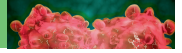


## BRIEF REPORT

## ENVIRONMENTAL MICROBIOLOGY



# The impact of fungi on soil protist communities in European cereal croplands

Florine Degruene<sup>1,2,3,4</sup> | Kenneth Dumack<sup>5,6</sup> | Masahiro Ryo<sup>7,8</sup> |  
 Gina Garland<sup>9,10,11</sup> | Sana Romdhane<sup>12</sup> | Aurélien Saghai<sup>13</sup> |  
 Samiran Banerjee<sup>14</sup> | Anna Edlinger<sup>9,15</sup> | Chantal Herzog<sup>9,16</sup> |  
 David S. Pescador<sup>17,18</sup> | Pablo García-Palacios<sup>19</sup> |  
 Anna Maria Fiore-Donno<sup>5,6</sup> | Michael Bonkowski<sup>5,6</sup> | Sara Hallin<sup>13</sup> |  
 Marcel G. A. van der Heijden<sup>9,16</sup> | Fernando T. Maestre<sup>20</sup> |  
 Laurent Philippot<sup>12</sup> | Michael Glemnitz<sup>7</sup> | Klaus Sieling<sup>21</sup> |  
 Matthias C. Rillig<sup>1,2</sup>

<sup>1</sup>Institute of Biology, Freie Universität Berlin, Berlin, Germany

<sup>2</sup>Berlin-Brandenburg Institute of Advanced Biodiversity Research, Berlin, Germany

<sup>3</sup>Eco&Sols, Univ Montpellier, CIRAD, INRAE, Institut Agro, IRD, Montpellier, France

<sup>4</sup>CIRAD, UMR Eco&Sols, Montpellier, France

<sup>5</sup>Terrestrial Ecology, Institute of Zoology, University of Cologne, Köln, Germany

<sup>6</sup>Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, Cologne, Germany

<sup>7</sup>Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, Germany

<sup>8</sup>Brandenburg University of Technology Cottbus–Senftenberg, Cottbus, Germany

<sup>9</sup>Agroscope, Plant-Soil Interactions Group, Zurich, Switzerland

<sup>10</sup>Agroscope, Soil Quality and Use Group, Zurich, Switzerland

<sup>11</sup>Department of Environmental System Sciences, Soil Resources Group, ETH Zurich, Zurich, Switzerland

<sup>12</sup>Department of Agroecology, University of Bourgogne Franche-Comté, INRAE, AgroSup Dijon, Dijon, France

<sup>13</sup>Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

<sup>14</sup>Department of Microbiological Sciences, North Dakota State University, Fargo, North Dakota, USA

<sup>15</sup>Wageningen Environmental Research, Wageningen University & Research, Wageningen, The Netherlands

<sup>16</sup>Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland

<sup>17</sup>Departamento de Farmacología, Farmacognosia y Botánica, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain

<sup>18</sup>Departamento de Biología y Geología, Física y Química Inorgánica, Escuela Superior de Ciencias Experimentales y Tecnología, Universidad Rey Juan Carlos, Móstoles, Spain

<sup>19</sup>Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, Madrid, Spain

<sup>20</sup>Environmental Sciences and Engineering, Biological and Environmental Science and Engineering Division, King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia

<sup>21</sup>Institute of Crop Science and Plant Breeding, Christian-Albrechts-University, Kiel, Germany

## Correspondence

Florine Degruene and Matthias C. Rillig,  
 Institute of Biology, Freie Universität Berlin,  
 Berlin, Germany.  
 Email: [florine.degrune@cirad.fr](mailto:florine.degrune@cirad.fr) and [rillig@zedat.fu-berlin.de](mailto:rillig@zedat.fu-berlin.de)

## Abstract

Protists, a crucial part of the soil food web, are increasingly acknowledged as significant influencers of nutrient cycling and plant performance in farmlands. While topographical and climatic factors are often considered to drive

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microbial communities on a continental scale, higher trophic levels like heterotrophic protists also rely on their food sources. In this context, bacterivores have received more attention than fungivores. Our study explored the connection between the community composition of protists (specifically Rhizaria and Cercozoa) and fungi across 156 cereal fields in Europe, spanning a latitudinal gradient of 3000 km. We employed a machine-learning approach to measure the significance of fungal communities in comparison to bacterial communities, soil abiotic factors, and climate as determinants of the Cercozoa community composition. Our findings indicate that climatic variables and fungal communities are the primary drivers of cercozoan communities, accounting for 70% of their community composition. Structural equation modelling (SEM) unveiled indirect climatic effects on the cercozoan communities through a change in the composition of the fungal communities. Our data also imply that fungivory might be more prevalent among protists than generally believed. This study uncovers a hidden facet of the soil food web, suggesting that the benefits of microbial diversity could be more effectively integrated into sustainable agriculture practices.

