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Southern Appalachian Peatlands Support High Archaeal Diversity

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Abstract

Mid-latitude peatlands with a temperate climate are sparsely studied and as such represent a gap in the current knowledge base regarding archaeal populations present and their roles in these environments. Phylogenetic analysis of the archaeal populations among three peatlands in the Southern Appalachians reveal not only methanogenic species but also significant populations of thaumarchaeal and crenarchaeal-related organ- isms of the uncultured miscellaneous crenarchaeotal group (MCG) and the terrestrial group 1.1c, as well as deep-branching Euryarchaeota primarily within the Lake Dagow sediment and rice cluster V lineages. The Thaum/Crenarchaea and deep-branching Euryarchaea represented approximately 24–83 % and 2–18 %, respectively, of the total SSU rRNA clones retrieved in each library, and methanogens represented approximate- ly 14–72 % of the clones retrieved. Several taxa that are either rare or novel to acidic peatlands were detected including the euryarchaeal SM1K20 cluster and thaumarchaeal/crenarchaeal-related clusters 1.1a, C3, SAGMCG-1, pSL12, and AK59. All three major groups (methanogens, Thaumarchaea/Crenarchaea, and deep- branching Euryarchaea) were detected in the RNA library, suggesting at least a minimum level of maintenance activity. Compared to their northern counterparts, Southern Appalachian peatlands appear to harbor a relatively high diversity of Archaea and exhibit a high level of intra-site heterogeneity.

Introduction

Wetland areas are major contributors to emission of the greenhouse gas methane [1–4] and as such are partially responsible for the resulting environmental changes associated with greenhouse gas accumulation [5–8]. Because methane released from these wetland areas is of microbial origin [9–13], it is important to increase our understanding of the associated microbial communities. The southern region of the United States is of special interest as mid-latitude wetlands are predicted to dramatically increase methane gas release as climate warming occurs [14–16].

Southern Appalachian peatlands are similar to their northern counterparts in biogeochemical cycling and vegetation [17–19], yet there are distinct differences in succession, climate, biogeographic history, and some flora [20]. Most notably, rates of carbon mineralization [21], decomposition [17], net primary productivity [22, 23], and greenhouse gas production [21] in Southern Appalachian peatlands significantly contrast those of northern sites. Southern peatlands are also characterized by noticeable spatial heterogeneity in water input, pH, and vegetation [20], as well as physical and chemical properties [17]. Like Big Run Bog in West Virginia, most bogs further south in North Carolina receive some water seepage and thus hydrologically resemble fens [17]. Yet, they differ from fens in that the water input is often acidic and oligotrophic (nutrient poor) [20]. In general, they are most similar to ombrotrophic (rain-fed) bogs, especially in overall vegetation, pH, and nutrient concentration, and thus are considered bogs [17, 20]. Additionally, most northern bogs have been accumulating peat for the last 9,000 years since the last glaciers retreated. However, bogs in Southern Appalachia have been found to contain much older peat that has been accumulating since the peak of the last glacial advance some 13,000–18,000 years ago [24, 25]. Yet due to the relatively high rates of decomposition, the peat in southern bogs is

generally much shallower. For example, Pineola Bog is thought to contain the deepest peat layer in North Carolina with approximately 2 m of accumulated peat [20], whereas McLean Bog in New York has approximately 6 to 8 m of peat [26].

To our knowledge, there have been no studies evaluating the microbial diversity in Southern Appalachian bogs. Further, northern bogs of Russia [27–29], Germany [30–32], Scandinavia [33, 34], Canada [35–37], and the United States [38, 39] have received the most attention, followed by southern bogs of West Virginia and Maryland, which have received some biogeochemical study [17, 19, 40–42]. Southern Appalachian peatlands harbor a diversity of important and rare flora and fauna, yet they have rarely been studied [20, 43, 44]. Thus, efforts are warranted to conserve, understand, and research these richly diverse ecosystems important in global climate change.

Methods

Site Descriptions

Potato "Tater" Hill lower bog is a roughly 4.05-ha bog at ca. 1,300 m elevation, located approximately 11 km northwest of Boone, NC (near N 36° 16′ 33.96″; W 81° 41′ 27.96″). Tater Hill experiences seep intrusions around the perimeter of the site; thus, the bog exhibits a noticeable biogeochemical gradient from higher to lower pH and from minerotrophic (spring- or stream-fed) to oligotrophic. Some adjacent seep areas are impacted by beaver dams. Tater Hill flora is marked by the presence of some bog species found throughout the United States, such as *Sphagnum* spp., *Drosera rotundifolia* (common sundew, found during this study), and *Vaccinium macrocarpon* (cranberry), as well as some species found only in the NC, TN, and VA areas, such as *Lilium grayi* (Gray's Lily) [45]. Other species found on site during the study period included the following: *Carex* ssp., *Juncus* ssp. (*Juncus acuminatus*, *Juncus tenuis*, and *Juncus marginatus*, among others), *Luzula acuminata* var. *carolinae*, *Luzula echinata*, *Oxypolis rigidior*, *Mimulus ringens*, *Oenothera* ssp., *Eriophyllum virginicum*, *Osmundastrum cinnamomea* (*Osmunda cinnamomea*), *Salix sericea*, *Rhododendron* ssp. (*Rhododendron vaseyi*, *Rhododendron calendulaceum*, and *Rhododendron maximum* among others), *Vaccinium* ssp., *Aronia prunifolia*, *Spiraea alba*, *Ilex verticillata*, and *Viburnum cassinoides* (Andrew Jenkins and Tim Metcalf, personal communication). Unlike the other study sites (described below), Tater Hill Bog is underlain by amphibolite metamorphic rock originating from ancient lava flows [46], and the soil is significantly richer and more minerotrophic, at least in some areas of the site. Consequently, Tater Hill Bog displayed both the highest pH on average (ca. 5.7), as well as the greatest variability (Table 1). Tater Hill is classified as a Southern Appalachian

bog (typic subtype) by the most recent southern classification scheme [47], although it would most likely be considered a moderate fen by northern classification methods [48]. Due to its more minerotrophic nature, Tater Hill Bog was selected to contrast the typical Southern Appalachian bogs, such as Sugar Mountain Bog, that are found throughout the region.

Pineola Bog is located adjacent to the Linville River in the Pisgah National Forest (near N 36° 01′ 30.72″; W 81° 53′ 47.04″), at ca. 1,080 m elevation. The site consists of approximately 47.35 ha, and, like Tater Hill Bog, it is reported to contain some species common to northern bogs such as *Vaccinium macrocarpon*, as well as other plants that are rare to the area: *Thelypteris simulata*, *V. macrocarpon*, *Carex trisperma*, *Arisaema triphyllum* ssp. *stewardsonii*, and *Sphagnum fallax* [49]. Pineola Bog contains the deepest (ca. 2 m) reported peat layer in the region [20]. Classified as a swamp–bog complex (typic subtype) according to the southern classification system [47], Pineola was found to harbor a canopy of *Acer rubrum*, *Tsuga canadensis*, *Quercus rubra*, *Betula alleghaniensis*, *Liriodendron tulipifera*, *Betula lenta*, and *Aesculus flava*, as well as an understory of *Rhododendron maximum*, *Kalmia latifolia*, *Viburnum cassinoides*, *Viburnum nudum*, *Sambucus canadensis*, *Clethera virginiana*, *Alnus serrulata*, *Lobelia cardinalis*, several *Carex* ssp., *Osmundastrum cinnamomea* (*Osmunda cinnamomea*), *Juncus acuminatus*, *Xanthorhiza simplicissim*, and *Sphagnum* spp. (Andrew Jenkins and Tim Metcalf, personal communication).

