Title: Alpha and Gammaproteobacterial Methanotrophs Co-Dominate the Active Methane Oxidizing Communities in an Acidic Boreal Peat Bog

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Abstract

The objective of this study was to characterize metabolically active, aerobic methanotrophs in an ombrotrophic peatland in the Marcell Experimental Forest, Minnesota, USA. Methanotrophs were investigated in the field and in laboratory incubations using DNA-stable isotope probing, expression studies on particulate methane monooxygenase (*pmo*A) genes, and amplicon sequencing of 16S rRNA genes. Potential rates of oxidation ranged from 14-17 μmol CH_4 g dry wt $soil^{-1}$ d^{-1}. Within DNA-SIP incubations, the relative abundance of methanotrophs increased from 4% *in situ* to 25-36% after 8 -14 days. Phylogenetic analysis of the ^{13}C-enriched DNA fractions revealed the active methanotrophs were dominated by the genera *Methylocystis* (Type II; Alphaproteobacteria), *Methylomonas,* and *Methylovulum* (Type I; Gammaproteobacteria). In field samples, a transcript-to-gene ratio of 1 to 2 was observed for *pmo*A in surface peat layers which attenuated rapidly with depth, indicating the highest methane consumption was associated with the 0-10 cm depth interval. Metagenomes and sequencing of cDNA *pmo*A amplicons from field samples confirmed the dominant active methanotrophs were *Methylocystis* and *Methylomonas*. Although Type II methanotrophs have long been shown to mediate methane consumption in peatlands, our results indicate members of the genera *Methylomonas* and *Methylovulum* (Type I) can significantly contribute to aerobic methane oxidation in these ecosystems.

43

44 **Introduction**

45 Methane is the third most important greenhouse gas and has 28 times the potential
46 of carbon dioxide to trap heat radiation on a molecular basis over a 100 year time scale
47 (1, 2, 3). Wetlands, such as peatlands, represent the largest natural source of methane to
48 the atmosphere (4). Aerobic methanotrophic bacteria live at the oxic-anoxic interface of
49 wetland soils and it has been shown that they consume as much as 90 % of the methane
50 produced belowground before it reaches the atmosphere, thus serving as a biofilter
51 regulating emissions (3, 5, 6, 7). The response of methane dynamics in wetlands to
52 global climate change is uncertain, and climate models would be improved through
53 quantification of the response of microbially-mediated mechanisms of methanotrophy to
54 temperature and moisture variation.

55 Aerobic methanotrophs are phylogenetically located in two phyla: the
56 Proteobacteria and Verrucomicrobia (8). The majority of characterized methane-
57 oxidizing organisms have been separated into Type I methanotrophs of the
58 Gammaproteobacteria and Type II methanotrophs of the Alphaproteobacteria (9, 10, 11).
59 The prevailing view has been that most methanotrophs only grow on methane or
60 methanol as a source of carbon and energy (4). However, more recently, a number of
61 Type II methanotrophs (*Methylocella*, *Methylocapsa*, and *Methylocystis*) have been
62 characterized as facultative methanotrophs capable of conserving energy for growth on
63 multi-carbon compounds such as acetate, pyruvate, succinate, malate, and ethanol (12).
64 Although members of the phylum Verrucomicrobia have been widely detected in
65 peatlands, none have been definitively linked to methanotrophy and thus more research is

66 needed to ascertain the role of Verrucomicrobia in the carbon cycle of peatlands (8, 13,
67 14, 15, 16).

68 Aerobic methane oxidation in Proteobacterial methanotrophs is catalyzed by the
69 enzyme methane monooxygenase (MMO), either particulate MMO (pMMO) or a soluble
70 MMO (sMMO). The genes *pmoA* (encoding the 27kDa subunit of pMMO) and *mmoX*
71 (encoding the alpha-subunit of the hydroxylase of sMMO) as well as 16S rRNA genes
72 have been used most often as molecular markers to characterize methanotrophs in
73 peatlands and other environments (6, 17, 18, 19, 20, 21, 22, 23). Previous studies point to
74 a co-dominance of Alpha- and Gammaproteobacteria, and in particular, the genera
75 *Methylocystis* and *Methylomonas*; however, the majority of past work in the field was
76 conducted at the DNA level, and less information is available on which microbial
77 populations are actively involved in methane oxidation *in situ*. Using a combination of
78 stable isotope probing (SIP) and a functional gene (*pmoA*) array, Chen *et al*. (24)
79 determined that *Methylocystis* populations predominated the active methanotrophs in a
80 range of peatlands in the UK. Phospholipid fatty acid stable isotope analysis (PLFA-SIP)
81 was also utilized in conjunction with mRNA analyses to probe the active methanotrophic
82 communities in peatlands in the UK, again finding a community dominated by
83 *Methylocystis* (6). Gupta *et al*. (7) also detected a predominance of Type II
84 methanotrophs (*Methylocystis*, *Methylosinus*, *Methylocapsa*, and *Methylocella*) in a
85 peatland in New York, USA, using SIP. As reviewed by Dedysh (25), a number of
86 acidophilic and acidotolerant Type II methanotrophs have been cultivated from peatlands.
87 The first acid-tolerant Type I methanotroph was only recently isolated and described by
88 Danilova *et al.* (26); however, cultivation of both types might suggest their involvement

in active methane oxidation.

The objective of this study was to identify the microorganisms actively involved in methane oxidation in climatically sensitive boreal peatlands using multiple, independent molecular approaches in the field and laboratory. Based on previous studies, it was hypothesized that the Alphaproteobacterial methanotrophs were most active in methane oxidation with only a minor contribution from the Gammaproteobacterial methanotrophs. This study was conducted at the Marcell Experimental Forest (MEF) in northern Minnesota, USA, where the U.S. Department of Energy (DOE) Oak Ridge National Laboratory and the USDA Forest Service are conducting a large-scale field climate manipulation known as Spruce and Peatland Response Under Climatic and Environmental Change (SPRUCE).

