

A SUB-ANTARCTIC PEAT MOSS METAGENOME INDICATES MICROBIOME RESILIENCE TO STRESS AND BIOGEOCHEMICAL FUNCTIONS OF EARLY PALEOZOIC TERRESTRIAL ECOSYSTEMS

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Premise of research. Abundant peat mosses and epibiotic microbiota, common in widespread modern peatlands, constitute complex biotic systems recognized to provide globally significant ecosystem services: organic carbon sequestration, methane oxidation, and nitrogen fixation. Because recent fossil and molecular diversification evidence indicates that peat mosses are >450 Myr old, they may be among Earth's earliest land plants. The biogeochemical effects of early vegetation, which may have occupied remote islands and experienced nutritional stress and episodes of high UV radiation, are poorly understood. To gain insight into taxonomic composition, biogeochemical function, and resilience of archaic peat moss microbiomes, we performed shotgun metagenomic sequencing of a similarly stressed modern peat moss.

Methodology. *Sphagnum fimbriatum* was sampled from Chilean sub-Antarctic Navarino Island, which is remote from nutrient pollution and subject to high UV beneath the southern ozone hole. 16S, 18S, 23S, and 28S ribosomal RNA sequences filtered from contigs assembled from long-read Roche 454 and deep short-read Illumina sequences were employed to infer prokaryotic and eukaryotic microbiome composition at generic and higher levels for comparison to peat moss microbiota reported for other locales.

Pivotal results. Comparison of bacterial diversity—including >75 genera represented by ≥100 sequence reads and >250 genera represented by ≥10 reads—to diversity reported from previous peatland studies indicated the operation of recognized peatland functions (carbon sequestration, methane oxidation, and nitrogen fixation), signifying stress resilience. Eukaryotic features included >45 protist genera, some of pivotal evolutionary significance; distinctive fungal associations; and ancient lineages of microscopic invertebrate animals, including spring-tails known to foster moss reproduction.

Conclusions. Metagenomic approaches provide a new, expanding window into early terrestrial vegetation and its biogeochemical effects, indicating that complex peat moss systems resilient to stressful environmental conditions likely occurred in deep time and persist to the present.

Keywords: metagenome, microbiome, Paleozoic, peatland, peat moss, UV.

Online enhancements: appendix tables and figures.

Introduction

Modern peat mosses dominate modern, widespread terrestrial/semiaquatic ecosystems known as *Sphagnum* peatlands, creating environmental conditions favorable to grass pink (*Calopogon*) orchids, insectivorous sundew and pitcher plants, and other distinctive vegetation (Crum 1992; Rydin and Jeglum 2006). Peat mosses are also recognized for hosting diverse bacterial epibionts, together forming consortia that play globally significant environ-

mental roles: organic carbon sequestration (Gorham 1991), nitrogen fixation (Rydin and Jeglum 2006), and methane oxidation (Kip et al. 2010). Peat mosses also provide a key habitat for diverse microscopic protists, fungi, and invertebrate animals (Hingley 1993) of ancient lineage (Porter and Knoll 2000; Parfrey et al. 2011; Chang et al. 2015). Together, peat mosses and associated microbiota represent complex biotic systems whose taxonomic and functional complexity is incompletely understood but if better known would illuminate both modern peatland function and the biogeochemical roles played by Earth's earliest vegetation.

The large, abundant, porose hyaline cells characteristic of peat moss leaves and stem surfaces (Crum 1992) provide a large surface area for microbial biofilm formation as well as habitat for eukaryotic components of the moss microbiota (Hingley

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1993). Newly reported Middle Ordovician microfossils that display such distinctive porose cells and consequently were interpreted as leaf and stem remains of peat moss (Cardona-Correa et al. 2016) are consistent with recent molecular diversification analyses indicating that peat mosses (and the related moss *Takakia*) represent early-diverging lineages of modern land plants that originated during the late Proterozoic–early Paleozoic (460–607 Ma; Laenen et al. 2014). These observations suggest that complex peat moss systems have played important global biogeochemical roles over much deeper time than previously thought, but those roles are as yet unclear.

The presence of peat mosses in the early Paleozoic fossil record is consistent with evidence (1) that peat mosses typically produce distinctive vegetative and reproductive tissues that are able to resist hydrolytic degradation (Kroken et al. 1996) long enough to reach anaerobic sediments and form fossils and (2) for diverse types of Ordovician microfossils interpreted as reproductive remains of bryophyte-like early land plants (Gray et al. 1982; Strother et al. 1996; Steeman et al. 2009; Rubinstein et al. 2010; Wellman and Strother 2015). The concept that peat mosses represented early terrestrial vegetation is also supported by numerous features of modern peat mosses known to foster success under stressful environmental conditions, including limiting levels of essential minerals, such as combined nitrogen and enhanced UV exposure.

Stress adaptations of modern peat mosses include efficient uptake of combined nitrogen (Fritz et al. 2014), mixotrophic capacity (Graham et al. 2010), structural responses to elevated temperature and UV (Cardona-Correa et al. 2015b), light-directed growth enabling reorientation after spatial disturbance (Cardona-Correa et al. 2015a), capacity for rapid asexual propagation into large landscape areas (Rydin and Jeglum 2006), effective sexual dispersal (Whitaker and Edwards 2010) occurring on continental to intercontinental scales (Sundberg 2013), and the formation of partnerships with beneficial bacteria that enhance plant nutrition, defense, or growth (Bragina et al. 2012, 2013, 2015; Müller et al. 2015).

