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Distinct microbial communities drive methane cycling in below- and above-ground compartments of tropical cloud forests growing on peat

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Abstract

Cloud forests are unique yet understudied ecosystems regarding CH_4 exchange despite their significance in carbon storage. We investigated CH_4 fluxes in peat soil and tree stems of two tropical cloud forests on Réunion Island, one featuring *Erica reunionensis* and the second a mix of *E. reunionensis* and *Alsophila glaucifolia*. The study examined microbiomes across below-ground (soil) and above-ground (canopy soil, leaves, and stems) forest compartments. Metagenomics and qPCR analyses targeted key genes in methanogenesis and methanotrophy in soil and above-ground samples, alongside soil physicochemical measurements. CH_4 fluxes from peat soil and tree stems were measured using gas chromatography and portable trace gas analyzers. Peat soil in both forests acted as a CH_4 sink (-23.8 ± 4.84 µg C m^{-2} h^{-1}) and CO_2 source (55.5 ± 5.51 µg C m^{-2} h^{-1}), with higher CH_4 uptake in sites dominated by endemic tree species *E. reunionensis*. In forest soils, a high abundance of n-DAMO 16 S rRNA gene ($3.42 \times 10^7 \pm 7 \times 10^6$ copies/g dw) was associated with nitrate levels and higher rates of CH_4 uptake and CO_2 emissions. NC-10 bacteria (0.1–0.3%) were detected in only the Erica forest soil, verrucomicrobial methanotrophs (0.1–3.1%) only in the mixed forest soil, whereas alphaproteobacterial methanotrophs (0.1–3.3%) were present in all soils. Tree stems in both forests were weak sinks of CH_4 (-0.94 ± 0.4 µg C m^{-2} h^{-1}). The canopy soil hosted verrucomicrobial methanotrophs (0.1–0.3%). The leaves in both forests exhibited metabolic potential for CH_4 production, e.g., exhibiting high *mcrA* copy numbers ($3.5 \times 10^5 \pm 2.3 \times 10^5$ copies/g dw). However, no CH_4-cycling functional genes were detected in the stem core samples. Tropical cloud forest peat soils showed high anaerobic methanotrophy via the n-DAMO process, while aerobic methanotrophs were abundant in canopy soils. Leaves hosted methanotrophs but predominantly methanogens. These results highlight the significant differences between canopy and soil microbiomes in the CH_4 cycle, emphasizing the importance of above-ground microbiomes in forest CH_4 gas budgets.

Keywords Peatlands, Soil microbiome, Phyllosphere microbiome, Canopy soil microbiome, Leaf microbiome, Stem microbiome

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Introduction

Methane (CH_4) is a significant greenhouse gas, ~25 times more potent than CO_2 in terms of its global warming potential over a 100-year period [1]. Microbial processes in soils are the major biological sources and sinks of CH_4 [2] with tropical forest soils being the net sinks (average uptake: 4.94 Tg CH_4 yr^{-1}) in global CH_4 budgets [3, 4]. Vegetation could also significantly contribute to the global CH_4 cycle via various pathways and processes [5, 6]. For example, tree stems act as conduits for soil CH_4 or function independently as sources or sinks [7], such as tree stems from upland forests are CH_4 sinks due to microbe-mediated methanotrophic activity on and within their woody surfaces and tissues [6, 8]. Tree leaves from various ecosystems are reported to be sources [9–12] and sinks of CH_4 [13, 14]. Similarly, the cryptogamic covers can also be the sources [15] or the sinks of CH_4 [16]. Metagenomics analyses have revealed that trees harbor the microbes responsible for both CH_4 production and consumption [17], therefore, the net CH_4 fluxes would depend on the balance between these processes [15, 18]. The fluxes from stems or other vegetative parts can also be influenced by the taxonomy, age, and other tree traits in addition to the soil type and seasons [19, 20]. Following the temperate upland forests, tropical forest soils are the significant net CH_4 sinks [3], but tropical wetland forests can emit CH_4 depending on the soil saturation [21, 22]. However, the precise patterns, magnitudes, and origins of these CH_4 fluxes in different forest ecosystems are not yet fully known [23–25]. Additionally, the microbial role in CH_4 fluxes from above-ground compartments in tropical forests remains less well understood, highlighting that more species- and ecosystem-specific field data is essential.

Tropical cloud forests are mid-elevation forests located in tropical regions, distinguished by their elevated humidity levels due to persistent or frequent ground-level cloud condensation. According to the hydro-climatic approach (based on cloud coverage), these forests constitute 13-14.5% of the total tropical and subtropical forest area between 35° S and 23.5° N latitude [26–28], and exhibit significantly high levels of endemism and biodiversity [29]. Furthermore, they play an essential role in various ecosystem services, including carbon (C) storage and water and climate regulation [30, 31]. These forests are often on peat soils [32], and tropical peatlands may act as sources of CO_2 or CH_4 depending on natural weather events such as drought or flooding, as well as anthropogenic interventions like drainage, land-use change, and deforestation [33–37]. The CO_2 and CH_4 emission levels from tropical peatlands are also typically higher than other global peatlands [38, 39]. CH_4 emissions may decrease with a lowering water table and reduced soil water content, making peat soil a net CH_4 sink [40]. Meanwhile, CO_2 emissions significantly increase, with the highest levels observed in tropical peat soils [41]. Given the significant amounts of carbon loss through emissions, tropical peatlands are becoming increasingly important to study; however, investigations on peat soils, particularly in tropical cloud forests, have remained limited.

Peatlands in cloud forests have a high capacity for carbon storage [42] and experience dynamic fluctuations in both ambient and soil moisture. These variations can alter oxic conditions above and below ground, influencing CH_4 fluxes. Generally, under anaerobic conditions, autotrophic methanogens produce CH_4 via the methyl–coenzyme M reductase (MCR) enzyme complex [43, 44]. Conversely, in aerobic conditions, methanotrophic microbes oxidize CH_4 using the enzymes particulate methane monooxygenase (pMMO) and the soluble methane monooxygenase (sMMO) [45, 46]. CH_4 can also be oxidized by anaerobic methanotrophs using nitrite/nitrate as an electron acceptor, coupling the anaerobic CH_4 oxidation with denitrification, and the process is called nitrite/nitrate-dependent anaerobic methane oxidation (n-DAMO) [47]. Two microbial groups mediate n-DAMO: bacteria belonging to the phylum NC-10 and archaea from the ANME-2d cluster [48, 49]. While microbial communities in soils and sediments have been extensively researched, there is a growing recognition of the importance of microbial community composition in above-ground compartments for CH_4 cycling. However, a significant gap exists in data from tropical regions, which limits our understanding of these above-ground compartments' role in the CH_4 cycling in tropical forests.

