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Metatranscriptomics sheds light on the links between the functional traits of fungal guilds and ecological processes in forest soil ecosystems

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Summary

- Soil fungi belonging to different functional guilds, such as saprotrophs, pathogens, and mycorrhizal symbionts, play key roles in forest ecosystems. To date, no study has compared the actual gene expression of these guilds in different forest soils.
- We used metatranscriptomics to study the competition for organic resources by these fungal groups in boreal, temperate, and Mediterranean forest soils. Using a dedicated mRNA annotation pipeline combined with the JGI MycoCosm database, we compared the transcripts of these three fungal guilds, targeting enzymes involved in C- and N mobilization from plant and microbial cell walls.
- Genes encoding enzymes involved in the degradation of plant cell walls were expressed at a higher level in saprotrophic fungi than in ectomycorrhizal and pathogenic fungi. However, ectomycorrhizal and saprotrophic fungi showed similarly high expression levels of genes encoding enzymes involved in fungal cell wall degradation. Transcripts for N-related transporters were more highly expressed in ectomycorrhizal fungi than in other groups. We showed that ectomycorrhizal and saprotrophic fungi compete for N in soil organic matter, suggesting that their interactions could decelerate C cycling.
- Metatranscriptomics provides a unique tool to test controversial ecological hypotheses and to better understand the underlying ecological processes involved in soil functioning and carbon stabilization.

Introduction

In forest ecosystems, tree species and forest management practices can greatly influence organic matter (OM) chemistry and structure, leading to variations in annual CO_2 uptake and long-term carbon storage (Kjønaas *et al.*, 2021; Augusto & Boča, 2022; Jörgensen *et al.*, 2022). Combined with climatic and soil conditions, tree species composition also has varying impacts on belowground microbial communities, soil microbial activities, fungal biomass, and storage of soil organic carbon (Tedersoo *et al.*, 2011; Joly *et al.*, 2016; Ma *et al.*, 2017; Mundra *et al.*, 2022). Fungi are major players in the two major life-supporting processes of trees in forest ecosystems, both directly by supporting tree nutrient uptake and growth and indirectly by driving decomposition (Högberg & Högberg, 2002; Six *et al.*, 2006; Fontaine *et al.*, 2011). In line with these major functions, fungi are divided into functional guilds, including saprotrophs, parasites (or pathogens), and mycorrhizal symbionts (Berbee *et al.*, 2017). Indeed, the ecological concept of functional redundancy is based on the observation that many species perform similar

*These authors contributed equally to this work.
[Correction added on 9 January 2024, after first online publication: the spelling of Raúl Castanera's name has been corrected in the author list.]

roles in different communities and ecosystems and may therefore be substitutable with little impact on ecosystem processes (Lawton, 1994; Louca *et al.*, 2018). This concept of ecology refers to the notion of ecological guilds (Root, 1967), whereby species are grouped according to their functional similarities within communities. For soil fungi, the mycorrhizal symbionts contribute to tree growth by providing water and nutrients, while the other two guilds specialize in the decomposition of cell walls from dead or living plants, involving distinct decay processes (Boddy & Watkinson, 1995; Olson *et al.*, 2012). As major agents of dead organic matter transformation, saprotrophic fungi play critical roles in nutrient cycling and carbon sequestration. Organic materials of plant and microbial origin are decomposed by extracellular hydrolytic and oxidative enzymes and metabolized by soil fungi to obtain energy, carbon, and nutrients. By contrast, ectomycorrhizal fungi represent a unique functional group of mutualistic fungi that rely on their host plants for sugars. Although ectomycorrhizal fungi cannot use organic matter as their primary C source, some ectomycorrhizal fungal species, such as *Cortinarius glaucopus*, are very efficient decomposers of soil organic matter (SOM) via hydrolytic and oxidative enzyme activities or they may facilitate SOM decomposition by stimulating the activities of free-living saprotrophic organisms (Lindahl & Tunlid, 2015; Bödeker *et al.*, 2016; Frey, 2019; Mayer *et al.*, s. d.). In addition, these fungi are highly diverse in many forest soils and multiple environmental factors and habitats directly or indirectly contribute to the high spatial and temporal diversity of soil fungal communities (Rincón *et al.*, 2015; Bahram *et al.*, 2018; van der Linde *et al.*, 2018). Although different forest soils promote the coexistence of different trophic guilds of fungi, the actual functional properties of these fungal groups and their synergistic or antagonistic interactions are often unclear.

Soil fungi also contribute to organic carbon stabilization in forest soils (Clemmensen *et al.*, 2013; Angst *et al.*, 2021; Buckeridge *et al.*, 2022). In addition to the influence of input chemistry on C storage in forest soils, other regulatory mechanisms of C sequestration also need to be investigated, particularly potential complementary and competitive interactions that could exist within and between fungal trophic guilds (Fernandez & Kennedy, 2016; Mayer *et al.*, s. d.). Moreover, competition for resources between saprotrophic and ectomycorrhizal fungi within a fungal community would influence the decomposition of soil biopolymers, illustrating the distribution of functions between these two guilds, particularly for organic nitrogen mining (Gadgil & Gadgil, 1971; Talbot *et al.*, 2013; Zak *et al.*, 2019). In addition, a significant fraction of plant C is converted into microbial biomass or other microbial products. During fungal decomposition, necromass is recycled by active soil microorganisms, including other fungi (Maillard *et al.*, 2023). A better understanding of the processes carried out by different fungal groups can be obtained using metagenomics and metatranscriptomics, which enable the description of *in situ* functional complementarities and competition between functional guilds. These methods may be used to test important ecological theories, such as the 'Gadgil effect' (Fernandez & Kennedy, 2016), and from a longer perspective, guide strategies to maximize carbon capture in forest soils.

The increasing availability of sequenced fungal genomes (Kiss *et al.*, 2019; Miyauchi *et al.*, 2020; Lebreton *et al.*, 2021) provides unprecedented opportunities to study both the functional and taxonomic diversities of soil communities through annotation of environmental metagenomic and metatranscriptomic datasets. This technology has been applied to soil eukaryotes for over a decade (Bailly *et al.*, 2007; Damon *et al.*, 2012). However, previous studies have provided limited information on the links between gene expression and functional traits because of scarce genomic resources available at the time of publication. Since then, only a few studies have used metatranscriptomics to evaluate the contribution of soil microorganisms to nutrient and C cycling in forest ecosystems. These surveys have mainly focused on the soil bacterial communities (Žifčáková *et al.*, 2017). Recent studies have characterized the metatranscriptome of ectomycorrhizal root tips *in situ* (Liao *et al.*, 2014, 2016; Erlandson *et al.*, 2022; Law *et al.*, 2022; Rivera Pérez *et al.*, 2022).

The objective of this study was to evaluate the usefulness of metatranscriptomics as a tool to study the functional divergences, redundancies, and complementarities of fungi belonging to ectomycorrhizal, saprotrophic, and pathogenic guilds within forest soils across various forest biomes. Using metatranscriptomics, we assessed the expression of genes related to SOM decomposition as well as nutrient scavenging and assimilation. We investigated the *in situ* competition for resources (e.g. carbohydrates and nitrogenous compounds) of these fungal guilds by sequencing environmental RNAs extracted from boreal, temperate, and Mediterranean forest soils to test the generality of the following assumptions. Our aim was to demonstrate that: (1) the level of mRNA transcripts can be used to describe the relative taxonomic distribution of active fungal communities, and that fungal assemblages vary greatly from one forest ecosystem to another; (2) there are specific links between taxonomic diversity and certain functional characteristics (i.e. functional diversity) of active fungi in soils; and (3) gene expression related to the decomposition of nutrient-free plant cell wall compounds (highly C-rich biopolymers) would be restricted to saprotrophs, whereas genes related to the decomposition of nutrient-containing microbial cell wall compounds would be expressed by both saprotrophic and biotrophic (ectomycorrhizal) fungi. To achieve these objectives, we compared the gene expression of the main trophic guilds using a dedicated fungal mRNA annotation pipeline, GenBank (Benson *et al.*, 2013), and the JGI MycoCosm databases (Grigoriev *et al.*, 2014). Finally, we wanted to show that metatranscriptomics is a relevant tool for providing functional indicators to study the diversity and function of the main fungal guilds in forest soils at refined taxonomic levels.

