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Viewpoints

New insight to the role of microbes in the methane exchange in trees: evidence from metagenomic sequencing

Summary

Methane (CH_4) exchange in tree stems and canopies and the processes involved are among the least understood components of the global CH_4 cycle. Recent studies have focused on quantifying tree stems as sources of CH_4 and understanding abiotic CH_4 emissions in plant canopies, with the role of microbial *in situ* CH_4 formation receiving less attention. Moreover, despite initial reports revealing CH_4 consumption, studies have not adequately evaluated the potential of microbial CH_4 oxidation within trees. In this paper, we discuss the current level of understanding on these processes. Further, we demonstrate the potential of novel metagenomic tools in revealing the involvement of microbes in the CH_4 exchange of plants, and particularly in boreal trees. We detected CH_4-producing methanogens and novel monooxygenases, potentially involved in CH_4 consumption, in coniferous plants. In addition, our field flux measurements from Norway spruce (*Picea abies*) canopies demonstrate both net CH_4 emissions and uptake, giving further evidence that both production and consumption are relevant to the net CH_4 exchange. Our findings, together with the emerging diversity of novel CH_4-producing microbial groups, strongly suggest microbial analyses should be integrated in the studies aiming to reveal the processes and drivers behind plant CH_4 exchange.

Introduction

The first evidence on aerobic methane (CH_4) emissions by terrestrial vegetation was provided by Keppler *et al.* (2006), estimating that plants – including woody and grass species – are a large source of CH_4. Since then, numerous studies (e.g. Keppler *et al.*, 2008; Wang *et al.*, 2008; Brüggemann *et al.*, 2009; Bruhn *et al.*, 2009, 2014; Martel & Qaderi, 2017, 2019) have confirmed aerobic CH_4 emissions from terrestrial plants. During the past decade, tree stems from tropical to boreal forests and trees growing under varying hydrological conditions have been found to emit CH_4 through multiple mechanisms behind the emissions (Carmichael *et al.*, 2014; Barba *et al.*, 2019). Although the CH_4 emissions from tree stems and from aerobic production in plant canopies are widely recognized, neither of these sources are yet included in the global CH_4 budget (Saunois *et al.*, 2020).

Overall, discussion on aerobic plant CH_4 production has mainly concentrated on plant physiology, which was recently reviewed by L. Li *et al.* (2020), whereas a more general view of the current understanding of tree-derived CH_4 fluxes, magnitudes, processes, and drivers has been presented by Carmichael *et al.* (2014), Covey & Megonigal (2019) and Barba *et al.* (2019). Potential microbial CH_4 production within the aboveground tree habitat remains less studied in comparison with other mechanisms. So far, the presence of the most-well known CH_4 producers – the methanogenic archaea – has been reported only from broadleaf tree stems: first, based on basic cultivation methods (Zeikus & Ward, 1974; Zeikus & Henning, 1975), and recently based on molecular biology (Yip *et al.*, 2019; H-L. Li *et al.*, 2020). Tree-canopy-derived CH_4 emissions are considered to be formed mostly by abiotic/plant physiological processes (Bruhn *et al.*, 2014; Lenhart *et al.*, 2015a), whereas the potential role of microbial CH_4 production has been overlooked – at least partly due to the assumption that the anaerobic methanogens would not thrive within the oxygen-producing canopy habitat. Recently discovered, aerobic CH_4-producing microbial groups – such as fungi (Lenhart *et al.*, 2012) and cyanobacteria (Bižić *et al.*, 2020) – have not yet been thoroughly considered as sources of CH_4 in living tree stems or canopies.

Atmospheric hydroxyl (OH) radicals are recognized as the main sink for atmospheric CH_4, whereas the largest biological sink is microbiological CH_4 oxidation that occurs mostly in soils (Kirschke *et al.*, 2013). CH_4 consumption in plants has been observed both in the field and in laboratory studies (Kirschbaum & Walcroft, 2008; Sundqvist *et al.*, 2012; Zhang *et al.*, 2014; Halmeenmäki *et al.*, 2017; Stępniewska *et al.*, 2018). Research has mainly concentrated on CH_4-rich environments, such as peatlands, where the importance of *Sphagnum* moss-associated methanotrophic bacteria is well recognized (e.g. Larmola *et al.*, 2010). Although boreal tree shoots have been shown capable of *in situ* CH_4 consumption (Sundqvist *et al.*, 2012), not much research has been done to reveal the mechanisms behind this process. Identification of within-tree methanotrophs (Doronina *et al.*, 2004; Van Aken *et al.*, 2004; Iguchi *et al.*, 2012) points to microbial CH_4 oxidation, but a possibility for a nonmicrobial sink cannot be ruled out either.

In this viewpoint, we discuss the magnitude and current process-level understanding of CH_4 exchange of trees, with an emphasis on the role of microbes in the so far considered *nonmicrobial* CH_4 production in plant tissues. We propose that the lack of microbial observations is not caused by the absence of these populations, but at least partly due to undeveloped methods with poor detection limits. To demonstrate the potential of novel molecular biology tools, we provide two types of metagenomics data from coniferous tree tissues: (1) functional genes detected through an extensive

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screening of public metagenome entries (National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA)) published so far; and (2) functional gene detection through a novel probe-targeted metagenome sequencing method. In addition, we present CH_4 flux data from Norway spruce (*Picea abies*) shoots indicating the occurrence of both CH_4 production and consumption in the tree canopy.

Through our findings from the tree canopies, we highlight the need for extending the discussions of aerobic CH_4 production from abiotic and plant physiological processes to plant–microbe interactions. Furthermore, we discuss how modern tools could advance our knowledge on the CH_4 cycling microbes from *detection of presence* to the understanding of *active processes*, and to the characterization of novel microbial groups involved in the tree CH_4 exchange.

Current understanding of the methane production in trees

Trees as sources of methane

Tree stem flux measurements indicate that trees across vegetation zones and growing habitats are mostly sources of CH_4 (Carmichael *et al.*, 2014; Barba *et al.*, 2019). The stem CH_4 emissions are proposed to form either through internal CH_4 formation within tree tissues (e.g. Wang *et al.*, 2016) or transport of soil-derived, microbially formed CH_4 emitted through tree stems (e.g. Rusch & Rennenberg, 1998), or a combination of these two as reviewed recently by Barba *et al.* (2019). The number of field-scale studies on canopy CH_4 exchange remain low despite the numerous laboratory studies reporting aerobic, presumably nonmicrobial CH_4 production in plant leaves first presented by Keppler *et al.* (2006). Existing field evidence on canopy CH_4 exchange indicates both emissions and uptake of CH_4 and calls for further studies (Sundqvist *et al.*, 2012; Machacova *et al.*, 2016; Pangala *et al.*, 2017; this study).

Aerobic methane production through nonmicrobial mechanisms

Increasing evidence suggests that aerobic CH_4 formation in plant leaves may be an integral part of cellular responses to changing redox conditions in all eukaryotes, and that this common biochemical CH_4 source may exist in all eukaryotes – plants, animals, fungi, and algae (Keppler *et al.*, 2009; Liu *et al.*, 2015). Several studies also suggest that aerobic nonmicrobial CH_4 formation occurs through reactive oxygen species (ROS) generation and a subsequent release of CH_4 from precursor compounds, such as pectic methyl groups, methionine, or other substrates (e.g. Keppler *et al.*, 2008; McLeod *et al.*, 2008; Vigano *et al.*, 2008; Bruhn *et al.*, 2009; Lenhart *et al.*, 2015a).