We selected two cloud forests with peat soils on the tropical island of La Réunion for this study during early spring (dry season). In these forests, we aimed to investigate the microbiomes associated with CH_4 cycling in different forest ecosystem compartments, including the soil, cryptogamic canopy soil, stem cores, and foliage. In addition, we also aimed to determine the CH_4 and CO_2 fluxes from the soil and tree stems. Our hypotheses were as follows: (1) The cloud forest peat soils and tree stems would absorb atmospheric CH_4 due to low soil moisture (2) In the nitrogen (N) rich peat soils of cloud forests, especially where nitrate is abundant, the n-DAMO process would be dominant; (3) The above-ground microbiomes of phyllosphere, cryptogamic canopy soil, and stems of these forests would exhibit distinct CH_4-cycling microbiome than soils because of varying oxic conditions.

Materials and methods

Sites description

Two cloud forests, located between 1500 and 1650 m above sea level within the montane cloud forest vegetation band, were examined on the tropical island of

La Réunion, France. The first site was the Plateau de Thym, a 25,000-year-old peatland [50, 51] situated in the Forêt de Bébour region of the commune of Saint-Benoît (21.097139° S, 55.548028° E). The second location is the Plaine des Cafres in the municipality of Le Tampon (21.145343° S, 55.569692° E).

The Plateau de Thym forest was primarily dominated by *Erica reunionensis* and *Hubertia ambavilla*. In the understory, the main species were *Erica galioides* and *Juncus effusus*, along with patchy populations of *Sphagnum* species. The Plaine des Cafres forest mainly comprised an endemic shrub species, *Erica reunionensis*, and an endemic tree fern species, *Alsophila glaucifolia*. Dominating the epiphytic vegetation included *Cordyline mauritiana* and various fern species, such as *Hymenophyllum inaequale*, *H. capillare*, and *Blechnum attenuatum*. The most prevalent species in the understory included *Embelia angustifolia*, *Anthoxanthum odoratum*, and *Cynorkis ridleyi*. The bryophyte layer was unevenly distributed and predominantly consisted of *Sphagnum* spp. The Plateau de Thym forest is referred to as the 'Erica forest' in the current study, while the Plaine des Cafres forest is referred to as the 'Mixed forest', primarily characterized by *E. reunionensis* or *A. glaucifolia*.

Measurements and sampling were conducted in November 2022, coinciding with the early spring dry season on Réunion Island. In the Erica forest, all points were selected within areas dominated by *E. reunionensis*. In contrast, the mixed forest of Plaine des Cafres contained six points in regions dominated by *E. reunionensis* and six points in areas dominated by *A. glaucifolia*.

Soil and stem gas flux sampling and measurement

To quantify the CH_4 and CO_2 fluxes at the soil surface, 65 L polyvinyl chloride chambers (with a surface area of 0.0196 m^2, a volume of 0.065 m^3, a diameter of 0.5 m, and a height of 0.4 m) were positioned over the soil using pre-installed chamber collars ($n = 24$). During a one-hour measurement period, gas samples were collected from the chambers and injected into 50 ml pre-vacuumed glass bottles at 20-minute intervals [52]. This sampling was done twice daily between 10:00 and 16:00 for two days. The change in concentration of fluxes over a one-hour measurement period was determined using gas chromatographs (GC-2014, Shimadzu, Kyoto, Japan) equipped with a Loftfield-type autosampler [53] in addition to an electron capture detector (GC-ECD) to measure the concentrations of CO_2 and a flame ionization detector (GC-FID) to measure CH_4.

To measure the CH_4 and CO_2 fluxes on the surfaces of tree stems, static chambers (total area = 0.0108 m^2 stem surface, total volume = 0.00119 m^3) were installed on the stems of *E. reunionensis* ($n = 6$ in Erica forest, $n = 10$ in mixed forest) and *A. glaucifolia* ($n = 6$ in mixed forest) at a height of approximately 20 cm above the ground [11]. The chambers were made of transparent plastic (Lock & Lock, Seoul, South Korea), and their bottoms were removed prior to installation. A neoprene band was affixed to the bottom edges to create an airtight seal between the tree surfaces and the chambers. Each chamber was then closed with an airtight removable lid, which offered a connection to a trace gas analyzer (LI-COR LI-7810 $CH_4/CO_2/H_2O$, Li-Cor Biosciences, Lincoln, NE, USA). CH_4 and CO_2 fluxes were measured by circulating the chamber headspace air between the chambers and the gas analyzer in a closed loop, with measurements taken at ten-minute intervals.

The quality of the manual chamber measurements for CO_2 and CH_4 fluxes was validated using the adjusted R^2 value from a linear regression of gas concentration over time. Fluxes were considered valid and accepted only when the adjusted R^2 value exceeded 0.9. The flux values were calculated by determining the least-squares linear regression of the gas concentration change in the static chambers' headspaces over the sampling time. Equations used for the calculation were adapted from a previous study [54].

Soil, canopy soil, and plant sampling

Soil samples were collected from the gas sampling points ($n = 24$) to analyze their chemical properties and for microbial analyses. Composite samples from the topsoil of the upper horizon (0–10 cm depth) were gathered in plastic grip-seal bags with properly disinfected equipment. Additionally, for microbial analyses, canopy soil from mixed forest ($n = 19$), which consisted of dead epiphytes and cryptogams, was collected and placed in grip-seal bags with active silica gel to absorb moisture. Canopy soil was only present in the mixed forest. Stem core samples ($n = 8$) from *E. reunionensis* were obtained from both cloud forest sites. Stems were drilled using a 3-threaded increment borer with 400 mm length and 5.15 mm diameter (Haglöf Sweden AB, Langsle, Sweden) from the point of gas sampling. Similarly, leaves of *E. reunionensis* ($n = 5 + 6$) from both forests and *A. glaucifolia* ($n = 6$) from mixed forest were collected from different branches of different individuals and packed with silica gel. The silica gel was regularly changed until all moisture from the samples was thoroughly removed.

Soil physical and chemical analyses

Soil temperature was measured at each sampling point using probes (Campbell Scientific, Logan, USA) at a depth of 10 cm. Soil water content (SWC) was assessed with the ProCheck moisture sensor (Decagon Devices, WA, USA). The Estonian Environmental Research Centre in Tartu conducted chemical analyses of the soil, which included measurements of total N, total C content, and

pH. For the determination of nitrate (NO_3^--N) levels in the soil samples, extraction was performed using 2 M KCl at a ratio of 1:10. These levels were then analyzed through flow-injection analysis following standard methods [55].

