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Niche and ecosystem preference of earliest diverging fungi in soils

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ABSTRACT

Within the supergroup Rotosphaeromycetes, or "Holomycota"/"Nucletmycea", there are several well-recognised unicellular clades in the earliest diverging fungi (EDF). However, we know little about their occurrence. Here, we investigated EDF in the rhizosphere and bulk soils from cropland, forest, orchard, and wetland ecosystems around the Beijing-Hebei area, China, to illustrate their niche and ecosystem preference. More than 500 new operational taxonomic units (OTUs) of EDF were detected based on the 18S rRNA genes. Microsporida and Aphelida constitute dominant groups, whereas Rozellosporida was quite rare. Although the EDF community was site-specific, the soil chemical characteristics, vegetation, and other eukaryotic microorganisms were the key factors driving the occurrence of EDF. Moreover, the stochastic process consisted the most of the EDF community assembly.

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1. Introduction

"Earliest Diverging Fungi (EDF)" have been well recognised to include Aphelida, Rozellosporida, Microsporida, and some FISH or sequence-based clades such as NCLC1 and BCG2 (Powell 1984; Jones et al. 2011; Capella-Gutiérrez et al. 2012; Tedersoo et al. 2018; Chambouvet et al. 2019). These fungi usually have a parasitic intracellular lifespan with the capacity for phagotrophy (Letcher et al. 2018; Karpov et al. 2018), but their free-living status is still limited known. Zoospores are the infection propagules to invade host cells by the encyst sticking to the surface and germinating to a thallus into the host cell for Aphelida, Rozellosporida, and probably NCLC1 (Karpov et al. 2014; Letcher et al. 2018; Chambouvet et al. 2019) or by the polar tube to penetrate the host cell for Microsporida (Keeling and Doolittle 1996; Wang et al. 2015; Corsaro et al. 2020). There are some concerns about the correct taxon names of EDF (Tedersoo et al. 2018). Additionally, there are conflicting views on whether Aphelida, Microsporida, Rozellida, and related lineages belong in "Fungi" (Ocaña-Pallarès et al. 2022; Merényi et al. 2022, preprint). This study ignored the controversy and treated EDF as fungi for convenience. Please be aware that the phylogeny of Fungi-Rotosphaerida has not yet been solved and the group "EDF" is probably polyphyletic (Chambouvet et al. 2019; Ocaña-Pallarès et al. 2022).

Previous studies have demonstrated the wide distribution and seasonal fluctuations of EDF with different dynamics in marine, freshwater, and other water bodies (Lara et al. 2010; Corsaro et al. 2014; Richards et al. 2015). However, knowledge of EDF species and populations in soils, as well as systematic and inter-ecological comparisons of EDF community structure were still lacking. Several pieces of research have revealed a wide distribution of EDF in terrestrial ecosystems or extreme habitats, such as forests, sediments, and frozen soil around glaciers, based on the detection of the environmental rRNA gene (Mohamed and Martiny 2011; Livermore and Mattes 2013; Brad et al. 2018; Jamy et al. 2020). As potential key

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contributors to nutrient cycling and energy transfer, EDF have received far less attention than other components of the soil microbiome (Oliverio et al. 2020). Plant biodiversity and species composition determine the functioning and stability of terrestrial ecosystems (van der Heijden et al. 1998) and shaped the rhizosphere microbiome (Carney and Matson 2006). However, the occurrence of EDF in different terrestrial ecosystems and their preference in the rhizosphere and bulk soils remain unknown. It is very important to make a clear investigation of where and how we can find EDF in terrestrial ecosystems. Along with the advancement of sequencing technology and the discovery of new EDF taxa, it has become possible to clarify the EDF distribution in certain regions.

The Beijing-Hebei area is rich in ecosystem types and has similar climatic and latitudinal conditions (Chen et al. 2020), allowing a convenient and simple comparison of biological and soil physicochemical properties that shape the EDF communities. Here, to address the questions above, we hypothesised that the EDF community structure is associated with ecosystems and driven by certain environmental factors and other eukaryotes. We also hypothesised that roots may recruit some eukaryotic organisms that are the host of EDF, hence the rhizosphere EDF may differ from the bulk soil. To investigate this, ecosystems with well-developed root networks should be considered. Thus we collected the bulk and rhizosphere soils from the forest, wetland, orchard, and cropland ecosystems in Beijing and Hebei, China, and extracted the environmental DNA, from which amplified 18S rDNA gene V4 district as amplicons. Concentrating on EDF and other fungal groups, we included Rotosphaerida in the analysis as the outgroup control to compare the differences between different fungal and non-fungal communities.

According to the niche and the neutral theories, the community assemblance of organisms is influenced by determinism (controlled by the factors from environment and other selection pressures) and stochastic (controlled by the intrinsic factors of the community itself randomly like the dynamics as speciation/extinction or rates of death/birth, etc.) processes (Zhou and Ning 2017; Ning et al. 2020), which differed among communities and environments (Li et al. 2019; Aslani et al. 2022). To gain a deeper understanding of the community processes, we also investigated the community assembly of EDF.

2. Materials and methods

2.1. Soil sampling and rhizosphere soil separation

We collected 135 samples from different ecosystems and habitats around Beijing, China, in November 2020. In each sample site, we collected the bulk and rhizosphere soil with five replicates, respectively, especially accompanied by samples from the sediment at Chaobai River (Table S1). For bulk soil samples, the top layer of the soil was removed by 5 cm and then the soil samples were collected using a soil probe (25 cm × 1.9 cm each column, and five columns were mixed up and made of one replicate) and a zig-zag strategy. For rhizosphere soil samples, each replicate was collected from different plants, with at least five root branches with the attached soil following our previous method (Hussain et al. 2018). Soil samples were packed into pre-autoclaved PE bags and delivered directly to the lab in a 4 °C refrigerator. To separate rhizosphere soil, roots were washed with 25 mL PBS in 50 mL centrifuge tubes with a vibrating shaker for 20 min and then were removed by pre-autoclaved tweezers. The tubes with soil were centrifuged in 2,000 × *g* for 25 min to keep the EM in the sediment without breaking the naked cells, and then the supernatant was carefully removed. The rhizosphere soil was transferred into a 1.5 mL centrifuge tube and preserved at −80 °C till utilisation.

2.2. DNA extraction, PCR amplification, and amplicon sequencing

Soil DNA was extracted using QIAGEN DNEasy PowerSoil Kit (0.25 g soil/tube) within 1 week after sampling, checking with PCR (primer pair C22FS-A1B2R, see Table S2) amplifying to ensure EDF existed in every site. The V4 region of the 18S rRNA gene was amplified using primer pair Ek-NSF573 - Ek-NSR951 (Mangot et al. 2013) with Vazyme 2 × *Taq* plus Master Mix and purified with Agencourt AMPure XP. The libraries were constructed with NEB Next Ultra II DNA Library Prep Kit. The amplicon libraries were qualified with Agilent 2100

and then sequenced with Illumina MiSeq (PE250). All the sequencing procedures would not stop until reaching at least 30,000 raw reads for each sample.

2.3. Soil chemical characters measuring

The bulk soil samples were divided into two portions with one freeze-dried to measure the soil moisture and the other not. The dried portion was used to measure total carbon (TC), total nitrogen (TN), soil organic matter (SOM), pH, total phosphorus (TP), total Sulphur (TS), total potassium (TK), and Salinity (SAL), while the other portion was for ammoniacal and nitrate nitrogen. TC and TN were measured using the VARIO MACRO cube elemental analyser (Shimadzu). Soil pH was detected using DELTA 320 pH metre (Mettler Toledo). NO_x-N and NH_3-N were detected by continuous-flow AutoAnalyzer 3 (BRAN + LUEBBE). TS and SAL were measured using already well-established methods (Butters and Chenery 1959; Feng et al. 2005). Soil organic carbon (SOC), TK, and TP were measured following the Standard Methods of China (GB9834–88, GB9836–88, GB9837–88) and SOM was estimated from SOC (Van Bemmelen 1890). The chemical characteristics of rhizosphere soil were considered the same as the bulk soil around.