Materials and Methods

Site Description and Sample Collection

Peat samples were collected at the S1 Bog located in the Marcell Experimental Forest (MEF; N 47°30.476'; W 93°27.162') north of Grand Rapids, MN (27). This site has been described in detail in other publications (14, 15). The S1 bog is acidic with an average pH of 3.5 – 4.0 and is oxygen limited with oxygen levels decreasing to below detection (limit of approximately 20 ppb) within the top five centimeters of the bog (14).

For use in DNA-SIP incubations, a 10 x 10 x 10 cm block of peat, approximately 1 liter in volume, was sampled using a sterilized bread knife in hollows from the S1 bog, transect 3, in July 2012. The collected peat was homogenized by hand in a sterile bag and stored at 4°C until use in experiments. Samples for nucleic acid extraction were collected

in triplicate with a Russian peat corer as described by Lin *et al.* (14, 15). Each core was sub-sectioned into 10 cm intervals and immediately placed on dry ice. Samples were then stored at -80°C in a portable freezer until nucleic acid extractions were performed. DNA and RNA extractions were performed with MoBio PowerSoil DNA and Total RNA Extraction kits, respectively, according to the manufacturer's instructions.

Microcosm incubations with ^{13}C-labelled methane

Ten grams of homogenized peat from the 0 – 10 cm depth interval of S1 peat bog midway along the third transect (S1T3M) were added to 150 mL serum bottles in duplicate for each treatment. This site was chosen for consistency with other field samples obtained from the S1 peat bog. Bottles were sealed with blue-butyl rubber stoppers and crimped with aluminum crimp seals. Samples were stored in the dark at room temperature (appx. 24ºC). Treatments included those for which the headspace was amended with either 1% (vol/vol) 99.9% ^{12}C-CH_4 (Sigma) or 1% (vol/vol) 99.9% ^{13}C-CH_4 (Sigma).This concentration is higher than *in situ* levels of methane to obtain enough labeled DNA for subsequent analyses. Headspace concentrations were monitored with a gas chromatograph – flame ionization detector (GC-FID) equipped with a methanizer over two weeks of incubation. Analysis of headspace gas (150 µL) was performed on a Shimadzu GC-2014 with a Supelco custom packed column (Packing 80/100 Hayesep Q). The flow rate was 30 mL/min with the injector and detector at 100°C, the column at 40°C, and the methanizer at 380°C. Samples were taken on the day of preparation (day 0) and subsequently after 3, 8, 11, and 14 days. The samples were not replaced due to the relatively short incubation and minimal headspace sampling. In parallel, ^{12}C and ^{13}C-CH_4 samples were sacrificed at the initiation of the experiment (T0), after eight days (T1), and

after fourteen days (T2). A subsample of 5 grams was removed from each sample and frozen at -80°C until DNA was extracted for further analysis.

Wet to dry weight was determined by weighing out ~5 grams of peat from the incubation. Samples were then dried in a drying oven at 60°C until a stable mass was obtained (appx. 7 days).

Stable Isotope Probing – ultracentrifugation and gradient fractionation

DNA was extracted from frozen peat samples with the Mo Bio Powersoil DNA kit according to the manufacturer's protocol and stored at -20°C until further analysis. Stable isotope probing was conducted as described previously (28, 29). In brief, extracted DNA was added to a cesium chloride solution and centrifuged by ultracentrifugation at 177,000 x g. After 40 hours, samples were removed from the ultracentrifuge and fractionated by needle fractionation into twelve or thirteen fractions and the density of each fraction was determined with a digital refractometer (Reichart AR200). ^{13}C-enriched DNA was expected within the "heavy" fractions (five to eight). DNA was precipitated from all fractions with polyethylene glycol and glycogen as a carrier (28, 29). Precipitated DNA was stored at -20°C until further analysis.

Microbial community characterization in SIP incubations.

All fractionated DNA samples from 8 and 14 day time points were fingerprinted with Automated Ribosomal Intergenic Spacer Analysis (ARISA). First, ARISA PCR was run on each sample with the S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18 primers (30). PCR reactions were performed with an initial denaturation step at 95˚C for 5 minutes followed by 30 cycles of 30 seconds at 94˚C, 1 minute at 52˚C, and 1 minute at 72˚C followed by a 10 minute final elongation step at 72˚C. PCR products were run on a

158 1.5% w/v agarose gel with 1xTris/Borate/EDTA (TBE) buffer and successful reaction
159 products were cleaned with the Mo Bio PCR cleanup kit following the manufacturer's
160 instructions. ARISA PCR products were then separated and analyzed using an Agilent
161 model 2100 Bioanalyzer and unique bands in heavy fractions were noted from ^{13}C-CH_4
162 samples to determine the success of the SIP incubation.
163 Quantitative PCR (qPCR) was used to determine the abundance of *pmoA* genes in
164 different DNA fractions retrieved from the ^{13}C-CH_4 incubated soil samples (after 14 days
165 of incubation). To minimize effects of inhibitors, DNA from SIP fractions was diluted to
166 1/20 of original concentrations. All fractions were analyzed with A189f/Mb661r PCR
167 primers as described by Kolb *et al.* (31) to target the abundance of *pmo*A genes in 20µL
168 reactions with 2 µL of template DNA (2.3 – 8.8 ng/µL) added to a master mix of 10 µL of
169 Sybr green to a final concentration of 1x, 1.6 µL each of forward and reverse primers to a
170 final concentration of 0.8 µM, and 4.8 µL of PCR grade water. Samples were run against
171 a standard curve in a StepOnePlus instrument with 96 wells with the following
172 parameters: an initial denaturation step of 5 minutes at 95°C and 40 cycles of
173 denaturation at 95°C for 15s, annealing at 64°C for 45s, extension at 72°C for 45s, and
174 data acquisition at 86.5°C for 16s. Quantity of *pmo*A genes was normalized to the
175 abundance of 16S rRNA genes, which were analyzed as described in Lin *et al.* (15) with
176 1/20 dilution of DNA.
177 DNA fractions from one ^{13}C-CH_4 sample from T1 (8 day incubation) and one ^{13}C-
178 CH_4 sample from T2 (14 day incubation) were sequenced on an Illumina MiSeq platform
179 at the Michigan State Sequencing Facility with the 515F/806R primer set (32). Sequences
180 were analyzed in QIIME 1.8 (33) as follows: overlapping reads were merged with fastq-