DNA extraction and metagenome sequencing

Prior to DNA extraction, the canopy soil and plant samples were finely crushed using a coffee grinder. The grinder components were sterilized with 70% ethanol between each sample to avoid contamination. The DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) was utilized to extract DNA from 0.25 g of soil and 0.12 g of canopy soil and plant material, adhering to the manufacturer's guidelines. All samples—soil, canopy soil, stem, and leaves—were homogenized with lysis buffer using the Precellys 24 Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000 rpm for 20 s. For the canopy soil and plant samples, the amount of lysis buffer (CD1) was increased to 50–70% to enhance the lysate yield. The concentration and quality of the DNA extracted were assessed using a Tecan AG Infinite spectrophotometer before storing the samples at − 20 °C.

Metagenome sequencing was performed on 36 samples from various ecosystems, including soils ($n = 12$), canopy soils ($n = 14$), and leaves ($n = 10$). Library preparation and sequencing were performed by Biomarker Technologies (BMK) GmbH (Münster, Germany). Briefly, the constructed library was sequenced on the Illumina NovaSeq X platform using a paired end (PE) 150 bp strategy. Details of metagenome data processing are described in the Supplementary material in the 'Extended methods' section.

Quantitative polymerase chain reaction (qPCR)

The bacterial and archaeal 16 S rRNA genes were quantified using RotorGene® Q equipment (Qiagen, Hilden, Germany). The *mcrA* gene, which encodes the MCR enzyme, was quantified to determine the abundance of methanogenic microbes. Similarly, the *pmoA* gene, which encodes the pMMO subunit, was quantified to assess the abundance of methanotrophic microbes. The n-DAMO 16 S rRNA gene was also quantified to evaluate the presence of nitrate/nitrite-dependent anoxic methane-oxidizing microbes.

Primers and qPCR program settings are described in Table S1. Each qPCR run included both samples and negative controls. The data obtained were analyzed using the RotorGene Series Software program (version 2.0.2, Qiagen, Hilden, Germany) and the LinRegPCR application (version 2020.0). Gene copy numbers were calculated from the samples' threshold cycles and normalized based on the samples' dry weight%. The results are expressed as gene copies per gram of dry weight of the sample (copies g^{-1} dw). Details of qPCR data processing are described in [56].

Data analysis

For statistical analyses, the primary factor for comparison in soil used was the type of forests (Erica and Mixed). However, within the Mixed forest, we also categorized the sites based on the dominance of tree species to examine the intra-forest variability. The sample type (canopy soil, leaf, and stem) was used as the main factor in the above-ground compartments. Gene copy number data was assessed for normality using Shapiro-Wilk normality tests in Jamovi [57], and was later normalized by $\log_{10}$ transformation. However, robust analyses were employed to account for any violation of the homogeneity of variances, and Welch's ANOVA was performed coupled with Games-Howell pairwise post hoc tests. The main effect tested was the ecosystem (forest type) in the case of soil fluxes and gene copy numbers in soil samples, while sample type was the main factor in the case of gene copy numbers in above-ground samples. Moreover, species was also tested as a factor regarding tree stem fluxes to observe species-specific differences. Welch's t-test was also employed in cases where ANOVA could not be performed due to insufficient data or the absence of variance in one group. Welch's ANOVA and t-tests were performed in Jamovi and using the *ggstatsplot* package in RStudio [58], and all figures (boxplots, PCA, and heatmap) were created using the *ggplot2* package in RStudio [59]. SEM was performed to observe ecological interactions regarding moisture-temperature regulation, microbial mediation of GHG fluxes, and biogeochemical feedback. For the structural equation model (SEM), we used *lavaan* and *semplot* packages in the R [60]. The Chi test and the comparative fit index (CFI) were used to check the model's performance.

Results

Soil and tree stem fluxes

The soil in both forests was a net sink of atmospheric CH_4 (Fig. 1a). Mean soil CH_4 flux in the Erica forest was measured at -22.4 ± 4.20 (mean ± SE μg C m^{-2} h^{-1}). At the location in the mixed forest, where *E. reunionensis* predominated, the uptake was recorded at -33.7 ± 15 μg C m^{-2} h^{-1}, while the mean CH_4 flux in the location dominated by tree fern *A. glaucifolia* was -16.7 ± 9.88 μg C m^{-2} h^{-1}. The mean soil CO_2 fluxes in the Erica forest were recorded at 61.2 ± 7.57 mg C m^{-2} h^{-1}. In the mixed forest, the soil at the site dominated by *E. reunionensis* produced a mean flux of 58.6 ± 13.7 mg C m^{-2} h^{-1}, whereas the site dominated by *A. glaucifolia* had a mean flux of 41.1 ± 7.93 mg C m^{-2} h^{-1} (Fig. 1b). The difference between the two forests was statistically insignificant

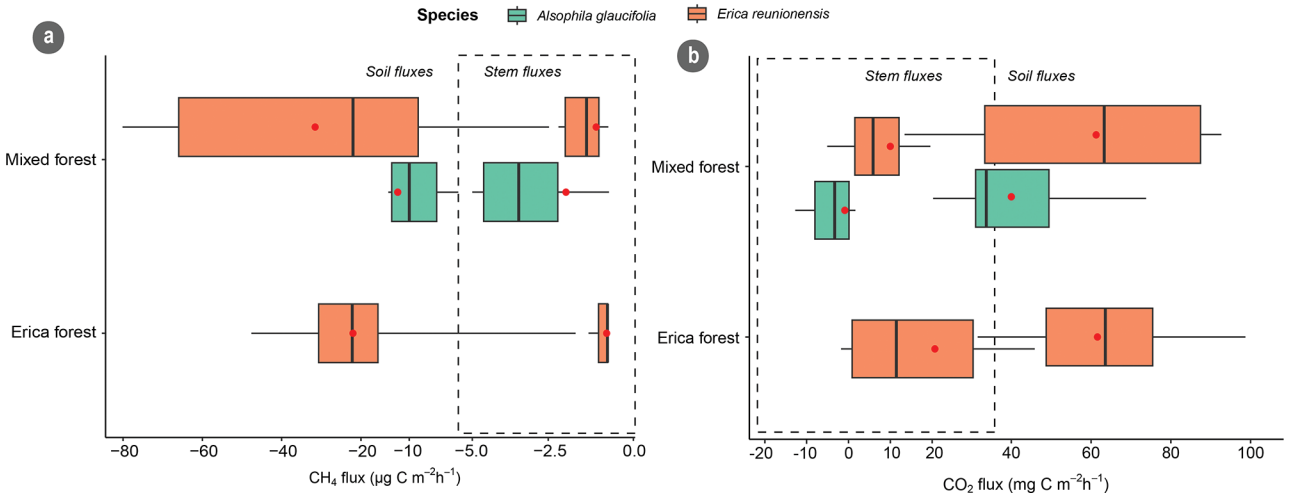

Fig. 1 Boxplot illustrating **(a)** CH_4 fluxes and **(b)** CO_2 fluxes from the soil and stems. The colors indicate the dominant plant species in soil and those related to stem fluxes. The box illustrates the interquartile range (IQR), encompassing the 25th and 75th percentiles of the data distribution. The lines extending from the box, referred to as whiskers, show the data range that falls within 1.5 times the IQR. The bars within the box indicate the median. The red dots represent the mean values

regarding the soil CH_4 ($F(1, 15.6) = 0.08$, $p = 0.782$) and CO_2 flux ($F(1, 21.9) = 1.07$, $p = 0.312$).

