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## Searching for Cold-Adapted Microorganisms in the Underground Glacier of Scarisoara Ice Cave, Romania

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# SEARCHING FOR COLD-ADAPTED MICROORGANISMS IN THE UNDERGROUND GLACIER OF SCARISOARA ICE CAVE, ROMANIA

## ISKANJE NA MRAZ PRILAGOJENIH MIKROORGANIZMOV V PODZEMNEM LEDENIKU LEDENE JAMI SCARISOARA (ROMUNIJA)

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### Abstract

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*Alexandra Hillebrand, Corina Itcus, Ioan Ardelean, Denisa Pascu, Aurel Perșoiu, Andreea Rusu, Traian Brad, Elena Popa, Bogdan P. Onac & Cristina Purcarea: Searching for cold-adapted microorganisms in the underground glacier of Scarisoara Ice Cave, Romania*

Scarisoara Ice Cave (Romania) hosts one of world's largest and oldest underground glacier. While no studies were carried out on the existence of microorganisms in this cave's ice block, our interest is to investigate the presence of microorganisms and their chronological distribution in the cave's subterranean ice in relationship with past climatic changes. Samples were collected from ice layers of different age (from present to ~900 cal. yrs. BP), and the diversity of embedded microbial communities was assessed by classical cultivation and molecular techniques. The microorganisms from ice-sediments were cultivated at 4 °C and 15 °C, in the presence and absence of light. Epifluorescence microscopy analysis indicates the presence of autotrophic prokaryotes and eukaryotes in sunlight-exposed ice and water samples. Total DNA was isolated from each ice sample and the bacterial and eukaryotic SSU-rRNA genes were amplified by PCR. The chemical composition and organic content of both deeply buried (>10 m inside the ice block) and surface (supra-glacial pond water) habitats were analyzed in relation to their age and organic composition. This study is the first to report on the presence of both prokaryotic and eukaryotic microorganisms in the subterranean ice block of Scarisoara Ice Cave, thriving in both organic-rich ice and clear ice layers. Phototrophic prokaryotes and eukaryotes were identified in sun-exposed recent ice. The compo-

### Izvleček

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*Alexandra Hillebrand, Corina Itcus, Ioan Ardelean, Denisa Pascu, Aurel Perșoiu, Andreea Rusu, Traian Brad, Elena Popa, Bogdan P. Onac & Cristina Purcarea: Iskanje na mraz prilagojenih mikroorganizmov v podzemnem ledeniku Ledene jami Scarisoara (Romunija)*

Študija obravnava pojavnost in časovno porazdelitev mikroorganizmov v jamskem ledu v Ledeni jami Scarisoara (Romunija), v kateri je eden največjih in najstarejših jamskih ledenikov. Pojavnost mikroorganizmov nas zanima predvsem v povezavi s preteklimi klimatskimi spremembami. Vzorcili smo v različnih plasteh ledu, starih od 900 let do recentnih in v njih določevali združbe mikroorganizmov z klasičnim gojenjem in z molekularnimi metodami. Mikroorganizme iz lednih sedimentov smo v temi in na svetlobi gojili na temperaturah 5 °C in 15 °C. Epifluorescentna mikroskopija je pokazala prisotnost avtotrofnih prokariotov in evkariotov v vzorcih ledu in vode, ki so bili izpostavljeni sončnemu obsevanju. Iz ledu smo izolirali celotno DNK ter bakterijske in evkariotske gene male ribosomske podenote (SSU-rRNA) namnožili s polimerazno verižno reakcijo (PCR). Kemično sestavo in organski delež globoko pokopanih (več kot 10 m globoko v ledu) in površinskih (vodni bazen na površini ledu) habitatov smo povezali z njihovo starostjo in organsko sestavo. Študija prva poroča o prokariotskih in evkariotskih mikroorganizmih v podzemnem ledu jame Scarisoara, ki uspevajo tako v organsko bogatih plasteh ledu kot v prosojnim ledu. V ledu, izpostavljenem sončni svetlobi, smo odkrili prisotnost fototrofnih prokariotov in evkariotov. Sestava na mraz prilagojenih mikroorganizmov v ledu se spremi-

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sition of cold-adapted ice embedded microbiota varied with the habitat age and organic content, as resulting from dissimilarities in growth curve profiles at two different temperatures. The presence of bacteria and eukaryotes in all the analyzed samples was asserted by PCR amplification of SSU-rRNA gene fragments. These findings can be further used to reconstruct changes in the microbial diversity over the past approximately 5000 years, in correlation with climatic and environmental changes recorded by the ice block.

**Keywords:** underground glacier, climate archives, microorganisms, psychrophiles, Scarisoara Ice Cave.

nja s starostjo in organsko sestavo habitata, na kar kaže tudi razlika rastnih krivulj pri dveh različnih temperaturah. Prisotnost bakterij in evkariontov v vseh analiziranih vzorcih smo potrdili tudi z PCR namnožitvijo genskih fragmentov SSU-rRNA. Opisane ugotovitve bodo uporabne za rekonstrukcijo sprememb pestrosti mikroorganizmov v odvisnosti od okoljskih sprememb, zabeleženih v jamskem ledu v zadnjih 5000 letih.