join (34) and quality filtering was performed with USEARCH 7.0, rejecting reads with an expected error greater than 0.5 (35). Read length was limited to approximately 250 base pairs after primer removal with the inclusion of only completely assembled reads. Subsequently, initial OTUs from the 16S rRNA gene sequence reads were picked *de novo* based on 97% similarity and representative sequences were picked from each OTU. Representative sequences were filtered by comparison to the Greengenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) at 60% similarity. An OTU table was compiled and filtered by removing phylotypes comprising less than 0.05% of the library. Taxonomy was assigned to the parsed OTU table with the Greengenes database. 16S rRNA gene sequences assigned to the phylum Proteobacteria were screened for methanotrophs with a maximum-likelihood phylogenetic tree. Sequences were aligned and identity determined at 95% similarity to nearest neighboring sequence in SILVA (36). The methanotrophs represented in DNA-SIP samples were identified with a nucleic acid maximum-likelihood tree with bootstrap analysis (1,000 replications). Analysis of variance and regression analysis of the shift in methanotroph community composition in heavy and light fractions was conducted in R to test for significant differences in methanotroph populations (37).

Metagenomic analysis of field samples

Libraries for metagenomic sequencing were generated from field DNA extracts using the Nextera DNA sample preparation kit (Illumina, Inc. San Diego, CA) as in Lin et al. (15). Libraries were size-selected using E-Gels (Life Technologies, Inc.) for an insert size range of 400-800 bp. Libraries were then quantified and quality checked using the Invitrogen Qubit and Agilent Bioanalyzer. FASTQ files from the metagenomic

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sequencing were loaded into the MG-RAST server for quality filtering and downstream analysis (38). The paired-end reads from each library were joined and then filtered with the default parameters. Protein and pathway search was performed with SEED annotations (e-value of 10^{-5}) in MG-RAST. The amino acid sequences derived from the gene calling were downloaded for searching the *pmo*A gene. A Hidden Markov Model (HMM) for the *pmo*A gene was created by using HMMER v3.0 tools (http://hmmer.janelia.org/), based on HMM training sequences downloaded from the functional gene pipeline and repository (http://fungene.cme.msu.edu/). All HMM search hits with e–values below a threshold of 10^{-5} were counted and retrieved. For the taxonomic assignment of gene sequences, the corresponding BLASTP search outputs were uploaded for analysis through the software Metagenome Analyzer (MEGAN) (39).

Quantification of pmoA genes and transcripts in field samples

Quantification of *pmo*A genes and transcripts was conducted with DNA and cDNA extracted from field samples, respectively, according to methods described above for analysis of SIP fractions. cDNA standards and cDNA of environmental RNA samples were synthesized using GoScript Reverse Transcription system following the manufacturer's protocol (Promega). The *pmo*A gene fragment used for constructing plasmid standards of qPCR was amplified from genomic DNA of *Methylococcus capsulatus Bath.* The plasmid standard was prepared according to Lin *et al*. (40). To prepare cDNA standards, plasmid DNA with a positive *pmo*A insert was linearized with *NcoI* restriction enzyme following the manufacturer's protocol (Promega), and purified by MinElute PCR purification kit (Qiagen Inc., CA). RNA was synthesized from the linearized plasmid DNA using the Riboprobe *in vitro* transcription system according to

the manufacturer's protocol (Promega).

Power SYBR® Green PCR Master Mix was used for all qPCR assays. Plasmid DNA or cDNA standards with inserts of specific gene fragments were used to establish standard curves that were included in each run. The standard contains different quantities of cloned gene fragments, spanning 7 orders of magnitude from 10^1 to 10^7 gene copies per PCR well. To minimize the effects of inhibitors in assays, peat DNA was diluted to 1/40 of original concentrations, and duplicate 20ul reactions each containing 2 µl of diluted DNA were run for each sample. The *pmo*A amplicons from the synthesized environmental cDNA were sent to the University of Illinois at Chicago (UIC) for DNA sequencing using a 454 platform.

Raw pmoA sequences were demultiplexed, trimmed, and quality filtered in CLCbio.

Sequence Accession Numbers

Gene sequences from the analysis of SIP fractions have been submitted to the GenBank database under accession number PRJNA286313. Metagenomes have been submitted to MG-RAST under identification numbers 4538779.3, 4538778.3, and 4538997.3. Amplicon sequences for the *pmo*A gene are in the process of submission and are available upon request.

Results

Abundance, activity and community composition of methanotrophs in the field.

The abundance of *pmo*A genes and transcripts decreased rapidly with depth in the peat column, decreasing by two orders of magnitude from 0 to 100 cm depth (Figure 1).

The transcript-to-gene ratio, a proxy for *pmo*A expression or the activity of methanotrophs, decreased to background levels below 40 cm depth.

Multiple lines of evidence allowed us to determine the dominant methanotrophs in the S1 bog soils (Figure 2). In all of the soils sampled, sequences affiliated with the genus *Methylocystis* comprised over 75 % of the *pmoA* sequences retrieved from metagenomes and sequenced amplicons derived from cDNA (Figure 2). Overall, the remaining *pmo*A sequences were mainly affiliated with the genus *Methylomonas*. At mid-depth (approximately 30 cm), *Methylosinus*-like sequences showed a higher relative abundance compared to *Methylomonas*-like sequences in the metagenomes, while *Methylomonas*-like sequences were second in relative abundance to *Methylocystis* in cDNA amplicons. However, this could be due to the slightly different depths sampled or temporal variability, since the metagenomes and cDNA amplicons were sampled in successive years. However, microbial community composition was shown to be temporally stable at the DNA level in extensive field studies of the S1 bog (14, 15).

