Distinct communities of Cercozoa at different soil depths in a temperate agricultural field

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Abstract

Protists are the most important predators of soil microbes like bacteria and fungi and are highly diverse in terrestrial ecosystems. However, the structure of protistan communities throughout the soil profile is still poorly explored. Here, we used Illumina sequencing to track differences in the relative abundance and diversity of Cercozoa, a major group of protists, at two depths; 10-30 cm (topsoil) and 60-75 cm (subsoil) in an agricultural field in Germany. At the two depths, we also distinguished among three soil compartments: rhizosphere, drilosphere (earthworm burrows) and bulk soil. With increasing depth, we found an overall decline in richness, but we were able to detect subsoil specific phylotypes and contrasting relative abundance patterns between top- and subsoil for different clades. We also found that the compartment effect disappeared in the subsoil when compared to the topsoil. More studies

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are now needed to describe and isolate these possibly subsoil specific phylotypes and better understand their ecology and function.

Keywords: protists, cercozoa, Illumina, soil biodiversity, vertical distribution

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Introduction

Soil microorganisms are essential components of terrestrial ecosystems as they substantially influence biogeochemical processes and support multiple soil functions (Falkowski, Fenchel and Delong 2008; Bardgett and van der Putten 2014; Rousk and Bengtson 2014; Bender, Wagg and van der Heijden 2016). It is well-known that the majority of soil microorganisms are concentrated in a thin upper-most layer of soils with a strong decrease in microbial biomass and diversity along soil depth (Fierer, Schimel and Holden 2003; Eilers *et al.* 2012; Stroobants *et al.* 2014; Sosa-Hernández *et al.* 2018). Accordingly, our understanding of the structure and dynamic of microbial communities has been restricted for a long time to the upper soil layer and communities living in deeper soil horizons are still poorly understood. Although occurring at low cell density, these subsoil microorganisms strongly contribute to the turnover of nutrients in soil when considering the large volume of subsoil compared to the thin, active topsoil layer (Kautz *et al.* 2013).

In arable soils the subsoil is generally more compact than the ploughed soil horizon (Hamza and Anderson 2005). As a consequence, the higher bulk density found in deeper soil layers generally results in a reduction of pore size and soil aeration causing local anoxic conditions (Berisso *et al.* 2012). In addition, deeper soil is less subjected to variations in the soil moisture than upper soil (Hupet and Vanclooster (2002). This has also been demonstrated in our field experiments where Perkons et al. (2014) have shown that pore volume and air capacity are lower in the subsoil than in the topsoil. Accordingly, protist communities may be morphologically and physiologically adapted to the conditions of the subsoil. A previous study provided first insight into the distribution pattern of morphological groups of protists (naked amoebae, flagellates and ciliates) within a soil profile (Scharroba *et al.* 2012). The authors reported, in general, a decline in density from the topsoil (0-10 cm) to deeper soil layers (40-70 cm). However, the used enumeration method does not allow a fine differentiation into phylogenetic groups and underestimates the actual diversity. High throughput sequencing methods offer new means to explore the diversity and the environmental response of soil protists in much greater detail.

Among the protists, the phylum Cercozoa (Cavalier-Smith 1998) together with the phylum Amoebozoa, constitutes the dominant protistan group in terrestrial habitats (Urich *et al.* 2008; Bates *et al.* 2013). This group is immensely diverse and new clades are still being discovered and described (Bass 2004; Bass *et al.* 2009a; Dumack *et al.* 2016). The Cercozoa encompass an array of various feeding habits and lifestyles including phagotrophy, autotrophy and parasitism (Cavalier-Smith and Chao 2003), underpinning multiple ecological roles in the functioning of terrestrial habitats.

Here, using the high throughput and resolution of Illumina sequencing, we tested for structural shifts in the community structure of Cercozoa between two soil depths, topsoil (10-30 cm) and subsoil (60-75 cm), in an agricultural field in Germany; additionally, we included key soil compartments (rhizosphere, bulk soil and drilosphere). These compartments are known to strongly influence and drive the structure of microbial communities (Garbeva, Van Veen and Van Elsas 2004; Berg and Smalla 2009; Uksa *et al.* 2014) which in turn might influence the community structure of Cercozoa.