The Middle Ordovician through the pre-Hirnantian Late Ordovician (470–445 Ma) period commonly associated with early terrestrial vegetation (Wellman and Strother 2015) featured a mild climate and attendant high sea levels, such that remote oceanic islands are thought to have been important centers of biotic evolution, dispersal, and migration (Cocks and Torsvik 2004). Emergent weathering-resistant quartzite islands are hypothesized to have been the terrestrial sources of 455–454 Ma multicellular remains interpreted as peat moss fragments that were extracted from nearby marine carbonates (Cardona-Correa et al. 2016). Early terrestrial vegetation may also have been influenced by episodes of increased exposure to UV-B radiation, which has been posited to result from outer space gamma ray bursts that reduced atmospheric ozone (Melott et al. 2004). Analogous conditions of stratospheric ozone depletion and attendant higher UV-B levels affect modern high-latitude regions, where ozone depletion has been linked to photocatalysis in polar air masses of ozone to oxygen by chlorine monoxide arising from pollutants. Although recent healing of the Antarctic ozone layer may have begun as the result of policy-directed reduction in anthropogenic halocarbons, volcanic eruptions continue to contribute to enlargement of the southern ozone hole (Solomon

et al. 2016). Biologists have expressed concern regarding continued UV-B effects on Antarctic and sub-Antarctic biota (Ballaré et al. 2001; Rozema et al. 2005), including peat moss systems (Robson et al. 2005). Sub-Antarctic Chile is of particular concern because this region is recognized for harboring exceptional bryophyte diversity that includes peat mosses (Rossi et al. 2008; Goffinet et al. 2012), has for decades been influenced by enhanced UV-B associated with the southern ozone hole, and is also vulnerable to the atmospheric effects of southern Andean volcanic eruptions (Sapkota et al. 2007).

UV radiation is known to influence communities of bacteria and other microbes in aquatic and terrestrial ecosystems; bacteria are regarded as particularly sensitive to UV because they are often too small to contain sufficient concentrations of UV-screening compounds (Ballaré et al. 2011; Häder et al. 2011). Experimental microcosm field studies revealed that relatively high UV radiation levels present in Patagonia, Argentina, generated substantial differences in bacterioplankton community composition by comparison to adjacent communities protected by UV screening materials, and these differences were postulated to have functional effects (Manrique et al. 2012). In addition, variation in the local distribution of sub-Antarctic corticolous lichens having cyanobacterial photobionts suggested vulnerability to UV exposure (Trest et al. 2015). Together, these observations indicate that the microbiota of peat mosses and other hosts living in environments influenced by the southern ozone hole likely experience UV stress.

To learn more about the microbiomes of UV-stressed peat mosses and to infer the biogeochemical roles played by early peat moss microbiota, we analyzed shotgun metagenomic DNA sequences derived from washed fragments of *Sphagnum fimbriatum* sampled from a sub-Antarctic peatland located on Navarino Island, XII Region Magallanes and Chilean Antarctica, Chile. In contrast to northern high-latitude peatland sites that may be enriched by anthropomorphic nitrogen deposition (Bragazza 2006; Zajac and Blodau 2016), Navarino Island is remote from concentrated sources of airborne industrial pollutants. Peat core studies from Navarino Island indicate that atmospheric dust deposits derive from continental Patagonia and have been consistent over the past 6000 yr (Sapkota et al. 2007).

Metagenomic sequence data were employed to assess *S. fimbriatum*-associated biotic diversity. 16S and 23S ribosomal DNA (rDNA) sequences were used to identify as many peat moss-associated bacteria as possible to the level of genus to facilitate inferences of functional diversity and for comparison to bacterial genera reported from culture and culture-independent studies of other peatland locales, to infer stress resilience of the sub-Antarctic peat moss system. A catalog of relatively abundant bacterial taxa that could not be identified to genus with the use of current taxonomic databases was also assembled, to indicate potential new biotic and functional diversity present in the sub-Antarctic system. 18S and 28S rDNA sequences filtered from the same metagenomic data set were used to explore the diversity of peat moss-associated eukaryotes, which have less commonly been the focus of high-throughput sequencing studies of plant hosts but are known to be important components of the microbiomes of an aquatic bryalean moss, freshwater green algae, and a terrestrial liverwort (Nakai et al. 2012; Graham et al. 2015; Knack et al. 2015).

Material and Methods

Collection Location

Random samples of a green peat moss later identified on the basis of microscopic leaf characters as *Sphagnum fimbriatum*, previously known to occur on Navarino Island (Goffinet et al. 2012), were collected by gloved operators who had extensive experience with peat moss field collection into sterile, transparent Whirl-Pak bags (Nasco, Fort Atkinson, WI) on January 11, 2014, from multiple microsites within an unshaded peatland located at S54°56.489, W67°37.377, on Navarino Island, XII Region Magallanes and Chilean Antarctica, Chile. This island occurs within the UNESCO Cabo de Hornos Biosphere Reserve (Goffinet et al. 2012), which is known for harboring a high diversity of bryophytes and lichens (Rossi et al. 2008), is remote from airborne industrial pollution (Sapkota et al. 2007), and has long been impacted by UV radiation arising from the southern ozone hole. NASA data for September 16, 2013, approximately 4 mo previous to sample collection, indicated an ozone level of 250–300 Dobson units (http://ozonewatch.gsfc.nasa.gov/Scripts/big_image.php?date=2013-09-16&hem=S). On the day of collection, UV-A + UV-B readings averaged 120 mW/cm², determined as described by Trest et al. (2015) for lichen biodiversity research performed on Navarino Island during the same time period by the same team. Water pH at the collection site was 4.7. In the general area of the collection site, precipitation is documented to average 467 mm, is distributed fairly evenly throughout the year, and includes some snow; the annual average temperature was 6°C (Trest et al. 2015).

Sample Processing and DNA Extraction and Sequencing

Individual moss leaves from different parts of hundreds of *S. fimbriatum* plants, together with fewer stem fragments and small terminal leaf clusters (capitula), were excised by a gloved operator within a few hours after collection, with the aid of alcohol-sterilized fine forceps, on the inside surface of a sterile Whirl-Pak freshly exposed by slicing one side seam apart with a sterilized single-edged razor blade. During this process, liverworts, the peat moss *Sphagnum magellanicum*, and vascular plants that also occurred at the site were specifically excluded from samples. Excised *S. fimbriatum* parts were immediately placed into DNA/RNA-free Eppendorf tubes containing collection site water that had been freshly filtered and delivered by means of a 50-mL sterile syringe equipped with a 0.2-μm syringe tip filter. Tubes containing mixtures of material excised from multiple plants and microsites were hand-agitated to dislodge loosely attached materials, then liquid was removed with the use of sterile pipettes and replaced twice more with filtered collection site water to complete a washing process designed to remove loosely associated microbiota (Graham et al. 2015; Knack et al. 2015). On days 4 and 7 during transit, each tube was briefly opened while held within an unzipped Whirl-Pak held upside down, to allow aeration without airborne contamination. No intact moss plants were transported, and except during brief periods of sampling for DNA extraction and microscopy, moss material was retained within closed Eppendorf transport tubes in a lit growth cabinet.

