Science of the Total Environment 892 (2023) 164405

Contents lists available at ScienceDirect

Science of the Total Environment

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

Soil development following glacier retreat shapes metagenomic and metabolomic functioning associated with asynchronous C and N accumulation

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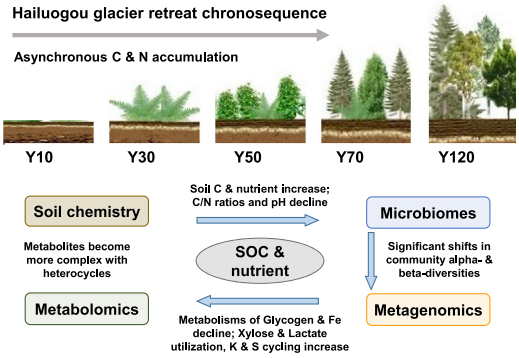
HIGHLIGHTS

- Alpha diversities of soil bacteria, protozoa and N fixers increased with soil ages.
- Soil development shifted the beta diversities and composition of microbial groups.
- Metagenomic C-related functioning differed greatly with soil chronosequence.
- Metabolomics show increased complexity of C metabolite structure with soil ages.
- Soil C and C/N ratios were most related to metagenomic and metabolomic functions.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Abasiofiok Mark Ibekwe

Keywords:
Hailuogou glacier forefield
Soil development
Metagenomics
Metabolomics
Soil C/N ratios

ABSTRACT

Glacier retreat caused by global warming may result in the variation of soil organic carbon and nutrient cycling. Yet, the dynamic change of soil microbial functional profiles, especially C metabolism-related, with soil development following glacier retreat are still unclear. In the present study, we investigated the soil microbial communities, metagenomic functioning, and metabolomic profiles along the Hailuogou Glacier forefield representing a 120-year chronosequence. The alpha diversity indices of soil bacteria, protozoa and *nifH* genes showed an upward trend with increased soil ages, and the beta diversity of soil archaea, bacteria, fungi, protozoa, *nifH* and *nirS* genes were significantly correlated with soil ages, in which increasing soil C and P while decreased C/N and pH significantly contributed to the differences of soil microbial communities among the analyzed environmental variables. The metagenomic functional genes related to the metabolisms of Glycogen and Cellulosome, Iron Acquisition and Metabolism were significantly decreased with chronosequence, while the utilization of Xylose and Lactate, Potassium Metabolism, Sulfur Metabolism showing an upward trend with soil ages, in which soil C/N ratios and pH were the most influential factors. In addition, soil C and C/N ratios were also significantly correlated to metabolomic compositions, in which the complexity of the metabolite structure increased with soil ages. Our results indicate that glacier

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http://dx.doi.org/10.1016/j.scitotenv.2023.164405
Received 20 April 2023; Received in revised form 18 May 2023; Accepted 20 May 2023
Available online 26 May 2023

retreat may lead to the asynchronous C and N accumulation along the chronosequence, thereby affecting the metagenomic and metabolomic functioning of soil microbial communities related to C metabolisms during soil development following glacier retreat.

1. Introduction

Mountains are unique terrestrial ecosystems, showing altitudinal variations in landscape, climate and biodiversity (Wang et al., 2022). Glaciers formed on mountains of high altitude are the critical component of the water cycle but sensitive to climate change (Xia et al., 2013). During recent decades, global warming has weakened the inherent stability of mountain glaciers and permafrost (Trisos et al., 2020; Tebaldi et al., 2021). Specially, a typical monsoonal marine glacier in the southeast of the Tibet Plateau, the center of high mountains in Asia (Zhi-guo, 2012) has suffered melting at a high rate (He et al., 2008; Xia et al., 2013). This may have significant impacts on local and regional ecosystem functions, such as energy flow and biogeochemical cycles. As the primary decomposers of soil carbon (C) and keystone regulators of nutrient cycling, the microbiota is presumed highly sensitive to glacier retreat and thereafter soil formation and development (Bradley et al., 2014). The diversity and distribution patterns of soil microorganisms are highly sensitive to rapid glacier retreat and subsequent ecological consequences. Recent observations have well-revealed microbial responses to glacier retreat (Jiang et al., 2018; Bai et al., 2020a; Wang et al., 2021a); however, a systematic and comprehensive understanding is still lacking, specifically pertaining to structural and functional evolution of the microbiota relating to C metabolisms and nutrient cycles across the transition from glacier retreat to soil development.

The newly exposed terrain after glacier retreat will be colonized by microbiota, consequently undergo various changes in soil physical and chemical properties, as well as the initial biogeochemical processes, especially soil C and nitrogen (N) cycles (Bradley et al., 2014). For example, soil formation after glacier retreat starts with the gradual accumulation of soil C in the upper layer (Schweizer et al., 2018) via chemoautotrophic and photoautotrophic microbial C fixation (Wang et al., 2021b). Heterotrophic microbes also gradually emerge and participate in soil C decomposition and turnover (Soong et al., 2019). During the early stage of soil development, soil N accrual is mainly through energy-costly and low-efficient N fixation by free-living microbes. As such, the rate of soil N accumulation is largely lower than the rate of soil C accumulation (Wang et al., 2021b), the so-called asynchronous accumulation of C and N. In the later stage of soil development following plant establishment, symbiotic N-fixing microbes become predominant, fix atmospheric N at high efficiency, and help alleviate N limitation in the soil environment (Zheng et al., 2020). The soil microbiome evolves along soil development and the baseline information about the magnitude and direction of evolving in diversity, composition, function, and metabolic profiling is critically needed to better project ecological consequences of glacier retreat under future global warming.

Recent advances in high-throughput sequencing approach allow for detection of rare members of the microbial community and therefore improve analytical resolution in diversity and composition (Caporaso et al., 2012). Shotgun metagenomic sequencing has been appreciated as a valid tool for an encyclopedic survey of microbial functions and metabolic pathways in the soil (Quince et al., 2017). Microbial functioning can also be estimated from metabolites of biochemical pathways by metabolomic analysis (Zhao et al., 2019; Li et al., 2020b; Withers et al., 2020; Brown et al., 2021). In the present study, we combined different -omics data to assess how microbial function was connected with microbial diversity along soil development after glacier retreat and hoped to offer biological trajectory that could more accurately predict soil multifunctions. Specifically, a field study based on a space-for-time substitution approach (Fernández-Martínez et al., 2017) was established in the Hailuogou Glacier, a typical monsoonal marine glacier in the Gongga Mountain on the southeastern edge of the Tibet Plateau. This ~120-year glacier retreat chronosequence has similar climate and well-developed soil profiles from similar parent materials (An et al., 2019; Li et al., 2020a; Wang et al., 2020). However, previous studies solely focused on certain microbial processes or enzyme activities, behind which the functional mechanism, microbial C metabolism-related in particular, is still in question. To fill these knowledge gaps, this study aimed to (i) explore whether the diversities of soil microbiota, including archaea, bacteria, fungi, protozoa, and N-cycling processors, linearly increase along the Hailuogou glacier retreat chronosequence; (ii) elucidate whether asynchronous accumulations of soil C and N drive metagenomic functional traits related to soil C and nutrient metabolisms with soil development; (iii) answer whether the structure complexities of low molecular weight metabolites show clear increasing pattern between the early and later successional stages after glacier retreat.

2. Materials and methods

2.1. Site description and soil sampling

The study site is located in the Hailuogou Glacier on the eastern slope of Gongga Mountain, Sichuan Province, China (29°36′N,101°53′E) with an elevation gradient of 2700–3600 m. Gongga Mountain peaks at 7556 m and is highest in the eastern part of the Tibetan Plateau and the Hengduan Mountains. The Hailuogou Glacier is a typical marine glacier with fast melting speed and no glacier advance during the last century. The mean annual precipitation (MAP) is 1910 mm, and the mean annual temperature (MAT) is 4.4 °C. The glacier retreat area includes sequential sites of soil formation and development signified by a complete succession of plant communities (An et al., 2019; Bai et al., 2020a). We chose five sites, with glacier retreat starting in 2010, 1990, 1970, 1950 and 1900 corresponding to 10, 30, 50, 70, and 120 year-old soil development, respectively, when soil sampling in September 2021 (Fig. S1). Soil ages were determined based on historic records of glacier front location and the retreat distance by field surveys (An et al., 2019; Li et al., 2020a; Wang et al., 2020). At each site, three replicate plots (5 m × 5 m) were made with a distance of approximately 100 m apart. At each plot, five soil cores (top 15 cm) were taken randomly and then pooled to form a composite soil sample. A total of 15 composite soil samples (5 sites × 3 replicates) were kept in a dry ice-filled cooler and taken back to the laboratory within 24 h. After visible plant material and stones were manually removed, soil sample was sieved through 2-mm mesh, and stored at 4 °C for soil chemical analyses and at −80 °C for soil DNA extraction and metabolomic measurements.

2.2. Soil chemical analyses

Basic chemical properties of soil sample were analyzed as previously reported (Zhang et al., 2022a), including soil pH, soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), NH_4^+ and NO_3^- content, available phosphorus (AP), and cation exchange capacity (CEC). Soil pH was analyzed with 1:5 (w/v) soil-to-water ratio suspension using the potentiostatic method. Soil NH_4^+- and NO_3^--N were extracted with 2 mol L^{-1} KCl and measured using a flow injection auto analyzer (FIAstar 5000 Analyzer, Foss Tecator, Denmark). The SOC and TN were determined by an elemental analyzer (Vario MAX cube elemental analyzer, Elementar, GER). The TP and AP contents were measured by phosphorus molybdenum blue spectrophotometry and colorimetric method, respectively.