Sugar Mountain Bog is a very small (roughly 2.83 ha) oligotrophic bog within a larger 86.60-ha natural area located near Pineola Bog (near N 36° 04′ 58.08″; W 81° 53′ 47.04″), at nearly 1,100 m elevation. This study site was chosen as a classic example of a Southern Appalachian bog of the typic subtype [47, 49]. Flora present during the study period (2010–2012) included *Sphagnum* spp., herbaceous plants such as *Galax urceolata*, *Carex* ssp., *Lilium grayi*, *Scirpus* sp., and *Osmundastrum cinnamomea*, in addition to shrubs such as *Spiraea alba*, *Salix sericea*, *Salix humilis*, *Viburnum cassinoides*, *Rhododendron maximum*, *Kalmia latifolia*, *Clethra accuminata*, *Hypericum densiflorum*, *Alnus serrulata*, *Rosa palustris*, and *Vaccinium corymbosum* (Andrew Jenkins and Tim Metcalf, personal communication). Rare and/or threatened plant species such as *V. macrocarpon*, *C. trisperma*, *Epilobium ciliatum*, *Lycopodiella inundata*, *Lilium grayi*, and *Platanthera grandiflora* have also been reported at the site [49]. This site exhibited the lowest pH at ca. 4.7 (Table 1). All three study sites were generally low in exchangeable ion concentrations. For example, sodium concentration was around 1–3 mg/L (ca. 90 μM) and potassium was around 20 μM, or 0.4–0.7 mg/L [50], significantly higher than some northern peatlands, such as McLean Bog (Cortland, NY) with reported values of 1–4 μM. In contrast to other peatlands throughout the world [51], aluminum, silicon, and iron were generally high (0.1–2.5, 14–33, and 0.4–11 mg/L,

Table 1 General characteristics of the peatland study sites

	Pineola	Tater Hill	Sugar Mountain
Wetland description	Oligotrophic forested swamp–bog complex	Minerotrophic Southern Appalachian bog	Oligotrophic Southern Appalachian bog
Temperature range (°C)	5.0–15.5	4.5–16.5	4.5–16.5
pH average (range)	5.0 (4.0–5.6)	5.7 (4.9–6.8)	4.7 (3.9–5.3)
Vegetation	*Sphagnum* spp., *Pinus palustris*, *Acer saccharum*, *Rhododendron maximum*, *Vaccinium macrocarpon*	*Sphagnum* spp., *Rhododendron* spp., *Vaccinium macrocarpon*, *Carex* spp., *Juncus* spp., *Lilium grayi*	*Sphagnum* spp., *Rhododendron maximum*, *Vaccinium corymbosum*, *Rosa palustris*, *Carex* spp.
Water table range (cm below surface)	2.5–15	0.0–7.5	0.0–7.5
Potential methane production (mmol/L CH_4 day^{-1})	0.006	0.029	0.014

The values are reported either as an average or as a range encompassing the entire sampling period between the summer of 2010 and the winter of 2012. Potential methane production rates were determined in a previous study

respectively), likely due to the nature of the bedrock in the area [52]. The potential methane production as determined in a previous study [50] was within the range for northern bogs (Table 1). All three sites had areas of low pH (pH 4 or 5), and all three had water tables relatively close to the peat surface (0–15 cm; Table 1).

Sampling Procedures

Each site was sampled during all four seasons from the summer of 2011 to the summer of 2012, and additional samples were also taken in the summer of 2010. During each sampling event, temperature, water table depth below the peat surface, and pH of the peat layer were measured. Peat samples were collected in airtight jars from 10 to 25 cm below the standing water table, corresponding to the depth of maximum methane production as determined at these sites previously (data not shown). Three plots were sampled within each site with the exception of Sugar Mountain, which was used for comparative purposes only. Samples were stored and transported at ambient temperature from study sites to the laboratory where they were subsampled for DNA analysis, dry weight calculations, soil slurries, and analyses of potential methane production. Additionally, aliquots of each peat sample were stored at − 80 °C for nucleic acid extraction and molecular analyses. Pore water was collected from each site during the summer of 2012 and sent for ICP analysis at Cornell University (Cornell University, Ithaca, NY, USA).

Nucleic Acid Extraction and cDNA Synthesis

Total genomic DNA was extracted from peat samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) per the manufacturer's instructions

and stored at − 20 °C or − 80 °C for molecular analysis or long- term storage, respectively. Total RNA was isolated according to the manufacturer's protocol using the TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY, USA) with slight modifications. Briefly, 0.3–0.5-g (fresh weight) aliquots of peat were added to 1.5-mL Eppendorf© tubes along with sterile ceramic beads and 750 μL of detergent/lysing solution and homogenized for 1 min at max speed using a MiniBeadbeater™ (Biospec Products, Bartlesville, OK, USA). Homogenized samples were centrifuged for 5 min at 12,000×*g* and the supernatant transferred to a sterile 1.5-mL Eppendorf© tube. A second lysing step was then performed by the addition of 1 mL of TRIzol reagent to supernatant with subsequent repetitive pipetting. Phase separation was achieved by the incubation of samples for 5 min at 20 °C, after which 0.2 mL of chloroform was added. Tubes were vigorously shaken and incubated at 20 °C for an additional 3 min. Samples were centrifuged at 12,000×*g* for 15 min resulting in a separation of the mixture into a lower red, phenol-chloroform phase containing proteins, an interphase containing DNA, and a colorless upper aqueous phase containing the desired RNA. RNA was then precipitated by carefully transferring the upper aqueous phase to a new, sterile 1.5-mL Eppendorf© tube along with 0.5 mL of isopropyl alcohol and incubating samples at − 20 °C for 10 min. Centrifugation was performed at 12,000×*g* for 10 min, after which the supernatant was discarded. The resulting RNA pellet was washed once with 1 mL of 75 % ethanol and centrifuged at 7,500×*g* for 5 min and the supernatant was discarded. The RNA pellet was dried at room temperature for 10 min and dissolved in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 min at 60 °C. RNA was stored at − 20 °C for no more than a week prior to analysis. RNA was treated with the RTS DNase Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA), and was deemed free of DNA via PCR. First strand cDNA synthesis was performed

using the OneStep RT-PCR Kit (Qiagen, Germany) according to the manufacturer's protocol.

SSU rRNA and SSU rRNA gene Amplification, Cloning, and Sequencing

Archaeal SSU rRNA genes were amplified from extracted DNA or from cDNA using the Archaea-specific primer set Ar109f (5′-ACKGCTCAGTAACACGT-3′) and Ar922r (5′-YCCGGCGTTGANTCCAATT-3′) [53] and ARMAN-specific primer sets ARM979F (5′-TATTACCAGAAGCGAC GGC-3′) and ARM1356R (5′-AGGGACGTATTCACCGCT CG-3′) [54] and ARMANF (5′-AGGCAGATGGCGGGGT AAC-3′) and ARMANR (5′-CGCATTGACGTTGAGCGCC-3′) (this study). The general archaeal 25-µL PCR mixture contained PCR Master Mix (0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2), 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 5 µM of each primer 109F and 922R, 3 ng of DNA, and dH_2O. The thermal profile for general archaeal species was as follows: initial denaturation at 98 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min. A final extension at 72 °C for 7 min ended the protocol. The ARMAN-specific thermal profile was as follows: a 45-s denaturation step at 98 °C followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 57 °C for 1 min, and extension at 72 °C for 1.5 min. The protocol ended with an additional extension step at 72 °C for 7 min. Amplified products were visualized by gel electrophoresis in 1 % agarose and stained with GelRed (Phenix Research Products, Candler, NC, USA). PCR reactions were conducted in triplicate and combined before cloning to reduce PCR bias. Amplicons were cloned using a TA cloning kit (TOPO 2.1; Invitrogen, Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol. A total of four libraries were generated from DNA extracted from each of the following samples (the total number of sequences for each library is listed in parentheses along with the sampling date): Tater Hill DNA (90; 2011), Pineola DNA (164; 2011/2012), and Sugar Mountain DNA (88; 2011). Additionally, a cDNA library of 89 sequences was created from samples collected at Pineola in the summer of 2012, resulting in a total of five libraries. PCR amplification of successful transformants was performed in a 25-µL reaction containing PCR Master Mix (0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl₂), 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 15 µM of each primer (M13F-M13R), cell material, and dH_2O. The PCR protocol began with an initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1.5 min, and extension at 72 °C for 3 min. A final extension at 72 °C for 7 min ended the protocol. PCR products were visualized on a 1 % agarose gel via gel electrophoresis to ensure the expected product sizes. Sequencing was