Ten days after field collection, DNA was extracted from washed peat moss parts using an MPBio FastDNA SPIN Kit for soil (MP Biomedicals, Solon, OH), modified by adding 10 mg mL⁻¹ lysozyme in the initial step. Pooled DNA was shotgun sequenced at the University of Wisconsin–Madison Biotechnology Center using (1) a Roche 454 sequencing platform (Flx+ chemistry) to acquire longer reads averaging 530 bp, a significant proportion of which were greater than 600 bp in length, totaling ~640 Mb; and (2) paired-end Illumina sequencing for greater depth in the form of shorter reads (table A1; tables A1–A3 are available online).

Bioinformatic Processing

Sequence reads were processed using the next-generation sequencing analysis pipeline of the SILVA ribosomal RNA (rRNA) gene database project (SILVAngs ver. 1.3; Ionescu et al. 2012; Klindworth et al. 2013; Quast et al. 2013). The SILVA Incremental Aligner (SINA ver. 1.2.10 for ARB SVN, rev. 21008; Pruesse et al. 2012) was applied against the SILVA small subunit (SSU) and large subunit (LSU) rRNA SEED. Reads shorter than 50 aligned bases and those with >2% ambiguities or homopolymers were excluded before further processing, as were likely contaminants and artifacts and those having low alignment quality scores. Identical reads were identified and unique reads clustered using cd-hit-est software (ver. 3.1.2; Weizhong and Godzik 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98 as operational taxonomic units (OTUs) for classification performed by local nucleotide BLAST search against the nonredundant version of the SILVA SSU and LSU reference data sets (rel. 123.1), using blastn version 2.2.30+ with standard settings (Camacho et al. 2009). To determine numbers of individual reads per taxonomic path (within the constraints imposed by possible sequencing biases and occurrence of multiple rRNA operons), the classification of each OTU reference read was mapped onto all reads assigned to the respective OTU. Reads for which there were no or weak ([% sequence identity + % alignment coverage]/2 < 93) BLAST hits remained unclassified and were labeled “no relative” in SILVAngs fingerprint and Krona charts (Ondov et al. 2011). Summary rarefaction curves are shown in figures A1 and A2 (available online). rDNA sequence data employed in this study were deposited in the NCBI Short Read Archive (<http://www.ncbi.nlm.nih.gov/sra>) as BioProject PRJNA 384422.

Comparisons to Bacterial Genera Inferred from Previous Peat Moss or Peatland Studies

To assess the degree to which the Navarino Island *S. fimbriatum* bacterial communities resembled those of other peat mosses or peatlands, including systems not located beneath the southern ozone hole, we counted Navarino Island bacterial genera that had been reported in five other studies chosen for particular features. Comparisons were accomplished at the generic level because genera represent the best-resolved taxonomic level commonly reported in high-throughput studies of microbiota, and genera are widely regarded to provide more accurate insight into potential functional roles than higher-level family-phyllum clas-

sification. We employed information from Dedysh (2011) because this report summarized bacterial genera that had been isolated and cultivated in the laboratory by numerous investigators who had sampled diverse peatland sites. Four additional reports were chosen because they report bacterial genera associated with peat mosses or peatlands surveyed with the use of culture-independent, high-throughput molecular methods similar to those we used. Bragina et al. (2012, 2015) employed 16S rDNA amplicons to survey peat moss-associated bacteria in alpine raised bogs of Austria. In a search for potential antibiotics, Müller et al. (2015) employed a metagenome produced for *S. magellanicum* sampled from an alpine bog to obtain gene sequences classifying with nonribosomal peptide synthetases and polyketide synthases; sequence data were used to infer the presence of bacterial genera likely to produce these compounds. The Quiroga et al. (2015) study employed high-throughput sequencing data to classify genera of bacterioplankton in pools in a Tierra del Fuego, Argentina, peat bog, nearer to our sample locale on Navarino Island.

Genera common to the present study and the five previous investigations are counted and annotated with appropriate literature citations in table A2, which lists all bacterial taxa (including genera) inferred to occur in the *S. fimbriatum* microbiota at rDNA sequence read levels >10. Taxa detected in lower read numbers are not included in table A2.

One caveat of our comparison between Navarino Island *S. fimbriatum* bacterial associates and those reported from other locales is that most past sequence-based studies of peat moss or peatland bacteria have relied on a single sequence marker—namely, 16S rDNA—whereas we utilized both 16S and 23S rDNA to infer bacterial taxa, finding that 23S rDNA detected diversity that was not apparent from 16S rDNA sequence data alone. For this reason, our comparison provides a conservative estimate of community overlap. In noting bacterial genera found in two or more of the specified locales, we focused on those generally associated with recognized peatland functions, namely, organic carbon sequestration, nitrogen fixation, and methane oxidation. The degree to which bacterial genera indicating these key functions occurred in the Navarino Island peat moss microbiome was used to estimate the level of community functional resilience to stresses (e.g., relatively high UV exposure) associated with this locale. Absence of bacterial genera associated with one or more key peatland functions would indicate lack of functional resilience.

Microscopy

At the time of DNA extraction, bright-field microscopy was performed with a Zeiss Axioplan fluorescence microscope, and images were recorded with a Nikon D300s digital camera and Camera Control Pro software (Nikon, Melville, NY). For SEM, moss leaves were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer at pH 7, dehydrated in an ethanol series to 95%, then critical-point dried. Material was attached to SEM stubs using carbon tape, fine forceps were used to expose inner hyaline cell walls with attached microbiota, and material was coated with iridium before examination using a Hitachi S-4800 ultra-high-resolution cold cathode field emission SEM operated at 5 kV at the University of Wisconsin–Milwaukee Electron Microscopy Laboratory.