Material and methods

Soil sampling design, amplicon generation and Illumina paired-end sequencing

The study site is an agricultural field located in the southwest of the state of North Rhine-Wesphalia, Germany. The soil has been characterized as Haplic Luvisol (FAO/ISRIC/ISSS, 1998). A detailed description of the soil profile is given by Vetterlein et al.(2013). Samples were collected from three plots within the same field, in each plot the top- (10–30 cm, i.e. in the plough layer) and subsoil (60–75 cm) were sampled, across three different compartments: drilosphere (soil directly influenced by earthworms), rhizosphere (soil directly influenced by roots) and bulk soil (without roots or earthworm burrows), i.e. 6 samples per plot and 18 samples in total. Sampling occurred in May 2011, at a time when chicory (*Cichorium intybus* L.) was grown at the field (see Uksa et al. (2014) for details).

DNA was isolated from 0.25 g of fresh soil using the PowerMax® soil DNA isolation kit (MO BIO Laboratories, Solana Beach, CA) according to the manufacturer's recommendations. We carried out a two-step PCR to amplify a fragment (c. 350 bp) of the V4 region of the SSU/18S gene using the primers sets designed by Fiore-Donno et al. (2018) for

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the specific amplification of Cercozoa. According to the authors, in the first PCR, the forward primers S616F_Cerco (5'-TTAAAAAGCTCGTAGTTG-3') and S616F_Eocer (5'-TTAAAAAGCGCGTAGTTG-3') were mixed in the proportions of 80% and 20%, and used with the reverse primer S963R_Cerco (5'-CAACTTTCGTTCTTGATTAAA-3'). In the second nested PCR, we used the same forward primer mix as in the first nested PCR together with the reverse primer S947R_Cerco (5'-AAGAAGACATCCTTGGTG-3'). Adaptors for binding the indexes (see below) were attached to the primers of the second PCR. All PCRs were performed using the Kapa HiFi PCR Kit (Kapa Biosystems, Woburn, MA, USA). We incorporated 1 µl of soil DNA template, which was 1/10 diluted for the first PCR and 1 µl of the resulting PCR product as a template for the second PCR. We employed the following final concentrations: Kapa HiFi PCR Kit (Kapa Biosystems, Woburn, MA, USA) 0.5 units, buffer 1X, dNTPs 0.3 mM each and primers 0.3 µM. The thermal program consisted of an initial denaturation step at 95°C for 3 min, 30 cycles (first PCR) and 24 cycles (second PCR) at 98°C for 20 s, 62°C for 30 s, 72°C for 30 s; and a final elongation step at 72°C for 5 min. Amplicons were afterwards purified using magnetic beads (GC Biotech, Alphen aan den Rijn, The Netherlands). The resulting purified DNA was tagged with indexes (to distinguish the sequences from each sample) and adaptors required for Illumina MiSeq sequencing. The libraries were purified using magnetic beads, pooled in equimolar amounts and sequenced on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv, Berlin, Germany) to generate 2x300 bp paired-end reads. Sequences were deposited at the European Nucleotide Archive (ENA) and can be obtained under the accession number PRJEB30791.

Data processing

The sequence data were processed according to a customized pipeline largely based on USEARCH (Edgar, 2010). The reads were assembled using PEAR, a pair-end read merger (Zhang *et al.* 2014) and primer sequences were removed using CUTADAPT (Martin 2011). Quality filtering was carried out by removing reads exceeding maximum expected errors = 1 using the *fastq_filter* command (Edgar and Flyvbjerg 2015) implemented in USEARCH.

Quality filtered sequences were clustered into zero-radius Operational Taxonomic Units (z-OTU) using the *unoise3* command in USEARCH. In contrast with classical OTU delineation at a 97% similarity, this command is expected to retrieve correct biological sequences, allowing for high taxonomic resolution

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(https://drive5.com/usearch/manual/uparse_otu_radius.html, 10 September 2018). For taxonomic assignment, z-OTU sequences were queried against PR2 (Guillou *et al.* 2013) using the naive Bayesian classifier (Wang *et al.* 2007) implemented in MOTHUR (Schloss *et al.* 2009) and a minimum bootstrap support of 60%. Singletons and non-cercozoan sequences were removed from the dataset as well as z-OTUs represented by < 0.005% of the total sequences.

