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MICROBIOLOGY

Using metabarcoding to assess Viridiplantae sequence diversity present in Antarctic glacial ice

PAULO E.A.S. CÂMARA, GRACIELE C.A. MENEZES, OTAVIO H.B. PINTO, MICHELINE C. SILVA, PETER CONVEY & LUIZ H. ROSA

Abstract: Antarctica contains most of the glacial ice on the planet, a habitat that is largely unexplored by biologists. Recent warming in parts of Antarctica, particularly the Antarctic Peninsula region, is leading to widespread glacial retreat, releasing melt water and, potentially, contained biological material and propagules. In this study, we used a DNA metabarcoding approach to characterize Viridiplantae DNA present in Antarctic glacial ice. Ice samples from six glaciers in the South Shetland Islands and Antarctic Peninsula were analysed, detecting the presence of DNA representing a total of 16 taxa including 11 Chlorophyta (green algae) and five Magnoliophyta (flowering plants). The green algae may indicate the presence of a viable algal community in the ice or simply of preserved DNA, and the sequence diversity assigned included representatives of Chlorophyta not previously recorded in Antarctica. The presence of flowering plant DNA is most likely to be associated with pollen or tissue fragments introduced by humans.

Key words: Algae, Angiosperms, DNA, biodiversity.

INTRODUCTION

Glaciers and ice sheets cover about 15 million km^2 globally, or about 10% of the Earth's land surface (Anesio & Laybourn-Parry 2012). A limited number of microorganisms (bacteria, various groups of algae, and fungi) are known to be able to survive the harsh conditions within ice (Sanyal et al. 2018, Perini et al. 2019). Many of these microorganisms exhibit a range of adaptations that protect their metabolism from the damaging effects of harsh environmental conditions such as extreme temperatures and lack of liquid water (Siddiqui & Cavicchioli 2006, Margesin & Miteva 2011), including some of potential biotechnological interest. Some microbial communities present in glacial ice are biochemically active (Price 2000, Anesio et al. 2009, Hodson et al. 2010). However, available reports are mostly restricted to bacteria and from studies in the Northern Hemisphere (Sheridan et al. 2003, Miteva & Brenchley 2005).

Antarctica contains most of the world's glacial ice (de Menezes et al. 2020), representing about 70% of freshwater globally (Sadaiappan et al. 2020). Formed by the accumulation of snow gradually compressed over many years, Antarctic glacial ice may provide a unique habitat for microorganisms that could have been trapped for many thousands of years (Abyzov 1993, Gunde-Cimerman et al. 2003), with the oldest ice yet drilled in Antarctica being dated to several hundred thousand years (Elzinga 2012).

In recent decades, parts of Antarctica have experienced the impacts of anthropogenic warming. In the Antarctic Peninsula region, the temperature increase already exceeds 1.5 °C over pre-industrial temperatures (Turner

et al. 2005), where it has led to widespread glacial retreat (Cook et al. 2017). This releases meltwater into the surrounding environment, potentially including viable biological material and propagules. Therefore, understanding the biological diversity contained in this habitat is a research priority.

To date, very few studies have addressed the biodiversity of ice-associated habitats. The majority have focused on bacteria and are mostly based on traditional culture methods (Margesin et al. 2002, Foght et al. 2004, Yallop & Anesio 2010) or direct observation (Porazinska et al. 2004, Stibal et al. 2006). The biodiversity and adaptations of species found in some ice-associated habitats, such as glacier surface cryoconite holes (Porazinska et al. 2004) and in surface snow (Davey et al. 2019) have received research attention, and there are a small number of studies of algal communities found in glacier ice, mostly in the Northern Hemisphere and focused on the ice surface (Takeuchi et al. 2015, Stibal et al. 2017, Onuma et al. 2018). The considerable differences in physical and chemical properties of surface snow and bare ice result in very distinct biological communities being present in these two environments (Yoshimura et al. 1997, Lutz et al. 2017).