Although plant ROS are produced during aerobic respiration and photosynthesis as a normal by-product of aerobic plant metabolism, ROS production can be induced by different environmental stressors (Huang *et al.*, 2019). Similarly, environmental stressors like ultraviolet (UV) radiation and elevated temperature (e.g. McLeod *et al.*, 2008; Vigano *et al.*, 2008; Bruhn *et al.*, 2009, 2014; Qaderi & Reid, 2009), physical injury of the plant – for example, leaf damage caused by cutting and hypoxia (Wang *et al.*, 2009) – water stress (Qaderi & Reid, 2009), and low light levels (Martel & Qaderi, 2017) have been observed to stimulate aerobic nonmicrobial CH_4 production in plants. Still, the biochemical pathways behind stress-induced CH_4 formation and its potential occurrence in natural conditions remain unknown.

Microbes as potential methane producers in trees

Different parts of the trees (leaves, stem, bark, roots) serve as unique habitats for a variety of microbial communities that can live either as *epiphytes* on the plant surface, or as *endophytes* inside the plant tissues – used here to include also pathogens, as rationalized by Griffin & Carson (2018). Together, different microbes, including bacteria, archaea, and fungi, form the tree *microbiome* (Terhonen *et al.*, 2019). Studies on tree microbiomes have largely concentrated on fungi and less on bacteria or archaea (Griffin & Carson, 2018; Harrison & Griffin, 2020) and tree-stem-associated prokaryotes especially are still poorly characterized (Baldrian, 2017). Geographically, endophyte studies have focused on the tropical and temperate regions, leaving boreal and alpine ecosystems poorly examined (Harrison & Griffin, 2020).

Many of the tree-associated microbes are important to the host plants via promoting plant growth and increasing resistance to stress and pathogens, whereas some of them can negatively affect plant growth (Frank, 2018; Terhonen *et al.*, 2019; Chaudhry *et al.*, 2020). Owing to varying conditions caused by both host metabolic processes and abiotic stress factors, such as drought and UV radiation, tree foliage microbiomes especially are highly dynamic systems (Chaudhry *et al.*, 2020). In addition, colonization patterns affect the microbiome composition: whereas some endophytes can stay with their host the whole plant life cycle (vertical transmission), most are estimated to originate from the environment through horizontal transmission either from the soil or through the air (Frank *et al.*, 2017).

On first thought, trees and other plant tissues seem to be mostly aerobic environments and, as such, unsuited habitats for anaerobic organisms, like methanogenic archaea (Kirschke *et al.*, 2013). As recently reviewed by Covey & Megonical (2019), however, anoxia can prevail inside both healthy and infected tree stems, leading to CH_4 production (Fig. 1b). In both situations, anoxia could be created through oxygen-consuming metabolic processes of the tree-associated endophytic microbes, such as fungi, and also through active stem and root respiration (Teskey *et al.*, 2008), helping to maintain favorable conditions for methanogenesis. In particular, fungal-mediated decay of heartwood (i.e. heart rot disease) has been suggested as an important driver of CH_4 emissions from living trees (Covey *et al.*, 2012). Moreover, anaerobic methanogens have also been detected in the roots of Norway spruce, Scots pine (*Pinus sylvestris*), silver birch (*Betula pendula*), and black alder (*Alnus glutinosa*) (Bomberg & Timonen, 2009; Bomberg *et al.*, 2011; Fig. 1c). As in the stems, root-associated archaea are predicted to benefit from the O_2 consumption of other microbes, and also

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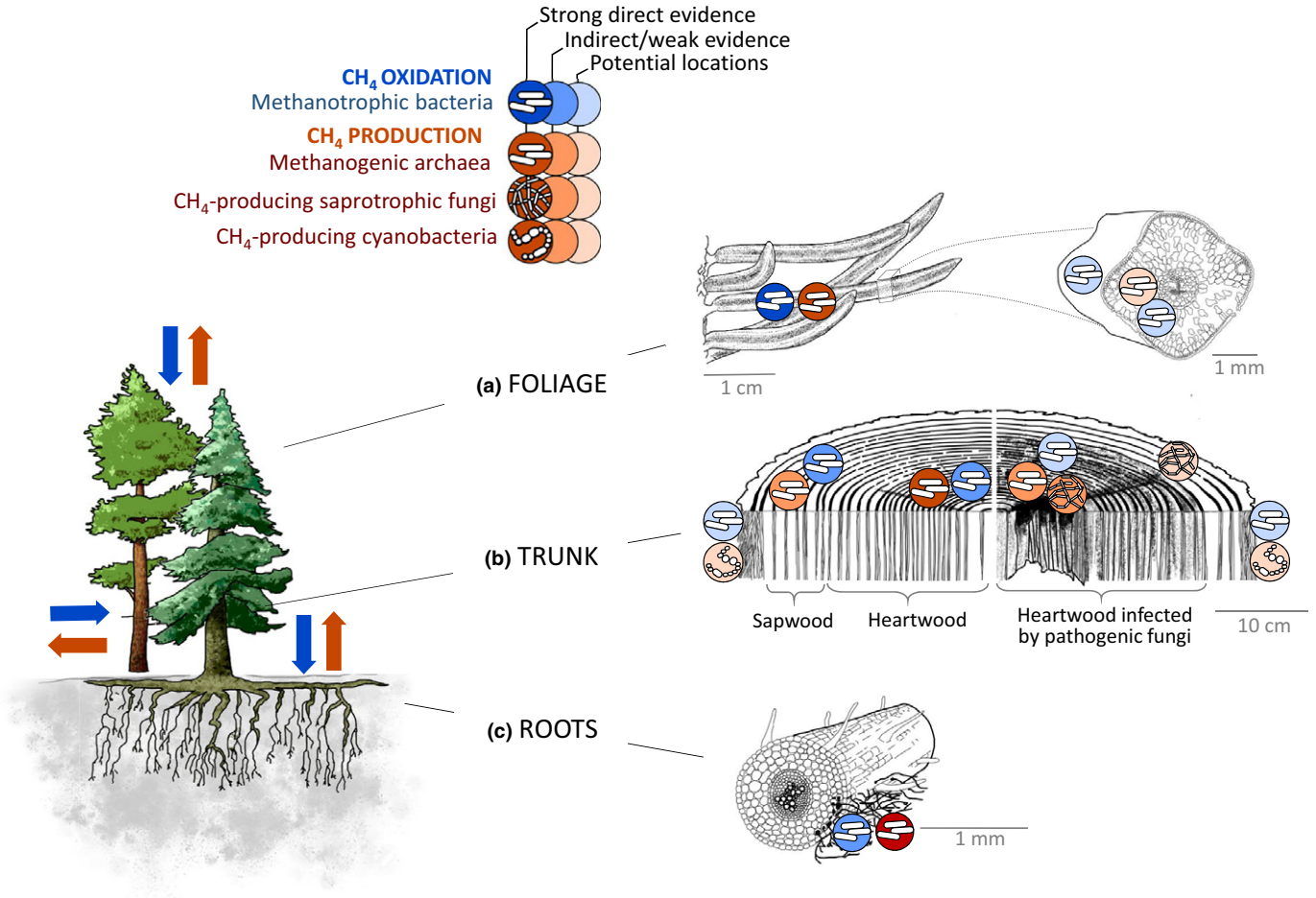

Fig. 1 Locations of different methane (CH_4)-producing and the so far known CH_4-consuming microbes, the methanotrophic bacteria, present within different tree compartments: (a) foliage, (b) trunk, (c) roots, as determined based on the references and new results presented in this viewpoint. The locations are marked with dots colored based on the level of scientific evidence: strong evidence indicates more than one study and/or detected in several tree species; indirect/weak evidence indicates only one study or one tree species, or detected activity (e.g. flux measurements); potential locations indicates potentially suitable conditions for the given microbial group. Nitrogenase-related CH_4 production was not included in the figure owing to the still low understanding of its potential occurrence in the tree habitat. Red arrows, CH_4 production; blue arrows, CH_4 consumption.

directly from the carbon (C) compounds exuded from the roots (Bomberg *et al.*, 2011).