2.4. Data analysis

The adapters of the amplicon data were removed by a Python script, and then the pair-end reads were joined by PEAR 0.9.8 (-p 0.0001 -v 10 -n 130 -t 100 -q 20 -g 2 -j 35 -u 0) (Zhang et al. 2014). Later the full-length data were imported into QIIME2 (Bolyen et al. 2019) for OTUs clustering and rough classification by using DADA2 (Callahan et al. 2016) with a trimmed length of 335 bp and VSEARCH (Rognes et al. 2016) to get the de-novo OTUs in 97% similarity, and the feature-classifier was trained with SILVA 138.1 database (Quast et al. 2012). All the Metazoan, Streptophyta, and non-ribosome OTUs were removed right after.

To ensure an accurate classification, different eukaryotic 18S rRNA gene sequences from GenBank were utilised for reference (Data S1 and Table S3) and were used to construct a maximum likelihood tree combined with EDF + Rotosphaerida ("LKM15" "Cryptomycota" "Aphelida" "Nucleariaand Fonticula Group"), and Unknown ("Unclassified" "Eukaryota;_") OTU sequences. The MSAs (multiple sequence alignments) were aligned by MAFFT v 7.4 (-E-INS-i) (Katoh et al. 2018) and trimmed by trimAl (–gt 0.02) (Capella-Gutiérrez et al. 2009). The trees for classification were built several times by IQ-TREE 2.0.6 (-m GTR+G+I -B 5000 -alrt 2500 -bnni -nstep 150 -T AUTO) (Hoang et al. 2018; Minh et al. 2020) and removed the traits not belonging to EDF till we met a comparable tree to previous studies, using a bunch of guide sequences (Data S2) constructed using all of the EM (Cocquyt 2009; Lara et al. 2010; Livermore and Mattes 2013; Bass et al. 2018; Brown et al. 2018; Lax et al. 2018; Letcher et al. 2018; Tedersoo et al. 2018; Chambouvet et al. 2019; Galindo et al. 2019; Stentiford et al. 2019; Burki et al. 2020; Mesentsev et al. 2020; Richardson et al. 2020; Seto et al. 2020; Siemensma and Dumack 2020; Strelow et al. 2020). Consequently, the sequences belonging to EDF or other taxa were replaced into pseudo-taxa or certain taxa manually (Table 1). The phylogenetic tree was visualised using TreeViewer 2.0.1 (https://github.com/arklumpus/TreeViewer). Two OTU tables, one for all eukaryotic microorganisms and the other for Rotosphaerida and EDF, were created. All the OTU tables were subsampled (1,315 for all Eukaryotes and 90 for the latter, hence some of the sites were removed) to calculate diversities using QIIME2 as well as the amplicon (http://github.com/microbiota/amplicon) and phyloseq (McMurdie et al. 2013) packages in R 4.0.3. Distance-based redundancy analyses were calculated by Canoco 5.0 (Šmilauer and Lepš 2014). The correlation coefficient between OTUs was obtained using Hmisc (Harrell and Harrell 2019) package in R for spearman's ρ. Also, the DNCImper (Vilmi et al. 2021) and iCAMP (Ning et al. 2020) packages in R were used to estimate stochastic and determination processes on EDF community assembling.

3. Results

3.1. Amplicon data and EDF OTU features

We got 13,064,542 clean reads which were denoised into 19,207 amplicon sequence variants (ASVs). After removing non-ribosome, Metazoan,

Table 1. Taxa constructed by this research describing EDF & Rotosphaerida monophyletic clusters and the OTU amount of each taxon.

Supergroups	Taxon-1	Taxon-2	OTUs
Rotosphaeromycetes	Rotosphaerida (14 OTUs)	Nucleariidia	0
		Parvularidia	8
		Pompholyxophrys-like	1
		Fonticulidia	0
		NUC1	4
		NUC2	1
		Marine-Roto-Group-1	0
	BCG2 (1 OTU)	BCG2-Group	1
	Rozellosporida (29 OTUs)	Rozellosporidia	29
	Microsporida (295 OTUs)	Morellosporidia	113
		Paramicrosporidia	49
		Nucleophagosporidia	13
		WIM27	64
		Microsporidia	56
		(UnknownLBM)	46
	BMG2 (9 OTUs)	BMG2-Group	9
	BHG1 (Unsure, 32 OTUs)	BHG1-Group	32
	BHG2 (Unsure, 1 OTU)	BHG2-Group	1
	NCLC1	NCLC1-Group	0
	Aphelida (188 OTUs)	E13	19
		TAGIRI-24	11
		Amoeboaphelidia	62
		A. collabense-like Group	0
		Aphelidia	11
		Paraphelidia	18
		AHG1	32
		AHG2	33
		D1P02G09	2

The species diversity of EDF and Rotosphaerida could be estimated from the OTUs numbers of each taxon. Nucleariidia, Fonticulidia, Marine-Roto-Group-1, NCLC1, and *A. collabense*-like Group were not detected in this research. EDF and Rotosphaerida belonged to the supergroup Nucletmycea or Holomycota but the name of Nucletmycea was combined from Nuclearia and -mycea. As the name of Nuclearia had changed to Rotosphaerida (Adl et al. 2019), we use the word "Rotosphaeromycetes" instead of Nucletmycea as the name of this supergroup as an alternation. Other groups were named after their original phylum or group names except Pompholyxophrys-like group because the OTU we detected was not clearly divided within genus *Lithocola* and *Pompholyxophrys*.
BHG1, BHG2 were settled at an unsure placement in the phylogenetic tree. Also, "Unknown Long-branch Microsporids (LBM)" were placed in Microsporidia but sometimes out of them, and the node bootstrap values were quite low. The OTU number of Microsporidia includes "True" Microsporidia (Group I – V, Metchnikovella, Amphiamblys, Chytridopsida, etc.), and "UnknownLBM". Despite the possible error, we still set them here to avoid fake negatives and thus we kept them in the following analysis.

and Streptophytan sequences, we finally acquired 555 EDF and 14 Rotosphaerida OTUs from 7,694 de-novo clustered OTUs with a 97% similarity for all eukaryotic microorganisms (EM). Among the hundreds of EM OTUs obtained, there were only dozens or a few EDF OTUs in each sample, and the number of EDF OTUs varied in different ecosystems, habitats, and sample sites (Table 1, Table S4). Usually, EDF OTUs were more encountered in ecosystems of forests and wetlands than those in orchards or croplands. Overall, the taxa of Microsporida (295 OTUs) and Aphelida (188 OTUs) were more dominant, while that of Rozellosporida, BMG2, and others were quite rare.

3.2. Phylogenetic tree of EDF

We constructed a phylogenetic tree of the 18S rRNA genes of EDF, other phylogenetic closed fungi, and outgroups of fungi referenced from GenBank, with our amplicon-based OTUs and former cloned sequences (Figure 1). Many OTUs are clustered into monophyletic clades, such as 113 OTUs within the clade containing genus *Morellospora* and *Mitosporidium*, and 64 OTUs