Rapid developments in molecular biological techniques in recent decades now allow the assessment of biological diversity in environmental samples based on the DNA sequences present, for instance through DNA metabarcoding using high throughput sequencing (HTS) (Rippin et al. 2018, Ruppert et al. 2019). This includes the possibility of detecting stages which are typically not detected in morphological surveys (e.g. pollen, spores, microscopic fragments and even single cells), as well as traces of environmental DNA, which may sometimes be preserved for many years (Barnes & Turner 2015). Conversely, these approaches do not allow assessment of viability or activity, while putative identifications depend on the level of diversity coverage achieved by existing databases (Darling & Mahon 2011). To date, only a few studies (Rippin et al. 2018, Garrido-Benavent et al. 2020, Fraser et al. 2018, Câmara et al. 2020) have applied HTS for assessing plant biodiversity in Antarctica, and none in terrestrial glacier ice. The aim of the current study was, therefore, to use HTS to characterize Viridiplantae DNA present within glacial ice from Antarctica.

MATERIALS AND METHODS

Sampling

We collected three glacial ice fragments of approximately 20 kg each were collected from the ablation zone of seven glaciers in the South Shetland Islands and the north-west Antarctic Peninsula during the austral summer of 2015/2016 (Figure 1, Table I). Immediately after collection, the external surface of the ice was sterilized following the protocol established by de Menezes et al. (2020), ice samples were then melted at room temperature (ca. 22°C) for 48 h in sterile conditions. The water obtained was filtered using 0.47 µm membranes (Millipore, USA). A total of 12-15 L of ice were filtered until the membranes were saturated (ca. 4-5 L per membrane), meaning a total of three membranes were obtained from each site. Membranes were stored at -20°C until DNA extraction.

DNA extraction, Illumina library construction and sequencing

Total DNA was extracted as described by Lever et al. (2015). For DNA cleaning we used the DNeasy Plant Mini Kit (QIAGEN, Carlsbad, USA) from step six, following the manufacturer's instructions. The three membranes from each sampling site were extracted separately, with the products

obtained then being combined to concentrate the DNA. DNA quality was analysed by agarose gel electrophoresis (1% agarose in 1× Trisborate-EDTA) and then quantified using Quanti-iT™ Pico Green dsDNA Assay (Invitrogen). The internal transcribed spacer 2 (ITS2) of the nuclear ribosomal DNA was used as a DNA barcode for molecular species identification (Chen et al. 2010, Richardson et al. 2015) using the universal primers ITS3 and ITS4 (White et al. 1990). Library construction and DNA amplification were performed using the Library kit Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2, following Illumina 16S Metagenomic Sequencing Library Preparation Part #15044223 Rev. B protocol. Paired-end sequencing (2 × 300 bp) was performed on a MiSeq System (Illumina) by Macrogen Inc. (South Korea).