Data analysis

All statistical analyses were performed using R version 3.5.0 (R Core Team 2018). Community analyses were performed with the package VEGAN (Oksanen *et al.* 2018). Differences in community composition were examined using the Bray-Curtis distance metric calculated from standardized z-OTU abundances (Hellinger method, *decostand* function). The significance of the experimental factors (depth and compartment) was tested using permutational multivariate analysis of variance (PERMANOVA) using the ADONIS function with 99,999 permutations, and the dispersion within each depth-related group was assessed using BETADISPER function and used as a measure of beta diversity (Anderson, Ellingsen and McArdle 2006). The major variance components of cercozoan community composition were visualized using principal coordinate analyses (PCO) based on standardized z-OTU abundances. A measure of alpha diversity was determined using two diversity metrics: Shannon index (*diversity* function) and richness (*specnumber* function), both calculated from standardized z-OTU abundances rarefied at the lowest number of sequences found (i.e. 44,432). The significance of the sampling factors on alpha diversity indexes was examined using again ADONIS based on Euclidean distances calculated from univariate Shannon and richness variables with 99,999 permutations. A pairwise comparison of Shannon and richness indexes among the different groups (topBULK, topDRIL, topRHIZ, subBULK, subDRIL, subRHIZ) was calculated using the *pairwise.t.test* function with correction for multiple testing (method="BH"). We binned the z-OTUs at the family level to infer differences in their relative abundance and richness between top- and subsoil. For each family, the effect of depth was examined using ADONIS based on Euclidean distances with 99,999 permutations. In order to visualize positive or negative depth-related responses of the individual family, the relative abundances and richness (number of z-OTUs) were first centered to remove the compartment effect and then scaled. The network representing the taxonomic diversity of Cercozoa was generated with Cytoscape 3.3.0 (Shannon *et al.* 2003) using the Allegro Fruchterman-Reingold algorithm (Fruchterman and Reingold 1991). The network is characterized by nodes

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(= z-OTUs) and edges (= taxonomic path from phylum to z-OTU level), whereas z-OTUs are placed at the level of the lowest possible taxonomic assignment. Specific z-OTUs from top- and subsoil were determined based on the presence/absence of these z-OTUs (based on non-rarefied abundance) in top- and subsoil samples, respectively, and visualized onto the taxonomic network. Rarefaction curves of each sample (based on non-rarefied abundance) were computed with the function *rarecurve*. And the species accumulation curve (based on non-rarefied abundance) was computed with the function *specaccum*.

Results

A total of 3,150,415 high-quality sequences (mean=175,023; sd=120,527) were retrieved after the data processing, which yielded a total of 700 z-OTUs (after z-OTUs < 0.005% were removed) (mean=315; sd=153). A table with the taxonomic assignment and the read count from each sample of z-OTU is provided (Table S1). Rarefaction and species accumulation curves (based on total z-OTUs) showed saturation and thus confirmed that the cercozoan richness per sample was not underestimated (Figure S1).

Within the phylum Cercozoa, Sarcomonadea was the dominant class representing 75% of the total sequence reads, followed by Thecofilosea (16% of total sequence reads), Imbricatea (4.2%) and Granofilosea (1.4%). Within the Sarcomonadea, the order Glissomonadida (50% of total sequences) dominated followed by Cercomonadida (25%). Within the other classes - Thecofilosea, Imbricatea and Granofilosea – the dominant orders were Cryomonadida (15%), Thaumatomonadida (1.8%) and Limnofilida (1.3%), respectively (Figure 1, A).

