Science of the Total Environment 957 (2024) 177015

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Microbial landscapes in *Trinervitermes trinervoides* termite colonies are affected by mound compartments and soil properties but not by symbiotic *Podaxis* fungi

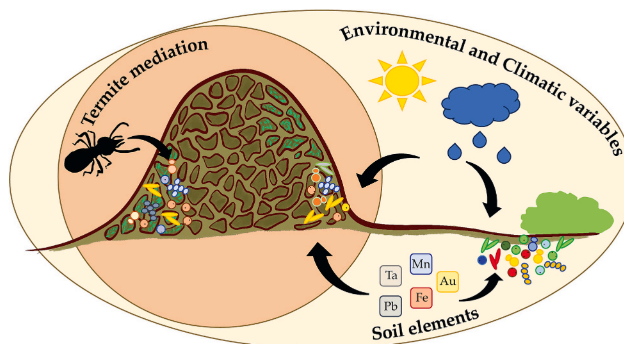
Kasun H. Bodawatta [a], Lorrie Maccario [b], Nils Peereboom [c], Benjamin H. Conlon [c], Guangshuo Li [c], Tamás Plaszkó [d,e], Celia Vinagre-Izquierdo [f], Knud A. Jønsson [g], Risto M. Vesala [h], Z. Wilhelm de Beer [i], Anders Priemé [b,d], Michael Poulsen [c,*]

[a] Section for Molecular Ecology and Evolution, Globe Institute, University of Copenhagen, Copenhagen, Denmark
[b] Section of Microbiology, Department of Biology, University of Copenhagen, Copenhagen, Denmark
[c] Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Copenhagen, Denmark
[d] Center for Volatile Interactions, Department of Biology, University of Copenhagen, Copenhagen, Denmark
[e] Section for Terrestrial Ecology, Department of Biology, University of Copenhagen, Copenhagen, Denmark
[f] Conservation and Evolutionary Genetics Group, Estación Biológica de Doñana (EBD-CSIC), Seville, Spain
[g] Department of Bioinformatics and Genetics, Swedish Museum of Natural History, Stockholm, Sweden
[h] Finnish Museum of Natural History, University of Helsinki, Helsinki, Finland
[i] Department of Biochemistry, Genetics and Microbiology, Forestry and Agriculture Biotechnology Institute, University of Pretoria, Pretoria, Gauteng 0001, South Africa

HIGHLIGHTS

- Termites and their symbionts impact soil microbiomes with ecosystem consequences
- We investigated factors shaping *T. trinervoides*-associated microbiomes in African savannahs
- Symbiotic *Podaxis* fungi do not affect microbiomes within and around mounds
- Termites, abiotic variables, and soil elements play strong roles in microbiome structure
- Soil microbiomes are governed by abiotic and geochemical properties, and secondarily by termite modulation

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Abasiofiok Mark Ibekwe

Keywords:
ICP-MS
MiSeq amplicon sequencing
Symbioses

ABSTRACT

Termites are important ecosystem engineers and play key roles in modulating microbial communities within and outside their mounds. Microbial diversity within termite mounds is generally lower than surrounding soils, due to termite-associated antimicrobial compounds and active sanitary behaviours. Microbial symbionts of termites can also influence the microbial landscape, by inhibiting or out-competing other microbes. Certain members of the arid habitat fungal genus *Podaxis* (Agaricomycetes; Agaricaceae) are symbiotic with savannah specialist grass-cutting termites, and have the potential to influence mound-associated microbiomes. To test this, we

* Corresponding author.
E-mail address: mpoulsen@bio.ku.dk (M. Poulsen).

https://doi.org/10.1016/j.scitotenv.2024.177015
Received 20 April 2024; Received in revised form 15 October 2024; Accepted 15 October 2024
Available online 24 November 2024
0048-9697/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Bacteria
Fungi

characterized fungal (ITS2) and bacterial (16S rRNA) communities within and outside 49 *Trinervitermes trinervoides* mounds with and without *Podaxis* fruiting bodies across a 1000 km transect in South Africa. We predicted that *Podaxis* would be a dominant member of the fungal communities in mounds and negatively impact microbial diversity. Further, we explored how environmental variables shaped microbial communities, including whether soil elemental composition affected *Podaxis* presence. As expected, we observed less diverse fungal communities, but not bacterial communities, within than outside mounds, while microbial communities differed by sampling regions and mound compartments. *Podaxis* sequences were present in 48 out of 49 mounds in low relative abundances, and neither fruiting body presence nor sequence abundance were associated with microbial diversity or composition. There was, however, an overall association between the presence of *Podaxis* fruiting bodies and elemental composition, with different elements displaying varying associations depending on geographic region. Both environmental variables and soil elements were associated with fungal and bacterial taxa, indicating that they are key drivers of microbial community composition. Taken together, our findings suggest that microbial landscapes in termite mounds are not strongly influenced by *Podaxis* but mainly driven by termite filtering and regional abiotic variables and elemental compositions.

1. Introduction

Termites are among the most successful invertebrates on the planet with an astounding 50 Mt of global carbon captured in termite biomass, surpassing even that of birds (Bar-On et al., 2018). Termites often dominate insect communities in terrestrial ecosystems, where they act as important ecosystem engineers, changing physical soil structures (Jouquet et al., 2007; Jouquet et al., 2016), modulating abiotic conditions (Sanderson, 1996), and affecting water and nutrient availabilities for other organisms (Dangerfield et al., 1998; Jouquet et al., 2011). Environmental modulations from termite activities extend beyond changes to the physicochemical properties of soils and include shifting microbial community compositions (Jouquet et al., 2005; Li and Greening, 2022). For example, microbial composition and richness in soil within termite mounds, galleries, and extended foraging sheaths tend to differ from those of the surrounding soil (Chen et al., 2021a; Chen et al., 2021b; Chiri et al., 2020; Li and Greening, 2022; Yan et al., 2021). These differences can be driven by several termite-modulating factors, including treatment of mound soil with antimicrobial compounds (Rosengaus et al., 2000), changing physicochemical properties (Van Thuyne et al., 2021; Van Thuyne and Verrecchia, 2021), including soil nutrients and pH (Chen et al., 2021b) and the release of termite waste products (e.g., CH_4, H_2, and CO_2) (Chiri et al., 2020; Chiri et al., 2021; Schmidt et al., 2023). Lastly, termite mounds and surrounding soil environments may overlap in microbial taxa due to the recruitment of microbes to termite mounds or the dissemination of termite-associated microbes to the environment (Li and Greening, 2022).

Termite species in the genera *Trinervitermes* and *Nasutitermes* (subfamily Nasutitermitinae; Blattodea, Termitidae) are mound-building termites that modulate the physical environment of their habitats (Sileshi et al., 2010) and cut grass for nutrition that is stored within mounds (Adam et al., 2008). Some species in these genera furthermore engage in symbioses with species of basidiomycete fungi in the genus *Podaxis* (Agaricomycetes; Agaricaceae), although the benefits or costs of association remain unknown (Conlon et al., 2016). *Podaxis* is frequently observed growing from the grass chambers of the mounds, even in healthy colonies (Conlon et al., 2016). Four termite-specific *Podaxis* species have been described (Li et al., 2023), and reductions in fungal genome size and metabolic capacities indicate adaptations to live symbiotically with termite hosts (Conlon et al., 2021). Despite termite-associated species potentially having a reduced chemical repertoire (Conlon et al., 2021), *Podaxis* is a prolific producer of specialised metabolites (Guo et al., 2022). However, the prevalence of *Podaxis* and potential effects of these antimicrobials on bacterial and fungal community compositions within and outside mounds are unknown.

Here, we investigated factors shaping *Trinervitermes*-associated microbial communities through MiSeq amplicon sequencing of fungal and bacterial communities in different locations within mounds (grass chambers and soil from within and outside mounds) from 49 *Trinervitermes trinervoides* colonies along an approximately 1000 km North-South transect in South Africa (Fig. 1A). First, we tested the hypothesis that termites and the nest environment would affect microbiome structure, which would lead to richness and compositional differences between grass chambers, mound soil, and outside soil, with mound wall soil being least rich due to soil density and the application of termite antimicrobial compounds. Second, we hypothesised that *Podaxis* would be more abundant within than outside termite mounds and dominate fungal communities in grass chambers where *Podaxis* grows from. If *Podaxis* is a key associate of *Trinervitermes*, we expected it to be central and a highly connected member of microbial networks. Third, if competition with *Podaxis* negatively impacts other microbes, we expected reduced microbial diversity in colonies with *Podaxis*, and differences in community structure between mounds with and without *Podaxis*. We tested for effects of *Podaxis* based on both visual presence/absence of *Podaxis* fruiting bodies on colonies and relative abundance of *Podaxis* sequences from amplicon sequencing data. In contrast, if termites and the nest environment more strongly influence microbiome structures than *Podaxis*, we expected that compositional differences would be unaffected by *Podaxis* presence. Lastly, given the extent of our transect, we hypothesised that microbial communities would be affected by differences in large-scale climatic or environmental variables, i.e., precipitation, temperature, elevation, habitat type, and soil elemental compositions, and predicted that their effects would be weaker in mounds, due to stable and buffered conditions, than surrounding soils.