**Ključne besede:** podzemni ledenik, klimatski arhiv, mikroorganizmi, psihrofili, Ledena jama Scarisoara.

## INTRODUCTION

The microbial diversity of cold environments was investigated from a large variety of exposed ice habitats (Priscu *et al.* 2007) such as polar ice sheets (Jungblut *et al.* 2010; Rehakova *et al.* 2010; Varin *et al.* 2010), alpine glaciers (Tscherko *et al.* 2003) and frozen lakes (Felip *et al.* 1995, Dieser *et al.* 2010), and Antarctic permanent lake ice (Priscu *et al.* 1998, Murray *et al.* 2012). Comparative geochemical and microbiological studies of ice cores drilled in polar and high mountain areas (Miteva *et al.* 2009) allowed for the identification of climate biomarkers. In ice layers of different ages from Antarctic (Abyzov *et al.* 1998; Xiang *et al.* 2005) and Alpine (Zhang *et al.* 2006) glaciers, the variation in microbial composition and abundance was correlated with climatic changes. However, very little is known to date about microorganisms communities living in ice deposits from caves, an isolated and light deprived cold environment that ensures advanced species' conservation, and no temporal-dependence studies of these microorganisms diversity were carried out on this type of habitat. Only a few studies report the isolation of bacteria (Margesin *et al.* 2004) and diatom flora (Lauriol *et al.* 2006) from ice caves, whereas the vast majority of ice caves studies targeted their palaeoclimatic information (Racovita & Serban 1990; Yonge & MacDonald 1999; Citterio *et al.* 2004; Kern *et al.* 2004; Holmlund *et al.* 2005; Luetscher 2005; Feurdean *et al.* 2011; Maggi *et al.* 2011). However, unraveling the ice cave biodiversity could complement the palaeoclimate record, in addition to the broad biotechnological potential of psychrophilic species isolated from this particular environment.

Among microorganisms commonly found in frozen environments, phototrophic prokaryotes and eukaryotes from light exposed environments play an important role in carbon and nitrogen enrichment of the environment by photosynthetic assimilation of atmospheric nitrogen and CO<sub>2</sub>, respectively (Vincent 2007; Morgan-Kiss *et al.*

2006; Jungblut *et al.* 2010; Namsaraev *et al.* 2010; Uetake *et al.* 2010). Their presence and role in perennial ice deposits from caves could be of particular interest for palaeoclimatic reconstruction.

Recently, the discovery of liquid water and ice on Mars (Levin & Weatherwax 2003; Kerr 2010), as well as the theoretical demonstration of the possible existence of ice caves on Mars (Boston 2004; Williams *et al.* 2010), increased the interest for such peculiar terrestrial environments and their biodiversity that mimic extraterrestrial conditions, thus giving an insight on exobiology aspects (Jakosky *et al.* 2003; Rampelotto 2010), but also into possible earlier environments on Earth.

Scarisoara Ice Cave (Romania), containing one of the oldest and largest subterranean ice block (Perșoiu 2011), presents an easily accessible and radiocarbon dated (~1200 cal. Yrs. BP) ice wall, with clear regular horizontal stratification (Fig. 1B) (Holmlund *et al.* 2005; Perșoiu & Pazdur 2011). This cave's ice block constitutes a chronological record of changes in climate and biodiversity embedded in yearly-accumulated ice layers.

A pioneering microbiological study carried out by Pop (1949) on Scarisoara Ice Cave reports the presence of nitrifying bacteria in the calcareous sediments from the Great Reservation. However, no microbiological studies were carried out to date on the underground ice block from this cave.

In this context, this work reports the identification of microorganisms belonging to Bacteria and Eukarya domains, including phototrophs (cyanobacteria and green algae), in recent, 400-years, and 900-years old ice sediments from Scarisoara Ice Cave. This work represents the first microbiological study of ice deposits from this underground glacier, contributing to an integrative characterization of a well-documented cave from geological, chronological, and palaeoclimatic perspectives.

## SITE DESCRIPTION

Scarisoara Ice Cave (1,165 m a.s.l., Apuseni Mts., Romania, Fig. 1A) is a short (700 m) and deep (105 m) cave, hosting one of the oldest (>5000 years) and largest (>100.000 m<sup>3</sup>) cave glacier in the world (Perşoiu 2011). The ice block formed mainly by the annual freezing in late autumn of ponded drip and rain/snowmelt water, overlapping the ice from previous year (Racovita 1927; Perşoiu *et al.* 2011a). As both rain and drip water carry relatively large amounts of organic matter (*e.g.* soil, pollen, invertebrates), the freezing process resulted in the genesis of a varve-like deposit, comprising both clear ice (1–20 cm thick) and sediment-rich layer, including organic material (Racovita & Onac 2000; Feurdean *et al.* 2011) and cryogenically precipitated calcite (Zak *et al.* 2008). Melting and sublimation due to geothermal heat and cold air circulation led to the gradual retreat of the sides of the ice block, resulting in the development of a vertical wall towards the Little Reservation, where ~1000 years old strata are exposed (Perşoiu & Pazdur 2011) (Fig. 1B).