Results

Shotgun metagenomic analyses provided evidence that the peat moss *Sphagnum fimbriatum*, collected from a peatland on remote sub-Antarctic Navarino Island and therefore subject to nutrient-availability challenges and enhanced UV exposure, possessed a complex microbiome including diverse bacteria and eukaryotes (figs. 1–3; tables A2, A3). As expected, a large number (1984) of 18S rDNA sequence reads classified as the genus *Sphagnum*, consistent with morphological evidence for identification of the sampled peat moss as *S. fimbriatum*.

Bacterial Taxonomic and Functional Diversity

Microscopy revealed the occurrence of bacteria of diverse morphotypes attached to inner wall surfaces of porose hyaline cells (fig. 1). More than 75 bacterial genera (fig. 2) and 28 additional

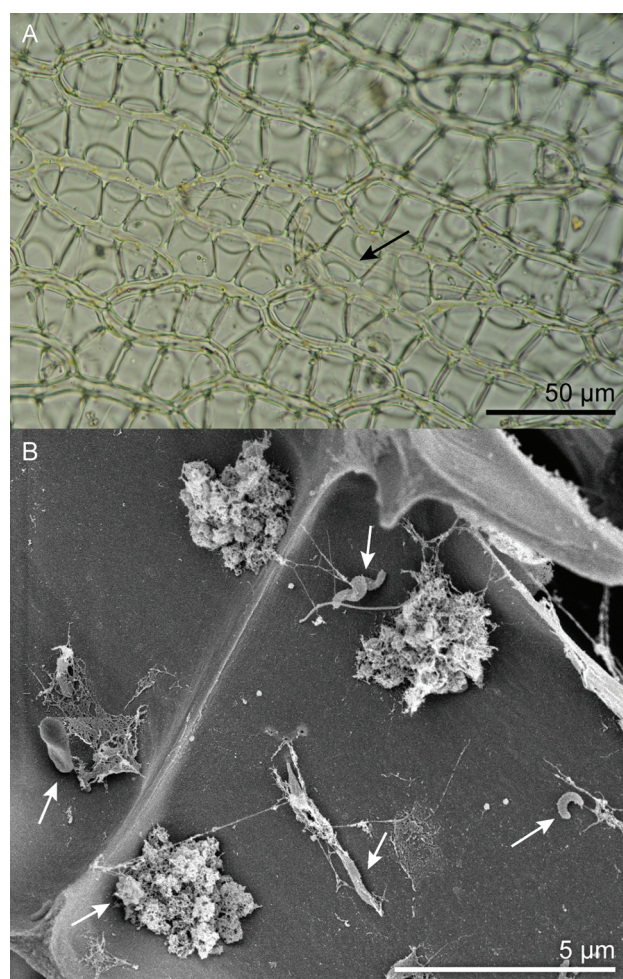


Fig. 1 Microscopic evidence for *Sphagnum fimbriatum* microbiota. A, Bright-field view of typical peat moss leaf showing porose hyaline cells containing bacterial and eukaryotic organisms, including fungal hyphae (arrow). B, SEM view of peat moss leaf showing structurally diverse microbiota (arrows) attached by mucilage to the inner surface of the wall of a porose hyaline cell that had been broken open immediately prior to metal coating.

