Novel bacterial lineages associated with boreal moss species

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Abstract

Mosses are critical components of boreal ecosystems where they typically account for a large proportion of net primary productivity and harbor diverse bacterial communities that can be the major source of biologically-fixed nitrogen in these ecosystems. Despite their ecological importance, we have limited understanding of how microbial communities vary across boreal moss species and the extent to which local environmental conditions may influence the composition of these bacterial communities. We used marker gene sequencing to analyze bacterial communities associated with eight boreal moss species collected near Fairbanks, AK USA. We found that host identity was more important than site in determining bacterial community composition and that mosses harbor diverse lineages of potential N₂-fixers as well as an abundance of novel taxa assigned to understudied bacterial phyla (including candidate phylum WPS-2). We performed shotgun metagenomic sequencing to assemble genomes from the WPS-2 candidate phylum and found that these moss-associated bacteria are likely anoxygenic phototrophs capable of carbon fixation via RuBisCo with an ability to utilize byproducts of photorespiration from hosts via a glyoxylate shunt. These results give new insights into the metabolic capabilities of understudied bacterial lineages that associate with mosses and the importance of plant hosts in shaping their microbiomes.

29 **Introduction**

30 Mosses, like their cousins, the vascular plants, associate with a broad diversity of microbes,
 31 including, bacteria, fungi, and other microbial eukaryotes (Lindo and Gonzalez, 2010). These moss-
 32 microbe associations are particularly relevant to terrestrial nitrogen (N) and carbon (C) cycling in
 33 northern ecosystems, where mosses are ubiquitously distributed and can be responsible for as much as
 34 50% of ecosystem net primary productivity (Turetsky *et al.*, 2012). Moss-associated N₂-fixing bacteria
 35 are often the primary source of ecosystem N inputs in boreal forests (DeLuca *et al.*, 2002) and moss-
 36 associated microbes can have important influences on ecosystem C dynamics via methane oxidation,
 37 especially in peatlands, one of the largest natural sources of atmospheric methane (Kip *et al.*, 2010).
 38 Together, the importance of moss-microbial communities to terrestrial biogeochemistry and unique
 39 features of bryophyte biology make boreal moss communities a useful system for investigating the
 40 interactions between host species identity, microbial community structure, and ecosystem function.

41 Mosses are ubiquitous across boreal forests, with distributions spanning ecologically important
 42 environmental gradients (Lindo and Gonzalez, 2010; Turetsky *et al.*, 2012). Moss diversity in these
 43 forests can be quite high (Geffert *et al.*, 2013), and different moss species often grow interspersed at a
 44 given location, creating many abundant and naturally occurring ‘common-garden’ experiments for
 45 testing how host identity and local environmental conditions influence the assembly of moss-associated
 46 microbial communities. Furthermore, mosses, unlike more commonly-studied vascular plants, have
 47 simpler leaf-tissue structures and do not possess roots thus representing a comparatively homogeneous
 48 host environment, reducing the need to control for inter-tissue spatial variation (a problem when
 49 studying vascular plants, e.g. Leff *et al.*, 2015). Mosses are also small enough that an entire plant can
 50 be sampled for microbial analyses, an impossible task for most larger plants. Together these traits make
 51 mosses a useful study system for investigating the impacts of environmental and host factors on

52 microbial community structure and the contributions of these moss-associated microbial communities
53 to ecosystem function.

54 Despite their potential biogeochemical importance and their utility as a model system, we still
55 know surprisingly little about the structure and function of those microbial communities associated
56 with boreal mosses. Much of the previous research has focused on a handful of abundant boreal moss
57 species, in particular, members of the peat-moss genus *Sphagnum*, and the dominant feather mosses,
58 *Pleurozium schreberi* and *Hylocomium splendens* (e.g. DeLuca *et al.*, 2002; Opelt *et al.*, 2007;
59 Zackrisson *et al.*, 2009; Ininbergs *et al.*, 2011; Bragina, Berg, *et al.*, 2012; Bragina *et al.*, 2015). While
60 these species are among the most abundant mosses in many boreal forests, the microbes living in
61 association with less abundant hosts may be equally as important contributors to key ecosystem
62 processes (Rousk *et al.*, 2015). Previous work on the abundant species makes it clear that boreal
63 mosses possess surprisingly diverse microbial communities, containing not only well-studied
64 *Cyanobacteria* but also novel and undescribed lineages within the *Alphaproteobacteria* sub-phylum
65 and the *Verrucomicrobia* phylum among others (Bragina *et al.*, 2015). However, it is unclear whether
66 local environmental factors govern the microbial community assembly, or whether a moss species hosts
67 a characteristic microbial community, regardless of its abiotic environment.

68 It is well-known that mosses can harbor N₂-fixing bacteria and N₂-fixation rates of *Sphagnum* and
69 the feather mosses, *Pleurozium schreberi* and *Hylocomium splendens*, can be quite variable across
70 season (DeLuca *et al.*, 2002), forest type (Zackrisson *et al.*, 2004), and moss species (Leppänen *et al.*,
71 2015) with reported rates ranging from 0.3-4 kg N ha⁻¹ yr⁻¹ (DeLuca *et al.*, 2007). A number of
72 previous studies have focused on selected moss-associated N₂-fixing bacterial taxa, particularly taxa
73 within the *Cyanobacteria* phylum (reviewed in Rousk, Jones, *et al.*, 2013), and how the composition or
74 diversity of these cyanobacteria relate to N₂-fixation rates (Ininbergs *et al.*, 2011). However,
75 *Cyanobacteria* are unlikely to be the only moss-associated bacteria capable of N₂-fixation. For

76 example, Bragina *et al.* (2012) found that nitrogenase sequence libraries from bacteria on two
 77 *Sphagnum* species were dominated by alphaproteobacterial sequences, indicating that non-
 78 cyanobacterial N₂-fixers may play a more important role than previously recognized. In short, it
 79 remains unclear which bacteria are responsible for N₂-fixation in boreal mosses and how the
 80 abundances of these N₂-fixing taxa may vary across different moss species.

81 Here we characterized the bacterial communities associated with eight common boreal moss
 82 species at three different sites using marker gene (16S rRNA gene) amplicon sequencing. We used this
 83 dataset to address the following questions: 1) How do bacterial communities vary across different moss
 84 species and different sites? and 2) Which microbial taxa from these communities are potential N₂-
 85 fixers? Based on our observation that many of the taxa found in association with the moss species were
 86 representatives of bacterial lineages for which little is known (including members of the candidate
 87 phylum WPS-2), we then assembled genomes from shotgun metagenomic data to determine the
 88 functional attributes of these abundant, ubiquitous, and previously undescribed members of moss-
 89 associated microbial communities.