Materials and Methods

Site description and soil sampling

In the present project, four coniferous forest sites from the same sequencing program (Community Science Program led by the U.S. Department of Energy Joint Genome Institute), displaying various bioclimatic parameters, were investigated: Aspurz (Aspz:

Spain, sub-Mediterranean forest), Champenoux (Chpx: France, temperate forest), Lamborn (Lbrn: Sweden, boreal forest) and Montmorency (Mrcy: Canada, boreal forest). A brief description is summarized in Table 1.

The Swedish Lamborn forest site (Lbrn) is located close to the Siljansfors and Jädraås experimental forests where previous studies have been conducted (Lindahl *et al.*, 2007; Marupakula *et al.*, 2021). The altitude is *c.* 300 m. The forest is dominated by *Pinus sylvestris* with ground vegetation of *Vaccinium myrtillus*. The stand age was *c.* 80 yr, with a mean stem density of 750 ha^{-1}, and some of the plots were subjected to fertilization. The soil was a sandy moraine/podzol with a well-developed eluvial (E) horizon. The Canadian Montmorency Experimental Forest (Mrcy) of Laval University is located 70 km north of Quebec City. Soils have been described as Orthic or Humo-Ferric Podzols. Climate is typical of boreal forests. The altitude varied between 600 and 1000 m. The predominant tree species was balsam fir (*Abies balsamea*). The French Champenoux (Chpx) forest site (altitude 256 m) is located within the Amance state forest in Lorraine. The dominant tree species were silver fir (*Abies alba* Mill.) with ground vegetation of *A. alba* and *Fraxinus excelsior* seedlings. Soil samples from the Champenoux forest site were collected during two seasons (spring and summer). The Spanish site of Aspurz (Aspz) is a mixed forest with a Mediterranean climate, where we distinguished monodominant patches of *Pinus sylvestris*. Spanish sampling was also carried out over two seasons (spring and fall).

As the thicknesses of forest floor, that is L, F and H layers (litter, humus, wood debris and organic matter compartments) were very different between the four sites, only samples corresponding to soil horizons (organic and/or organo-mineral) were sampled for our metatranscriptomic analysis. A total of 51 samples of soil were collected from the four sites with a 10 cm diameter corer: Aspz ($n = 12$), Chpx ($n = 18$), Mrcy ($n = 9$) and Lbrn ($n = 12$). Soils were homogenized using a sieve (5 mm mesh diameter) to remove large roots and debris and then transferred to a freezer at −80°C. RNA extraction was performed within weeks of sampling and freezing and immediately sent to Joint Genome Institute (JGI) for sequencing (see below). In addition, sampling was carried out under site-specific conditions (seasonal effects, nitrogen fertilization, and forest dieback), which are beyond the scope of this analysis. Nevertheless, to avoid obscuring potential sources of intrasite variability, all the samples ($n = 51$) were divided into subsites (according to treatments) designated by the letters A to J. For each sample, detailed information concerning soil parameters and site-specific sampling conditions is given in Supporting Information Table S1.

RNA isolation, amplification, and RNA sequencing

Total RNA was extracted from 1.5 g of sieved soil material using the PowerSoil Total RNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. The quantity and quality of total RNA were assessed using the Experion RNA StdSens Analysis kit (Bio-Rad) and Agilent RNA Nano kit on an Agilent 2100 Bioanalyzer system. One microgram of the extracted RNA was amplified with one round of the MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. aRNA quality and quantity were monitored using NanoDrop1000 (Thermo Scientific, Waltham, MA, USA) and the Experion RNA StdSens Analysis kit (Bio-Rad). RNA extraction and sequencing were performed within 2 yr after sample collection.

Stranded cDNA libraries were generated at the JGI using the Illumina Truseq Stranded mRNA Library prep kit, following the manufacturer's instructions. About 10 ng or less of RNA was fragmented using divalent cations and high temperature. The fragmented RNA was reverse-transcribed using random hexamers and SSII (Invitrogen) followed by second-strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, adapter ligation, and 8–10 cycles of PCR. The prepared libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed with other libraries, and the pooled libraries were sequenced at JGI using an Illumina HiSeq sequencing platform with the TruSeq paired-end cluster kit v.4 and Illumina's cBot instrument to generate a clustered flow cell for sequencing, following a 2 × 150

Table 1 Location and climatic description of the four coniferous forest sites.

Site name	Aspz	Chpx	Mrcy	Lbrn
Location	Aspurz	Champenoux	Montmorency	Lamborn
Country	Spain	France	Canada	Sweden
Coordinates	42.70°N 1.14°W	48.75°N 6.35°E	47.27°N 71.21°W	60.96°N 15.80°E
Collection year	2012	2013	2016	2011
Sample number	12	18	9	12
Host tree*	*Pinus sylvestris*	*Abies alba*	*Abies balsamea*	*Pinus sylvestris*
MAT	12.0	13.7	0.4	7.0
MAP	913	778	1583	700
Altitude	615	254	777	273
Bioclimate	Submediterranean	Temperate	Boreal	Boreal

Site description including complete name, geographic origin, coordinates, year of collection, dominant tree species (Host tree), mean annual temperature (MAT; °C) and mean annual precipitation (MAP; mm), altitude (meters) and bioclimate.
*Predominant tree species.

indexed run. In total, 51 samples (Aspz:12, Chpx:18, Lbrn:12, and Mrcy:9) were sequenced by JGI using Illumina Hiseq.

Bioinformatics and statistical analyses

The raw reads were cleaned using BBTools suite. Softwares (tools from the BBTools suite), user guide and parameters are freely available: (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/). BBDuk program was used to remove contaminants, trim reads that contained adapter sequence and quality trim the entire tail end of reads once the quality drops to 0 at a single position. BBDuk was used to remove reads that contained 1 or more 'N' bases, had an average quality score across the read < 10 or had a minimum length < 52 bp or 33% of the full read length. Reads mapped with BBMap to masked human, cat, dog and mouse references at 93% identity and reads aligned to common microbial contaminants were separated into independent files. Ribosomal RNA reads and reads containing known spike-ins were removed. Five co-assemblies of the filtered reads were performed with all samples from each site (or subsites with the Lbrn site) using Megahit v.1.1.3 with default parameters (Li *et al.*, 2015). Contigs shorter than 500 bp were excluded. For each of the 51 samples, filtered reads were mapped onto the corresponding *de novo* co-assembly using Bowtie v.2.3.0 (Langmead *et al.*, 2009). Mapped read counts were determined using SAMtools v.1.7 (Li *et al.*, 2009). Contigs supported by fewer than five mapped reads in at least three different samples were discarded. The remaining contigs were annotated with a Blast-like approach using Diamond v.0.9.19 (Buchfink *et al.*, 2015) and parameters '--more_sensitive –max-target-seq 1 --max-hsps 1 --evalue 1e−10' and three different databases: NCBI-NR (April 2021 version, Sayers *et al.*, 2022), gene repertoires of MycoCosm genomes (all publicly available genomes in February 2022; Grigoriev *et al.*, 2014), and MEROPS database (January 2020 release, Rawlings *et al.*, 2014). A total of 1117 649 fungal contigs (per co-assembly: median 348 k, min 188 k, max 421 k) corresponding to over 1 billion reads (1111 million; per sample: median 18 M, min 7 M, max 46 M) were used to compute Transcript per Kilobase Million (TPM) values (Trapnell *et al.*, 2012).