2.3. Soil DNA extraction, amplicon sequencing and community analysis

Soil DNA was extracted from ~0.6 g soil sample using Qiagen PowerSoil DNA Isolation Kit (Mo Bio Laboratories) following the manufacturer's

protocols. DNA concentrations were determined (50 ng μL^{-1}) and purity was confirmed by the ratio of absorbance at 260 and 280 nm (260/2801.70–1.90) using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, United States). We conducted amplicon sequencing and microbial community analysis following protocols in our previous publications (Chen et al., 2018; Chen et al., 2019a; Zhang et al., 2022a). The amplicon primers used for the microbial community analyses are given in Table S1 (Zhang et al., 2015; Jiang et al., 2017; Hirakata et al., 2019; Cavalieri et al., 2020; Raes et al., 2020; Dai et al., 2021).

All the PCR amplifications were conducted with Illumina overhang adaptors as follows: initialization at 95 °C for 3 min, and 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, followed by a final elongation of 10 min at 72 °C. A negative control with no template was included in the PCR. Triplicate PCR reactions were performed per sample; amplicon size and concentration were checked by 1 % agarose gel electrophoresis. Subsequently, amplicons were polled in equidensity ratios using GeneTools Analysis Software (Version4.03.05.0, SynGene) for cleaning up using E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, USA). Purified DNA fragments were added at both ends with unique index (barcode) sequences using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA), followed by the test of quality. An equimolar mixture of the purified amplicons were sequenced (250 bp × 2) on an Illumina Nova6000 platform.

Raw reads were processed using Quantitative Insights into Microbial Ecology (QIIME) standard operation procedure (Caporaso et al., 2010). Forward and reverse primers were removed by using Cutadapt (Martin, 2011); paired-end sequencing data was then joined and demultiplexed using QIIME with quality filter score at default Phred > 19. Chimeras of trimmed and filtered sequences were identified and removed by using USEARCH (Edgar, 2010). Operational taxonomic units (OTU) were clustered at >97 % similarity. Representative sequences for each OTU were selected for taxonomic assignment. Archaeal, bacterial, and fungal OTUs were assigned using the Ribosomal Database Project (RDP) database method (Cole et al., 2014) against Greengenes database (version_16.8) (McDonald et al., 2012) for 16S rRNA sequencing data and UNITE database (version_12.11) (Koljalg et al., 2013) for ITS sequencing data. Protozoal OTUs were generated from 18S rRNA gene sequencing reads and assigned using the Protist Ribosomal Reference (PR2) database (version_4.12) (Guillou et al., 2013), and further filtered to represent only protozoa taxa by excluding the potential sequences of fungi, viridiplantae, metazoan, etc. Bacterial *nifH* and *nirS* gene OTUs were assigned using the FunGene database (version_9.9.11) (Fish et al., 2013). All chimeric and singletons were removed after clustering at 97 % similarity for downstream analysis. All the sequencing raw data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA849388.

The same primers and PCR protocols were used to conduct quantitative real-time PCR on each soil DNA sample with three analytical replicates (CFX96 Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA) to determine the copy numbers of the 16S, 18S rDNA, ITS regions of rDNA, and *nirS* and *nifH* genes (Chen et al., 2019b; Zhang et al., 2022b) .

2.4. Metagenomic sequence process

We sequenced metagenomic data following protocols in our previous publications (Dai et al., 2020; Zhang et al., 2022a). Shotgun metagenomes were prepared using 1 μg DNA per sample by the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's instructions. Barcodes were added to each sample to give the attribute for sequences. Purified barcoded libraries were sequenced on an Illumina Nova6000 platform. Final libraries were validated on an Agilent Bioanalyzer (Agilent, Santa Clara, USA) using a DNA7500 chip and concentration was determined on an Invitrogen Qubit (Waltham, USA) with the broad range double stranded DNA assay. All metagenome sequences were deposited and processed in the Rapid Annotation using Subsystems Technology for Metagenomes (MG-RAST) (Keegan et al., 2016) under project accession number mgp100910 following Trimmomatic (version 0.36) employed to remove low-quality reads for subsequent analysis (Bolger et al., 2014). Soil metagenomic sequences were annotated using the RefSeq database for taxonomic assignment (taxonomy) and the SEED Subsystems database for functional classification (function) according to the default setting recommended by the server (maximum e-value cutoff was $1e^{-5}$, minimum identity cutoff was 60 %, and minimum alignment length was 50) (Meyer et al., 2019). Based on the metagenomic taxonomic and functional annotations, gene abundances were directly obtained from the server for each sample at each taxonomic or functional level.

2.5. Metabolite extraction, profiling, and analysis

Soils samples (100 mg) were freeze-dried and then extracted using methanol solution (methanol: water = 3: 1, with isotopically-labelled internal standard mixture). Sequential extraction procedures included three repeats of homogenization at 35 Hz for 4 min and ice-bath ultrasonic vibration for 5 min, incubation for 1 h at −40 °C, and then centrifugation at 12,000 rpm for 15 min at 4 °C (Zhang et al., 2022a). The supernatant was transferred and tested in 2 mL injection bottle, and analyzed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to QExactive HFX mass spectrometer (Orbitrap MS, Thermo). The quality control (QC) samples were prepared by mixing the same amount of supernatant from all of the sample.

For reliable analysis, the original data set was managed by (1) filtering out single peaks of presumable noise, outlier peaks and peaks of extremely low area, only the peak area data with one group of null values not >50 % or all groups of null values not >50 % were retained (2) filling in the missing values by numerical simulation and minimum half method; (3) normalization based on internal standard (IS) was used for normalization (Dunn et al., 2011). The raw data were converted to the mzXML format using ProteoWizard for peak detection, extraction, alignment, and integration. Then, an MS2 database (BiotreeDB, version 2.1) was applied in metabolite annotation. The cutoff for annotation was set at 0.3. Finally, the soil metabolomic data were log transformed and calculated for the Euclidean distance between samples for subsequent statistical analyses as previously described (Veach et al., 2020).

2.6. Community assembly process and co-occurrence network analysis

Microbial community assembly was processed according to the previous descriptions (Zhang et al., 2022b). The nearest taxon index (NTI) and net relatedness index (NRI) were calculated using the R package “picante” (Kembel, 2010). A positive NTI or NRI value (>1.96) indicates that the community was more phylogenetically clustered, while a negative NTI or NRI value (<−1.96) indicates that the community was phylogenetically overdispersed. Observed values were compared with 999 null values generated by a null model as −1 × (observed values − mean of null values)/standard deviation of null values to indicate whether the observed phylogenetic distance was deviated from a random expectation. The values of beta NTI and beta NRI were also calculated as the values of <|1.96| suggest neutral assembly processes while the values of >|1.96| indicate deterministic processes.

The protocol to analyze co-occurrence networks was explained previously (Chen et al., 2021; Dai et al., 2021; Chen et al., 2022b). Co-occurrence network analysis was performed using Molecular Ecological Network Analysis Pipeline (Zhou et al., 2011; Deng et al., 2012). The network with default settings was constructed with a data matrix of normalized relative abundances multiplied by 10^6 (meeting the requirements of the pipeline): (1) Only OTUs with more than half of the tested samples are retained; (2) only used 0.01 in blanks with paired valid values; (3) perform logarithmic transformation with Pearson correlation coefficients; and (4) choose “Decrease the cut-off from top” as the calculation order and “Regress Poisson distribution only” as the scan speed. To associate a pair of species, a default cut-off value (similarity threshold, S_t) for the similarity

matrix was generated. Global network properties, centrality of individual nodes, and module separation and modularity calculations were then run using greedy modularity optimizations based on the default setting. The export and visualization of network files were carried out by Cytoscape software (Shannon et al., 2003). Indices including positive links, negative links, the average degree (avgK), coefficient (avgCC), average path distance (GD), geodesic efficiency (E), and harmonic geodesic distance (HD) were calculated to evaluate the topological properties of the microbial community networks. The modularity characterizes modular structures in the networks, and the values of >0.4 indicate that the module density is noticeably higher than the overall average.

2.7. Statistical analyses

The numbers of microbial OTUs and metagenomic taxonomic or functional genes were standardized to the relative abundance through dividing by total numbers. Based on the relative abundances, we calculated Bray-Curtis similarity matrix to construct a triangular pairwise Bray-Curtis similarity matrix to examine the community structures and functional profiles in PRIMER 7 (Plymouth Routines in Multivariate Ecological Research Statistical Software, v7.0.13, PRIMER-E Ltd., UK). The Euclidean distance was used to calculate the metabolomic distance matrix. The PERMANOVA (permutational multivariate analysis of variance), dbRDA (distance-based redundancy analysis), and DistLM (marginal tests performed by distance-based linear model analysis) were further conducted to evaluate the significant difference among soil ages and the association of soil chemical properties with the distribution of composition structures of microbial communities and metagenomics in PRIMER 7. Linear discriminant analysis effect size (LEfSe) was used to show the significant differences of major bacterial, fungal and protozoal taxonomic groups. Heatmaps were presented by HeatMapper (Babicki et al., 2016). Non-metric multidimensional scaling (NMDS) analysis were used to show the phylogenetic distance of the microbial communities.

3. Results

3.1. Soil properties along the glacier retreat chronosequence

Soil chemical properties changed significantly over soil development, but time patterns were properties-dependent (Fig. 1). Soil organic C (SOC), total nitrogen (TN), total phosphorus (TP), NH_4^+ and available phosphorus (AP) increased during the first 50 years and then gradually stabilized. On the contrary, soil pH and C/N ratio declined from ~8.5 and >30:1, respectively, in newly-developed soils (i.e., 10- and 30-years old) to ~6.5 and ~15:1 in the aged soils (i.e., ≥50 years old). Soil NO_3^- and cation exchange capacity (CEC) changed little except for an ephemeral surge in the 50-year-old soil. However, there were no significant difference in the abundance of archaea, bacteria, fungi, and protozoa, *nifH*-containing or *nirS*-containing community along the glacier chronosequence (Table S2).