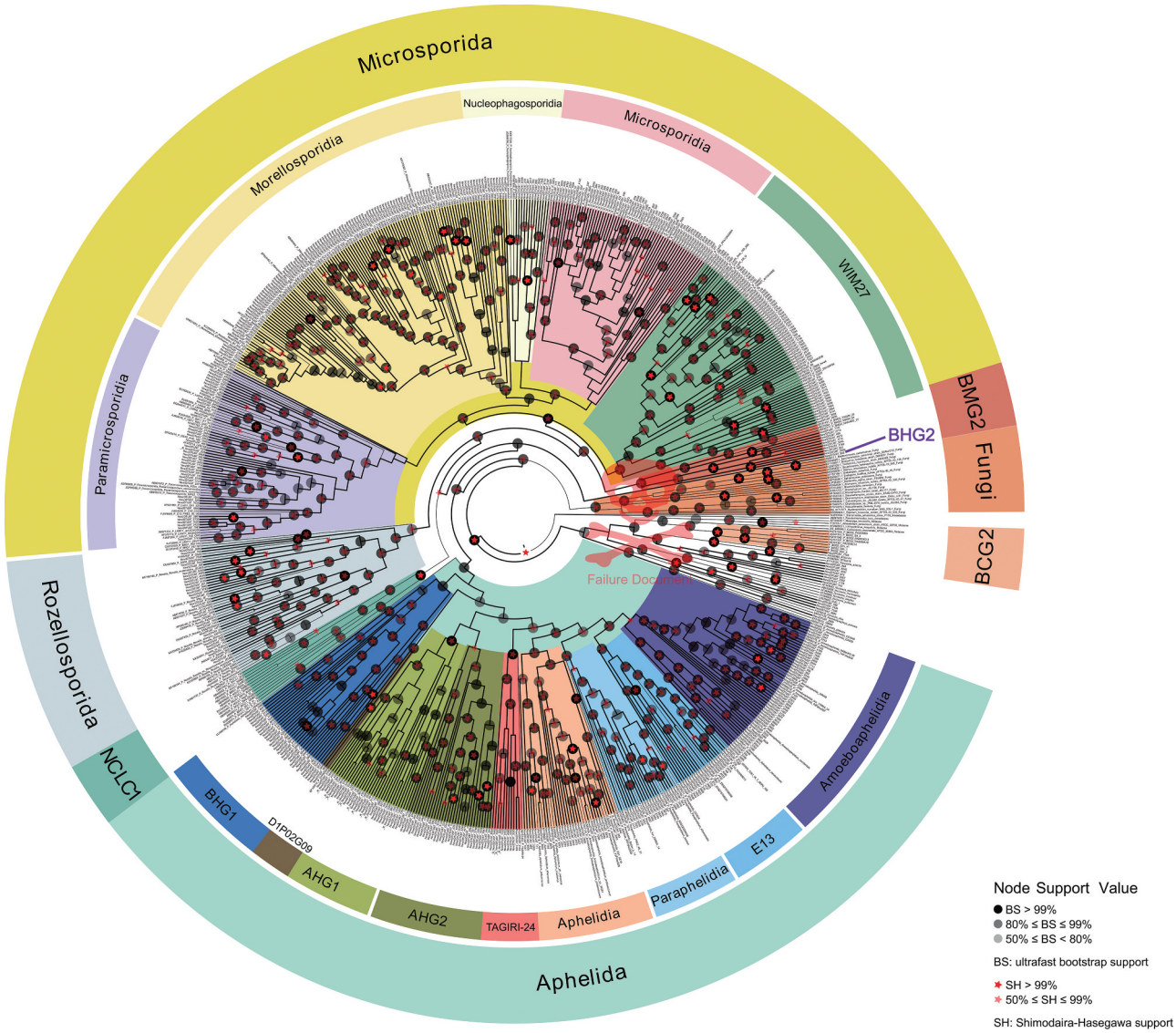

Figure 1. Phylogenetic tree of OTUs belong to EDF and Rotosphaerida constructed with related referenced 18S rRNA gene sequences (Maximum-likelihood method with ultra-fast bootstrap and SH-alrt). All of the OTU sequences were thickened. The aim of this tree is to cluster different OTUs into phylogenetic groups, but the relations between these clusters or clades should not be treat seriously owing to the different lengths of OTUs and the reference sequences, meanwhile Amoebozoa as the outgroup. The tree was artificially divided into several monophyletic groups and named as "taxa" in Table 1 according to both phylogenetic clusters and different ecology preference.

within Laz IX and WIM27, which should be treated as independent "taxa". Therefore, taxa were constructed as follows: Rotosphaerida, BCG2, BHG1, Aphelida, BHG2, NCLC1 (though not detected in this study), Rozellosporida, BMG2, and Microsporida. We divided Aphelida into nine sub-taxa, i.e. Amoeboaphelidia, E13, Paraphelidia, Aphelidia, TAGIRI-24, D1P02G09, AHG1, AHG2, and *Aphelida collabense*-like groups. Meanwhile, Microsporida were divided into WIM27, Paramicrosporidia, Nucleophagosporidia, Microsporidia, and Morellosporidia. However, we left the BMG2 group outside of Microsporidia. We suggest Rozellosporida out of Microsporida because they have no polar tubes or fibres during the parasitic procedure and their phylogenetic relationship and ecological preference were also different from Microsporida (Letcher et al. 2018; Powell and Letcher 2019). The BHG1 group was settled alone because of its unsteady topology, in and out of Aphelida, when constructing the tree with different parameters. Besides, the BCG2 group was not clearly

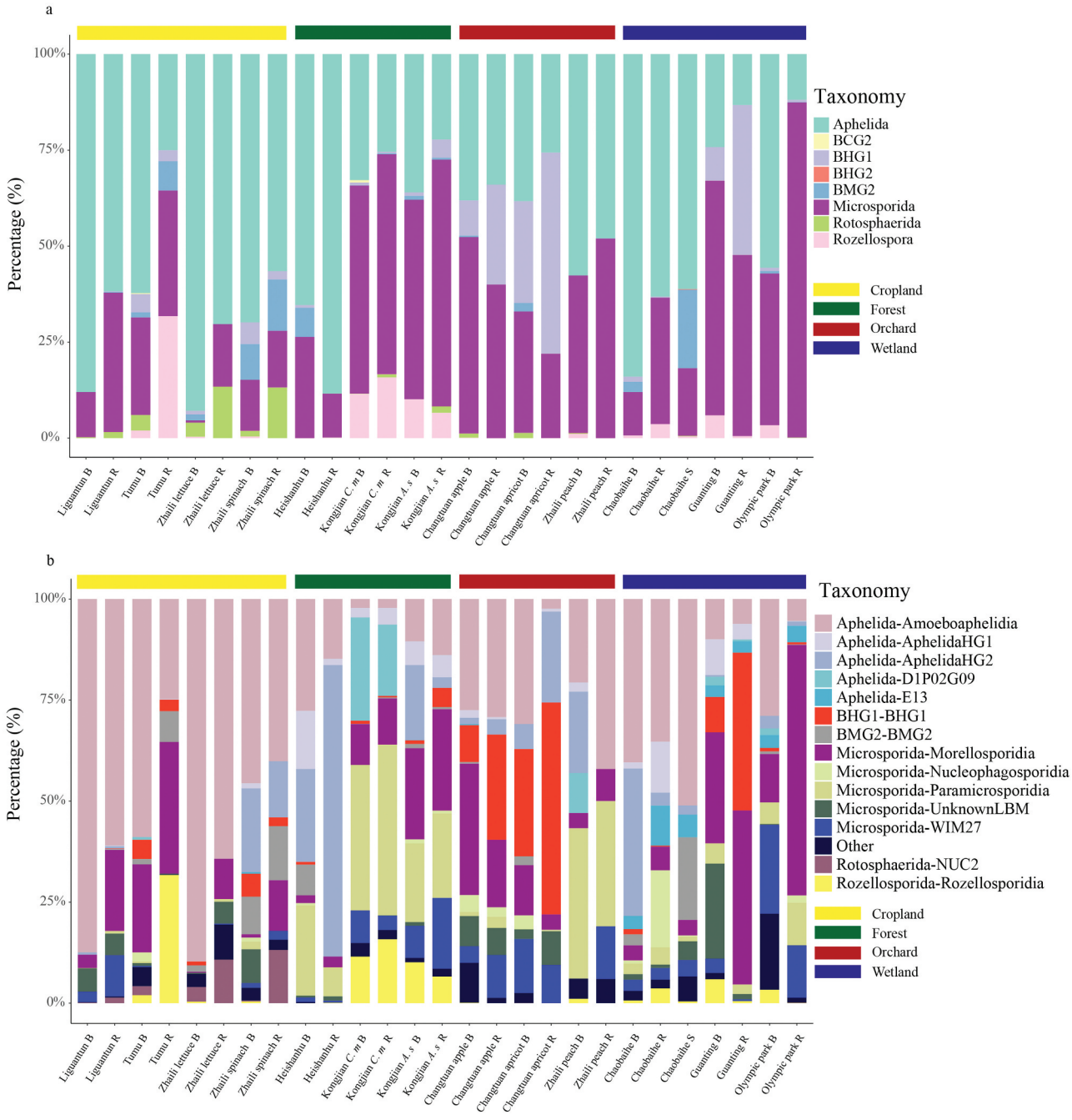

Figure 2. OTU Relative abundance of different taxa of EDF and Rotosphaerida. Each bar represented a special habitat. Bars belong to ecosystems like cropland, forest, orchard, and wetland were represented by coloured bars upward each of the graph. The most abundant 14 taxa were coloured differently and the rest were grouped as "Other". (a) Relative abundance of pseudo taxa 1. (b) Relative abundance of pseudo taxa 2. Habitats B: Bulk soil; Habitats R: Rhizosphere; Habitats S: Sediment; *C.M.*: *Corylus mandshurica*; *A. S.*: *Armeniaca sibirica*.

divided from the Rotosphaerida clade, which was different from previous works (Bass et al. 2018; Tedersoo et al. 2018) and might be due to the long-branch attraction.