The following steps were performed using R, particularly the tidyR suite (Wickham *et al.*, 2019). All scripts are available in the Supporting Information. Conflicting taxonomic affiliations (produced by MycoCosm or NCBI-NR) were solved based on a higher diamond bitscore, with an advantage given to MycoCosm in the case of the tied scores. Contigs that were not annotated as fungi were discarded, and read counts were subjected to TPM transformation. Functional annotations were based on (1) KOG and signal peptide annotations from MycoCosm genomes, (2) CAZyme annotation from CAZyDB and (3) the MEROPS database. Functional gene families linked to important ecological traits related to N and C cycling processes (Treseder & Lennon, 2015; Romero-Olivares *et al.*, 2021) have been termed 'foraging genes' and defined on the basis of CAZyme, signal peptide, and KOG annotations. Families of genes encoding CAZymes acting on plant cell walls (PCW with 82 families), fungal cell walls (FCW with 30 families), and other microbial cell walls (neither PCW nor FCW with 95 families) were defined using *de novo* family annotation and existing classification (Drula *et al.*, 2022). Proteases were defined by matches to the MEROPS database, excluding inhibitors, nonpeptidases, and unassigned annotations. Lipases were defined based on the KOG classification using the 'Lipid transport and metabolism' KOG class and lipase matches in KOG deflines. Secreted proteins were identified based on MycoCosm SignalP annotation for proteins with 0.8 SigP probability. Categories of transporters (i.e. carbon-, nitrogen-, and inorganic ion-related) were defined based on MycoCosm KOG annotation using corresponding KOG classes and matching KOG deflines.

The main functional fungal guilds (ectomycorrhizal, saprotrophic, and pathogenic) were defined based on taxonomic affiliation and the Fungal Traits database (Põlme *et al.*, 2020). Reliability of the fungal guild annotation was assessed comparing the predicted gene annotations of unpublished fungal genomes to their expected one, as described in Methods S1. To allow a better comparison between the level of expression of each function by a given trophic guild, guild-normalized TPM was calculated per contig as the ratio of contig TPM/summed TPM of all contigs related to the guild TPM.

Ordination plots were obtained using vegan R package v.2.5.6 (Oksanen, 2022). Barplots and boxplots were generated using Ggplot2 software. Differential expression between fungal guilds was tested using Wilcoxon tests based on TPM and/or guild-normalized TPM, with FDR-adjusted *P*-values. Supporting R script and package version details are available in Methods S2.

Results

Based on transcript levels, ectomycorrhizal (ECM) and saprotrophic (SAP) fungi were the two most active fungal guilds in the four forest soils analyzed (Fig. S1). However, their relative expression levels showed significant variation between sites or subsites, that is between samples from the same site (SAP: 20–68%, median = 46%; ECM: 3–67%, median = 26%). Transcription by endophytic, ericoid, and arbuscular mycorrhizal fungi was also site-dependent. Transcripts from arbuscular mycorrhizal fungi were more abundant at the French (Chpx) and Canadian (Mrcy) sites, whereas transcripts from ericoid mycorrhizal fungi were mainly identified at the Swedish (Lbrn) site. Endophytic transcripts were more abundant in Spanish (Aspz) and Lbrn sites than in the other two sites. The Aspz site demonstrated strong subsite variation, but overall, transcripts of endophytic, ericoid, and arbuscular mycorrhizal fungi represented less than a few percent of the total. Transcripts from potential fungal pathogens represented a relatively even proportion of fungal mRNA, ranging from 15 to 20% in almost all forest samples. Given that the total number of fungal transcripts varied significantly between samples within each guild, comparative analyses between guilds were based on guild-normalized TPM to ensure an unbiased comparison between the functional expression profiles of saprotrophic, ectomycorrhizal, and potential pathogenic (PAT) fungi. This reflects the level of investment (transcript quantification) in a

given function inside each guild, independent of guild abundance in the different samples.

Ectomycorrhizal fungal genera counted 240 895 contigs, vs 685 530 contigs for saprotrophs. Communities of saprotrophic fungi had a higher diversity of fungal genera ($n = 815$) than ectomycorrhizal fungi ($n = 152$). However, the major ectomycorrhizal fungal genera (> 2% normalized TPM in at least six samples) accounted for > 80% of the total abundance of ectomycorrhizal transcripts, whereas the major saprotrophic fungal genera accounted for < 40% of the total abundance of saprotrophic transcripts (60% for the major saprotrophic phylum). We observed strong site specificity, characterized by the dominance of transcripts from certain ectomycorrhizal fungal genera (Fig. 1a). For example, transcripts of *Russula* were the most abundant in the French forest, *Piloderma* transcripts dominated both boreal sites, and *Cortinarius* transcripts were abundant in the Spanish and Swedish sites. Conversely, saprotrophic fungal genera rarely reached such high abundances, except for *Mycena* and

Fig. 1 Community profiles of the main ectomycorrhizal (ECM) and saprotrophic (SAP) fungal genera (top and bottom, respectively) expressed through cumulative abundance of fungal guild-normalized Transcript per Kilobase Million (TPM) values. Main genera are those with guild-normalized TPM over 2% in at least six samples, minor genera were grouped as Others. ECM genera were colored by taxonomic Order. Due to their higher diversity, minor SAP genera included in abundant Phyla (over 2% in at least six samples) were regrouped by Phylum in 'Other (Phylum-name)'. SAP genera were colored according to their taxonomic Phylum.

Filobasidium, which represented > 10% of the total abundance of saprotrophic transcripts in rare samples (Fig. 1b).

Using normalized taxonomic abundance of transcripts, non-metric multidimensional scaling (NMDS) revealed the grouping of samples in accordance with their geographical origin (Fig. 2a). However, closer analysis revealed taxonomic heterogeneity within the sites, with well-separated subgroups corresponding to intra-site treatment and/or soil layers identified as subgroups A to J (Table S1). The within-site differences, which were related to experimental of each site (although not the focus of this study), were sometimes higher than the between-site differences. A similar NMDS analysis was applied to the euKaryotic Orthologous Groups (KOG) classification, providing an overview of the community composition based on functions related to major physiological processes and cellular components. In contrast to the taxonomic variation between the sites (Fig. 2a), there was limited functional dissimilarity (KOG classes) between samples from the four forest sites (Fig. 2b). In the third step, we targeted