The climate of the cave is rather stable in the warm period (April–September), the air temperature ( $T_{\text{air}}$ ) remaining constant at 0 °C in the Great Hall, the Church and Little Reservation, and slowly rising (up to 4 °C) towards the deepest parts of the cave (Racovita 1994; Perşoiu *et al.* 2011b). During the cold period (October–March), the air temperature variations in the vicinity of the cave's entrance (the Great Hall) closely (<1 hour delay) follow the external ones, dropping to –14 °C, while in the inner sections of the cave the positive temperatures are preserved. High (>95 %) relative humidity values are recorded in the entire cave throughout the year, with relatively low values occurring in winter in the Great Hall and its surrounding area, when the outside cold and drier air sinks into the cave and replaces the warm and moist air therein.

The ice block is located in the Great Hall at the bottom of the shaft. A reduced section of the ice block surface (~10 m<sup>2</sup>) from the Great Hall is exposed to sunlight, thus promoting the development of an abundant popu-

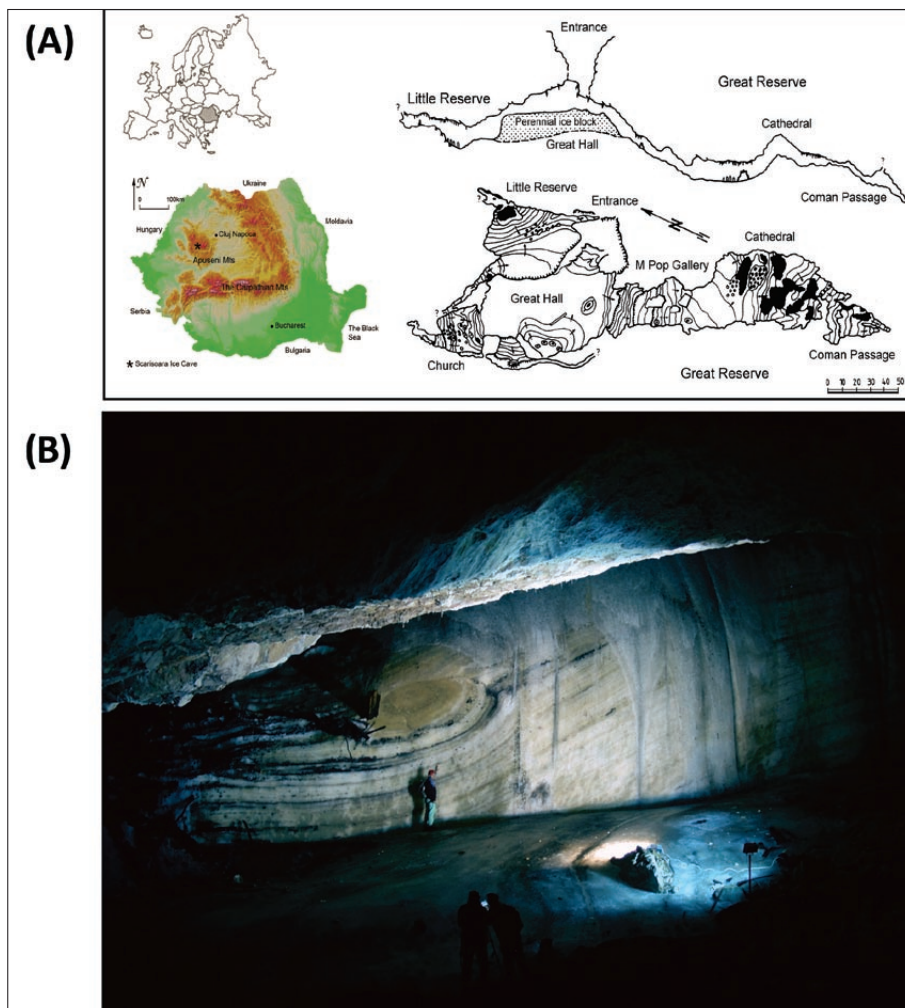


Fig. 1: Scarisoara Ice Cave map and Little Reservation ice wall. (A) Cave's location and map with cross-section. Sampling location (box): Great Hall and Little Reservation; (B) Ice wall of Little Reservation showing horizontal stratification (Photo: A. Perşoiu).



Fig. 2: Ice sampling from Scari-soara Ice Cave. (A) Little Reservation, light-deprived locations. Samples were collected from single layers within the ice wall; (B) Great Hall, light-exposed locations. Samples were collected vertically from one annual-layer of the ice floor; (C) Sterile ice collection in 1-liter flask; (D) sun-exposed supra-glacial pond (SGP) at the entrance of Great Hall containing phototrophic (micro)organisms (green) (Photos: C. Purcarea).

lation of phototrophic (micro)organisms living in the supra-glacial pond at the water-ice interface (Fig. 2D).

#### SAMPLING

Ice samples were collected during the cold period from five different sites within the glacial part of the cave (Great Hall and Little Reservation, Fig. 1A), corresponding to ice layers of different ages, organic content and light exposure regime (direct sunlight exposure, diffuse light exposure, and darkness).