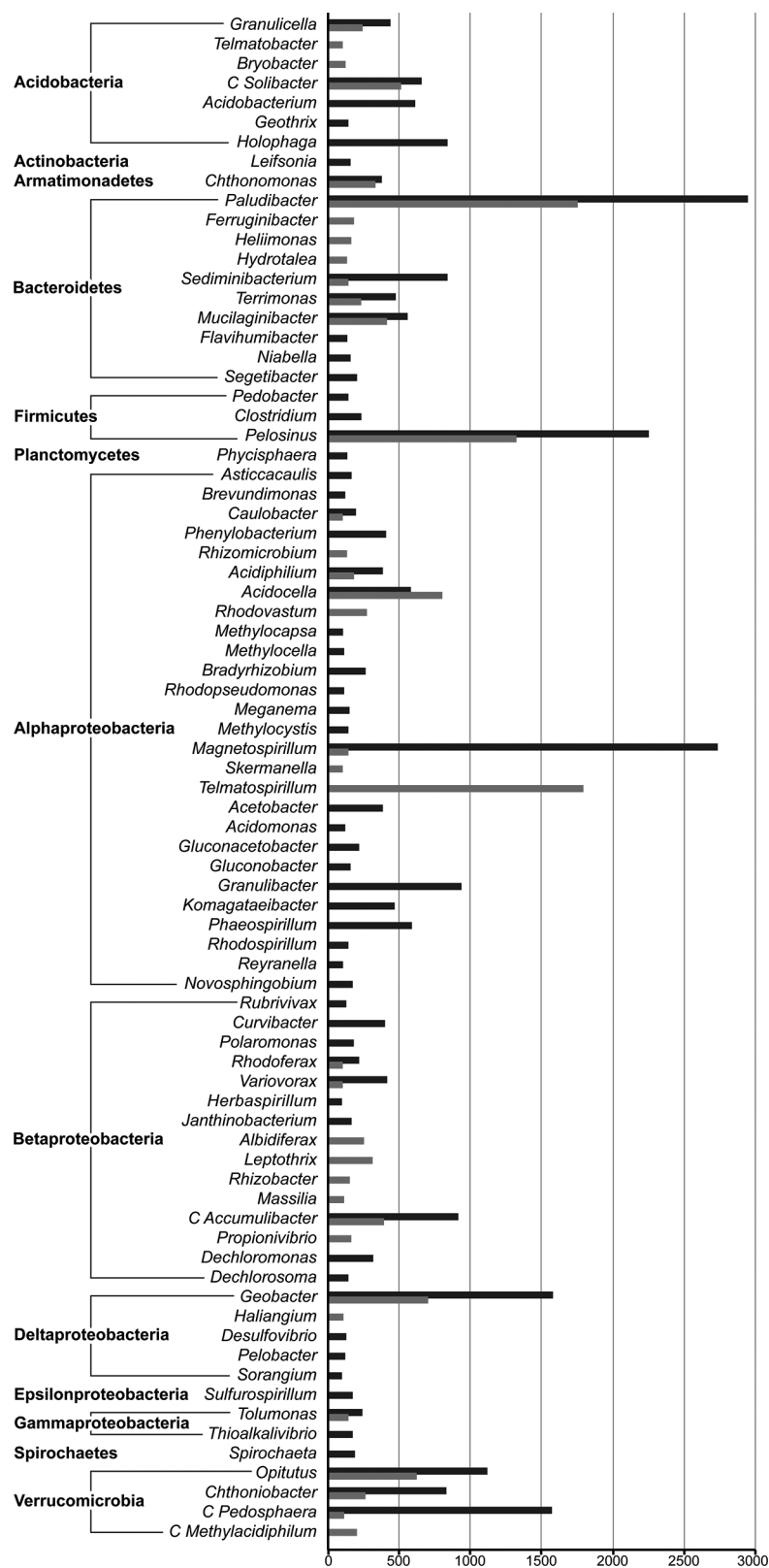


Fig. 2 Relatively abundant bacterial genera, that is, those defined by >100 16S or 23S ribosomal DNA (rDNA) reads. Light gray bars indicate 16S rDNA read abundance; dark gray bars indicate 23S rDNA read abundance. “C” indicates *Candidatus* (provisional genera). Some genera were detected only by 16S rDNA sequences, some were detected only by 23S rDNA sequences, and some were detected by both, with read abundances differing. A color version of this figure is available online.

bacterial groups of higher taxonomic level (family and higher) that could not be classified more precisely with existing databases (fig. 3) were represented by at least 100 16S and/or 23S rDNA sequence read numbers and therefore were considered relatively abundant. An additional >200 bacterial genera and >100 higher bacterial taxa (family-phylum) were detected at ≥ 10 reads (table A2). Further biodiversity may be reflected by 1607 16S and 9098 23S rDNA high-quality reads that could not be classified into known bacterial phyla using existing reference databases (see table A1).

Relatively abundant bacterial genera included *Bradyrhizobium* (fig. 2), many species of which are known to fix nitrogen. Diverse additional genera associated with nitrogen fixation—for example, *Azorhizobium*, *Azospirillum*, *Mesorhizobium*, and *Rhizobium*—were detected at lower read abundances (table A2).

Sequence reads classifying into bacterial genera that included known methane oxidizers (Alphaproteobacteria *Methylocapsa*, *Methylocella*, and *Methylocystis* and Verrucomicrobia *Candidatus Methyacidiphilum*)—known to promote peat moss growth by generating carbon dioxide, which is potentially useful in overcoming carbon limitation—were also relatively abundant in the Navarino Island system (fig. 2). Additional bacterial genera commonly associated with methane oxidation (*Methylibium*, *Methylosinus*) were detected at lower read abundances (table A2).

Other relatively abundant Navarino Island peat moss-associated bacterial genera are functionally linked to carbon cycling processes: *Tolumonas* and *Phenylbacterium* are known for the capacity to break down phenolic compounds, *Komagataeibacter* is a known microbial cellulose producer indicating carbon sequestration, and *Opitutus* and *Chthoniobacter* have