The stems of *E. reunionensis* in the Erica forest exhibited mean CH_4 fluxes of -0.31 ± 0.17 and -0.96 ± 0.67 μg C m^{-2} h^{-1} in the mixed forest, while the mean CH_4 flux from the tree fern *A. glaucifolia* stem was -1.53 ± 1.10 μg C m^{-2} h^{-1}. Overall, stems in both forests were weak sinks for CH_4. However, for CO_2, the mean flux from *E. reunionensis* in the Erica forest was 20.9 ± 8.30 mg C m^{-2} h^{-1}, while in the mixed forest, it was 10.5 ± 2.59 mg C m^{-2} h^{-1}. The CO_2 fluxes from the *A. glaucifolia* stems were lower and recorded at -1.10 ± 2.47 mg C m^{-2} h^{-1}. The difference between the two forests was statistically insignificant regarding the overall stem CH_4 ($F(1, 17.47) = 2.10$, $p = 0.165$) and CO_2 flux ($F(1, 5.80) = 2.93$, $p = 0.140$). The difference between species-specific CO_2 fluxes was significant between *E. reunionensis* and *A. glaucifolia* ($F(1, 19.46) = 12.69$, $p = 0.002$), while it was insignificant for CH_4 fluxes ($F(1, 6.52) = 0.47$, $p = 0.516$).

Microbial abundance of methanogens and methanotrophs in soil

The gene copy numbers of *mcrA* (methanogenesis) were significantly higher in mixed forest soil as compared to the Erica forest soil ($F(1, 21.6) = 16.4$, $p < 0.001$, Fig. 2a). The *pmoA* (aerobic methanotrophy) genes were abundant in the mixed forest soils while not detected in the Erica forest soils (hence Welch's t-test was performed instead of ANOVA), but the difference was statistically insignificant (Welch's $t(11.0) = -2.07$, $p = 0.062$). However, the difference was statistically significant when $\log_{10}$ transformed values of *pmoA* abundance were compared in Welch's t-test (Welch's $t(11.0) = -23.63$, $p < 0.001$). In contrast, the n-DAMO 16 S rRNA (anaerobic methanotrophy) gene was found in greater abundance in the Erica forest soil ($F(1, 15.2) = 10.9$, $p = 0.005$, Table S2).

The metagenomic analyses show that key genes involved in CH_4 oxidation, *pmoAB*, encoding pMMO, are observed in only soil samples, irrespective of the forest type, while *mmoC*, encoding soluble methane monooxygenase (sMMO) are present across all samples (Fig. 3). Furthermore, among key genes for methanol oxidation, *xoxF* encoding lanthanide-dependent methanol dehydrogenase, was found to be the most abundant in all soil samples. These circumstances are likely associated with the abundance of n-DAMO 16 S rRNA quantified from qPCR. Across different forests, all soil samples show a similar pattern of relative abundance of key genes for methanogenesis (including *mcr*/*mcrA*, *mtbC*, *mtmC*, *mtr*/*mtrABCR*, and *mttBC*). It was observed that *mtrAB*, involved in CH_4 production via hydrogenotrophic or acetoclastic methanogenesis, showed a higher relative abundance than *mtbC*, *mtmC*, and *mttBC* (Fig. 3), which is associated with CH_4 production via the methylotrophic pathway.

Microbial abundance of methanogens and methanotrophs in above-ground samples

In the mixed forest, high gene copy numbers of the *mcrA* gene were found in canopy soil samples (Table S3). The *pmoA* gene was also detected in the canopy soils but in less abundance than the *mcrA* and n-DAMO 16 S rRNA genes. Additionally, n-DAMO exhibited high gene copies in the canopy soils as well. The gene copy numbers were higher in canopy soils than in leaves and stems for *mcrA*, *pmoA*, and n-DAMO 16 S rRNA (Fig. 2d-f).