90 **Experimental Procedures**

91 **Sample Collection**

92 To characterize and compare the bacterial communities associated with different moss host species
 93 and their potential contributions to N₂-fixation, we collected samples of eight boreal moss species (the
 94 closely related pleurocarpous mosses *Pleurozium schreberi* (Brid.) Mitt., *Hylocomnium splendens*
 95 (Hedw.) Shimp, *Sanionia uncinata* (Hedw.) Loeske, and *Tomenthypnum nitens* (Hedw.) Loeske, and the
 96 successively more distantly related species, *Aulacomnium palustre* (Hedw.) Schwägr. and *A. turgidum*
 97 (Wahlenb.) Schwägr., *Dicranum elongatum* Schleich. ex Schwägr., and *Sphagnum capillifolium* (Ehrh.)
 98 Hedw.). The samples were collected from three black spruce (*Picea mariana*)-dominated sites that were

each at least a kilometer apart within the arboretum of the University of Alaska Fairbanks during late July 2014. The selected moss species are both common in boreal forests and represent lineages spanning the phylogenetic diversity of mosses. At each of the three sites we collected one sample for each of the eight species (except *Aulacomnium turgidum* and *Dicranum elongatum*, which could only be found at two sites), resulting in a total of 22 samples for analysis. Each sample was divided into four subsamples of ten stems each for microbial community analysis, isotopic enrichment, natural abundance voucher, and taxonomic voucher specimens (See Table S1). For each species within each site, a clump of ramets were removed from a monospecific patch and carefully sorted and cleaned with a gloved hand. Brown or decaying material was removed from the bottom so that each ramet was approximately 5 cm in length and included the apical meristem. Each sample was then divided into four subsamples of ten stems each for microbial community analysis, isotopic enrichment, natural abundance voucher, and taxonomic voucher specimens (See Table S1). Samples collected for microbial analyses were placed in a cooler on blue ice in the field and returned to the lab where they were frozen (-20°C) within 2 h of collection. Samples for the N₂-fixation rate assays were also placed in the coolers until the measurements were started, usually within 2 h of sample collection. Natural abundance voucher specimens were returned to the lab and dried at 60°C for 48 h within 2 h of collection.

N₂-Fixation Measurements

To quantify field rates of N₂-fixation for each sample, we used an isotopic enrichment approach modified from Ruess *et al.* (2009, 2013). Each sample was placed in a 60 mL translucent polycarbonate syringe that was depressed to contain 10 ml of air. We then added 10 mL of ¹⁵N₂ (98% enriched, Cambridge Isotope Laboratories, Inc., U.S.A.) and sealed the syringe with a stopcock. Sealed syringes from all sites were placed in site A, where they were incubated for 24 h under similar light and temperature conditions. After 24 h, mosses were removed from the syringes and placed in a 60 °C oven for 48 h. Both enriched incubation samples and natural abundance samples were ground to a

123 homogenous powder. Nitrogen and carbon concentrations and atom% ^{15}N and ^{13}C values for both
 124 enriched incubation samples and natural abundance voucher specimens were measured on a Costech
 125 ECS4010 coupled to a Thermo Scientific Delta V Advantage Isotope ratio mass spectrometer in Mack's
 126 lab at Northern Arizona University. Nitrogen fixation was calculated by comparing the ^{15}N values from
 127 enriched and control samples (see Jean *et al.* (2017) for details).

128 **Amplicon-Based Bacterial Community Analysis**

129 To analyze the bacterial communities associated with each of the collected moss specimens, we
 130 PCR amplified and sequenced a portion of the bacterial and archaeal 16S rRNA marker gene. First, to
 131 minimize any bias from micro-spatial differences along the moss tissue, we homogenized each sample
 132 (ten moss stems per sample, approximately 0.25 g of tissue) with liquid N_2 under sterile conditions. We
 133 then extracted DNA from each homogenized moss sample using the MoBio Power Soil DNA extraction
 134 kit (MoBio Laboratories, Carlsbad, CA). After extracting DNA, we used the 515f / 806r primers to
 135 PCR amplify the V4-V5 region of the 16S rRNA gene (Caporaso *et al.*, 2012). For each sample, we
 136 used a unique primer pair that included a 12-bp barcode and Illumina sequencing adapters to allow for
 137 multiplexed sequencing. To minimize amplification of mitochondrial and chloroplast DNA, we used
 138 PNA (peptide nucleic acid) clamps during PCR amplification (Lundberg *et al.*, 2013). During both
 139 DNA extraction and PCR amplification, we included negative controls to check for potential
 140 contaminants introduced during those steps. We prepared the samples for sequencing by normalizing
 141 the concentrations of PCR products across all samples using the ThermoFisher Scientific SequalPrep
 142 Normalization plate (Thermo Fisher Scientific Inc. USA) and pooled the amplicons together. We
 143 sequenced all samples on the Illumina MiSeq platform running the 2 x 150 bp paired-end chemistry at
 144 the University of Colorado Next Generation Sequencing Facility. The *Hylocomium splendens*
 145 amplicon sequences were sequenced on a separate run. Because we cannot control for run-to-run
 146 variation we have excluded these samples from downstream statistical analyses. A cursory analysis

147 indicates that the *Hylocomnium* microbiome is statistically distinguishable from the other mosses in our
148 sample, however the community was not marked by novel species relative to the other samples we
149 analyzed.

150 We used the approach described by Leff *et al.* (2015) to analyze the 16S rRNA sequence data.
151 Briefly, we removed adapters from the raw reads using cutadapt (paired, -O 1) (Martin, 2011) and
152 demultiplexed the reads using a custom in-house python script (“prep_fastq_for_uparse.py” at:
153 <https://github.com/leffj/helper-code-for-uparse/>). Then, using USEARCH v.8 (Edgar, 2010), we
154 merged, quality filtered (“maxee rate” = 0.005) and dereplicated the reads to create a fasta file
155 containing each unique amplicon sequence. We also removed singletons (sequences found only once
156 across the entire sample set) from the dereplicated reads. We created a de novo database from our
157 sequences using the UPARSE pipeline (Edgar, 2013) (implemented with USEARCH v.8) by clustering
158 reads at ≥97% sequence similarity level (which we refer to as ‘phylotypes’). As a quality-control
159 measure, we removed representative sequences from phylotypes that were less than 75% similar to any
160 sequence in the Greengenes database (version August 2013) (McDonald *et al.*, 2012). We assumed
161 these highly divergent sequences to be chimeric, a product of non-specific amplification, or of
162 insufficient quality.

163 To generate phylotype counts, we mapped the merged reads back to the *de novo* database
164 (command “-usearch_global”, -id 0.97) and used a custom python script
165 (“create_otu_table_from_uc_file.py” at: <https://github.com/leffj/helper-code-for-uparse/>) to generate a
166 table of phylotype counts per sample from the USEARCH mapping output. We classified the reads
167 with the RDP Naïve Bayesian classifier (Wang *et al.*, 2007) against the Greengenes database
168 (McDonald *et al.*, 2012) and removed any residual chloroplast and mitochondrial sequences. To control
169 for differences in read depth (sequencing “effort”) across samples, we randomly selected 6000 bacterial
170 and archaeal 16S rRNA gene reads per sample prior to downstream analyses. The 6000 read cut-off

171 was chosen based on the read depth of the sample with fewest reads after mitochondria and chloroplast
172 removal. The filtered reads can be found on FigShare (DOI:
173 <https://doi.org/10.6084/m9.figshare.5594527>).

174 To measure differences between communities across host species and sites, we calculated pairwise
175 Bray-Curtis dissimilarities on the square-root transformed phylotype table and tested for differences
176 between species and sites using a PERMANOVA test. All statistical analyses were conducted in R (R
177 Core Team, 2017, package “Vegan”, R Core Team, 2017). We used metaMDS (R package “Vegan”) to
178 generate NMDS plots from the dissimilarity matrix.