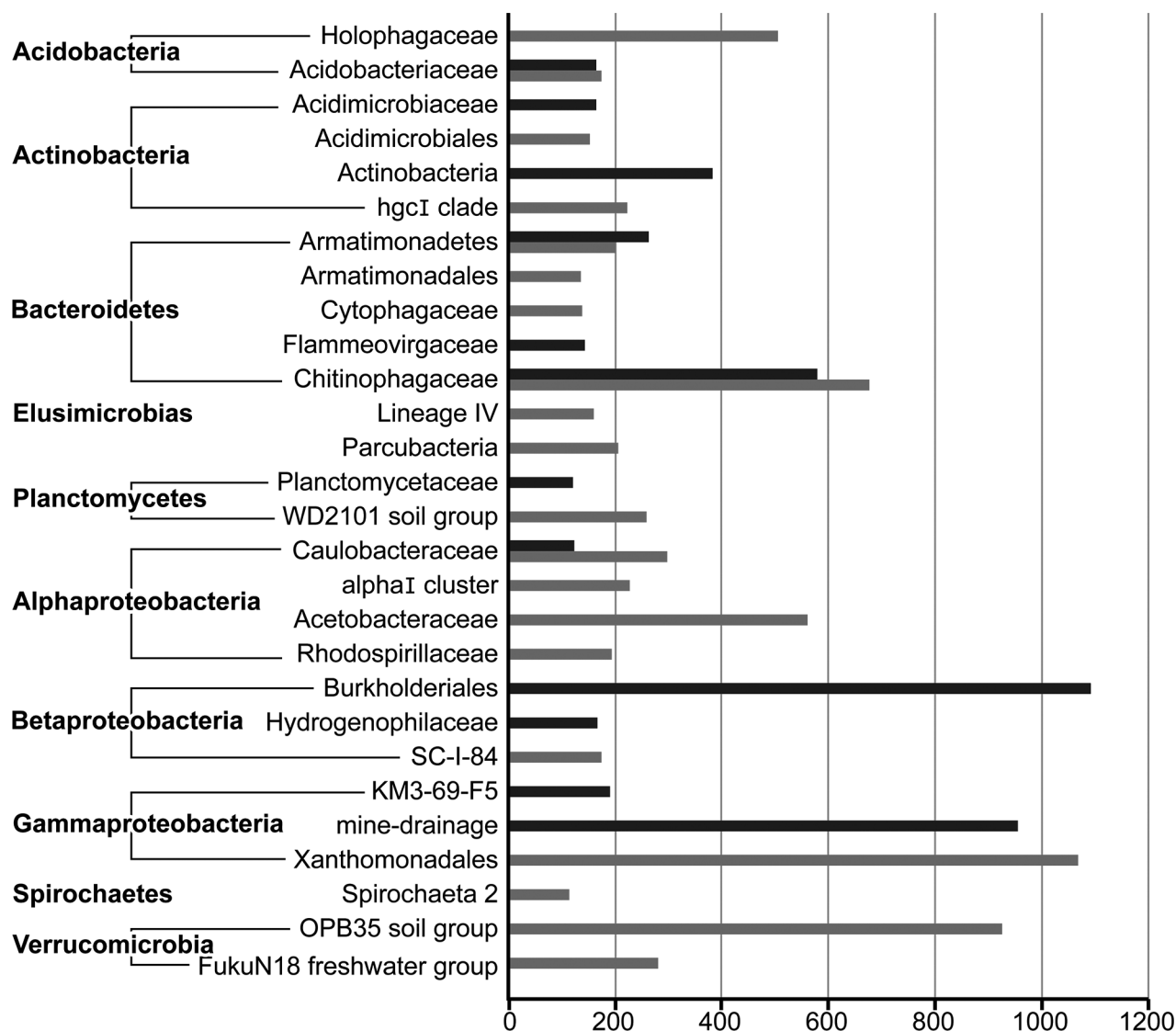


Fig. 3 Relatively abundant bacterial taxa not identifiable to the generic level, represented by >100 16S or 23S ribosomal DNA (rDNA) reads, indicating potential new taxonomic diversity. Light gray bars indicate 16S rDNA read abundance; dark gray bars indicate 23S rDNA read abundance. A color version of this figure is available online.

recognized polysaccharide degradation functions. Additional relatively abundant bacterial genera indicating mineral accumulation or redox reactions of potential biogeochemical significance include the phosphate-accumulator *Candidatus Accumulibacter*, the iron reducers *Acidiphilium* and *Albidiferax*, the sulfate reducer *Desulfovibrio*, the elemental sulfur reducer/denitrifier *Sulfurospirillum*, the chemolithoautotrophic sulfur oxidizer *Thioalkalivibrio*, and *Geobacter*, an organic and metal oxidizer that produces nanowires of ecological and technological interest. Surprisingly large populations were inferred from a high abundance of sequence reads indicating *Magnetospirillum*, notable for cellular presence of iron-containing (magnetite) magnetosomes.

Comparative Bacterial Diversity

At least 60 bacterial genera inferred to occur in the Navarino Island *S. fimbriatum* microbiome, including more than half of the relatively abundant genera listed in figure 2, had been listed in five previous reports of peatland or peat moss microbiota of other locales by investigators using culture methods or culture-independent methods similar to ours (table A2).

Bacterial genera previously cultivated from peat mosses or peatlands that were also detected in our study of Navarino Island *S. fimbriatum* included *Granulicella*, *Telmatobacter*, *Bryobacter*, *Geothrix*, *Holophaga*, *Candidatus Microthrix*, *Conexibacter*, *Mucilaginibacter*, *Sediminibacterium*, *Dyadobacter*, *Pedobacter*, *Bacillus*, *Clostridium*, *Schlesneria*, *Singulisphaera*, *Caulobacter*, *Bradyrhizobium*, *Rhodopseudomonas*, *Rhizomicrobium*, *Acidisoma*, *Acidocella*, *Telmatospirillum*, *Sphingomonas*, *Brevundimonas*, *Beijerinckia*, *Methylocapsa*, *Methylocella*, *Methyloferula*, *Hyphomicrobium*, *Methylocystis*, *Mesorhizobium*, *Burkholderia*, *Geobacter*, *Anaeromyxobacter*, *Beggiatoa*, *Rahnella*, *Serratia*, and *Opitutus*.

Additional bacterial genera detected with the use of high-throughput molecular tools from both this study and Austrian alpine bogs included *Nostoc*, *Asticcacaulis*, *Phenyllobacterium*, *Rhodoblastus*, *Magnetospirillum*, *Azospirillum*, *Novosphingobium*, *Afpia*, *Methylobacterium*, *Rhizobium*, *Stappia*, *Acidomonas*, *Komagataeibacter*, *Rubrivivax*, *Janthinobacterium*, *Ralstonia*, *Steroidobacter*, and *Chthoniobacter*. Additional bacteria genera detected both in this study and from pool waters of a sub-Antarctic peat bog in Patagonia, Argentina, included *Flavobacterium*, *Curvibacter*, *Limnohabitans*, *Methylotenera*, *Legionella*, and *Pseudomonas*. Bacterial genera detected in five previous studies of peat mosses or peatlands of other locales were noted on our list of Navarino Island *S. fimbriatum* bacteria detected at >10 rDNA reads (table A2).

Eukaryotic Diversity

Nearly 100 18S rDNA sequence reads were classified with the moss *Takakia*, and nearly 3400 reads could not be classified more definitively than the phylum Bryophyta, possibly indicating bryophyte diversity as yet unrepresented in reference 18S rDNA databases. Navarino Island moss metagenomic data also suggested the presence of at least 45 identifiable genera of protists and fungi representing major eukaryotic supergroups (table A3), in addition to diverse higher taxa that could not be identified to the generic level with available database resources. Some microscopic eukaryotes were also detected by

microscopic analysis (see fig. 1A). Of the high-quality sequence reads (table A1), 414 18S and 2435 28S rDNA types could not be classified into known eukaryotic supergroups, suggesting the possible occurrence of new protist biodiversity.

By comparison to unicellular prokaryotic taxa having limited genetic redundancy, the relative abundances of eukaryotes are more difficult to infer from high-throughput sequence read numbers because eukaryotes have been influenced by whole-genome duplication and gene duplication events, and many species of microscopic eukaryotes are multicellular. For these reasons, read abundance numbers for eukaryotic taxa cannot be assumed to represent organismal population sizes; hence, 18S/28S rDNA read abundances >100 are noted in table A3 for “apparent abundance.”

Unicellular eukaryotes notable for apparent abundance included the heterotrophic amoebozoan *Phalansterium*. The Navarino Island peat moss microbiome included sequences indicating the rhizarian *Paulinella* and additional paulinellid amoebas that are relevant to the evolutionary origin of primary endosymbiosis. Also inferred was *Sphaerastrum*, a centrohelid protist that is important as a close heterotrophic relative of the primarily autotrophic haptophytes and therefore relevant to secondary plastid origins. Nuclearioid amoebas (protists closely related to fungi) and the choanoflagellate *Monosiga* (protist relative of the animal kingdom) were also inferred from Navarino Island peat moss metagenomic sequence data. Diverse algal lineages were represented; the most abundant representative identifiable to the genus level was the early-diverging plastidless cryptomonad *Goniomonas*.