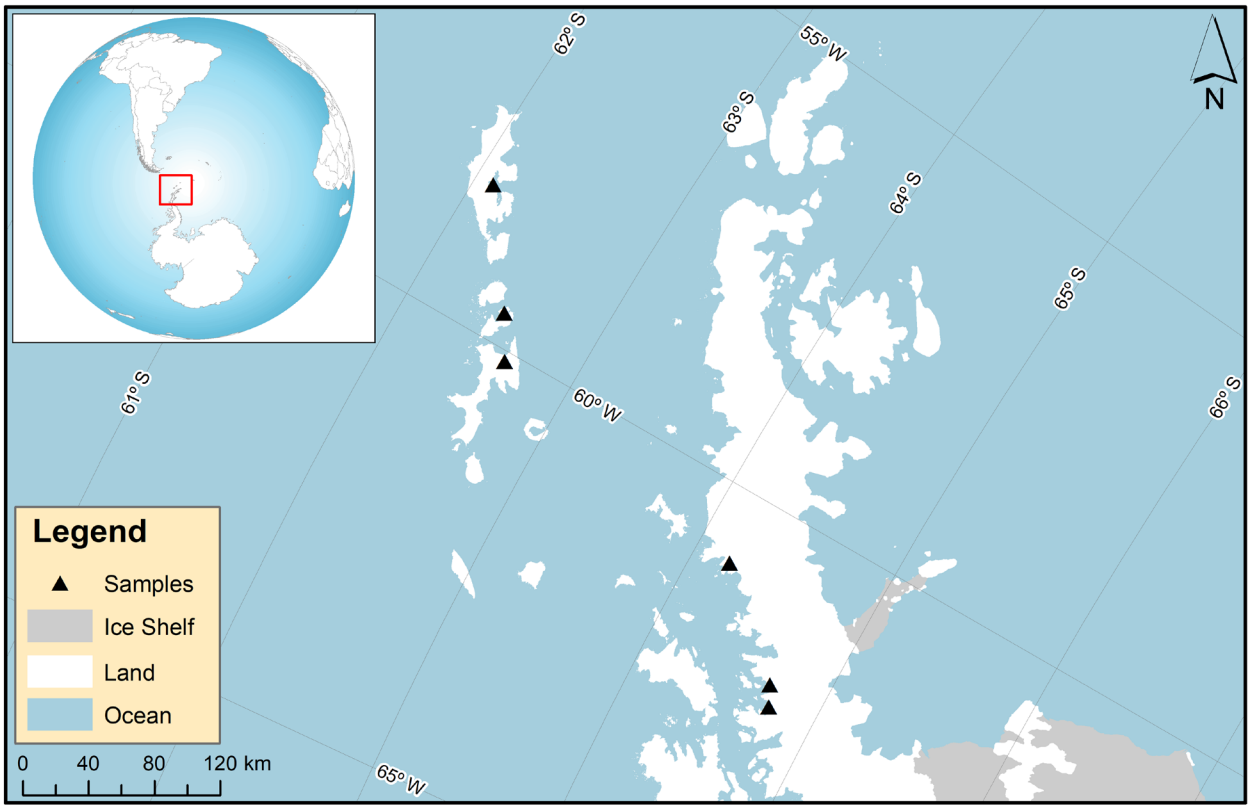

Figure 1. Map showing the sampling locations in the South Shetland Islands, KGI = King George Island, Grw = Greenwich Island, Livin = Livingston Island, Pen1 = Antarctic Peninsula 1, Pen2 = Antarctic Peninsula 2, Arctow = Arctowski Peninsula.

Table I. Sites were glacial ice was sampled in South Shetland Islands and Antarctic Peninsula.

Glacier	Site	Coordinates	Code
King George Island	Ajax/Stenhouse	62°06'S, 058°27"W	KGI
Greenwich Island	Fuerza Aerea	62°30'S 59°38"W	Grw
Livingston Island	Huron	62°37'50"S 60°06'50"W	Livin
Antarctic Peninsula 1	Sikorsky	64°12'S 60°53"W	Pen1
Antarctic Peninsula 2	Leonardo/Blanchard	64°42'S, 61°58'W	Pen2
Arctowski Peninsula	Rozier/Woodbury	64°45'S 62°13"W	Arctow

Data analysis

Raw fastq files were filtered using BBDuk version 38.34 (BBMap – Bushnell B. –sourceforge.net/projects/bbmap/) to remove Illumina adapters, known Illumina artefacts, and the PhiX Control v3 Library. Quality read filtering was carried out using Sickle version 1.33 -q 30 -l 50 (Joshi et al. 2011), to trim 3' or 5' ends with low Phred quality score, and sequences shorter than 50 bp were discarded. The remaining sequences were imported to QIIME2 version 2019.10 (https://qiime2.org/) for bioinformatics analyses (Bolyen et al. 2019) and the pipeline was executed for merged pair-ended sequences with the following plug-ins: vsearch join-pairs (Rognes et al. 2016), vsearch dereplicate-sequences, quality-filter q-score-joined (Bokulich et al. 2013), vsearch cluster-features-de-novo 97% identity limit, vsearch uchime-denovo. Taxonomic assignments were determined for operational taxonomic units (OTUs) using the feature-classifier (Bokulich et al. 2018) classify-sklearn against the PLANiTS2 database (Banchi et al. 2020) trained with Naïve Bayes classifier. We follow the definition of Viridiplantae of Leliaert et al. (2012).

