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Assessing cryptic non-fungal eukaryotic diversity associated with rocks at Lions Rump, South Shetland Islands, Maritime Antarctica

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Abstract

Maritime Antarctica experiences less extreme environmental conditions than much of the Antarctic continent and has further been impacted by considerable warming in recent decades. While inventories exist of macroscopic Antarctic biodiversity, and there is some information available on culturable microorganisms, much less is known about the presence of other cryptic eukaryotic organisms. DNA metabarcoding provides a method for assigning the DNA of multiple different organisms simultaneously from environmental samples. In this study, we used DNA metabarcoding to investigate the environmental DNA (eDNA) diversity of non-fungal eukaryotic organisms associated with rocks in the South Shetland Islands. Five sampling points were selected from a stratigraphic profile at Mazurek Point, King George Island. Collected rock samples were pulverized, total DNA was extracted and amplicons were generated using ITS2 primers, then these were sequenced using an Illumina MiSeq system. Sequences representing five kingdoms and nine phyla were retrieved. Viridiplantae was the most diverse and abundant group, with 42 assigned taxa, followed by Chromista, with 22 assigned taxa. The precise lithology did not influence the assigned diversity. The majority of assigned taxa are widespread and plausibly present in the area, but some are not known from Antarctica, including some from tropical regions. The latter assignments probably result from the limitations of the databases used, although in some cases they may indicate evidence of anthropogenically associated or naturally dispersed DNA-containing material.

Keywords: Antarctica; DNA metabarcoding; eDNA; King George Island

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Introduction

Antarctica hosts some of the most pristine biomes remaining on Earth and, at the same time, faces some of the most extreme environmental conditions (Convey & Biersma 2024). However, parts of the continent, in particular the Maritime Antarctic, which includes the Antarctic Peninsula and South Shetland Islands (SSIs), have experienced considerable increases in air and marine temperatures, as well as widespread glacial retreat and other environmental changes, since the second half of the twentieth century (Turner *et al.* 2009, Convey & Peck 2019, Kreczmer *et al.* 2021). Even without this warming, the Maritime Antarctic region, particularly the SSIs, is characterized by the least extreme conditions in

Antarctica (Convey & Biersma 2024). Regional warming in the Maritime Antarctic has resulted in reports of vegetation expansion at a local scale (Fowbert & Smith 1994, Cannone *et al.* 2016, 2017, 2022). However, even though this region is known as Antarctica's most vegetated region, only ~1.34% of its ice-free ground has been estimated to be currently vegetated (Fretwell *et al.* 2011).

Câmara *et al.* (2023), in a metabarcoding study of airspora, investigated a 40° latitudinal transect from Brazil to the SSIs, generating data that suggested that long-distance aerial dispersal may play a role in colonizing new environments (see also Rosa *et al.* 2020, Câmara *et al.* 2024), in concert with previous aerobiological studies (e.g. see Marshall *et al.* 1996, Pearce *et al.* 2016, Kleinteich *et al.* 2017). Some surface habitats in Antarctica such as rock surfaces are generally not suitable for macroscopic plant vegetation, although they do provide habitats for epilithic mosses (Ochyra *et al.* 2008) and lichens (Øvstedal & Smith 2001), as well as microorganisms such as fungi (Gonçalves *et al.* 2019) and algae (van Thielen & Garbary 1999). Câmara *et al.* (2022) applied DNA metabarcoding to rocks obtained from the Ellsworth Mountains at the base of the Antarctic Peninsula in Continental Antarctica,