**Samples 1-S and 1-L**, collected from the Great Hall (Fig. 2B), consist of one-year-old ice from two different microclimatic environments: sample **1-S** was collected near the entrance, a less isolated section of the cave, where the ice resulted from the freezing of seepage and rain/snowmelt water accumulated in a sun-exposed lake (Fig. 2D), whereas **sample 1-L** was collected from a similarly formed ice layer located in the central area of the hall, therefore less influenced by the outside environment, where only diffuse light is present. The microclimatic conditions during the ice genesis were similar for these two locations, consisting of an initial phase of water accumulation at constant air temperature of 0 °C, followed by a freezing phase trapping microorganisms and organic matter in the newly formed ice (Perșoiu *et al.* 2011a).

**Samples 400-O, 900, and 900-O**, collected from the Little Reservation (Fig. 2A), originate from ice layers of different age and organic matter content. **400-O** and **900-O** were collected from two organic-rich ice layers located at 1125.5 and 1345.4 cm bellow the pres-

ent-day surface of the ice block, whereas **sample 900** was collected from a layer of clear ice, with no visible organic content, at 1355.4 cm bellow surface. The ages of the two ice layers of **400-O** and **900-O/900** samples are 399 and 887 cal. yrs. BP, respectively (Perșoiu & Pazdur 2011).

Ice samples collection (Fig. 2) was carried out with a manually driven 3 cm diameter auger under sterile conditions, by flaming both the auger and the ice surface with a portable gas burner for 5 s, to avoid contamination with recent microbial species (Fig. 2A and 2C). About 5 cm of ice were removed from the ice block surface in both the sun- and light-exposed areas from the Great Hall (Fig. 2B), and the ice samples were collected vertically (Fig. 2B) and transferred in the presence of open flame to 1-L sterile flasks (Fig. 2C). Samples collected from the Little Reservation (Fig. 2A) were processed in a similar manner, after removing a deeper ice layer (20 cm) from the surface of the wall, due to a greater contamination risk with recent ice. The drilling was carried out horizontally, parallel to the ice lamination (Fig. 2A). The ice samples were stored at -20 °C.

Water samples (SGP) were also collected under sterile conditions in 1-L flasks from the supra-glacial pond formed during the warm period in a sun-exposed area at the entrance of the Great Hall (Fig. 2D), and stored at 4 °C until processed.

## MATERIAL AND METHODS

### CHEMICAL ANALYSIS

The chemical analysis of ice and supra-glacial sunlight exposed water samples was performed to determine the organic content, nutrients and salinity of the corresponding habitat of each microenvironment of microbial community. The chemical oxygen demand (COD) and the concentrations ( $\text{mg L}^{-1}$ ) of nitrates, calcium ions, sulfates, and chlorides were determined spectrophotometrically (Clesceri *et al.* 1999) using a Specord 200 spectrophotometer (Analytic Jena). The chemical oxygen demand (COD) was determined by CCOCr volumetric analysis using the small scale tube dichromate method based on the ISO 15705:2002 standard. Chlorides were determined by the argentometric method based on the SR ISO 9297:2001 standard. Sulfates were analyzed by precipitation with barium chloride, using a turbidimetric method (EPA 375.4). The nitrates concentration was measured spectrophotometrically after reaction with sulfosalicylic acid and alkaline treatment (SR ISO 7890-3:2000). Calcium content was determined using the EDTA titration method according to SR ISO 6058:2008.

### MICROORGANISMS CULTIVATION

Heterotrophic microorganisms from ice samples were cultivated in Luria-Bertani (LB) liquid medium supplemented with 1 % glucose (LBG) (Sambrook & Russell, 2001) at 4 °C and 15 °C. This rich medium favoring bacterial cultivation, also promotes the growth of diverse heterotrophic prokaryotes. Melted ice samples (10  $\mu\text{l}$ ) were inoculated in 3-ml liquid medium, and the cultures were incubated for 32 days at the two different temperatures, under static conditions. The microbial communities growth curves were monitored at  $\text{OD}_{600}$  using a FluStar Omega plate reader (BGM Labtech).

The presence of cultivable phototrophic prokaryotes (cyanobacteria) and eukaryotes (filamentous algae), was investigated by cultivation on BG<sub>11</sub> liquid medium at 7 °C in the presence of light under static conditions (Rippka *et al.* 1979).

### EPIFLUORESCENCE AND LIGHT MICROSCOPY

Heterotrophic and phototrophic microorganisms were analyzed by epifluorescence/light microscopy using AXIO Scope A1 microscope with Axio Cam ERC 5s (Zeiss). The cells were either untreated or stained with SybrGreen (SG) for fluorescence labeling of the entire microbial population, or with ethidium homodimer (HD) (del Giorgio & Gasol, 2008) indicating altered plasmatic membrane (dead cells) for cell viability assessment. SGP water samples were fixed with 2 % formaldehyde immediately after collection, and analyzed by light microscopy using 0.1 % crystal violet staining (Sarchizian & Ardelean 2012).