Local anaerobic microenvironments could exist also in the needles and leaves of trees, where the activity of endophytes, and thus O_2 consumption, is enhanced by the fresh, photosynthesis-derived C compounds (Fig. 1a). This type of interaction has been reported at least from gramineous plants (Minamisawa *et al.*, 2004), where anaerobic nitrogen (N)-fixing clostridia are supported by other, nondiazotrophic endophytes. Although direct canopy-derived evidence is still lacking, anaerobic N fixation occurs also in coniferous needles (Moyes *et al.*, 2016), demonstrating the potential for other anaerobic processes as well.

Although oxygen effectively inhibits archaeal CH_4 production (Fetzer *et al.*, 1993; Yuan *et al.*, 2009), at least some methanogens can still tolerate oxic conditions – as previously observed, for example, in upland soils (Peters & Conrad, 1996; Angel *et al.*, 2012). Lyu & Lu (2018) evaluated the mechanisms behind this tolerance in their recent meta-analysis of both genomic and environmental data. They found strong evidence of two distinct methanogen clusters, with one of them harboring expanded category of oxygen tolerance features, including ways to combat ROS-derived oxidative stress. This split into clusters was largely in line with the classification into phylogenetic methanogen orders and their evolutionary history in relation to atmospheric O_2 levels. Moreover, global analysis of methanogens detected in oxic habitats gave further evidence that these particular methanogens have the potential to survive in the presence of oxygen (Lyu & Lu, 2018) – and thus possibly even within the canopy habitat.

In addition to methanogenic archaea, tree-derived CH_4 could be produced by other microbial groups, better suited for a life in aerobic conditions. First, saprophytic fungi produce CH_4 at least in nonliving wood material (Lenhart *et al.*, 2012) . The fungal CH_4 was shown to derive from methionine (Lenhart *et al.*, 2012), a precursor compound linked with plant stress-induced aerobic CH_4 production (Lenhart *et al.*, 2015a). On the other hand, Lenhart *et al.* (2012) also suggested that the fungal CH_4 production can be connected to chloromethane (CH_3Cl)

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formation and the type of substrates available, which might limit this process to the wood-decay fungi. As some of the wood-decaying fungi can also infect living trees (Asiegbu *et al.*, 2005), and since needles harbor complex fungal microbiomes (Pirttilä & Wäli, 2009), their role in the tree stem and canopy CH_4 exchange may be significant, but this remains to be resolved (Fig. 1b).

Another recently discovered CH_4-producing group are cyanobacteria, which were linked to this process both under oxic and anoxic conditions (Bižić *et al.*, 2020). CH_4 production was suggested to occur through general cell metabolism, such as photoautotrophic C fixation, and mechanisms that are dependent on photosynthetic products during light, and on storage compounds during dark. Since photosynthesis-performing chloroplasts in plants are known to have evolved from cyanobacteria through endosymbiosis (Raven & Allen, 2003), these bacteria could be connected to CH_4 production of land plants as well (Fig. 1a,b). This relation further underlines the decadal discussion on mechanistic understanding of aerobic CH_4 formation in plants (e.g. Keppler *et al.*, 2009; Liu *et al.*, 2015). So far, the most direct link between cyanobacteria and tree-related CH_4 emissions are the cyanobacteria-containing cryptogamic covers, such as lichens, which can grow on tree stems and have shown small CH_4 emissions in laboratory incubations (Lenhart *et al.*, 2015b; Fig. 1b). Finally, CH_4 is also produced during the process of N fixation when it involves the iron nitrogenase, and to a lesser extent the vanadium nitrogenase (Zheng *et al.*, 2018). These enzymes are found in various species representing both archaea and bacteria (McRose *et al.*, 2017). This finding is interesting owing to recent indications that endophytic diazotrophs are essential for coniferous trees growing on nutrient-poor soil (Moyes *et al.*, 2016; Puri *et al.*, 2020) and also considering cryptogamic covers, where cyanobacteria utilize these enzymes (Bellenger *et al.*, 2020). Furthermore, another nitrogenase-type enzyme system, found in various microbial groups, was recently reported to produce CH_4 from dimethyl sulfide (North *et al.*, 2020), produced, for example, by various bacteria in terrestrial environments (Carrión *et al.*, 2015, 2017). Although the link between all of these nonarchaeal groups and CH_4 production in the living trees is uncertain, the aforementioned findings suggest that CH_4 formation in terrestrial ecosystems is a far more widespread trait than previously thought and also warrants their evaluation in the tree CH_4 studies.

Methanogenic microbes in canopies of coniferous trees

Compared with potential aerobic CH_4 production in plants, the role of methanogenic archaea is often left undetermined in current CH_4-exchange studies focusing on the photosynthesizing plant parts. This situation stems at least partly from practical reasons, as we have lacked methods with adequate resolution to identify microbial populations behind locally relatively small, but globally significant emissions, and methods that allow linking previously unrelated organisms with particular functions, like CH_4-production. PCR-based methods, such as amplicon sequencing, are a standard tool in microbial ecology. Yet, coverage limitations often make them unfit for the analysis of rare, poorly characterized endophytes. Accordingly, only two PCR-based studies (and only two molecular analyses in general) have been published on tree-dwelling methanogens, and only from tree stems (Yip *et al.*, 2019; H-L. Li *et al.*, 2020). The recent developments in high-throughput sequencing techniques have led to the rise of metagenomic methods, which can potentially revolutionize the analysis of various microbiomes, such as tree endophytes. Compared with PCR, metagenomic approaches entail a far wider perspective: whole microbiomes within plant tissues can be sequenced, and with the right analytical tools even genes from novel taxa can be revealed.

We evaluated two different metagenomic sequencing approaches in revealing potential CH_4-producing microbes in trees, with a focus on boreal tree canopies. First, we conducted an extensive meta-analysis of methanogenic functional genes in already published data-entries in the SRA (https://www.ncbi.nlm.nih.gov/sra) related to pine and spruce tissues (a detailed description is given in Supporting Information Methods S1), similarly as previously for the methanogens in the SRA-data from peatlands (Bräuer *et al.*, 2020). In brief, gene fragments of CH_4-producing archaea (*mcrA*, coding for the methyl-coenzyme M reductase) were searched with HMMER (hidden Markov model search of gene structures) from the published SRA database (NCBI), and phylogenetics of them were analyzed against obtained cultured and candidate divisions of functional genes. Second, we utilized a novel 'probe-targeted capture' method (Aalto *et al.*, 2020; Siljanen *et al.*, 2021) to analyze genes related to CH_4 cycling from Norway spruce needles collected from eastern Finland (Kuopio). The same method was used recently for the detection of N-cycling microbes in plant biomass (Aalto *et al.*, 2020). Here, capture reaction was carried out with 12 190 unique probes, which were designed based on the currently known *mcrA* gene diversity in the public databases (Siljanen *et al.*, 2021). Before the analysis, spruce branches were incubated in aerobic conditions in a medium of sodium acetate containing diluted nitrate mineral salts for 14 d with 100 ppm CH_4 in the headspace to enhance the detection of both methanogens and methanotrophs (results for the methanotrophs are reported later in this paper).