Overall, soil depth was the main factor explaining differences in cercozoan community composition (PERMANOVA, R^2=0.20, $F_{1,14}$=4.295, p<0.001), followed by compartment – rhizosphere, bulk soil and drilosphere, (PERMANOVA, R^2=0.16, $F_{2,14}$=1.742, p<0.05) (Figure 2). Analysis of homogeneity of depth-related group variance revealed higher beta diversity in the subsoil (mean distance to centroid: 0.451) when compared to the topsoil (mean distance to centroid: 0.324) (BETADISPER, $F_{1,16}$=12.335, p<0.01). Similarly, soil depth was the major factor explaining alpha diversity indexes, while the compartment showed a more moderate effect (Table 1, A). Overall, the topsoil showed the highest values of alpha diversity (Shannon index=4.71±0.37, richness=371±130), compared to the subsoil (Shannon index=3.56±0.54, richness=172±71). In the two depths, the rhizosphere showed systematically the lowest values of alpha diversity indexes when compared to the bulk soil and the drilosphere (Table 1, B).

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At the family level, different depth-specific responses (relative abundance and richness) of individual cercozoan groups were revealed (Figure 3). When significant ($p_{value}<0.05$), these responses did vary in strength (more or less pronounced) and/or in direction (increased or decreased with increasing depth). For example, Nudifilidae and Allapsidae showed opposite patterns of depth-specific response in relative abundance. While relative abundance of Nudifilidae *decreased* with increasing depth, the relative abundance of Allapsidae *increased* with increasing soil depth. However, in general, most cercozoan families showed a *decrease* of their relative abundance and/or richness with increasing depth (Figure 3, blue background). Overall only a few groups including Sandonidae, Rhogostomidae, Thaumatomonadidae, Limnofilidae and Allapsidae showed an *increase* of their relative abundance and/or the diversity with increasing depth (Figure 3, green background).

At the z-OTU level, we showed that almost all z-OTUs occurred in the two soil layers, but some were apparently restricted to topsoil (163 z-OTUs, 23% of total z-OTUs), and only a few were exclusively present in the subsoil (13 z-OTUs, 1.9%) (Figure 1, B). These depth specific phylotypes did not show a specific phylogenetic pattern, since they belonged to different clades.

Discussion

Here, we present the first evidence of structural differences in cercozoan communities occurring in top- and subsoil, and the existence of subsoil specific cercozoan phylotypes based on a metabarcoding approach. In comparison with earlier inventories based on morphological traits (Ekelund, Rønn and Christensen 2001; Scharroba *et al.* 2012), our high-throughput sequencing approach offers a much higher phylogenetic resolution.

When considering all the samples together, the Glissomonadida, encompassing small bacterivorous flagellates, was the dominant group of Cercozoa, which is in agreement with previous soil surveys (Bates *et al.* 2013; Bugge Harder *et al.* 2016; Fiore-Donno *et al.* 2018). The second dominant order of Cercozoa in our soil samples was Cercomonadida, a group of amoeboflagellates usually larger in size than that of Glissomonadida (Bass *et al.* 2009b).

Structural differences in cercozoan communities between top- and subsoil occurred in terms of alpha and beta diversity. First, considering all the samples together, we noticed a decrease in alpha diversity with increasing depth (Table 1). This confirmed previous studies based on enumeration methods (Euringer and Lueders 2008; Scharroba *et al.* 2012). This general

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decrease in alpha diversity can be related to a depletion in the microbial food resources with increasing depth, but also related to a compositional difference in the microbial food web (Fierer, Schimel and Holden 2003; Eilers *et al.* 2012; Stroobants *et al.* 2014; Sosa-Hernández *et al.* 2018). Second, beta diversity increased in the subsoil, i.e. the composition in cercozoan communities differed more among samples in the subsoil when compared to the topsoil (Figure 2). One reason that could explain this pattern is that in the topsoil ploughing mixed the three compartments, increasing the chance of finding the same communities in all three compartments. In contrast, ploughing would not affect the subsoil, where the three compartments remained isolated from each other.