In addition to sequences not yet classifiable to the genus level that may represent new fungal biodiversity, the Navarino Island peat moss microbiome was inferred to include the early-diverging unicellular *Rozella* (Cryptomycota) and ascomycete genera *Neocudoniella*, *Varicosporium*, *Acidea*, *Galera*, *Tetrachaetum*, and *Pezoloma*. The Navarino Island *S. fimbriatum* microbiome also included sequences that classified into several archaic lineages of microinvertebrate animals, including oligochaete (segmented) worms, cyclopoid and harpacticoid copepods, collembolans (springtails), chaetonotid gastrotrichs, nematodes, and ploimid and adinetid rotifers (table A3).

Discussion

Ribosomal Markers and Bacterial Diversity

The detection of many Navarino Island *Sphagnum fimbriatum*-associated bacterial genera (and higher taxonomic groups) by only 16S or only 23S rDNA as well as differences in 16S versus 23S rDNA read numbers for genera detected by both markers indicated the utility of employing both markers for taxonomic surveys. Among the bacterial genera represented by >100 reads, diverse functionally significant bacterial genera—putative methanotrophs *Methylocystis*, *Methylocapsa*, and *Methylocella*; nitrogen-fixing *Bradyrhizobium*; phenolic degrader *Phenyllobacterium*; cellulose-producer *Komagataeibacter*; and sulfur-cycle participants *Desulfovibrio*, *Sulfurospirillum*, and *Thioalkalivibrio*—would have been missed had we utilized only 16S rDNA to report relatively abundant bacterial diversity. The exceptional abundance of magnetite-producing *Magnetospirillum*, which might be relevant to the production of “bog iron”

deposits of archeological significance, was apparent only with the use of 23S rDNA. These observations argue for the routine use of 23S as well as 16S rDNA sequences for bacterial taxonomic surveys of peatlands and other habitats.

Evidence for Microbiome Stress Resilience

Many of the relatively abundant Navarino Island *S. fimbriatum*-associated bacterial genera (as well as additional bacterial genera that were less abundant) had previously been recognized to generally function in peatland N₂ reduction (fixation) and methane oxidation (Raghoebarsing et al. 2005; Kip et al. 2010, 2012; Belova et al. 2013; Larmola et al. 2014) and appeared in the five relatively recent reports judged particularly relevant to our study that we used for direct comparisons (Dedysh 2011; Bragina et al. 2012, 2015; Müller et al. 2015; Quiroga et al. 2015). Amplicon studies of the nitrogen fixation marker gene *nifH* likewise indicated the presence of diverse taxonomic groups (Kox et al. 2016) that were also inferred for the Navarino Island *S. fimbriatum* microbiome. Although Esson et al. (2016) reported that a boreal North American peat bog was codominated by alpha- and gammaproteobacterial methanotrophs, methanotrophic genera classifying with Alphaproteobacteria and Verrucomicrobia seemed more important than Gammaproteobacteria in the Navarino Island system, with *Methylocystis* important at both sites.

At least some of the nitrogen-fixing bacterial genera detected in the Navarino Island peat moss system (e.g., *Rhizobium*) are known to produce vitamin B₁₂, which has been experimentally determined to enhance the growth of methane-oxidizing bacteria (Iguchi et al. 2011). Microbial methane oxidation is of biogeochemical significance because it reduces the amount of the powerful greenhouse gas methane entering Earth's atmosphere, and it also fosters peat moss growth by generating carbon dioxide that mosses can absorb and use in photosynthesis (Raghoebarsing et al. 2005; Kip et al. 2010), helping these plants cope with the CO₂ limitation that can occur in waterlogged locales. Methanotrophs can also be important contributors to nitrogen fixation (Larmola et al. 2014).

Other peat moss-associated bacteria have been hypothesized to foster moss growth by producing fungicidal, bactericidal, or growth-promoting compounds. About half of the genera cited to function in these ways in a recent review (Kostka et al. 2016) were also detected in the Navarino Island *S. fimbriatum* microbiome. Additional bacterial genera hypothesized to foster plant growth were detected at relatively abundant levels in the Navarino Island system: *Janthinobacterium* is known for producing a blue-violet pigment (violacein) having hypothesized antimicrobial (and antitumor) properties (Valdes et al. 2015), and *Methylobacterium* and *Variovorax* are thought to produce plant growth-promoting substances (Satola et al. 2013; Iguchi et al. 2015). These results suggest the possibility that peat moss systems might be useful sources of plant growth-promoting bacteria of potential agricultural utility.

The relatively abundant Navarino Island peat moss bacterial genera *Tolomonas* and *Phenylbacterium* are known for the capacity to break down phenolic compounds, a potentially significant biogeochemical function in peatlands because phenolic polymers are major constituents of peat moss cell walls (Kroken et al. 1996), and increased production of these UV-absorbing molecules has been experimentally linked to UV stress (Cardona-

Correa et al. 2015b). Relatively abundant cellulose-producing (C-sequestering) bacteria, such as *Komagataeibacter*, and polysaccharide-degrading bacteria, such as *Opitutus* and *Chthoniobacter*, may also be more important in peatland organic carbon sequestration and degradation than previously recognized. These results also suggest the potential for peat moss systems to serve as sources of microbes for industrial biomass deconstruction, such as in the production of renewable biofuels.

Our inference that at least 60 of the bacterial genera (including methane oxidizers and nitrogen fixers) detected in the Navarino Island *S. fimbriatum* microbiome had been detected previously in diverse peatlands or peat moss microbiota by numerous investigators using culture methods (reviewed in Dedysh 2011) or culture-independent methods similar to ours (Bragina et al. 2012, 2015; Müller et al. 2015; Quiroga et al. 2015) indicated that the Navarino Island peat moss system maintained a complex microbiota indicating globally significant biogeochemical functions (e.g., carbon sequestration, nitrogen fixation, and methane oxidation) associated with peatlands. Overall, such comparisons indicate that the Navarino Island *S. fimbriatum* microbiome includes bacterial taxa and functions present in less-stressed peat moss systems, suggesting taxonomic and functional resilience of the sub-Antarctic peat moss microbiota to modern stressful conditions likely to have occurred in earlier time periods. This evidence for resilience may help to explain why peat mosses have apparently survived on Earth for hundreds of millions of years and through global mass extinction events. Evidence for functional resilience of the Navarino Island *S. fimbriatum* microbiome offers hope that peat moss-microbe systems will persist through present and future climate perturbation. Because peat mosses are abundant and recognized for substantial global effects, future investigations focused on their functional resilience may yield further insight into the past and future.