3.3. EDF community features in soil ecosystems

The EDF biodiversity monitored by the relative abundance of OTUs varied in different ecosystems and sample sites, which reached up to 9.8% of all the eukaryotic microorganisms in the strawberry greenhouse at Liguantun although the average was 1.2% among the other samples (Figures 2–3, Figure S1). Aphelida and Microsporida were the most dominant taxa of EDF, and their relative abundances in the EDF community were 50.0% and 35.4%, respectively, followed by Amoeboaphelidia (32.3%), Morellosporidia

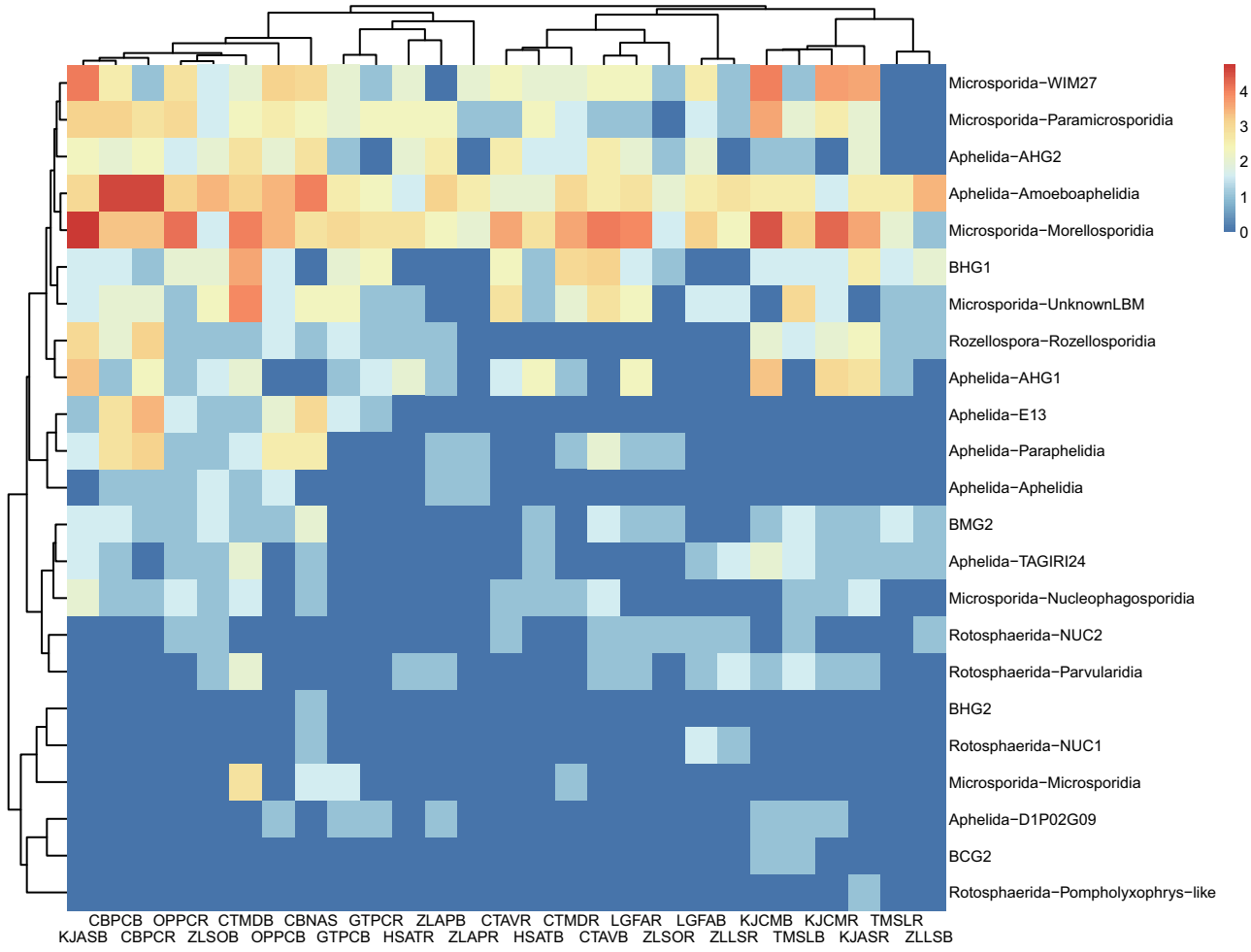

Figure 3. Heatmap for observed features (types of different OTUs) in taxa 2 of each habitat. The observed features were "log1p" transformed using package Vegan in R and the score was coloured as the legend. The taxa and habitats were clustered by UPGMA method using Bray-Curtis distance. Names of habitats were abbreviated into five characters, in which the first two represented geographical sites, the middle two for vegetation, and the last one for rhizosphere (habitats R), bulk soil (habitats B), or sediment (habitats S). All of the full names and details of sample habitats can be checked in Table S1.

(15.2%), Paramicrosporidia (9.5%), and AHG2 (9.3%). On the contrary, Rozellosporida, BMG2, BHG2, and BCG2 were quite rare although Rozellosporida and BMG2 were widespread taxa. Only 1 BCG2 OTU was detected in the primaeval forest at Kongjian and a tomato greenhouse at Tumu; and 1 BHG2 OTU in the sediment of Chaobai River. The EDF OTUs detected were most founded in the wetland (249), followed by forest (208) and Orchard (173), and lowest in cropland (122), indicating that the richness of EDF were different among ecosystems. Interestingly, the unique OTUs counted were detected to be the lowest in cropland (36.1%) compared with wetland (68.7%), forest (66.4%), and orchard (57.2%) (Figure S2). For the niche preference, the unique EDF OTUs counted were the highest in sediment (71.13%, though unbalanced sampled), followed by bulk (54.68%), and the lowest in the rhizosphere (41.06%) soils (Figure S2).

We measured the α diversities of EDF including observation features, Shannon indexes, and Pielou evenness of different ecosystems and niches (Figures 4a–4f, and Figure S3 for observation features distribution). Overall the α diversities hit the lowest in croplands, where the observation features were significantly lower than in other ecosystems (Kruskal–Wallis' test, $P < 0.05$) but the Shannon indexes and Pielou evenness were not. Forest, orchard, and wetland ecosystems have no significant differences from each other. As to niches, the observation features of sediment were larger than those of bulk soil, and the latter was significantly larger than the rhizosphere (Kruskal–Wallis' test, $P < 0.05$), rather than the other