2. Materials and methods

2.1. Experimental design and termite barcoding

Trinervitermes trinervoides mounds were sampled in November 2019 during three days at the onset of spring rains over a 1000 km transect in South Africa (Fig. 1A). In total, we sampled 49 colonies. For each colony, the number of *Podaxis* fruiting bodies were noted (Table S1). At each mound we collected from three locations within mounds: mixture of grass and soil from grass chambers (Grass), soil from north and south-facing inside walls that predictably differ in sun exposure (North and South, respectively), and two from outside mounds: the outside mound wall (Wall) and 5 m away from the mound (Outside). The inner wall samples were collected 5–10 cm from the outer wall surface and the grass chamber (where present). From here on "Sample location" refers to these mound compartments and outside soils. Termite workers were collected for species identification (Fig. 1D). Soil and grass samples were collected in cryotubes containing 1 ml of RNAlater® and stored at −20 °C. From the same sampling locations, we collected dry soil in 15 ml

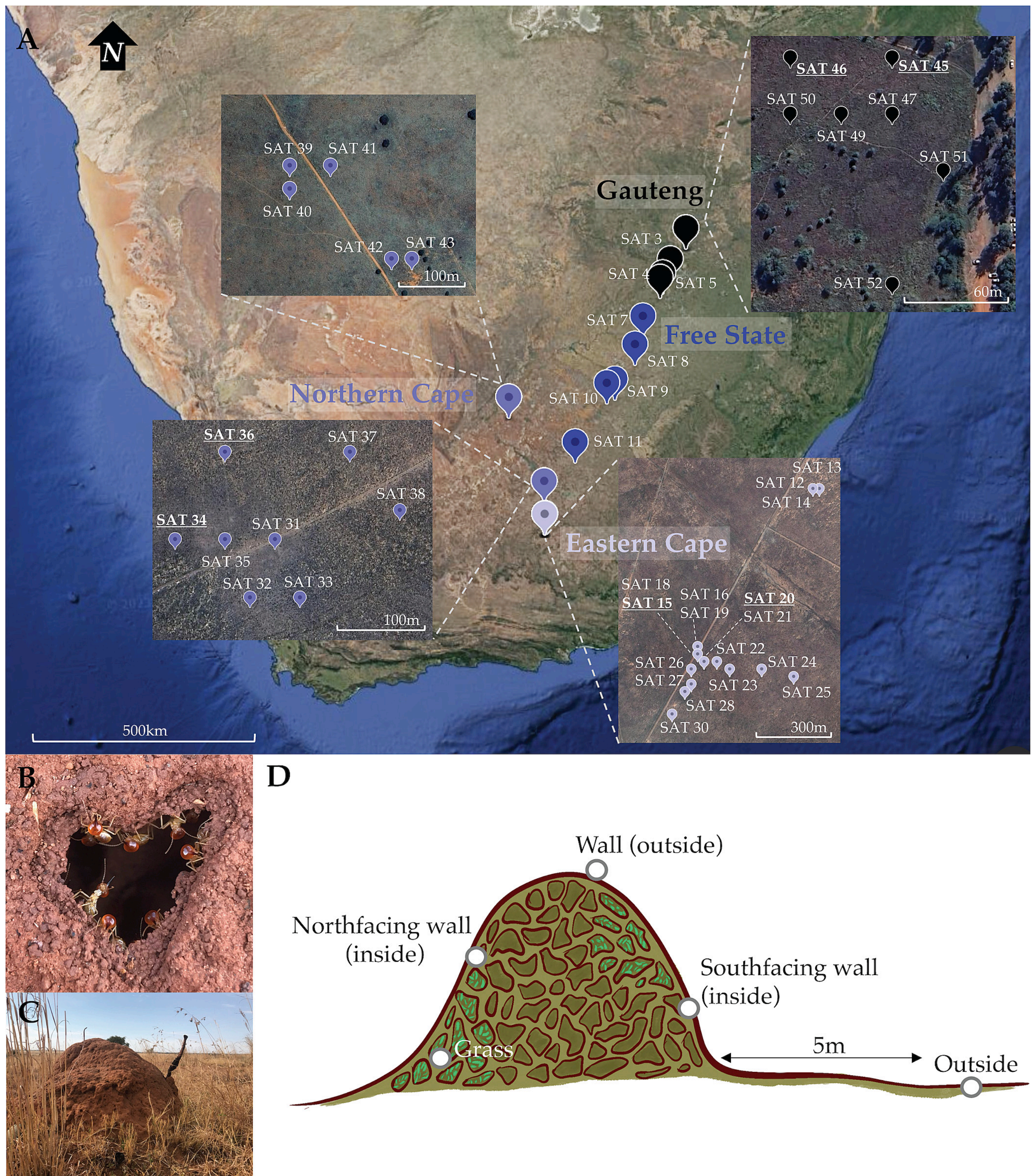


Fig. 1. Sampling localities and *Trinervitermes trinervoides* mound sample locations. **A.** Map indicating the sampling sites of 49 *Trinervitermes* mounds across South Africa. Colours represent four provinces from which samples were collected (Gauteng, Free State, Northern Cape, and Eastern Cape). Satellite maps were acquired from Google Earth. Mounds used for soil biogeochemical analyses are underlined. **B.** Image showing the *Trinervitermes trinervoides* soldiers. **C.** Mound of *Trinervitermes trinervoides* with a fruiting body of *Podaxis*. **D.** Schematic *Trinervitermes* mound indicating sample locations in mounds for soil elemental analyses and bacterial and fungal community characterisation.

falcon tubes for mineral analysis from one mound with and one without *Podaxis* fruiting bodies from three provinces (Fig. 1A).

We confirmed that all termite colonies were *T. trinervoides* by extracting DNA from one worker/colony using a Chelex extraction (c.f. Sinotte et al., 2021). We then used A-tLeu 5′-CAGATAAGTGCATTGGATTT-3′ (Miura et al., 1998) and B-tLys 5′- GTTTAAGAGACCAGTACTTG-3′ (Liu and Beckenbach, 1992) primers to amplify a region in the mitochondrial cytochrome *c* oxidase II (COII). PCRs were performed and PCR products were purified and sequenced following Sinotte et al., 2021. Forward and reverse sequences were aligned in Geneious prime v. 2019.1.1 (Biomatters Ltd., New Zealand) using Geneious' own algorithm after primers and low-quality ends were trimmed. Consensus sequences were aligned using MUSCLE v. 3.8.425 (Edgar, 2004) and closest matches were always *T. trinervoides* in a BLASTn search in GenBank (Table S1).

2.2. Microbial community characterisation

DNA from 250 mg of soil or soil-grass mix was extracted using the Qiagen DNeasy PowerSoil Pro Kit (Hilden, Germany) according to the manufacturer's instructions. DNA was further purified using Genomic DNA Clean & Concentrator (Zymo Research, USA). Concentration of extracted DNA was checked using NanoDrop ND-1000 (Thermo Scientific, Germany).

Amplicon sequencing libraries were prepared using a two-step PCR, targeting the bacterial 16S rRNA gene V3-V4 regions and fungal ITS1-ITS2. PCRs were performed for 30 cycles using primers Uni341F (5′-CCTAYGGGRBGCASCAG-3′) and Uni806R (5'-GGACTACNNGGGTATCTAAT-3′) originally published by (Yu et al., 2005) and modified as described by (Sundberg et al., 2013) for bacteria and gITS7 ('5-GTGARTCATCGARTCTTTG-3′) (Ihrmark et al., 2012) and ITS4ngs ('5-TCCGTAGGTGAACCTGCGG-3′; (Tedersoo et al., 2015) for fungi. PCR amplification products were purified using HighPrep PCR clean-up (MagBio Genomics, USA) using a 0.65:1 (beads:PCR reaction) volumetric ratio. A second PCR reaction was performed to add Illumina sequencing adapters and sample-specific dual indexes using PCRBIO HiFi (PCR Biosystems Ltd., UK) for 15 cycles. The second PCR products were purified as described for the first PCR. Sample concentrations were normalized using the SequalPrep Normalization Plate (96) Kit (Thermofisher, USA), following the manufacturer's instructions. The libraries were then pooled up-concentrated using DNA Clean and Concentrator-5 Kit (Zymo Research, USA). The final 9pM library was sequenced following the manufacturer's instructions on an Illumina MiSeq platform at the Section of Microbiology - University of Copenhagen, using Reagent Kit v3 (2×300 cycles) (Illumina, USA).

MiSeq amplicon sequences of bacterial and fungal communities were analysed separately in Qiime2–2022.8 (Bolyen et al., 2019). Paired-end sequences were denoised using the DADA2 pipeline (Callahan et al., 2016) and assigned to amplicon sequence variants (ASVs) at 100 % similarity. Bacterial 16S rRNA ASVs were assigned to taxonomy using the SILVA 138.1 bacterial database (Quast et al., 2013), and fungal ITS sequences were assigned to taxonomy using the UNITE database (Nilsson et al., 2019). We removed archaeal, chloroplast and mitochondrial sequences. Bacterial and fungal phylogenies were generated using the align-to-tree-mafft-fasttree command with Qiime2. We removed any sample with sequencing depth below 5000 reads, leading to the removal of 40 samples from the bacterial dataset and 19 samples from the fungal dataset. Some extraction and sequencing controls contained bacterial (two extraction controls) and fungal sequences (seven extraction and two sequencing controls). To identify whether these sequences were true contaminants, we ran the decontam package (Davis et al., 2018) on ASV tables with the default parameters (threshold = 0.1) and prevalence method. This identified 339 ASVs in the 16S rRNA dataset and 307 ASVs in ITS dataset as contaminants. We removed these ASVs from the subsequent analyses.

2.3. Effects of sample location on fungal and bacterial diversity

Statistical analyses were conducted using R v. 4.2.2 (R Core Team, 2022). We calculated the bacterial and fungal Chao1 richness estimate, Shannon's diversity index and the core dominance index using the microbiome package (Lahti and Shetty, 2017) within the Phyloseq package (McMurdie and Holmes, 2013). The core dominance index indicates the prevalence of the most abundance taxa within each microbiome, providing insights into the skewness of microbial communities. Additionally, we calculated faith's phylogenetic diversity (PD) using the picante package (Kembel et al., 2010). The influence of sampling province and sample location on microbial alpha diversities were investigated using the generalized linear mixed models (glmms) in the lme4 package (Bates et al., 2015). Alpha diversities were used as the dependent variables, while sampling province and sample location and their interaction were used as independent variables, and colony ID was included as a random effect. The models were validated by investigating the residual distributions in qq plots. Pair-wise differences between independent variables were investigated using emmeans package based on the output of the glmm without the interaction (Lenth, 2023). ASV sharing between different sample locations were examined through generating upset plots in UpsetR package (Conway et al., 2017).

Microbial community composition dissimilarities were investigated by calculating Bray-Curtis distances within the Phyloseq package (McMurdie and Holmes, 2013). Community similarities were visualized using principal coordinate analyses plots (PCoA). The influence of sampling province, sample location and their interaction was tested using Permutational multivariate analyses of variance tests (PERMANOVAs) with the adonis2 function in the vegan package (Oksanen et al., 2022), setting colony ID as a 'strata' parameter and running 10,000 permutations. The pairwise differences were investigated using the wrapper package pairwise.adonis (Martinez, 2020).

To investigate how microbial co-occurrence patterns differed between termite mound-associated and outside sample locations, we conducted co-occurrence networks using the SPIEC-EASI (Kurtz et al., 2015) and igraph (Csardi and Nepusz, 2006) packages. These networks were generated individually for each sampling location, using only fungal and bacterial ASVs with >250 sequences. Stability of the networks were kept below 0.05 by adjusting the lambda.min.ratio. From these networks we acquired parameters such as modularity (levels of clustering within the network), ratio of negative to positive interactions between microbial ASVs, and degree (the number of interactions a microbial taxon has with others).

2.4. Podaxis prevalence in microbial communities

Fungal amplicon sequences were manually curated to identify *Podaxis* sequences, as the UNITE database did not provide genus level hits to *Podaxis*. To do so, sequences were blasted to the NT database (Sayers et al., 2022) in GenBank by BLAST+ (Camacho et al., 2009) and the five results with highest scores were selected to determine taxonomic classification. All ITS reads belonging to the Agaricaceae family were selected and ITS sequences aligning to *Podaxis* were downloaded from NCBI and aligned using MAFFT v73 (Katoh and Standley, 2013).