Many factors, including extraction, PCR and primer bias, can affect the number of reads obtained (Medinger et al. 2010), and thus lead to misinterpretation of absolute abundance (Weber & Pawlowski 2013). However, Giner et al. (2016) concluded that such biases did not affect the proportionality between reads and cell abundance, implying that more reads are linked with higher abundance (Deiner et al. 2017, Hering et al. 2018). Therefore, for comparative purposes we used the number of reads as a proxy for relative abundance.

RESULTS

The calculated rarefaction curves indicated that the sampling gave an accurate representation of the local OTU diversity in the sites where such a curve was possible to calculate (Fig. 2), for Arctowski Peninsula the curve had not completely stabilized. For sites with only one taxon (e.g. Livingston Island) it was not possible to produce a rarefaction curve.

A total of 2,007,454 paired-end DNA reads were generated in the sequencing run of which 704,819 reads remained after quality filtering, representing 16 OTUs (Table II). These were assigned to 11 Chlorophyta (green algae) and five Magnoliophyta (flowering plants). Some OTUs could only be resolved at higher taxonomic level (family, order or division). The unassigned ranks refer to groups not present in the consulted databases (Fig. 3), mostly representing Fungi.

Sequences assigned to a number of flowering plants (Magnoliophyta) were also identified (Table II), with four OTUs from King George Island and one from the Antarctic Peninsula region. The alga *Koliella longiseta* had the highest number of reads, followed by *Chlamydomonas nivalis*. The most widespread taxon (present in four sites) was also *K. longiseta*.

DISCUSSION

Hodson et al. (2008) recognised two glacial ecosystems, supraglacial, including organisms living on the ice surface, and subglacial, including organisms living within the ice layers, with the former community being more diverse and abundant.

The sequence data obtained here revealed the presence of a quite diverse algal assemblage. *Koliella longiseta*, a freshwater species, was the most abundant OTU and was also present in four of the six glaciers glacial ice samples