### GENOMIC DNA EXTRACTION AND PCR AMPLIFICATION

The occurrence of bacteria and eukaryotes in ice samples was investigated by PCR amplification of SSU-rRNA specific genes. 200 ml of sediment-rich samples 400-O and 900-O, and 800 ml of 1-S, 1-L, and 900 ice samples were thawed at 4 °C and filtered through 0.22  $\mu\text{m}$  sterile MF-membranes (Millipore) using a vacuum-driven stainless steel filtering system (Millipore) and Laboport vacuum pump Type N86KN.18 (KNF LAB). The cells collected on the filter were treated with 15 units of mutanolysin (Fermentas) for 1 hour at 37 °C, and the genomic DNA was further isolated using DNeasy Blood and Tissue kit (Qiagen). DNA concentration and purity were measured using the NanoDrop 1000 (Thermo Scientific).

Bacterial 16S-rRNA and eukaryotic 18S-rRNA gene fragments were amplified by PCR using specific primers (Tab. 1). The reaction mixture contained 100 ng genomic DNA, 2  $\mu\text{M}$  or 0.4  $\mu\text{M}$  of each bacterial or eukaryotic primers, respectively, 0.2 mM dNTP, 1 x *Taq* DNA polymerase buffer, 2 mM  $\text{MgCl}_2$ , and 1 unit *Taq* DNA polymerase (Fermentas).

The amplification reaction consisted of (1) initial denaturation at 95 °C for 2 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 90 s at 72 °C, and a final extension step of 5 min at 72 °C for Bacteria, and (2) denaturation at 94 °C for 130 s, followed by 35 cycles of 30 s at 94 °C, 45 s at 56 °C, 130 s at 72 °C, and a final extension step of 7 min at 72 °C for Eukarya. The DNA fragments were analyzed by 1 % agarose gel electrophoresis.

Tab. 1: Primers used for 16S/18S-rRNA gene amplification

| Specificity | Primers             | Sequence                                     | Reference                 |
|-------------|---------------------|--|---------------------------|
| Bacteria    | B8F<br>1525R        | AGAGTTTGATCCTGGCTCAG<br>AAGGAGGTGATCCAGCCGCA | Roling <i>et al.</i> 2001 |
| Eukarya     | Euk1A<br>Euk516R-GC | CTGGTTGATCCTGCCAG<br>ACCAGACTTGCCCTCC-#      | Diez <i>et al.</i> 2001   |

# GC clamp sequence is CGCCCGGGGCGCGCCCGGGCGGGGCGGGGGCACGGGGGG

## RESULTS

## CHEMICAL ANALYSIS OF ICE SAMPLES AND SUPRA-GLACIAL POND WATER

The chemical oxygen demand and a partial salinity and nutrients concentrations of supra-glacial pond water (SGP), and 400-O, 900-O and 900 ice samples were determined and analyzed in correlation with the age and organic content of the samples. The results (Tab. 2) showed a decrease of the COD and nitrate content with the age of ice between samples 400-O and 900-O by 4.3-fold and 3.6-fold, respectively. The presence of organic substrate in the 900-O sample is associated with higher COD (6-fold), nitrate (2.4-fold) and calcium (1.8-fold) concentrations than in sample 900 (clear ice). Sulfate and chloride concentrations showed minor variations with the age and organic content of the samples. Meanwhile, SGP exhibited a comparable organic content (COD) with that of sample 900, and slightly higher (1.3-1.7-fold) chloride concentrations than samples 400-O, 900-O, and 900.

## CULTIVATION OF HETEROTROPHIC PROKARYOTES FROM ICE SAMPLES

The presence of cold-adapted bacteria in 1-S, 1-L, 400-O, 900 and 900-O ice samples was investigated by cultivation in LBG medium at 4 °C and 15 °C. Under these conditions, the results (Fig. 3) indicated that all samples contained cultivable microorganisms, and that the growth dynamics of the corresponding microbial communities is temperature-dependent.

At 4 °C, the microbial growth curves had different profiles for recent and old ice samples, with a faster growth and a shorter lag time in the cases of 1-L and 400-O (Fig. 3A), while at 15 °C (Fig. 3B), the species from the same samples had relatively more uniform growth profiles (Fig. 3A). The cultivable microorganisms at 15 °C (Fig. 3B) from all samples presented a shorter lag time (4–12 days) than the corresponding ones (7–24 days) growing at 4 °C (Fig. 3A). Both microbial com-

munities from 900-O and 900 samples exhibited similar growth profiles, suggesting a relatively homogenous microbiota within this ice layer regardless their organic