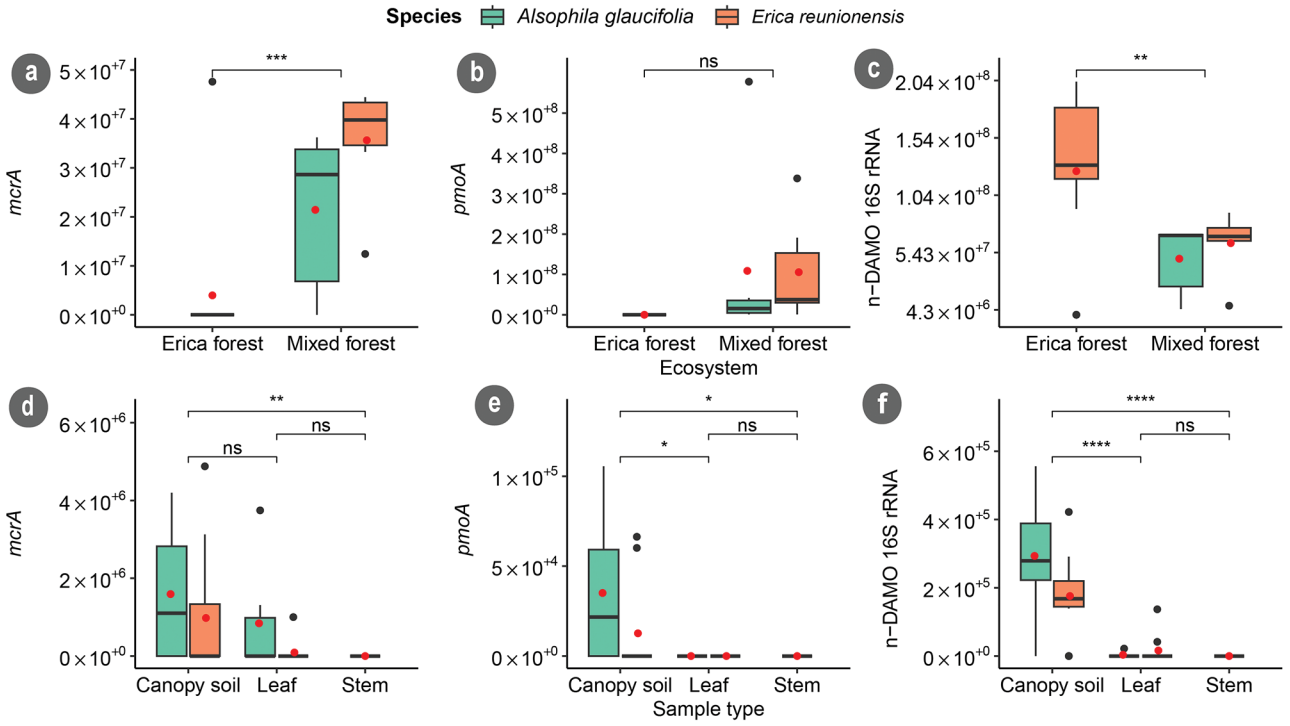

Fig. 2 Abundances of genes (g^{-1} dry weight) involved in methanogenesis (*mcrA*) and methanotrophy (*pmoA* and n-DAMO 16 S rRNA) in soil (**a-c**) and plant samples (**d-f**) quantified in qPCR. Different colors represent the species dominant in the sites in a-c, while the related species in d-f. The box depicts the interquartile range (IQR), which captures the 25th and 75th percentiles of the data. The whiskers that extend from the box represent the data range within 1.5 times the IQR. Inside the box, a line indicates the median value of the data set, while the red dots represent the mean values. Black dots are the outliers. The pairwise relationships are indicated by *ns* (not significant), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$)

The leaves of *A. glaucifolia* showed a high abundance of the *mcrA* gene. In the Erica forest, the *mcrA* gene was detected in the leaves of *E. reunionensis*. The *pmoA* gene was not found at the detectable limit for qPCR in any above-ground sample from this forest; however, the n-DAMO 16 S rRNA gene was detected in two leaf samples. However, the *mcrA* gene was not detected in the leaves of *E. reunionensis* in this forest.

The metagenomics analyses of the above-ground samples revealed that the leaves of *E. reunionensis* had a higher relative abundance of the *mtr* gene as compared to the leaves of *A. glaucifolia*. However, the leaves of *A. glaucifolia* contained a higher abundance of *mtrA* and *mtrB* genes (Fig. 3). All these genes are involved in methanogenesis and were consistently abundant in the above-ground samples. Canopy soils showed the highest read counts of the methanogenic genes (*mcrA*, *mtr*, *mtrA*, and *mtrB*) as compared to leaves or soil samples.

In the cloud forests' soils, the ratio of the quantified *mcrA* gene to the product of *pmoA* and n-DAMO 16 S rRNA genes was found to be less than one, indicating a relatively higher abundance of methanotrophic genes in the soil as compared to methanogenic gene *mcrA* (Fig. 4a). However, in plant samples and canopy soil, the *mcrA* gene exhibited a higher abundance compared to the *pmoA* and n-DAMO genes.

Relative abundance of key CH_4-cycling microbial genera.

Based on the classification of small subunit (SSU) rRNA gene sequences obtained from metagenomes, more CH_4-cycling microbial genera (e.g., methanogens, methylotrophs, and methanotrophs) were detected in soil and canopy soil compared to leaf samples (Fig. 5a). It should be noted that these estimations may be biased by the potential fragmentation of SSU rRNA sequences and the limited coverage inherent to metagenomic data. The relative abundance of alphaproteobacterial methane oxidation bacteria (alpha-MOB) (0.1–3.3%) was observed across all samples from both forests, while verrucomicrobial MOB (the relative abundance of 0.1–3.1%) was observed in soil and canopy soil samples across Mixed forests. The NC-10 phylum (the relative abundance of 0.1–0.3%, Fig. 5a), associated with the n-DAMO process, was detected only in soil samples from Erica forest dominated by *E. Reunionensis*. Regarding leaf samples obtained from different forests, the abundance of CH_4 cycling microbial genera was detected only in leaf samples from the mixed forests dominated by *A. glaucifolia*, showing the presence of alpha-MOB and methylotrophs. Furthermore, the relative abundance of alpha-MOB shows a negative relationship with CH_4 fluxes, while positive relationships were observed between the relative

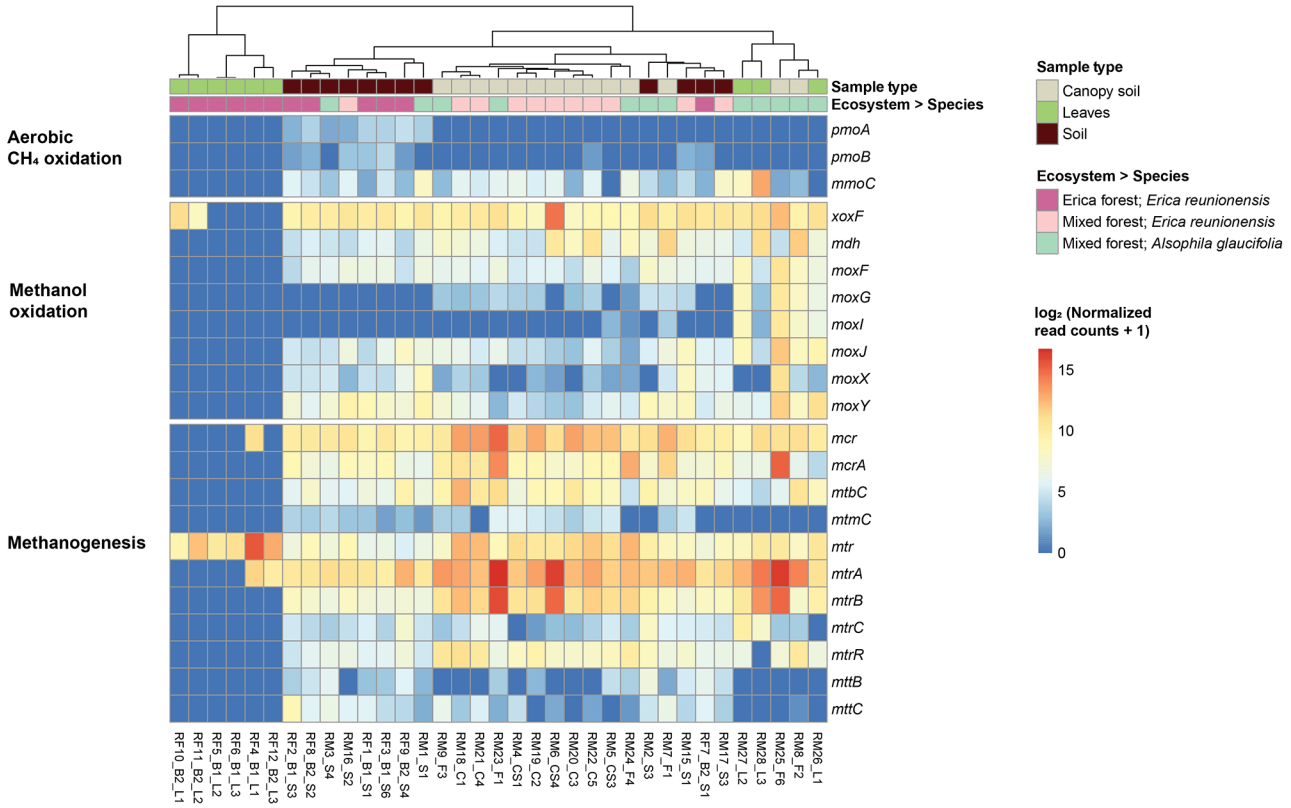

Fig. 3 Functional gene abundance across soil, canopy soil, and leaves samples. The row-scaled heatmap represents the $\log_2$-transformed normalized read counts for key functional genes for methanogenesis, aerobic methanotrophy, and methanol oxidation