Peat Moss as Habitat for Microeukaryotes

Our observations also indicated that nutrient- and UV-stressed peat moss systems may be among the environments that serve as habitat for diverse archaic eukaryotic lineages. Peat moss systems may therefore prove to be useful sources of isolates for phylogenomic studies aimed at clarifying early eukaryotic diversification. Although diverse microeukaryotes have been reported from microscopic studies of peat mosses (Hingley 1993), eukaryotic components of peat moss microbiomes have not generally been the focus of high-throughput sequencing efforts; the present study may be the most taxonomically comprehensive high-throughput analysis of peatmoss-associated microbiota yet performed.

Although microscopy-based analyses of protist diversity may suffice for some microeukaryotic groups, diversity assessment of other taxa may require the use of sequencing methods. Moss-dwelling ciliates of King George Island—the largest of the Southern Shetland Islands, whose maritime Antarctic environmental conditions may resemble those of Navarino Island—included *Colpoda*, *Halteria*, *Stylonychia*, *Oxytricha*, *Paramecium*, and *Vorticella* (Mieczan and Tarkowska-Kukuryk 2014), in common with the Navarino Island peat moss system. On the basis of ribosomal marker read abundances, the single most abundant eukaryotic algal genus in the *S. fimbriatum* microbiome was the

early-diverging, nonphotosynthetic, plastidless unicellular cryptomonad *Goniomonas*, which is of evolutionary importance as a close relative of Viridiplantae (Kim and Archibald 2013; Burki et al. 2015). We did not detect this inconspicuous protist by microscopic methods; molecular methods were essential to detect this key protist.

An internal transcribed spacer–based investigation of fungi associated with the bryophytes of King George Island reported two strains of “zygomycetes,” 16 of ascomycetes, and one of basidiomycete (Yu et al. 2014). None of the fungal genera inferred to occur in the Navarino Island *S. fimbriatum* microbiome was cited in a recent compilation derived from previous studies of fungi (primarily pathogens) associated with peat mosses (Kostka et al. 2016), and none of the fungal genera cited in that review was present in the Navarino Island *S. fimbriatum* microbiome. In view of the recognized significance of fungi in global carbon cycles, these observations argue the need for high-throughput sequencing studies focused on peat moss–associated fungi and their functions.

Although springtails, detected in our metagenomic analysis, are known to facilitate fertilization in mosses (Rosenstiel et al. 2012), the ecological roles played by microinvertebrates in peatlands are otherwise poorly known. It is possible that at least some of the peat moss–associated bacteria observed in this and other studies may actually be components of invertebrate microbiomes. Paracubacteria (notable for reduced genomes indicating symbiotic associations) and nitrogen-fixing Elusimicrobia lineage IV (which includes intracellular symbionts of animals) might prove to be such examples. Comprehending the microbiomes of early-diverging animals could shed light on the early evolution of animal microbiomes. Our metagenomic sequencing results suggested that at least some peat moss–associated microinvertebrates, such as relatively abundant springtails, may be sufficiently numerous to collect for separate microbiome analyses.

Sub-Antarctic Peat Moss–Microbiome Systems as Models of Early Terrestrial Ecosystems

The antiquity (460–600 Ma) of the *Sphagnum* lineage indicated by molecular diversification evidence (Laenen et al. 2014), the 454–455 Ma peat moss–like vegetative remains (Cardona-Correa et al. 2016), and the occurrence in the Navarino Island *S. fimbriatum* microbiome of diverse, early-diverging lineages of bacteria, protists, fungi, and invertebrate animals suggest that modern nutrient- and UV-stressed peat moss systems might represent reasonable models of early terrestrial plant communities. The earliest terrestrial ecosystems are poorly understood, although they are considered important in comprehending the evolutionary development of modern biogeochemical cycles. Models suggest that early moss-like plants might have strongly influenced early

Paleozoic atmospheric chemistry and climate (Graham et al. 2004; Lenton et al. 2012, 2016; Porada et al. 2016). The present study detected a strong signal (nearly 100 18S rDNA sequences) for the presence of the equally archaic moss *Takakia*, whose ancestral lineage is thought to have diverged at about the same time as that of peat mosses (>450 Ma; Laenen et al. 2014). Although the molecular signal found in our metagenomics study might represent some classification anomaly of unknown type, future studies might include searches of Navarino Island for this moss genus, currently thought to be restricted in geographical occurrence to western North America and central and eastern Asia (Schofield 1985; Zhang et al. 2016).

Early land vegetation is also thought to have included liverworts and lichens that together with associated microbiota likewise likely had global environmental effects. For example, comparisons of modern cryptogamic ground covers imaged by X-ray tomography to 407 Ma Rhynie Chert (Scotland) remains indicated that lichens and liverworts, such as *Blasia* and their associated microbiota, provide better analogues of Early Devonian plant/soil associations than do (relatively late-diverging) bryalean mosses, such as *Racomitrium* (Mitchell et al. 2016).

The observations reported here support previous hypotheses—based on metagenomic data for a liverwort and related streptophyte algae (Knack et al. 2015)—that in moving onto land, early plants might have benefitted from functional suites of microbiota similar to those present in the microbiomes of modern representatives of algal ancestors. Microbiomes that included nitrogen fixers and other functional suites might have helped photosynthetic hosts to cope with environmental stresses and also initiated terrestrial biogeochemical effects that extend into the present. These key roles include carbon sequestration and organic carbon deconstruction as well as nitrogen fixation, methane oxidation, and numerous less widely recognized types of mineral redox reactions. The new metagenomic data presented here also indicate that modern peat mosses and the earliest plants might have served as habitat for diverse early-diverging protists, fungi, and invertebrate animals for hundreds of millions of years.

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