Fig. 1 Neighbor-joining tree of methanogenic euryarchaeotal SSU rRNA gene sequences retrieved from Pineola Bog, Tater Hill Bog, and Sugar Mountain Bog. Only representative sequences of each operational taxonomic unit (OTU) from all DNA and cDNA libraries were included. The number of sequences represented by each OTU is given in *parentheses* in the following arrangement: (1) DNA sequences from Pineola Bog (2011), (2) DNA sequences from Tater Hill Bog (2011), (3) DNA sequences from Sugar Mountain Bog (2011), (4) RNA sequences from Pineola Bog (2012), and (5) DNA sequences from Pineola Bog (2012). Alignments were created using the online SILVA aligner. Dendrogram was created using the PHYLIP package. *Thermotoga maritima* and *Aquifex pyrophilus* were used as outgroups. Bootstrap values represent 100 replicates and values are shown for node values both ≥ 50 and supported by maximum likelihood analysis
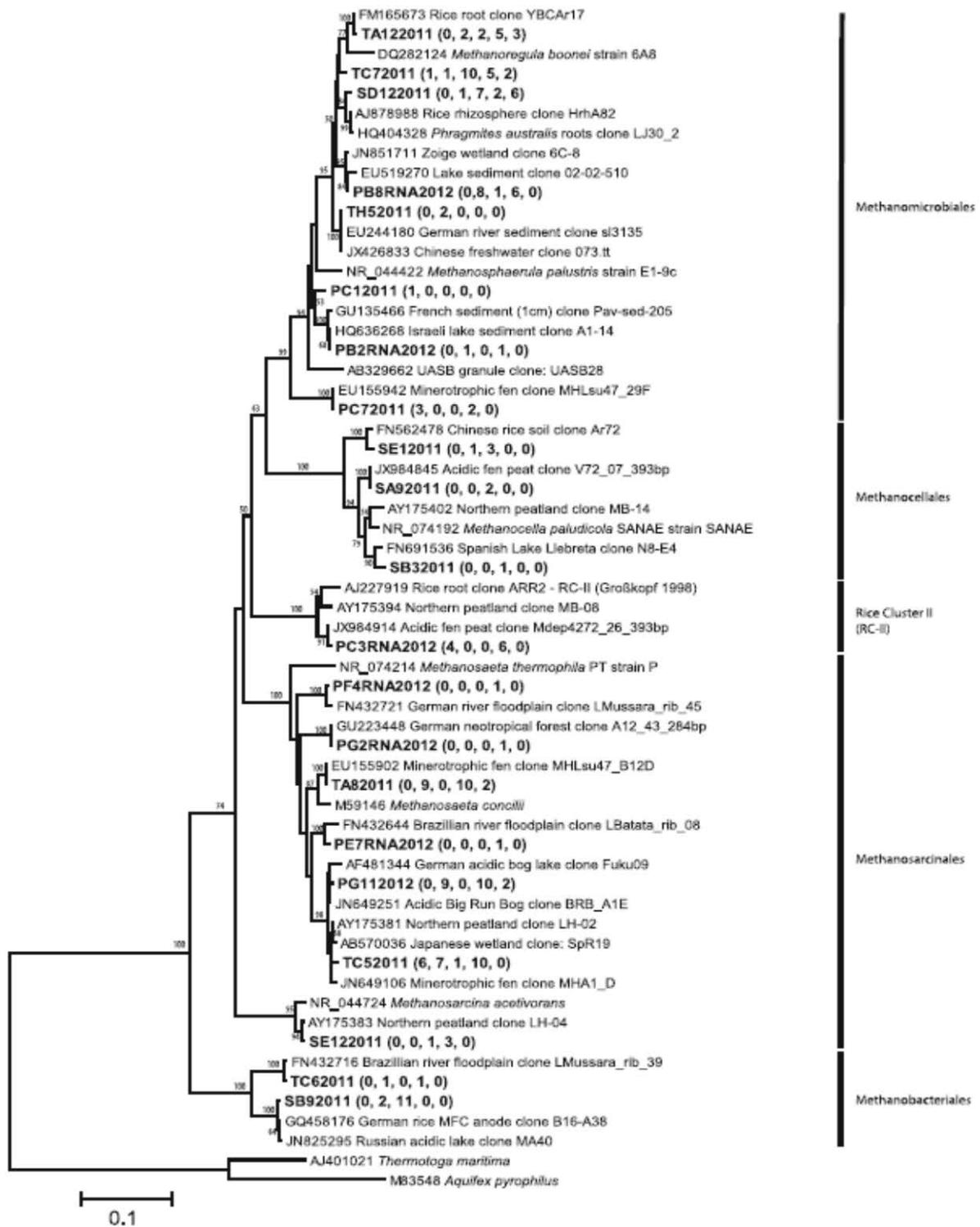
conducted using a Sanger platform at Beckman-Coulter Genomics (Danvers, MA, USA).

Sequence Analysis and Phylogenetics

All forward and reverse sequences containing ≥ 400 bp were imported into Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA), and contiguous sequences were assembled from respective forward and reverse sequences from each clone. Resulting contigs were screened for chimeras using DECIPHER [55] and were compared to existing sequences using the BLAST tool (http://blast.ncbi.nlm.nih.gov/) from the NCBI database. Sequence files along with a selection of top hits from the BLAST comparison were aligned using the SILVA aligner of the ARB program (http://www.arb-silva.de/ aligner/). Sequence editing was performed using the BioEdit tool (Ibis Biosciences, Carlsbad, CA, USA). Phylogenetic trees were constructed by neighbor joining (NJ) and by the maximum-likelihood method. A distance matrix was generated using the Phylip package (http://evolution.gs.washington. edu/phylip.html) and used for subsequent operational taxonomic unit (OTU) assignment and NJ tree construction. The DOTUR computer program was used to generate OTUs, rarefaction curves, as well as the species richness estimates (ACE and Chao 1) for each data set. A 97 % SSU rRNA gene sequence similarity threshold was used to define each OTU. Good's nonparametric coverage was estimated using the equation $[1 - (n/N)] \times 100$, where "N" is the total number of clones evaluated and "n" is the number of singleton OTUs [56]. NJ tree construction and subsequent maximum likelihood tree construction was performed using the Phylip package.