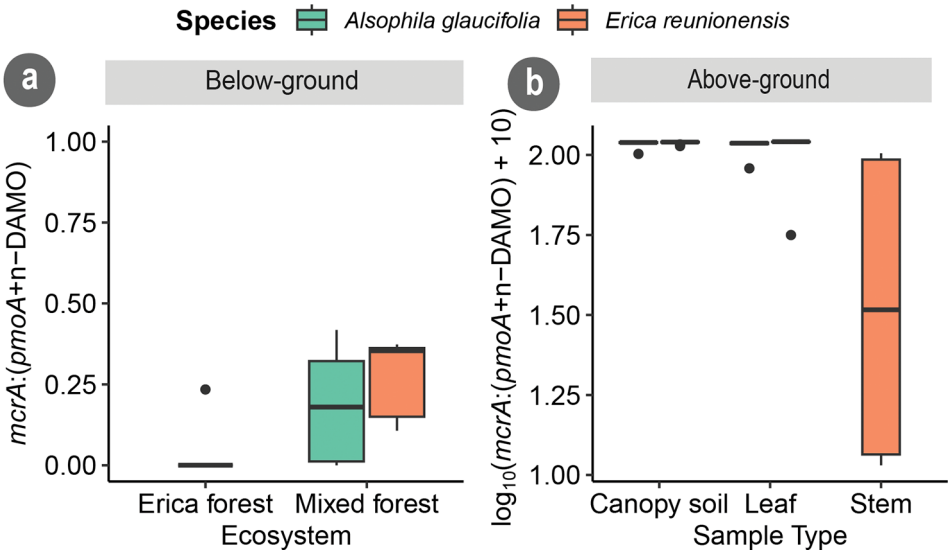

Fig. 4 The ratios of the microbial methanogenic (*mcrA*) to methanotrophic genes (*pmoA* and n-DAMO 16 S rRNA) **(a)** in peat soil and **(b)** in above-ground samples

abundance of NC-10 phylum and soil NO_3^- and NH_4^+ (Fig. 5b).

The SEM (Fig. 5c) had CFI = 0.984, Root Mean Square Error of Approximation (RMSEA) = 0.046, and Standardized Root Mean Square Residual (SRMR) = 0.109, demonstrating an excellent fit. It revealed a significant negative covariance between CH_4 and CO_2 (Estimate = -0.39, $p = 0.038$), indicating an inverse relationship between these two gas fluxes. SWC and the soil temperature were also inversely related (Estimate = -0.50,