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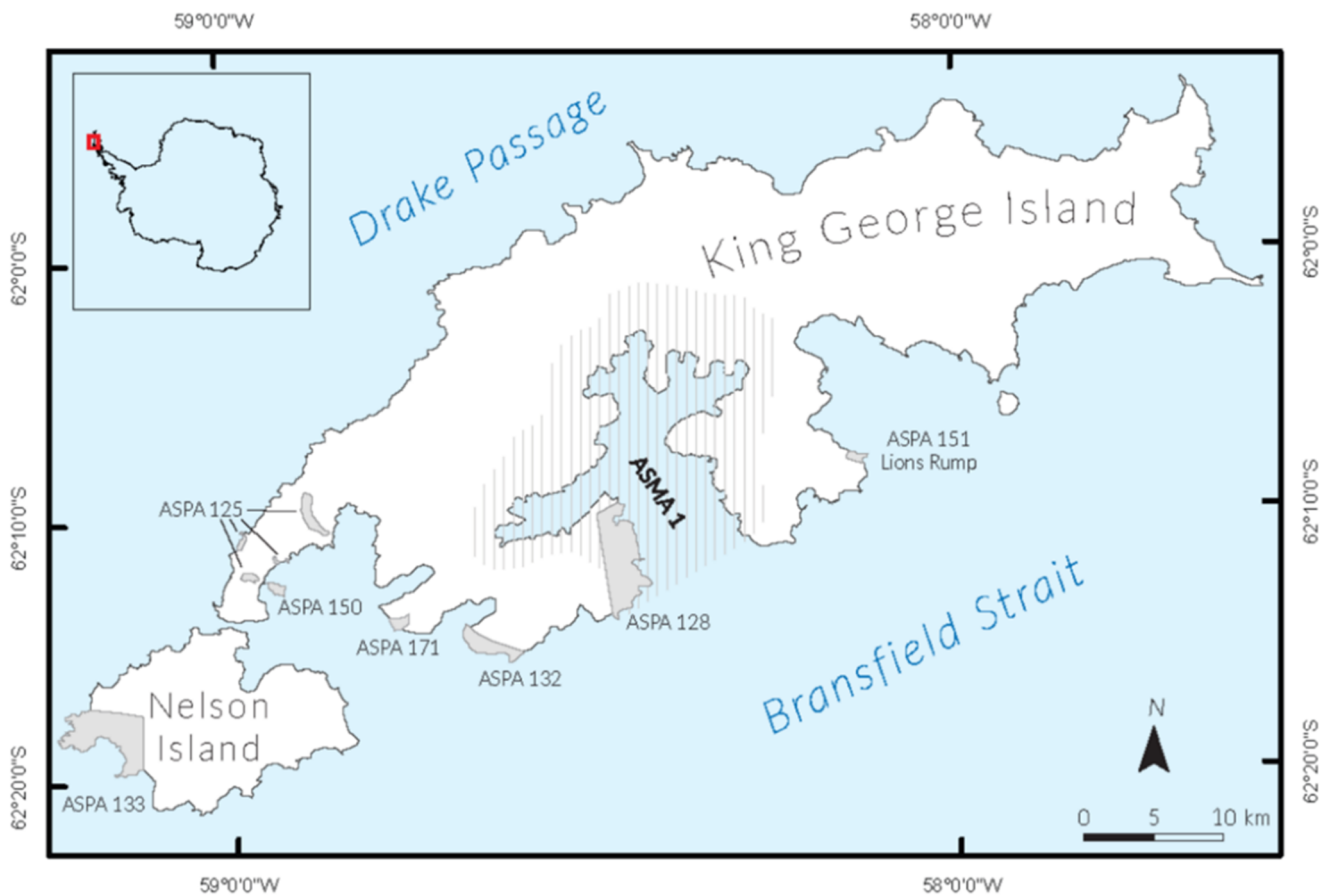


Figure 1. Map showing the location of Lions Rump (Antarctic Specially Protected Area (ASPAs) 151) on King George Island. Obtained from the ASPA 151 management plan (<https://www.ats.aq/devph/en/apa-database/55>). ASMA = Antarctic Specially Managed Area.

where climatic conditions are much harsher than in the SSIs, and reported sequence assignments of 48 non-fungal eukaryotic taxa, including algae, bryophytes and flowering plants (even though the latter two groups do not occur in this mountain range).

DNA metabarcoding enables the detection of DNA from environmental samples containing multiple different organisms simultaneously (known as environmental DNA, or eDNA; Rippin *et al.* 2018, Ruppert *et al.* 2019), including stages that are typically not detected in morphological surveys (e.g. pollen, spores, small fragments, single cells). This has been considered as an effective tool for detecting the presence and diversity of eukaryotic (including plant) DNA in extreme environments (Fraser *et al.* 2018, Rippin *et al.* 2018, Garrido-Benavent *et al.* 2020, Câmara *et al.* 2022), although we recognize from the outset that the assignment of a DNA sequence as belonging to a particular taxon does not confirm the presence of that specific taxon (commonly due to coverage and accuracy limitations in available sequence databases) or that of a living or viable organism or propagule. In this study, we used DNA metabarcoding to investigate the cryptic diversity of non-fungal eukaryotes associated with rocks obtained at Lions Rump on King George Island in the SSIs.