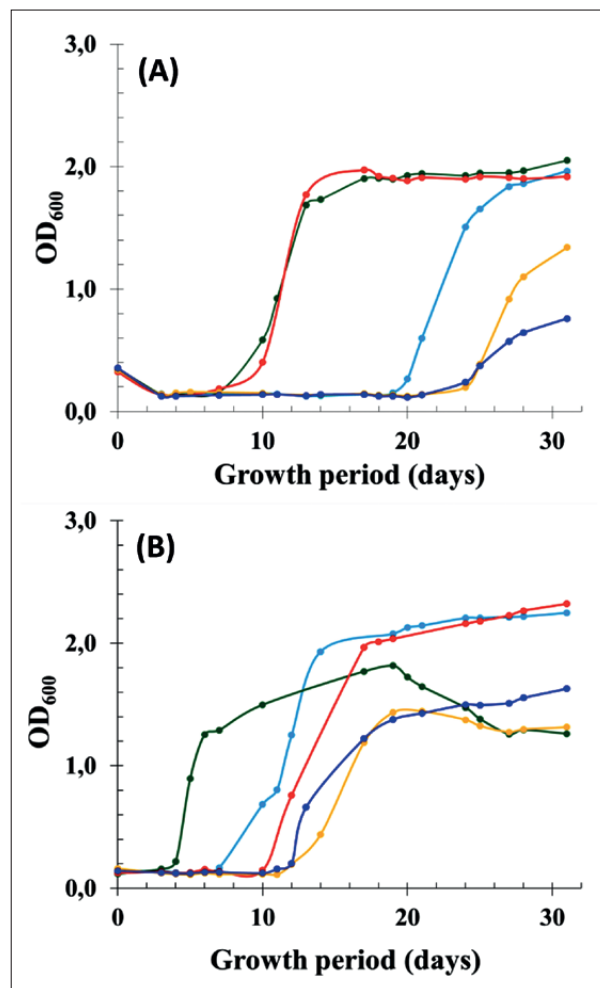


Fig. 3: Growth curves. Ice samples 1-S (light blue), 1-L (green), 400-O (red), 900 (dark blue) and 900-O (yellow) were cultivated for 32 days in LBG medium at (A) 4 °C, and (B) 15 °C.

Tab. 2: Chemical analysis of water and ice samples.

| Property / Compound |   | Concentration<br>(mg L <sup>-1</sup> ) |        |        |       |
|---------------------|---|--|--------|--------|-------|
|                     |   | SGP                                    | 400-O  | 900-O  | 900   |
| Organic content     | Chemical oxygen demand (COD)              | 30.75                                  | 740.88 | 170.52 | 28.77 |
| Nutrients           | Nitrates (NO <sub>3</sub> <sup>3-</sup> ) | 0.937                                  | 0.382  | 0.106  | 0.044 |
| Salinity            | Calcium ions (Ca <sup>2+</sup> )          | 50.9                                   | 17.0   | 16.0   | 8.9   |
|                     | Sulfates (SO <sub>4</sub> <sup>2-</sup> ) | 3.6                                    | 3.4    | 3.7    | 2.8   |
|                     | Chlorides (Cl <sup>-</sup> )              | 7.2                                    | 5.7    | 5.0    | 4.3   |



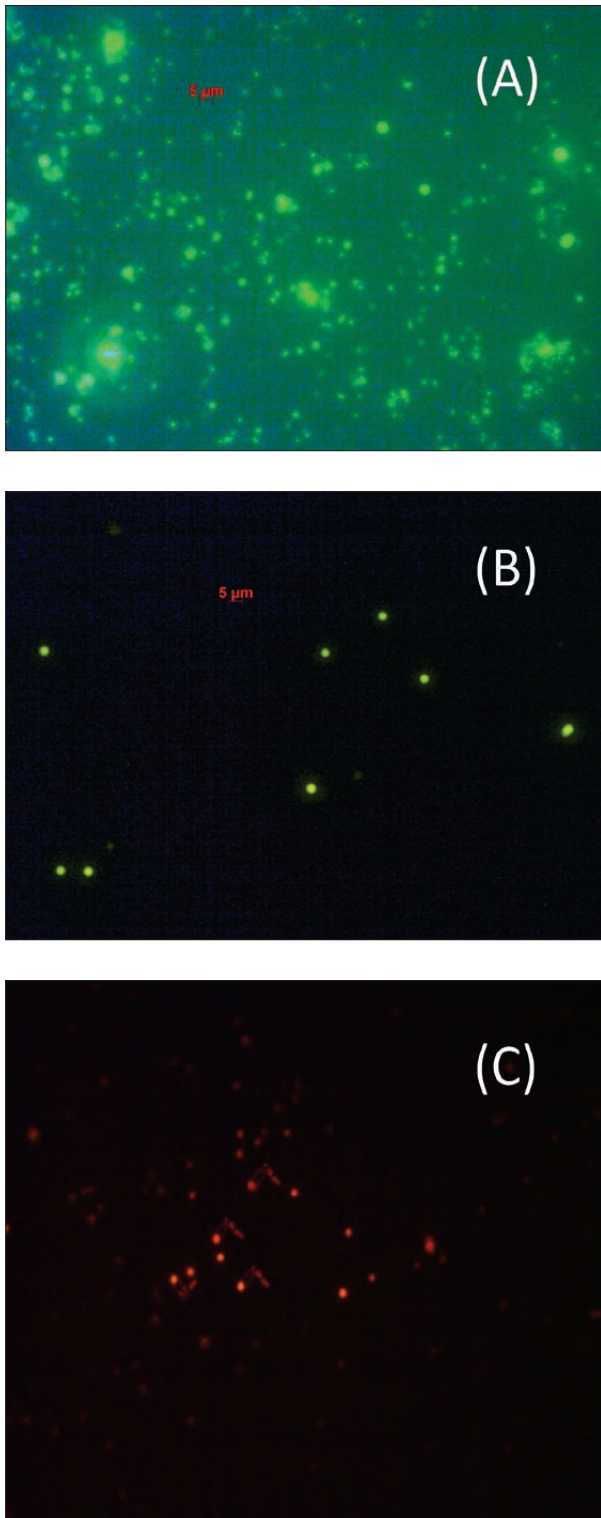


Fig. 4: Epifluorescence microscopy of prokaryotes from 1-S ice sample culture in BG11. Staining with (A) SybrGreen (total cell number); (B) ethidium homodimer (dead cells). (A) and (B) represent the same microscopic field; (C) chlorophyll natural fluorescence (cyanobacteria).