179 **Identifying Nearest Isolated Representatives and Classifying Putative N₂-fixing Bacteria**

180 To identify the nearest isolated representatives of the 30 most abundant bacterial phylotypes, we
181 used the RDP (Ribosomal Database Project) SeqMatch tool which finds the nearest relatives by
182 comparing the percent of shared sub-sequences between a query and the RDP isolate database (Cole *et al.*, 2014). We then downloaded the nearest-neighbor representative sequences from RDP and used
183 MUSCLE (Edgar, 2004) to create an alignment of the isolate sequences and representative sequences
184 from the 30 most abundant bacterial phylotypes, using FastTree (Price *et al.*, 2010) to generate the final
185 tree. We defined “putative N₂-fixing phylotypes” as those phylotypes whose nearest relative ($\geq 97\%$
186 similarity in the 16S rRNA gene region) has been found to be capable of N₂-fixation when cultivated in
187 isolation under laboratory conditions (four phylotypes). Alternatively, if the nearest neighbor was <
188 97% similar but came from a lineage which included only previously-described representative strains
189 known to be capable of N₂-fixation, the phylotype was also defined as a “putative N₂-fixer” (two
190 phylotypes).
191

192 Shotgun Metagenomic Sequencing and Analysis

193 Based on the preponderance of bacterial lineages identified from the 16S rRNA amplicon analyses
 194 that came from poorly described and novel bacterial lineages (see below), we generated shotgun
 195 metagenomic libraries from the samples of 3 moss species *Aulacomnium turgidum* (2 samples),
 196 *Pleurozium schreberi* (3 samples), and *Tomenthypnum nitens* (3 samples). We prepared the
 197 metagenomic libraries following the method described in Baym *et al.* (2015). All 8 libraries were then
 198 sequenced on the Illumina NextSeq platform running the 2 x 150 bp chemistry at the University of
 199 Colorado Next Generation Sequencing Facility. Each sample had an average of 58 million reads. After
 200 sequencing, we filtered the raw reads with Sickle (-q 20 -l 50) (Joshi and Fass, 2011) and used Metaxa2
 201 (Bengtsson-Palme *et al.*, 2015) on the filtered reads to verify that the 16S rRNA amplicon data was
 202 consistent with the taxonomic composition of the bacterial communities as inferred from the
 203 metagenomic data.

204 To assemble near complete genomes from the shotgun metagenomic data, we used the
 205 metagenomic *de novo* assembler, MEGAHIT (Li *et al.*, 2014) (default settings: --min-count 2 --k-min
 206 21 --k-max 99 --k-step 20) to co-assemble all the filtered reads without regard to sample origin
 207 (pooled-assembly). Then we sorted the assembled contigs into bins each representing a preliminary
 208 genome using the software MaxBin (Wu *et al.*, 2014) (-min_contig_length 1000 -max_iteration 50
 209 -prob_threshold 0.9 -markerset 40). Since MaxBin can take advantage of differences in abundance of
 210 identical contigs across samples, we were able to leverage differential abundances of reads from
 211 individual bacterial taxa across the pooled assembly to identify contigs belonging to the same
 212 organisms, thus facilitating the binning process. After binning, we used Bowtie2 (Langmead and
 213 Salzberg, 2012) to map the filtered reads back to each bin. We used CheckM (Parks *et al.*, 2015) to
 214 verify the completeness and contamination of each bin (Table S2). Briefly, CheckM measures
 215 metagenomic completeness and contamination on the basis of presence and number of conserved
 216 single-copy marker genes (genes which typically are present in bacterial genomes only once). The

number of marker genes present compared to the number of expected marker genes for a particular bacterial lineage is used as a measure of completeness while the number of copies of a marker gene indicates contamination. We considered bins that were greater than 70% complete and less than 10% contaminated to be “high-quality” bins (as per the top two levels of quality identified in Parks *et al.* (2015).

Because few of our high-quality bins contained assembled 16S rRNA sequences (a common problem, see Miller *et al.*, 2011), we used three separate methods to confirm the taxonomic identity of bins. First, we used the program EMIRGE (Miller *et al.*, 2011) to assemble full-length 16S rRNA sequences from the raw metagenomic reads and used USEARCH (Edgar, 2010) to match contigs from the bins to the EMIRGE-assembled full-length 16S rRNA sequences. Second, to verify this result with a technique that did not rely on genome assembly from metagenomic data, we used Metaxa2 (Bengtsson-Palme *et al.*, 2015) to independently identify fragments of the 16S rRNA gene in the binned contigs and classified those fragments against the Greengenes database using USEARCH (Edgar, 2010). Finally, we used the automatically-generated concatenated marker gene phylogeny available from CheckM to confirm that bins were clustered on a tree in the appropriate clade as indicated by analysis of their 16S rRNA gene sequences. After identifying the organisms represented by the bins, we used the Joint Genome Institute’s Integrated Microbial Genomes (IMG) analysis pipeline (Markowitz *et al.*, 2014) to identify and assign functions to the genes in the assembled bins. The assembled metagenomic reads are available through the IMG portal (IMG submission ID 115847).

Results and Discussion

Moss Species Identity Drives Microbiome Composition

Our analyses of moss-associated bacterial 16S rRNA amplicons show that mosses host diverse and species-specific bacterial communities. After quality filtering, but prior to chloroplast and

mitochondrial filtering, we obtained 7947 to 16590 16S rRNA gene reads per sample with an average sequencing depth of 11430 reads. The chloroplast and mitochondrial sequences that were removed made up only a small percentage of the reads (10% and 5%, respectively). Samples had an average richness of 924 phylotypes per sample and were dominated by eight bacterial phyla. Archaeal sequences were extremely rare and made up less than 0.001% of all reads. We found similar archaeal abundances in the primer-independent metagenomic data suggesting that the low archaeal abundance in the amplicon data was not a product of primer biases. The dominant bacterial phyla, as measured by average read abundance across all samples, were *Proteobacteria* (44.8% of reads across all samples), *Acidobacteria* (10.8%), *Verrucomicrobia* (9.8%), *Bacteroidetes* (9.3%), *Cyanobacteria* (6.5%), Candidate phyla WPS-2 (5.7%), *Planctomycetes* (5.2%), and *Actinobacteria* (4.2%) (Figure 1). The most abundant phylotypes identified across the entire sample set included those assigned to the *Acetobacteraceae* (9.5%), *Acidobacteriaceae* (8.2%), *Sinobacteraceae* (8.2%) and *Nostocaceae* (5.2%) families as well as many phylotypes that could only be classified to the phylum or class level of resolution (including those in the WPS-2, and *Verrucomicrobia* phyla, Figure 1). These phyla and families have been found in other microbial studies of mosses; notably *Acetobacteraceae* and *Acidobacteriaceae* were found to be abundant in sphagnum mosses (Bragina, Berg, *et al.*, 2012) and *Nostocaceae* have long been studied in association with feather mosses (DeLuca *et al.*, 2002) and *Sphagnum* (Bay *et al.*, 2013; Kostka *et al.*, 2016). Members of the WPS-2 and *Verrucomicrobia* phyla have previously been found in association with sphagnum mosses in bogs (Bragina *et al.*, 2015).