Real-Time Quantitative PCR

Due to the high percentage of clones belonging to the Crenarchaeota, abundance of Crenarchaeota and total Archaea were assessed for the Pineola and Tater Hill sites. Quantitative PCR (qPCR) was conducted with general archaeal primers 967F and 1060R [39], crenarchaeal-specific primers 771F and

FM165673 Rice root clone YBCAr17
TA122011 (0, 2, 2, 5, 3)
DQ282124 *Methanoregula boonei* strain 6A8
TC72011 (1, 1, 10, 5, 2)
SD122011 (0, 1, 7, 2, 6)
AJ878988 Rice rhizosphere clone HrhA82
HQ404328 *Phragmites australis* roots clone LJ30_2
JN851711 Zoige wetland clone 6C-8
EU519270 Lake sediment clone 02-02-510
PB8RNA2012 (0,8, 1, 6, 0)
TH52011 (0, 2, 0, 0, 0)
EU244180 German river sediment clone sl3135
JX426833 Chinese freshwater clone 073.tt
NR_044422 *Methanosphaerula palustris* strain E1-9c
PC12011 (1, 0, 0, 0, 0)
GU135466 French sediment (1cm) clone Pav-sed-205
HQ636268 Israeli lake sediment clone A1-14
PB2RNA2012 (0, 1, 0, 1, 0)
AB329662 UASB granule clone: UASB28
EU155942 Minerotrophic fen clone MHLsu47_29F
PC72011 (3, 0, 0, 2, 0)

Methanomicrobiales

FN562478 Chinese rice soil clone Ar72
SE12011 (0, 1, 3, 0, 0)
JX984845 Acidic fen peat clone V72_07_393bp
SA92011 (0, 0, 2, 0, 0)
AY175402 Northern peatland clone MB-14
NR_074192 *Methanocella paludicola* SANAE strain SANAE
FN691536 Spanish Lake Llebreta clone N8-E4
SB32011 (0, 0, 1, 0, 0)

Methanocellales

AJ227919 Rice root clone ARR2 - RC-II (Großkopf 1998)
AY175394 Northern peatland clone MB-08
JX984914 Acidic fen peat clone Mdep4272_26_393bp
PC3RNA2012 (4, 0, 0, 6, 0)

Rice Cluster II (RC-II)

NR_074214 *Methanosaeta thermophila* PT strain P
PF4RNA2012 (0, 0, 0, 1, 0)
FN432721 German river floodplain clone LMussara_rib_45
GU223448 German neotropical forest clone A12_43_284bp
PG2RNA2012 (0, 0, 0, 1, 0)
EU155902 Minerotrophic fen clone MHLsu47_B12D
TA82011 (0, 9, 0, 10, 2)
M59146 *Methanosaeta concilii*
FN432644 Brazillian river floodplain clone LBatata_rib_08
PE7RNA2012 (0, 0, 0, 1, 0)
AF481344 German acidic bog lake clone Fuku09
PG112012 (0, 9, 0, 10, 2)
JN649251 Acidic Big Run Bog clone BRB_A1E
AY175381 Northern peatland clone LH-02
AB570036 Japanese wetland clone: SpR19
TC52011 (6, 7, 1, 10, 0)
JN649106 Minerotrophic fen clone MHA1_D
NR_044724 *Methanosarcina acetivorans*
AY175383 Northern peatland clone LH-04
SE122011 (0, 0, 1, 3, 0)

Methanosarcinales

FN432716 Brazilian river floodplain clone LMussara_rib_39
TC62011 (0, 1, 0, 1, 0)
SB92011 (0, 2, 11, 0, 0)
GQ458176 German rice MFC anode clone B16–A38
JN825295 Russian acidic lake clone MA40

Methanobacteriales

AJ401021 *Thermotoga maritima*
M83548 *Aquifex pyrophilus*

0.1

957R [57], and E1/E2 methanogen-specific primers A-gE372 and A-gE540aR [39]. qPCR was performed on duplicate field samples in duplicate 20-μL reaction volumes containing the following: 9 μL SYBR Green Master Mix, 9.2 μL dH$_2$O, and 0.2 μM of each primer, on an Applied Biosystems™ 7300 Real-Time PCR System (Carlsbad, CA, USA). Reactions were performed using two different dilution factors to account for PCR inhibitors. Reaction conditions were as follows: initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and elongation at 72 °C for 2 min. A final dissociation step followed that consisted of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Efficiencies were between 80 and 110 %. Calibration curves for quantification were generated using one of the following standards: (1) plasmid DNA containing the SSU rRNA gene from OREC-R1096 (Crenarchaea, range of 10^2–10^6 target copies/μL) or (2) genomic DNA extracted using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA, USA) from *Methanoregula boonei* 6A8 (Archaea, range of 10^2–10^6 target copies/μL). Circular plasmid standards have been reported to cause overestimation of sample cell number using quantitative PCR (Hou et al. 2010). To circumvent this potential issue, plasmid DNA standards were linearized by restriction digest using BssHII (New England BioLabs, Ipswich, MA, USA).

Results

Phylogenetic Analysis of Methanogenic Archaea

A large portion of the Euryarchaeota sequences recovered from the clone libraries at all three sites were related to the Methanosarcinales and the Methanomicrobiales. The globally abundant Methanoregulaceae-associated cluster of Methanomicrobiales dominated the methanogenic communities of the DNA libraries from both Sugar Mountain Bog (2011) and Pineola Bog in 2012 (Fig. 1 and Table 2). In contrast, the methanogenic communities in Tater Hill Bog and Pineola Bog in 2011 were dominated by the Methanosarcinales order of methanogens, which represented approximately 30 % and 26 % of the overall archaeal community from each site, respectively (Table 2). The cDNA library from Pineola 2012 also harbored an abundance of Methanosarcinales (roughly 40 % of the archaeal population; Table 2). Methanobacteriales clones ranged in abundance from 0 to 14 % and were more abundant in the Sugar Mountain Bog samples compared to the other two sites. Members of the rice cluster II (RC-II) were also detected in Pineola Bog, but not in the other two sites. Compared to other Euryarchaeota or Crenarchaeota, methanogenic species were found to dominate the overall archaeal community in both Tater Hill (52 % of the total sequences) and Sugar Mountain Bog (50 %), as well as the cDNA library (71 %) from Pineola Bog (Table 2).

Deep-Branching Euryarchaea

Many deep-branching Euryarchaeotal lineages were also identified in all three study sites from both DNA and RNA libraries. Among the species identified, rice cluster V (RC-V), marine benthic group D (MBG-D), and the Lake Dagow sediment (LDS) cluster were the most commonly represented groups across sites (Fig. 2 and Table 2). The RC-V group was the most abundant of the deep-branching lineages, constituting approximately 8 %, 13 %, 1 %, and 3 % of the overall archaeal community from Tater Hill Bog, Sugar Mountain Bog, and Pineola Bog 2012 DNA and RNA, respectively. However, the MBG-D was also abundant and clones from this clade were highly represented (5 %) in the DNA library from Pineola Bog in 2011.

Crenarchaea, Thaumarchaea, and Deep-Branching ARMAN-2 Archaea

Crenarchaeaota represented at least 24 % of the sequences in each library; however, the greatest number of taxa were found in the Pineola Bog site (Fig. 3 and Table 2). Clones in the MCG cluster 1.3 were the most abundant among the crenarchaeal clones for all sites and represented ca. 22–31 % of the total clones in the DNA libraries and 10 % of the RNA library (Fig. 3 and Table 2). Terrestrial group 1.1c, also partially known as Rice Cluster IV, was fairly abundant in Pineola Bog DNA libraries as well (13–39 %) and was also detected (ca. 1 %) in both the Tater Hill Bog DNA and Pineola Bog RNA libraries. Clones affiliated with the deep peat lineage were most abundant (2–12 %) in the Pineola samples and were also detected (1 %) in Tater Hill Bog, though not in Sugar Mountain Bog (Table 2). Overall, thaumarchaeal and crenarchaeal-related sequences were found to dominate the archaeal community among both DNA libraries of Pineola Bog and represented roughly 70–83 % of the sequences in these libraries (Table 2).