These structural differences in cercozoan communities were also apparent when the z-OTUs were binned at the family level (Figure 3). The families showing a shift between the two depths likely featured specific traits that confer them the ability to dominate under the physical, chemical and biological conditions occurring either in the top- or in the subsoil. For example, Rhogostomidae (Figure 3, #3), Trinematidae (#4) and Fiscullidae (#5) feed mostly on eukaryotes, e.g. yeasts or algae (Seppey *et al.* 2017; Dumack, Pundt and Bonkowski 2018). Accordingly, we found these families (except Rhogostomidae) in higher relative abundance in the top soil where light-dependent algae occur. More importantly, the root plant parasites *Spongospora* and *Polymyxa* (Endomyxa: Plasmodiophorida; (Neuhauser *et al.* 2014) were more abundant in the topsoil where the roots mainly occur.

Despite most of the cercozoan families declining in relative abundance and richness with increasing depth (Figure 3), a few families surprisingly showed the opposite pattern with higher relative abundance and/or richness in the subsoil when compared to the topsoil. These were small (4-6 µm) bacterivorous flagellates such as Allapsidae (Howe *et al.* 2009) (Figure 3, #1) more likely to survive in the small soil pores typical of the compacted subsoils. The amoeboid Limnofilidae (#2) have a similarly small body size, characterized by very thin pseudopods able to capture bacteria in very small soil pores, but presumably their ability, for some members, to occur in oxygen-limited environments could be the most important trait for their increased relative abundance in subsurface soils (Bass *et al.* 2009a).

Although depth was the main factor driving structural differences in cercozoan community composition, we also showed significant structural differences among the three compartments, drilosphere, rhizosphere and bulk soil. The drilosphere and rhizosphere are hotspots of microbial activity in soil (Beare *et al.* 1995; Tiunov and Scheu 1999; Tiunov *et al.* 2001). Until today it seemed undisputable that these hotspots harbour specific microbial

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communities due to the distinct availability of resources (Andriuzzi *et al.* 2016). Previous studies on bacterial communities from the same samples as here found significant compartment effects on community composition (2014, 2015), which can explain the structural differences of their predators. The distinction among the three microhabitats highlights the importance of environmental filters, represented implicitly by microhabitats in our design, shaping the diversity of cercozoan communities in soil. Microhabitats, e.g. drilosphere and rhizosphere, harbored distinct communities compared to bulk soil, but subsoils harbored a specific cercozoan community independent of those microhabitats, demonstrating that subsoil conditions buffered the filtering effect of these microhabitats. Among these environmental filters, earthworms translocating organic particles on which bacterial and protistan cells are generally attached, might contribute to the distribution of protist within the soil profile (Andriuzzi *et al.* 2016).

The presence of a few subsoil-specific z-OTUs suggested specific lifestyles that confer advantages in terms of surviving in the oxygen-deprived and resource-limited deep soil layers. Numerous anoxic groups of protists are only known from environmental sequences, and thus not yet described (see for instance 'Novel Clade 12' in Bass et al. (2018)). Traditional culture-based approaches are still required to fill this gap in our knowledge (Dumack *et al.* 2016). We give, here, a useful hint that subsoil may be a so far neglected habitat for searching for new taxa showing specific adaptations.

Conclusion

Our study provides new insight into the community structure of soil Cercozoa at two soil depths using high-throughput sequencing. Even if, in our study, the relative abundance and richness decreased with increasing depth, a few families were more prevalent in the subsoil, suggesting they could be subsoil endemics. However, even though we could relate the presence of some groups to their lifestyle and feeding mode, we are still far from understanding their role in terrestrial ecosystems and which traits help some Cercozoa thrive in their respective habitats. Thus, our data can be the starting point of follow up studies to isolate representatives of the putative subsoil endemics and determine their physiology in greater details.