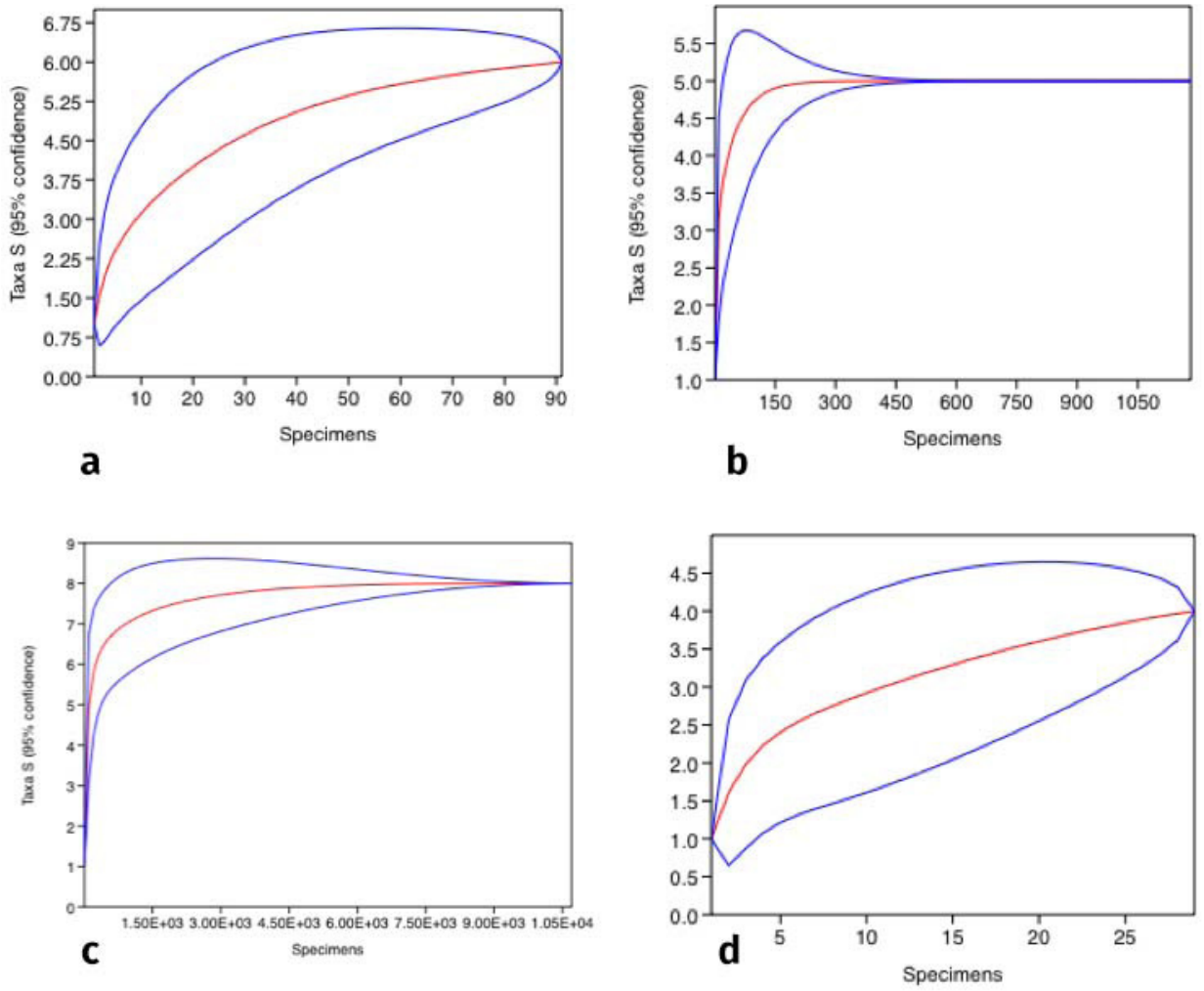

Figure. 2. Rarefaction curves for samples from (a) King George Island (b) Greenwich Island, (c) Antarctic Peninsula 2, (d) Arctowski Peninsula. Due to low numbers of taxa detected, it was not possible to generate rarefaction curves for the remaining samples. Blue lines represent 95% confidence limits.

obtained. The species was previously reported by Câmara et al. (2020) in a metabarcoding study of soil samples from Deception Island, also in the South Shetland Islands. Representatives of this genus are known to occur on alpine glacier surfaces and in snow (Hindák 1996). The second most abundant OTU was *C. nivalis*, a freshwater/terrestrial species, which was present at two sampling locations. *Clamydomonas nivalis* is also a well-known snow alga (Remias et al. 2005) and has been reported from mountains and snowfields globally (Guiry & Guiry 2021). *Chloromonas alpina* is also a snow alga, but has not previously been reported from Antarctica, with records from Europe, India and Australia/New Zealand (Guiry & Guiry 2021). *Myrmecia bisecta* is a terrestrial alga that has been reported in a metabarcoding study on Deception Island (Câmara et al. 2020) and also in a traditional culture study of soil near Bellingshausen Station on King George Island (Andreyeva & Kurbatova 2014). *Raphidonema nivale* is another snow alga that is also found in terrestrial and freshwater habitats, with wide occurrence in Europe, North and South America, Asia and Australia (Guiry & Guiry 2021), and

Table II. Assigned plant OTUs present in glacier ice obtained from six different sampling locations, with the number of reads for each. KGI = King George Island, Grw. = Greenwich Island, Pen1 = Antarctic Peninsula 1, Pen2= Antarctic Peninsula 2, Arctow.= Arctowski Peninsula and Living. = Livingston Island.

TAXA	KGI	Grw.	Pen. 1	Pen. 2	Arctow.	Livin.
Phylum Chlorophyta						
Chlamydomonadales	0	151	0	14	01	0
Chlamydomonas nivalis (F.A. Bauer) Wille	0	685	0	1074	0	0
Chlorelalles						
Chloromonas alpina Wille	0	0	0	62	0	0
Prasiolales						
Myrmecia bisecta Reisigl	0	18	0	0	0	0
Koliella longiseta (Vischer) Hindák	0	307	203	9209	11	0
Raphidonema nivale Lagerheim	0	0	0	249	0	0
Ulotrichales						
Chlorothrix sp.	03	32	0	166	0	0
Monostroma angicava Kjellman	0	0	0	0	0	20
Planophila sp.	0	0	0	0	2	0
Ulothrix sp.	0	0	0	4	0	0
Urospora sp.	23	0	0	99	0	0
Phylum Magnoliophyta						
Fabaceae						
Cenostigma sp.	3	0	0	0	0	0
Fagaceae						
Nothofagus pumilio (Poepp. & Endl.) Krasser	56	0	0	0	0	0
Myrtaceae						
Eugenia boliviana (D. Legrand) Mattos	5	0	0	0	0	0
Plantaginaceae						
Plantago lagopus L.	0	0	0	0	15	0
Rosaceae						
Malus sp.	1	0	0	0	0	0