Additionally, sequences were retrieved from several lineages less commonly reported in bogs, including marine group 1.1a, and groups C3, SAGMCG-1, pSL12, and AK59. Most notably, the proportion of sequences clustering in the marine group 1.1a rivaled that of the deep peat lineage, with greater than 5 % of the sequences from all three sites falling in this cluster. Interestingly, a sequence closely related to the newly described microarchaea *Candidatus* Microarchaeum acidiphilum (ARMAN-2) [54] was retrieved from the Pineola 2011 clone library. Using sequence data available for ARMAN-2 and related sequences retrieved from a Finnish peatland [58], we designed ARMAN-2-specific primers and retrieved sequences for a second OTU (Fig. 3). These sequences are shown to group phylogenetically as a deep-branching archaeal relative (Fig. 2), putatively among Crenarchaeota [59].

Table 2 Phylogenetic representation of the Archaea present in all three sample sites as estimated from clone sequences retrieved

	Tater Hill Bog, NC	Sugar Mountain Bog, NC	Pineola Bog, NC		
			DNA 2011	DNA 2012	RNA 2012
Methanogens					
Methanomicrobiales	17.86 %	25.64 %	6.58 %	10.89 %	23.33 %
Methanocellales	1.19 %	7.69 %	0.00 %	0.00 %	0.00 %
Rice cluster II	0.00 %	0.00 %	5.26 %	0.00 %	6.67 %
Methanosarcinales	29.76 %	2.56 %	7.90 %	3.96 %	40.00 %
Methanobacteriales	3.57 %	14.10 %	0.00 %	0.00 %	1.11 %
	52.38 %	50.00 %	19.74 %	14.85 %	71.11 %
Deep Euryarchaea					
Rice cluster V	8.33 %	12.82 %	2.63 %	0.99 %	3.33 %
Lake Dagow sediment cluster	3.57 %	2.56 %	1.32 %	0.99 %	1.11 %
Marine Benthic Group D	0.00 %	0.00 %	5.26 %	0.00 %	0.00 %
Rice cluster III	0.00 %	2.56 %	0.00 %	0.00 %	0.00 %
SM1K20	1.19 %	0.00 %	1.32 %	0.00 %	0.00 %
	13.09 %	17.95 %	10.53 %	1.98 %	4.44 %
Crenarchaeal-related					
Deep Peat Group	1.19 %	0.00 %	11.84 %	3.97 %	2.22 %
Thaumarchaea Grp C3	1.19 %	1.28 %	0.00 %	0.00 %	0.00 %
Miscellaneous Crenarchaeotal Group (MCG)	0.00 %	0.00 %	6.58 %	0.00 %	1.11 %
MCG Grp 1.3	26.19 %	21.80 %	30.26 %	30.69 %	10.00 %
Thaumarchaea pSL12	0.00 %	0.00 %	1.32 %	0.00 %	0.00 %
Thaumarchaea AK59	0.00 %	0.00 %	2.63 %	0.00 %	0.00 %
Grp. 1.1a	4.76 %	7.69 %	2.63 %	7.92 %	7.78 %
Grp. 1.1b	0.00 %	0.00 %	0.00 %	0.00 %	1.11 %
SAGMCG-1	0.00 %	1.28 %	0.00 %	0.99 %	1.11 %
Grp. 1.1c	1.19 %	0.00 %	13.16 %	38.61 %	1.11 %
ARMAN	0.00 %	0.00 %	1.32 %	0.99 %	0.00 %
	34.52 %	32.05 %	69.74 %	83.17 %	24.44 %

Potentially Active Archaeal Groups

In order to investigate the potential activity among archaeal groups, a cDNA clone library from extracted RNA was constructed in the summer of 2012 and compared to a DNA control library. It was revealed that Methanosarcinales-related sequences represented ~40 % of all sequences obtained (Table 2). Methanomicrobiales and RC-II were also represented in the library (23 and 7 %, respectively) as well as thaumarchaeal and crenarchaeal-related groups, suggesting at least a minimum level of maintenance activity sometime near the time of sampling, and verifying that crenarchaeotal-related organisms are not only present but most likely active, functional members of the community. Among the crenarchaeal sequences in the RNA library from Pineola Bog in 2012, group 1.3 was the most abundant, representing 10 % of the clones, and group 1.1a was the next most abundant archaeal group, representing ca. 8 % (Table 2). In contrast to the 2012 DNA library from Pineola Bog, the RNA library contained a lower abundance of sequences clustering in group 1.1c, approximately only 1 % of clones.

Quantification of Crenarchaea

The estimated quantity of archaeal cells showed high variation between different samplings, but did not appear to show any seasonal trend (Electronic Supplementary Material Fig. S1). For Pineola Bog samples, the median total number of Archaea was estimated at 1×10^8 cells per gram of wet soil (range was from 6×10^7 to 1×10^9) and the mean number of Crenarchaea was estimated at 3×10^8 cells per gram of wet soil (range was from 3×10^6 to 1×10^9). Tater Hill estimates were consistently one half to two orders of magnitude lower than Pineola estimates. For Tater Hill samples, the median estimated number of total Archaea was 4×10^7 cells per gram of wet soil (range was from 1×10^6 to 3×10^8), whereas the median numbers were estimated at 1×10^6 cells per gram of wet soil (range was from 6×10^4 to 3×10^7) (Electronic Supplementary Material Fig. S1).

Overall, the estimated number of crenarchaeal target sequences rivaled that of total archaeal sequences on most of the sampling dates.

Sequence Coverage and Species Richness

Good's estimates of sample coverage consistently ranged from 70 to 85 % across all libraries, suggesting that the majority of the major microbial taxa were most likely represented (Table 3).Rarefaction curves were generated for each library as well as for all libraries combined and the curves approached a horizontal asymptote (Electronic Supplementary Material Fig. S2). After sequencing approximately 440 clones across five libraries, fewer than 150 archaeal taxa were identified, indicating that the sequencing depth capacity of clone libraries was nearly exhausted. ACE and Chao1 estimates of species richness both revealed that Tater Hill Bog harbored the most diverse population of Archaea, whereas Pineola Bog and Sugar Mountain Bog were more similar to each other (Table 3).

Discussion

Community Composition of the Methanogenic Archaea

Among the methanogen-related clones identified, members of the Methanomicrobiales dominated in the oligotrophic bogs, Pineola and Sugar Mountain Bog. The majority (63 %) of the Methanomicrobiales clones retrieved clustered with *Methanoregula boonei* strain 6A8, the only formerly described cultured representative of acidophilic methanogens in the E2 group [60, 61]. Originally called the R-10 group [62, 63], this group is ubiquitous and predominates in ombrotrophic bogs [33, 64, 65]. Peat-associated organisms in the E2 group appear to be adapted to acidic, nutrient-poor environments with low concentrations of exchangeable cations, and clones from this cluster were most abundant in the most acidic site, Pineola Bog (pH 4.7).