Materials and methods

Sampling

The studied material was collected during the summer of 2021–2022 at Lions Rump, Mazurek Point, King George Island

($62^{\circ}08'30.81''S$, $58^{\circ}07'34.25''W$; Fig. 1). Lions Rump is an ice-free area located at the western side of the entrance to King George Bay, ~30 km from the nearest research station. It has an area of ~1.61 km² and was designated in 2002 as an Antarctic Specially Protected Area (ASPAs) 151 in recognition of its important geological features as well as significant populations of penguins and many other species of marine birds and mammals. Terrestrial vegetation is also present but not recognized as a 'major' value in the ASPA management plan.

For the purposes of this study, five sampling locations were selected from the Mazurek Point Formation (Troedson *et al.* 2002) and numbered S1–S5. They were obtained from different heights in a stratigraphic profile (9.0, 24.0, 34.7, 48.8 and 57.2 m, respectively) and were placed in sealed sterile bags and frozen on ship from soon after collection until being processed (see Rabelo *et al.* (2024) for a detailed description of this collection, storage and subsequent processing).

DNA extraction and sequencing

Samples were pulverized using a sterilized drill and a mortar. Total DNA was extracted using the FastDNA Spin Kit for Soil (MPBio, OH, USA), following the manufacturer's instructions. DNA quality was analysed using agarose gel electrophoresis (1% agarose in 1× Trisborate-EDTA) and then quantified using the Quanti-iT™ Pico Green dsDNA Assay (Invitrogen). We selected the internal transcribed spacer 2 (ITS2) of the nuclear ribosomal DNA

(Chen *et al.* 2010, Richardson *et al.* 2015, Câmara *et al.* 2022) as a barcode, which has been widely used to identify a diverse range of eukaryotic organisms, including fungi, animals, protozoans, chromists and plants (Ruppert *et al.* 2019) and has proved effective in recent eDNA studies of Antarctic diversity (Carvalho-Silva *et al.* 2021, Ogaki *et al.* 2021, Câmara *et al.* 2022). Polymerase chain reaction (PCR) amplicons were generated using the primers ITS3 and ITS4 (White *et al.* 1990) and were sequenced commercially using high-throughput sequencing by Macrogen, Inc. (South Korea) on an Illumina MiSeq sequencer (2 × 300 bp).

Data analyses and taxa identification

Quality analysis was carried out using *BBDuk* v. 38.87 in *BBmap* software (Bushnell 2014) with the following parameters: Illumina adapters removing (Illumina artefacts and the PhiX Control v3 Library); *ktrim* = 1; *k* = 23; *mink* = 11; *hdist* = 1; *minlen* = 50; *tpe*; *tbo*; *qtrim* = *rl*; *trimq* = 20; *ftm* = 5; *maq* = 20. The remaining sequences were imported to *QIIME2* version 2023.9 (<https://qiime2.org>) for bioinformatics analyses (Bolyen *et al.* 2019). The *qiime2-dada2* plugin was used for filtering, dereplication, turning paired-end fastq files into merged files and removing chimeras, using default parameters (Callahan *et al.* 2016). Taxonomic assignments of amplicon sequence variants (ASVs) were determined using the *qiime2-feature-classifier* (Bokulich *et al.* 2018) *classify-sklearn* against different databases, with a sequence similarity threshold set to 97%. First, ASVs were classified against the PLANITS2 database (Banchi *et al.* 2020). After this step, ASVs that remained unclassified were filtered and *classify-sklearn* classified against the UNITE Eukaryotes ITS database version 9.0 (Abarenkov *et al.* 2020). Finally, the remaining unclassified ASVs were filtered and aligned against the filtered National Center for Biotechnology Information (NCBI) non-redundant nucleotide sequences (nt) database (October 2024) using *BLASTn* (Camacho *et al.* 2009) with default parameters; the nt database was filtered with the following keywords: 'ITS1', 'ITS2', 'Internal transcribed spacer' and 'internal transcribed spacer'. Taxonomic assignments were performed using *MEGAN6* (Hudson *et al.* 2016). For simplicity, we henceforth refer to the assigned ASVs as 'taxa'. Venn diagrams were prepared as described by Bardou *et al.* (2014). For comparative purposes, we consider reads as a proxy for relative abundance (Deiner *et al.* 2017, Hering *et al.* 2018, Carvalho-Silva *et al.* 2021, Câmara *et al.* 2022). Rarefaction curves were generated using the software *PAST* 3.26 (Hammer *et al.* 2001).