3.2. Microbial alpha- and beta-diversities along the glacier retreat chronosequence

The species richness and Shannon index were calculated to compare alpha diversities of soil microbial communities among different soil ages along the Hailuogou glacier retreat chronosequence (Fig. 2a). Species richness and Shannon index of the bacterial, protozoal or N fixer community showed an upward trend with soil development, while both metrics of the archaeal community and fungal Shannon index followed a U-shaped pattern. Alpha diversity metrics of the *nirS*-containing denitrifier community remained unchanged as soil aged.

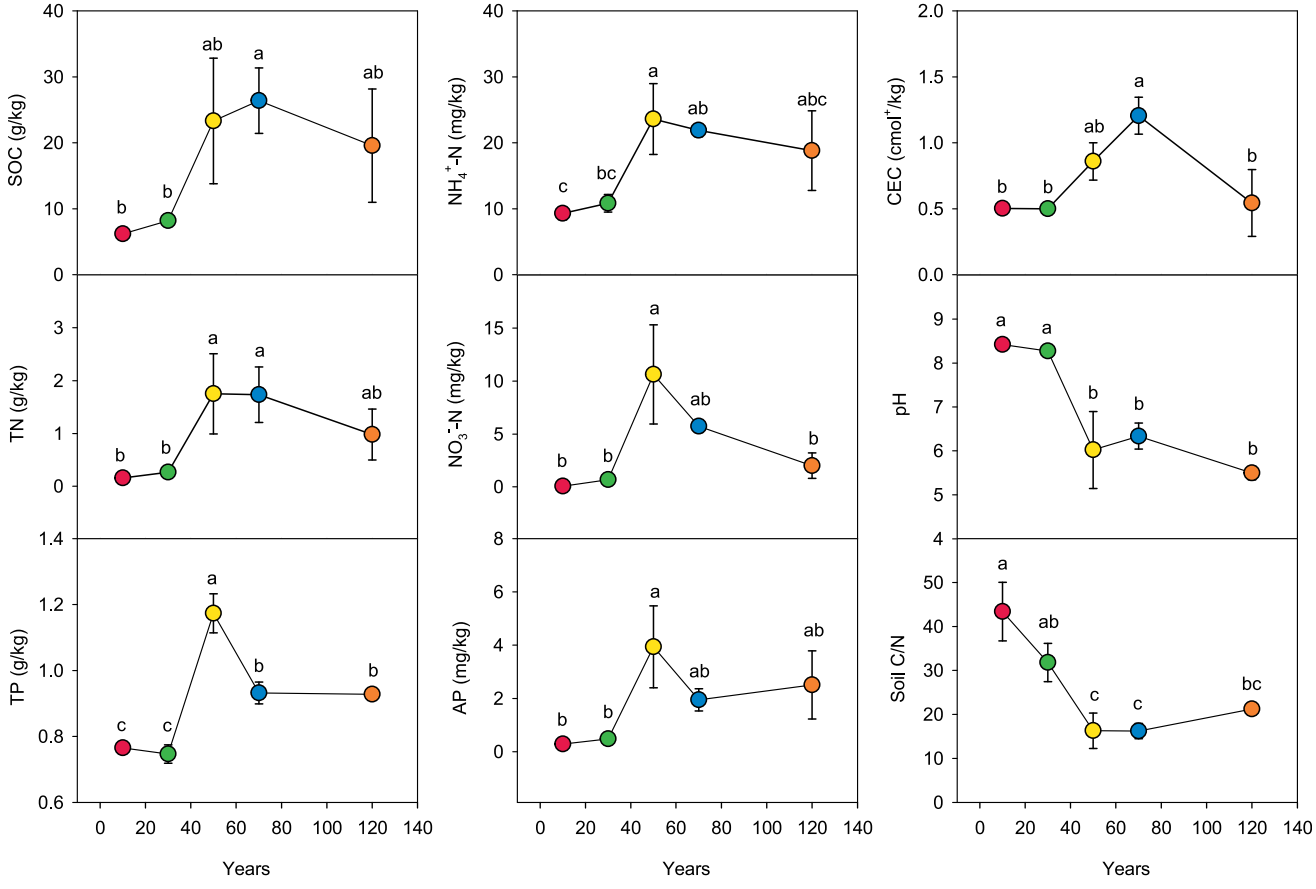

Fig. 1. Soil chemical properties of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence. Different letters represent significant differences of ANOVA at $P < 0.05$.

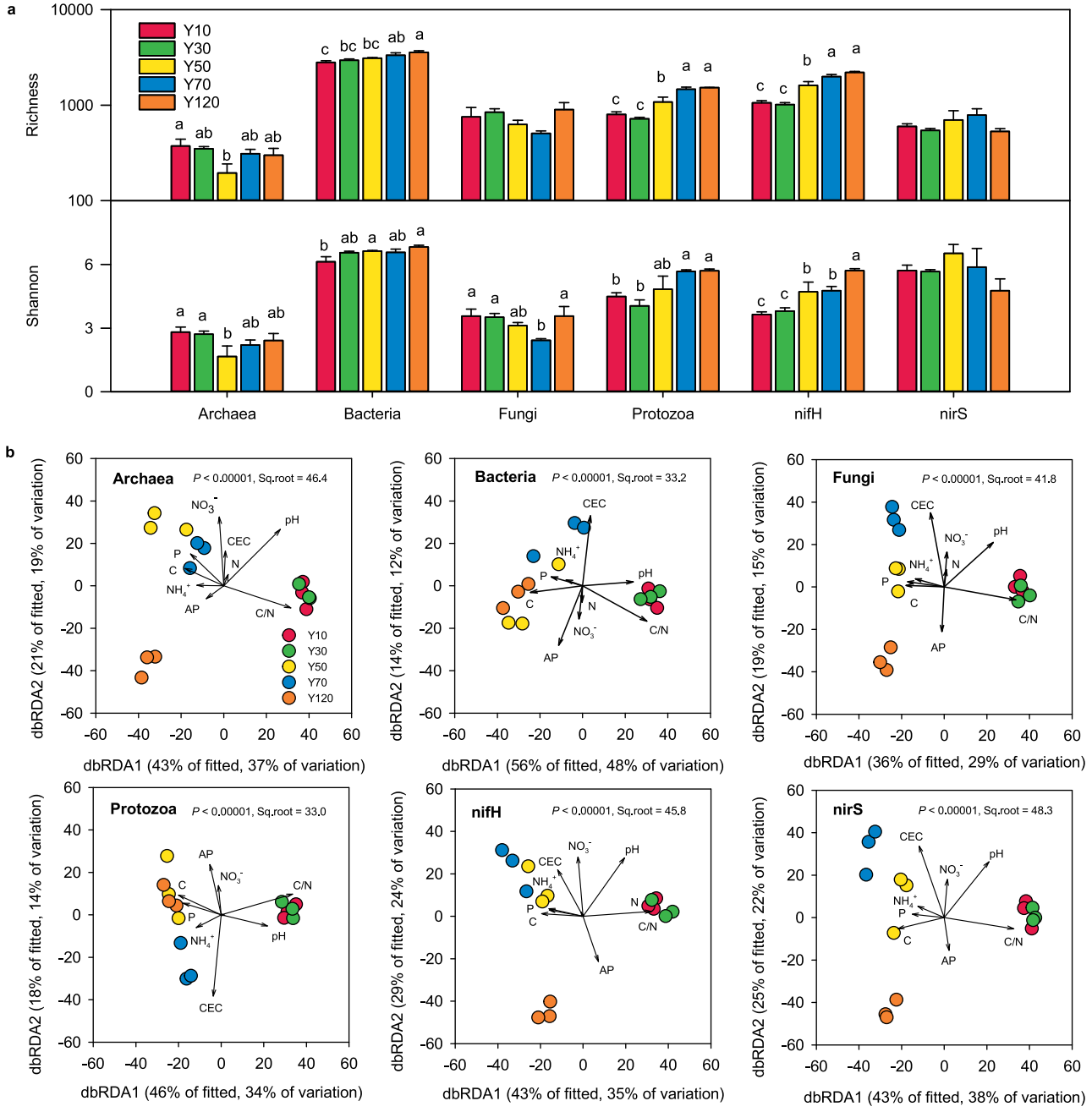

Fig. 2. a. Alpha diversities of archaea, bacteria, fungi, protozoa, N fixers (*nifH*), and bacterial denitrifiers (*nirS*) in the soil of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence. Different letters represent significant differences of ANOVA at $P < 0.05$. Sequence numbers were rarefied to 3078, 29,437, 62,702, 30,252, 26,101, and 41,136 for archaea, bacteria, fungi, protozoa, *nifH*, and *nirS* respectively. **b**. Distance-based redundancy analysis (dbRDA) of archaea, bacteria, fungi, protozoa, N fixers (*nifH*), and bacterial denitrifiers (*nirS*) in the soil of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence with soil chemical properties as explanatory variables. Fitted and total variations explained by each dimension are given in percentages. Significant PERMANOVA *P* values and percentages of variation explained by soil ages (Sq.root) are also given.

The beta diversities of archaea, bacteria, fungi, and protozoa also varied with soil development (PERMANOVA, $P < 0.00001$), with younger soils (10- and 30-years old) being clustered together and well-separated from older soils (≥50 years old) (Fig. 2b), with the percentages of explained variation around 29–48 %. This separation was primarily attributed to the change in soil C/N ratios and pH. The distribution of microbial communities in 50- and 70-years old soils were positively associated with soil C and P. The beta-diversity of archaeal, fungal, *nifH*-containing, or *nirS*-containing community in the 120-year-old soil also diverged from that in the 50- or 70-year-old soil (PERMANOVA, $P < 0.05$), which were related to increasing AP.