Clones affiliated with the family Methanosaetaceae in the Methanosarcinales order were particularly abundant (30 %) in Tater Hill Bog and in the cDNA library from Pineola Bog (~40 %), but were also fairly abundant (~3 %) in Sugar Mountain Bog and Pineola Bog (3–8 %) DNA libraries. Organisms of the Methanosaetaceae family are known to be aceticlastic (using acetate as their primary metabolic substrate), with a high affinity for acetate but low utilization rate [66–71]. Even though acetate was not specifically measured in this study, it can be assumed that acetate concentration is most likely fairly low in Pineola Bog and Tater Hill Bog given that the dominant methanogenic group present in the peat were those clustering in the Methanosaetaceae with *Methanosaeta concilli*. In contrast, only a few clones (four total) clustered with *Methanosarcina acetivorans* (Fig. 1), which is known to have a lower affinity for

acetate and to require concentrations above 1 mM [70, 71]. Although acetate concentrations have been shown to increase transiently in some sites [72, 73], these studies rarely demonstrated acetate concentrations above 1 mM. The presence of *Methanosarcina*-related sequences would suggest that acetate concentrations may occasionally reach metabolically available concentrations (ca. 1 mM) either spatially or temporally as previously shown [74]. This is not surprising as *Methanosarcina* spp. are readily enriched from peat [75, 76].
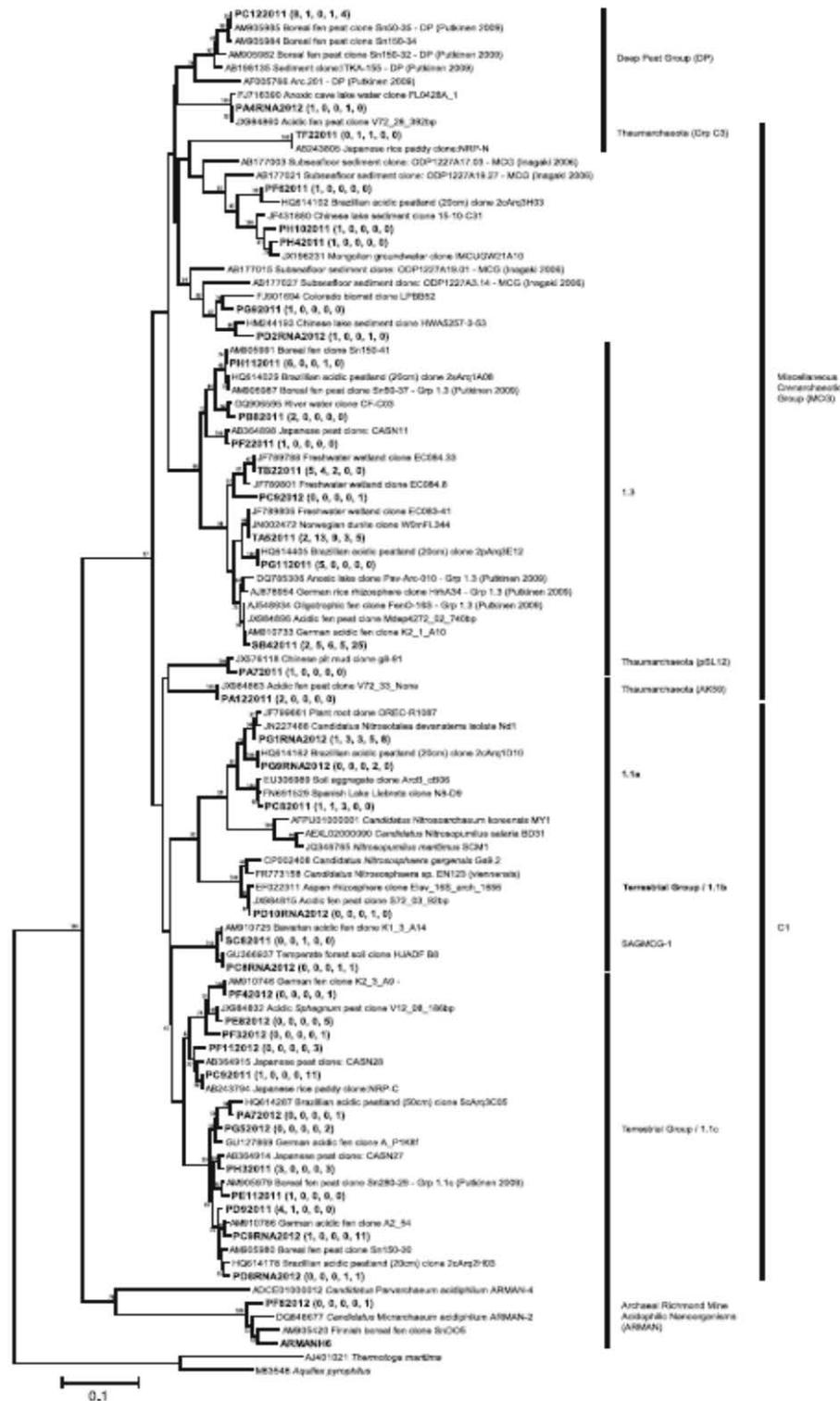
Closely following the Methanosaetaceae in dominance were the Methanobacteriales, a hydrogenotrophic group of methanogens. These organisms are commonly found in boreal [9] and temperate peatlands [38, 39, 77–81]; however, they are generally less abundant than the Methanomicrobiales. Early studies of Methanobacteriales enrichment cultures from peat suggested these members were less tolerant of acidity [31, 82]. However, subsequent laboratory incubations of low pH and low temperature demonstrated a substantial increase in members of the Methanobacteriaceae, suggesting that these organisms may be important in low-pH, low-temperature environments [29]. Sugar Mountain Bog had the largest number of clones in the Methanobacteriales (14 %), perhaps because it has the lowest pH of the sites. Finally, clones affiliated with hydrogenotrophic organisms of the RC-I/ Methanocellales were detected in Sugar Mountain Bog (~8 %) and Tater Hill Bog (1 %), and those affiliated with RC-II were found in two of the Pineola libraries (5–7 %). Overall, at least four orders of methanogens were detected in agreement with other researchers who generally find a dominance of the E1/E2 group of Methanomicrobiales [33, 62, 65, 83, 84] and *Methanosaeta*/*Methanosarcina* species [9, 33, 38, 63, 81], and occasionally detect other groups such as rice cluster I (RC-I) [64, 65] and RC-II [85, 86].

Deep-Branching Euryarchaeota

In addition to the methanogenic component of the archaeal communities of each study site, roughly 12 % of the clones retrieved fell within the deep-branching Euryarchaeota. These