Stable isotope probing incubations.

Within microcosm incubations, the most rapid methane oxidation rates were observed within the first three days of incubation at room temperature (approximately 24°C). Rates ranged from 13.8 to 17.3 µmol CH_4 g dwt $^{-1}$ d^{-1}. Samples amended with ^{13}C-CH_4 and ^{12}C-CH_4 demonstrated potential consumption rates of 15.1 ± 2.3 µmol CH_4 g dwt^{-1} d^{-1} and 15.9 ± 1.6 µmol CH_4 g dwt^{-1} d^{-1}, respectively. Rates of methane consumption were calculated in Excel utilizing the linest function from three point linear regions in methane depletion with time. After two weeks, nearly all of the methane in the headspace

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had been consumed (Figure 3). Samples were sacrificed for DNA-SIP analysis after eight days for time point one, after peak methane consumption rates were observed, and after fourteen days for time point two, when nearly all of the methane had been consumed.

Fingerprinting of the fractionated DNA was conducted with automated ribosomal intergenic spacer analysis (ARISA) which showed a clear shift in microbial communities, indicating incorporation of ^{13}C into DNA within "heavy" fractions 7 and 8 (Supplementary Figures 1 & 2). Potential enrichment of active methanotrophs within these fractions was supported by a relative enrichment in *pmo*A gene abundance as determined by qPCR (Supplementary Figure 3). Within ^{13}C-enriched fractions from DNA-SIP incubations, an enrichment of Proteobacteria was observed relative to the ^{12}C-enriched fractions. The relative abundance of methanotrophs in the overall community increased from approximately 4 % in the field samples (data not shown) to 36 % in the ^{13}C-enriched fractions after 8 days of incubation (Figure 4). The shift in the abundance of the methanotrophic community between ^{13}C-enriched fractions and ^{12}C-fractions was shown to be significant with ANOVA analysis (F-value = 7.144, df = 3, p = 0.0439). Phylogenetic analysis of 16S rRNA gene sequences showed a co-dominance of Alphaproteobacterial and Gammaproteobacterial methanotrophs, and phylotypes were most closely related to the rRNA genes of the genera *Methylocystis*, *Methylomonas*, and *Methylovulum* (Figure 4, 5). None of the phylotypes were closely related to cultivated members of each genus. Environmental sequences obtained in other peat bogs were similar to the sequences enriched in ^{13}C; however, phylotypes most closely related to *Methylomonas* and *Methylovulum* in particular remained phylogenetically distinct while phylotypes related to *Methylocystis* were phylogenetically similar to sequences obtained

in other acidic forest and peat soils (Figure 5). Methanotrophic genera *Methylocella* and *Methyloferula*, possessing only the soluble methane monooxygenase, were not detected.

Discussion

The community composition and activity of methanotrophic bacteria was interrogated in field samples from the S1 bog at Marcell Experimental Forest in Minnesota using several cultivation-independent approaches. Expression of a key gene in the methane oxidation pathway (*pmoA*, encoding a subunit of particulate methane monooxygenase) was used as a proxy for the activity of methanotrophs in the bog. Although *pmo*A genes could be detected throughout the peat column, indicating the presence of methanotrophs, transcript abundance decreased with depth and no *pmoA* transcripts were detected below the 30-40 cm depth interval. Biogeochemical characteristics of the S1 peat bog have been described in detail by Tfaily *et al.* (27) including distinct layers within the peat column encompassing the acrotelm (0-30 cm), mesotelm (30-75 cm), and catotelm (75 cm and deeper). Oxygen diffusion is limited within the bog due to the height of the water table but may extend lower in the acrotelm due to zones of aeration within the rhizosphere of plant roots (3, 14). Thus, in parallel with the availability of oxygen, methanotroph activity was highest at the surface and limited to the acrotelm and mesotelm. Although few studies have examined *pmo*A expression in wetlands, Freitag *et al*. (41) observed that transcript-to-gene ratios reflected methane dynamics in a UK peatland. Transcript-to-gene ratios of this study were in agreement with those determined by Freitag *et al*. (41). It should be noted that this study does not address microbial groups that mediate anaerobic methane oxidation (AOM),

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during which methanotrophy is coupled to utilization of alternate electron acceptors such as sulfate, nitrate, or nitrite (42, 43, 44). Given the scarcity of these alternate electron acceptors in the S1 bog (14), anaerobic methane oxidation would not be favored. However, further studies of AOM are warranted in this ecosystem.

Independent lines of evidence revealed the identity of active methanotrophs in the surface (0 to 10 cm depth), where the highest methane oxidation activity was detected. Results from metagenomes and next generation sequencing of *pmo*A cDNA amplicons from field samples indicated a predominance of the Type II methanotroph, *Methylocystis*, at the surface. Since the sequencing of cDNA amplicons was conducted on the same samples as those used for qPCR, the community composition should be directly comparable to gene expression determinations. *Methylocystis* comprised over 75 % of the metabolically active methanotrophs, with the Type I methanotroph *Methylomonas* making up the remainder of the active community. Thus, these groups appear to be the most abundant and the most active in mediating aerobic methanotrophy in the S1 bog. Previous metagenomic analysis of field samples from the S1 peat bog suggested the potential involvement of *Methylocystis* and, to a lesser extent, *Methylomonas* in methane oxidation processes (14, 15). The data presented in this study suggest not only active involvement of both of these genera in methane oxidation but also the involvement of a second Gammaproteobacterial methanotroph, *Methylovulum*.