To further classify the *Podaxis* sequences to putative species, we generated a phylogenetic tree using ITS sequences from known *Podaxis* species (Li et al., 2023) and the *Podaxis* amplicon sequences from our fungal dataset. We generated the phylogenetic tree using BEAST v. 1.8.4 (Drummond et al., 2012) by running the analysis for 100 million generations using a relaxed uncorrelated lognormal distribution for the molecular clock model, and assuming a birth-death speciation process as a tree prior. Tracer v. 1.6 (Rambaut et al., 2014) was used for convergence diagnostics, and TreeAnnotator v. 1.8.3 (Drummond et al., 2012) was used to summarize final out put trees into a maximum clade credibility (MCC) tree. Fifty million generations were removed as burn-in.

We then tested for differences in richness and relative abundance of

Podaxis sequences between sample locations using glmms, keeping mound as a random factor. Subsequently, based on the ITS phylogeny, we categorized MiSeq amplicon sequences into multiple clusters and investigated whether richness of each cluster differed between sampling locations.

2.5. Effects of Podaxis presence on microbial communities

Using linear models, we examined whether *Podaxis* presence (visible fruiting bodies and MiSeq sequences, respectively) was associated with overall alpha diversity of fungal and bacterial communities. We then investigated whether the presence/absence of *Podaxis* fruiting bodies and the relative abundance of *Podaxis* ITS sequences (in categories: 0 %, <2 %, 2–10 %, 10–20 % and > 20 %) influenced microbial community compositions using PERMANOVAs. All these analyses were conducted individually for each sampling location.

2.6. Effect of environmental variables and soil elements on microbial communities

We gathered climatic and environmental information for each mound using Google Earth Engine – GEE (Gorelick et al., 2017), a cloud-based platform for geospatial analysis and remote sensing. We collected monthly average temperature and precipitation for October 2019 using ERA5-Land Monthly Aggregated database (Muñoz, 2019), bands "Total_Precipitation_Sum", elevation data from Hole-filled SRTM for the globe v.4, SRTM 90 m DEM Digital Elevation Database (Jarvis et al., 2008), and habitat type and landcover use data from Terra and Aqua combined Moderate Resolution Imaging Spectroradiometer (MODIS) Land Cover Type (MCD12Q1) v.6.1 (Friedl and Sulla-Menashe, 2022), band "LC_Prop1" with Food and Agriculture Organization of the United Nations (FAO) Land Cover Classification System (LCCS) (Latham et al., 2002). First, we investigated the impact of environmental parameters on microbial alpha diversities on different sample locations using glmms. Monthly precipitation and temperature correlated positively (Pearson correlation = 0.9115, $t = 9.138$, df = 17, $p < 0.0001$), so we only included monthly temperature, elevation, habitat type and land cover in our models. Subsequently, the effect of abiotic parameters on microbial diversity was assessed using a random forest analysis with the ranger R package (Wright and Ziegler, 2017). To build the model, we used land cover, habitat type, monthly temperature and precipitation, elevation, along with sampling location, province, and the presence of *Podaxis* as predictor variables. The respective diversity metrics (Chao 1 richness estimate, Shannon's diversity index, or PD) were used as outcome variables. Variable importance was determined by visualizing impurity (the Gini index) of the predictors. Finally, we investigated which environmental parameters affected microbial community compositions using the distance-based ReDundancy Analysis (dbRDA) with the Bray-Curtis dissimilarities in the microeco package (Liu et al., 2021). The significant contributions by different variables were investigated with the envfit function. These models were done independently for each sample location.

Soil elements, particularly metals, can influence both taxonomic and functional diversity of bacterial and fungal communities (Luo et al., 2022; Stefanowicz et al., 2008). To test how soil elements affected microbial communities, we performed elemental analyses on 29 samples from one mound with *Podaxis* fruiting bodies and one without from each of three of the four provinces (Fig. 1A). We performed quadruple ICP-MS (PerkinElmer TotalQuant) at the ICP-MS laboratory at The Geological Survey of Denmark and Greenland (GEUS; https://eng.geus.dk/products-services-facilities/laboratories/icp-solution-laboratory) to quantify 65 different soil elements (Table S2).

The compositional differences in soil elements were analysed and visualized using principal component analyses (PCA) within the vegan package (Oksanen et al., 2022). We first investigated whether elemental composition differed between sampling provinces, sampling locations and presence/absence of *Podaxis* fruiting bodies using PERMANOVA similar to for the microbiome analyses. We were not able to test associations between soil minerals and the presence of *Podaxis* ITS sequences, since almost all termite mounds contained *Podaxis*. We further investigated the loading values of PCA plots (independently for each sampling province) to identify whether similar elements were associated with mounds with *Podaxis* fruiting bodies. Subsequently, we tested for differences in element amounts between sampling provinces with the cal_diff function using the Wilcoxon Rank Sum Test in the microeco package (Liu et al., 2021).

We then investigated whether the overall soil elemental compositions aligned with the bacterial and fungal community similarities through Mantel correlation tests in the vegan package (Oksanen et al., 2022). To identify specific associations between bacterial and fungal genera with different soil elements, we conducted Pearson's correlations using the cal_cor function within the microeco package and p values were adjusted using false discovery rate (Liu et al., 2021). Mantel tests were conducted on the full dataset, while Pearson's correlations were done individually by sampling province.

3. Results

3.1. Fungal and bacterial community compositions

We retained 195 of 245 collected samples after quality filtering, and a total of 10,171,447 (mean ± SD: 52,161 ± 21,742) fungal ITS sequences after removing contaminant ASVs, which were assigned to 14,268 ASVs (Table S3). Ascomycota accounted for 72.7 % of the sequences with 7469 ASVs, followed by Basidiomycota (14.4 %). Around 10 % of the ASVs were unassigned at the phylum level (Table S3). The majority of fungal ASVs were unique to sample location, with only 416 being found across all locations (Fig. S1A). Grass chambers contained the least unique ASVs while outside soils contained the most. For bacterial (16S rRNA) communities, we retained 179 samples after quality filtering and removal of contaminants, with 3,578,670 (mean ± SD: 19,992 ± 9486) sequences belonging to 23,647 ASVs (Table S4). Bacterial communities were dominated by Actinobacteriota (43.3 %), followed by Proteobacteria (19.7 %), Firmicutes (14.2 %), and Bacteroidota (5.4 %). As for fungal communities, only a fraction (1158) of these ASVs were shared between different sample locations and outside soils had most unique ASVs (Fig. S1B).

3.2. Effects of sample location and sampling province on fungal and bacterial diversity

Alpha diversities of fungal communities (Chao 1 richness estimate, Shannon's diversity index, Phylogenetic diversity: PD, core dominance) differed significantly between the sample locations, but sampling province did not significantly affect diversity (Fig. 2A-C, Table S5). Pairwise differences indicated that outside soils are richer and more diverse in fungi than sample locations within mounds (Fig. 2A, Table S6). Phylogenetic diversity (PD) of fungal communities, however, only differed significantly between grass chambers and outside soils, while other sample locations had intermediate PDs (Fig. 2B, Table S6). Core dominance was higher within than outside mounds, indicating that the most abundant fungal taxon within nests accounts for a larger proportion of the fungal community (Fig. 2C, Table S6). Sample location, but not province, significantly affected bacterial alpha diversities (Table S5). However, pair-wise comparisons revealed no clear patterns in alpha diversities between sample locations within or outside mounds (Fig. 2D-E, Table S6).

Fungal community composition was significantly impacted by sample location (PERMANOVA: pseudo-$F_4 = 3.535$, $R^2 = 0.0645$, $p < 0.0001$) and province (pseudo-$F_3 = 4.321$, $R^2 = 0.0591$, $p < 0.0001$) (Fig. 2G). There was also a significant interaction between the two variables (pseudo-$F_{12} = 1.423$, $R^2 = 0.0779$, $p < 0.0001$), indicating that

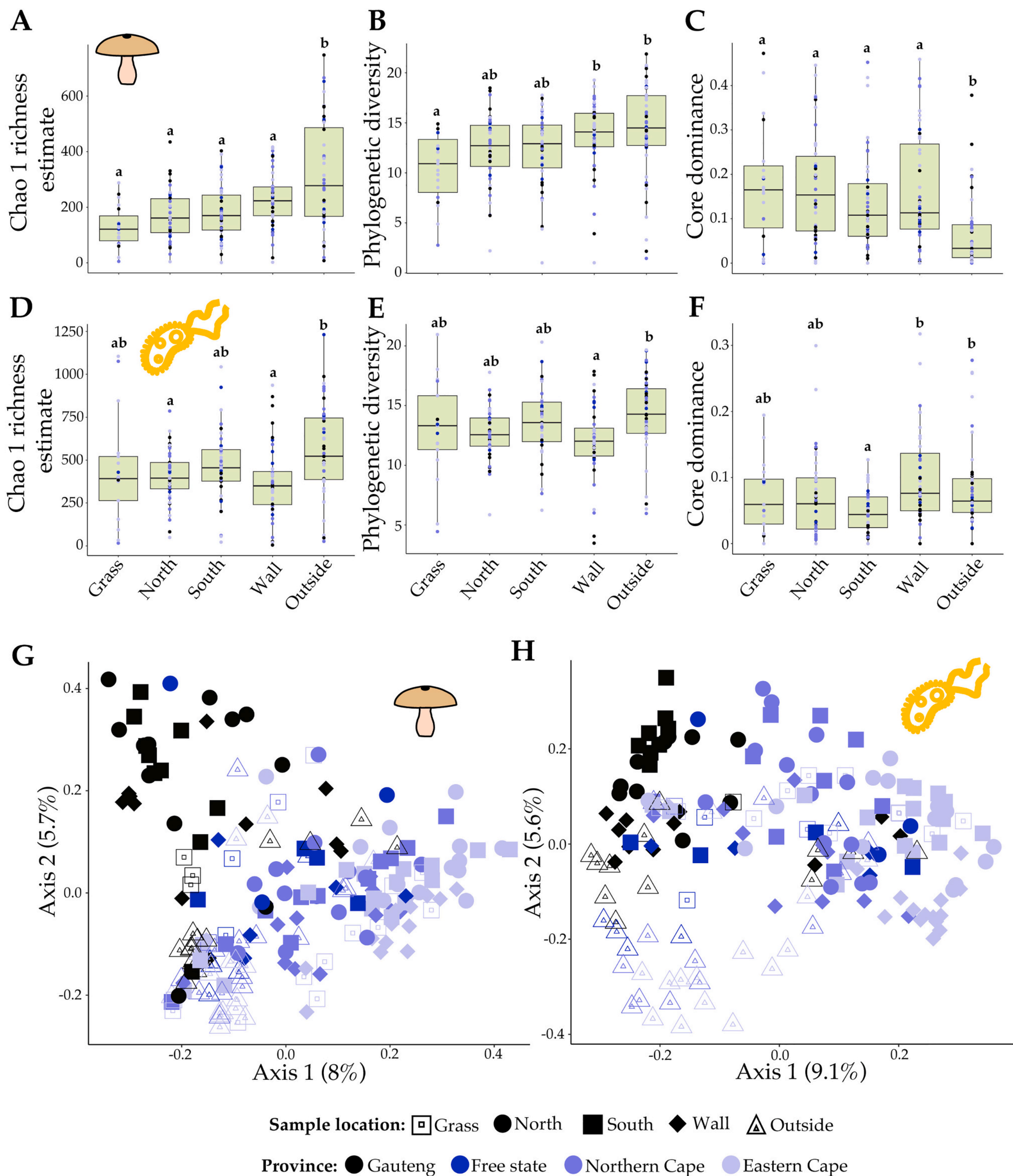


Fig. 2. Reduced fungal but not bacterial diversity within *T. trinervoides* mounds compared to outside soil and compositionally different fungal and bacterial communities between sample locations and provinces. Boxplots shows alpha diversity by sample location in fungal (A-C) and bacterial (D—F) communities. Lower case letters above boxes indicate significant differences of pair-wise testing based on estimated marginal means (full results in Table S6). Ordination plots showing the compositional differences in fungal (G) and bacterial (H) communities by sample and geographic locations.