Interestingly, both of our approaches revealed known methanogen species within the spruce canopies (Figs 1a, 2, S1; Table 1). An SRA database search revealed signs of methanogenic *mcrA* genes in both pine and spruce trees (Fig. 2; Tables S1, S2). However, although SRA sequences gave indications of a wide diversity (orders Methanosarcinales, Methanomicrobiales, and Methanobacteriales for spruce-derived entries and Methanomicrobiales for pine-derived entries), the number of quality-checked sequences was small (Table 1).

Our captured metagenome analysis included Norway spruce needles only from one location (Kuopio, Finland) and, as a smaller sample set, was expected to express lower *mcrA* diversity than the global database search. Still, by providing much higher length sequences (average 250 bp vs 100 bp of the SRAs) specifically enriched by *mcrA*-targeting probes, this method was able to give more reliable evidence on the presence of methanogenic archaea

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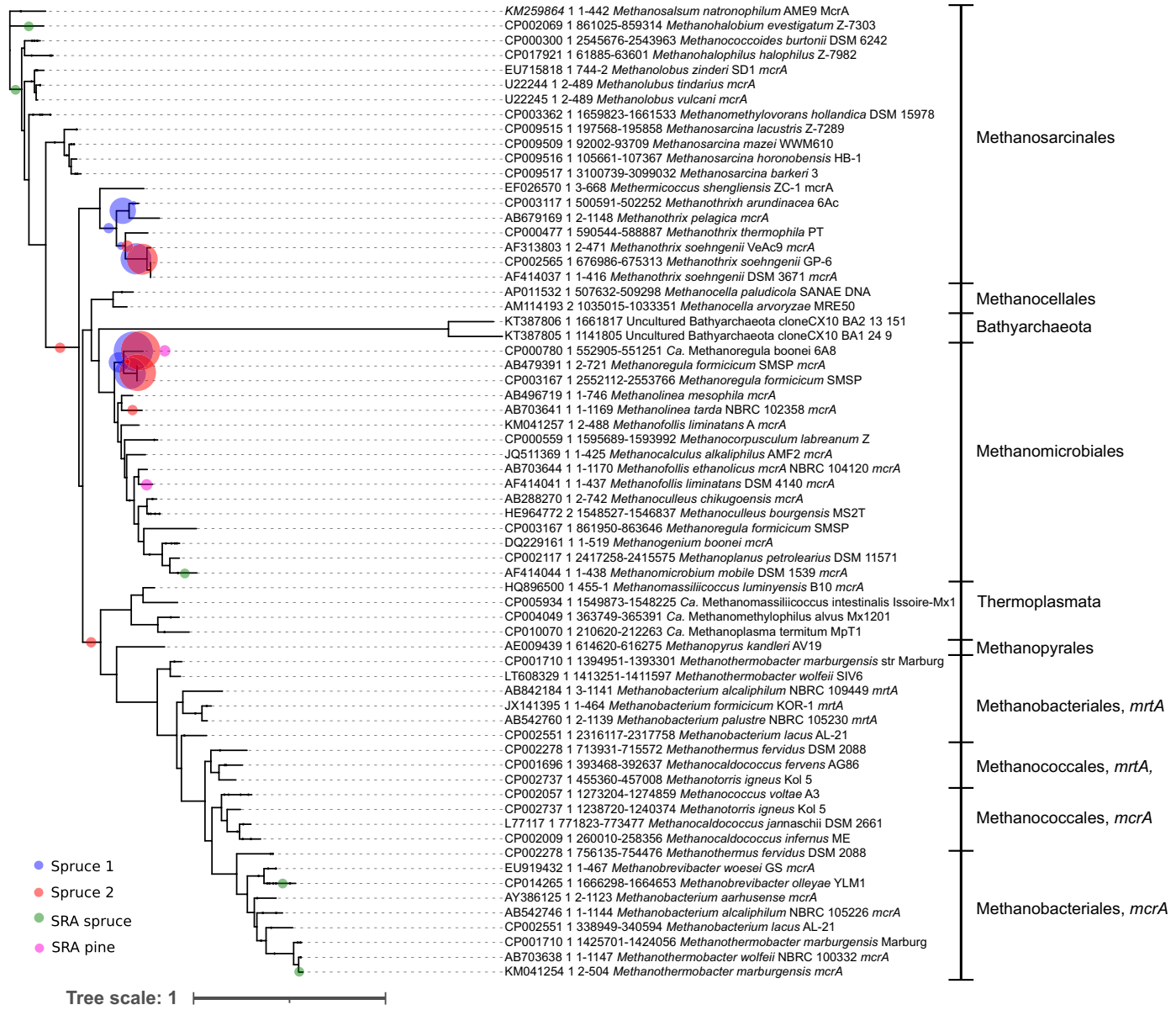

Fig. 2 Phylogenetic placements of the methanogenic *mcrA* gene (coding for the alpha subunit of the methyl-coenzyme M reductase (MCR))sequences retrieved through the Sequence Read Archive (SRA) database search and captured metagenomic sequencing from two pooled spruce needle samples (spruces 1 and 2) among the known methanogen and Bathyarchaeotal *mcrA*and *mrtA* (coding for the isozyme of the MCR) sequences inferred using the iTOL-tree (Letunic & Bork, 2019) with RAxML (Stamatakis, 2014). In the SRA-database search, 4822 pine and 1215 spruce SRA files in total were screened with HMMER for *mcrA* genes (details in the Supporting Information Methods S1). Only the best phylogenetic placement is shown for each query sequence, and only sequences with likelihood-weight ratios > 0.2 are included. Size of the placement icons reflects the relative number of sequences for a given placement within each sample. As an exception, the smallest size icon is used for positions with one to five sequence placements. The total number of sequences for each sample type is listed in Table 1. Original reference tree with 189 sequences together with bootstrap values is in Fig. S1. *Ca.*, *Candidatus*.

within the conifer habitat (Fig. 2; Table 1). The vast majority of the sequences belonged to orders Methanomicrobiales and Methanosarcinales within the class Methanomicrobia. Within the former order, most sequences were related to the genus *Methanoregula* (1799 out of 4066 sequences from spruce 1 matched with *Candidatus* Methanoregula boonei with a likelihood-weight ratio LWR > 0.95; scale for LWR: 0–1). Within Methanosarcinales, spruce *mcrA* sequences grouped with *Methanothrix* (formerly *Methanosaeta*) species (*Methanothrix soehngenii* linked with LWR > 0.95 with 303/4066 sequences from spruce 1 and 101/1865 from spruce 2). All of these well-described genera/species are common inhabitants in, for example, the waterlogged layers of peatlands, where they perform anaerobic methanogenesis as the last step of organic matter degradation (Bräuer *et al.*, 2020). The largest groups found, *Methanoregula* and *Methanothrix*, produce CH_4 by reducing carbon dioxide (CO_2) with hydrogen (H_2), or by splitting of acetate to CH_4 and CO_2, respectively. Accordingly, the detection of the *Methanothrix*

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Table 1 Number of quality-controlled (likelihood-weight ratio LWR > 0.20) sequences acquired/retrieved in this study together with their LWR values from the phylogenetic placement analysis (details in Supporting Information Methods S1).