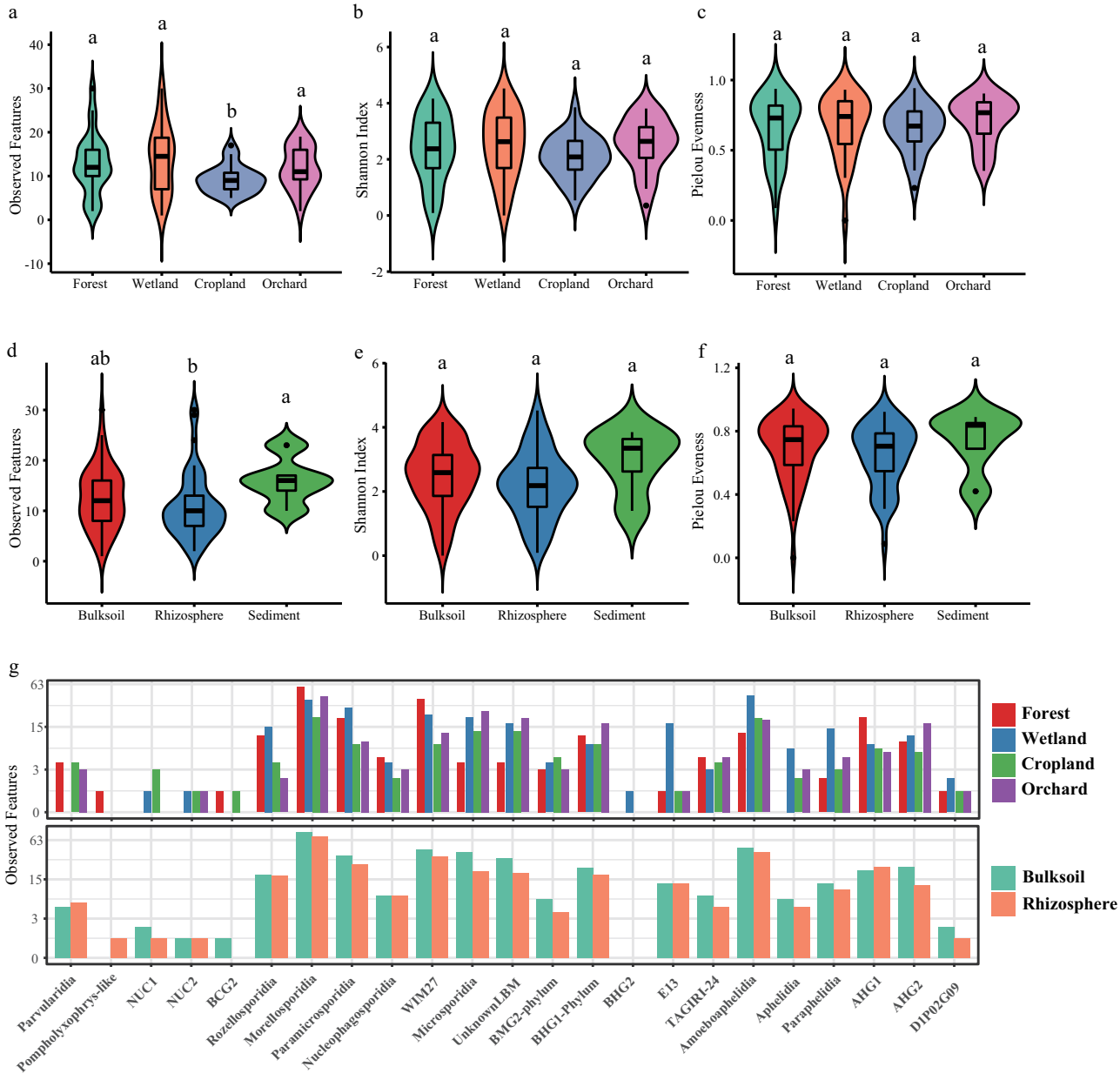

Figure 4. The alpha diversities of EDF community among different ecosystems and spatial niches of root. Kruskal-Wallis tests were used as statistical tests with threshold *P*-value <0.05. (a–c) Observed Features, Shannon Index, and Pielou Evenness of EDF among different ecosystems. (d–f) Observed Features, Shannon Index, and Pielou Evenness of EDF among different spatial niches. (g) Accumulative Observed Features in EDF and Rotosphaerida taxa-2 level (this was the original data, which did not subsample to the same volume), the y-axis was "log1p" transformed.

indexes. A non-subsampled phylogeny-based observation feature (Figure 4g) revealed different taxa preferences among ecosystems and niches where Morellosporida, WIM27, and AHG1 were more in forests, while Paramicrosporidia, E13, Aphelidia, Paraphelidia, and Amoeboaphelidia were more in wetlands. Furthermore, almost all of the taxa have more observation features in bulk soil than in the rhizosphere except Parvularidia and AHG1.

The β diversities of EDF were also detected using principal coordinate analysis (PCoA) with Bray-Curtis distances (Figures 5a–5b) showing the community dissimilarity between different ecosystems and niches. The communities were significantly distinguished (Adonis $P < 0.05$) among ecosystems and niches. However, the differences between groups were quite small (Permutations = 999, Adonis $R =$ 0.125 for ecosystems and Adonis $R = 0.028$ for niches)

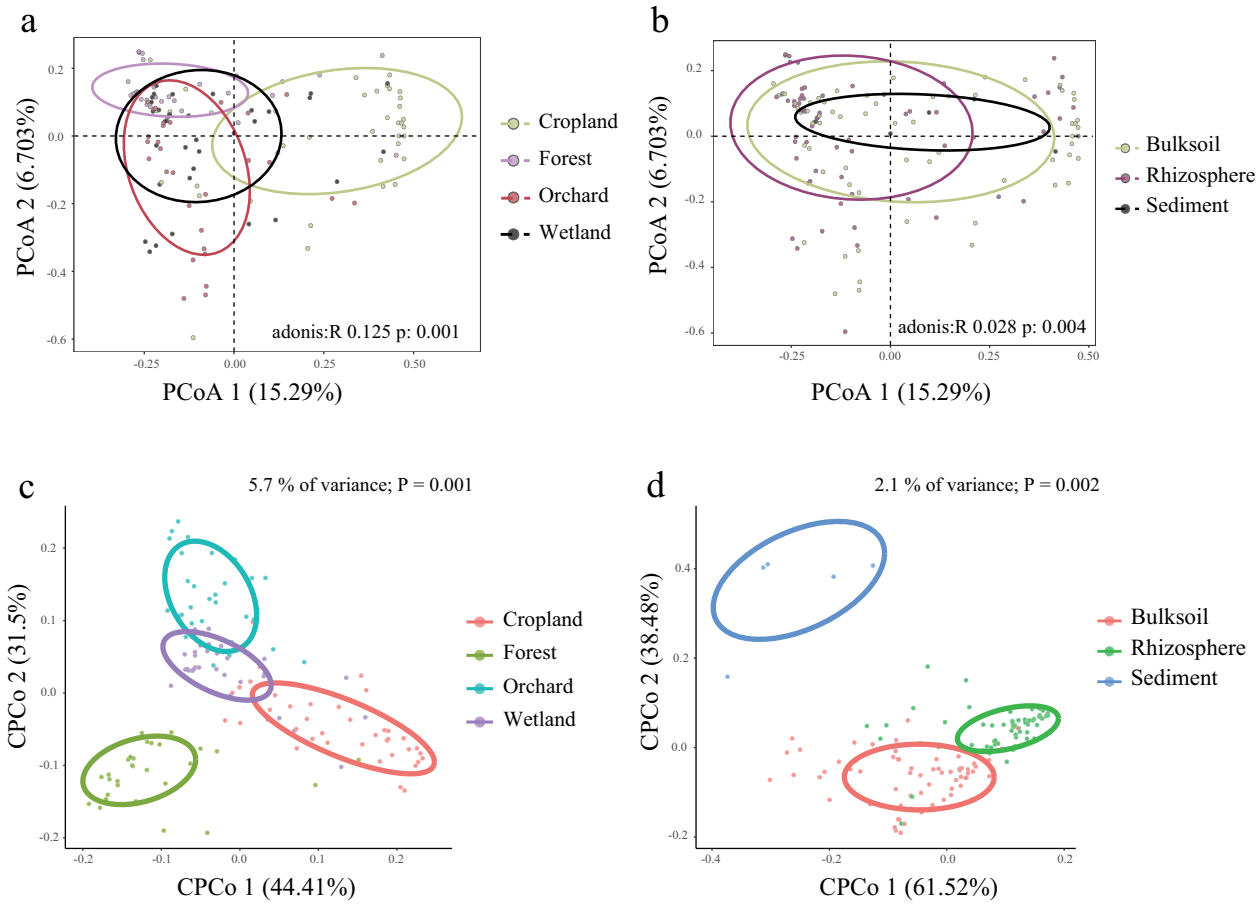

Figure 5. The PCoA and CPCoA of EDF+Rotosphaerida community. The analyses were using Bray-Curtis distance of homogenised (subsampled) relative abundance. Results were checked using Adonis test. The confidence eclipses were drawn in the 95% confidence. (a–b) PCoA among different ecosystems and niches, respectively; (c–d) CPCoA among different ecosystems and niches, respectively.

and the confidence ellipses (CI = 95%) were overlapped in PCoA. Therefore, we raised constrained principal coordinates analysis (CPCoA) to reveal further details (Figures 5c–5d, Figure S4). Ecosystems explained only 5.7%, with the first two constrained principal coordinates explaining 75.91%, and niches explained only 2.1% with the first two constrained principal coordinates explaining all of the constrained community dissimilarities. Factors such as ecosystems and niches cannot explain the community component well. However, we found that EDF were site-specific due to the higher percentage of variances explained if the CPCoA were more locally constrained, like ecosystems-spatial niches (10.1%), geographical sites (14.3%), vegetations (18%), and ecosystems-geographical sites (15.9%) (Figure S5). The vegetations were not an independent factor in this research but still indicated a strong explanation. Moreover, the CPCoA explained 31% of variances when considering all of the sample categories (each sample site was distinguished by bulk and rhizosphere soils, data not shown).