fungal communities within a sample location also differ by province. Pair-wise comparisons revealed that fungal communities of all sample locations differed significantly from each other except for the north and south side of the inner wall (Fig. 2G; Table S7). Fungal communities also differed significantly between all provinces (Fig. 2G; Table S7).

Bacterial community compositions were significantly influenced by sample location (PERMANOVA: pseudo-F_4 = 3.789, R^2 = 0.0737, p < 0.0001) and province (pseudo-F_3 = 4.666, R^2 = 0.0681, p < 0.0001), and there was a significant interaction between the two (Bray-Curtis: pseudo-F_{12} = 1.469, R^2 = 0.0857, p < 0.0001) (Fig. 2H). Pair-wise differences revealed that samples from inside mounds differed significantly from those from outside, but there were no significant

community-level differences between sample locations within mounds (Fig. 2H; Table S7). As for fungal communities, bacterial community structure differed significantly between provinces (Fig. 2H; Table S7).

Fungal community networks within mounds were more modular than outside locations (Fig. 3A), and grass chambers hosted a highly modular fungal network. This implies that there are more ecologically similar fungal taxa within than outside mounds. The proportion of negative to positive interactions were also lower within (mean ± SD: 0.1083 ± 0.0369) than outside (mean ± SD: 0.2049 ± 0.0578) mounds (Table S8). This suggests that there are more positive associations between fungal taxa within mounds. Overall, the number of interactions each fungal taxon has with other fungal taxa, i.e., the degree, significantly differed by sampling location (Linear model: $F_4 = 377.9$; $p < 0.0001$, Fig. S4). Pairwise differences revealed that degree levels differed significantly among all sampling locations, except between the inner north and south walls (Table S8). Fungal taxa within grass chambers had the lowest degree levels (mean ± SD: 9.313 ± 4.003) suggesting reduced interactions between taxa (Fig. S2A). This contrasts the high degree between fungal taxa in the outside soil (mean ± SD: 41.96 ± 25.08).

Similar to fungal communities, the modularity of bacterial communities within grass chambers was higher than for other locations (Fig. 3B). In contrast to fungal communities, modularity in the outside soil bacterial communities was higher than locations within termite mounds (Table S8). The proportion of negative to positive interactions did not differ between different sample locations (Table S8). The degree of ASVs differed significantly between sampling locations (Linear model: $F_4 = 238.5$; $p < 0.0001$; Fig. S2B), with grass chamber ASVs experiencing on average the lowest degree (mean ± SD: 9.339 ± 3.786). Pairwise comparisons revealed that degree levels differed significantly between all sample locations (Fig. S2B; Table S9).

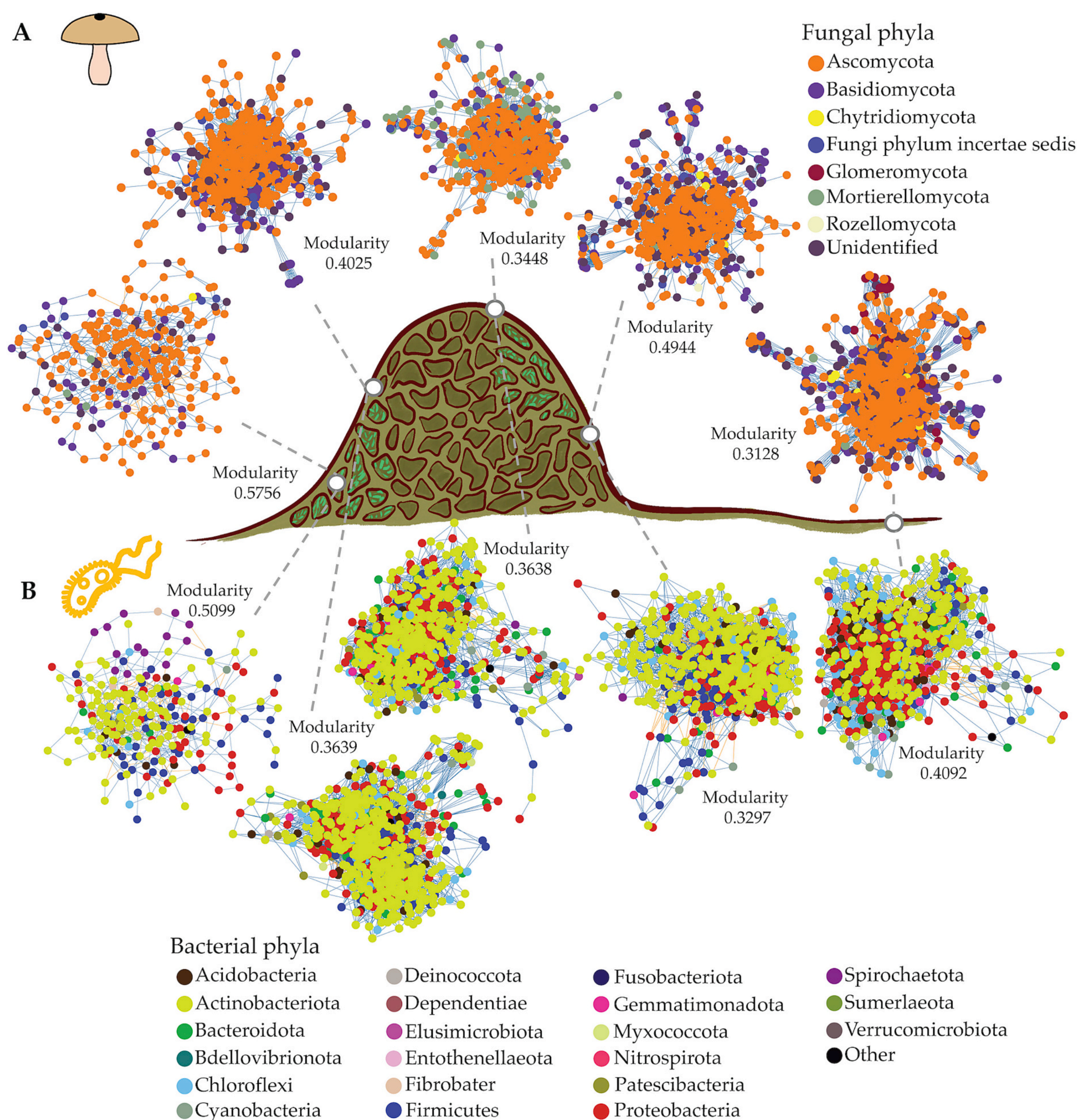


Fig. 3. Microbial network structures of fungal (A) and bacterial (B) communities differed notably inside and outside mounds. Network nodes are coloured according to microbial phylum and edges indicate whether associations are positive (blue) or negative (orange). The modularity is indicated near each network.

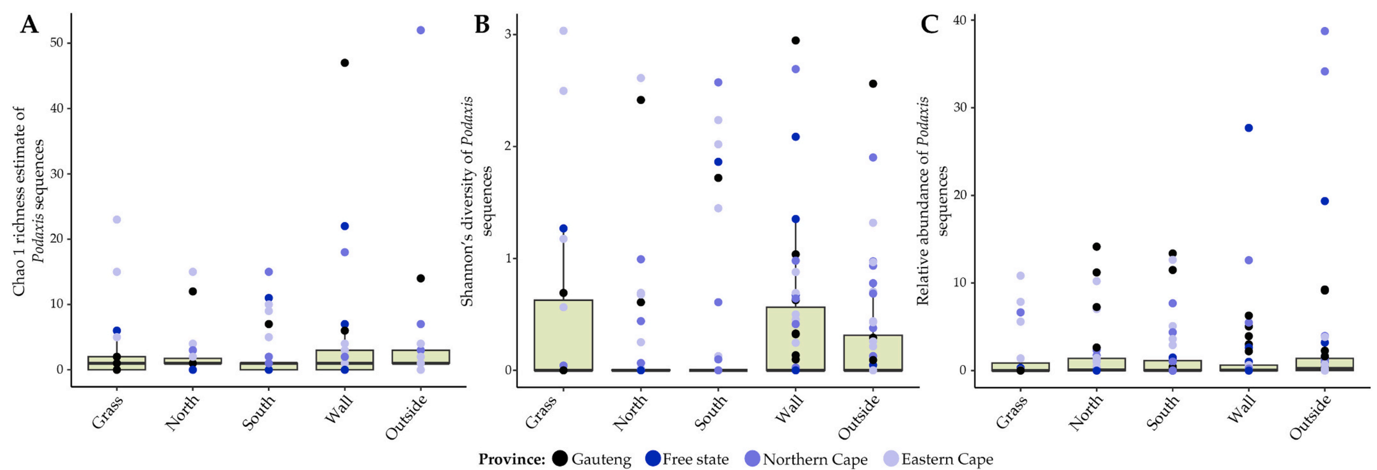


Fig. 4. Chao1 richness estimate, Shannon's diversity index and the relative abundance of *Podaxis* ASVs did not differ between sample locations (Table S10). Points are coloured according to the sampling province.