Field results were confirmed in the laboratory using a combination of stable isotope probing and next generation sequencing of 16S rRNA genes in a series of microcosm incubations. The active methanotrophic community was composed of a combination of *Methylocystis, Methylomonas,* and *Methylovulum*, which were observed

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to shift with time (Figure 4). As indicated previously, the presence of *Methylocystis* was not surprising given the well-documented presence, activity, and cultivated isolates from this methanotroph in acidic peatland ecosystems (6, 17, 21, 24). The presence and abundance of *Methylomonas* and *Methylovulum* were more surprising. While *Methylomonas* has been detected in amplicon sequences and cultured from peatlands, this genus has not been definitively linked to active methane oxidation in acidic boreal peatlands (23, 26, 45, 46). Through the use of SIP, *Methylomonas* has been shown to be active in methane oxidation in other environments such as a cave system, a soda lake, and landfill cover soil (47, 48, 49) that are more neutral to alkaline in pH. Studies on methanotrophs in peatlands have utilized a variety of methods including both cultivation-dependent and cultivation-independent such as diagnostic microarrays, PLFA-SIP, clone libraries, and DNA-SIP; however, this study utilized DNA-SIP experiments where the ^{13}C-enriched DNA obtained was directly sequenced in combination with metagenomic and cDNA analysis of field samples. Within the top 10 cm of the S1 bog, potential rates of methanogenesis only reach 0.025 μmol CH_4 g dwt^{-1} d^{-1} (27). If these potential rates are representative of *in situ* rates of methanogenesis, the methane concentrations in the headspace of SIP incubations after 14 days were more representative of the natural environment, lending greater significance to the observed shifts in populations of methanotrophs present (Figure 3, 4). The combination of amplicon sequencing of SIP enrichment samples and metagenomic sequence analysis of field samples, coupled with analysis of multiple time points enabled analysis of the Gammaproteobacterial (Type I) methanotroph community which can now be considered to be key active methane oxidizers in an acidic peatland ecosystem.

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Perhaps most remarkable is the presence of *Methylovulum* in the active methanotrophic community. The first isolate of this genus, *Methylovulum miyakonense,* was obtained in 2011 and to date no new species within this genus have been characterized (50). Although originally isolated from forest soil, *M. miyakonense* was also isolated from peatland soil (51), suggesting *Methylovulum* is present in other peatlands. The cultivation of a *Methylovulum*-like methanotroph from another acidic peat bog by Kip *et al.* (45) further supports this possibility. However, the strains did not appear to grow under acidic conditions, begging the question of the extent of the role *Methylovulum* might be playing in acidic peatland soil (50, 51). To our knowledge, these are the first data directly linking *Methylovulum* to active methane cycling in peatlands. Although the relative abundance of *Methylovulum* was low in the SIP incubations, there was a distinct enrichment in the ^{13}C-enriched samples compared to the ^{12}C-enriched samples suggesting active methane consumption (Supplementary Figure 4). While microcosm experiments may induce enrichment of organisms normally low in abundance *in situ*, other methanotrophic organisms detected at low abundance in the metagenomes, such as *Methylosinus*, were not enriched with ^{13}C over the course of the incubation, suggesting *Methylovulum* actively participates in methane consumption, if at low abundance.

Several possibilities may explain a seemingly neutrophilic methanotroph participating actively in methane oxidation in an acidic soil environment. One previous suggestion is the existence of neutral microenvironments, such as the plant endosphere, within the bog system, providing a specific ecological niche for *Methylovulum* (51). The *Methylovulum* 16S rRNA gene sequences detected in our experiments were not closely related to *M. miyakonense*, suggesting the existence of as yet uncultivated members of this genus that may be acido-tolerant or acidophilic. *Methylovulum* from other environments has also been identified as potentially psychrotolerant and capable of oxidizing methane at low concentrations, suggesting adaptability of this organism to the changing environmental

conditions at the surface of boreal peatlands (52). This would not be the first example of an organism seemingly suited to one particular environment playing a role in a wholly different environmental system. Rahman *et al.* (53) showed in 2011 that *Methylocella*, a facultative methanotroph isolated from acidic soil, resides in many diverse environments encompassing a pH range of 4.3 to 10.0. Based on this example, it is not necessarily surprising to find a methanotroph commonly found in more neutral environments actively participating in methane oxidation in the acidic peat soil. Rather this would encourage further probing of the active microbial community, potentially with a transcriptomic approach, to more fully assess which microorganisms are present and active in each environmental system.

An important step in analyzing the potential impacts of changing climate on the methane cycle in peatlands is to first identify the microorganisms actively involved in methane cycling. These data take a step toward that goal by identifying the active methane oxidizing bacteria at the S1 bog in the Marcell Experimental Forest. Active methane oxidizers include representatives from both Alphaproteobacteria and Gammaproteobacteria, and we show for the first time that *Methylovulum* and *Methylomonas* are directly involved in methane oxidation at the surface of the peat bog. Using these data, the key bacteria involved in methane oxidation can be targeted for cultivation for future studies to examine the physiology of these organisms and subsequently the potential effects of climate change on this methane oxidizing community in boreal peat bogs.

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599 **Figure Legends**
600 **Figure 1:** *pmo*A gene and transcript abundance in copies*g dwt^{-1} at depth from the S1
601 peatland. Samples tested were from duplicate soil cores collected in July 2013. Error bars

represent standard deviation.

Figure 2: Methanotroph community composition at depth in the Spruce S1 peat bog based on metagenomic and cDNA analysis of *pmo*A. Methanotrophs detected included *Methylocystis* (dark grey), *Methylomonas* (light grey), and *Methylosinus* (white). Samples for metagenomic analysis were collected at Spruce in July 2012 (SP0712) and samples for cDNA analysis were collected at Spruce in July 2013 (SP0713).