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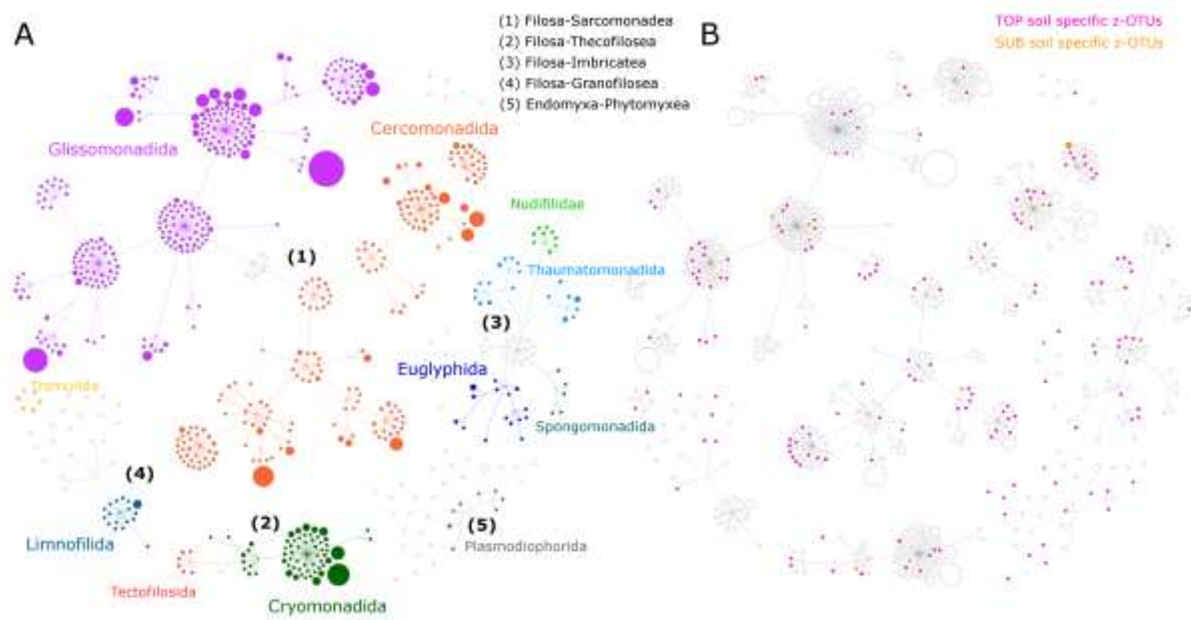

Figure 1. (A) Nodes correspond to z-OTUs and nodes size corresponds to their relative abundance in the dataset. Edges (lines connecting the nodes) represent the taxonomic path from class to z-OTU level, whereas z-OTUs are placed at the level of the lowest possible assignment. Nodes and edges were color-coded based on their assignment at the order level. Only orders represented by more than 0.1% of the total sequences were color-coded. Floating nodes were z-OTUs unclassified at the class level. Each class was identified by a number (1 to 5) **(B)** Same network, but only nodes were color-coded based: z-OTUs specific of the top soil are in pink and z-OTUs specific of the sub soil are in yellow.