3.3. *Podaxis* prevalence in microbial communities

We observed *Podaxis* fruiting bodies on 16 of the 49 colonies. In the cleaned ITS dataset, we identified 337 ASVs belonging to *Podaxis* that were distributed across 148 of the 195 samples (75.9 %) and included at least one sample in 48 of the 49 (97.9 %) termite mounds. The relative abundance of *Podaxis* in fungal communities ranged from 0.004 % to 77.5 % (mean ± SD: 3.87 % ± 10.17 %) (Fig. 4C). On average, fungal communities contained 2.62 (SD: ± 6) *Podaxis* ASVs. Neither sample type nor province affected the diversity or relative abundance of *Podaxis* ASVs (Fig. 4, Table S10). Within the co-occurrence networks, *Podaxis* only co-occurred with a few other fungal taxa (Fig. S3), indicating limited impact of *Podaxis* on fungal network structure.

The ITS tree demonstrated 14 clusters of *Podaxis* sequences (Fig. S4). Despite converging based on the tracer file, we did not obtain strong posterior probability support for many of the nodes in the tree, likely due to the poor resolution of short reads. Some previously published ITS sequences belonging to distinct *Podaxis* taxa clustered with our ITS sequences in distinct groups (Fig. S4), indicating that the placement of the MiSeq sequences can be trusted. However, none of the clusters showed significant differences in Chao1 richness between sample locations or provinces (Table S11), indicating that diverse *Podaxis* taxa can be detected both within and outside *T. trinervoides* mounds.

3.4. The influence of *Podaxis* on microbiomes

The presence of *Podaxis* fruiting bodies was not associated with fungal community richness, diversity, or PD in grass chambers, inner south wall, mound wall, or outside soils (Table S12). However, fungal richness was significantly lower in the inner north wall when *Podaxis* fruiting bodies were present (Table S12). The relative abundance of *Podaxis* sequences also did not associate with fungal community richness, diversity, or PD in neither grass chamber, mound wall, nor outside soils (Table S12). There was, however, a significant positive association between *Podaxis* relative abundance and fungal community Shannon's diversity in the inner north wall, *Podaxis* relative abundance associated negatively with the Chao1 richness in the inner south wall, and PD was negatively associated in the inner south wall and outside soil (Table S12). Alpha diversity indexes of bacterial communities were not impacted by the presence of *Podaxis* fruiting bodies (Table S12). However, the relative abundance of *Podaxis* was significantly negatively associated with bacterial community richness, diversity, and PD in the inner north wall. Bacterial alpha diversity in other locations did not associate with *Podaxis* relative abundance (Table S12). Thus, the presence of *Podaxis* appears to have little or no influence on fungal and bacterial community diversities in *Trinervitermes* termite mounds.

We also did not observe statistical differences in fungal community composition in mounds with *Podaxis* fruiting bodies for any of the sample locations (Fig. 5A, Table S13). However, despite the absence of obvious visible effects of the categorized relative abundance of *Podaxis* on fungal community composition (Fig. 5B), PERMANOVA tests revealed significant effects for the inner south wall, the outside wall, and the outside soil (Table S13). However, pairwise tests did not reveal significant differences between relative abundance of *Podaxis* in outside wall communities. In the inner south wall, the composition of fungal communities differed statistically between samples depending on the relative abundance of *Podaxis*: 0 % and 2–10 % (Pairwise PERMANOVA; $F = 1.526$, $p = 0.0204$), 0 % and 10–20 % ($F = 1.858$, $p = 0.0258$), <2 % and 10–20 % ($F = 2.503$, $p = 0.0006$), and 2–10 % and 10–20 % ($F = 2.149$, $p = 0.0222$). In the outside soil community, only the relative abundance groups of 0 % and < 2 % ($F = 2.207$, $p = 0.0209$) and < 2 % and 2–10 % ($F = 2.606$, $p = 0.0009$) were significantly different in fungal community structure (Fig. 5B). In summary, the absence of a consistent effect of *Podaxis* abundance on fungal communities supports that *Podaxis* is likely not critically important for structuring *Trinervitermes*-associated fungal communities.

For bacterial communities, we only observed a significant effect of *Podaxis* fruiting body presence on the outside soil communities (Table S13; Fig. S5). We did observe significant effects of different relative *Podaxis* abundances on bacterial community structure in the inner south wall and outside soils. Pairwise tests, however, did not reveal significant differences between levels of *Podaxis* relative abundance on inner wall communities. For outside soils, bacterial communities differed significantly between samples with *Podaxis* relative abundance of <2 % and 2–10 % (Pairwise PERMANOVA: $F = 2.639$, $p = 0.0009$) and 0 % and 2–10 % ($F = 2.078$, $p = 0.0279$).

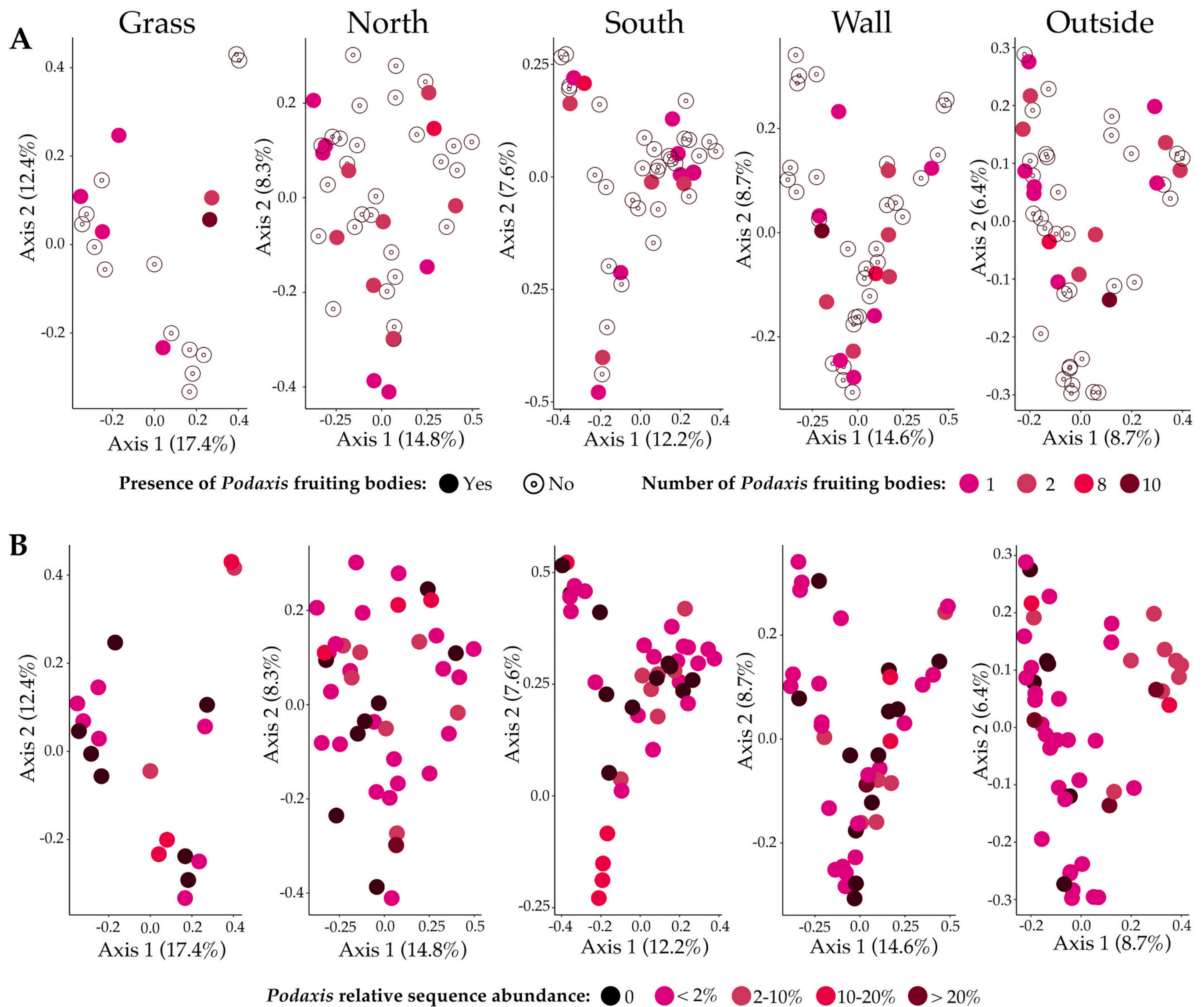


Fig. 5. *Podaxis* presence either as fruiting bodies (A) or ITS sequences (B) does not consistently affect fungal communities across sample locations. **A.** PCoA plots showing the influence of the presence of *Podaxis* fruiting bodies on fungal community similarity based on Bray-Curtis distances. The colours represent the number of fruiting bodies recorded at each mound. **B.** Same PCoA plots showing the influence of the different levels of the relative abundance of *Podaxis* in the ITS dataset on fungal communities. Colours represent the different categories of *Podaxis* relative abundance.

3.5. Effect of environmental variables and soil elements on microbial communities

None of the environmental variables (average monthly temperature, land cover, habitat type, or elevation) showed clear associations with any of the alpha diversity indexes of the fungal or bacterial communities (Table S14). Aligning with our glmms, random forest models revealed that the most important factor influencing bacterial and fungal alpha diversity was sampling location (Fig. S6). Notably, the importance of this variable was substantially greater for fungal communities, while elevation also played a prominent role for bacterial communities. The remaining environmental factors had nearly equal, but less substantial, importance (Fig. S6). dbRDA plots and envfit analysis revealed that the majority of the environmental variables influenced microbial community compositions significantly across sample locations in both bacterial and fungal datasets (Table S15; Fig. 6). Overall, the influence of elevation, monthly precipitation, and temperature tended to be in the opposite direction of those of percentage landcover and shrubland habitat types on microbial community structure (Fig. 6). However, all environmental variables were associated with sampling provinces, indicating that the effects of sampling province were driven by environmental conditions.