again recorded through metabarcoding in soil on Deception Island (Câmara et al. 2020).

Among the Ulotrichales, *Chlorothrix* is a genus of marine algae which has only three European species recognised. DNA sequences assigned to this genus have recently been reported from Deception Island (Câmara et al. 2020). *Monostroma angicava* is also a marine species, reported from Japan, China, Korea and Norway but not previously from Antarctica. The closely related species *M. kuroshiense* F. Bast. and *M. nitidum* Wittrock are widely cultivated in Asia as food and have been used for treating viral infections (Kazłowski et al. 2012). *Planophila* is a genus of terrestrial or subaerial algae with only three recognised European species, although again reported in soil from Deception Island (Câmara et al. 2020). The genus *Ulothrix* includes about 39 accepted species, with representatives occurring in both marine and freshwater environments, cosmopolitan in temperate and colder regions (Guiry & Guiry 2021), and sequences assigned to this genus have again been reported in soil from

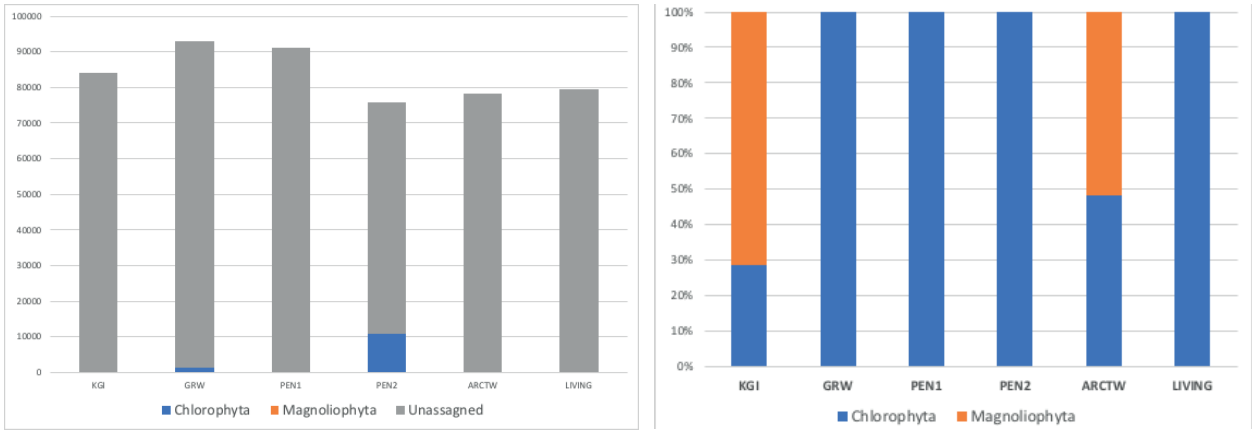

Figure 3. Histogram showing a) Tthe total number of reads obtained from each sampling site, including unassigned reads; and b) Percentage of groups represented, with the exclusion of unassigned reads.

Deception Island (Câmara et al. 2020), *Ulothrix australis* Gain has been previously reported from the Antarctic Peninsula (Papenfuss 1964). *Urospora* is a widespread marine genus with about 10 accepted species, including reports from the Arctic (Lee 1980), and with the species *U. penicilliformis* (Roth) Areschoug reported from the Antarctic Peninsula, Wilkes Land and the South Shetland Islands (Papenfuss 1964). The presence of sequences assigned to marine species could suggest seawater contamination although. As the studied glaciers are all coastal and the sampled ice surfaces were cleaned before DNA extraction, it is also possible that organisms (or propagules or fragments) were transferred in marine aerosols and became trapped in the ice.