3.3. Compositional dynamics of the soil microbial community

The archaeal community was dominated by three phyla, Nanoarchaeota (46 %), Crenarchaeota (40 %), and Thermoplasmatota (11 %) (Fig. 3). The relative abundance of Crenarchaeota and its genus *Nitrosotalea* increased with soil development if excluding the 50-year-old soil. However, Thermoplasmatota were more abundant in 10- and 30-year-old soils than ≥50-year-old soils ($P < 0.05$).

Of the bacterial community, Proteobacteria (41 %) was the most abundant phylum, followed by Bacteroidetes (16 %), Acidobacteria (15 %), Planctomycetes (5 %), Chloroflexi (5 %), Actinobacteria (3 %), and

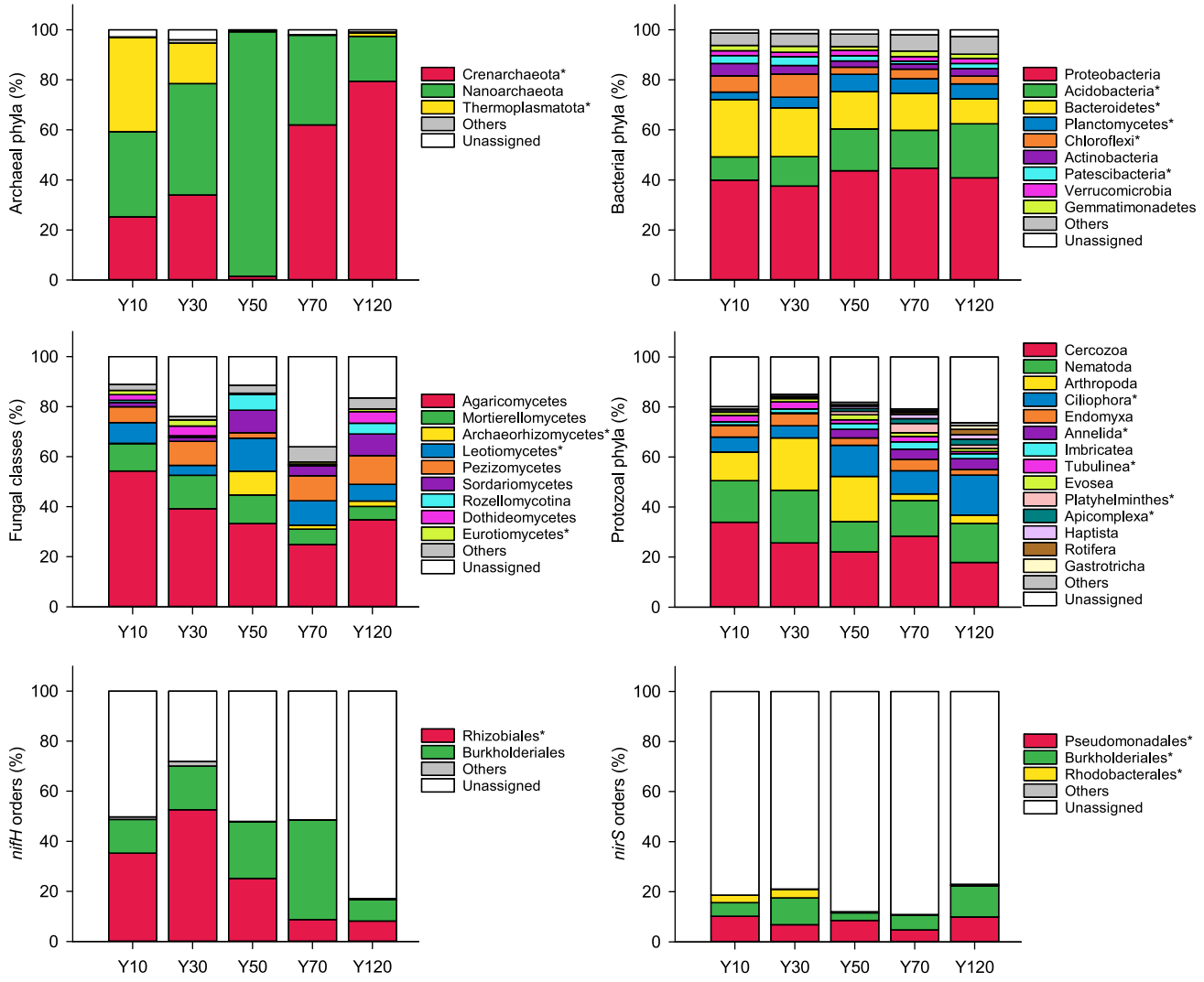

Fig. 3. Relative abundance of major compositions of archaea, bacteria, fungi, protozoa, N fixers (*nifH*), and bacterial denitrifiers (*nirS*) in the soil of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence. The asterisk indicates significant difference among soil ages based on the LEfSe analysis.

Patescibacteria (2 %) (Fig. 3). The relative abundance of Bacteroidetes (and its genus-*Flavobacterium*), Chloroflexi, and Patescibacteria declined with soil development. By contrast, Acidobacteria (its genera- *Bryobacter* and *RB41*) and Planctomycetes were more abundant in aged soils than 10- and/or 30-year-old soils ($P < 0.05$). So were for Alphaproteobacteria (its genera-*Reyranella*, *Bradyrhizobium*, *Pseudolabrys*, and *Rhodoplanes*) and Deltaproteobacteria (its genus-*Haliangium*) (Fig. S2, $P < 0.05$).

The fungal community was mainly composed of three phyla (~45 % Basidiomycota, ~10 % Mucoromycota, and ~8 % Ascomycota) and five classes (37 % Agaricomycetes, 9 % Mortierellomycetes, 8 % Leotiomycetes, 8 % Pezizomycetes, and 5 % Sordariomycetes) (Fig. 3). Soil development-associated differences in the fungal community manifested mainly at the class level, with more abundant Eurotiomycetes (its genera-*Cladophialophora*, *Exophiala*, and *Minimelanolocus*), but less abundant Leotiomycetes (its genera-*Pilidium* and *Pezicula*) and Archaeorhizomycetes (its genus-*Archaeorhizomyces*) in 10- and/or 30-year-old soils than older soils (Fig. S3, $P < 0.05$).

The top abundant protozoal phyla were Cerxozoa (16 %), Arthropod (11 %) and Cilliophora (10 %) (Fig. 3). The relative abundance of Cilliophora (its genera- *Cyrtolophosis*, *Cyclidium*, *Uroleptus*, and *Urostyla*), Annelida (its genus- *Cernosvitoviella*), Platyhelminthes (its genera-*Geocentrophora* and *Baicalellia*), or Apicoplexa (its genus-*Stenophora*) was significantly greater in older soils (i.e., ≥ 50 years old, $P < 0.05$), but Tubuline along with its class Echinamoebida and order Vermamoeba was more abundant in 10- and/or 30-year-old younger soils (Fig. S4, $P < 0.05$).

The *nifH*-containing community included ~26 % Rhizobiales, ~20 % Burkholderiales and ~53 % unassigned OTUs. As soil aged, Rhizobiales became less abundant ($P < 0.05$). Of 17 % OTUs of the *nirS*-containing bacterial community that could be assigned, Pesudomonadales, Buekhpldeiales, and Rhodobacterales accounted for 8 %, 7 % and 1 %, respectively. Rhodobacteriales were present mainly in 10- and 30-year-old soils ($P < 0.05$).

Due to the high diversities and significant difference in the bacterial, fungal and protozoal communities among different soil ages, the co-occurrence patterns of predominant OTUs were further analyzed to explore the potential roles of microbial interactions in community assembly processes. The topological characteristics of the bacterial and fungal networks showed that the numbers of nodes and links, the average degree (avgK), the average clustering coefficient (avgCC), the geodesic efficiency, and the density (D) were greater in soil ages of 50–70 years than 10–30 years (Table S4, Fig. S5). The protozoal networks only had greater numbers of nodes and links in 50–70 year-old soils in comparison with soil ages of 10–30 years. The values of the nearest taxon index (NTI) and the net relatedness index (NRI) of bacterial, fungal, and protozoal communities across different soil ages were >1.96, but only bacterial communities showed significant difference in 10–30-year-old soils in comparison with other soil ages (Fig. S6a, $P = 0.005$ and $P < 0.001$). The non-metric multidimensional scaling (NMDS) analysis of bacterial βNTI and βNRI revealed that soil ages significantly affected the microbial phylogenetic community turnover patters along glacier retreat chronosequence (Fig. S6b, PERMANOVA, $P = 0.00004$).

3.4. Microbial metagenomic functional profiles

Both metagenomic taxonomy and function were significantly affected by different soil ages along the Hailuogou glacier retreat chronosequence (Fig. 4a), although taxonomy (PERMANOVA, $P < 0.00001$) showed more significant effects than function (PERMANOVA, $P = 0.01130$). The taxonomic compositions and functional profiles of 10- and 30-year-old soils were separated from other soil ages, which were positively related to soil C/N ratios.