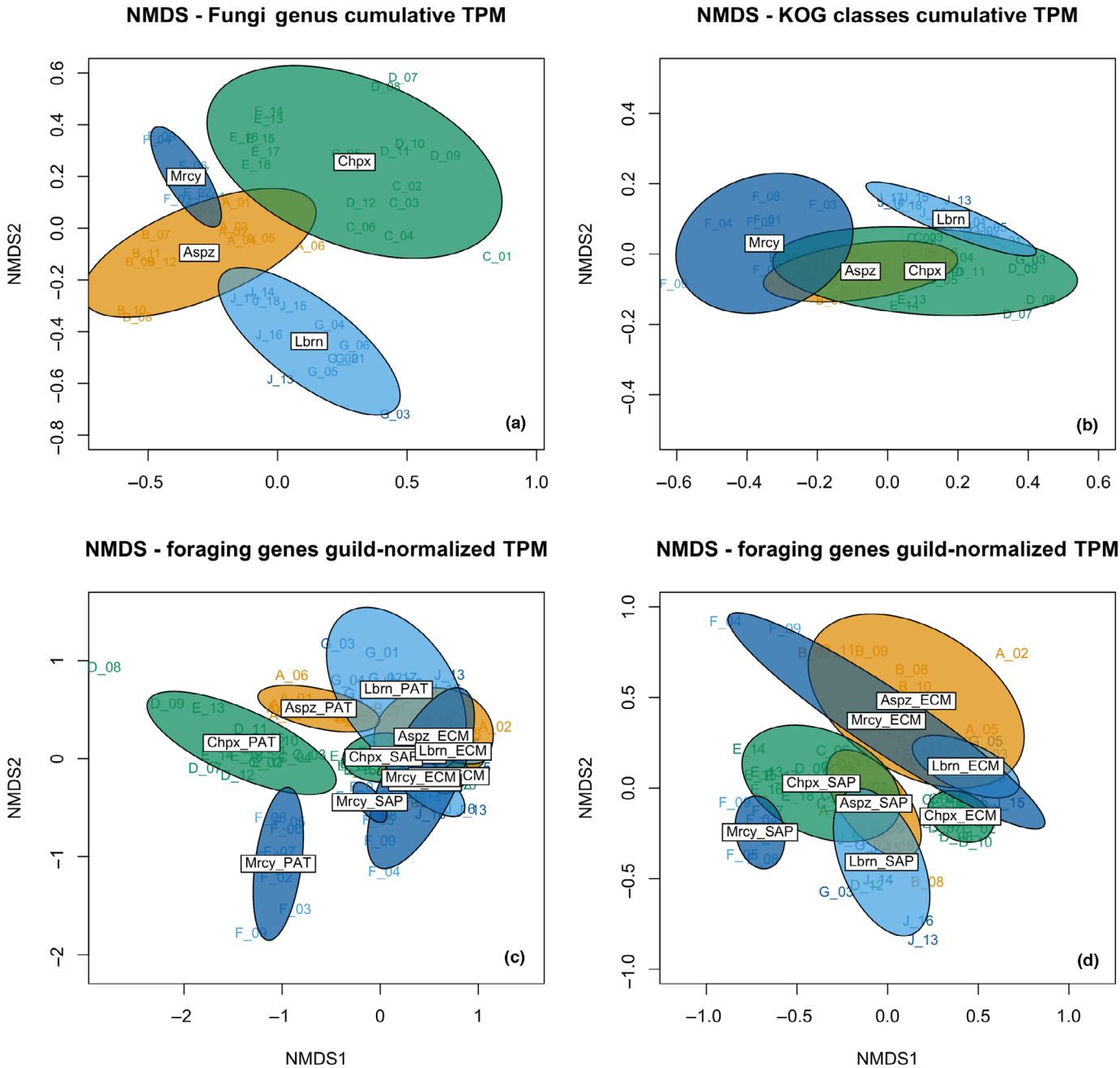

Fig. 2 Dissimilarity analyses (NMDS) applied to (a) fungal genus TPM, (b) foraging gene classes TPM (c) foraging genes guild-normalized TPM of the ECM, SAP and PAT fungal guilds and (d) foraging genes guild-normalized TPM for ECM/SAP fungal guilds only. For each sample, taxonomic genus summed TPM were calculated before the Bray–Curtis NMDS analysis. Samples are colored by site. Aspz, Aspurz (yellow); Chpx, Champenoux (green); Lbrn, Lamborn (light blue); Mrcy, Montmorency (dark blue) and labeled by site, or site and fungal guild (ECM, ectomycorrhizal; PAT, pathotrophic fungi; SAP, saprotrophic) for plots (c, d).

transcriptomic data corresponding to foraging gene classes and focused on differences in expression between trophic guilds and thus separated samples by ecological category using guild-normalized abundances (Fig. 2c). These so-called 'foraging genes' encode secreted CAZymes, secreted proteases, ureases, and membrane transporters (carbon compounds, nitrogen compounds, and inorganic ions). In this analysis, the abundance of pathogenic fungal transcripts showed strong dissimilarity, in contrast to the saprotrophic and ectomycorrhizal transcripts (Fig. 2c). Saprotrophic and ectomycorrhizal data formed clusters with little variation related to their geographic origin, unlike the pathogenic transcripts that were distinct between sites. Finally, we excluded pathotrophic transcripts to focus on the differences between ectomycorrhizal and saprotrophic fungi using guild-normalized abundances in foraging gene categories (Fig. 2d). Site effects appeared minor compared with the guild effect, which clearly separated the samples. These four analyses indicated a strong site-specific structuring of fungal taxonomic diversity (Fig. 2a), and functional patterns related to SOM decomposition processes remained highly distinct between fungal guilds, irrespective of fungal diversity between sites, illustrating the conservation of the distribution of foraging functions among guilds (Fig. 2c,d), independent of the high intersite taxonomic diversity.

The three main trophic guilds, ectomycorrhizal, saprotrophic, and pathogenic fungi, displayed contrasting expression levels for eight foraging gene classes (Fig. 3). The secreted plant cell wall (PCW) CAZymes were expressed at a higher level by saprotrophs than by ectomycorrhizal fungi (2 : 1 median ratio) or pathogens, whereas secreted fungal cell wall (FCW) CAZymes were highly expressed by both ectomycorrhizal and saprotrophic fungi, with a significantly higher expression for ectomycorrhizal fungi compared with saprotrophs (Fig. 3). Among these secreted PCW-degrading enzymes, we identified 46 families of glycoside hydrolases (GH), auxiliary activities (AA), carbohydrate esterases (CE), and other CAZymes involved in PCW degradation that were significantly more highly expressed in saprotrophs than in the other two guilds (Table S2). Interestingly, ureases and secreted FCW CAZymes were highly expressed by ectomycorrhizal fungi and at