Figure 3: The consumption of methane with time in the stable isotope probing incubations. Circles represent ^{12}C-CH_4 treatments whereas triangles represent ^{13}C-CH_4 amended treatments. The observed methane consumption rates ranged between 13.85 and 17.26 μmol CH_4 g dwt^{-1} d^{-1} (calculated based on three-point linear region within each sample distribution). Peat utilized was from the 0 – 10 cm depth interval in hollows from the S1 bog, collected in July 2012.

Figure 4: The relative abundance of Alphaproteobacterial (Type II) and Gammaproteobacterial (Type I) methanotrophs based on 16S rRNA genes in ^{13}C-enriched fractions (H) compared to light fractions (L) after 8 days (T1) and 14 days (T2) of incubation. The difference between methanotrophic communities in heavy and light fractions was significant based on ANOVA analysis (F-value = 7.144, df =3, p-value = 0.0439).

Figure 5: Phylogeny of methanotrophs within SIP fractions from 8 and 14 day incubations (diamonds) showing organisms within the Alphaproteobacteria, *Methylocystis sp.*, and the Gammaproteobacteria, *Methylomonas* and *Methylovulum sp.* based on 16S rRNA gene analysis. This phylogenetic tree was prepared with the maximum-likelihood method with bootstrap analysis of nucleic acid sequences (1000

625 replications).

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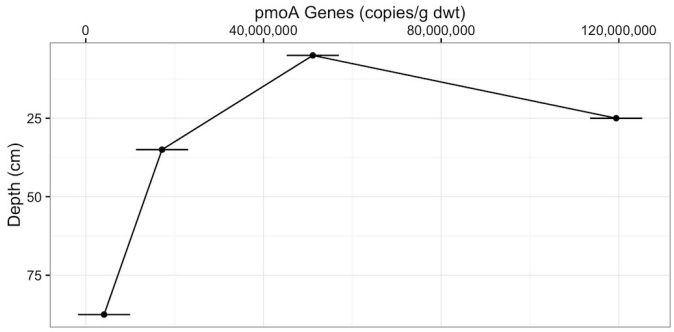