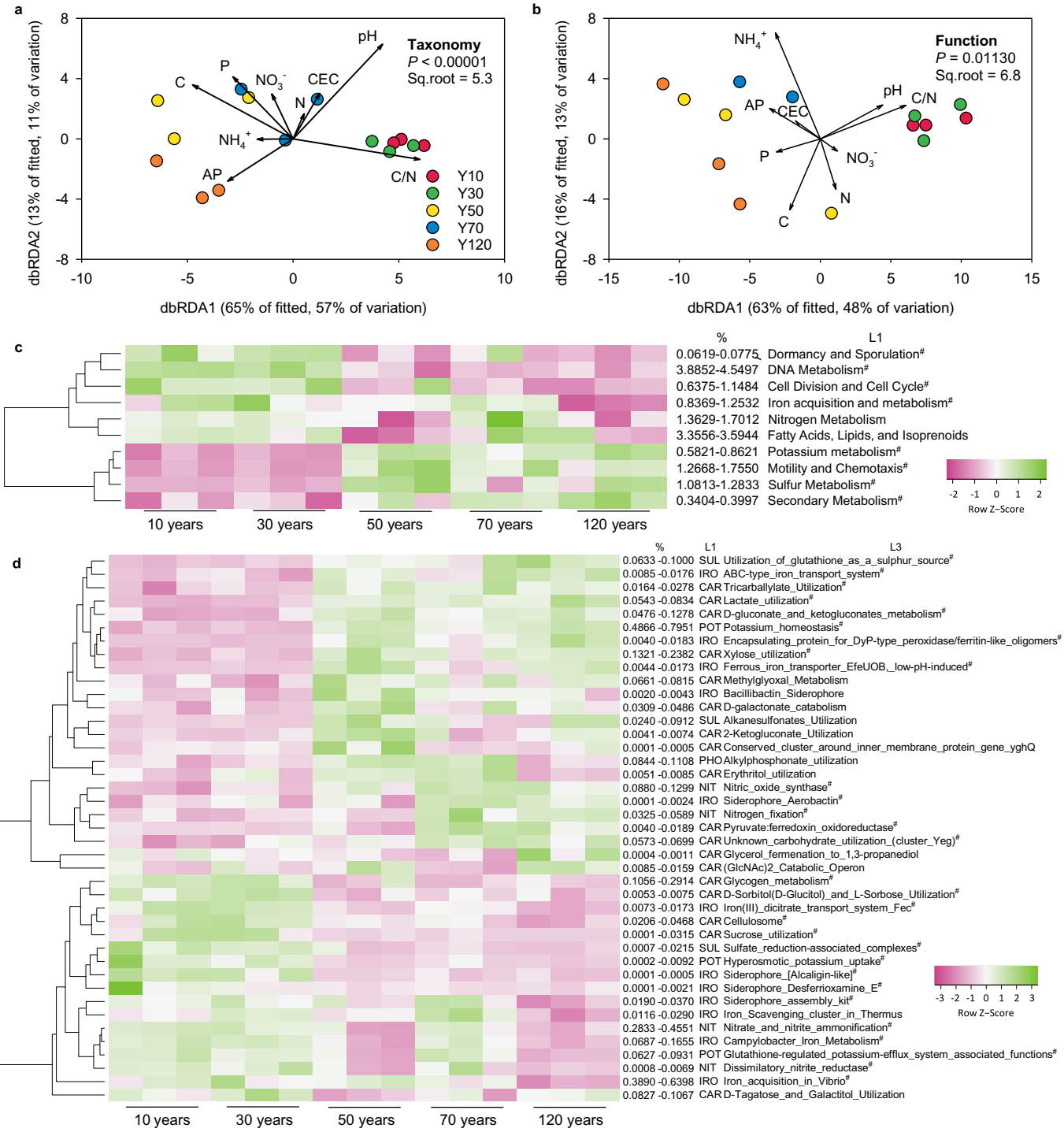

Fig. 4. a. Distance-based redundancy analysis (dbRDA) of soil metagenomes annotated in databases of RefSeq (taxonomy) and SEED Subsystems (function) in the soil of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence with soil chemical properties as explanatory variables. Fitted and total variations explained by each dimension are given in percentages. Significant PERMANOVA *P* values and percentages of variation explained by soil ages (Sq.root) are also given. **b**. Heatmaps showing relative abundance of metagenomic functional composition categorized by SEED Subsystems at level 1 that significantly differed in the soil of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence. The octothorpe indicates significant linear relationships of functional categories with soil ages based on the Pearson correlation analysis. **c**. Heatmaps showing relative abundance of metagenomic functional composition categorized by SEED Subsystems at level 3 within carbohydrate metabolisms (CAR), iron acquisition and metabolism (IRO), nitrogen metabolism (NIT), phosphorus metabolism (PHO), potassium metabolism (POT), and sulfur metabolism (SUL) that significantly differed in the soil of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence. The octothorpe indicates significant linear relationships of functional categories with soil ages based on the Pearson correlation analysis.

In metagenomic taxonomy, 120-year-old soils were further separated from 70-year-old soils, which were mainly associated with increasing AP. Specifically, for metagenomic taxonomy, Bacteroidetes, Betaproteobacteria, and Gammaproteobacteria were significantly less abundant in 10- and/or 30-year-old than other soil ages ($P < 0.05$), showing a decline trend with glacier retreat chronosequence, while Acidobacteria gradually increased with soil ages and reached the greatest point in 120-year-old soils ($P < 0.05$, Fig. S7).

Microbial genes relevant to Dormancy and Sporulation, DNA Metabolism, Cell Division and Cell Cycle, and Iron Acquisition and Metabolism were significantly more abundant in younger than ≥50-year-old soils (Fig. 4b, $P < 0.05$). However, genes relevant to Potassium Metabolism, Motility and Chemotaxis, Sulfur Metabolism, and Secondary Metabolism were less abundant in younger soils than older soils. The relative abundances of 18 genes involved in the carbohydrates metabolism shifted with soil development (Fig. 4c, $P < 0.05$). For instance, genes for the metabolisms of Glycogen and Cellulosome declined as soil aged, but genes for the utilization of Xylose and Lactate increased. A total of 7 functional categories associated with Iron Acquisition and Metabolism, e.g., Iron Acquisition in Vibrio and Siderophore assembly kit, were more abundant in younger soils than ≥50-year-old soils ($P < 0.05$), while 5 iron-related functions, mostly of

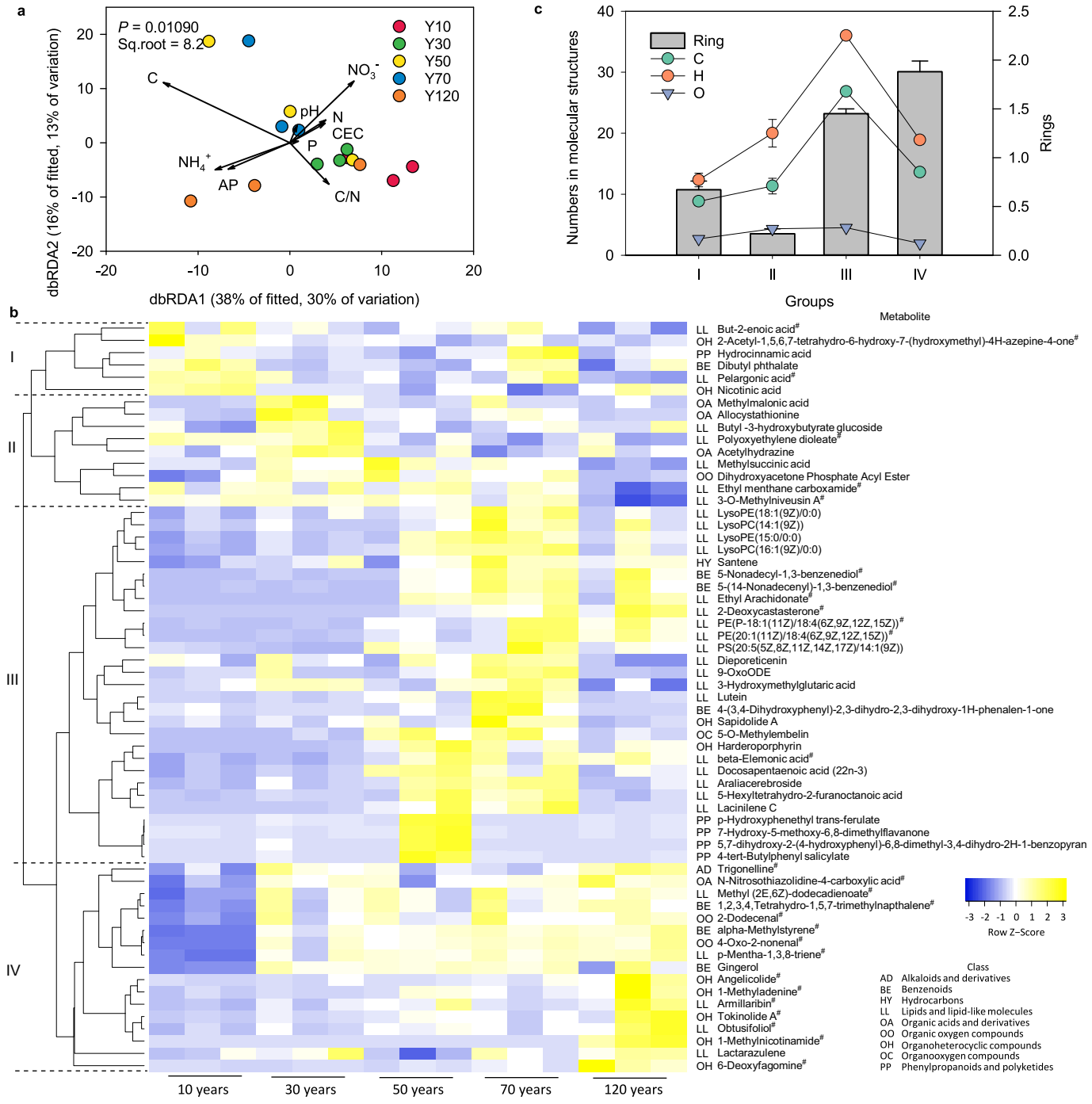

Fig. 5. a. Distance-based redundancy analysis (dbRDA) of metabolites with soil chemical properties as explanatory variables. Fitted and total variations explained by each dimension are given in percentages. Significant PERMANOVA *P* values and percentages of variation explained by soil ages (Sq.root) are also given. **b**. Heatmaps showing soil metabolites that significantly differed in the soil of different ages (10, 30, 50, 70, and 120 years) along the Hailuogou glacier retreat chronosequence. The octothorpe indicates significant linear relationships of functional categories with soil ages based on the Pearson correlation analysis. **c**. The numbers of C, H, O, and ring structures in molecules of soil metabolites clustered into four groups along the Hailuogou glacier retreat chronosequence.

lower abundance, decreased with soil ages. For Nitrogen Metabolism, genes encoding Nitric oxide synthase and N fixation increased, while genes encoding Nitrate and nitrite ammonification, and Dissimilatory nitrite reductase decreased with soil development ($P < 0.05$). In addition, the functions for Utilization of glutathione as a sulfur source, and Potassium homeostasis were less abundant in younger than 50-year-old soils ($P < 0.05$).