Moss species identity was more important than site in determining the composition of the moss-associated bacterial communities. Across all samples, moss species contributed to 63% of the variation in community composition (Permanova $R^2 = 0.63$, $p < 0.001$), while site was not a significant source of variation (Permanova $R^2 = 0.076$, $p = 0.21$) (Figure 2). This finding is consistent with studies in vascular plants which find that species identity is generally more important than site in shaping phyllosphere community composition (Redford *et al.*, 2010; Laforest-Lapointe *et al.*, 2016). However,

265 sites in this study were situated within a relatively small area. It is possible that across larger scales,
 266 differences in local environmental conditions may have a stronger influence on the observed bacterial
 267 communities. Across all moss species studied here, *Sanionia uncinata* harbored particularly distinct
 268 bacterial communities. More than half (59%) of phylotypes found on *Sanionia uncinata* were not found
 269 in any of the other moss species and, of the 30 most abundant phylotypes highlighted in Table S3, only
 270 77% were present on *S. uncinata* compared to more than 90% found across every other species (Figure
 271 3). While most of the mosses were dominated by *Acetobacteraceae* (*Alphaproteobacteria*),
 272 *Acidobacteriaceae* (*Acidobacteria*), and *Methylophilales* (*Verrucomicrobia*), the moss species
 273 *Sanionia uncinata* had a low abundance or complete absence of these taxa. Instead, *Sanionia uncinata*
 274 was dominated by *Comamonadaceae* (*Betaproteobacteria*), *Nostocaceae* (*Cyanobacteria*) and
 275 *Chitinophagaceae* (*Bacteroidetes*). The distinctiveness of the *S. uncinata* microbiome does not appear
 276 to reflect any aspect of phylogenetic relatedness among the sampled moss species. For instance, the
 277 pleurocarpous *S. uncinata* has much closer phylogenetic affinities with *P. schreberi* and *T. nitens*, yet
 278 the phylogenetically distant *Sphangnum capillifolium* hosts a microbiome much more similar to *P.*
 279 *schreberi* and *T. nitens* than does *S. uncinata*. The factors driving these differences between *S. uncinata*
 280 and the other moss species remain uncertain.

281 Evidence for multiple groups of N₂-fixing bacteria in boreal mosses

282 Using stable-isotope enrichment, we measured N₂-fixation rates and found that all of the moss
 283 species were actively fixing N₂ (Figure S1), though rates varied by a factor of 100 across samples (from
 284 0.98 to 100 µg N • g dry weight moss⁻¹ day⁻¹). Importantly, we find that many moss microbiomes which
 285 were assumed to be non-N₂-fixing (the genus *Dicranum*, for example, Gundale *et al.* (2011) do, in fact,
 286 appear to associate with bacteria capable of fixing N₂ at rates comparable to those of feather mosses,
 287 which are well-known to host bacteria capable of sustaining relatively high rates of N₂-fixation
 288 (DeLuca *et al.*, 2002; Turetsky *et al.*, 2012). Consistent with other studies, *Pleurozium schreberi* and

289 *Sanionia uncinata* had the highest average rates of N₂-fixation at 46.1 and 52.4 µg N • g dry weight
 290 moss⁻¹ day⁻¹, respectively. Our measured rates of fixation are within the expected range previously
 291 reported for *Sphagnum* mosses and *Pleurozium schreberi* (Vile *et al.*, 2014). However, because we used
 292 a δ¹⁵N method as opposed to an acetylene reductase assay, and because of the lack of reported N₂
 293 fixation studies for some of the moss species in our study, it is difficult to directly compare all of our
 294 measurements of N₂-fixation rates to those reported previously (e.g. DeLuca *et al.*, 2002; Zackrisson *et*
 295 *al.*, 2004; Gundale *et al.*, 2012).

296 Given that all of the sampled moss species had microbial communities with measurable N₂-fixation
 297 activities, we then used the 16S rRNA gene survey data to determine which taxa might be contributing
 298 to the N₂-fixation. We found three cyanobacterial and three alphaproteobacterial lineages that were
 299 reasonably abundant across nearly all moss species, with all of these lineages having previously been
 300 identified as capable of N₂-fixation. Specifically, the putative N₂-fixers in our samples were from the
 301 *Nostocaceae*, *Acetobacteraceae*, *Bradyrhizobiaceae*, and *Methylocystaceae* families (Figure 3). Previous
 302 studies have focused on the role of *Cyanobacteria* in bryophyte-associated N₂- fixation, but one study
 303 in *Sphagnum*, found that 50% of bacterial cells colonizing *Sphagnum* were from *Alphaproteobacteria*
 304 and that the *nifH* gene libraries for these species were dominated by alphaproteobacterial, rather than
 305 cyanobacterial sequences (Bragina, Maier, *et al.*, 2012). Interestingly, these two groups of putative N₂-
 306 fixers showed contrasting patterns of abundance between *Sanionia uncinata* and *Pluerozium schreberi*
 307 (the two moss species with the highest rates of N₂-fixation). These two hosts showed relatively similar
 308 average rates of N₂-fixation (52.4 µg N g⁻¹day⁻¹ and 46.1 µgN g⁻¹day⁻¹), yet *Sanionia uncinata* was
 309 dominated by *Cyanobacteria* (79%) and showed low relative abundances of the alphaproteobacterial
 310 N₂-fixers (7.2%), while *Pleurozium schreberi* showed the opposite pattern (6.8% cyanobacterial, 21.4%
 311 alphaproteobacterial). Together, these results suggest that multiple bacterial taxa may contribute to the
 312 measured N₂-fixation activity in these boreal moss species and, even though cyanobacterial N₂-fixers
 313 have received the bulk of the attention in previous studies (e.g. Ininbergs *et al.*, 2011; Rousk, Rousk, *et*

314 *al.*, 2013), they are not the only N₂-fixing bacteria that associate with boreal mosses. To identify which
315 of these putative N₂-fixing lineages are responsible for the measured N₂-fixation activities in these
316 moss species, future work using stable isotope probing-based approaches would be necessary (e.g.
317 Buckley *et al.*, 2007; Jehmlich *et al.*, 2010).

318 **Boreal mosses harbor abundant, undescribed bacterial lineages**

319 The mosses we studied hosted an unexpected abundance of understudied bacterial lineages.
320 Abundant phylotypes for which there were no close matches (<97% 16S rRNA sequence similarity) to
321 previously cultivated and described bacteria are summarized in Table S1. Of these phylotypes, several
322 of the most abundant phylotypes were from the phylum *Verrucomicrobia* and candidate phylum WPS-
323 2. These phylotypes had no closely related cultivated representatives beyond the phylum-level of
324 resolution, yet they were consistently among the most abundant phylotypes in many of the samples
325 (Table S1, Figure S2). Further analysis of the metagenomic data confirmed the observed high relative
326 abundances of WPS-2 and *Verrucomicrobia* and highlight that the abundances observed with the
327 amplicon-based analyses were not a product of primer biases (Figure S3).

328 *Verrucomicrobia* are widely distributed in soils (Brewer *et al.*, 2016), acidic geothermal
329 environments (Op den Camp *et al.*, 2009), and have also been found in boreal mosses, particularly in
330 those mosses growing in moist, acidic environments such as bogs and peatlands (Dedysh, 2011; Sharp
331 *et al.*, 2014; Bragina *et al.*, 2015). However, the ecologies of these moss-associated taxa remain poorly
332 known. Among our samples, the most common verrucomicrobial divisions were the orders
333 *Methylacidiphilales* (3.24%) and *Spartobacteria* (3.79%). One phylotype from *Methylacidiphilales*,
334 was the third most abundant phylotype overall. Several earlier studies hypothesized that bacteria from
335 *Methylacidiphilales* contribute to both N₂-fixation and methanotrophy in peatland environments
336 (Bragina *et al.*, 2015; Ho and Bodelier, 2015). We searched the assembled metagenomic contigs for the
337 methanotrophic marker gene (*pmoA*), and we were unable to find any evidence of the methanotrophic

338 marker gene (*pmoA*) associated with *Methylacidiphilales* in our metagenomic sequence data. It is
 339 therefore unlikely that *Methylacidiphilales* in these samples are oxidizing methane unless they are
 340 using genes that are not currently recognized in databases as being linked to methane oxidation.