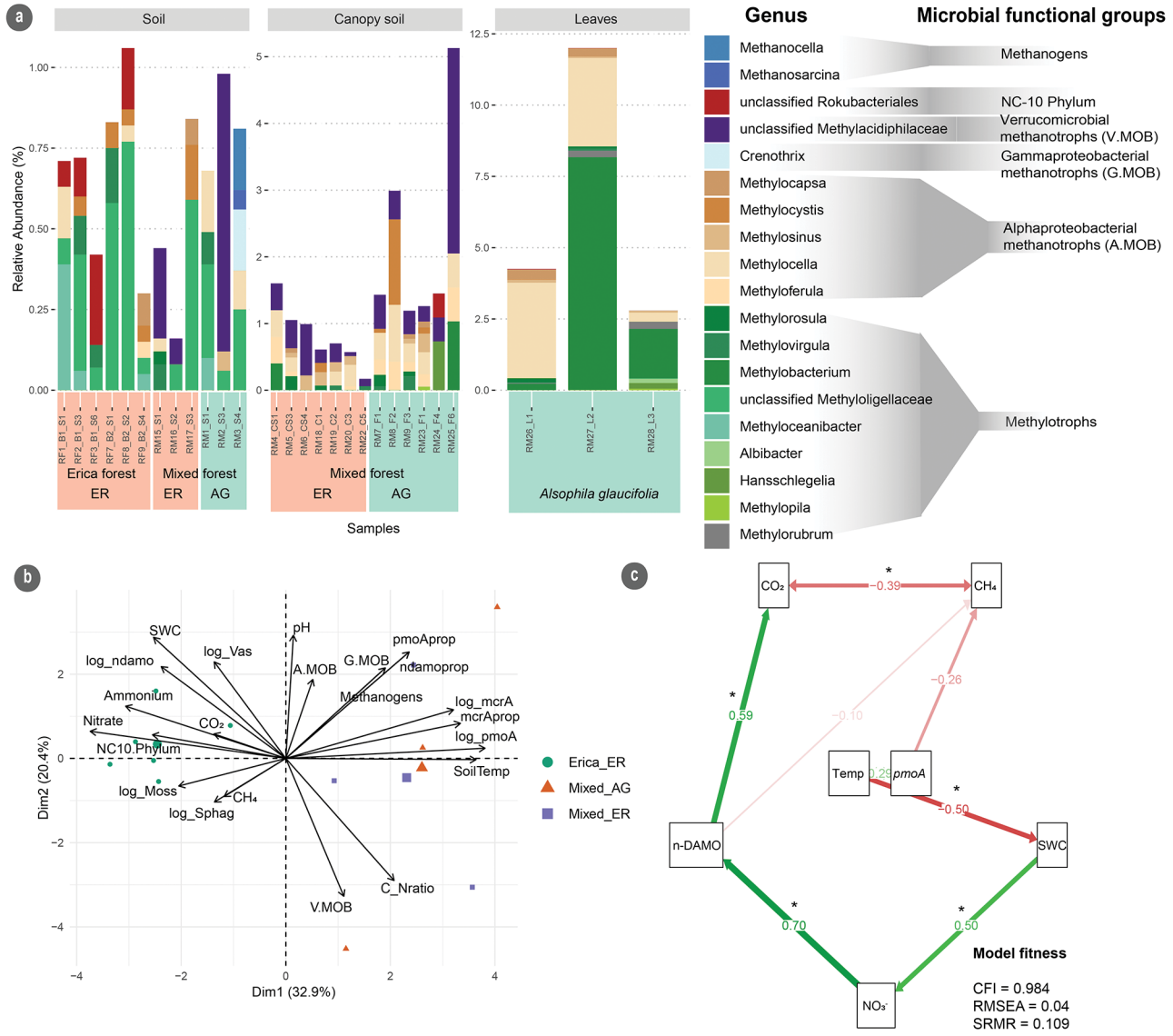

Fig. 5 **(a)** Relative abundance of small subunit rRNA (SSU) sequence of key CH_4 cycling microbial genera (including methanogenic archaea, methanotrophs, and methylotrophs) in soil, canopy soil, and leaves across tropical cloud forests: Erica Forest dominated by *E. reunionensis* (Erica_ER), Mixed Forest dominated by *E. reunionensis* (Mixed_ER), and dominated by *A. glaucifolia* (Mixed_AG). **(b)** Clustering of the forest sites based on principal component analysis biplot of soil variables, including physiochemical properties, gene copy numbers, and microbial functional groups. Big triangles represent the mean values, and colors represent the forest sites. **(c)** Structural Equation Model (SEM) for the soil CH_4 and CO_2 fluxes, including key controlling factors, i.e., SWC, soil NO_3^-, n-DAMO, and *pmoA* gene abundances. Red lines show negative correlations, while green lines show positive correlations. Asterisks represent the significant relationships

$p = 0.004$). The n-DAMO abundance had an insignificant negative correlation with CH_4 while a positive correlation with CO_2 (Estimate = -0.40, $p = 0.05$). Soil NO_3^- had a significant positive effect on n-DAMO abundance (Estimate = 0.70, $p = 0.0001$). The relationship between *pmoA* and CH_4 was also negative (Estimate = -0.262, $p = 0.130$), yet there was no correlation between *pmoA* and CO_2 resulting from soil respiration.

Discussion

The cloud forest peat soils are CH_4 sinks

In this study, we observed CH_4 uptake by the peat soil of the cloud forests. Meanwhile, these soils emitted CO_2 (Fig. 1). The mean uptake values in the sites dominated by *E. reunionensis* were consistent in both forests and greater than the sites dominated by *A. glaucifolia*. Soil and tree stem CH_4 fluxes were similar to those from a previous study conducted on the same island but in a different ecosystem [16]. Soil moisture in other ecosystems is a critical factor influencing the forest soil's capacity to

function as a CH_4 sink [61]. In our study, the SWC varied between 0.2 and 0.6 (m^3 m^{-3}); however, no correlation was found between SWC and CH_4 flux values. Conversely, the SWC showed a positive correlation with all microbial genes (*mcrA*, *pmoA*, and n-DAMO 16 S rRNA) as well as the relative abundance of key CH_4-cycling microbes (NC-10 and methylotrophs), indicating that microbial abundance was higher in moderately moist soil (Fig. 5b). The high soil moisture affects both methanogens and methanotrophs abundance and community structures [62]. Previous studies have demonstrated that, globally, temperature affects the soil emission of CH_4 rather than its uptake [3, 63]. Elevated temperatures often result in drier soil conditions, creating more oxic conditions in soil that enhance CH_4 oxidation through methanotrophic processes. However, very low SWC can also decrease the activity of these microbes. Research indicates that at temperatures below or above the optimum (ca. 25 °C), both the abundance [64] and the activity of methanotrophs tend to decrease [65]. The influence of temperature increase has also been shown to shift CH_4-cycling community composition in upland forest soils [66]. Our study demonstrated negative correlations between soil temperatures and gene abundances (Fig. 5b), indicating that warmer and drier soils contained fewer microbes involved in both methanogenesis and methanotrophy. The highest gene abundances were found at the SWC between 0.5 and 0.6 m^3 m^{-3}, which is in accordance with the previous studies in tropical forests [67].

In soils of cloud forest, the n-DAMO 16 S rRNA gene showed a strong positive correlation with SWC and NO_3^- levels (Fig. 5c). The relative abundance of NC-10 phylum also had a positive relationship with soil NH_4^+ and NO_3^- levels, indicating the potential for nitrate-dependent methanotrophy in soil. In this pathway, the microbes involved would obtain oxygen from the reduction process of the nitrogen oxides (NO_3^- and NO_2^-) to N_2 and O_2 (denitrification) and use this oxygen to oxidize the CH_4 [48, 49]. In peatlands rich in inorganic nitrogen, most of the n-DAMO process occurs within the topsoil [68]. We observed elevated soil NO_3^- levels in forest soils dominated by *E. reunionensis*. This could be due to high archaeal nitrification rates, coupled with *E. reunionensis* preference for NH_4^+ as its N source [69]. Consequently, soils in the forests dominated by *E. reunionensis* provide a conducive environment for the n-DAMO process. In both erica and mixed forests, the soil CH_4 sink values remained similar in sites dominated by *E. reunionensis*. This indicates that CH_4 oxidation is primarily influenced by the NO_3^- levels in the soil, suggesting that n-DAMO may serve as a key driver of this process.

SEM analysis was utilized to understand the processes explaining CH_4 fluxes, particularly focusing on the contributions of aerobic methanotrophy and n-DAMO in CH_4 oxidation. In general, the *pmoA* gene-driven methanotrophy is considered the main player for CH_4 oxidation, but due to recent research on the n-DAMO process' contribution to CH_4 oxidation in peat soils [68], we were interested in investigating this in our data. The SEM analysis revealed a negative correlation between n-DAMO gene abundance and CH_4 flux values while positively correlated with soil CO_2 flux values, indicating CH_4 oxidation. The correlation was also positive between NO_3^- and n-DAMO gene abundances, showing that the main source of oxygen was NO_3^- in this CH_4 oxidation pathway. We also observed a negative correlation between the abundance of *pmoA* and CH_4 flux, but it did not show any contribution to CO_2 production via oxidation (Fig. 5c). This shows that n-DAMO was a major contributor to the overall CH_4 oxidation process, followed by the aerobic CH_4 oxidation. Moreover, under sporadic anaerobic conditions, which could occur due to increased soil moisture (in the wet season or precipitation), n-DAMO can remain the dominant process until the ammonia oxidation (nitrification) is ceased due to prevailing anoxic conditions in the soil, and when the remaining soil NO_3^- is denitrified. In such conditions, if the pH becomes less acidic, the activity of n-DAMO also can increase [70].