3.5. Microbial metabolomic profiles

Of total 601 identified metabolites, 61 differed significantly among soils of different ages (Fig. 5b, $P < 0.05$), including 30 lipids and lipid-like molecules (LL), 9 organoheterocyclic compounds (OH) and 7 benzenoids (BE). The dbRDA ordination analysis showed that metabolomic compositions were significantly affected by soil development, despite being less significant when compared to age effects on microbial communities and metagenomic taxonomic profiles (Table S6, Fig. 5a, PERMANOVA $P = 0.01090$). However, soil C rather than pH or C/N ratios became the most influential factor. Metabolites were clustered into four groups, based on patterns with soil development (Fig. 5b). Group I and II had the lower C number as well as lower number of aromatic rings or heterocycles in their molecular structures than Group III and/or Group IV (Fig. 5c). The complexity of soil metabolic structures increased with soil ages along glacier retreat chronosequence.

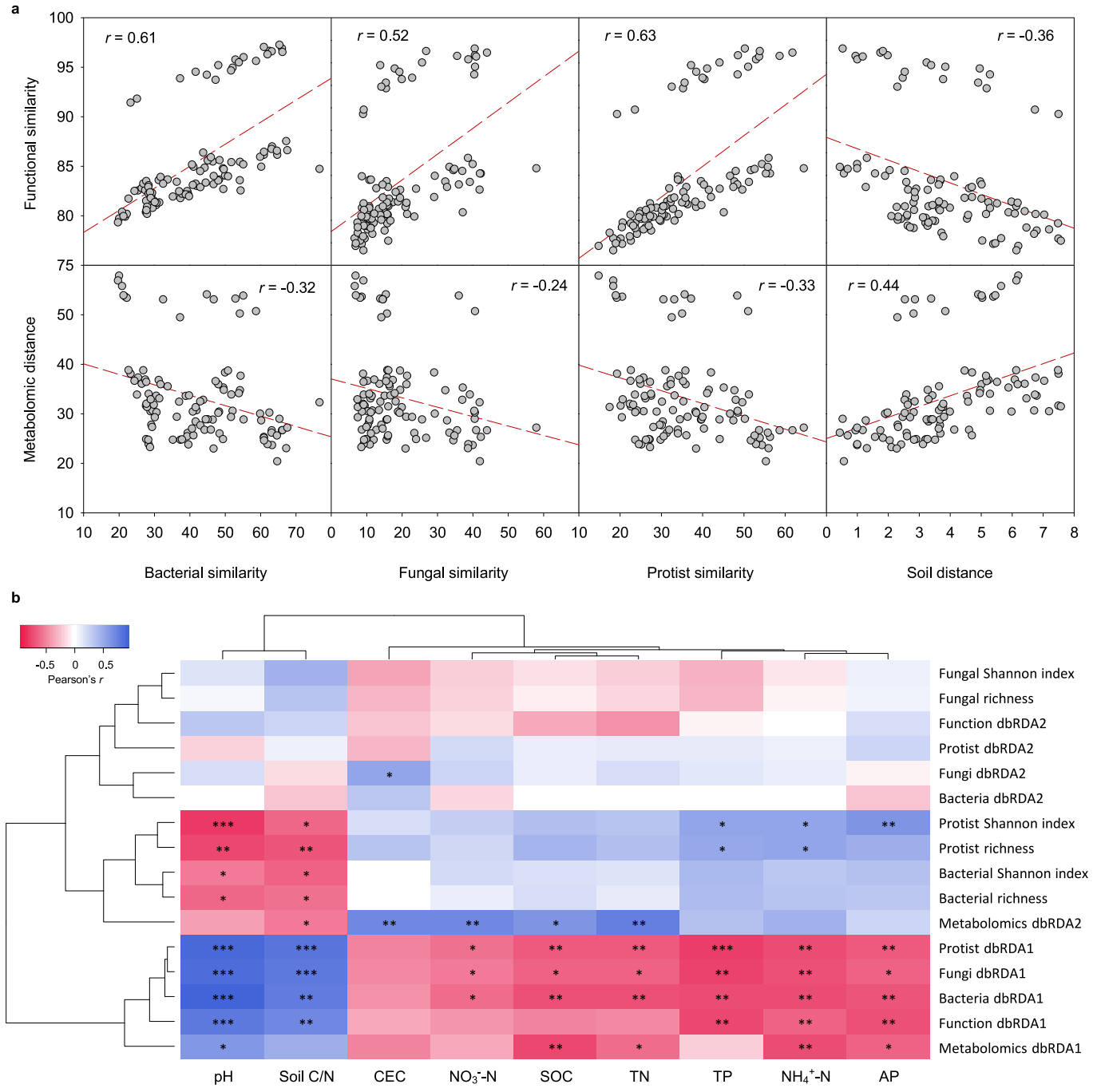

Fig. 6. a Relationships between microbial community structure and functional or metabolomic beta diversities. Pearson's correlations between similarity matrix of beta-diversity of community with functional similarity and metabolomic distance are shown. **b**. Heatmaps showing the relationships between the soil chemical properties and the beta diversities of functions, metabolites and microbial community. The octothorpe indicates significant linear relationships of metabolites and community with soil properties based on the Pearson correlation analysis.

Specifically, Group I contained 6 metabolites, including 2 organic oxygen compounds (OO), 2 LL, 1 BE, 1 phenylpropanoids and polyketides (PP), for example Pelargonic acid and But-2-enoic acid, whose abundance were greater in 10-year-old soils ($P < 0.05$). Group II contained 9 metabolites (5 LL, 3 OA, 1 OO), such as Methylmalonic acid and Methylsuccinic acid, which were more abundant in 30-year-old soils ($P < 0.05$). There were 29 metabolites clustered into Group III, including 18 LL, 4 PP, 3 BE, 2 Organoheterocyclic compounds (OH), 1 Organooxygen compounds (OC), 1 Hydrocarbons (HY), which were significantly more abundant in 50- and/or 70-year-old soils ($P < 0.05$). The organic compounds in Group III were more complex in their structures than Group I and II, for example 2-Deoxycastasterone, Lutein, beta-Elemonic acid, and Lacinilene C, etc. In 120-year-old soils, a total of 17 metabolites classified into Group IV was greater in abundance in comparison with other sites ($P < 0.05$), including 5 OH, 5 LL, 3 BE, 2 OO, 1 AD, and 1 OA. Especially, soil metabolites belonging to OH, such as Angelicolide, 1-Methyladenine, Tokinolide A, Obtusifoliol, 1-Methylnicotinamide, and 6-Deoxyfagomine, were more abundant in soil ages of 120 years ($P < 0.05$).

Compared metagenomic functional profiles, the metabolites showed less significant correlation with microbial communities (Fig. 6a). As represented by Pearson's associations between alpha- and beta-diversity metrics and soil properties (Fig. 6b), soil C/N ratios and pH had negative relationship with bacterial and protozoal alpha-diversities and significant associations with bacterial, fungal, and protozoal beta-diversities, which were opposite to the correlations of SOC, TN, TP, NH_4^+ and AP. In addition, metagenomic functional composition was correlated to soil C/N ratios, pH, TP, AP and NH_4^+, while metabolomic profiles were significantly associated with SOC, NH_4^+, TN, AP, and pH, indicating that soil parameters, especially soil C and C/N ratios, could be related with not only soil microbial compositions but also metagenomic functions and metabolites in the soil development after glacier retreat.

4. Discussion

4.1. Asynchronous soil C and N accumulation along soil chronosequence

During the soil chronosequence, the accumulation of the organic matter is crucial for the soil formation (Viereck, 1966). Similar trend for SOC was found in the early stage of glacier succession in our study (Fig. 1). During the initial primary succession of 0–30 years of soil ages, soil development is mainly regulated under the circumstance of pioneer plants and N fixing bacteria. Subsequently in 30–70 years, *Populus purdomii* became the dominant plants of high photosynthetic rate with herb and shrub colonization (Dandan et al., 2015). Thus, with glacier retreat succession, the development of plant vegetation gradually improve the availability of below-ground nutrients due to the root exudate and plant residues inputs (Jones et al., 2009; Li et al., 2015). In our study, NH_4^+, NO_3^-, and TN also significantly increased from the early to later soil development stages (Fig. 1) by mediation of N-fixing microbes associated with plant root systems (Bradley et al., 2014), as soil N is a limiting factor for plant vegetation (Perry et al., 2010; Xu et al., 2012). However, our results also showed a decline trend of soil C/N ratios along soil development chronosequence (Fig. 1), which can affect C and N mineralization that is essential for the microbial decomposition of plant organic inputs (Avnimelech, 1999; Lei et al., 2013). Thus, SOC and TN accumulating rapidly, and more importantly asynchronously, could potentially shift the microbial community and functioning between the early and later stage of glacier succession. In addition, our results showed that TP, AP, and CEC all increased with decreasing pH from the early to later stage of glacier succession (Fig. 1). Organic acid from plant residues and the dissolution of carbonate could cause the decrease of soil pH (Lei and Tang, 2008), which leads to the mineral weathering (Prietzel et al., 2013) and P solubility increase (Mccutcheon et al., 2020) as well as a significant enhancement of soil capacity to preserve fertility (Parfitt et al., 1995).