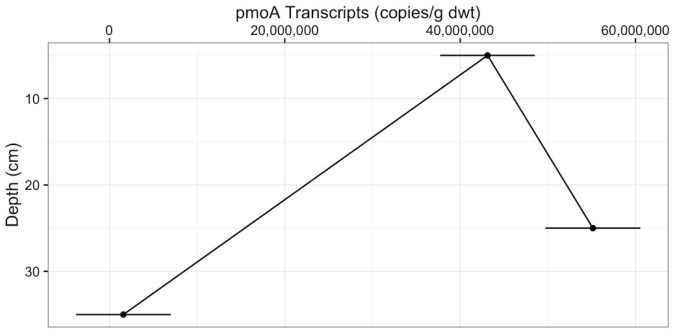

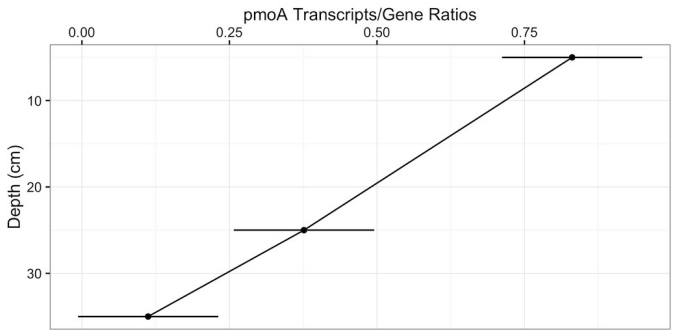

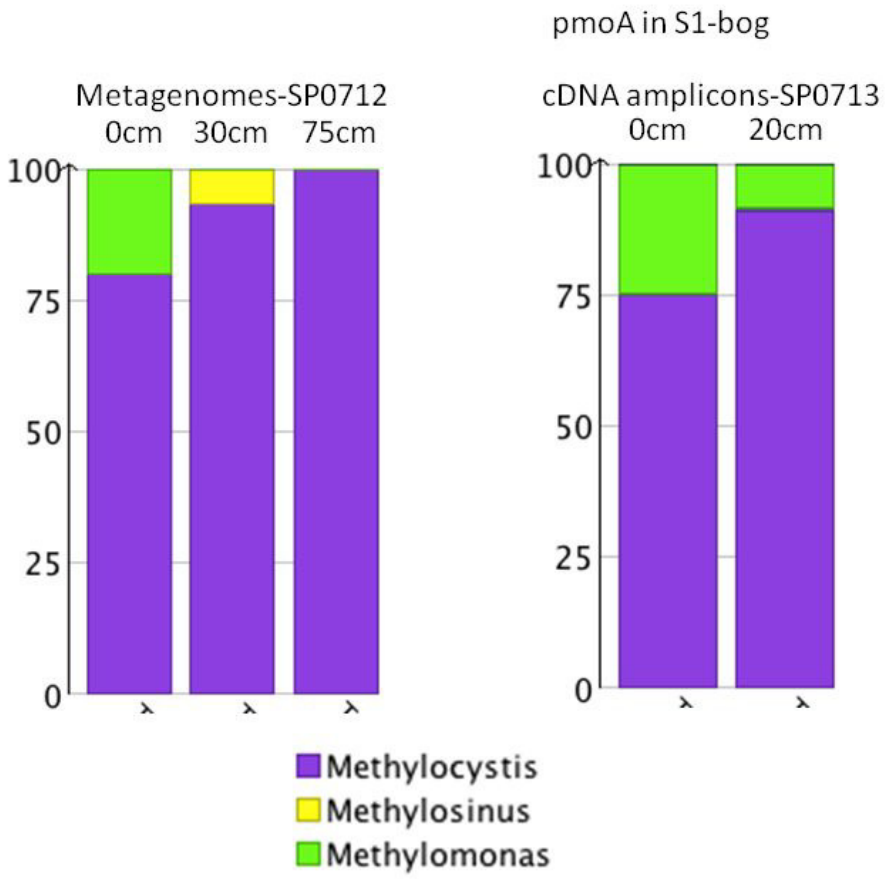

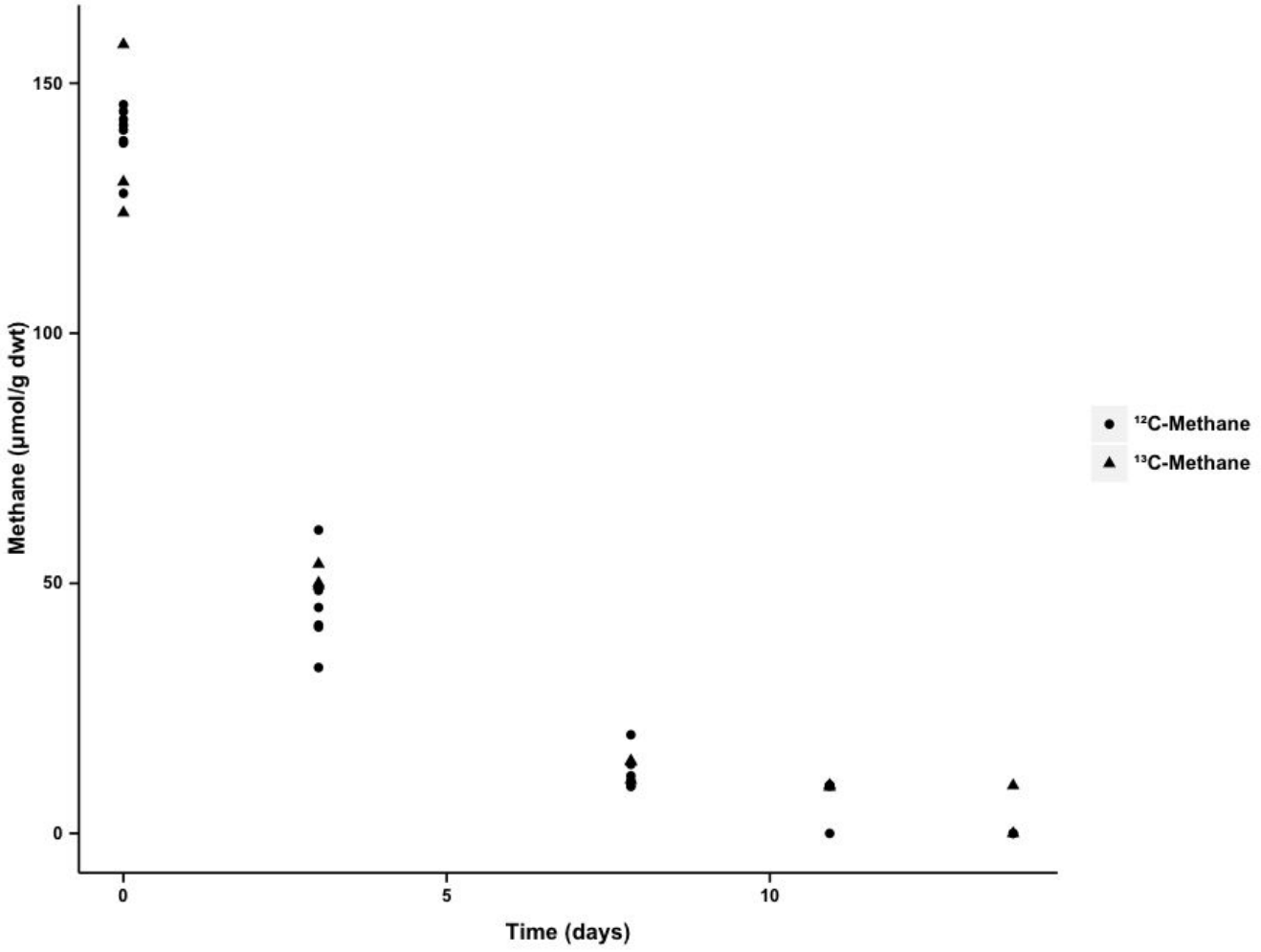