Sample and analysis type	Marker gene	Host tree species (plant part used in the sequencing)	No. of sequences	LWR range	LWR mean ± SD
Spruce 1, capture sequencing	*mcrA*	*Picea abies* (needles)	4066	0.26–1.00	0.83 ± 0.21
	mmoX		3782	0.20–1.00	0.67 ± 0.20
	Psm_mmoX		1	0.55	—
Spruce 2, capture sequencing	*mcrA*	*Picea abies* (needles)	1856	0.20–1.00	0.56 ± 0.22
	mmoX		270	0.21–0.99	0.69 ± 0.24
	Psm_mmoX		nd	nd	nd
SRA database, pine	*mcrA*	*Pseudotsuga menziesii* (megagametophyte)	2	0.22–0.29	0.26 ± 0.05
	mmoX	*Pinus taeda* (needles), *Pseudozuga menziesii* (needles)	4	0.24–0.43	0.35 ± 0.08
	Psm_mmoX	*Pinus sylvestris* (needles), *Pinus canariensis* (cambial cells), *Pinus contorta* (foliage), *Pseudozuga menziesii* (megagametophyte)	4	0.22–0.97	0.56 ± 0.35
SRA database, spruce	*mcrA*	*Picea abies* (megagametophyte)	6	0.21–0.36	0.28 ± 0.06
	mmoX	*Picea abies* (megagametophyte)	2	0.27–0.62	0.45 ± 0.24
	Psm_mmoX	*Picea abies* (megagametophyte)	50	0.02–1	0.57 ± 0.28

In the Sequence Read Archive (SRA)-database search, 4822 pine and 1215 spruce SRA files in total were screened with Hmmer for *mcrA*, *mmoX*, and *pmoA* genes. Average lengths for capture and SRA sequences were 250 bp and 100 bp, respectively. No traditional *pmoA* gene fragments were detected.
nd, not detected.
Psm_mmoX, *Pseudomonas* sp.-related novel *mmoX*.

sequences may have been enhanced by the spruce incubation treatment with acetate in the growth medium. However, our results still reflect the taxa present within untreated needles. In soils, both acetoclastic and hydrogenotrophic methanogenic pathways are sustained by the activity of other microbiota, such as syntrophic microbes (Bräuer *et al.*, 2020). This is likely the case also in the needle habitat and needs to be investigated through a wider analysis of the whole microbiome and related microbe–microbe interactions. It should be noted that plant metabolic processes could also serve as a source of substrates for the microbial methanogenesis. For example, acetate is continuously recycled within the plant cells (Zhang *et al.*, 2017) and could, thus, be available for the acetoclastic methanogens such as the *Methanothrix* species.

Considering the largely oxygenic conditions within spruce needles, detection of Methanomicrobiales and Methanosarcinales is fitting: they belong to the specific cluster of methanogens suggested to contain enhanced O_2 tolerance mechanisms (Lyu & Lu, 2018). For them to be actually active in CH_4 production, at least temporally anoxic microhabitats are needed – potentially involving the oxygen-consuming activity of other endophytes.

Methane consumption within tree stems and canopies: an unrecognized methane sink?

Evidence of methane consumption by trees from flux measurements

Despite numerous CH_4 flux studies on tree stems, consumption of CH_4 in stems has been rarely reported (Barba *et al.*, 2019; Welch *et al.*, 2019; Moldaschl *et al.*, 2021). As the net CH_4 exchange is the sum of both production and consumption processes, it remains unclear whether consumption exists but is mostly overcome by a higher CH_4 production rate. The few existing studies on tree canopy CH_4 exchange show that tree canopies can act as both sources and sinks of CH_4 (Sundqvist *et al.*, 2012; Machacova *et al.*, 2016; Halmeenmäki *et al.*, 2017; Pangala *et al.*, 2017; this study). Based on field measurements, canopy CH_4 consumption has, to our knowledge, been reported only by Sundqvist *et al.* (2012). All the tree species they measured – coniferous trees: Norway spruce and Scots pine; and broadleaf trees: birch (*Betula pubescens*) and rowan (*Sorbus aucuparia*) – were observed to mostly consume CH_4 with an average CH_4 consumption rate of $-11.2\ \mu g\ h^{-1}\ m^{-2}$ leaf area (LA) at lower branches of the trees during autumn period. Sundqvist *et al.* (2012) estimated that, with the uptake rate they measured, the tree canopy CH_4 sink could be of similar strength to the soil sink.

Here, we present results from two Norway spruce field campaigns, which further demonstrate the functioning of shoots as both sinks and sources of atmospheric CH_4 (Fig. 3; details in Methods S1). The average CH_4 exchange rate was $-0.3\ ng\ g^{-1}\ DW\ h^{-1}$ for mature spruce trees (Skogaryd, Sweden), and $1.4\ ng\ g^{-1}\ DW\ h^{-1}$ for 2 to 3-yr-old tree saplings (Helsinki, Finland). The scale and variation of the fluxes was clearly higher in the young samplings, possibly reflecting their growth phase and dynamic conditions during the spring period. However, our fluxes from both mature spruce shoots and from the saplings were markedly smaller than those measured by Sundqvist *et al.* (2012). As interpreted from Sundqvist *et al.* (2012, Fig. 1), their CH_4 fluxes from mature spruce shoots ranged from *c.* -40 to $32\ \mu g\ h^{-1}\ m^{-2}$ LA, which scales to a range of *c.* -200 to $160\ ng\ g^{-1}\ DW\ h^{-1}$ (Hager & Sterba, 1985). This high variability between the studies underlines the need for more flux measurements from tree canopies, and the need to consider both CH_4 production and consumption when evaluating the role of trees in the forest CH_4 balance. Most importantly, to uncover the drivers of these processes, potential involvement of microbes should be studied using simultaneous collection of tree tissue samples.

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Evidence of methane consumption by trees from detection of tree-associated methanotrophs