Results

A total of 187 664 DNA reads from non-fungal eukaryotic taxa were obtained (fungal data are presented by Rabelo *et al.* 2024), representing 70 assigned taxa. The rock sample with the highest numbers of reads and assigned taxa was S3 (33 taxa), followed by S5 (24), S1 (22), S4 (21) and S2 (16). Only three taxa were shared across all five samples (order Chlamydomonadales, *Chloromonas* sp. and *Prasiola* sp.; Figs 2 & 3). Representatives of five kingdoms and nine phyla were assigned (Table I). The kingdom Viridiplantae was the most diverse group, with 42 assigned taxa, followed by Chromista, with 22 assigned taxa. The most abundant individual taxa were also representatives of Viridiplantae (*Prasiola*, *Chloromonas* and Chlamydomonadales). Rarefaction curves all reached a plateau, suggesting that the data obtained provide a good representation of the sequence diversity in the crushed rock samples (Fig. 4).

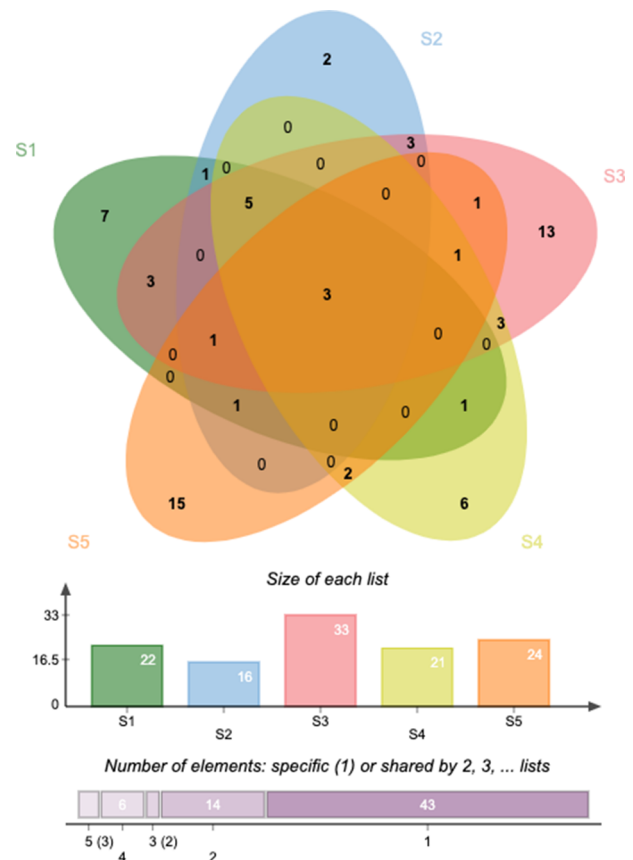


Figure 2. Venn diagram showing the numbers of non-fungal eukaryotic taxa detected in all five crushed rock samples and the numbers shared between samples from Lions Rump, King George Island, examined in this study.

Discussion

Our data confirm that DNA metabarcoding provides an effective tool to describe the DNA sequence diversity associated with a rock substrate from Antarctica. The large majority of the assigned DNA diversity detected in this study represents common and widespread taxa globally, although some could only be assigned to higher taxonomic ranks (e.g. class), limiting any inferences that can be made. We accept that the assignment of a DNA sequence does not confirm the presence of a viable organism, and such research is also limited by the quality and coverage of the available sequence databases. As the samples examined were immediately stored on collection in sterile containers and frozen before being processed in sterile flow hoods, we consider that the DNA obtained is unlikely to result from sample contamination, supported by the blanks and controls not suggesting contamination. In addition, if contamination were a contributing factor, we would expect more plausible contaminants to be present in multiple individual samples.

The presence of assigned marine taxa is unsurprising, as this coastal study site commonly experiences marine spray during periods of strong winds. Assignments to unknown taxa ranged from 3% to 10%, probably representing taxa not currently included in the consulted databases, and possibly including unsequenced or undescribed taxa. Future studies applying a wider range of markers (e.g. 16S, 18S, Cox1) are required to generate a more comprehensive taxon list.