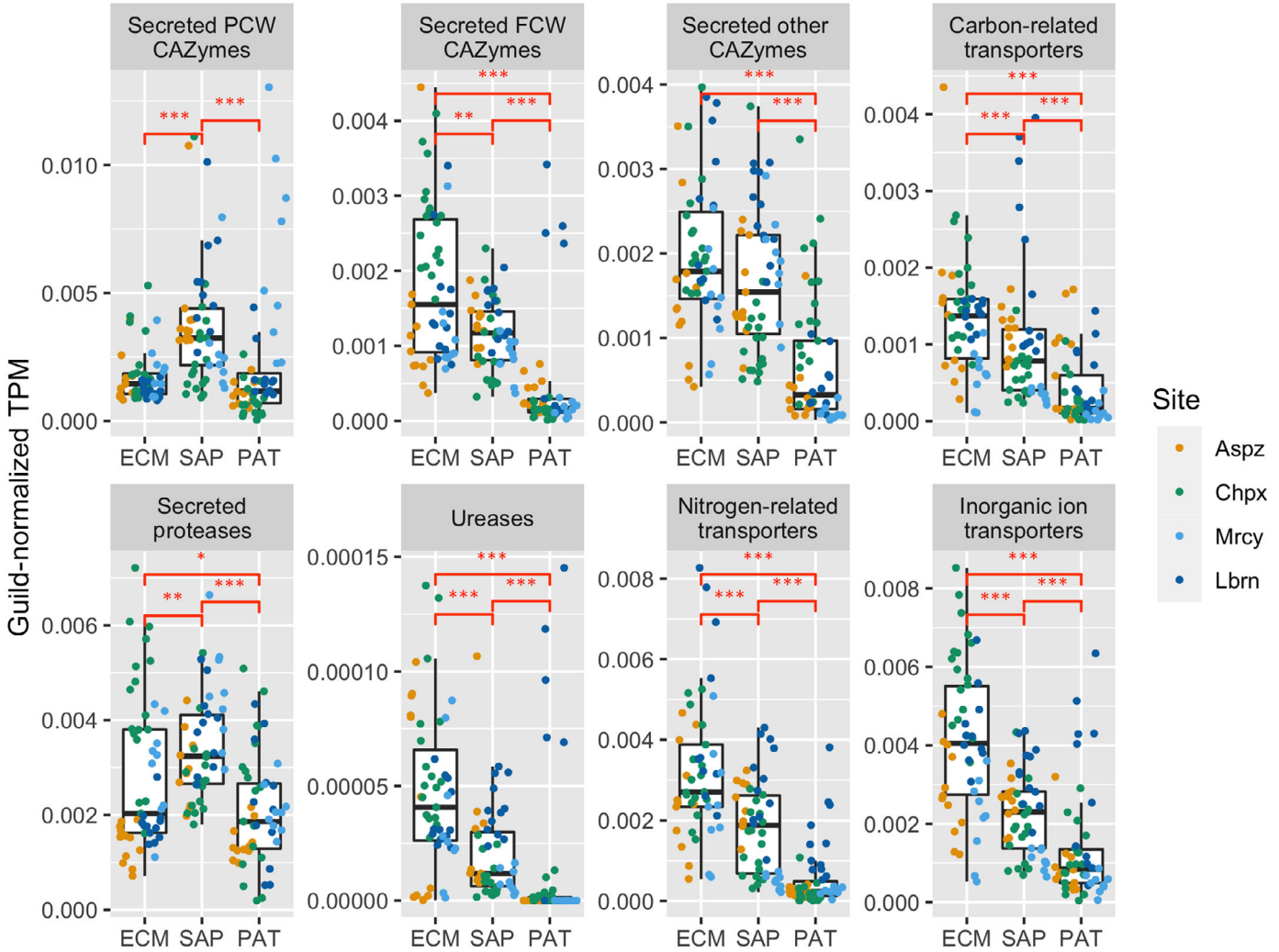

Fig. 3 Guild-normalized abundances of foraging gene classes, per fungal guild (ECM, ectomycorrhizal; SAP, saprotrophic; PAT, pathotrophic fungi), and foraging gene classes. Boxplots show distribution across all sites; individual sample values are plotted and coloured by site (Aspz, Aspurz; Chpx, Champenoux; Lbrn, Lamborn; Mrcy, Montmorency). Significant differences between guild-normalized distributions are indicated in red (Wilcoxon test with FDR adjustment; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$).

significantly higher levels than saprotrophs and pathogens. Genes for secreted proteases were slightly, but significantly, more expressed by saprotrophic fungi than by ectomycorrhizal fungi (3 : 2 median ratio) or pathogens, but displayed high variability in ectomycorrhizal fungi (Fig. 3).

A detailed analysis of these potential 'foraging indicators' revealed that six secreted PCW CAZyme families (out of 82 identified families, giving a 7% proportion) were expressed at significantly higher levels by ectomycorrhizal than saprotrophic fungi (Fig. 4). Expression of genes for secreted GH2 (ß-galactosidase), GH9 (endo-β-1,3(4)-glucanases), and GH88 (glucuronyl hydrolases) displayed strong differences and were almost specific to ectomycorrhizal fungi, while the Auxiliary Activity (AA) families AA5 (copper radical oxidases) and AA7

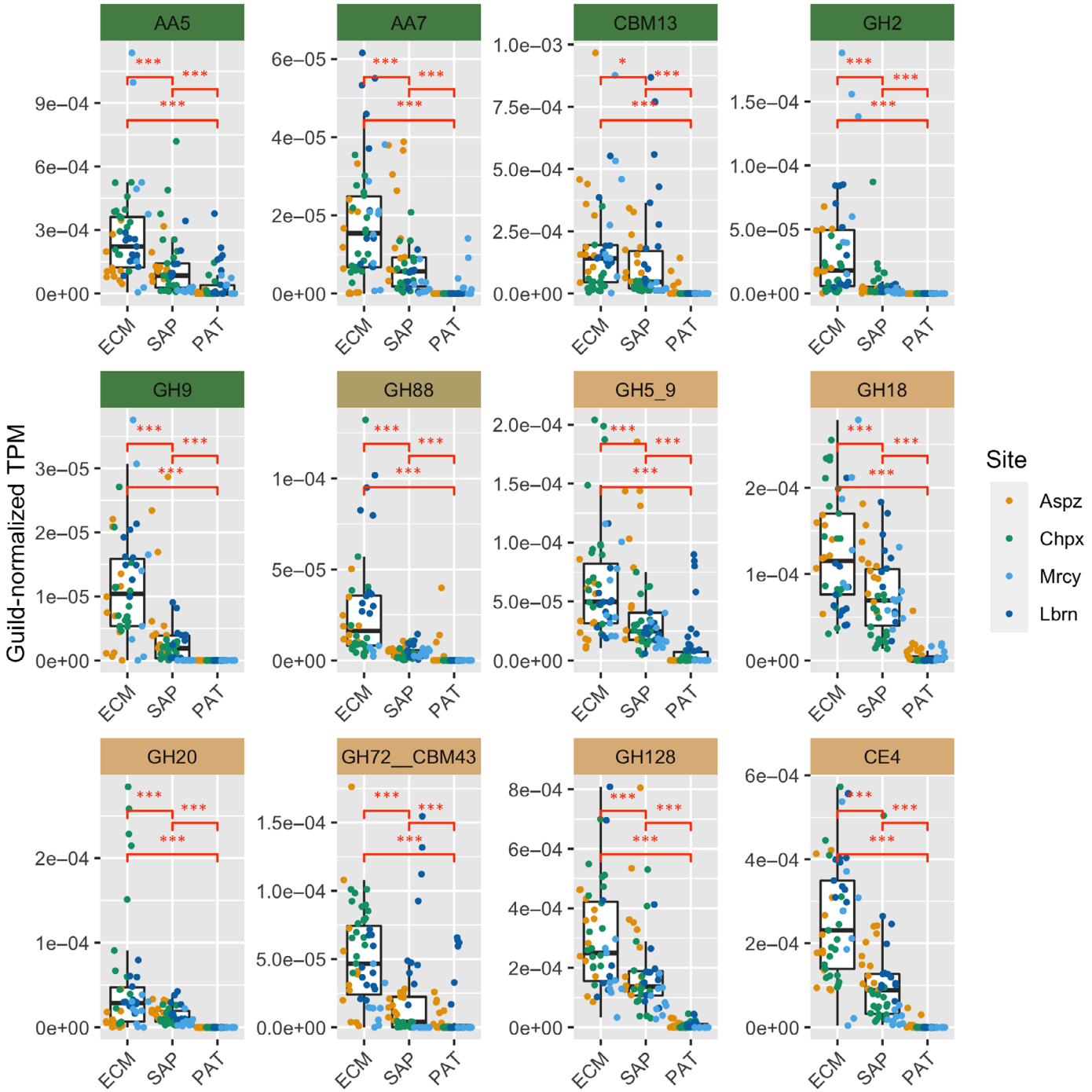

Fig. 4 Boxplot distribution of secreted CAZymes significantly overexpressed by ECM fungi compared with SAP fungi, per guild (ECM, ectomycorrhizal; SAP, saprotrophic; PAT, pathotrophic fungi), and CAZyme families. Boxplots show distribution across all sites; individual sample values are plotted and coloured by site (Aspz, Aspurz; Chpx, Champenoux; Lbrn, Lamborn; Mrcy, Montmorency). Significant differences between guild-normalized distributions are indicated in red (Wilcoxon test with FDR adjustment; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$). Plant cell wall CAZymes are indicated with a green label and fungal cell wall CAZymes with a brown label. GH88 may be involved in both cell wall degradation (khaki color).