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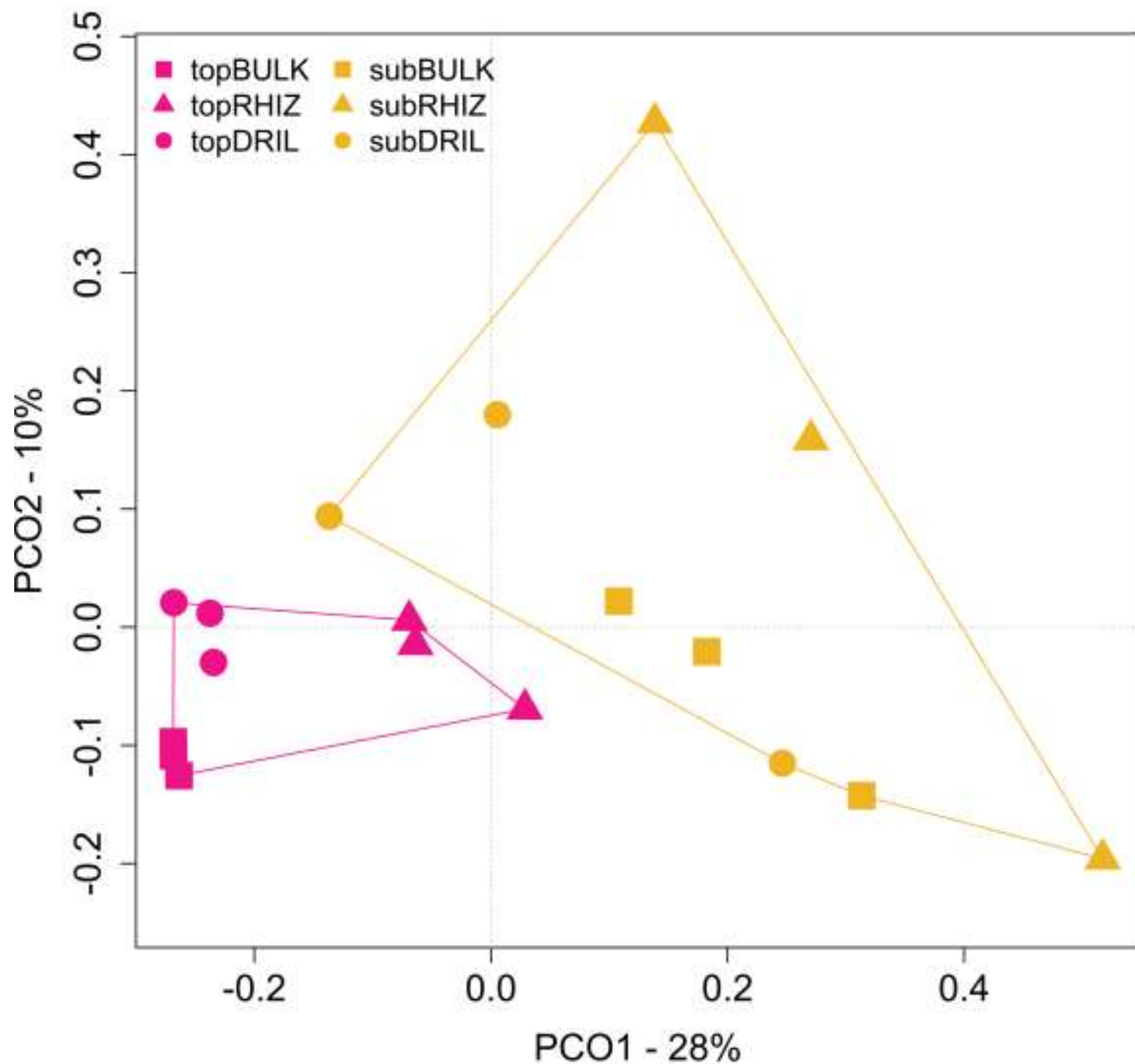

Figure 2. Principal coordinate analysis (PCO) based on Bray-Curtis distances calculated from standardized z-OTU abundances and showing dissimilarity in cercozoan community composition among samples (n=18). The first axis explained 28% of the total variance and the second axis explained 10 % of the total variance. The samples were first discriminated by the soil depth (top=top soil; sub=sub soil), then by the compartment (BULK=bulk soil; RHIZ=rhizosphere; DRIL=drilosphere).