SB52011 (0, 0, 2, 0, 0)
AJ867612 Swiss lake water clone M01
AM495334 German rice root clone LL1Root_09
TB62011 (0, 1, 1, 0, 0)
GU257278 German freshwater sediment clone OTRo_D1_78
AB686512 Japanese rice field soil clone: R8
AM712540 Permafrost peat clone Sv-86 - RC-V (Bhattarai 2012)
FR820765 Spanish Lake Llebreta clone ANNA-C8
PA102012 (0, 0, 0, 0, 1)
AY531695 German lake sediment clone Dg2002_T_16 - RC-V (Chan 2005)
PE4RNA2012 (0, 0, 0, 1, 0)
GU257225 German freshwater sediment clone OTRo_D1_9
JF262363 Yellowstone lake clone YLA079
PD112011 (1, 0, 0, 0, 0)
DQ310451 Canadian Mackenzie River clone mrR2.67 - RC-V (Galand 2005)
JN820176 Cave ferromanganese deposit clone G06
HE613643 Spanish ferromanganese biofilm clone 09T2A-G5
SG12011 (0, 0, 1, 0, 0)
AJ867624 Swiss lake water clone M33
TF52011 (0, 1, 0, 0, 0)
EU244238 Canadian Lake Mackenzie clone CaS1b.b08
SF112011 (0, 0, 1, 0, 0)
SC102011 (0, 0, 1, 0, 0)
AM503250 German rice root clone LL_Koral_38
JN853711 Swiss lake sediment clone KS8_3a
AM503238 German rice root clone LL_Koral_23 - RC-V (Bhattarai 2012)
AY133914 German Lake Dagow sediment clone LDS26
TC12011 (0, 1, 0, 0, 0)
PE12011 (1, 0, 0, 0, 0)
JQ315353 Russian oil field clone FwtA_otu6_3
EU244296 Canadian Mackenzie River clone CaR3b.f12
PA6RNA2012 (0, 0, 0, 1, 0)
JQ792392 Canadian oligotrophic lake sediment clone ALAKE10_44
SG32011 (0, 0, 1, 0, 0)
GU257074 German freshwater sediment clone OTRo_A1_20
SE52011 (0, 0, 1, 0, 0)
TG72011 (0, 1, 1, 0, 0)
FR865353 Chinese rice field soil clone t30d34La7
SH52011 (0, 0, 1, 0, 0)
AJ227951 German rice soil clone ABS3 - RC-V (Großkopf 1998)
DQ310411 Canadian Mackenzie River clone mrR1.22
TE122011 (0, 1, 0, 0, 0)
GU257184 Freshwater sediment clone OTRo_O1_23
JF835243 Brazilian river water clone K32F05
PD3RNA2012 (0, 0, 0, 1, 0)
GU047745 Wetland clone AL.59
DQ310383 Canadian Mackenzie River clone mrR1.51 - RC-V (Galand 2005)
TA42011 (0, 1, 0, 0, 0)
AB107814 Japanese paddy soil clone:TN-1
AB380105 Japanese soil clone: IU-FSC-17_Ar-A025
TE42011 (0, 1, 0, 0, 0)
AJ548932 Oligotrophic fen clone FenF-16S
JN825241 Russian acidic lake clone DA65
PG62012 (0, 0, 0, 1, 1)
JN825231 Russian acidic lake clone DA36
AY133915 German lake sediment clone LDS27 - LDS (Bhattarai 2012)
JF431710 Chinese lake sediment clone 30-10-T6
TD22011 (0, 1, 1, 0, 0)
AM503240 German rice root clone LL_Koral_26
TG12011 (1, 2, 1, 0, 0)
DQ310396 Canadian Mackenzie River clone mrR1.39
EF014581 Canadian Lake Mackenzie clone CaS1s.50
EU155986 Minerotrophic fen clone MH1100_E48
PA92011 (4, 0, 0, 0, 0)
AM851081 Alpine lake sediment clone Cad915-20
DQ301914 Acidic peatland clone CBd-306F
JF789814 Freshwater wetland clone EC081.16
AJ576232 Landfill leachate clone GZK56 - VADIN (Paul 2012)
EU910624 Sediment clone G16 - TMEG-1 (Paul 2012)
AJ576219 Landfill leachate clone GZK24 - TMEG-2 (Paul 2012)
FM957953 Chinese rice paddy soil clone NA5AR42 - RC-III (Paul 2012)
SG102011 (0, 0, 2, 0, 0)
JN605146 Estuarine sediment clone 87B915n4 - SM1K20 (Breuker 2013)
TE102011 (0, 1, 0, 0, 0)
FJ479771 Zoige wetland clone 7C-6
PC52011 (1, 0, 0, 0, 0)
EF014531 Canadian Mackenzie River clone CaR3s.23
AJ401021 *Thermotoga maritima*
M83548 *Aquifex pyrophilus*

Rice Cluster V (RC-V)

Lake Dagow Sediment Group (LDS)

Marine Benthic Group D (MBG-D)

VADIN

TMEG

Rice Cluster III (RC-III)

SM1K20

0.1

Fig. 3 Neighbor-joining tree of crenarchaeotal SSU rRNA gene sequences retrieved from Pineola Bog, Tater Hill Bog, and Sugar Mountain Bog. Only representative sequences of each operational taxonomic unit (OTU) from all DNA and cDNA libraries were included. The number of sequences represented by each OTU is given in *parentheses* in the following arrangement: (1) DNA sequences from Pineola Bog (2011), (2) DNA sequences from Tater Hill Bog (2011), (3) DNA sequences from Sugar Mountain Bog (2011), (4) RNA sequences from Pineola Bog (2012), and (5) DNA sequences from Pineola Bog (2012). Alignments were created using the online SILVA aligner. Dendrogram was created using the PHYLIP package. *Thermotoga maritima* and *Aquifex pyrophilus* were used as outgroups. Bootstrap values represent 100 replicates and values are shown for node values both ≥50 and supported by maximum likelihood analysis

Table 3 Similarity-based OTUs (97 %) and estimates of species richness and library coverage

Year of sample	Sample site (library type)	Number of sequences	Cluster distance 0.03			
			OTU	ACE	Chao 1	Good's coverage
2011	Pineola (DNA)	78	31	68	88	76 %
2011	Tater Hill (DNA)	90	37	153	107	70 %
2011	Sugar Mtn. (DNA)	88	34	98	73	75 %
2012	Pineola (DNA)	93	25	46	45	85 %
2012	Pineola (RNA)	91	34	90	76	77 %

species are typically related to the Thermoplasmata and contain candidate methanogenic groups such as MBG-D [87] as well as verified methanogens affiliated with rice cluster III (RC-III) such as the human-associated *Methanomassiliococcus huminyensis*. The majority of the sequences were found to belong to the RC-V, which represented approximately 3 %, 8 %, and 13 % of the total archaeal sequences recovered from Pineola Bog, Tater Hill Bog, and Sugar Mountain Bog, respectively (Table 2). The RC-V group is an environmental cluster found ubiquitously throughout freshwater, inland habitats [88], and members of this group are probably not methanogenic [89]. Roughly 1–2 % of the total clones retrieved here clustered in the LDS (ca. 2 %), the MBG-D (up to 1.5 %), RC-III (ca. 1 %), and SM1K20 clusters. Previous studies have retrieved clones affiliated with RC-III [53], RC-V [88], MBG-D [39], and the LDS cluster [58] from peat. To the best of our knowledge, clones affiliated with SM1K20 have not previously been detected in peat, and, in fact, the top hits were affiliated with wetland, riverine, and estuarine rather than peatland environments. Interestingly, it has been inferred from molecular and phylogenetic analyses that organisms in MBG-D and RC-III contain mcrA and are likely to be involved in methanogenesis [87]. However, more recent metagenomic analyses indicate a role for MBG-D organisms in peptide degradation [90]. Unfortunately, the physiological roles of organisms within other clusters, such as RC-V and SM1K20, remain unknown, although sequences in the SM1K20 have been associated with marine environments characterized as organic-lean [91].

Crenarchaea, Thaumarchaea, and Deep-Branching ARMAN-2 Archaea

Crenarchaeal and thaumarchaeal sequences were also recovered from each study site. Pineola Bog was revealed to contain the most abundant crenarchaeal/thaumarchaeal population with ~70–83 % of all DNA-derived sequences obtained associating with these phyla (Table 2), including two OTUs found to be related to the deeply branching *Candidatus* Microarchaeum acidiphilum ARMAN-2 [54]. Among

terrestrial environments, Crenarchaea/Thaumarchaea have typically been shown to represent only a small portion of the overall archaeal population (~0.5 to 3 %) [57]. However, deep marine sediments with colder temperatures and slow decomposition rates have been demonstrated to harbor a dominant crenarchaeal/thaumarchaeal community [92, 93], and several clusters within these phyla have been associated with slowly decomposing, anoxic, low-energy environments [91]. Further, thaumarchaeal and crenarchaeal-related sequences can represent a relatively large percentage of the total Archaea in some peatlands, including roughly 70 % of archaeal DGGE fragment sequences identified from 18 distinct peatlands throughout Alaska and Massachusetts [94], roughly half of archaeal SSU rRNA clones from an acidic peatland in Scandinavia [80], 90 % of the Archaea in a peatland in Brazil [95], and approximately 40 % of the T-RFLP bands in a Canadian Peatland [85]. Similarly, thaumarchaeal and crenarchaeal sequences were found to dominate a forested acidic peatland in Slovenia [96] and a forested peat swamp in Malaysia [97].