(glucooligosaccharide oxidases) showed a 50% increase in guild-normalized TPM. In addition, six secreted FCW CAZyme families (out of 30 identified; 20%) were highly expressed by ectomycorrhizal fungi with a median ratio of > 2 : 1, and these gene families were almost all related to chitin degradation (Fig. 4). By contrast, 50 secreted PCW CAZyme families (out of 82; 61%) and 13 secreted FCW CAZyme families (out of 30; 43%) were significantly more highly expressed by saprotrophs than ectomycorrhizal fungi (Table S2). However, although a larger number of FCW families were more highly expressed by saprotrophic fungi than by ectomycorrhizal fungi, the summed expression of all FCW families was significantly higher for ectomycorrhizal fungi.

To compare the expression levels of fungal foraging genes with those of genes associated with nutrient transport and assimilation, we identified transporter gene families that differed significantly in their expression between the three functional guilds. Genes encoding membrane transporters for carbon compounds (carbon-related), nitrogen compounds (N-related), and inorganic ions were significantly more highly expressed in ectomycorrhizal fungi than in saprotrophic fungi (Fig. 3). The strongest differences were observed for inorganic ion transporters (2 : 1 median ratio in favor of ECM) and N transporters (*c.* 3 : 2 median ratio). Detailed expression levels for these three major classes of transporters were compared between guilds (Fig. 5). Surprisingly, ectomycorrhizal fungi showed significantly higher expression levels in the soil for all C-related transporters (labeled in orange), except for fatty acid transporters, which were almost exclusively expressed by saprotrophs. For N-related transporters (blue labels), the expression levels of amino acid transporters were not significantly different between ectomycorrhizal and saprotrophic fungi, even though both groups had significantly higher expression levels than the pathogenic fungi. However, the expression of other N-related transporters was clearly and significantly higher in ectomycorrhizal fungi than in saprotrophs, especially for the urea transporters with a 10-fold higher guild-normalized TPM in ectomycorrhizal fungi. Inorganic ion transporters (yellow labels) displayed more heterogeneous patterns. Indeed, whereas Ca^{2+} transporters were more highly expressed by saprotrophs, phosphate, Cu^{2+}, Mg^{2+}, and Zn^{2+} transporters were significantly more expressed by ectomycorrhizal fungi than by pathogens and saprotrophs. Finally, Mn^{2+} and Fe^{2+} transporters did not differ in their expression between saprotrophs and ectomycorrhizal fungi.

Finally, considering only the transcripts of the 12 secreted CAZyme families with significantly higher expression in ectomycorrhizal fungi (Fig. 4), as well as ureases and urea transporters (Figs 3, 5), we found that these ectomycorrhizal transcripts were predominantly associated with eight fungal orders (Fig. S2). We found that Atheliales (i.e. ectomycorrhizal corticioid fungi such as *Piloderma*, *Tylospora*, and *Byssocorticium*) expressed high levels of many genes involved in FCW degradation (e.g. CE4 and AA7). Genes for GH2, GH88, and GH72_CBM43 were strongly expressed by Russulales, whereas Endogonales expressed GH18 more strongly than other orders. Finally, the gene expression of other selected CAZyme families, as well as those of ureases and urea transporters, were shared by different taxa or by 2–3 geographically abundant orders (Figs 1a, 5).

Discussion

Using metatranscriptomics, we monitored the activities of pathogenic, saprotrophic, and ectomycorrhizal fungi in forest soil

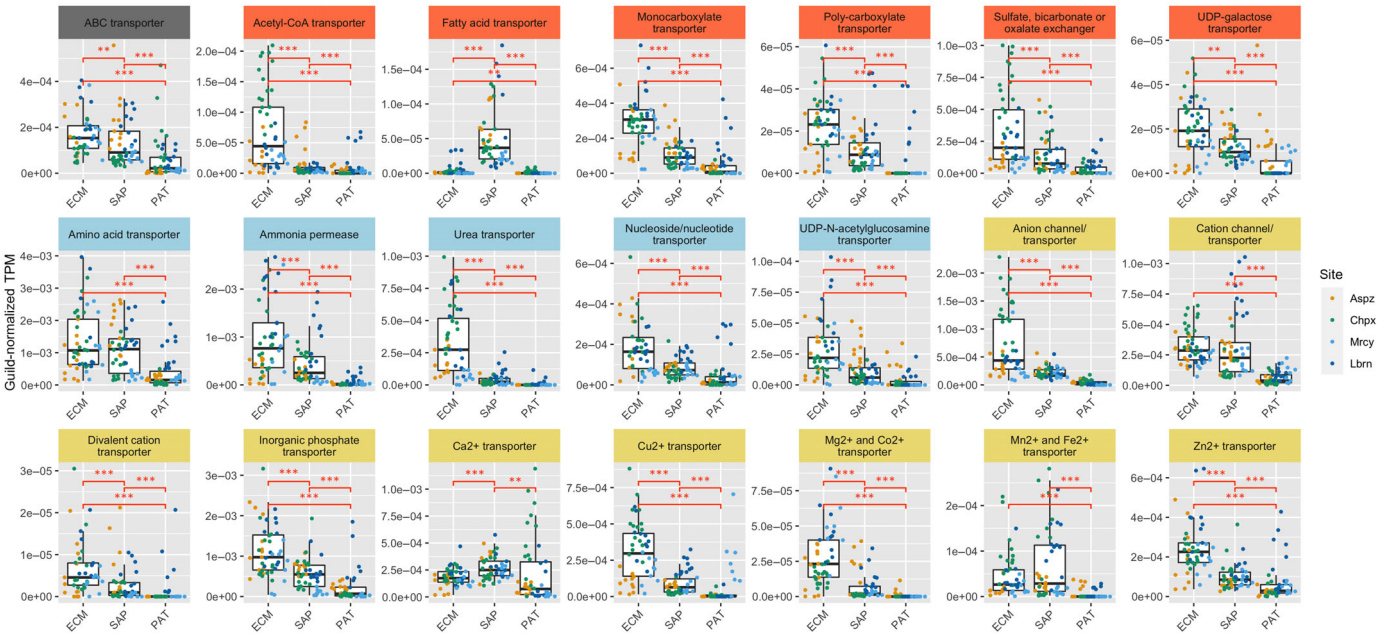

Fig. 5 Boxplot distribution of transporters expression, per fungal guild (ECM, ectomycorrhizal; SAP, saprotrophic; PAT, pathotrophic fungi) and transporter type. Boxplots show distribution across all sites; individual sample values are plotted and colored by site (Aspz, Aspurz; Chpx, Champenoux; Lbrn, Lamborn; Mrcy, Montmorency). Significant differences between guild-normalized distributions are indicated in red (Wilcoxon test with FDR adjustment; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$). Carbon-related, nitrogen-related and inorganic ion transporters are respectively labelled in orange, blue, and yellow.

resource mobilization and assimilation, providing an indirect assessment of functional traits. Environmental RNAs were extracted from four contrasted forest soils in Europe and North America. We assigned transcripts to potential gene functions and associated taxonomic and functional groups. We further discuss how this high-throughput technology can allow us to test microbial ecology concepts under field conditions.