4.1.1. Shifts in soil microbial diversities along soil chronosequence

Soil microbial diversity is an essential driver of soil ecosystem functioning (Kennedy and Smith, 1995). In the present study, we found that alpha-diversity of bacteria increased with soil ages (Fig. 2a), which is consistent with other findings. For instance, a significant increase of alpha diversity of bacteria has been observed along an 18-year long chronosequence in Forni Glacier forefield (Franzetti et al., 2020). It has been also suggested that the alpha-diversity of bacterial communities from recently deglaciated region were lower in Midtre Lov'enbreen glacier covering 1900-year retreating period (Venkatachalam et al., 2021). Besides, we also found the increase in alpha diversity of N fixers and protozoa along soil ages (Fig. 2a). During the glacier retreat, the improvement of nutrition availability (Wang et al., 2021b) and soil humidity (Foissner, 1999; Mayzlish and Steinberger, 2004) benefits the N fixers and protozoal community. However, in our study, alpha-diversity of either archaea or fungi showed no significant increasing pattern (Fig. 2a). It has been reported that alpha diversity of fungal community were not significantly different along 34-year and 66-year long chronosequence respectively in the Tierra del Fuego glacier forefield (Fernández-Martínez et al., 2017). A possible explanation of inconsistent patterns of microbial alpha-diversities could be the heterogeneity of their ecological niches (Bradley et al., 2014). For instance, due to the different preferences of vegetation, the heterogeneity of bacterial community increased while fungal community remained static along Lyman Glacier chronosequence representing 70-year deglaciation (Brown and Jumpponen, 2014).

The analysis of beta-diversity could enhance the understanding on the mechanisms of microbial community assembly (Li et al., 2018). In this study, the beta-diversities of soil archaea, bacteria and fungi significantly correlated with soil ages were found (Fig. 2b). It has been demonstrated a strong effect of glacier chronosequence on the community structure of bacteria, archaea and fungi (Zumsteg et al., 2012). It has been also shown that soil ages affected the structures of bacteria and fungi in Forni Glacier forefield (Franzetti et al., 2020). Present finding that the beta-diversities of protozoal, N fixers, and bacteria denitrifier communities significantly shifted along soil ages in Hailuogou glacier further filled the gaps of relevant research. Meanwhile, soil pH, SOC, and C/N ratios can significantly explain the structure variation of soil microbial communities with soil ages (Table S3). It is well known that soil pH has notable influences on microbial structures (Xun et al., 2015; Fierer and Noah, 2017). SOC as C source to meet the soil microbial colonization is crucial in shaping the C cycling in the deglaciated forefield system (Bradley et al., 2014), and thus soil C/N ratios reveal the microbial growth and nutrient limitation mediated by C and N cycles along the glacier forefield chronosequence (Göransson et al., 2011). Additionally, we observed that AP was positively correlated to the community separation of archaea, fungi, *nifH*, and *nirS* in 120-year-old soils (Fig. 2b). The availability of phosphate is related to soil pH and plant root (Penn and Camberato, 2019; Bai et al., 2020b). Thus, stationary soil pH and SOC in 120-year-old soils (Fig. 1) probably made AP significantly affect the development of microbial community structures in later soil age.

4.2. Soil microbial composition varied along glacier chronosequence

Based on the microbial analysis of high-throughput sequencing, our analysis can provide dynamic variation of microbial composition in Hailuogou glacier forefield (Fig. 3). Our results showed a shift from an archaeal community with Nanoarchaeota, Crenarchaeota, and Thermoplasmatota equally distributed in the 10- and/or 30-year-old soils to a Crenarchaeota-dominated one in the 70- and/or 120-year old soils. The rhizosphere-inhabiting Crenarchaeota had been found predominantly colonize in the later stage, but can also adapted to the early succession stage of Damma glacier forefield (Zumsteg et al., 2012). Meanwhile, it has been reported that the abundance of Crenarchaeota that dominated archaeal communities in later soil succession were positively related to decreasing soil pH along 150-year long chronosequence in the Styggedalsbreen glacier forefield (Mateos-Rivera et al., 2016), and *Nitrosotalea* belonging to Crenarchaeota was also observed to be favored in lower-pH environment (Jiao et al., 2019). Taken together, these results indicated that Crenarchaeota is the most adaptable community of archaea to the change of soil development in the glacier deglaciated system.

In bacterial community, Proteobacteria became the dominant phylum due to their various metabolic diversity (Zumsteg et al., 2012; Pessi et al., 2015; Ma et al., 2022), including phototrophs, photoheterotrophs and chemolithotrophs contributing to their widespread distribution throughout glacier retreat chronosequence (Kersters et al., 2006). Therefore, although there was no significant variation of Proteobacteria among soil ages, we still observed that *Reyranella*, *Bradyrhizobium*, *Pseudolabrys*, and *Rhodoplanes* belonging to Alphaproteobacteria and *Haliangium* of Deltaproteobacteria increased along glacier retreat chronosequence (Fig. S2). At phylum levels, Acidobacteria, Nitrospira, and Planctomycetes showed an upward trend, but Bacteroidetes, Chloroflexi and Patescibacteria decreased along glacier retreat chronosequence. It has been suggested that a wide range of transporters for nutrient uptake contributed to the distribution of Acidobacteria in complex environment (Kielak et al., 2016), and thus increased cell specialization and enzymes stability at more extreme pH possibly explained why Acidobacteria communities thrived in lower-pH soil environment. *Nitrospira*, the largest genera of Nitrospirae, is critical in the N-cycling due to its function of nitrification in microbial communities (Daims et al., 2015). The diversified metabolism of Planctomycetes that could be aerobic, anaerobes, mesophilic and neutrophilic allowing them colonize in different ecosystems such as acidophilic habits (Lage and Bondoso, 2014). Therefore, these bacterial groups showed a positive correlation with soil ages. On the contrary, it has been suggested that *Flavobacterium*, belonging to Bacteroidetes, could produce photoprotection and membrane integrity for the survival of glacier bacteria in cold environment (Liu et al., 2021). In addition, Patescibacteria could participate in the C and N cycling in cold environment with or without host requirements, which was essential for the nutrient cycling in glacial habitats (Rathore et al., 2022). Moreover, Chloroflexi were typically photoheterotrophic bacteria, which acted as an important role in the photosynthetic source of organic C in cold environment (Liu et al., 2017). Thus, glacier retreat caused a significant decline of Bacteroidetes, Chloroflexi, and Patescibacteria compared to their bacterial counterparts.

In general, the fungal communities at class level were dominated by Agaricomycetes. The widespread distribution of Agaricomycetes can be explained by the mycorrhizal association of Agaricomycetes with angiosperms and Pinaceae (Hibbett and Matheny, 2009) throughout the soil development chronosequence. Though Archaeorhizomycetes, Eurotiomycetes, and Leotiomycetes are all members of Ascomycota, their responses to soil ages were divergent along glacier chronosequence (Fig. S3). Leotiomycetes could participate in the C cycling by lichenization that utilizes green algal or cyanobacterial photobionts through the symbiotic process (Prieto et al., 2019), while Archaeorhizomycetes could obtain carbohydrates without recognizable symbiotic process in forest roots and rhizospheres (Rosling et al., 2011). Therefore, lower soil C/N ratios and higher CEC in the 50- and 70-year old soils compared to 10- and 30-year old soils may partially explain the increase of the relative abundance of *Pilidium and Pezicula* belonging to Leotiomycetes, and *Archaeorhizomyces* of Archaeorhizomycetes.

There is a paucity of information concerning protozoal response to glacier retreat. We observed the increase of *Cyrtolophosis*, *Cyclidium*, *Uroleptus*, and *Urostyla*, belonging to Cilliophora, and *Cernosvitoviella* of Annelida along glacier chronosequence (Fig. S4). Specifically, Cilliophora could affect nutrient cycles served as phagotrophs feeding on bacteria, fungi and other protists (Foissner et al., 2008). In addition, it has been shown that Annelida could help the decomposition of organic matter to increase availability of mineral nutrients for plant colonization (Edwards and Bohlen, 1996). Therefore, the increasingly complex microbial food webs and nutrient levels with soil ages could partially explain the increase of Cilliophora and Annelida. We further identified a series of responsive protozoal taxa to glacier retreat, such as Echinamoebida at the class level, Vermamoeba at the order level, and *Geocentrophora*, *Baicalellia*, *Stenophora* at the genus level, providing a high-resolution investigation of the associated protozoa with glacier retreat in the mountain ecosystems.

The N fixation is considered fundamental in the accumulation of soil total N in the initial stages of soil development (Telling et al., 2011). The present study showed that the major *nifH* carrier, Rhizobiales, decreased with soil ages contradicting with the upward trend of alpha diversity of N fixers (Fig. 2), and therefore the N fixers from unassigned OTUs here representing a larger proportion contributed to the overall diversity increase. Denitrification is a critical process of N cycling where nitrate is reduced to N_2O and/or N_2 by microbial communities (Brankatschk et al., 2011). The *nirS* carrier Rhodobacterales showed a decrease trend with soil ages. Likewise, it has been reported that the *nirS* decreased along 25–129-year-long chronosequence in the Rotmoosferner glacier forefield, which attributed to the increasing organic matter with soil development (Kandeler et al., 2006). Similarly, most OTUs of bacterial denitrifiers have not been assigned, so detailed investigation of microbial functional communities relating to N cycling is still needed for future studies.