Methanotrophs vary in their preference for the concentration of CH_4 and can thus be divided into high (atmospheric CH_4) and low-affinity oxidizers – although some of them oxidize CH_4 both in high and low concentrations (Chowdhury & Dick, 2013; Ho *et al.*, 2019). High-affinity methanotrophs are responsible for the CH_4 sink of the upland soils, whereas low-affinity populations thrive, for example, in waterlogged soils with high *in situ* CH_4 production (Knief *et al.*, 2003; Chowdhury & Dick, 2013). Trees could potentially provide microhabitats for both types of methanotrophy. In the canopies, CH_4 concentrations are likely closer to atmospheric concentrations, and thus they might harbor high-affinity-type oxidizers as both epi- and endophytes. Our results, however, point to the presence of methanogenic activity in the needles, which might support low-affinity oxidizers. In line with this, Iguchi *et al.* (2012) reported isolation of common methanotrophs, *Methylomonas* sp. and *Methylocystis* sp., from Norway spruce and *Pinus parviflora* needles (Fig. 1a). Although their CH_4 oxidation capacity was not tested, the isolates were obtained using a high CH_4 concentration (20%). Likewise, Doronina *et al.* (2004) were able to isolate a *Methylocystis*-related strain from the needles of *Picea pungens*. All the isolates mentioned also grew with methanol (CH_3OH), and thus represented the so-called facultative methanotrophs, potentially supported by CH_3OH formation in the plant physiological processes (Dorokhov *et al.*, 2018). More recently, similar facultative methanotrophs were detected through 16S ribosomal RNA (rRNA) gene sequencing from the needles of *Pinus radiata* (Rúa *et al.*, 2016) and Norway spruce (Haas *et al.*, 2018). Tree stems have been shown to occasionally hold very high CH_4 concentrations (Covey *et al.*, 2012) and might, thus, serve as a habitat for low-affinity oxidizers, similar to, for example, *Sphagnum* mosses in peatlands (e.g. Larmola *et al.*, 2010; Putkinen *et al.*, 2012). This is supported by the detection of both 16S rRNA genes related to common alpha and gammaproteobacterial methanotrophs and methanogens (as discussed earlier in the paper) in the stems of *Populus deltoides* (Yip *et al.*, 2019; Fig. 1b). In addition to living stems, methanotrophs have been found in fallen logs infected by fungi (Mäkipää *et al.*, 2018) – another tree habitat where *in situ* CH_4 production likely takes place (Covey *et al.*, 2012), and, in unquantifiable amounts, in Scots pine roots (Halmeenmäki *et al.*, 2017, Fig. 1c).

Based on these findings, and the CH_4 consumption detected in the field measurements, we suggest that methanotrophs are present in the conifer habitat and that their in-depth characterization is possible with the improved metagenomics tools now available. For this purpose, we used the same two metagenomic approaches as with the methanogens (details in Methods S1; Bräuer *et al.*, 2020). First, the SRA database was searched for methanotrophic functional genes *pmoA* and *mmoX*, coding for the particulate and soluble forms of methane monooxygenase (MMO), respectively. Second, the same genes were targeted with the capture enrichment approach (Aalto *et al.*, 2020) to detect methanotrophs in spruce shoots, which were first incubated to enhance the detection of CH_4-cycling microbes (as described in the section Methanogenic microbes in canopies of coniferous trees; Dunfield *et al.*, 2003; Dedysh *et al.*, 2005). Capture reaction included 640 unique probes for *mmoX* and 19 900 probes for *pmoA* (Siljanen *et al.*, 2021).

Both analyses revealed similar patterns: monooxygenase (MO) genes were found, but except for two pine-derived SRA-sequences, similar to alphaproteobacterial *mmoX* genes, they were not related to *pmoA* or *mmoX* genes of known methanotrophs (Figs 4, S2; Tables 1, S1, S2). Almost all other SRA sequences with proper likelihood weight values (Table 1) were from the same project targeting the genome of the host tree, *P. abies*, with sequenced DNA deriving from the spruce megagametophyte (Nystedt *et al.*, 2013) – likely reflecting the lack of microbiome targeting analyses in general. Except for one actinobacterial propane MO (PMO) match, these SRA sequences were related to novel *Pseudomonas* sp.-related MO genes, which, in addition to butane, have been linked with CH_4 oxidation (Cooley *et al.*, 2009). By contrast, *P. abies*-derived sequences, captured with *mmoX* probes, all grouped either with actinobacterial or proteobacterial PMOs. As with the *mcrA* analysis, the quality of the short SRA-database-retrieved sequences was lower than the ones produced in the capture sequencing (Table 1). No similarities to 'traditional' *pmoA* genes were found with the capture approach.

Taken together, our analysis revealed only minor indications of currently known, 'traditional' methanotrophs in the analyzed conifers. However, the novel MOs, detected both in the SRA and in the captured metagenomics data, might have the potential to consume CH_4 in the tree canopies. In general, understanding of the alkane/alkene monooxygenases is far from complete and their functioning within the trees has not been examined. Recent analysis indicates that PMO and MMO enzymes share a common ancestor but have evolved in different directions. Consequently, only MMO and butane MO (BMO) seem to be capable of breaking the C–H bond of CH_4 (Osborne & Haritos, 2019). PMOs and BMOs can primarily break the molecule at the secondary C, which is estimated to require a maximum cleavage energy of 400 kJ mol^{-1}. Breaking of the C–H bond of CH_4, with an estimated cleavage energy requirement of 431 kJ mol^{-1}, would at least be a lot less energetically efficient by the PMOs than by MMOs. Yet, we cannot rule out the possibility of PMOs or BMOs oxidizing CH_4 as a co-substrate or unspecifically, as previously shown with BMO from *Pseudomonas butanovorans* (Cooley *et al.*, 2009).

Evidently, we need a deeper understanding of the tree-associated CH_4 consumption mechanisms and microbial communities involved. Excluding the two alphaproteobacterial *mmoX* SRA-fragments, our metagenomic approaches could not detect methanotrophs related to previous needle isolates (Doronina *et al.*, 2004; Iguchi *et al.*, 2012). This likely reflects the well-known challenge to cultivate single strains from complex environmental communities: the aforementioned facultative methanotroph isolates likely represent strains adapted to higher CH_4 concentrations (low-affinity oxidizers). They have proven easier to grow in the laboratory conditions than high-affinity methanotrophs – the first strain able

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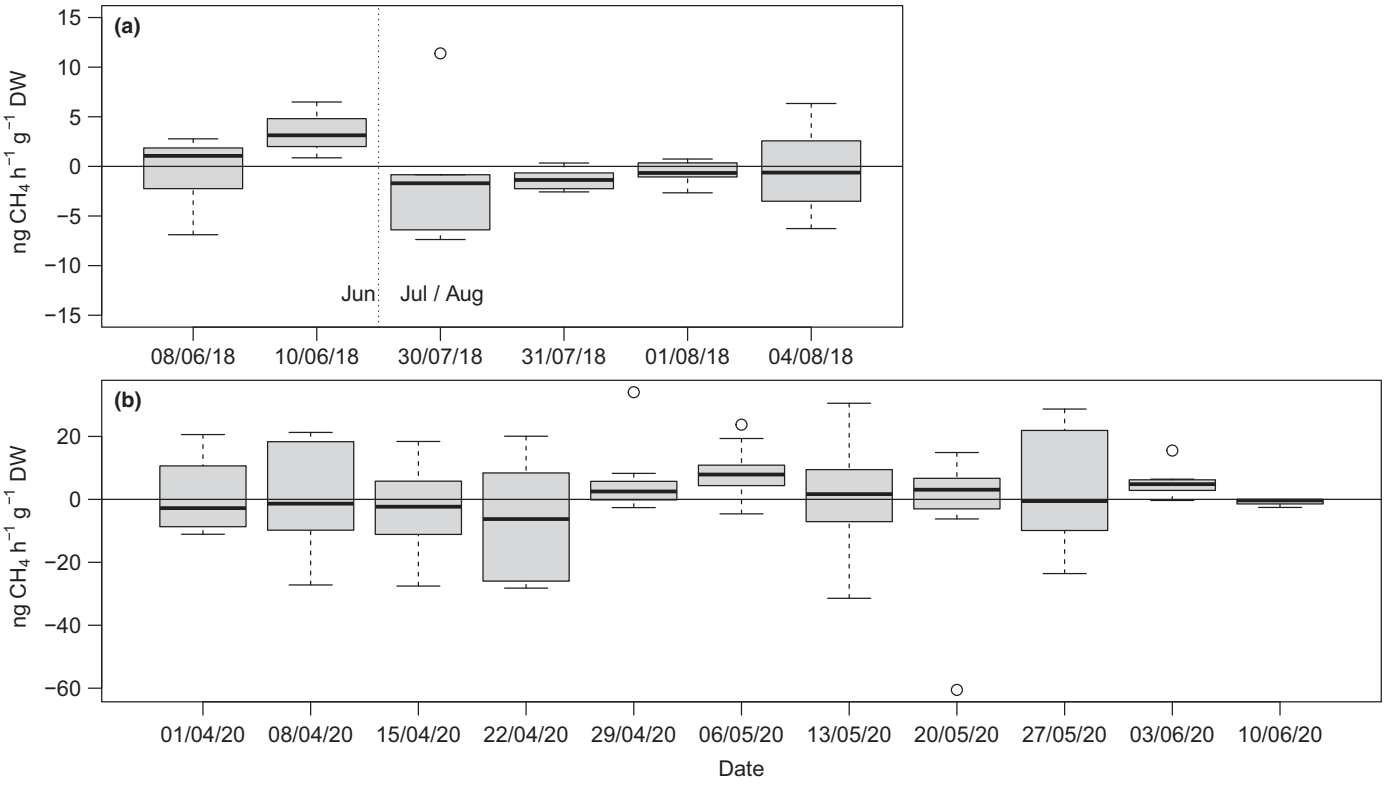