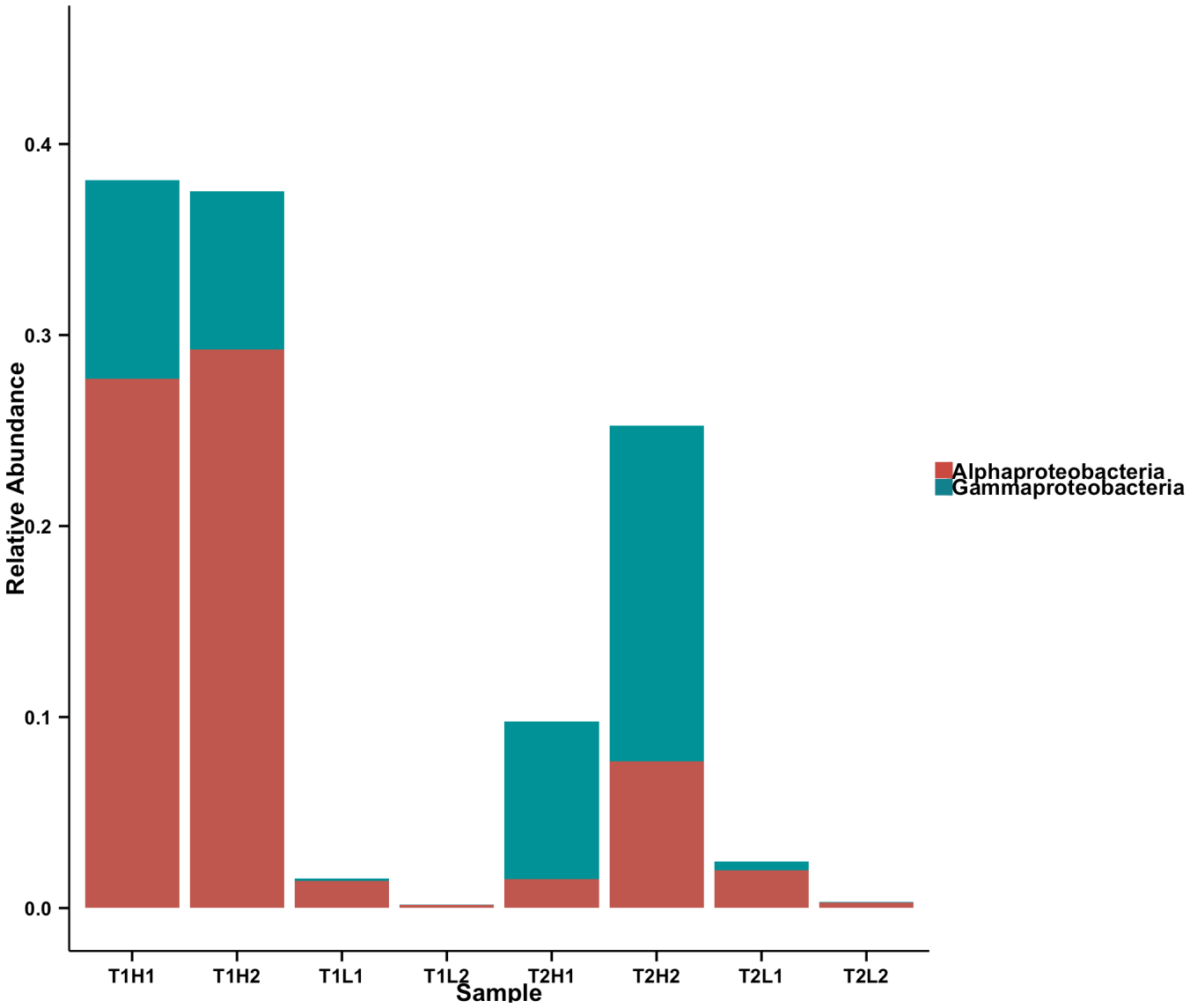

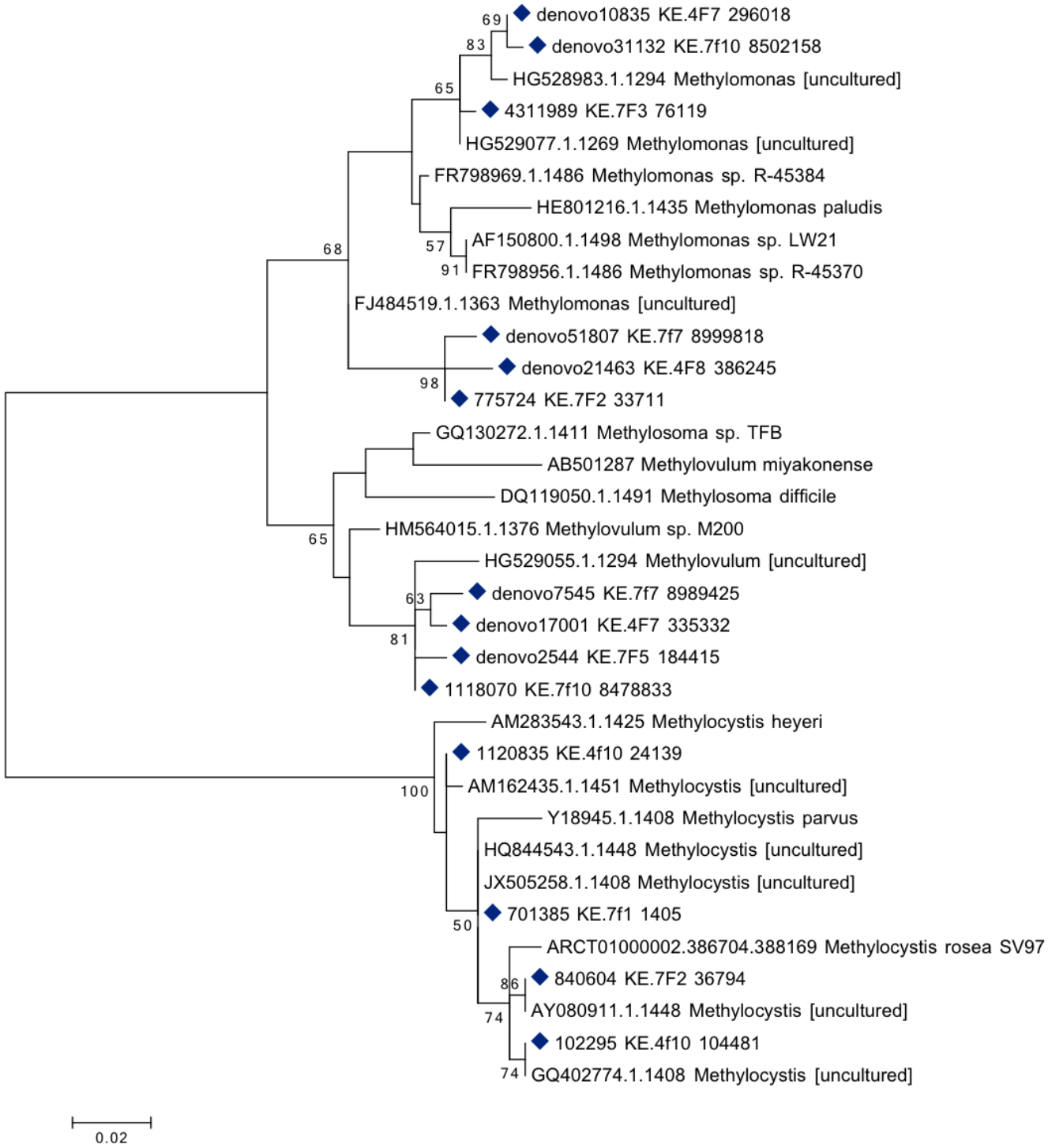

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