341 In addition to relatively high abundances of poorly-described verrucomicrobial phylotypes, the
 342 mosses were also dominated by a single phylotype from the candidate phylum WPS-2 which
 343 represented 3.5% of the 16S rRNA sequences analyzed (Figure 3), making it one of the most abundant
 344 phylotypes in six of the seven moss species (with the exception of *Sanionia uncinata*). No members of
 345 WPS-2 have ever been cultured, and no published genome exists for any member of this candidate
 346 phylum. Given how little we know about this phylum, we can only speculate about the ecology of this
 347 group from a few previous studies in which members of this phylum have been detected.
 348 Representatives from WPS-2 have previously been found in acidic and cold environments, including
 349 alpine bog vegetation (Bragina *et al.*, 2015), mineral deposits from a low-temperature acidic spring
 350 (Grasby *et al.*, 2013), and acidic natural gas extraction shale (Trexler *et al.*, 2014). Interestingly, Trexler
 351 *et al.* (2014) also observed that WPS-2 seems to co-occur with methanotrophs in aquatic mosses and
 352 speculated that WPS-2 may be using derivatives of methanotrophy such as carbon dioxide,
 353 formaldehyde, or formate. However, we found only two instances of the methane oxidation marker
 354 gene (*pmoA*) in our shotgun metagenomes (out of more than 926 million reads), therefore it seems
 355 unlikely that the highly abundant members of the WPS-2 phylum found in our samples associate with
 356 methanotrophs.

357 **Draft genome of WPS-2 recovered from metagenomic data**

358 To learn more about the abundant and poorly studied taxa found in these mosses and to provide
 359 further insight into the functional attributes of these bacteria, we chose moss samples with particularly
 360 high abundances of diverse taxa of interest (*Methylacidiphilae* and WPS-2) for metagenomic analysis.
 361 We assembled 61 genome bins from these moss-associated bacterial communities, seven of which

362 passed contamination and completeness standards (Table S2). Despite the apparent abundance of
 363 *Methylophilae*, none of the recovered bins were from this phylum. The inability to recover highly
 364 abundant strains from metagenomes is fairly common and can be a product of high strain variation
 365 (intraspecific variation) (Miller *et al.*, 2011).

366 Four phyla were represented in the recovered genome bins: *Acidobacteria* (1 bin), *Proteobacteria*
 367 (1 bin, *Alphaproteobacteria*), *Cyanobacteria* (2 bins), and WPS-2 (3 bins). The three bins from WPS-2
 368 ranged from 84 – 89% complete and represent the first genomic data available for members of the
 369 candidate phylum WPS-2. We estimate that the full genome sizes for these WPS-2 representatives are
 370 3.88, 3.41, and 4.34 Mbp based on the presence of lineage-specific single-copy marker genes.

371 Because WPS-2 is one of the most abundant groups across our samples and we know so little about
 372 the functional attributes of this group, we used gene annotation to try to reconstruct the potential
 373 metabolic attributes of the most complete genome bin obtained for WPS-2. The gene annotations
 374 revealed that WPS-2 is likely an anoxygenic phototroph, capable of carbon fixation, and able to
 375 metabolize the bi-products of photorespiration making it well-suited to life on the surface of a plant
 376 (Figure 4). We identified key genes involved in anoxygenic photosynthesis including those encoding
 377 the M and L subunits of anoxygenic photosynthetic reaction centers (*pufM* and *pufL*) as well as the
 378 gene for the Y subunit of chlorophyllide a reductase (*bchY*), a universal marker gene for BChl-
 379 containing anoxygenic phototrophs (Yutin *et al.*, 2005, 2009). Anoxygenic photosynthesis was first
 380 recognized for its importance in marine environments, but recently it has also been recognized as a
 381 common trait of phyllosphere bacteria found on the surface of vascular plants (Atamna-Ismaeel *et al.*,
 382 2013). There are three types of anoxygenic phototrophs that contain *pufM*-type reaction centers: purple
 383 sulfur bacteria, purple non-sulfur bacteria, and aerobic anoxygenic phototrophs (AAP) (Yurkov and
 384 Hughes, 2017). Of these three groups, we hypothesize that WPS-2 is an aerobic anoxygenic phototroph
 385 since abundant sulfur sources are expected to be extremely limited in boreal forests and the exposed

environment of the moss phyllosphere is unlikely to provide the anaerobic environment necessary to support purple non-sulfur bacteria. If WPS-2 is truly an AAP, it would be one of the few observed lineages to possess RuBisCo. Until recently, all other known AAP were thought incapable of RuBisCo-facilitated carbon fixation (Hughes *et al.*, 2017) however, a recent study (Graham *et al.*, 2017) has found four Alphaproteobacteria in a marine metagenomic dataset that appear to possess the ability to fix carbon via the Calvin-Benson-Bassham cycle.

If WPS-2 is an AAP possessing RuBisCo, how might it live in close association to moss and their cyanobacteria without being out-competed for light and carbon resources? WPS-2 appears to possess several traits which may allow it to effectively associate with mosses. As an anoxygenic phototroph, WPS-2 would not compete with its host for light resources since anoxygenic phototrophs absorb light in a complementary spectrum to that of plants and cyanobacteria, with a maximal absorption peak between 500 and 550nm (the region where chlorophyll has its minimum absorption) (Atamna-Ismaeel *et al.*, 2012). Interestingly, an absorption peak in this area of the spectrum may also give WPS-2 a photosynthetic advantage under light snow cover since the wavelengths with the greatest transmittance through snow are also in that region of the light spectrum (Perovich, 2007).

In addition to its complementary absorption spectrum, WPS-2 may be able to take advantage of the byproducts of its host's photorespiration to supplement its C requirements. Photorespiration takes place when RuBisCo acts on O₂ rather than CO₂ producing phosphoglycolate, a potent inhibitor of the Calvin cycle. Thus, in plants and other carbon fixers who use the Calvin cycle, metabolizing phosphoglycolate is important. The moss-associated WPS-2 phylotypes appear to possess the enzymes necessary for converting phosphoglycolate into glyoxylate and passing it into the TCA cycle. This process, known as the "glyoxylate bypass", is a modified TCA cycle that allows organisms to bypass the normal electron-generating steps of the cycle and devote it entirely to biomass production (Kornberg, 1966). Since WPS-2 is likely phototrophic, during times of high light it is unlikely to need its TCA cycle for energy

410 generation and could instead take advantage of its host's sugars for biomass production. In such a
 411 situation, WPS-2 would only need to use RuBisCo under conditions when sugars from the plants were
 412 scarce, such as early in the growing season or under snow pack. The complementary absorption
 413 spectrum of the moss-associated members of the WPS-2 phylum and their ability to metabolize
 414 glyoxylate appear to make this group uniquely equipped for living commensally on their moss hosts.