Fig. 3 Spruce shoot methane (CH_4) fluxes (median, quartiles and interquartile ranges) measured at (a) Skogaryd Research Catchment spruce forest, Sweden, in June–August 2018, and (b) Helsinki yard saplings, Finland, in April–June 2020. Shoot fluxes were measured using manually operated transparent shoot chambers, as in Machacova *et al*. (2016), connected to an online CH_4/CO_2 greenhouse gas analyzer (UGGA; ABB - Los Gatos Research, San Jose, CA, USA). In total, there were 34 separate shoot flux measurements with mature trees in Skogaryd and 89 separate shoot flux measurements with spruce saplings in Helsinki. Details of the measurement setup and data processing are given in the Supporting Information Methods S1. Note the different *y*-axis scales in the two graphs.

to grow in atmospheric CH_4 concentration was isolated by Tveit *et al*. (2019). Sequence reads gained in our study could represent so far uncultivated CH_4 oxidizers adapted to low/trace level concentrations of CH_4 (induced by our incubation with CH_4 at 100 ppm). It should be noted that, owing to the presence of acetate in the growth media, our incubation might have favored facultative methanotrophs adapted to the use of this alternative C source.

Future directions for moving beyond descriptive studies

Currently, we still need more research even on the *presence* of CH_4-producing and consuming microbes in the aboveground tree habitat. As reviewed in this paper, the few existing studies on this topic have been largely based on either cultivation, which is biased towards distinct species thriving in the laboratory, or on the sequencing of universal 16S rRNA genes, which lacks information on specific functions and the sensitivity for the rare species. Modern metagenomic tools have the potential for more detailed characterization of tree microbiomes, giving insights to both taxonomy and function. Still, based on the project descriptions behind the retrieved SRA-database entries, metagenomic sequencing is still mostly targeting the host tree genomes more than the associated microbiomes. In addition, owing to the large genome size of the host tree compared with the epi- and endophytes, microbiome sequencing through the regular shotgun approach is hindered by a low signal-to-noise ratio (Schneider *et al*., 2021). In that sense, targeted capture metagenomics shows greater potential to uncover even rare microbial genes among the plant-cell DNA, as we showed here for the spruce shoots.

Though the metagenomic tools can generate a vast amount of genomic data, linking unknown DNA fragments to given functions and species is limited by the low amount of annotated reference sequences/genomes in the databases (Kaul *et al*., 2016; Schneider *et al*., 2021). To solve this, traditional cultivation approaches are still needed to complement the sequencing methods. Successful isolation of the relevant microbial strains would allow evaluation of the role of putative enzymes in the CH_4 cycle, such as *Pseudomonas*-related MOs. With pure cultures, full bacterial/archaeal genomes can be acquired, allowing the analysis of not only CH_4 metabolism but also other traits related to, for example, survival in the plant habitat and interactions with the host (Frank, 2018). Genomes of uncultivated organisms can be derived also through single-cell methods (Rinke *et al*., 2014), and by building them from metagenomic data (i.e. metagenome assembled genomes; Parks *et al*., 2017). As an alternative, novel genes/enzymes can be connected to particular functions with the help of metagenomics-based functional screening approaches (Ngara & Zhang, 2018) and by the use of isotope applications, such as nanoscale secondary ion mass spectrometry and stable isotope labeling, or their combinations (Musat *et al*., 2016).

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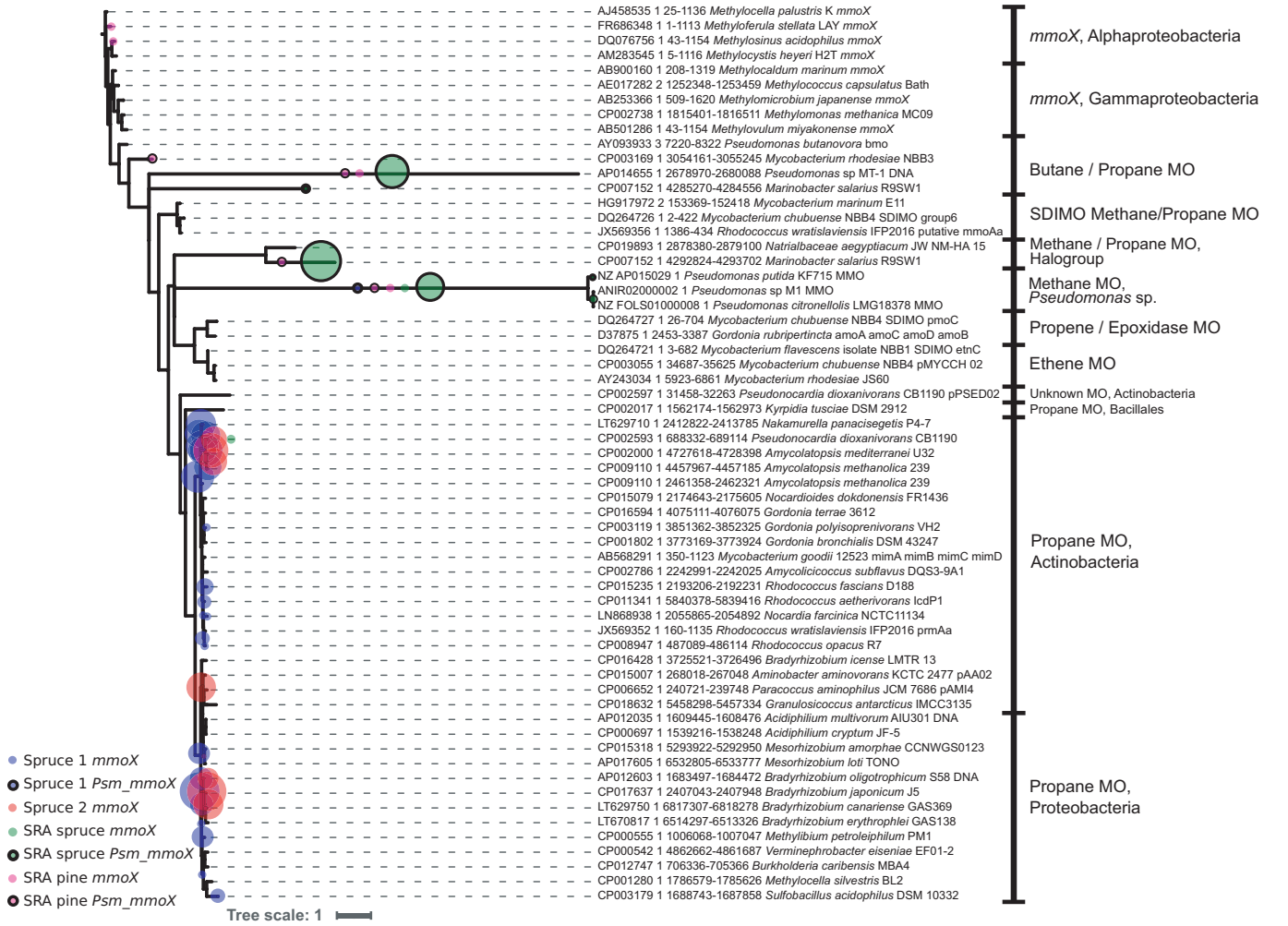