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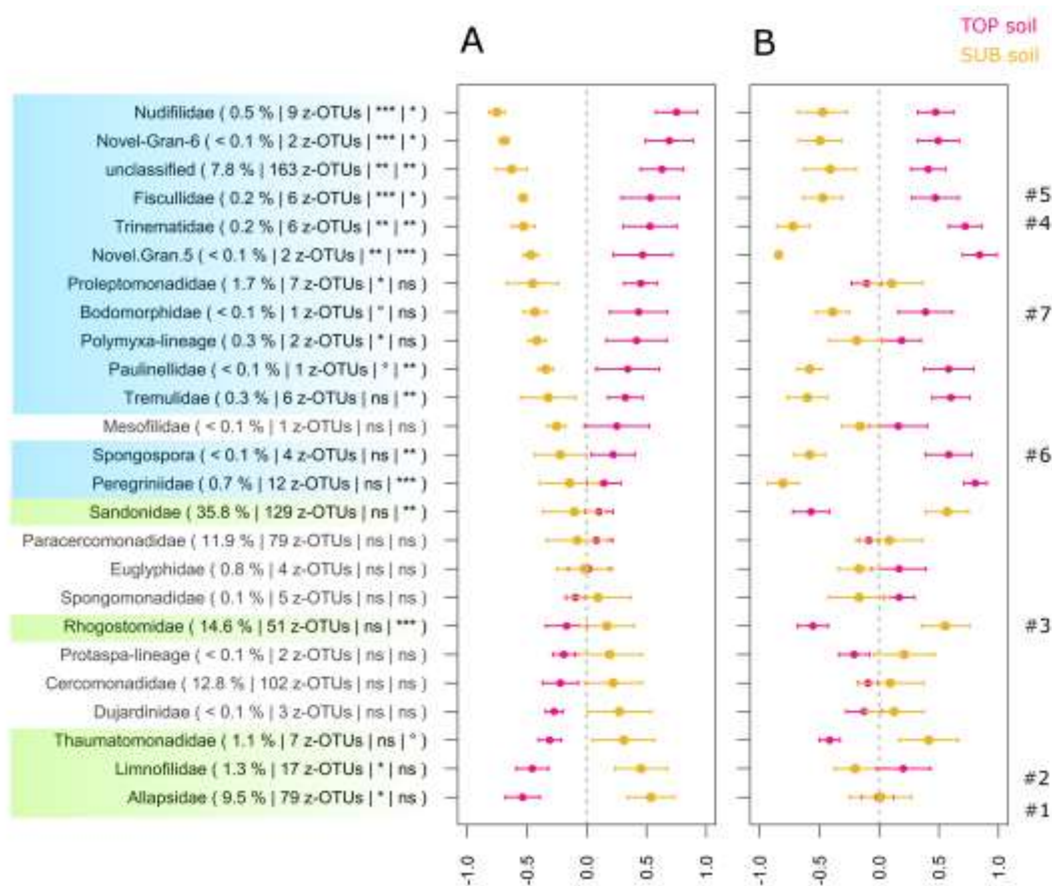

Figure 3. Data were scaled (mean=0, sd=1), representing values greater or smaller than the average across all samples. Values in brackets represent the total relative abundance (first argument), the total number of z-OTUs (second argument), the significance of the PERMANOVA test of the effect of depth on the relative abundance (third argument) and the significance of the PERMANOVA test of the effect of depth on the z-OTU richness (fourth argument). Significance level: $p<0.001$ (***); $p<0.01$ (**); $p<0.05$ (*); $p<0.1$ (°). When significant, the depth-specific response of each family is color-coded; in blue is a *decrease* of their relative abundance and/or richness with increasing depth, and in green is an *increase* of their relative abundance and/or the diversity with increasing depth. The # numbers identified the groups that are discussed in the main text (see Discussion).

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Table 1. Effect of depth and compartment on the richness and Shannon index (A). Mean and standard deviation of the richness and Shannon indexes in the three compartments in the top- and subsoil respectively (B).

	Richness		**Shannon index**	
	$F_{(x,y)}$ (p_{val})	**R^2**	**$F_{(x,y)}$ (p_{val})**	**R^2**
***Permanova*[A]**				
Depth	$52.785_{(1,14)}$ (p<0.001)	0.50	$52.790_{(1,14)}$ (p<0.001)	0.63
Compartment	$19.014_{(2,14)}$ (p<0.001)	0.36	$8.355_{(2,14)}$ (p<0.01)	0.20

***α-diversity*[B]**	**mean±sd**	**mean±sd**
topBULK	490±11[a]	5.06±0.05[a]
topDRIL	407±81[a]	4.81±0.12[a]
topRHIZ	214±21[b]	4.26±0.22[b]
subBULK	203±40[a]	3.55±0.31[a]
subDRIL	219±51[a]	4.03±0.48[a]
subRHIZ	93±43[b]	3.10±0.45[b]

(A) The permanova was performed using the function *adonis* (vegan package) with 99,999 permutations. Values are the Fratio (F), the statistical significance (p_{value}) and the coefficient of determination (R^2).

(B) Values represent the mean and standard deviation (sd) of richness and Shannon indexes calculated by group (Top=topsoil, sub=subsoil, BULK=bulk soil, DRIL=drilosphere, RHIZ=rhizosphere). The pairwise comparisons among the groups were calculated using the function *pairwise.t.test* with corrections for multiple testing (method: "BH"). Between two values, similar letters mean no difference, and different letters mean difference at $p_{val}<0.05$.

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