Many of the taxa assigned (Table I) are likely to be found in the local environment and/or have previously been reported from Antarctica. Furthermore, the non-fungal eukaryotic diversity of our

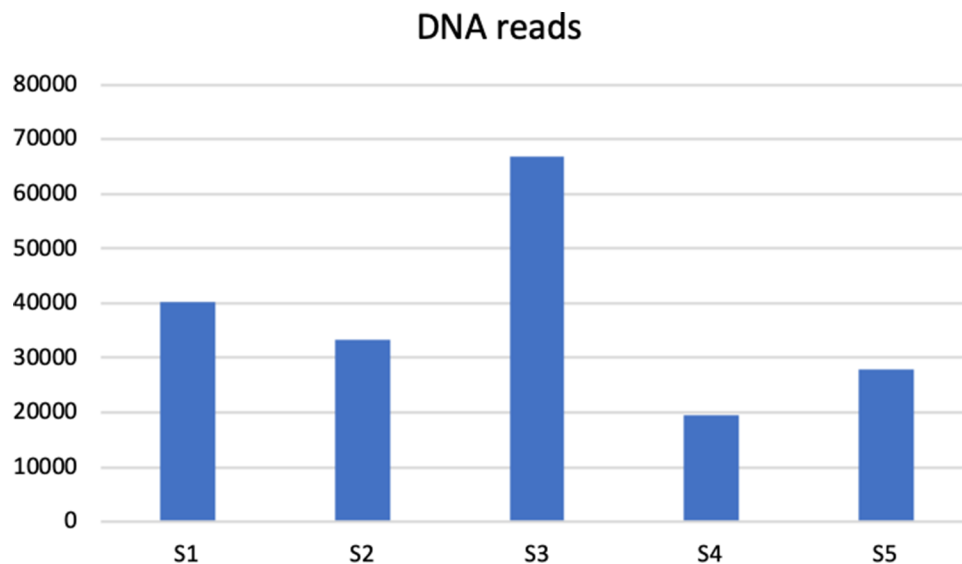


Figure 3. Numbers of DNA reads obtained from each of the five crushed rock samples obtained from Lions Rump, King George Island, in this study (y-axis = DNA reads, x-axis = sample number).

study location is poorly known, and no comprehensive biodiversity assessment has been made. The management plan for ASPA 151 (<https://www.ats.aq/devph/en/apa-database/55>) is vague in its description of the biodiversity present (e.g. noting that ‘knowledge of freshwater algae in this area is poor’) and provides no references, making it more difficult to address more precisely what is expected to be present. We expect that the local vegetation is quite similar to the overall King George Island description of Ochyra *et al.* (2008). Other assigned taxa are not known from Antarctica, and the inclusion of some tropical taxa (e.g. *Callicostella*, *Guazuma ulmifolia*) provides some support for the proposal of Câmara *et al.* (2023, 2024) that some biological material can be transported in the air column over long distances. However, such assignments could also indicate anthropogenic influence or contamination, such as those of *Brassica*, *Malus*, *Musa* and *Solanum*, which are human foodstuffs commonly present onboard vessels and at camping sites and research stations. Small fragments of such contaminants can be dispersed long distances, and their DNA can remain detectable after many years (Nobile *et al.* 2019, Ferreira *et al.* 2024). Although we have no means of confirming the origin of such ‘exotic’ DNA, it is pertinent to note that King George Island is one of the most visited locations in Antarctica, hosting the highest concentration of research stations and facilities on the continent, multiple tourist landing locations and even an airstrip.

Assignments to taxa that are associated with or causes of disease are notable. For instance, members of the genus *Myzocytiopsis* are widely known as parasites of nematodes, but they can also infect rotifers and amphipods (Rocha *et al.* 2017), while members of *Pythium* are plant parasites responsible for a wide range of diseases affecting hosts including soybean, peanut, tomato and maize, and they can even affect humans (Calvano *et al.* 2011). The finding of the latter genus could also be linked with the import of human food to Antarctica.

The sequence diversity differed between samples obtained at different stratigraphic positions, but this may simply have resulted from the single sample obtained from each position not capturing variability at each. Sample S3 (34.7 m), the part of the stratigraphic profile closest to the top of the profile close to the edge of the escarpment, generated the greatest diversity and relative abun-

dance. The least diverse and abundant assemblage was that of sample S2 (24.0 m), located in the part of the profile that is driest and steepest. However, lithology appeared to have little or no influence on diversity detected, but a larger sample size would be required to draw further conclusions. The most diverse sample, S3, and the least diverse sample, S2, were both conglomerates - a coarse-grained rock type characterized by high porosity. Meanwhile, sample S4, which was finer grained and consequently had lower porosity, showed diversity similar to that of sample S1, another conglomerate. The sample with the lowest relative abundance, S4, was a siltstone - a low-porosity rock (see Rabelo *et al.* 2024).