Soil elemental composition was significantly different between sampling province (PERMANOVA: $F_2 = 32.66$, $R^2 = 0.6665$, $p < 0.0001$), but not sample locations ($F_4 = 1.031$, $R^2 = 0.0421$, $p = 0.4184$). The levels of multiple elements differed between the three sampling provinces (Table S16), and geographically closer provinces were similar in elemental composition (Fig. 7A). Intriguingly, elemental composition was significantly associated with the presence of *Podaxis* fruiting bodies in mounds ($F_4 = 6.441$, $R^2 = 0.0657$, $p = 0.0051$), albeit with low explanatory power, implying that fruiting body formation of *Podaxis* may be influenced by the presence of certain soil elements. However, we detected *Podaxis* ITS sequences in all colonies used in the elemental analyses (Fig. 7B). Given the differences by province, we visualized the elemental composition of mounds at each province separately. This revealed that elemental composition differed between termite mounds with and without *Podaxis* fruiting bodies, despite geographic proximity, and loading values indicated that different elements contribute to the

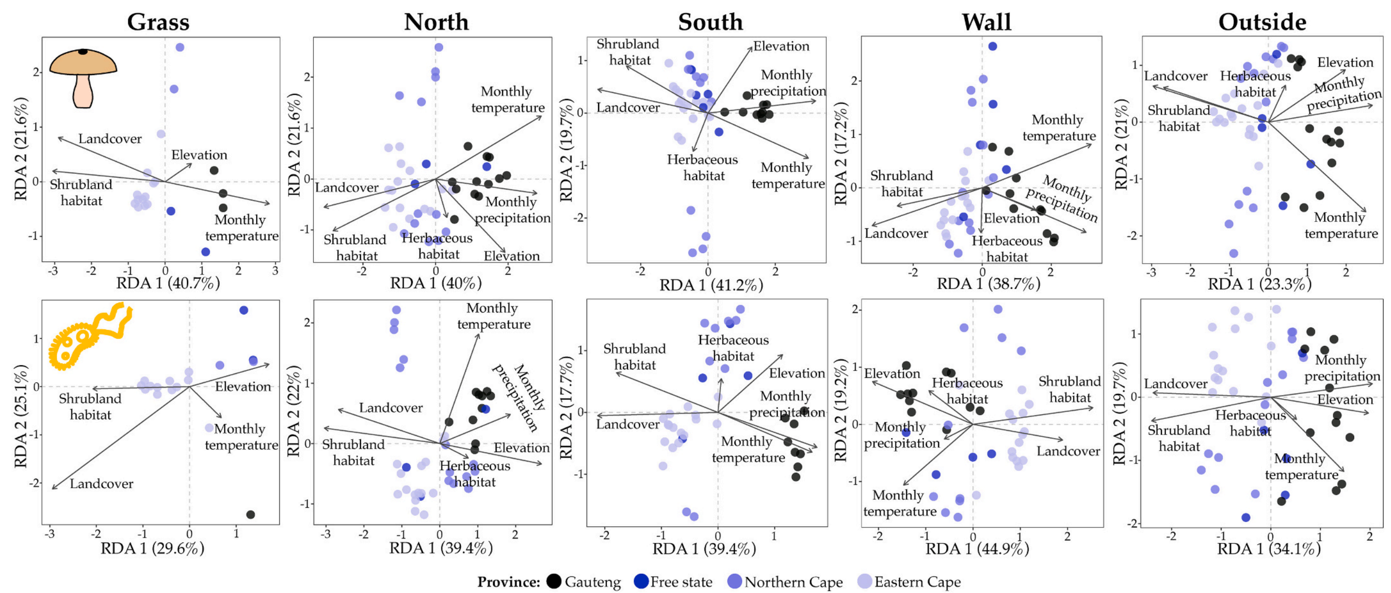


Fig. 6. Distance-based ReDundancy Analysis (dbRDA) plots of bacterial and fungal communities in different sampling locations. Arrows represent the influence of different environmental variables on driving the observed community compositions. The length of arrows are associated with the effect of each variable. Statistical results of envfit analyses corresponding to the influence of environmental variables can be found in Table S14.

separation of provinces (Fig. S6). However, given our low sample size, we are unable to determine whether these differences are truly associated with *Podaxis* fruiting body presence.

Mantel tests revealed a marginally non-significant correlation between the similarity in elemental composition and the similarities in fungal communities (Mantel $r = 0.1271$, $p = 0.0629$) (Fig. 7C), indicating a potential role of soil element composition in structuring the fungal communities. We did not find such an association between soil elements and bacterial communities (Mantel $r = 0.0853$, $p = 0.1649$) (Fig. 7D). However, specific correlations between microbial genera and soil elements from different provinces revealed multiple significant associations (Tables S17 and S18). We detected 125 significant correlations between fungal genera and soil elements in Gauteng, 10 in Northern Cape, and 19 in Eastern Cape (Table S17). In Northern Cape, *Podaxis* was significantly positively correlated with gold (Au), while it was significantly positively correlated with manganese (Mn) in the Eastern Cape (Table S17). For bacterial communities, there were 247 significant correlations in Gauteng, 137 in Eastern Cape, and 155 in Northern Cape (Table S18). Taken together, this indicates that soil elemental composition may impact the overall fungal communities, while different elements appear to have specific impacts on bacterial genera.

4. Discussion

We tested a series of hypotheses to better understand the biotic and abiotic drivers that influence African savannah soil microbiomes, and the role that *Trinervitermes* termites and their symbionts may play in governing these microbial landscapes. In support of our first hypothesis and aligning with previous work on other termite species (Baker et al., 2020; Chen et al., 2021b; Jouquet et al., 2011; Soukup et al., 2021), we found compositionally different microbial communities between mound compartments, emphasizing the role of termites in changing microbial communities in their immediate soil habitats. Previous work has attributed these effects to termite-driven modulation of soil properties and heterogeneity (Baker et al., 2020; Chen et al., 2021a). Despite only observing *Podaxis* fruiting bodies on a subset of mounds, we detected *Podaxis* ASVs in most samples, including in 83.3 % of samples from outside mounds. This contradicted our second hypothesis that *Podaxis* would be more prevalent within mounds (and particularly in grass chambers) than surrounding soil. We also did not find support in favour of our hypothesis that *Podaxis* presence would impact mound-associated microbial communities, neither based on fruiting body presence/absence nor based on *Podaxis* prevalence in the amplicon dataset. This may be because members of the fungal genus only rarely dominate communities, as only 17 within-mound samples had >10 % relative abundance of *Podaxis*. We did, however, find some support for the hypothesis that differences in soil element composition between provinces associate with fungal community compositions and have taxon-specific effects on bacteria. In the following, we discuss our findings in light of how abiotic and biotic factors ultimately affect fungal and bacterial microbiomes of savannah soils from local to ecosystem scales.

Our findings support that environmental conditions and abiotic soil properties across geographical sites are principal determinants of fungal and bacterial community compositions. This aligns with global abiotic drivers of soil microbiomes, where habitat type and environmental variables such as precipitation and temperature strongly influence fungal and bacterial soil communities (Cowan et al., 2022; Islam et al., 2020; Vasar et al., 2022). The additional effects of specific soil elements influencing either communities as a whole or specific microbial taxa imply that microbial community compositions are governed by complex multifaceted drivers. This ultimately led to distinct separation of microbial communities by sampling province, implying that different geographic regions will exhibit distinct microbial communities as a consequence of specific biogeochemical cycles and microbial soil processes.

Against this geographic backdrop, termite activities further shape microbial community compositions and diversity as the separation between regions was lower in samples collected within termite mounds than outside soils. A series of previous works have eluded to how termite engineering activities change mound soil properties (Brossard et al., 2007; Jouquet et al., 2007), how mound-adjecent soils contain microbial taxa of termite origins (Jouquet et al., 2005; Li and Greening, 2022), and how mound soils are infused by compounds from termite saliva (Wood, 1988). Our findings of reduced within-mound fungal diversity suggest that these factors affect particularly fungal taxa, which has also been documented within mounds of Australian *Amitermes meridionalis* grass-cutting termites (Chen et al., 2021b). We did not observe comparable impacts on bacterial diversity, which contrasts findings from other termite taxa, where bacterial diversity was found to be either higher

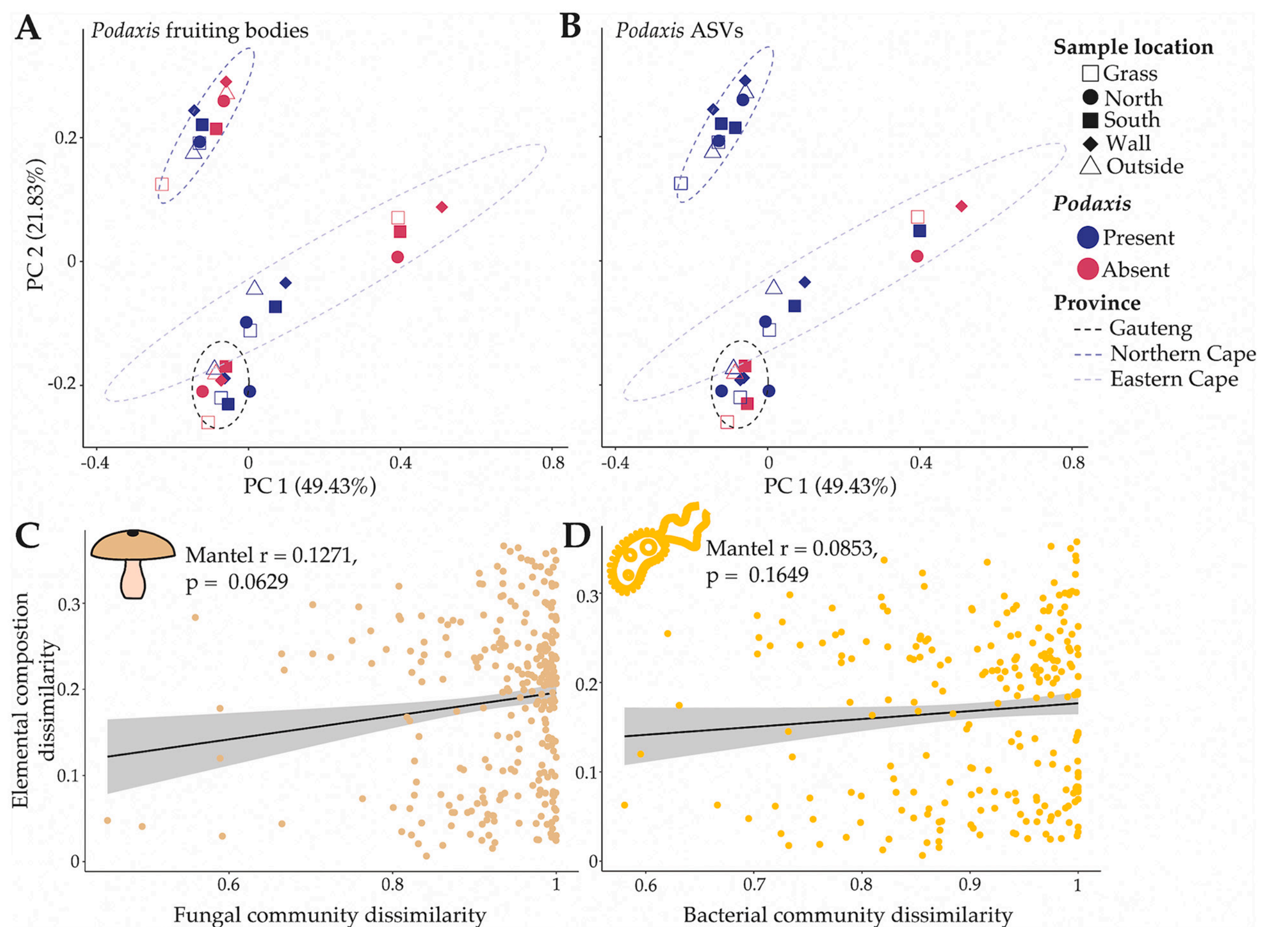


Fig. 7. Soil elemental composition differed markedly across sampling provinces and the elemental composition similarity associated significantly with the fungal community similarity, not with the bacterial community similarity. **A** and **B**. PCA plots of soil elemental composition differences between sampling provinces and sample locations. Colours represent the presence/absence of *Podaxis* fruiting bodies (A) and *Podaxis* ITS sequences (B). C and D represent the associations of soil elemental composition dissimilarity with fungal (C) and bacterial (D) community dissimilarity. Statistics for these associations were done with Mantel correlations, but trend line represent the linear relationships (grey area around the trend line represent the standard error of the datapoints).