3.4. Factors shaping the EDF community

To evaluate the effects and strength of chemical factors which influenced the EDF communities and drove the dissimilarity, db-RDA (distance-based redundancy analysis, Figure 6, Tables S5–S6) using Bray-Curtis distance was implemented with SOM, TC, TN, TC/TN, TK, TP, TS, NO_x-N, NH_3-N, SAL, and SM measured. The result demonstrated the content of chemical elements forming the community of different ecosystems, as the chemical factors could explain 25.9% (where the first two redundancy principal coordinates explained 45.0%) of the community dissimilarity, and the effects were significantly distinguished ($P = 0.002$). TC/TN, SOM, and TP made the strongest shaping effects (explained by 4.3%, 3.8%, and 2.9% of the total variance, respectively, with FDR-adjusted $P < 0.01$) on EDF communities. TP, TK, and

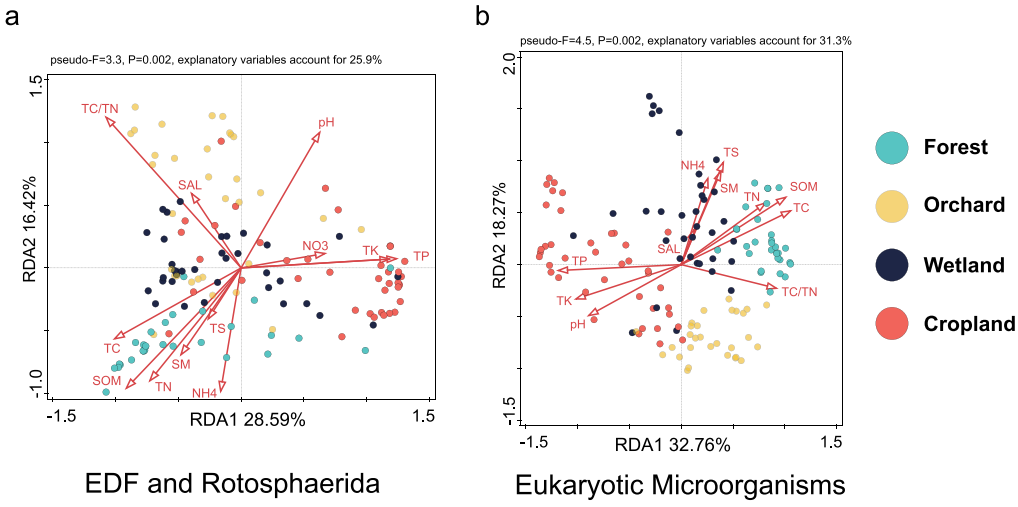

Figure 6. The db-RDA of EDF+Rotosphaerida and Eukaryotic Microorganisms influenced by soil chemical compounds. Each sample site was showed in the figures as coloured dots. TC: Total carbon; TN: Total nitrogen; SOM: Soil organic matter; SM: Soil moisture; NH_4: NH_3-Nitrogen (NH_3-N); NO_3: NO_x-Nitrogen (NO_x-N); TC/TN: Carbon to nitrogen ratio; SAL: Salinity; TK: Total potassium; TP: Total phosphorus.

NO_x-N strongly shaped the community in croplands but orchard communities fitted in higher TC/TN and SAL environments. TC, SOM, and TN have a positive effect on the community in forests and EDF fitted a lower pH herein. However, in wetlands, the community similarity was driven by multi-factors and no single effect can be explained largely.

Spearman's correlation analysis showed that the taxon relative abundance of EDF was significantly affected by soil chemical factors ($P < 0.01$, Figure 7). The diagram revealed that the relative abundance of Aphelida was positively correlated to Alveolata, Archaeplastida (here only refers to small-size Chlorophyta), Hemimastigophora, Holozoa, Rhizaria, and Stramenopiles but strongly negative to others. Microsporida had a similar trend along with Aphelida but was also positively related to Amoebozoa, CRuMs, Cryptista, and Haptista but not Archaeplastida. Also, a positive correlation was detected between BHG1 and Amoebozoa, CRuMs, Cryptista, Haptista, Holozoa, and other EMs. BMG2 had positive correlations with Alveolata. Rotosphaerida were positively related to Rhizaria, Aphelida, and other EMs. There was no significant correlation between Rozellosporida, BCG2, and BMG2 to other taxa. For soil chemical factors, Microsporida showed a positive response to TC, SOM, TN, TS, SAL, and TC/TN but negative to TK. Rozellosporida were positive to TC, SOM, TN, NH_3-N, and SM but negative to pH and TK. BHG1 had a strong positive relationship with TC/TN but was negative with NO_x-N, NH_3-N, and TK. Rotosphaerida response was distinguished from EDF, showing positive relation to NO_x-N, and NH_3-N but negative to TC/TN.

3.5. Stochastic process accessed more in EDF community assemblance

To unearth the community formation processes, DNCI (dispersal-niche continuum index) (Vilmi et al. 2021) was utilised in this research to estimate the stochasticity and the determinism process impacting the EDF community formation among ecosystems and habitats (Figure 8). The results revealed a major effect of stochastic dispersal on the formation among different ecosystems, which was probably common for small EM communities (Bahram et al. 2015). On the contrary, the rhizosphere microenvironment often led to a certain process of species selection for the DNCI factors near zero or being positive. The stochasticity effect accounted for the main part among ecosystems regardless of the phylogenetic resemblance as aforementioned in some of the habitats. However, in the same habitat, the difference in rhizosphere soil sometimes paid a determinism effect, especially when comparing the sites in different habitats. In