Identification of the most active taxa in fungal communities from different forest soils

Metatranscriptomic data from environmental eukaryotic mRNA allow contig-based taxonomic assignments of active fungal communities without the biases that are inevitable in rDNA metabarcoding, for example distortions in the relative abundance of species (Bellemain *et al.*, 2010; Rammitsu *et al.*, 2021), copy number imbalance (Lofgren *et al.*, 2019), and marker length biases (Castaño *et al.*, 2018). By focusing on eukaryotic mRNAs extracted from soil, our protocol required an amplification step of *c.* 10 cycles, but with low amplification bias, because the target polyA region is universally present in fungal transcripts. Conversely, as fresh soil samples must be stored very quickly in dry ice or liquid nitrogen to avoid RNA degradation, metatranscriptomics is a difficult technology to apply to field ecology. Although taxonomic annotation remains relatively accurate, metatranscriptomics is not yet able to describe fungal communities at the species level. It is likely that some contigs have been assigned with imprecise taxonomic information, particularly due to the still relatively low completeness of reference databases for certain fungal clades. However, classification at the genus or family level is very robust, as illustrated with our *in silico* quality controls, and of sufficient quality to obtain a good ecological guild assignment. In some cases, the assignment of certain contigs to the guilds studied can be ambiguous, because some fungal species may have dual lifestyles (Bergero *et al.*, 2000; Martino *et al.*, 2018). Finally, we have focused our analyses on three major guilds: pathogens, saprotrophic and ectomycorrhizal fungi. Within the Dikarya, the taxonomic information obtained using metatranscriptomics allowed good separation of the fungal communities inhabiting the four forest soils (i.e. genus level) and representative of the communities already described in the literature. The ectomycorrhizal fungal genera found in our studied climatic areas corresponded to fungal genera described in previous studies at the same sites using more conventional approaches (Marupakula *et al.*, 2021), or in close geographic areas with the same tree species (Buée *et al.*, 2011; Collado *et al.*, 2021). Notably, our metatranscriptomic data depicted a higher diversity of saprotrophic fungal genera than ectomycorrhizal fungal genera. The proportions of Mucoromycota, Chytridiomycota, and Mortierellomycota were much higher than those of metabarcoding surveys conducted in comparable geographic areas (Coince *et al.*, 2014; Pérez-Izquierdo *et al.*, 2017, 2021; Castaño *et al.*, 2018). Their cumulative proportion was higher than that of Basidiomycota at the Chpx and Mrcy sites. These findings suggest that the importance of the role of Dikarya (Basidiomycota and Ascomycota) in soils is probably overestimated by studies using metabarcoding approaches compared with other fungal phyla including specific genera, such as *Mucoromycota* and *Mortierella* genus, and *Cladochytrium* genus and other *Chytridiomycota*.

Linking taxonomic diversity to functional characteristics within fungal communities

In ecology, there is great interest in measuring ecophysiological traits to predict species complementarity and the differences that affect ecosystem functions (Koide *et al.*, 2014). Various researchers have attempted to predict the activity of fungal species by measuring enzymatic activities that drive biogeochemical cycles in laboratory microcosms and *in situ* targeting single guild (Courty *et al.*, 2005; Mathieu *et al.*, 2013; Talbot *et al.*, 2015). Some authors have also used molecular markers targeting a specific gene family as an indicator of a functional trait such as SOM decomposition (Luis *et al.*, 2004; Kellner *et al.*, 2008; Weber *et al.*, 2012; Barbi *et al.*, 2016, 2020). However, this type of approach does not make it possible to compare the relative contribution of the different fungal guilds (e.g. saprotrophic and ectomycorrhizal species) to an overall functional process such as litter decomposition or to the mobilization of nutrients from various environmental matrices. Currently, only meta-omics methods, particularly those using mRNA sequence analysis, offer new possibilities for linking the identification of taxonomic affiliations and information on functional traits.

Using eukaryotic mRNA sequencing, we observed a conserved pattern of key gene terms (KOG families) related to biological processes, molecular functions, and cellular components in the soil metatranscriptomes of the four studied forests. In addition, selected families of transcripts were taxonomically annotated to describe the relative participation of different taxa in various processes, illustrating contrasting traits of fungal subcommunities despite the relative homogeneity of the overall KOG family expression. By extracting transcripts of 'foraging genes' and linking them to members of guilds with different lifestyles (Põlme *et al.*, 2020), we demonstrated that proteolytic and hydrolytic activities related to SOM decomposition and nutrient transport processes segregated the three studied functional guilds, irrespective their taxonomic composition and geographic origin. Metatranscriptomic studies have shown taxonomic changes within microbiomes depending on the hosts or environments studied (Turner *et al.*, 2013; Žifčáková *et al.*, 2017; Schneider *et al.*, 2021; Wei *et al.*, 2022). However, the links between functions and taxa remain extremely difficult to apply because of the gap between the genomic resources available and the real microbial diversity observed. Grouping the fungal functions expressed by guilds makes it easier to establish links between taxonomic diversity and the functions expressed by the different taxa. Finally, as microbial beta diversity is very high, this approach confirms that identical guilds, made up of different taxa, can express the functions that characterize them at similar levels. Therefore, our results support the concept of functional redundancy as defined by Lawton (1994) and Louca *et al.* (2018). Although the major functional guilds of fungi are relatively well defined, to our knowledge, this concept has never been validated *in situ* in an environmental matrix, such as soil.

Partitioning of organic matter decomposition functions among the three major forest fungal guilds

Using guild-normalized TPM to ensure an unbiased comparison between the functional expression profiles of saprotrophic, ectomycorrhizal, and pathogenic fungi, we showed that genes involved in decomposition processes were weakly expressed by pathogenic fungi compared with ectomycorrhizal and saprotrophic species in forest soils. Indeed, biotrophic fungal pathogens likely express specific PCWDEs only during host infection, that is penetration and growth inside the living cells of the host and during tissue destruction (O'Connell *et al.*, 2012; Dhillon *et al.*, 2021). Conversely, ectomycorrhizal and saprotrophic fungi were more active decomposers in the forest soils. It was not surprising to measure a significantly higher expression of secreted PCWDEs in saprotrophs than in ectomycorrhizal fungi and pathogens, since saprotrophs depend on dead OM to meet their metabolic demands. By contrast, the expression of FCW CAZymes was significantly higher in ectomycorrhizal fungi than that in saprotrophs and pathogens. Surprisingly, we measured a somewhat higher expression of genes related to proteolytic activities (secreted proteases) in saprotrophs compared with ectomycorrhizal fungi, which have been proposed as key contributors to protein degradation and organic N mobilization in forest soils (Chalot & Brun, 1998; Nehls *et al.*, 2001; Nygren *et al.*, 2007). However, it has recently been suggested that the Fenton reaction could be an important component of the N acquisition machinery of several ectomycorrhizal fungi, such as *Paxillus involutus*, enhancing proteolytic activity after substrate oxidation (Op De Beeck *et al.*, 2018). Because we also observed comparable transcription of amino acid transporter genes in saprotrophic and ectomycorrhizal fungi, this dual oxidative-proteolytic system may be more efficient than a proteolytic system alone for releasing N from proteins complexed in soil OM, as suggested by Op De Beeck *et al.* (2018). Finally, the distinct functional patterns of the fungal guilds in the four forest sites indicated the functional complementarity of these ecological groups in OM decomposition. Overlapping transcription profiles also suggest a potential for competition between these two fungal guilds for soil organic N resources, particularly proteins, chitosan, and chitin (Lindahl & Tunlid, 2015; Frey, 2019). Such competition for organic N may be the basis for the 'Gadgil effect', in which ectomycorrhizal fungi are hypothesized to hamper decomposition by restricting the access of saprotrophs to soil nutrients. The extent of the 'Gadgil effect' has been proposed to depend on both the quality of the substrate and the composition of the ectomycorrhizal fungal community (Fernandez *et al.*, 2020) and may be further deciphered using metatranscriptomic approaches.