Diversity of the thaumarchaeal- and crenarchaeal-associated taxa was relatively high, with retrieved sequences clustering in terrestrial groups 1.1b and 1.1c as well as marine group 1.1a, SAGMCG-1, and groups 1.3, C3, pSL12, AK59, and the deep peat lineage. Sequences clustering in terrestrial group 1.1c, miscellaneous crenarchaeotal group (MCG) 1.3, and the deep peat lineage have been found in acidic peat soil in Finland [58, 98] and in Slovenia [96], and members of both the terrestrial group and MCG were also detected in Brazil [95]. It has been shown that group 1.1c can be selected in low pH conditions and can predominate in acidic (ca. pH 4) soil types [99–103]. Thus, it is not too surprising to find the 1.1c group in the acidic (pH 4.7–5.7) peat studied here.

Functional Role of the Crenarchaeal-Related Organisms

Despite current understanding that mesophilic Thaumarchaeota and Crenarchaeota are ubiquitous in cold, circumneutral, oxic oceanic waters [104, 105] as well as in terrestrial [106] and freshwater environments [107], little is known about their metabolic functions [58, 95, 103, 108]. Culturing studies indicate that some mesophilic Crenarchaea in marine and estuarine

environments may be involved in ammonia oxidation [109] and indeed crenarchaeal amoA genes have been discovered in a wide range of environments [110–112]. Of the groups represented in our sequences, only two are known to carry amoA, 1.1a and 1.1b [113]. Group 1.1a is less common in peatlands and the relatively large proportion of clones in this group (>5 %) suggests that nitrogen cycling may be somewhat important in these sites, despite being considered a minor process in most nutrient-poor, carbon-rich peat environments [114]. Overall, the majority of the clones retrieved clustered in group 1.3 (MCG; >20 %) and terrestrial group 1.1c (ca. 6 %). Although speculative, it is likely that the dominant Thaumarchaea and Crenarchaea in peatlands may be involved in anaerobic fermentation. Indeed, genomic analyses of *Nitrosopumilus maritiumus* and *Cenarchaeum symbiosum* demonstrated the presence of predicted transporters for amino acids, peptides, and glycerol [115], and recent metagenomic analyses indicate a role for MCG organisms in peptide degradation [90], at least in deep-sea environments. Overall, crenarchaeal populations present in peatlands represent potentially new functional groups of archaea, possibly playing important roles in the biogeochemical cycling of nutrients in these systems and as such warrant further study. Given that ARMAN-2 was originally identified from acid mine drainage [116], it is interesting to speculate as to the role this organism may be playing in acidic peatland environments such as Pineola Bog in this study or Siikaneva fen in Finland [58].

Potentially Active Archaeal Groups

The over-representation of methanogen-related sequences in the RNA library compared to the two DNA libraries may suggest that the methanogens were more active than the thaumarchaeal and crenarchaeal-related organisms. Alternatively, the abundance of methanogen-related rRNA may reflect a combination of larger cell volume and/or number of ribosomes per cell, since members of the MCG are reported to be quite small (0.4–0.5 μm coccoids) [93]. In contrast, *Methanosaeta* spp. are 2–6 μm long by 0.8–1.3 μm wide [117] and *Methanosarcina* spp. cells are 1.5–2 μm coccoids [118]. By volume, *Methanosaeta* spp. and *Methanosarcina* spp. cells would be anywhere from 15 to 200 times larger than the typical crenarchaeotal cell and could contain orders of magnitude greater numbers of ribosomes, suggesting that in our study, Crenarchaeota probably do represent a significant proportion of the active microbial community by cell number, despite the fact that only 24 % of the sequences retrieved from the rRNA library fell within this group. Overall, our findings support a growing body of evidence suggesting that Thaumarchaea and Crenarchaea may be numerically abundant in many different peatlands worldwide.

Archaeal Abundance

The numerical abundance of crenarchaeal-related sequences in both Tater Hill and Pineola was confirmed with qPCR analyses. Results estimated a median of roughly 10^8 total archaeal or crenarchaeal cells per gram of wet soil in Pineola Bog and roughly 10^7 total archaeal or crenarchaeal cells per gram of wet soil in Tater Hill Bog, in line with previous studies in two northern peatlands, which found up to 10^9 total archaeal cells per gram of dry soil (approximately $5-6\times10^7$ cells per gram of wet soil with approximately 5 % dry weight) [39]. The archaeal communities of the wetlands studied here appeared to vary significantly in estimated cell numbers between samples, perhaps reflecting the site heterogeneity that is known to be a hallmark of southern peatlands. Overall, the oligotrophic bog site (Pineola, pH 5) demonstrated consistently greater numbers of estimated archaeal cells (median of 10^8) when compared to the more circum-neutral site (Tater Hill, pH 5.7), which had a median number of 4×10^7 estimated total Archaea per gram of wet soil. Because it has been demonstrated that water table level has a significant impact on the archaeal community of wetlands [8, 119–123], this could be the result of the slightly more stable water table level for Pineola. Given its forested topography, the site is protected from sudden flooding due to heavy rainfall and also from drying during the warmer months due to evaporation. The largest discrepancy between the sites was observed during the fall sampling season, when Tater Hill Bog demonstrated a dip in archaeal numbers perhaps due to the open nature of the site that results in a decrease in the amount of leaf litter deposited during this time of increased bacterial decomposition [124–128].

Sequence Coverage and Species Richness

Results suggest that orders of magnitude deeper sequencing efforts would be required to discover a significantly higher diversity and identify rare community members, as is often revealed through next-generation sequencing [129]. Further, Chao1 and ACE richness estimates suggest a high diversity of archaea were present, despite the relatively low pH conditions. The relatively high archaeal sequence diversity and large number of distinct archaeal taxa obtained here likely result from two main features. First, the degree of spatial heterogeneity in Southern Appalachian peatlands [20] is likely a contributing factor since environmental heterogeneity is known to contribute to microbial diversity [130]. Additionally, low pH is known to limit diversity in soils [131], including peatland soils [83]. The overall pH averages measured for Pineola and Sugar Mountain (5.0 and 4.7, respectively) are at the higher end of that found in other oligotrophic bogs, which typically have pH values below 5, or sometimes as low as 2.6 [48]. It is worth mentioning, however, that certain groups of Archaea

are often affiliated with low pH environments, such as group 1.1c Thaumarchaea [103] and the E1/E2 (or R10) group, or Methanoregulaceae [39, 60, 62, 84]; however, the overall diversity is generally low in acidic environments [33]. Indeed, among the sites measured here, the location with the highest pH, Tater Hill (average pH of 5.7), had the largest species diversity estimates of 153 using ACE and 107 using Chao1, compared to 46–98 using ACE and 45–88 using Chao1 for libraries generated using samples from the other two sites with average pH values of 4.7 and 5.0. In line with our studies, microbial diversity was shown to increase along a gradient from pH 4.2 in an ombrotrophic bog to 5.1 in a mesotrophic fen in Finland [64]. Similarly, a fen in Minnesota was found to have higher diversity than that of a nearby bog [132].

Conclusions

This is one of the first molecular studies of temperate Southern U.S. peatlands and as such presents a unique perspective into variables specific to warmer climates as a proxy for potential climate warming events. High diversity among archaeal groups was evidenced by the recovery of high numbers of taxa, including multiple taxa that are either rare or novel for acidic peatlands, as well as by the high Chao1 estimates of ca. 80 (~50–110). Evidence for spatial and temporal variability was apparent in fluctuations in diversity indices between sample dates (e.g., Pineola 2011 vs. 2012) and in high variation of chemical data, such as pH within the sites (for example, see Table 1). Similar to other studies, the highest diversity was detected in the minerotrophic, higher pH (5.7 vs. 4.7–5.0) site. The discovery of a high abundance of crenarchaeotal sequences in Pineola Bog adds to a growing body of literature suggesting that these organisms may be more prevalent in peatlands than has previously been understood. However, the function of these organisms has yet to be elucidated.

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