415 **Conclusions**

416 We found that moss-associated bacterial communities are strongly structured by moss species
 417 identity with different moss species harboring distinct bacterial communities regardless of collection
 418 site. Surprisingly, all of the moss species studied possessed microbial communities that were capable of
 419 N₂-fixation and hosted a broad diversity of putative N₂-fixing bacterial lineages. However, it remains
 420 unclear which lineages are responsible for the measured N₂-fixation rates and whether distinct bacterial
 421 lineages are driving N₂-fixation in different moss species. Future work should focus on how microbial
 422 community variation between host species may influence N₂-fixation rates and how interactions both
 423 between bacteria and between bacteria and the moss host might contribute to these rates. We also found
 424 that mosses can harbor a number of poorly-described bacterial lineages, including a high relative
 425 abundance of bacteria assigned to the candidate phylum WPS-2 with previously unknown ecological
 426 and metabolic characteristics. Using shotgun metagenomic analyses, we were able to assemble a nearly
 427 complete genome representative of this WPS-2 lineage and the genomic analyses suggest that WPS-2 is
 428 an anoxygenic phototroph that is uniquely adapted to living in close-association with mosses in this
 429 ecosystem.

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 436

References

- Atamna-Ismaeel, N., Finkel, O., Glaser, F., von Mering, C., Vorholt, J.A., Koblížek, M., et al. (2012) Bacterial anoxygenic photosynthesis on plant leaf surfaces. *Environ. Microbiol. Rep.* **4**: 209–216.
- Atamna-Ismaeel, N., Finkel, O.M., Glaser, F., Sharon, I., Schneider, R., Post, A.F., et al. (2013) Microbial rhodopsins on leaf surfaces of terrestrial plants. *Environ. Microbiol.* **14**: 140–146.
- Bay, G., Nahar, N., Oubre, M., Whitehouse, M.J., Wardle, D.A., Zackrisson, O., et al. (2013) Boreal feather mosses secrete chemical signals to gain nitrogen. *New Phytol.* **200**: 54–60.
- Baym, M., Kryazhimskiy, S., Lieberman, T.D., Chung, H., Desai, M.M., and Kishony, R.K. (2015) Inexpensive multiplexed library preparation for megabase-sized genomes. *PLoS One* **10**: 1–15.
- Bengtsson-Palme, J., Hartmann, M., Eriksson, K.M., Pal, C., Thorell, K., Larsson, D.G.J., and Nilsson, R.H. (2015) metaxa2: Improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data. *Mol. Ecol. Resour.* **15**: 1403–1414.
- Bragina, A., Berg, C., and Berg, G. (2015) The core microbiome bonds the Alpine bog vegetation to a transkingdom metacommunity. *Mol. Ecol.* **24**: 4795–4807.
- Bragina, A., Berg, C., Cardinale, M., Shcherbakov, A., Chebotar, V., and Berg, G. (2012) *Sphagnum* mosses harbour highly specific bacterial diversity during their whole lifecycle. *ISME J.* **6**: 802–13.
- Bragina, A., Maier, S., Berg, C., Müller, H., Chobot, V., Hadacek, F., and Berg, G. (2012) Similar diversity of Alphaproteobacteria and nitrogenase gene amplicons on two related *Sphagnum* mosses. *Front. Microbiol.* **2**: 1–10.
- Brewer, T., Handley, K., Carini, P., Gibert, J., and Fierer, N. (2016) Genome reduction in an abundant and ubiquitous soil bacterium “*Candidatus Udaeobacter copiosus*.” *Nat. Microbiol.* **2**: 16198.
- Buckley, D.H., Huangyutitham, V., Hsu, S.F., and Nelson, T.A. (2007) Stable isotope probing with ¹⁵N₂ reveals novel noncultivated diazotrophs in soil. *Appl. Environ. Microbiol.* **73**: 3196–3204.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **6**: 1621–1624.
- Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., et al. (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **42**: 633–642.
- Dedysh, S.N. (2011) Cultivating uncultured bacteria from northern wetlands: Knowledge gained and remaining gaps. *Front. Microbiol.* **2**: 1–15.
- DeLuca, T.H., Zackrisson, O., Gentili, F., Sellstedt, A., and Nilsson, M.C. (2007) Ecosystem controls on nitrogen fixation in boreal feather moss communities. *Oecologia* **152**: 121–130.
- DeLuca, T.H., Zackrisson, O., Nilsson, M.-C., and Sellstedt, A. (2002) Quantifying nitrogen-fixation in feather moss carpets of boreal forests. *Nature* **419**: 917–920.
- Edgar, R.C. (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.

- 473 Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:
474 2460–2461.
- 475 Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat.*
476 *Methods* **10**: 996–8.
- 477 Geffert, J.L., Frahm, J.P., Barthlott, W., and Mutke, J. (2013) Global moss diversity: spatial and
478 taxonomic patterns of species richness. *J. Bryol.* **35**: 1–11.
- 479 Graham, E., Heidelberg, J.F., and Tully, B. (2017) Undocumented Potential For Primary Productivity In
480 A Globally-Distributed Bacterial Photoautotroph. *bioRxiv*.
- 481 Grasby, S.E., Richards, B.C., Sharp, C.E., Brady, A.L., Jones, G.M., Dunfield, P.F., and Williamson,
482 M.-C. (2013) The Paint Pots, Kootenay National Park, Canada — a natural acid spring analogue
483 for Mars 1, 2. *Can. J. Earth Sci.* **50**: 94–108.
- 484 Gundale, M.J., Deluca, T.H., and Nordin, A. (2011) Bryophytes attenuate anthropogenic nitrogen inputs
485 in boreal forests. *Glob. Chang. Biol.* **17**: 2743–2753.
- 486 Gundale, M.J., Nilsson, M., Bansal, S., and Jäderlund, A. (2012) The interactive effects of temperature
487 and light on biological nitrogen fixation in boreal forests. *New Phytol.* **194**: 453–463.
- 488 Ho, A. and Bodelier, P.L.E. (2015) Diazotrophic methanotrophs in peatlands: the missing link? *Plant*
489 *Soil* 419–423.
- 490 Hughes, E., Head, B., Maltman, C., Piercey-Normore, M., and Yurkov, V. (2017) Aerobic anoxygenic
491 phototrophs in gold mine tailings in. *Can. J. Microbiol.* **63**: 212–218.
- 492 Ininbergs, K., Bay, G., Rasmussen, U., Wardle, D.A., and Nilsson, M.C. (2011) Composition and
493 diversity of *nifH* genes of nitrogen-fixing cyanobacteria associated with boreal forest feather
494 mosses. *New Phytol.* **192**: 507–517.
- 495 Jean, M. (2017) Broadleaf litter and environmental effects on bryophytes in boreal forests of Alaska.
- 496 Jehmlich, N., Schmidt, F., Taubert, M., Seifert, J., Bastida, F., von Bergen, M., et al. (2010) Protein-
497 based stable isotope probing. *Nat. Protoc.* **5**: 1957–1966.
- 498 Joshi, N.A. and Fass, J.N. (2011) Sickle: A sliding-window, adaptive, quality-based trimming tool for
499 FastQ files (Version 1.33) [Software].
- 500 Kip, N., Winden, J.F. Van, Pan, Y., Bodrossy, L., Reichart, G., Smolders, A.J.P., et al. (2010) Global
501 prevalence of methane oxidation by symbiotic bacteria in peat-moss ecosystems. *Nat. Geosci.* **3**:
502 617–621.
- 503 Kornberg, H.L. (1966) The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem. J.* **99**:
504 1–11.
- 505 Kostka, J.E., Weston, D.J., Glass, J.B., Lilleskov, E.A., Shaw, A.J., and Turetsky, M.R. (2016) Tansley
506 insight The *Sphagnum* microbiome: new insights from an ancient plant lineage. *New Phytol.*