## INTRODUCTION

Despite being an integral part of the soil microbiome, heterotrophic protists (hereafter referred to as “protists”) have long been overlooked in ecological and environmental studies (Chandarana & Amaresan, 2022; Singer et al., 2021). Protists are abundant and diverse in soil ecosystems (Bonkowski et al., 2019), with densities ranging between  $10^4$  and  $10^8$  per gram of soil (Adl & Coleman, 2005). They occupy a key position in the soil food web, where they actively participate in the turnover of nutrients (Bonkowski & Clarholm, 2015) and other key soil processes (Geisen et al., 2021). More recent studies have emphasized their role as potential fungivores (Estermann et al., 2023; Geisen, 2016; Geisen et al., 2016), and bacterivorous protists are crucial in enhancing the plant-mycorrhiza symbiosis (Estermann et al., 2023). Soil protists are therefore increasingly associated with improved plant performance which makes them a key natural resource for agroecosystem productivity. Indeed, the management of croplands aimed at optimizing the benefits offered by soil protists requires a better understanding of the biotic and abiotic factors determining their diversity and community assembly across various climatic zones.

Previous studies conducted in natural ecosystems have revealed that soil protists are not randomly distributed, but instead exhibit predictable spatial patterns (Fenchel et al., 2019; Lara et al., 2016; Rozmoš et al., 2022), largely determined by climate and topography (Bates et al., 2013; Oliverio et al., 2020). Interestingly, Oliverio et al. (2020) detected co-large-scale occurrence patterns between protist and bacterial taxa, indicating that bacteria and protists may be interlinked across broad geographical scales; this may be explained by protistan-bacterial interactions or shared environmental preferences of specific taxa. Indeed, the community composition of bacteria emerged as an important driver of the community composition of soil protists in a large-scale

environmental survey of natural ecosystems (Seppey et al., 2020). This suggests that the drivers of the community assembly of soil protists are likely a combination of protistan–bacterial interactions and abiotic factors (soil, climate, and topography). This assumption is supported by two lines of evidence: (1) the existence of strong trophic interactions between protists and bacteria within the soil food web, and (2) a growing body of experimental work showing selective feeding of specific protistan taxa on bacterial prey (Nguyen et al., 2021). While protists are commonly considered bacterivores, there is increasing evidence of facultative fungivory (Amacker et al., 2022; Geisen et al., 2016), suggesting that protistan-fungal interactions may also contribute to shaping the community composition of protists (Dumack et al., 2018).

Here, we combined 18S rRNA gene metabarcoding to machine learning and structural equation modelling (SEM) to assess the importance of fungi relative to bacteria and abiotic factors (soil and climate) in predicting the community composition of soil Cercozoa in croplands across a 3000 km latitudinal gradient in Europe (Xiong et al., 2019). Cercozoa is a large phylum, in terms of species number and abundance in soil (Garland et al., 2021); it includes different feeding strategies (e.g. bacterivory and omnivory – intended as feeding on both bacteria and other eukaryotes but mostly fungi (Bass & Cavalier-Smith, 2004)) and can be specifically targeted in metabarcoding (Estermann et al., 2023), and the identified taxa can be functionally annotated (Fiore-Donno et al., 2017).

Accordingly, we hypothesize that Cercozoa and Fungi are strongly correlated across large geographical distances in croplands. While traditional factors such as soil, climate, and topographical features explain a major fraction of the community composition of Cercozoa, we assume that part of this spatial variability is explained by the fungal community. By examining the relationship between Cercozoa and Fungi along a large geographic gradient (3000 km), we aim to elucidate the



extent to which fungal communities serve as predictive indicators for the community composition of Cercozoa, a major group of microbial consumers in soil. Given the pivotal role of Cercozoa in soil ecosystems, this study aims to provide valuable insights into ecosystem dynamics and trophic interactions.

## EXPERIMENTAL PROCEDURES

### Site description, soil collection, and processing

A complete description of the sampling campaign is provided in Garland et al. (2021). Briefly, soil samples were collected across 156 cropland sites on a North–South gradient in Europe, including sites in Sweden ( $n = 31$ ), Germany ( $n = 36$ ), Switzerland ( $n = 38$ ), France ( $n = 29$ ), and Spain ( $n = 22$ ). To reduce variation between cropland sites as much as possible, we primarily targeted fields planted with wheat (*Triticum aestivum*) ( $n = 121$ , 78% of sites). When wheat fields were not available, another cereal was chosen instead (i.e., barley, *Hordeum vulgare* [ $n = 26$ ]; oat, *Avena sativa* [ $n = 6$ ]; rye, *Secale cereale* [ $n = 1$ ]; or triticale, *Triticosecale* sp. [ $n = 1$ ]). We sampled soils primarily from conventionally managed plots that engage in tillage and inorganic fertilization practices. Long-term precipitation and temperature information for each site was downloaded from the WorldClim database (<https://www.worldclim.org/>, last accessed: June 2019). This information was then used to calculate the mean annual precipitation (MAP) and mean annual temperature (MAT).