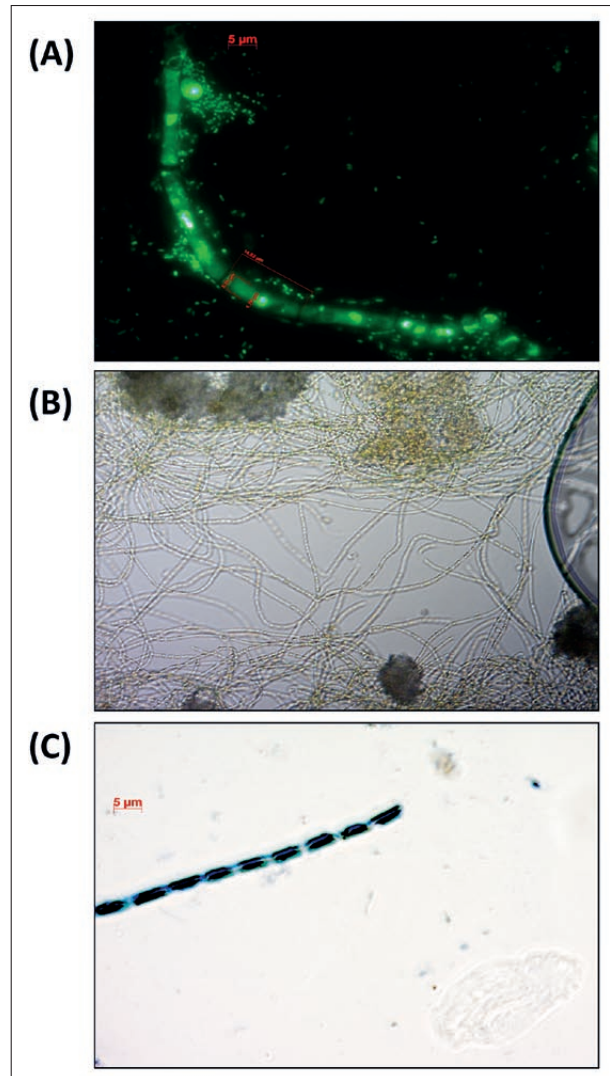


Fig. 5: Filamentous eukaryotic microorganisms from 1-S culture in BG11 and SGP. Cells resulted from cultivation of 1-S ice sample in BG11 at 7 °C for 2 months were analyzed by (A) epifluorescence microscopy using 10xSybrGreen, (B) light microscopy, without staining. (C) SGP water sample fixed with 2 % formaldehyde stained with 0.1 % crystal violet were analyzed by light microscopy.

content. In addition, microbiota from 900 and 900-O presented a slower growth at 4 °C as compared with that of recent and 400-year old ice, indicating a reduced diversity and/or abundance in older strata.

Taken together, the cultivation characteristics of the ice-contained microbiota under different growth conditions suggest differences in the population composition of cold-adapted microbial communities from all the analyzed ice deposits of Scarisoara Ice Cave.

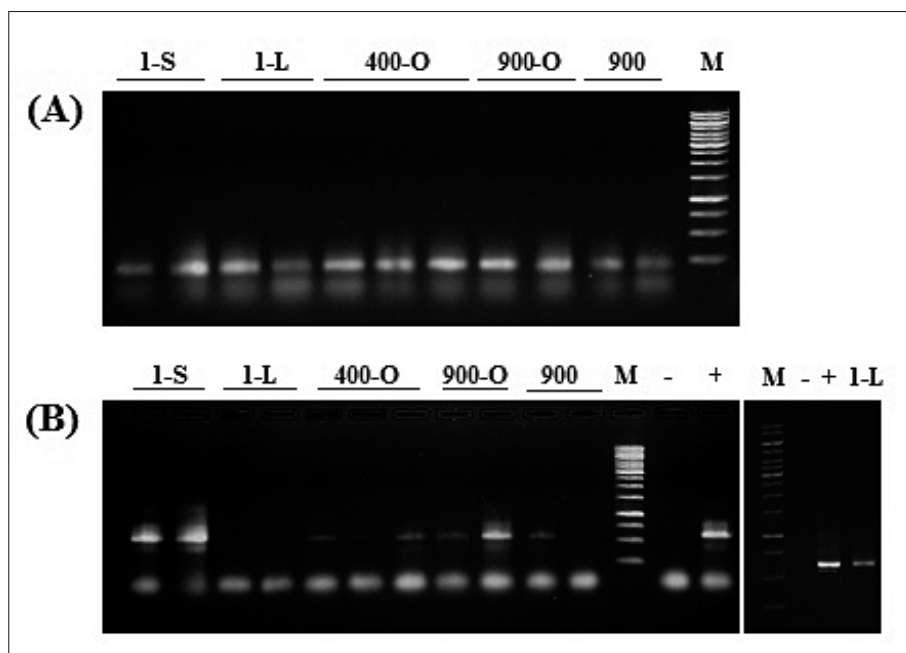


Fig. 6: PCR amplification of SSU-rRNA genes from ice samples. (A) bacterial 16S-rRNA gene fragments using 8F and 1512R primers, (B) eukaryotic 18S-rRNA gene fragments using Euk1A and Euk516R-GC primers.