- 507 Laforest-Lapointe, I., Messier, C., and Kembel, S.W. (2016) Tree phyllosphere bacterial communities :
508 exploring the magnitude of intra- and inter-individual variation among host species. **4**: 1–18.
- 509 Langmead, B. and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**:
510 357–359.
- 511 Leff, J.W., Del Tredici, P., Friedman, W.E., and Fierer, N. (2015) Spatial structuring of bacterial
512 communities within individual *Ginkgo biloba* trees. *Environ. Microbiol.* **17**: 2352–2361.
- 513 Leppänen, S.M., Rissanen, A.J., and Tirola, M. (2015) Nitrogen fixation in *Sphagnum* mosses is
514 affected by moss species and water table level. *Plant Soil* **389**: 185–196.
- 515 Li, D., Liu, C.M., Luo, R., Sadakane, K., and Lam, T.W. (2014) MEGAHIT: An ultra-fast single-node
516 solution for large and complex metagenomics assembly via succinct de Bruijn graph.
517 *Bioinformatics* **31**: 1674–1676.
- 518 Lindo, Z. and Gonzalez, A. (2010) The Bryosphere : An Integral and Influential Component of the
519 Earth’s Biosphere. 612–627.
- 520 Lundberg, D.S., Yourstone, S., Mieczkowski, P., Jones, C.D., and Dangl, J.L. (2013) Practical
521 innovations for high-throughput amplicon sequencing. *Nat. Methods* **10**: 999–1002.
- 522 Markowitz, V.M., Chen, I.M.A., Chu, K., Szeto, E., Palaniappan, K., Pillay, M., et al. (2014) IMG/M 4
523 version of the integrated metagenome comparative analysis system. *Nucleic Acids Res.* **42**: 568–
524 573.
- 525 Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads.
526 *EMBnet.journal* **17**: 10–12.
- 527 McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., et al. (2012) An
528 improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of
529 bacteria and archaea. *ISME J.* **6**: 610–618.
- 530 Miller, C.S., Baker, B.J., Thomas, B.C., Singer, S.W., and Banfield, J.F. (2011) EMIRGE:
531 reconstruction of full-length ribosomal genes from microbial community short read sequencing
532 data. *Genome Biol.* **12**: R44.
- 533 Op den Camp, H.J.M., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S., et al. (2009)
534 Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia.
535 *Environ. Microbiol. Rep.* **1**: 293–306.
- 536 Opelt, K., Chobot, V., Hadacek, F., Schönmann, S., Eberl, L., and Berg, G. (2007) Investigations of the
537 structure and function of bacterial communities associated with *Sphagnum* mosses. *Environ.*
538 *Microbiol.* **9**: 2795–2809.
- 539 Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015) CheckM:
540 assessing the quality of microbial genomes recovered from isolates, single cells, and
541 metagenomes. *Genome Res.* **25**: 1043–55.

542 Perovich, D.K. (2007) Light reflection and transmission by a temperate snow cover. *J. Glaciol.* **53**:
543 201–210.

544 Price, M.N., Dehal, P.S., and Arkin, A.P. (2010) FastTree 2 - Approximately maximum-likelihood trees
545 for large alignments. *PLoS One* **5**:.

546 R Core Team (2017) R: A Language and Environment for Statistical Computing.

547 Redford, A.J., Bowers, R.M., Knight, R., Linhart, Y., and Fierer, N. (2010) The ecology of the
548 phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves.
549 *Environ. Microbiol.* **12**: 2885–93.

550 Rousk, K., Jones, D.L., and Deluca, T.H. (2013) Moss-cyanobacteria associations as biogenic sources
551 of nitrogen in boreal forest ecosystems. **4**: 1–10.

552 Rousk, K., Rousk, J., Jones, D.L., Zackrisson, O., and DeLuca, T.H. (2013) Feather moss nitrogen
553 acquisition across natural fertility gradients in boreal forests. *Soil Biol. Biochem.* **61**: 86–95.

554 Rousk, K., Sorensen, P.L., Lett, S., and Michelsen, A. (2015) Across-habitat comparison of diazotroph
555 activity in the subarctic. *Microb. Ecol.* **69**: 778–787.

556 Ruess, R.W., Anderson, M.D., Mcfarland, J.M., Kielland, K., Olson, K., and Taylor, D.L. (2013)
557 Ecosystem-level consequences of symbiont partnerships in an N-fixing shrub from interior
558 Alaskan floodplains. *Ecol. Monogr.* **83**: 177–194.

559 Ruess, R.W., Mcfarland, J.M., Trummer, L.M., and Rohrs-Richey, J.K. (2009) Disease-mediated
560 declines in N-fixation inputs by *Alnus tenuifolia* to early-successional floodplains in interior and
561 south-central Alaska. *Ecosystems* **12**: 489–502.

562 Sharp, C.E., Smirnova, A. V., Graham, J.M., Stott, M.B., Khadka, R., Moore, T.R., et al. (2014)
563 Distribution and diversity of Verrucomicrobia methanotrophs in geothermal and acidic
564 environments. *Environ. Microbiol.* **16**: 1867–1878.

565 Trexler, R., Solomon, C., Brislawn, C.J., Wright, J.R., Rosenberger, A., Keddache, M., et al. (2014)
566 Assessing impacts of unconventional natural gas extraction on microbial communities in
567 headwater stream ecosystems in Northwestern Pennsylvania. *Front. Microbiol.* **5**: 1–13.

568 Turetsky, M.R., Bond-Lamberty, B., Euskirchen, E., Talbot, J., Frolking, S., McGuire, a. D., and
569 Tuittila, E.S. (2012) The resilience and functional role of moss in boreal and arctic ecosystems.
570 *New Phytol.* **196**: 49–67.

571 Vile, M.A., Kelman Wieder, R., Živković, T., Scott, K.D., Vitt, D.H., Hartsock, J.A., et al. (2014) N₂-
572 fixation by methanotrophs sustains carbon and nitrogen accumulation in pristine peatlands.
573 *Biogeochemistry* **121**: 317–328.

574 Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naïve Bayesian classifier for rapid
575 assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **73**:
576 5261–5267.

- Wu, Y.-W., Tang, Y.-H., Tringe, S.G., Simmons, B. a, and Singer, S.W. (2014) MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. *Microbiome* **2**: 26.
- Yurkov, V. and Hughes, E. (2017) Aerobic Anoxygenic Phototrophs: Four Decades of Mystery. In, Hallenbeck, P.C. (ed), *Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects*. Springer International Publishing, Cham, pp. 193–214.
- Yutin, N., Suzuki, M.T., and Béjà, O. (2005) Novel primers reveal wider diversity among marine aerobic anoxygenic phototrophs. *Appl. Environ. Microbiol.* **71**: 8958–8962.
- Yutin, N., Suzuki, M.T., Rosenberg, M., Rotem, D., Madigan, M.T., Süling, J., et al. (2009) BchY-based degenerate primers target all types of anoxygenic photosynthetic bacteria in a single PCR. *Appl. Environ. Microbiol.* **75**: 7556–7559.
- Zackrisson, A.O., Deluca, T.H., Nilsson, M., Sellstedt, A., Berglund, L.M., and Zackrisson, O. (2004) Nitrogen fixation increases with successional age in boreal forests. *Ecology* **85**: 3327–3334.
- Zackrisson, O., Deluca, T.H., Gentili, F., Sellstedt, A., and Jäderlund, A. (2009) Nitrogen fixation in mixed *Hylocomium splendens* moss communities. *Oecologia* **160**: 309–319.