(Baker et al., 2020) or lower (Chen et al., 2021b) within mounds than in surrounding soils. These apparently conflicting results could be driven by species-specific differences in how termite activities affect soil properties, and particularly the extent to which the termites deposit gut bacteria in mound soils.

Termite governance of microbial landscapes within mounds were somewhat apparent within our microbial network structures. More modular fungal networks (more compartmentalized interactions between groups of taxa), along with lower ratios of negative to positive interactions within termite mounds, suggest interactions between more ecologically similar fungal taxa within than outside mounds (c.f. Hernandez et al., 2021). This could be driven by the filtering properties of uniform mound environments (e.g., regarding temperature, water and nutrient contents, and termite effects) only permitting fungal taxa with a narrower ecological niche to persist. Bacterial co-occurrence networks were more modular in grass chambers and outside soils than mound wall communities. This is likely to be driven by differences in nutrient availability, as more modular bacterial networks have been observed in nutrient-richer surface habitats than nutrient-poorer subterranean caves (Reboleira et al., 2022). Collectively, these findings point to a stronger role of termites in structuring the diversity and composition of fungal than bacterial communities, with the latter seemingly being more affected by nutrient levels. However, caution must be taken when interpreting networks as they indicate potential associations based on amplicon data rather than realized interactions between microbes.

Contrary to our expectations, *Podaxis* did not dominate the grass chamber communities and also did not appear to affect mound microbiomes, neither when evaluating fruiting body presence nor the abundance of ITS sequences. However, we should note that only one of 49 sampled colonies was apparently free from *Podaxis*, limiting the power of our analyses. Future work extending comparisons of elements and *Podaxis* presence is needed to unambiguously establish factors governing its presence and abundance with termite colonies. The near omnipresence of *Podaxis* with *T. trinervoides* suggests that evaluating presence/absence of *Podaxis* might not be sufficient to decipher what governs its presence, nor effects on other microbes, which could instead potentially be achieved through comparisons to termite species that do not host *Podaxis*. Expanding sampling beyond South Africa would also allow more extensive exploration of the combined effects of habitat, elements, abiotic conditions, and climate, on termite symbiosis with *Podaxis*.

While we tentatively conclude that *Podaxis* does not affect microbial communities of termite mounds, future work to delve deeper into the prevalence of the fungal genus in these environments may help unravel interactions. There are at least seven species of *Podaxis* in South Africa, of which four appear exclusively associated with *T. trinervoides* and three are free-living (Conlon et al., 2016; Conlon et al., 2021; Li et al., 2023). The low resolution of short ITS sequences was insufficient to robustly assign ASVs to species in our study, precluding establishing if symbiotic species exclusively exist within termite colonies. Given the prevalence of the fungal genus across mound and environmental samples, and given the prolific metabolic potential of species of *Podaxis* with both lifestyles

(Guo et al., 2022), future work should address the extent to which species segregate by niches and explore their interactions with respective fungal and bacterial communities.

In conclusion, our findings imply that soil microbial communities in African savannah ecosystems are primarily governed by geography, driven by regional climatic and elemental composition effects, and secondarily modulated by termite ecosystem engineering activities. Sequential effects at these scales are logical and imply that conceivable changes of soil processes are likely to be affected by changing climates, but also by termite species assemblies that interact with and impact soil properties and microbial communities. These interconnected scales should be explored further, as climate, plant cover and other ecosystem-level changes should predictably also affect termite-mound microbiomes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.177015.

CRediT authorship contribution statement

Kasun H. Bodawatta: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Lorrie Maccario:** Writing – review & editing, Resources, Methodology, Formal analysis, Data curation. **Nils Peereboom:** Writing – review & editing, Methodology, Data curation. **Benjamin H. Conlon:** Writing – review & editing, Methodology, Data curation. **Guangshuo Li:** Writing – review & editing, Methodology, Data curation. **Tamás Plaszkó:** Methodology, Formal analysis. **Celia Vinagre-Izquierdo:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Knud A. Jønsson:** Writing – review & editing, Resources, Methodology, Formal analysis. **Risto M. Vesala:** Writing – review & editing, Methodology. **Z. Wilhelm de Beer:** Writing – review & editing, Resources, Methodology, Investigation, Data curation, Conceptualization. **Anders Priemé:** Writing – review & editing, Resources, Methodology. **Michael Poulsen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by The Danish Council for Independent Research (DFF – 7014-00178) to MP. AP and TP were supported by the Danish National Research Foundation (Center for Volatile Interactions - VOLT, DNRF168). We thank Olga Nielsen and Tonny Bernt Thomsen for carrying out ICP-MS analyses, Luka Alexander Civa for help with sorting ITS sequences, and members of the Social and Symbiotic Evolution Group at the UCPH for critical comments to a previous version of this manuscript. GL was supported by a PhD scholarship from the China Scholarship Council.

Data availability

Fungal (PRJNA1100193) and bacterial (PRJNA1100180) amplicon sequences are available from the SRA Archive in GenBank. Termite COI sequences can be found in Table S1. Original datasets and R scripts used for microbiome analyses can be found in Zenodo online repository: https://doi.org/10.5281/zenodo.13895044.

References

Adam, R.A., Mitchell, J.D., van der Westhuizen, M.C., 2008. Aspects of foraging in the harvester termite, *Trinervitermes trinervoides* (Sjöstedt) (Termitidae: Nasutitermitinae). African Entomol. 16 (153–161), 9. https://doi.org/10.4001/1021-3589-16.2.153.

Baker, C.C.M., Castillo Vardaro, J.A., Doak, D.F., Pansu, J., Puissant, J., Pringle, R.M., et al., 2020. Spatial patterning of soil microbial communities created by fungus-farming termites. Mol. Ecol. 29, 4487–4501. https://doi.org/10.1111/mec.15585.

Bar-On, Y.M., Phillips, R., Milo, R., 2018. The biomass distribution on earth. Proc. Natl. Acad. Sci. USA 115, 6506–6511. https://doi.org/10.1073/pnas.1711842115.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48. https://doi.org/10.18637/jss.v067.i01.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Brossard, M., López-Hernández, D., Lepage, M., Leprun, J.C., 2007. Nutrient storage in soils and nests of mound-building *Trinervitermes* termites in Central Burkina Faso: consequences for soil fertility. Biol. Fertil. Soils 43, 437–447. https://doi.org/10.1007/s00374-006-0121-6.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al., 2009. BLAST+: architecture and applications. BMC Bioinf. 10, 421. https://doi.org/10.1186/1471-2105-10-421.

Chen, Q.-L., Hu, H.-W., Yan, Z.-Z., Li, C.-Y., Nguyen, B.-A.T., Sun, A.-Q., et al., 2021a. Deterministic selection dominates microbial community assembly in termite mounds. Soil Biol. Biochem. 152, 108073. https://doi.org/10.1016/j.soilbio.2020.108073.

Chen, Q.-L., Hu, H.-W., Yan, Z.-Z., Li, C.-Y., Nguyen, B.-A.T., Zheng, Y., et al., 2021b. Termite mounds reduce soil microbial diversity by filtering rare microbial taxa. Environ. Microbiol. 23, 2659–2668. https://doi.org/10.1111/1462-2920.15507.

Chiri, E., Greening, C., Lappan, R., Waite, D.W., Jirapanjawat, T., Dong, X., et al., 2020. Termite mounds contain soil-derived methanotroph communities kinetically adapted to elevated methane concen- trations. ISME J. 14, 2715–2731. https://doi.org/10.1038/s41396-020-0722-3.

Chiri, E., Nauer, P.A., Lappan, R., Jirapanjawat, T., Waite, D.W., Handley, K.M., et al., 2021. Termite gas emissions select for hydrogenotrophic microbial communities in termite mounds. Proc. Natl. Acad. Sci. USA 118, e2102625118. https://doi.org/10.1073/pnas.2102625118.

Conlon, B.H., de Beer, Z.W., de Fine Licht, H.H., Aanen, D.K., Poulsen, M., 2016. Phylogenetic analyses of *Podaxis* specimens from southern Africa reveal hidden diversity and new insights into associations with termites. Fungal Biol. 120, 1–12. https://doi.org/10.1016/j.funbio.2016.05.011.

Conlon, B.H., Gostinčar, C., Fricke, J., Kreuzenbeck, N.B., Daniel, J.-M., Schlosser, M.S. L., et al., 2021. Genome reduction and relaxed selection is associated with the transition to symbiosis in the basidiomycete genus *Podaxis*. iScience 24, 102680. https://doi.org/10.1016/j.isci.2021.102680.

Conway, J.R., Lex, A., Gehlenborg, N., 2017. UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinformatics 33, 2938–2940. https://doi.org/10.1093/bioinformatics/btx364.

Cowan, D.A., Lebre, P.H., Amon, C.E.R., Becker, R.W., Boga, H.I., Boulangé, A., et al., 2022. Biogeographical survey of soil microbiomes across sub-Saharan Africa: structure, drivers, and predicted climate-driven changes. Microbiome 10, 131. https://doi.org/10.1186/s40168-022-01297-w.

Csardi G, Nepusz T. The igraph software package for complex network research. InterJournal, Complex Systems 2006. https://igraph.org.

Dangerfield, J.M., McCarthy, T.S., Ellery, W.N., 1998. The mound-building termite *Macrotermes michaelseni* as an ecosystem engineer. J. Trop. Ecol. 14, 507–520. https://doi.org/10.1017/S0266467498000364.

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6, 226. https://doi.org/10.1186/s40168-018-0605-2.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973. https://doi.org/10.1093/molbev/mss075.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. https://doi.org/10.1093/nar/gkh340.

Friedl, M., Sulla-Menashe, D., 2022. MODIS/Terra+Aqua Land Cover Type Yearly L3 Global 500m SIN Grid V061. In: Center NELPDAA (Ed.).

Gorelick, N., Hancher, M., Dixon, M., Ilyushchenko, S., Thau, D., Moore, R., 2017. Google earth engine: planetary-scale geospatial analysis for everyone. RSE 202, 18–27. https://doi.org/10.1016/j.rse.2017.06.031.