Rabelo *et al.* (2024) reported the assignment of 198 fungi taxa from the same samples, compared with only 70 other eukaryotic taxa found in the present study. This could reflect that fungi are commonly very diverse in various Antarctic substrates (Ogaki *et al.* 2021, Rosa *et al.* 2022), but it could also represent an artefact of the primers used, as our study used ITS2 alone. As there are no universal barcoding markers for all organisms, using a wider range of markers would probably reveal more taxa from different groups (e.g. 18S for protists and COX1 for metazoans). Furthermore, although the interpretations of Rabelo *et al.* (2024) focused on endolithic organisms, it is not possible here to differentiate for most microbial taxa whether they might represent true endoliths or those present on the rock surface. However, none of the taxa reported here have been reported as true endoliths, although there is limited information on endolithic taxa in Antarctica other than fungi and associated photobiont algae, as well as bacteria (Hughes & Lawley 2003, Martins *et al.* 2020).

Câmara *et al.* (2022) investigated plant sequence diversity derived from seven rock surface samples obtained in the Ellsworth Mountains at the base of the Antarctic Peninsula in Continental Antarctica using the same methodology and marker (ITS2) as here. They reported 48 distinct assignments, of which 40 were flowering plants, a group not present in that region and that reaches the southern distributional limit of the two native Antarctic species on northern Alexander Island, $\sim 10^\circ$ of latitude further north. These assignments also included some for taxa commonly associated with human activities (e.g. *Musa*, *Curcubita*, *Pimpinella*), as well as others with no such association but with known wind dispersal

Table 1. Numbers of DNA sequences assigned to specific taxa from each of the five crushed rock samples obtained from Lions Rump, King George Island. Habitat (Hab.) and distribution (Distr.) descriptors: As = Asia; An = Antarctica; Au = Australia; B = brackish; Bi = bipolar; C = cosmopolitan; Eu = Europe; F = freshwater; Fk = Falkland/Malvinas Islands; M = marine; ME = Middle East; Neo = Neotropical; Sa = South America; St = subtropical; T = terrestrial; Tr = tropical; W = widespread.

	Hab./Distr.	S1	S2	S3	S4	S5
KINGDOM CHROMISTA (22 taxa)						
PHYLUM CILIOPHORA	W/C	339	0	0	0	0
Class Oligohymenophorea	W/C	8966	3627	310	268	0
<i>Homalogastra</i> sp.	M/C	1205	0	388	0	0
Order Sessilida	W/C					
<i>Opercularia</i> sp.	F/W	0	0	0	215	0
<i>Pyxidium tardigradum</i>	T/W	171	100	434	68	0
Order Sporadotrichida	W/C	1037	1056	0	625	0
Family Oxytrichidae	W/C	0	600	0	0	0
<i>Laurentiella</i> sp.	F/W	0	0	21	0	0
<i>Protogastrostyla</i> sp.	M, B/?	0	0	0	0	324
<i>Sterkiella</i> sp.	F/?	247	0	0	0	0
Order Haptorida	W/C					
Family Spathidiidae	W/C	0	386	0	0	0
<i>Bryophyllum</i> sp.	W/C	0	0	33	0	0
Class Nassophorea						
<i>Nassula</i> sp.	W/C	0	0	0	1018	0
Class Phyllopharyngea						
<i>Podophrya fixa</i>	W/C	134	0	0	0	0
Class Spirotrichea						
Order Urostylida	W/C	0	0	167	0	0
<i>Anteholosticha</i> sp.	W/C	632	1194	0	0	2285
Family Urostylidae	W/C	0	0	0	0	392
PHYLUM OOMYCOTA						
<i>Pythium caudatum</i>	F, T/W	0	0	452	0	0
<i>Pythium</i> sp.	W/C	38	0	336	0	0
<i>Myzocytiopsis</i> sp.	W/C	29	0	0	0	0
KINGDOM PROTOZOA (2 taxa)						
PHYLUM CERCOZOA	W/C	0	497	0	0	0
PHYLUM HETEROLOBOSEA						
<i>Neovahlkampfia damariscottae</i>	M, B/NA	253	0	72	0	0
KINGDOM VIRIDIPLANTAE (42 taxa)						
PHYLUM CHLOROPHYTA	W/C	0	0	1884	234	283
Order Chlamydomonadales	W/C	2178	1894	7968	1409	379
<i>Chlamydomonas leiostraca</i> ^a	F, T/W	3777	3980	868	181	0
<i>Chlamydomonas</i> sp.	W/C	0	0	29	0	0
<i>Chlorococcum</i> sp.	W/C	0	0	58	50	0
<i>Chloromonas fonticola</i>	F/?	314	714	60	376	0
<i>Chloromonas</i> sp.	W/C	4900	2081	9668	501	1078