Author Contributions

The project was conceived by N.F., M.C.M, and S.F.M. The manuscript was written by H.H.M. and N.F. with contributions from all co-authors. M.C.M. collected the samples. J.S. and S.M. performed the isotopic analysis. L.R.L. and S.F.M. identified the moss species and prepared the voucher specimens. H.H.M. and N.F. performed the molecular analyses.

Conflict of Interest Statement

The authors declare no conflict of interest in this study.

Materials and Correspondence

Requests for materials and correspondence should be addressed to N.F. or H.H.M.

Figure Captions:

Figure 1: Heat map showing the relative abundances of the most abundant bacterial families across the 7 moss species. Phylum-level classifications for each family are noted on the left. Higher relative abundances are indicated in brighter colors. Families with no official classification are noted as “Unclassified Family.” Host moss species are indicated along the x-axis and ordered according to overall bacterial community similarity.

Figure 2: Non-metric multidimensional scaling (NMDS) plot of bacterial community dissimilarities across samples (measured using the Bray-Curtis distance metric). Samples from the same host species tend to cluster together (A), while those from the same site were spread across ordination space (B). A PERMANOVA test revealed that species identity contributes to 63% of the variation in community composition ($R^2 = 0.63$, $p < 0.001$), while site is not a significant source of variation between these communities ($R^2 = 0.08$, $p = 0.21$).

Figure 3: A phylogenetic tree of the top 30 bacterial phylotypes (circles) and their N₂-fixing potential. Putative N₂-fixers (black circles) were identified by their relatedness to known N₂-fixers (see text).

The putative N₂-fixers observed in our moss samples include representatives from the cyanobacterial and alphaproteobacterial groups. The phylotypes represented here were chosen as they were consistently the most abundant phylotypes across all moss species and together account for 35% of 16S rRNA gene reads in the dataset. Each inner colored ring represents a different species of moss, from the inside ring to the outer ring they are: *Sanionia uncinata* (blue), *Dicranum elongatum* (yellow), *Aulacomnium turgidum* (bright red), *Pleurozium schreberi* (dark red), *Sphagnum capillifolium* (green), *Aulacomnium palustre* (purple), *Tomenthypnum nitens* (gray). The opacity of the colored rings represents the relative abundance of the different phylotypes (log transformed and scaled between 0 and 1) within each moss species. The black outer ring represents the total relative abundance of each phylotype across all host species (also log transformed and scaled between 0 and 1).

Figure 4: Diagram highlighting the inferred metabolic pathways linked to anoxygenic phototrophy and carbon cycling between the WPS-2 bacterial phylotype found to be abundant in mosses and the moss host cells. In the bacterial cell, light in a complementary spectrum to that absorbed by plants can be used to fuel cellular processes through anoxygenic phototrophy. CO₂ fixation via RuBisCo in the bacterial cell may also occur, a novel feature for an aerobic anoxygenic phototroph. In the host cell, photorespiration (the action of RuBisCo on O₂ rather than CO₂) can produce a Calvin Cycle-inhibiting byproduct, 2-phosphoglycolate, that the bacterial cell may have the ability to metabolize for biomass production through conversion to glyoxylate, a process known as the “glyoxylate bypass”. This process represents a possible mechanism for a mutualistic or commensal interactions between these WPS-2 bacteria and the moss host.

Proteobacteria	<i>Acetobacteraceae</i>	0.058	0.069	0.128	0.125	0.135	0.164	0.013
	<i>Sinobacteraceae</i>	0.093	0.079	0.076	0.094	0.098	0.135	0.014
	<i>Comamonadaceae</i>	0.021	0.026	0.026	0.015	0.021	0.001	0.146
	<i>Caulobacteraceae</i>	0.015	0.015	0.028	0.031	0.028	0.026	0.015
	<i>Sphingomonadaceae</i>	0.009	0.017	0.007	0.016	0.012	0.002	0.083
	<i>Unclassified Family 1</i>	0.031	0.018	0.019	0.012	0.004	0.017	0.003
	<i>Methylocystaceae</i>	0.015	0.019	0.009	0.023	0.018	0.009	0.003
	<i>Burkholderiaceae</i>	0.015	0.01	0.008	0.028	0.023	0.008	<0.005
	<i>Rhodospirillaceae</i>	0.015	0.012	0.025	0.01	0.01	0.014	0.004
	<i>Unclassified Family 4</i>	0.015	0.016	0.018	0.008	0.008	0.006	0.014
	<i>Unclassified Family 3</i>	0.011	0.012	0.01	0.012	0.018	0.005	0.008
	<i>Xanthomonadaceae</i>	0.027	0.015	0.008	0.01	0.003	0.005	0.007
	<i>Coxiellaceae</i>	0.012	0.006	0.016	0.013	0.003	0.022	0.001
	<i>Bradyrhizobiaceae</i>	0.008	0.013	0.007	0.011	0.004	0.005	0.008
	<i>Unclassified Family 2</i>	0.007	0.005	0.011	0.003	0.003	0.007	0.011
	<i>Hyphomicrobiaceae</i>	0.005	0.008	0.008	0.001	<0.005	0.006	0.012
Acidobacteria	<i>Acidobacteriaceae</i>	0.063	0.056	0.052	0.129	0.158	0.122	0.006
	<i>Solibacteraceae</i>	0.018	0.014	0.021	0.02	0.016	0.03	0.002
Verrucomicrobia	<i>[Chthoniobacteraceae]</i>	0.034	0.029	0.037	0.051	0.051	0.015	0.049
	<i>Unclassified Family 1</i>	0.042	0.037	0.03	0.041	0.034	0.02	0.001
	<i>auto67_4W</i>	0.032	0.022	0.02	0.003	<0.005	0.009	0.002
	<i>Opitutaceae</i>	0.009	0.011	0.014	0.004	0.002	0.011	0.008
Bacteroidetes	<i>Chitinophagaceae</i>	0.035	0.054	0.036	0.022	0.017	0.015	0.087
	<i>Sphingobacteriaceae</i>	0.027	0.029	0.015	0.033	0.058	0.019	0.025
	<i>Cytophagaceae</i>	0.005	0.011	0.011	0.001	<0.005	0.003	0.046
Cyanobacteria	<i>Nostocaceae</i>	0.091	0.069	0.046	0.028	0.039	0.001	0.082
	<i>Unclassified Family 1</i>	0.015	0.009	0.007	0.011	0.001	0.013	0.001
WPS-2	<i>Unclassified Family 1</i>	0.064	0.065	0.06	0.077	0.075	0.062	0.001
Planctomycetes	<i>Unclassified Family 1</i>	0.018	0.017	0.022	0.018	0.019	0.025	0.01
	<i>Isosphaeraceae</i>	0.013	0.016	0.012	0.022	0.03	0.023	0.002
	<i>Gemmataceae</i>	0.015	0.016	0.014	0.006	0.003	0.025	0.01
Actinobacteria	<i>Conexibacteraceae</i>	0.004	0.005	0.001	0.012	0.011	0.03	<0.005
	<i>Microbacteriaceae</i>	0.004	0.013	0.004	0.009	0.012	0.003	0.007

Aulacomnium palustre

Tomenthypnum nitens

Sphagnum capillifolium

Aulacomnium turgidum

Pleurozium schreberi

Dicranum elongatum

Sanionia uncinata

- No evidence for N₂-fixation
● Putative N₂-fixer

Rings

Sanionia uncinata

Dicranum elongatum

Aulacomnium turgidum

Pleurozium schreberi

Sphagnum capillifolium

Aulacomnium palustre

Tomenthypnum nitens

Total Mean Relative
Abundance