Guo H, Daniel J-M, Seibel E, Burkhardt I, Conlon BH, Görls H, et al. Insights into the metabolomic capacity of *Podaxis* and isolation of podaxisterols A–D, ergosterol derivatives carrying nitrosyl cyanide-derived modifications. J. Nat. Prod. 2022; 85: 2159–2167. https://doi.org/10.1021/acs.jnatprod.2c00380.

Hernandez DJ, David AS, Menges ES, Searcy CA, Afkhami ME. Environmental stress destabilizes microbial networks. ISME J. 2021; 15: 1722–1734. https://academic.oup.com/ismej/article/ 15/6/1722–1734/7474624.

Ihrmark, K., Bodeker, I.T., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., et al., 2012. New primers to amplify the fungal ITS2 region-evaluation by 454-

sequencing of artificial and natural communities. FEMS Microbiol. Ecol. 82, 666–677. https://doi.org/10.1111/j.1574-6941.2012. 01437.x.
Islam, W., Noman, A., Naveed, H., Huang, Z., Chen, H.Y.H., 2020. Role of environmental factors in shaping the soil microbiome. Environ. Sci. Pollut. Res. 27, 41225–41247. https://doi.org/10.1007/s11356-020-10471-2.
Jarvis, A., Reuter, H.I., Nelson, A., Guevara, E., 2008. Hole-Filled SRTM for the Globe Version 4, Available from the CGIAR-CSI SRTM 90m Database.
Jouquet, P., Ranjard, L., Lepage, M., Lata, J.C., 2005. Incidence of fungus-growing termites (Isoptera, macro- termitinae) on the structure of soil microbial communities. Soil Biol. Biochem. 37, 1852–1859. https://doi.org/10.1016/j.soilbio.2005.02.017.
Jouquet, P., Bottinelli, N., Lata, J.-C., Mora, P., Caquineau, S., 2007. Role of the fungus-growing termite *Pseudacanthotermes spiniger* (Isoptera, Macrotermitinae) in the dynamic of clay and soil organic matter content. An experimental analysis. Geoderma 139, 127–133. https://doi.org/10.1016/j.geoderma.2007.01.011.
Jouquet, P., Traoré, S., Choosai, C., Hartmann, C., Bignell, D., 2011. Influence of termites on ecosystem functioning. Ecosystem services provided by termites. Eur. J. Soil Biol. 47, 215–222. https://doi.org/10.1016/j.ejsobi.2011.05.005.
Jouquet, P., Bottinelli, N., Shanbhag, R.R., Bourguignon, T., Traoré, S., Abbasi, S.A., 2016. Termites: the neglected soil engineers of tropical soils. Soil Sci. 181, 157–165. https://doi.org/10.1097/SS.00000000000001 19.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010.
Kembel, S.W., Cowan, P.D., Helmus, M.R., Cornwell, W.K., Morlon, H., Ackerly, D.D., et al., 2010. Picante: R tools for integrating phylogenies and ecology. Bioinformatics 26, 1463–1464. https://doi.org/10.1093/bio- informatics/btq166.
Kurtz, Z.D., Muller, C.L., Miraldi, E.R., Littman, D.R., Blaser, M.J., Bonneau, R.A., 2015. Sparse and compositionally robust inference of microbial ecological networks. PLoS Comput. Biol. 11, e1004226. https://doi.org/10.1371/journal.pcbi.1004226.
Lahti, L., Shetty, S., 2017. Tools for Microbiome Analysis in R. https://microsud.github.io/Tools-Microbiome-Analysis/.
Latham, J.S., He, C., Alinovi, L., DiGregorio, A., Kalensky, Z., 2002. FAO methodologies for land cover classification and mapping. In: Walsh, S.J., Crews-Meyer, K.A. (Eds.), Linking People, Place, and Policy. Springer, Boston, MA, USA.
Lenth, R.V., 2023. Emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.8.8.
Li, G.S., Leal-Dutra, C.A., Cuesta-Maté, A., Conlon, B.H., Peereboom, N., Beemelmanns, C., et al., 2023. Resolution of eleven reported and five novel *Podaxis* species based on ITS phylogeny, phylogenomics, morphology, ecology, and geographic distribution. Pers.: Mol. Phylogeny Evol. Fungi. 51, 257–279. https://doi.org/10.3767/persoonia.2023.51.07.
Li, H., Greening, C., 2022. Termite-engineered microbial communities of termite nest structures: a new dimension to the extended phenotype. FEMS Microbiol. Rev. 46: 46, fuac034. https://doi.org/10.1093/femsre/fuac034.
Liu, C., Cui, Y., Li, X., Yao, M., 2021. Microeco: an R package for data mining in microbial community ecology. FEMS Microbiol. Ecol. 97. https://doi.org/10.1093/femsec/fiaa255.
Liu, H., Beckenbach, A.T., 1992. Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. Mol. Phylogenet. Evol. 1, 41–52. https://doi.org/10.1016/1055-7903(92)90034-e.
Luo, Y., Zhang, D., Guo, Y., Zhang, S., Chang, L., Qi, Y., et al., 2022. Comparative insights into influences of co-contamination by rare-earth elements and heavy metals on soil bacterial and fungal communities. J. Soils Sediments 22, 2499–2515. https://doi.org/10.1007/s11368-022-03241-9.
Martinez, Arbizu P., 2020. pairwiseAdonis: pairwise multilevel comparison using adonis. R package version 0.4.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.
Miura, T., Maekawa, K., Kitade, O., Abe, T., Matsumoto, T., 1998. Phylogenetic relationships among subfamilies in higher termites (Isoptera: Termitidae) based on mitochondrial COII gene sequences. Ann. Entomol. Soc. Am. 91, 515–523. https://doi.org/10.1093/aesa/91.5.515.
Muñoz Sabater J. ERA5-Land monthly averaged data from 1981 to present. In: (CDS) CCCSCSCDS, editor, 2019.
Nilsson, R.H., Larsson, K.H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., et al., 2019. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. 47, D259–D264. https://doi.org/10.1093/nar/gky1022.
Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, et al. vegan: Community Ecology Package, 2022.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.
R Core Team, 2022. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rambaut A, Suchard MA, Xie D, Drummond AJ. Tracer v1.6., 2014. https://bioweb.pasteur.fr/packages/pack@Tracer@v1.6.
Reboleira, A.S., Bodawatta, K.H., Ravn, N.M.R., Lauritzen, S.-E., Skoglund, R.Ø., Poulsen, M., et al., 2022. Nutrient-limited subarctic caves harbour more diverse and complex bacterial communities than their surface soil. Environ. Microbiome 17, 41. https://doi.org/10.1186/s40793-022-00435-z.
Rosengaus, R.B., Lefebver, M.L., Traniello, J.F.A., 2000. Inhibition of fungal spore germination by *Nasutitermes*: evidence for a possible antiseptic role of soldier defensive secretions. J. Chem. Ecol. 26, 21–39. https://doi.org/10.1023/A:1005481209579.
Sanderson, M.G., 1996. Biomass of termites and their emissions of methane and carbon dioxide: a global database. Glob. Biogeochem. Cycles 10, 543–557. https://doi.org/10.1029/96GB01893.
Sayers, E.W., Bolton, E.E., Brister, J.R., Canese, K., Chan, J., Comeau, D.C., et al., 2022. Database resources of the national center for biotechnology information. Nucleic Acids Res. 50, D20–D26. https://doi.org/10.1093/nar/gkab1112.
Schmidt, S., Bos, N., Murphy, R., KN, A., SK, S., Meyling, N.V., et al., 2023. Make the environment protect you from disease: elevated CO_2 inhibits antagonists of the fungus-farming termite symbiosis. Front. Ecol. Evol. 11, 1134492. https://doi.org/10.3389/fevo.2023.1134492.
Sileshi, G.W., Arshad, M.A., Konaté, S., Nkunika, P.O.Y., 2010. Termite-induced heterogeneity in African savanna vegetation: mechanisms and patterns. J. Veg. Sci. 21, 923–937. https://doi.org/10.1111/j.1654-1103.2010.01197.x.
Sinotte, V.M., Conlon, B.H., Seibel, E., Schwitalla, J.W., de Beer, Z.W., Poulsen, M., et al., 2021. Female-biased sex allocation and lack of inbreeding avoidance in *Cubitermes* termites. Ecol. Evol. 11, 5598–5605. https://doi.org/10.1002/ece3.7462.
Soukup, P., Větrovský, T., Stiblik, P., Votýpková, K., Chakraborty, A., Sillam-Dussès, D., et al., 2021. Termites are associated with external species-specific bacterial communities. Appl. Environ. Microbiol. 87. https://doi.org/10.1128/aem.02042-20 e02042-20.
Stefanowicz, A.M., Niklinska, M., Laskowski, R., 2008. Metals affect soil bacterial and fungal functional diversity differently. Environ. Toxicol. Chem. 27, 591–598. https://doi.org/10.1897/07-288.1.
Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., et al., 2013. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol. Ecol. 85, 612–626. https://doi.org/10.1111/1574-6941.12148.
Tedersoo, L., Anslan, S., Bahram, M., Põlme, S., Riit, T., Liiv, I., et al., 2015. Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. MycoKeys 10, 1–43. https://doi.org/10.3897/mycokeys.10.4852.
Van Thuyne, J., Verrecchia, E.P., 2021. Impacts of fungus-growing termites on surficial geology parameters: a review. Earth Sci. Rev. 223, 103862. https://doi.org/10.1016/j.earscirev.2021.103862.
Van Thuyne, J., Darini, I., Mainga, A., Verrecchia, E.P., 2021. Are fungus-growing termites super sediment-sorting insects of subtropical environments? J. Arid Environ. 193, 104566. https://doi.org/10.1016/j.jaridenv.2021.104566.
Vasar, M., Davison, J., Sepp, S.-K., Mucina, L., Oja, J., Al-Quraishy, S., et al., 2022. Global soil microbiomes: a new frontline of biome-ecology research. Glob. Ecol. Biogeogr. 31, 1120–1132. https://doi.org/10.1111/geb.13487.
Wood, T.G., 1988. Termites and the soil environment. Biol. Fertil. Soils 6, 228–236.
Wright, M.N., Ziegler, A., 2017. Ranger: a fast implementation of random forests for high dimensional data in C++ and R. J. Stat. Softw. 77, 1–17. https://doi.org/10.18637/jss.v077.i01.
Yan, Z.-Z., Chen, Q.-L., Li, C.-Y., Nguyen, B.-A.T., Zhu, Y.-G., He, J.-Z., et al., 2021. Termite mound formation reduces the abundance and diversity of soil resistomes. Environ. Microbiol. 23, 7661–7670. https://doi.org/10.1111/1462-2920.15631.
Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89, 670–679. https://doi.org/10.1002/bit.20347.