Selection of functional indicators to understand the underlying mechanisms of soil fungal ecological processes

By assessing the frequency of genes encoding for specific enzymes that contribute to OM decomposition (CAZymes) or facilitate N uptake (transporters), different studies have attempted to link genomic resources to functional traits in fungi (Floudas *et al.*, 2012; Treseder & Lennon, 2015; Romero-Olivares *et al.*, 2021). Indeed, it has been suggested that gene copy number modulates gene expression and may contribute to significant phenotypic variations (Martino *et al.*, 2018; Miyauchi *et al.*, 2020; Romero-Olivares *et al.*, 2021). Using our metatranscriptomics data, we were able to discuss the theoretical relationships between *a priori* knowledge of the functions facilitated by major fungal guilds (i.e. traits) and data obtained from fungal gene repertoires. Based on this theoretical framework, our study validates refined functional indicators for further exploration.

Analysis of the mean expression values of all PCWDEs provides information that matches the picture obtained from fungal genomic data, confirming that saprotrophic fungi are the primary fungal drivers of the decomposition of plant OM in forests. Indeed, saprotrophic fungi have a larger repertoire of genes encoding PCWDEs than ectomycorrhizal fungi do (Kohler *et al.*, 2015; Miyauchi *et al.*, 2020). Likewise, 46 PCWDE families were significantly more highly expressed by saprotrophic fungi than ectomycorrhizal fungi in our study. Some of these CAZyme gene families, such as CBM1, GH7, and AA9, have already been identified as molecular markers of OM decomposition by saprotrophs (Treseder & Lennon, 2015; Miyauchi *et al.*, 2020). While these three gene families are indeed strongly expressed in saprotrophic taxa, other PCWDE families, such as CE5, CE15, GH11, GH28, GH62, and GH74, encoding cutinases, xylanases, xylosidases, galacturonases, glucanases, or xyloglucanases, were also strongly expressed, almost exclusively by saprotrophs. Furthermore, Miyauchi *et al.* (2020) reported that the proportion of microbial cell wall degrading enzymes (MCWDEs), acting on chitin, glucans, mannans, and peptidoglycans, was similar between ectomycorrhizal and saprotrophic fungi, in contrast to PCWDE. In the present study, we showed that among the CAZyme genes most strongly and significantly expressed by ECM, 75% corresponded to FCWDE. In particular, we highlight the high expression of AA5 (oxidases), GH5_9 (β-1,3-glucanases), GH18 (chitinases), GH20 (β-N-acetylglucosaminidases), and GH128 (β-1,3-glucanases) families within the ectomycorrhizal guild. Moreover, recent studies have confirmed that certain ectomycorrhizal fungal species use GH18 and GH20 to depolymerize chitin by hydrolytic mechanisms and that degradation of FCW, and in particular chitin, by ectomycorrhizal fungi might be a key functional trait of N cycling in forest soil (Lindahl & Taylor, 2004; Maillard *et al.*, 2023). However, some traits (i.e. gene families) seemed to be expressed only by a limited number of ectomycorrhizal taxonomic groups, such as the AA7 (carbohydrate oxidase) family, which is expressed only by Atheliales or Agaricales, regardless of the forest type.

We also used the expression of gene families encoding amino acid, urea, and N-acetyl-glucosamine transporters, as well as ammonia permeases, as indicators of N uptake potential, and other transporter genes as indicators of the potential to take up several other key nutrients. With particularly high expression levels in ectomycorrhizal fungi, we found significant difference in the expression of N transporters (ammonium, nitrate, and amino acids) between the three fungal guilds. Through the study of gene expression, our results temper the conclusions of some genomics studies (Romero-Olivares *et al.*, 2021). Indeed, when studying the distribution of gene frequencies between the genomes of the

main ecological guilds of fungi, these authors did not find quantitative differences in nitrogen transporters (ammonium, nitrate and amino acids) between pathogenic, saprotrophic, and ectomycorrhizal fungi. Remarkably, we also measured higher levels of transcripts encoding urease and urea transporters in ectomycorrhizal fungi. Urea, which was an important fertilizer in forest soils (Saarsalmi & Mälkönen, 2001; Fox, 2004; Albaugh *et al.*, 2007), may be a significant source of N for ECM fungi and their host trees. Morel *et al.* (2008) have suggested that the ectomycorrhizal species *Paxillus involutus* is capable of using urea as an N source. Nicolás *et al.* (2019) confirmed urea uptake by *P. involutus* by measuring the upregulation of urea transporters in this fungus but not in *Laccaria bicolor*, another ectomycorrhizal basidiomycete. These results suggest that urease and urea transporters are relevant functional indicators to study urea uptake by ectomycorrhizal fungi as a functional trait of N nutrition in mycorrhizal symbiosis. Similarly, genes encoding glycosylamines and N-acetyl-glucosamine transporters were expressed at significantly higher levels in ectomycorrhizal fungi than in saprotrophs and pathogens, which is in line with various ectomycorrhizal-secreted CAZymes encoding β-N-acetylhexosaminidases, chitin deacetylases, and chitooligosaccharide oxidases. In addition, the expression of genes encoding transporters of P and other elements (Cu, Mg, Co, and Zn), or nonspecific cation and anion transporters, was significantly higher in ectomycorrhizal fungi than in saprotrophic and pathogenic fungi. Interestingly, the expression of genes coding for fatty acid transporters was significantly higher in saprotrophic fungi, suggesting a central role for this group in soil lipid recycling. Although saprotrophic fungi mobilize C from OM, the expression of fungal sugar transporter genes, such as carboxylate transporters, is significantly higher in ectomycorrhizal fungi than in saprotrophs and pathogens. Plant roots or soil microbes can release carboxylates, which are capable of mobilizing P or other micronutrients, such as Mn (Lambers *et al.*, 2015a,b). Potentially, these elements are assimilated as chelates by the carboxylate channels of ectomycorrhizal fungi.

Thanks to the increase in fungal genomic resources, we have shown that metatranscriptomics may provide information from a broad suite of functional markers to track fungal functions in soils. By combining genome sequences with landscape-scale metatranscriptomics of soil dynamics *in situ*, we were able to link the composition of fungal communities with important soil processes at an ecosystem scale. Focusing on the transcriptional regulation of genes encoding secreted enzymes, we obtained a more holistic picture of enzyme production involved in OM decomposition, and novel indicators known to encode important functions were identified. For the first time, we demonstrated that transcriptional patterns could be translated into decomposition strategies, focusing on competition for N mobilization between fungal guilds for the transformation of recalcitrant carbon pools, hydrolytic degradation of plant and microbial cell walls. Our results emphasize the added value of metatranscriptomics for interdisciplinary research to forecast the effects of trophic relationships between functional guilds on processes, functions, and resilience in forest ecosystems. This ecological validation of metatranscriptomic data is of great conceptual importance because the potential vulnerability of soil trophic interactions subjected to environmental changes may be identified, allowing better prediction of the cascading effects of global change.

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Competing interests

None declared.

Author contributions

FMM coordinated this project. FMM and MB designed this study. CM, LF, RF, BL, AO and AS provided the sample soils and carried out the mRNA experiments. RC, JBI, LR, FJC, MA and AGP participated in the selection of the sampling sites, sampling, and preparation of the samples from Navarra. LA performed bioinformatics and statistical analyses of the data. AC, VS, AC, KL, BF, BK, YY and IVG performed coordinated RNA sequencing at the JGI. LA performed KOG contig annotations, and VL and BH performed CAZyme annotations. MB, LA and FMM analyzed and interpreted the data. MB wrote the manuscript with help from FMM, LA and other authors. LA and MB contributed equally to this work.

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Data availability

Raw data are available on the SRA. Links to the SRA Accessions for each sample are provided in Table S1 (columns BioSample and BioProject).

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Supporting Information

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

Fig. S1 Fungal trophic guilds transcript TPM abundances across sites.

Fig. S2 Distribution of the expression of ECM-overexpressed functions.

Methods S1 Taxonomic and functional guild annotation quality assessment.

Methods S2 Supporting R script and package version details.

Table S1 Sample description.

Table S2 Overexpressed CAZyme families.

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