Fig. 4 Phylogenetic placements of the methanotrophic *mmoX* gene (coding for the alpha subunit of the soluble methane monooxygenase (sMMO))-type sequences retrieved through the Sequence Read Archive (SRA)-database search and captured metagenomic sequencing from two pooled spruce needle samples (spruces 1 and 2) among the known monooxygenases for methane and other short alkenes inferred using the iTOL-tree (Letunic & Bork, 2019) with RAxML (Stamatakis, 2014). In the SRA-database search, 4822 pine and 1215 spruce SRA files in total were screened with Hmmer for *mmoX* genes (details in Supporting Information Methods S1). Only the best phylogenetic placement is shown for each query sequence, and only sequences with likelihood-weight ratios > 0.2 are included. Size of the placement icons reflects the relative number of sequences for a given placement within each sample. As an exception, the smallest size icon is used for positions with one to five sequence placements. Total number of sequences for each sample type is listed in Table 1. Original reference tree with 92 sequences together with bootstrap values is in Fig. S2. MO, monooxygenase; *Psm_mmoX*, *Pseudomonas* sp.-related *mmoX*-type sequences (potentially coding for the sMMO); SDIMO, soluble di-iron monooxygenase.

To discern the true contribution of microbes on the tree CH_4 exchange, we need to analyze *active species/genes* through methods such as metatranscriptomics. Like metagenomics, metatranscriptomics is challenging to apply to endophytes owing to the vast amount of plant-derived background noise and the limited amount of reference data, but it has been successfully used to examine fungal endophytes in Norway spruce roots and needles (Schneider *et al.*, 2021). Considering the tree CH_4 cycle, expression analyses of the whole microbiome would enable detailed studies on the interactions between CH_4 cycling and other community members – potentially unfolding the role of other microbes; for example, as providers of methanogenic substrates or in controlling the O_2 level. Simultaneous analysis of both the host and the microbiome (dual RNA sequencing; Kaul *et al.*, 2016) would uncover connections between microbial and plant metabolism, and their relation to the CH_4 exchange. Moreover, this type of dual expression analysis would give clues on *why*, for example, methanogens would inhabit the tree tissues: only for their own benefit or as a part of a mutualistic association potentially aiding the survival of the host (L. Li *et al.*, 2020). It should be noted that RNA sequencing studies concentrating on the plant host often bypass the microbes by targeting only polyadenylated messenger RNA (mRNA) sequences not present in the prokaryotes. Probe-targeted capture is also a promising approach within the metatranscriptomics, although careful optimization is still needed due to the low amount of microbial transcripts within the plants. The next step from the transcript analysis would be metaproteomic analysis of actual, working enzymes – the most reliable molecular markers for active, metabolic processes (Kaul *et al.*, 2016). Its value has been demonstrated, for example, in the study of diatzotrophic methanotrophs (Bao *et al.*,

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2014) and other C-cycling endophytes (Knief *et al.*, 2012) within rice plants.

Despite methodological breakthroughs, 'omics methods may still require excessive resources for large-scale experiments and field sampling campaigns. Thus, an important aim should be to utilize the 'omics-derived microbiome data for the development of more cost-effective, high-throughput analyses, such as quantitative PCR assays with primers targeting the most important community members. This type of approach has been applied, for example, by Sessitsch *et al.* (2012), who combined metagenomics with mRNA amplicon sequencing to reveal N-cycling activity of the rice root endophytes. In addition, 'omics data could be utilized to design probes for applications such as fluorescence *in situ* hybridization (Wagner & Haider, 2012), which could be used to visualize the detailed locations of CH_4-cycling microbes within the different tree tissues (Fig. 1).

To get the full advantage of the microbiome analyses, one should carefully consider the experimental setup, including where, when, and how to collect and how to process the tree tissue samples. Tree microbiomes differ, for example, between different heights of the same tree (Herrmann *et al.*, 2021), between young and old trees (Carper *et al.*, 2018; Koivusaari *et al.*, 2018), and between the seasons (Haas *et al.*, 2018) – highlighting the need to cover various spatiotemporal aspects. Owing to the extensive variability, high numbers of both biological and technical replicates are necessary to reveal the effects of particular abiotic and biotic variables (Bullington *et al.*, 2021). Finally, as the ultimate aim is to gain a holistic understanding of the individual drivers behind the tree and forest CH_4 balance, it is essential to combine expertise and methodology from various fields in the same studies and field campaigns.

Conclusions

The role of microbes in the tree CH_4 exchange is still not adequately addressed. Our results demonstrate the potential of probe-targeted metagenomic tools in uncovering genes of rare but functionally important microbes within the large genomic pool of the plant host. To our knowledge, this is the first study to report the presence of archaeal methanogens in conifer needles. Future studies should strive to reveal the functioning of not only methanogens, but also other CH_4-producing microbial groups within the tree tissues. Furthermore, CH_4 consumption, and the microbes potentially responsible for it, should be more strongly considered when evaluating the forest CH_4 budget. In the end, we need multifaceted experiments, aiming to evaluate microbial activity alongside other CH_4-forming processes, the flux rates, and environmental variables. Only then will we be able to estimate the contribution of microbes to the CH_4 balance at the ecosystem scale, and eventually at regional to global level.

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Author contributions

AP, HMPS, AL, MP and MT designed the concept of this paper. HMPS and IP performed the experiments with spruce samples and carried out the DNA extractions. HMPS screened the SRA database and used captured metagenomics for spruce DNA, HMPS and AP analyzed the sequence data, and AP processed the sequencing data into figures. IH and ST measured the tree canopy fluxes and processed the data. AP, AL, KP and MP wrote the first draft of the manuscript; all co-authors contributed to reviewing and editing the manuscript. AP and HMPS share the first authorship (equal contribution).

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Data availability

The sequence data produced in this study is deposited to the NCBI SRA database under the BioProject link PRJNA685973.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phylogenetic tree of the reference *mcrA* sequences.

Fig S2 Phylogenetic tree of the reference monooxygenase gene sequences.

Methods S1 Additional methodological details.

Table S1 List of the pine SRA entries retrieved in the database search.

Table S2 List of the spruce SRA entries retrieved in the database search.

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Key words: boreal forests, captured metagenomics, methane exchange, methanogenic archaea, methanotrophic bacteria, plant microbiome, tree.

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