#### PRESENCE OF CULTIVABLE PHOTOTROPHIC PROKARYOTES AND EUKARYOTES IN SUNLIGHT EXPOSED ICE

The existence of cultivable phototrophic microorganisms in SGP and 1-S samples collected from the same sunlight-exposed location before and after water freezing, was investigated. 1-S ice samples (10 ml) were incubated with 90 ml BG<sub>11</sub> liquid medium at 7 °C for up to 2 months in the presence of light and analyzed by epifluorescence microscopy.

In the case of 1-year old sunlight-exposed ice (1-S) cultivated in BG<sub>11</sub>, the results (Fig. 4) revealed the presence of a high number of prokaryotic cells (Fig. 4A). The majority of these cells are viable, as resulted from the reduced number of dead cells from the same microscopic field (Fig. 4B). Moreover, this culture contains a high number of cyanobacteria exhibiting chlorophyll auto-fluorescence (Fig. 4C).

In addition to prokaryotic phototrophs, this microbial populations contained cultivable filamentous eu-

karyotes with clearly visible nuclei (Fig. 5A). Moreover, light microscopy of the untreated cells reveals the presence of chlorophyll-containing filamentous phototrophic eukaryotes in this culture (Fig. 5B). Meanwhile, in the supra-glacial pond at the ice/water interface (SPG), the presence of algae is clearly evidenced (Fig. 5C).

#### PCR AMPLIFICATION OF SSU-RRNA FROM ICE SAMPLES

The presence of bacteria and eukaryotes in ice layers of different age was assessed by PCR amplification of the corresponding 16S/18S-rRNA gene fragments in all the collected samples. Under these conditions, the results obtained using total DNA extracted from at least two duplicates for each ice sample confirmed the presence of bacterial (Fig. 6A) and eukaryotic (Fig. 6B) species in all the recent and old ice layers investigated. The 18S-rRNA gene amplification, using a GC-clamp eukaryotic primer set (Tab. 1), was further confirmed by DGGE analyses (to be published elsewhere).

## DISCUSSION

In this first microbiological study of the biodiversity within the underground ice block from Scarisoara Ice Cave (Romania), we identified the presence of microorganisms thriving in as old as 900 years ice layers of both rich and poor organic content. These microor-

ganisms contain both bacterial and eukaryotic microorganisms.

Epifluorescence microscopy analysis of recent (one year old) ice and supra-glacial water from areas of the cave exposed to sunlight revealed the presence of

prokaryotic and eukaryotic phototrophs, cyanobacteria and filamentous algae, respectively. Bacterial communities from recent, 399 and 887 cal. BP old samples (1-S, 1-L, 400-O, 900-O, and 900), displayed a different composition in cultivable psychrophilic/psychrotolerant microorganisms from ice layers of different age, organic content and sunlight exposure, as resulting from their variable growth curves patterns at 4 and 15 °C.

The chemical composition of the present-day and old habitats of these microorganisms showed major variations with respect to COD, nitrate, and calcium ions concentrations, as a function of both age and organic content of the ice. The substantial decrease of these concentrations between the 399 and 887 cal. BP old ice layers is expected to be associated with a lower microbial content in the latter one. This hypothesis is sustained by the reduced bacterial growth of samples 900 and 900-O as compared to 400-O in LBG at both 4 °C and 15 °C, suggesting a lower representation of psychrophilic / psychrotolerant cultivable strains from microbial communities within the older ice sample than in the more recent ice layers.

While these samples differ by age, there is also a clear distinction in terms of external climate during the deposition of the ice layers from which samples 400-O, 900 and 900-O were collected. Explicitly, samples 900

and 900-O originate from an ice layer deposited during the Medieval Warm Period (MWP), when the significant development of *Fagus sylvatica* forest indicates warmer and drier summers (Feurdean *et al.* 2011). In contrast, sample 400-O was collected from a layer formed during the Little Ice Age (LIA), a colder and wetter period, when *Picea abies* forests (and associated acidic soils) dominated the landscape around the cave's entrance (Feurdean *et al.* 2011). The wetter conditions that prevailed during the LIA over the MWP led to frequent inflow of large volumes of water inside the cave, which carried organic matter and nutrients that could have further improved the habitability of ponded water before freezing, hence favoring an increased biodiversity of the microorganisms' communities.

This study, reporting on the presence of cultivable microorganisms in ~1000-year old subterranean ice from Scarisoara Ice Cave (Romania), and the age-dependent variations of the microbial communities within this habitat, is the first such environmental survey of an ice cave. While, to date, this particular type of ecological niche received a very limited attention, the present study made the first step in unraveling the diversity of microbiota thriving in underground ice deposits from this cave in correlation with past climatic changes.

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