(Continued)

Table I. Continued.

	Hab./Distr.	S1	S2	S3	S4	S5
Order Chlorellales	W/C	0	0	0	0	261
Family Chlorellaceae		0	0	489	0	0
<i>Auxenochlorella symbiontica</i>	F/ME	0	0	140	0	0
<i>Chlorella</i> sp.	W/C	0	0	0	0	76
<i>Compactochlorella</i> sp. ^a	F/?	0	0	0	0	102
<i>Meyerella</i> sp. ^a	F	0	0	0	0	11 949
<i>Muriella terrestris</i>	T/W	0	0	0	0	1612
Order Prasiolales						
<i>Desmococcus olivaceus</i>	T/W	0	0	0	143	49
<i>Prasiola</i> sp.	W/C	13 212	5883	24 074	3472	10
<i>Raphidonema</i> sp.	F/W	0	0	0	0	118
Order Sphaeropleales						
<i>Neocystis</i> sp.	F/W	0	0	0	0	776
<i>Tetrademus distendus</i> ^a	F/?	0	7859	0	0	0
Order Trebouxiales						
<i>Coccomyxa</i> sp.	W/C	0	0	0	0	147
<i>Lobosphaera</i> sp.	T, F/W	0	0	0	0	4046
<i>Myrmecia pyriformis</i>	F/W	0	0	0	0	463
<i>Trebouxia potteri</i>	F/Eu	0	0	115	0	0
<i>Trebouxia solaris</i>	F/Eu	0	0	181	0	0
Order Ulotrichales						
<i>Chlorothrix</i> sp.	M/W	0	0	0	1087	0
<i>Planophila</i> sp.	M, F/W	0	0	0	634	592
PHYLUM BRYOPHYTA						
Family Amblystegiaceae						
<i>Sanionia</i> sp.	T/Bi	0	0	0	0	161
Family Bryaceae	T/W	0	0	0	6102	0
Family Dicranaceae						
<i>Dicranum</i> sp. ^a	T/W	0	0	59	0	0
Family Pilotrichaceae						
<i>Callicostella</i> sp. ^a	T/Tr	20	0	0	0	0
Family Rhizogoniaceae						
<i>Pyrrhobryum latifolium</i> ^a	T/As, Au	629	841	446	0	72
PHYLUM MAGNOLIOPHYTA						
Family Asteraceae ^a	T/W	0	0	361	0	0
Family Brassicaceae						
<i>Brassica</i> sp. ^a	T/W	8	0	0	163	0
Family Malvaceae						
<i>Guazuma ulmifolia</i> ^a	T/Neo	162	0	0	0	0
<i>Sida cordata</i> ^a		0	0	61	0	0
Family Musaceae						
<i>Musa</i> sp. ^a	T/Tr	97	152	0	0	0

(Continued)

Table I. Continued.

	Hab./Distr.	S1	S2	S3	S4	S5
Family Poaceae	T/W	0	0	0	0	71
<i>Deschampsia parvula</i> ^a	T/Sa, Fk	83	0	0	0	0
<i>Zoysia japonica</i> ^a	T/As	0	0	0	248	0
<i>Zoysia sp.</i> ^a	T/Tr/St	0	0	14	720	0
Family Rosaceae						
<i>Malus domestica</i> ^a	T/W	0	11	0	0	0
Family Solanaceae						
<i>Solanum sp.</i> ^a	T/W	0	0	0	53	0
KINGDOM HOLOZOA (1 taxon)						
<i>Ichthyosporia sp.</i>		0	0	0	0	202
KINGDOM METAZOA (3 taxa)						
PHYLUM ARTHROPODA						
Family Isotomidae	T/W	0	0	0	160	0
<i>Folsomia sp.</i>	T/W	0	0	0	0	174
PHYLUM TARDIGRADA						
<i>Ramajendas frigidus</i>	T/An	0	0	23	0	0
UNKNOWN		1770	2292	6490	937	922
<i>Total</i>		40 201	33 205	66 814	19 624	27 820

^a Previously unreported taxon for Maritime Antarctica.