Among the sequences assigned to flowering plants (Magnoliophyta), the most abundant OTU was the Fagaceae *Nothofagus pumilio*, a southern beech tree species native to the Andes of Patagonia, Tierra del Fuego and Navarino Island, where it is widespread and abundant. Pollen of this tree genus is widely reported in palynological studies of both ice and sediment cores obtained in Antarctica. The Plantaginaceae *Plantago lagopus* is a herb distributed in Europe, Asia and South America and used as a herbal tea and in medication for blood pressure (Galisteo et al. 2005). *Eugenia boliviana* an endemic Mytaceae from the Bolivian (Andes) and Southern Brazil, *Cenostigma* is a neotropical legume genus with medicinal uses and the Rosaceae *Malus* (apple) is a widely cultivated for food (Jackson 2003). None of these taxa are considered as invasive, and the association of several with food or medicinal uses may suggest a human role in their presence in the current study. This inference may also be supported by the majority of flowering plant OTUs being found on King George Island, which is one of the most intensively human impacted locations in Antarctica. Indeed, the *Malus* OTU was present in ice obtained very close to the Brazilian Antarctic Station Comandante Ferraz.

The presence of DNA assigned to OTUs of flowering plants most likely reflects the presence of pollen or tissue fragments, or brought by humans, especially taxa used as food.

CONCLUSIONS

This study is the first to use a metabarcoding approach to assess DNA sequence diversity present in Antarctic glacial ice. The data obtained confirm the presence of Viridiplantae DNA in the ice. The OTU diversity detected suggests that the

ice may contain a community of green algae, though cannot differentiate whether members of this are active or viable, or are represented by preserved DNA. Chlorophyta records include cold environment taxa not previously reported from Antarctica. The presence of DNA assigned to OTUs of flowering plants most likely reflects the presence of pollen or tissue fragments, or an association with human contamination.

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PAULO E.A.S. CÂMARA[1,2]
https://orcid.org/0000-0002-3944-996X

GRACIELE C.A. MENEZES[3]
https://orcid.org/0000-0002-9427-1893

OTAVIO H.B. PINTO[4]
https://orcid.org/0000-0002-8382-2987

MICHELINE C. SILVA[1]
https://orcid.org/0000-0002-2389-3804

PETER CONVEY[5,6]
https://orcid.org/0000-0001-8497-9903

LUIZ H. ROSA[3]
https://orcid.org/0000-0001-9749-5182

[1]Universidade de Brasília, Departamento de Botânica, Instituto de Ciências Biológicas, Campus Universitário Darcy Ribeiro, s/n, 70910-900 Brasília, DF, Brazil

[2]Universidade Federal de Santa Catarina, Pós-graduação em Plantas, Fungos e Algas, Campus Universitário, s/n, Sala 208, Bloco E, Córrego Grande, 88040-900 Florianópolis, SC, Brazil

[3]Universidade Federal de Minas Gerais, Departamento de Microbiologia, Instituto de Ciências Biológicas, Av. Antônio Carlos, 6627, Pampulha, 31270-000 Belo Horizonte, MG, Brazil

[4]Universidade de Brasília, Departamento de Biologia Celular, Instituto de Ciências Biológicas, Campus Universitário Darcy Ribeiro, s/n, 70910-000 Brasília, DF, Brazil

[5]British Antarctic Survey, NERC, High Cross, Madingley Road, Cambridge CB3 0ET, U.K.

[6]University of Johannesburg, Department of Zoology, PO Box 524, Auckland Park 2006, Johannesburg, South Africa

Correspondence to: **Paulo Eduardo Aguiar Saraiva Câmara**
E-mail: paducamara@gmail.com

Author contributions

PEASC, LHR and PC designed the experiment, GCAM performed lab work, OHBP and PEASC did the bioinformatic and PEASC, MCS, PC and LHR did data analyses. All author contributed equally to the writing of this manuscript.