishniacii* is in agreement with the capability

to respond to cold. Membrane fluidity can be regulated by changing the lipid composition (Ernst et al. 2016), and the number of double bonds in phospholipid fatty acyl chains are increased at low temperature to maintain the appropriate physical state of the membrane (Chintalapati, Kiran and Shivaji 2004). Recombinant expression of Δ -12 fatty acid desaturase in *S. cerevisiae* enhances their survival at low temperatures (Shi et al. 2012). The complete set of desaturases described for Filobasidiales, including the presence of Δ -12 fatty acid desaturases, indicate the ability of these species to regulate the membrane fluidity under extreme cold conditions. Moreover, when we individually compared gene redundancy of a variety of enzymes involved in lipid metabolism, we determined that all the *Naganishia* species used in this work, as well as the closely related *F. wieriingae* genomes lack sterol 3-beta-glucosyltransferase. This enzyme catalyzes the synthesis of sterol glycosides and acyl sterol glycosides and is ubiquitous in fungi (Grille et al. 2010). The attachment of a sugar moiety to the 3-hydroxyl group of a sterol increases the size of the hydrophilic head of the lipid changing its biophysical properties and it has been observed that a decrease in sterol glycosides and acylated sterol glycosides content was correlated with an increase in resistance to freezing stress (Thieringer et al. 1998). Lack of this enzyme probably serves as a complementary trait contributing to the cold tolerance of *N. vishniacii*.

Analysis of amino acids usage

When comparing differences in the amino acid usage, we found a considerable enrichment of glutamine for *N. vishniacii*, a phenomenon particularly evident in the group of highly conserved CEGs and higher in magnitude than on its psychrophilic cognere *N. adeliensis* (Scorzetti et al. 2000). Increased flexibility has been proposed to be the main structural feature of cold-adapted enzymes to compensate for the reduction in chemical reaction rate inherent to low temperatures (Gerday et al. 2000; Gianese, Argos and Pascarella 2001). For this reason, a higher usage of small residues is proposed to be preferred by these enzymes (Adekoya et al. 2006). In this way, the energetic cost of conformational changes required to interact with substrates could be reduced. Increased catalytic efficiency is a key feature for low-temperature enzymes that have to compensate for slow metabolic rates that would occur at low temperatures due to the low kinetic energy available to overcome reaction barriers (Georlette et al. 2004).

The relationship between glutamine usage in enzymes and temperature has been previously described in Archaeas, following an almost linear trend over the complete range of optimum growth temperatures from psychrophiles to hyperthermophiles (Saunders et al. 2003). Notably, *N. vishniacii* glutamine percentage was the highest among all the compared species.

Also, a marked trend in reducing proline content was observed in *N. vishniacii* when compared with *C. neoformans* and also with both *N. albida* strains, but not with *N. adeliensis*. Although this change was not statistically significant, it is remarkable as a similar trend has already been reported as an attribute of cold-adapted enzymes (Feller and Gerday 1997). Proline usage in peptides restricts backbone rotations and reduces flexibility (Siddiqui and Cavicchioli 2006), and for that reason proline is a more common amino acid in proteins adapted to high temperatures (Aghajari et al. 1998; Spiwok et al. 2007; Sælensminde, Halskau and Jonassen 2009; Tronelli et al. 2007). Depletion of Pro in *N. vishniacii* and in *N. adeliensis* may be in line with adapting to their freezing environments. To our knowledge,

this is the first time that changes in amino acids usage related to cold adaptation are reported for yeasts.

FINAL REMARKS

Understanding adaptations to the environment has been one of the main questions in biology from the time of Darwin to the present. Scientists seek to understand how evolution shapes these characteristics in all organisms and what are the particular changes that make organisms more suited to their environments. Comparative genomics is shedding light on the understanding not only of the phylogenetic relationships between species, but also of the molecular mechanisms involved in sequence evolution, speciation, genome rearrangements, the origin of new genes as well as the evolution of those involved in different adaptations (Koonin, Aravind and Kondrashov 2000; Miller et al. 2004; Wolfe 2006). Availability of an increasing range of yeasts genomes is a powerful source of information for determining the genetic basis for phenotypic variation, the mechanisms of genomic rearrangements and those involved in speciation and isolation. They also help to elucidate processes that take place on short timescales, such as recent adaptation of species to their environments (Wolfe 2006; Marsit et al. 2016; Riley et al. 2016; Libkind et al. 2020). In this work we explored the genome of the extremophile yeast *N. vishniacii* and were able to reveal a plethora of genomic traits that may be the underlying basis of its capability to grow in the extreme environments of Antarctic dry soils.

To the best of our knowledge, since this species was first isolated from the Beacon supergroup sandstone (Vishniac and Hemphling 1979a), no report of its presence has been made outside this zone in Antarctica (Buzzini and Margesin 2014). Even though many authors have proposed much of the microbiota of Antarctica as potentially originating elsewhere and colonizing Antarctica by aerial dispersion (Vincent 2000; Schmidt et al. 2017). The ability of *N. vishniacii* to withstand daily and yearly environmental extremes of its peculiar habitat and its repeated occurrence in this place only, suggest it may constitute an endemic species, since emigrant recolonization of vacant sites, or sites with unstable biodiversity, is expected to be random with respect to the taxon (Vishniac and Hemphling 1979b). Even though lack of systematic and culture-independent studies in this region, make biogeography, ecological relevance and patterns of spatial distribution of fungi an unrevealed matter (Dreesens, Lee and Cary 2014), presence of specific traits of adaptation in *N. vishniacii*, much of them postulated in this work for the first time, supports a tight association of this species to this place.

Comparative genomics allowed the identification of a set of candidate genes probably involved in cold adaptation and also different trends during the evolution of *N. vishniacii* genome in relation to its capability to cope with extreme cold conditions, including genes that are unique, in higher copy number, carrying signatures of positive selective pressure, involved in specific metabolic pathways, and with unique changes in amino acids composition. To the best of our knowledge, such a combination of genomic features for cold tolerance has not been previously reported in fungi. While the genome sequence analyses are the first steps to understand the adaptation of a given organism to life under extreme conditions, further integrative *omic* studies are required to infer the possible underlying mechanisms.

AUTHOR CONTRIBUTIONS

P.N. carried out experiments, assembled and merged genomes, performed annotation and bioinformatics analysis, analyzed data and wrote the manuscript. N.B. assisted, performed and supervised bioinformatics analysis. S.H. annotated genome and submitted genomic data to GenBank. H.S. performed the original genome assembly. C.D. sequenced genome. K.B. and I.G. coordinated genome sequencing, annotations and data submission. L.C. collected the samples and isolated the yeast. M.M., L.C., D.L. and N.B. conceived the study and contributed with the writing and revision of the manuscript. M.M. also assisted with data analysis, bioinformatics analysis and experiments. D.L. and M.M. provided the funding.

ACKNOWLEDGMENTS

The authors thank the following researchers for permission to use genome data prior to publications: Rikiya Endoh and the Genome Information Upgrading Program of the National BioResource Project of the MEXT, Japan for *S. phenolicus*, Dan Close for *S. terricola*, and Kyria Boundy-Mills for *F. wieringae*. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract number DE-AC02-05CH11231.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSyr](https://femsyr.onlinelibrary.wiley.com/doi/10.1111/femsyr.10002) online.

FUNDING

This work was partially supported by Consejo Nacional de Investigaciones Científicas y Técnicas [PIP11220130100392CO], Fondo para la Investigación Científica y Tecnológica [PICT 2542 to D.L., PICT 0565 to M.M.] and Universidad Nacional del Comahue [B199].

Conflicts of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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