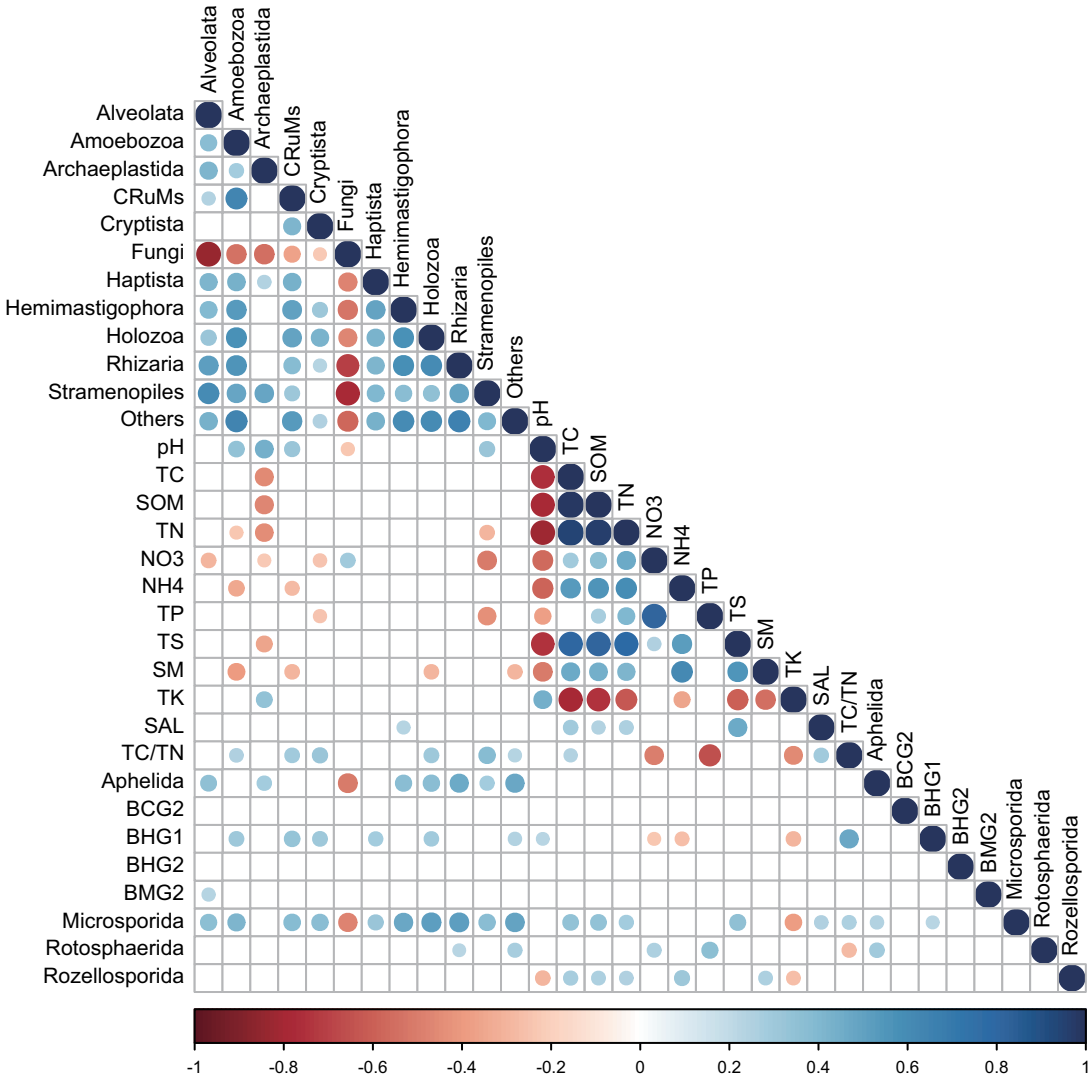

Figure 7. The spearman correlation among the relative abundance of different taxa and the physiochemical characters. The colour and size represented the spearman correlation value. The correlations were not visualised, i.e. no coloured circles when the *P*-value >0.05. TC: Total carbon; TN: Total nitrogen; SOM: Soil organic matter; SM: Soil moisture; NH_4: NH_3-Nitrogen (NH_3-N); NO_3: NO_x-Nitrogen (NO_x-N); TC/TN: Carbon to nitrogen ratio; SAL: Salinity; TK: Total potassium; TP: Total phosphorus.

forests and wetlands, the determinism effect occasionally dominates the differentiation of some rhizosphere niches. Meanwhile, the two effects were more balanced in the forest. To ensure this result, iCAMP (Ning et al. 2020) was also utilised to analyse the process with a comparison to DNCI and reflected a similar trend with a greater role of stochastic processes (66.9% in Guanting Reservoir to 94.4% in croplands), especially as drift, in the construction of EDF communities from most of the rhizosphere and bulk soil among sample sites (Figure S6). The dispersal limitation process was more prevelant in forests and wetlands, but in Guanting, the homogeneous selection peaked at 46.7% in the rhizosphere, suggesting site-specific community construction at that place (which is also hinted in our PERMANOVA result, Table S7).

4. Discussion

In this research, we created a reference-based phylogenetic tree of EDF, separated it into various phylum-level taxa, and thoroughly examined the characteristics of EDF communities in rhizosphere and bulk soils of four ecosystems around Beijing based on prior studies on EDF core

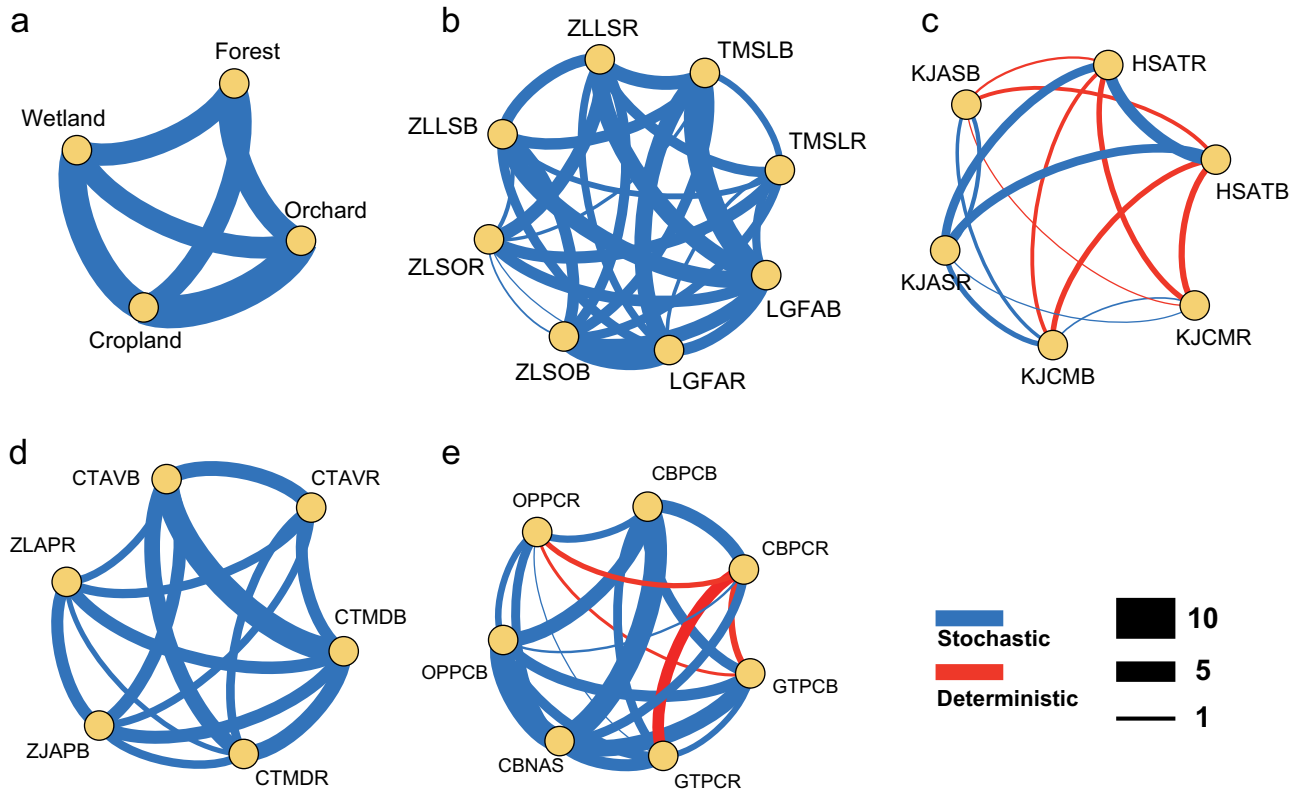

Figure 8. DNCI networks of dispersal and niche-drive effects influencing the EDF communities' assembly among different ecosystems considering the habitats and the rhizosphere-niches. The analyses were manipulated by DNCI score, which is negative (blue in this figure) when the dispersal effect prevail, and positive (red in this figure) when the niche effect take over. The thickness of each linkages represented the absolute value of DNCI score, and would be very thin when the stochasticity and determinism effects met a balance. Effects were stronger while the linkages were thicker. (a–e) DNCI among ecosystems, croplands, forests, orchards, and wetlands, respectively. All of the full names and details of sample habitats can be checked in Table S1.

taxonomic species. With the guide tree, there were roughly 500 new EDF OTUs detected, which are composed of the majority of soil EDF communities, indicating that EDF are widely distributed across soil ecosystems. We found that the habitats, soil chemical characteristics, and vegetations are the major drivers to shape the EDF community. The wide distribution and abundance of EDF in different ecosystems and niches imply the essential ecological roles that should be explored.