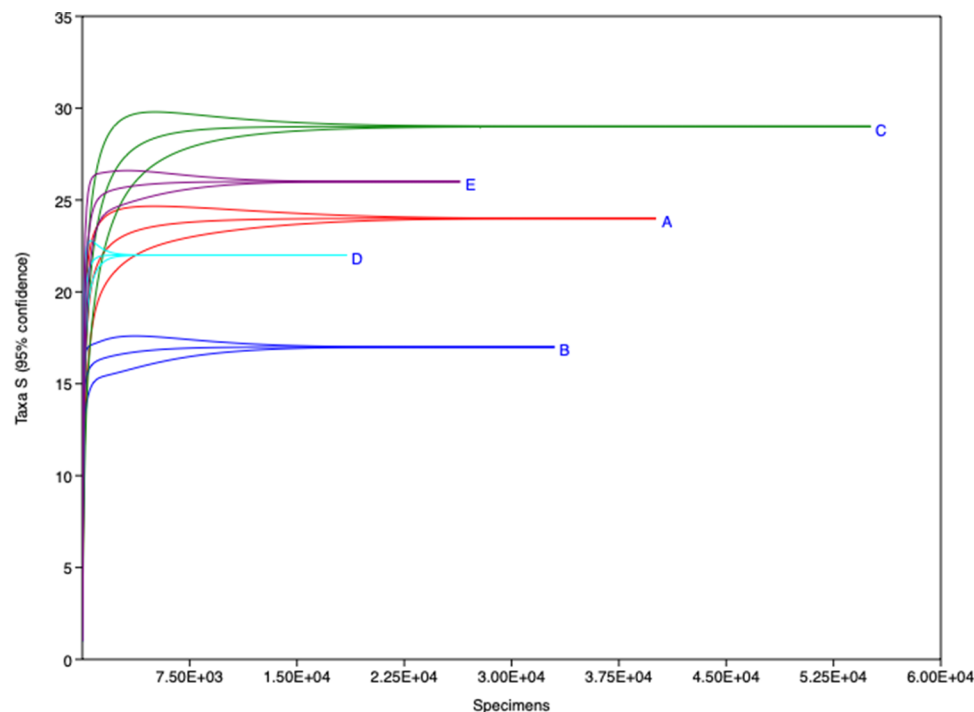


Figure 4. Rarefaction curves of sequence assignments ('taxa') obtained from each of the five crushed rock samples based on taxa profile (0.03 similarity) and showing 95% confidence limits (A = S1, B = S2, C = S3, D = S4 and E = S5).

of pollen, such as the grass family Poaceae. Fraser *et al.* (2018) investigated nine sites near Mount Erebus on Ross Island, also in Continental Antarctica, using three different markers, and they also reported sequence assignments to exotic species, including trees (ash) and human foodstuffs such as soy and wheat.

The use of morphology alone to study endolithic and hypolithic communities has been employed before. Hughes & Lawley (2003) investigated endolithic communities in the Dry Valleys and reported only prokaryotes and fungi. Khan *et al.* (2011), using molecular tools, reported the presence of green algae and mosses

on hypolithic communities also in the Dry Valleys, but mostly only at a higher-rank taxonomic level.

Conclusions

Our study indicates that rock surfaces in the Lions Rump ASPA host a significant diversity of DNA sequences representing eukaryotic taxa. Many of these are plausible indicators of native taxa, while others suggest potential human influence, and some others may indicate the presence of potentially pathogenic taxa. While further directed studies are required to confirm the presence of living or viable organisms or propagules, the continued use of metabarcoding approaches will represent an important contribution to monitoring habitats in the Antarctic Peninsula region as climatic conditions become progressively more environmentally favourable, particularly in the context of identifying the establishment of new incoming organisms.

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Competing interests. The authors declare none.

Author contributions. PEASC and LHR designed the study and secured funds, NGR and MC-S performed laboratory work, FACL performed the bioinformatics analyses, MAC, SMS, GS and PAS performed the fieldwork, VNG performed the analyses and PC contributed to data interpretation and the development of the manuscript. All authors contributed to the final writing of the text.

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