While the Erica forest soil contained the highest gene copies of the 16 S rRNA gene and relative abundance of NC-10 phylum bacteria, the mixed forests had *pmoA*-containing verrucomicrobial MOB and alphaproteobacterial MOB as their dominant methanotrophic groups in different samples, followed by methylotrophs (Figs. 2 and 5a). Verrucomicrobial MOB are a very common type of methanotroph in tropical soils [71], and some of these bacteria have also been found to be active in extremely acidic environments [72, 73]. Our studied sites also had acidic pH ranging between 3 and 5. Alphaproteobacterial MOB are also very common methanotrophs, usually living in the junction zones of oxic and anoxic parts of soils, and can survive the shortage or the excess of CH_4 by showing facultative as well as mixotrophic capabilities [74, 75]. MOB can vary through the soil depths, but the highest potential is found to be in the top 15 cm [76], which was also similar to the depth where we sampled. In both cloud forest soils, the *mcrA* to *pmoA* and n-DAMO 16 S rRNA gene ratios remained consistently below 1 (Fig. 4). This highlights an overall dominance of methanotrophs in these soils, further supported by the relative abundance of methanotrophic groups.

The role of above-ground compartments in CH_4 cycling

In our study, we observed that the stems of both species in the two forests functioned as weak sinks for CH_4. Despite the absence of methanotrophic genes in their core samples, stems of *A. glaucifolia* absorbed more CH_4 than those of *E. reunionensis*. This may indicate

that the microorganisms living on the bark are responsible for the methanotrophy instead of those living inside of stems. Notably, the canopy soil on the surfaces of *A. glaucifolia* stems contained a significant abundance of *pmoA* and n-DAMO genes. Previous research has indicated that microbial communities residing on stem surfaces or within wood crevices can oxidize atmospheric CH_4 [8], and it has been reported in many upland forests globally where the wood was found to be a significant CH_4 sink [6]. Additionally, *pmoA* and n-DAMO genes were also detected within the canopy soils on the stems of *E. reunionensis*, indicating their potential to oxidize CH_4. No functional gene was detected in the stem core samples which shows that the microbial activity is more likely to occur on the stem surfaces, cryptogams, and in the wood crevices. This finding is inconsistent with the findings of a study where methanogenic communities were detected in the heartwood of *Populus deltoides* [18]. The variability between different tree species in terms of functional gene presence or abundance may arise from the diverse tree physiology traits (stem moisture content, stem density, and bark characteristics) [77].

Like soil, canopy soil also had fewer *pmoA* than n-DAMO genes (16 S rRNA). Nitrogen deposition and microbial nitrification have been reported in forest canopies in different ecosystems [78–80]. The NO_3^- in the canopy can be utilized by the n-DAMO in canopy soils to oxidize the CH_4. The canopy soil also had a significant abundance of the methanogenesis genes *mtrA*, *mtrB*, *mcr*, and *mcrA*, which indicates that during anoxic conditions, the stems that carry the cryptogamic canopy soil may be a source of CH_4 as the ratio of methanogenic to methanotrophic gene abundances was greater than one in the canopy soils (Fig. 4b).

The surfaces of leaves, or the phyllosphere, host a variety of microbial communities that play a significant role in ecosystem functions and contribute to nutrient cycling [81]. This field of study needs exploration because its contributions to global greenhouse gas budgets are largely overlooked. Our study found the *mcrA* and *pmoA* genes through qPCR in several individual samples, although their gene copy numbers varied across the samples. Notably, all leaves of *A. glaucifolia* from the mixed forest exhibited a significant abundance of the *mcrA* and *mtr* genes in qPCR as well as metagenomics (Fig. 3). Furthermore, the ratio of *mcrA* to the combined levels of *pmoA* and n-DAMO was higher in these leaves than in all other above-ground samples (Fig. 4). This finding suggests that the phyllosphere of *A. glaucifolia* has metabolic potential to emit CH_4. Additionally, the *mtr* gene was consistently found abundant in all leaf samples from the cloud forests, further indicating a potential for methanogenesis in the phyllosphere of other leaves. A similar trend was also found in the phyllosphere of Norway spruce (*Picea abies*) in the boreal ecosystem, where metagenomic analyses revealed the presence of methanogenic archaea and methanotrophic bacteria in the phyllosphere [17]. In addition to the detection of methanogenic genes in leaves by qPCR, the analysis of the SSU rRNA revealed that the leaves of *A. glaucifolia* also contained methanotrophic genera (Fig. 5a), highlighting a potential role of leaves in both microbial CH_4 production and consumption. Previously, the 16 S rRNA gene sequencing has revealed significant differences in the communities of methanotrophs found in the phyllosphere of different tree species [82]. The presence of methanotrophic microbes in the phyllosphere has been confirmed by isolation and culturing previously; however, like ours, many studies could not amplify the *pmoA* gene in the DNA extracted from leaves [83].

This study combined qPCR and metagenomics approach to target the phyllosphere microbiome, and the findings regarding the presence of methanogens and methanotrophs are unprecedented in terms of CH_4 cycling in tropical forests. This warrants further research focusing on the phyllosphere microbial communities and the shoot flux measurements to estimate the activity of methanogens or methanotrophs based on the oxic or anoxic conditions resulting from different climatic conditions.

Conclusion

Our study found that peat soil and tree stems in cloud forests act as sinks for CH_4 and exhibit high methanotrophic potential. However, the microbial methanotrophic genera detected in the soil differ from those in the above-ground forest compartments. In the peat soil, NC-10 bacteria were in high abundance in the forest featuring *E. reunionensis*, which suggests that the anaerobic methanotrophy (n-DAMO) may primarily drive CH_4 oxidation there. Meanwhile, peat soil and the canopy soils in the mixed forest are mainly dominated by aerobic alphaproteobacterial and verrucomicrobial methanotrophs, respectively. Additionally, leaves from both types of forests show a high abundance of the *mtr* gene, while leaves of *A. glaucifolia* exhibited an abundance of the *mcrA* gene alongside the detected methanotrophic genera. Our results indicate that soil in the Erica forest, which is abundant in the NC-10 phylum, can act as a CH_4 sink even under anaerobic conditions induced by precipitation (during the wet season). In contrast, the phyllosphere in these forests can release CH_4 under similar conditions due to the presence of methanogens.

Supplementary Information

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Supplementary Material 1

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Author contributions
FAK, ÜM, and ME conceptualized and designed this study. FAK, ÜM, ME, RR, MÖ, KS, CAP, and KK participated in sampling. FAK, RK, and ME performed laboratory procedures, data analyses, bioinformatics, and visualization. FAK prepared the first draft of the manuscript, and all co-authors, including MM and SAMT, participated in the editing and preparation of the final draft.

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Data availability
The metagenomic sequencing data that support the findings of this study has been deposited in NCBI's SRA under Bioproject number PRJNA1217467 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1217467. The processed metagenomic data is accessible at https://www.ebi.ac.uk/biostudies/studies/S-BSST1945.

Declarations

Competing interests
The authors declare no competing interests.

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