4.1. New insights showed by EDF phylogeny

The phylogeny of EDF has been studied from limited locations (Lara et al. 2010; Richards et al. 2015; Voigt et al. 2021). In this research, we acquired over 500 18S rRNA gene-based OTUs from the Beijing area to make a further understanding of EDF, Rotosphaerida, and Fungal phylogeny. We named EDF taxa following the International Code of Zoological Nomenclature (Ride et al. 1999) which has already been used in the "true" Microsporiids to avoid confusion and referred to a recent work about the Phytophagea and Opisthophagea hypothesis (Galindo et al. 2023). In addition, we found that some sequences were affiliated outside of the known taxa in the phylogenetic trees such as AHG1, AHG2, BHG1, and BHG2. The tree topology within Microsporidia and Aphelida was complex, and the fine structure often varies with the adjustment of tree parameters. Therefore, we classified Microsporida into Microsporidia, Nucleophagosporidia (named after genus *Nucleophaga*), Morellosporidia (named after genus *Morellospora* and included genus

Mitosporium), Paramicrosporidia (named after genus *Paramicrosporium*) and WIM27 groups; and traditional Aphelida into Aphelidia, Paraphelidia, Amoeboaphelidia, E13, TAGIRI-24, AHG1, AHG2, D1P02G09, and the singleton *Aphelidium collabens* - like groups according to the largest monophyletic clade. "true" Microsporidia according to Bass et al. (2018) was embedded within pan-microsporidia which is closely related to Nucleophagosporidia. To avoid false negatives, we identified long-branch sequences attracted to Microsporidia as "UnknownLBM" (red shaded in Table 1), with the actual number of "true" Microsporidia OTUs may be less than 10.

4.2. Preference of EDF and Rotosphaerida communities in soil

In this study, EDF widely existed with a varied relative abundance among ecosystems. The most obvious indicators of the EDF community were Aphelida and Microsporida, which are the most abundant lineages in all the ecosystems. The relative abundance of Aphelida sometimes reached over 90% of EDF and over 10% of EM in croplands, peculiarly for the taxa that were previously found aquatic (Letcher and Powell 2019; Seto et al. 2020) and mainly belonged to Amoeboaphelidia. However, the E13 group was dominant in wetlands, possibly due to the amoeba-like and flagellated zoospores having different mobilities in varied niches (Letcher et al. 2013). Microsporida was much more abundant in forests, orchards, and wetlands than in cropland, and sometimes consists of around 50% except in the newly developed young forest in Heishanhu. Furthermore, Paramicrosporidia preferred forests and orchards with arbour vegetation over others. We also noted that Morellosporidia was usually more common in the rhizosphere of croplands. However, the preference for higher taxa levels was not detected. Rozellospora was previously detected in aquatic ecosystems (Letcher and Powell 2018), but they can also be found in forests and other soil environments in this survey. Besides, Rotosphaerida was overall rare in most of the samples, probably due to their aquatic preference (Galindo et al. 2019).

We set the sediment of Chaobai River as a contrast to the EDF community in soil and calculated the EM relative abundance among sites (Figure 2 and Figure S1). Interestingly for EDF, the relative abundance in sediment was similar to that in bulk or rhizosphere soil except for more BMG2. We found that the EM community in this research has a similar trend compared to previous works (Zhao et al. 2018; Jamy et al. 2020), as well as more Stramenopiles but much fewer Fungi in the sediment than in ordinary soil. EDF had different relative abundances among sites or habitats considering all the EM. The effect of sediment on EDF community structure was less than that of Stramenopiles and other Fungi. All these facts indicated that EDF has a special environment preference different from the other taxa in EM.

4.3. Biotic and abiotic factors shaping EDF communities

It is important to investigate the biotic and abiotic drivers to shape EDF communities and other taxa. Compared with the α and β diversities of EM (Figures S5, S7, S8), EDF showed the same trend as all of the EM but with a lower standard deviation in α diversity, while all of the factors could explain less variance for EDF than that for EM in β diversity. Extensive distribution with less influence by environments indicated the wide fitness of EDF in ecosystems.

Although the whole EDF were widely distributed, most of the EDF OTUs were rare and only exist in specific sites (Figures S2 and S9, Tables S4 and S8). The EDF community composition was more significantly influenced by environmental factors and vegetation than those of the rhizosphere, which was consistent with other research (Asiloglu et al. 2021). Compared to all EM, these factors fall short of adequately explaining the EDF community structure (Figures 5, S4, S8). However, sample categories (i.e. sample sites + rhizosphere/bulk soil) could explain up to 31% of the EDF community dissimilarity from a site-dependent view, indicating that EDF species were highly site-specific in soil environments.

The environmental factors have a significant influence on EDF communities (Figure 6 and Table S6), such as TC/TN ratio, which was higher

in the orchards in Changtuan (19.46) than that in other sites (<15). The TC/TN ratio was much lower in arbour-leading ecosystems (9.96), probably due to this kind of vegetation has fast mineralisation and N release (Brust 2019), as well as the more developed mycorrhizae (Read et al. 2004). Regarding other soil characteristics, the Tumu tomato greenhouse has a much higher TP (2.60 g/kg) than other sites (0.62–1.80 g/kg), which might have a special impact on the EM (Shimano 2007) so as EDF. The enrichment of Rotosphaerida in the rhizosphere in tomatoes (Figure 2) could be explained by much more chemical fertiliser input (Guo et al. 2018), or possibly the enrichment of pathogenic bacteria subsequently resulted in their phagotrophic and bacterivore habits (Xiong et al. 2020; Asiloglu et al. 2021).

4.4. A stochastic process for EDF community assembling

Both deterministic (e.g. vegetation, environmental properties, and pathogenic microorganisms) and stochastic progress significantly contribute to the construction of microbial communities (Ceja-Navarro et al. 2021). Unlike rhizosphere bacteria, eukaryotic microbes are distributed widely across diverse environments and associated with the rhizosphere microenvironment to regulate phytohormones, plant nutrient uptake, and pathogen populations (Rosenberg et al. 2009; Gao et al. 2019). However, not all EM communities were under strong selection pressure (Asiloglu et al. 2021; Ceja-Navarro et al. 2021). In our results, the EDF community was mostly assembled with stochastic progress, especially drift and dispersal limitation regardless of the possible seasonal influence (Li et al. 2019; Ceja-Navarro et al. 2021). The drift process means random changes or diversification with weak selection/dispersal and the dispersal limitation process means the migration of individuals to new sites is restricted, thus the structure of the community differs (Zhou and Ning 2017). Since the living strategy of EDF mostly depends on other EM, their migration was closely connected to their hosts. The strongly site-specific lifestyle (Figures S2 and S9) of EDF also corroborated the role of drift and dispersal limitations.

5. Conclusions

Here, we elucidated the extensive occurrence and abundance of the EDF among soil ecosystems and the special preference for niches and ecosystems of different groups of EDF. In the Beijing-Hebei area, Aphelida and Microsporida consisted the most in soil ecosystems. Especially, some EDF groups, rather than the whole EDF, had a particular preference for rhizosphere soil. Most EDF communities are probably stochastically assembled by drift. The chemical and biotic factors, especially for the TC/TN, SOM, TP, and vegetation, are the main drivers to shape the EDF communities. This is the first work systematically investigating EDF in terrestrial ecosystems using hand-correct amplicon-based 18S rDNA gene phylogeny. Also, this work is an initial exploration of the soil ecology distribution of EDF. There are still many subsequent directions to be studied. Overall, EDF is the new research hotspot for construction and clarifying the evolutionary history of Fungi and Opisthokonta. As ancient diverging organisms, EDF with its peculiar progress of community assemblance should be a novel comprehension of parasitism and need more investigation.

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Data availability statement

The raw amplicon sequencing data for this article can be accessed online at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA848264.

Originality-Significance Statement

The earliest diverging fungi (EDF) are widespread and phylogenetically important. Understanding their occurrence and

diversity in diverse ecosystems and niche preferences will provide essential data to explore their impact on other organisms, the origin, the evolution of fungi, and biotechnological application.

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