The co-occurrence network patterns in this study showed that the nonrandom community assembly may be a general characteristic of microbes. In addition, the associated community topology properties showed that the bacterial, fungal, and protozoal networks in soil ages of 50–70 years were more interconnected (Table S4, Fig. S5), suggesting that the microbial communities are able to develop into a more efficient system along increasing glacier chronosequence (Deng et al., 2012). On the contrary, the network in soil ages of 10–30 years was relatively simple and had the highest modularity except protozoa (Table S4), indicating that the bacterial and fungal communities were more dynamic with largest number of taxa but no common co-occurrence across sites in the initial soil ages (Layeghifard et al., 2017). The reason why the networks differed along soil ages may be due to microbial taxa being in dormant or inactive stages (Fierer and Jackson, 2006), and therefore our results were consistent with other studies that microbial community networks become more interconnected along soil development chronosequence (Morriën et al., 2017; Mapelli et al., 2018; Gaiero et al., 2021), further supporting the succession theory (Odum, 2014) that the lifeforms in the continuously developing ecosystem are becoming more and more strongly integrated with each other (Dong et al., 2022).

Regarding the community setting, both NTI and NRI > 2 provides the evidence for phylogenetic clustering of bacterial, fungal and protozoal communities (Kembel, 2009) (Fig. S6a). The degree of phylogenetic clustering in bacterial community was more strengthened compared to fungi and protozoa along glacier chronosequence ($P = 0.005$ and $P < 0.001$). Moreover, the NMDS of bacterial community assembly based on βNTI/βNRI analysis revealed that soil ages were critical in bacterial phylogenetic community turnover patters (Fig. S6b), due to their strong relationships with microbial co-occurrence networks (Dong et al., 2022), and thus with the ecosystem development, the increasing plant cover and soil nutrient as well as associated increase in SOC and decline in soil C/N ratios attract the microbes sharing similar environmental preferences to play an important role in community competition.

4.3. Significant changes in functional genes were detected along soil chronosequence

Metagenomic taxonomic and functional analyses are used to improve the understanding of community compositions, functions and their links to environmental properties of microbial communities (Simon and Daniel, 2011). Based on our results, soil ages are significantly associated with not only microbial community compositions but also metagenomic taxonomy and function in Hailuogou glacier (Fig. 4a). Generally, the variation of metagenomic taxonomy was more significant to soil properties influence than function (Fig. 4a, Table S5), suggesting certain degree of functional redundancy as microbial functional diversity could remain relatively more stable when exposed to environmental disturbances (Chen et al., 2022a).

Specifically, we found that the functional genes involved in Dormancy and Sporulation, and Cell Division and Cell Cycle were relatively more abundant in the early succession stage (Fig. 4b). Dormancy is the ability of microorganisms to reduce metabolic activity and maintain viability under adverse conditions, which can be mediated by sporulation factors and toxin-antitoxin systems (Kearns and Shade, 2018). It has been reported that dormancy strategies in low-nutrient environment like bulk soil, were

dominated by sporulation, while in nutrient-rich environment like rhizosphere were dominated by toxin-antitoxin systems (Ling et al., 2022). Meanwhile, certain metagenomic functions, such as DNA Metabolism, Cell Division and Cell Cycle, and Iron Acquisition and Metabolism, in particular iron acquisition in Vibrio and Siderophore assembly kit, showed a decreased trend with soil chronosequence (Fig. 4c), possibly reflecting the strong growth to reproduction with intense microbial metabolisms of DNA activity and iron necessity in early soil ages. In addition, our results further demonstrated that the genes involved in motility and chemotaxis were greater in abundance in the soil ages of 50–70 years, as it has been found that bacteria could facilitate rapid colonization through chemotaxis in nutrient-rich environment of later soil ages (Cremer et al., 2019). Besides, with soil chronosequence, secondary metabolites are crucial for the mediation of microbial interactions (Junkins et al., 2022), and therefore we showed that the more connected network of soil microbial communities along glacier chronosequence may correlate with the increase of metagenomic genes involved in Secondary Metabolism. Potassium Metabolism and Sulfur Metabolism, for example the utilization of glutathione that served as the ubiquitous nucleophile for the conversation of electrophilic substances (Deponte, 2013) and regulated the potassium homeostasis by glutathione-dependent potassium efflux system (Hartl et al., 2020), also showed an increase trend in respond to glacier retreat, so we concluded that the microbial selection of potassium and sulfur were relatively stronger than iron in the nutrient-rich environment of aged soils.

Moreover, the significant shifts of carbohydrate metabolism in our study reflect the active transformation of soil organic matter in the different stages of soil development. For example, soil microbiota chooses intracellular-storage glycogen as C source in the early soil ages, but as succession proceeds, the degradation of more complex carbohydrate, such as xylose and lactate, significantly increases. For the functional genes related to N metabolism, the same decline trends were detected in the metabolisms of nitrate and nitrite ammonification, and dissimilatory nitrite reductase. Consistent with our result, it has been shown that nitrate ammonification is negatively related with higher SOC that repressed the catabolism of *nrf*, the key gene responsible for the process (Schmidt et al., 2011). It has been pointed out that the ammonification of nitrate and nitrite may compete with denitrification under anaerobic environment (Dong et al., 2009), so the improved aerobic conditions along glacier chronosequence may contribute to the alleviation of competition between microbial processes for limited oxygen content. Besides, denitrification pathway is mediated by diverse polyphyletic microbial communities, and several genes encoded the nitrate reductase, such as *narG*, and nitrite reductases, such as *nirS,* can be simultaneously activated in the dissimilative reduction of nitrate to dinitrogen gas (Levy-Booth et al., 2014). The decreased trend of nitrite denitrification was synchronized with the *nirS* carrier (Fig. 3) along the glacier forefield in this study. For the nitrate reductase encoded by *narG*, it has been reported that decreased trend of nitrate denitrification was opposite with the *narG* carrier along the Rotmoosferner glacier forefield. These results suggested that the change of microbial function may not always be the same as the community (Kandeler et al., 2006). Therefore, a more comprehensive investigation about the relationship between microbial communities and functions on the C and N cycles along the glacier forefields should be taken into consideration.

4.4. Metabolites become more complex with heterocycles along soil chronosequence

Our results revealed that soil ages had significant impacts on metabolomic compositions, which was significantly associated with SOC (Table S6, Figs. 5a and 6). Generally, SOC is considered as a diverse series of biomolecules representing the decomposition products of plant and microbial biomass, and therefore soil microbes play a key role in the SOC paradigms for efficient metabolisms (Hall et al., 2020). Therefore, we found that SOC was positively correlated to microbial community, which in turn had a strong selection for certain soil metabolites with more complex structures. In our study, lipids and lipid-like molecules are the largest pool among identified metabolites (Fig. 5b). This could be ascribed to the significantly decreased pH along soil chronosequence as the more acidic soils with thicker O horizons have been found to be associated with greater lipid relative abundance (Hall et al., 2020). Additionally, it has been suggested that the interaction between soil microbiota and environment conditions may lead to the syntheses of polymeric molecules involving a greater diversity of bond types and thus more stable bonds for the persistence of organic C turnovers (Kleber, 2010), which partially explains why the structures of soil metabolite tended toward an increasing complexity with more heterocycles along soil development chronosequence in our study (Fig. 5c).

Our results also showed a strong positive linear relationship between soil parameters and/or microbial communities with microbial functions of metagenomics and/or metabolomics (Fig. 6a). It has been indicated that the changes in soil community composition affect the soil biodiversity, thus regulating the ecosystem functions and sustainability (Wagg et al., 2014). We also found that soil chemical properties significantly influenced microbial communities' structure (Fig. 6b). To sum up, during the soil formation and development after glacier retreat, the chronosequence-dependent soil properties significantly shaped soil microbiota composition, thereby affecting the change of microbial diversity, and ultimately causing fluctuations in ecological functions in microbial metagenomics and metabolomics.

5. Conclusions

Soil microbial roles in response to glacier retreat in microbial successional trajectories, metagenomic taxonomy and function, and metabolomic profiling are unraveled along the Hailuogou Glacier chronosequence. We revealed that SOC and available nutrient exhibited greater influence on the microbial communities during the soil development. Initially, SOC accumulated fast and available nutrient, such as TN, increased later while soil C/N and pH declined with soil ages, leading to the asynchronous C and N accumulation to drive the significant shifts in microbial community alpha- and beta-diversities as well as the compositions along the glacier chronosequence. The bacterial, fungal and protozoal networks are more interconnected in later soil ages, while the degree of phylogenetic clustering in community was more strengthened along glacier chronosequence. Moreover, succession ages significantly affected the microbial function related to the metabolisms of carbohydrates, such as decreased the metabolisms of Glycogen but increased the utilization of Xylose and Lactate. Functions related to iron metabolism decreased while potassium and sulfur increased with soil ages. Furthermore, the strong interactions between soil microbiota and soil environment resulted in the increasing complexity of metabolites with soil ages. These results provide important insights into the impact of soil development after glacier retreat on structuring soil microbial communities and functions in glacier forefield, which could help enhance our understanding into the influence of global warming on the microbial mediation of ecological functions related to energy flow and nutrient cycling in mountain ecosystem.

CRediT authorship contribution statement

H·C and W·S conceived and designed the research. Y·H, Q.F, Y.Q and J.Q performed the experiments and contribute the data collection. Y·H, J.Z, J.L and Q.L complied and analyzed the data. Y·H, Q.F, and Y.Q wrote the initial draft with significant contribution from H·C. X.Y, W·W, R.C, Z.Y, Z.D, Y.Q and H.C contributed to the revision of the manuscript. All authors read and approved the final manuscript.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

This work was financially funded by the National Natural Science Foundation of China (42277282), Basic and Applied Basic Research Foundation of Guangdong Province (2022A1515010861), Shenzhen Science and Technology Program (JCYJ20220530150201003), and Young Teachers Team Project of Fundamental Research Funds for the Central Universities, Sun Yat-sen University (22qntd2702).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164405.

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