The soils were sampled during the flowering period of each crop, which ranged from May in the Southern sites to July in the Northern sites. At each site, soil samples were taken to a depth of 20 cm. A subset of samples was kept intact and used to measure bulk density and soil aggregation. The remaining soil cores were homogenized and sieved to 2 mm. A portion of this soil was air-dried for further processing of soil physical and chemical properties, a portion was kept refrigerated at 4°C for microbial biomass and a third portion was frozen at –20°C for DNA extraction and identification of bacterial, fungal and cercozoan OTUs. All samples were then shipped to a single location so each measurement could be made in the same laboratory, with the same equipment, and at the same time to minimize analytical variation.

### DNA extraction, amplification, sequencing, and data processing of cercozoan communities

In addition to the detailed description already available in Garland et al. (2021), we provide a brief description

of the protocols used for Cercozoa, as they are the main purpose of this study. The V4 region of the SSU/18S rRNA gene (c. 350 bp) was amplified by carrying out a two-step PCR using the specific primer sets (Estermann et al., 2023). The forward primers S616F\_Cerco (5'-TTAAAAGCTCGTAGTTG-3') and S616F\_Eocer (5'-TTAAAAGCGCGTAGTTG-3') were mixed in the proportions of 80% and 20%, and used with the reverse primer S963R\_Cerco (5'-CAACTTCGTTCTTGATTAAA-3'). In the second nested PCR (with 1  $\mu$ L of amplicon as the template), we used the same forward primer mix with the reverse primer S947R\_Cerco (5'-AAGAAGACATCCTTGGTG-3'). The resulting libraries were purified, 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 2  $\times$  300 bp paired-end reads.

The sequence data were processed using a customized pipeline largely based on USEARCH10 (v.11). The reads were merged using PEAR (Dumack et al., 2019), and primer sequences were trimmed using CUTADAPT allowing for one mismatch (Zhang et al., 2014). Error filtering was done using a maximum expected error of one and by using USEARCH fastq\_filter (Martin, 2011), and operational taxonomic units (OTUs) were clustered at 97% identity. On-the-fly denovo chimera and singletons were removed using UPARSE (Edgar & Flyvbjerg, 2015). Quality-filtered reads were mapped against the OTU centroid sequences using USEARCH otutab (maxrejects 0, maxaccepts 0, top\_hit\_only). For the taxonomic assignment, OTU centroid sequences were queried against PR2 (Edgar, 2013) using the naive Bayesian classifier implemented in Mothur (Guillou et al., 2013) and a minimum bootstrap support of 80%. Non-cercozoan sequences were removed from the dataset as well as low-abundance OTUs represented by <0.01% of the total sequences. A summary describing the sequencing details on the retrieved sequences and OTUs (Table S1) as well as the accumulation curves (Figure S1) and rarefaction curves (Figure S2) are provided in the Appendix S1.

## Statistical analyses

### Beta diversity and data analysis of soil parameters

Methods associated with all the data used in the present study are fully described in Garland et al. (2021). The community composition analysis was performed with the package vegan (version 2.5–6). All bacterial, fungal, and cercozoan OTU tables (provided in Garland et al., 2021) were standardized using Hellinger transformation (function *decostand*). Dissimilarities in



community composition (i.e., beta diversity) of Cercozoa, fungi and bacteria were examined using Principal Coordinate Analysis (PCoA) based on the Bray–Curtis dissimilarity index. The two first principal coordinates of the PCoA of the microbial groups were extracted and used in the statistical analysis (PCo1 and PCo2) as a measure of beta diversity. Dissimilarities in the community composition of Cercozoa and fungi between the 156 samples were displayed in an ordination plot (function *ordiplot*). The Bray–Curtis dissimilarity matrix was used in the distance–decay analysis to examine the spatial variation in community composition over increasing geographic distance. The soil abiotic parameters, measured as previously described (Garland et al., 2021) included soil pH, soil moisture (SM), clay content, total nitrogen ( $N_{\text{tot}}$ ), total phosphorus ( $P_{\text{tot}}$ ) and total carbon ( $C_{\text{tot}}$ ). Climatic variables included the mean average temperature (MAT) and precipitation (MAP) as described above. Latitude values were converted into geographic distance by multiplying individual values by 111,000 m, which is generally accepted as an approximation of 1° latitude. The computed geographic distances were used in the distance–decay analyses.

## Random forest analysis

To estimate the relative importance of the predictors, we used a random forest (RF) algorithm with permutation-based variable selection (Schloss et al., 2009), which offers an unbiased estimate of variable importance (Hapfelmeier & Ulm, 2013). The algorithm builds a non-parametric model by automatically finding nonlinear associations between predictors and the response variable, and it estimates variable importance even among highly correlated predictors (Hothorn et al., 2006; Strobl et al., 2008). The original R script used was that of (Schloss et al., 2009), which is based on the functions *ctree* and *cforest* as implemented in the R package “party”. The RF algorithm was applied to estimate the relative importance score ( $R^2$ -fitting) of the biotic and abiotic factors in predicting the community composition (i.e., beta diversity) of soil Cercozoa. We used the first (PCo1) and second (PCo2) principal coordinates to represent the dissimilarity in community composition in the RF model. In the RF model, latitude and longitude were included to control for spatial autocorrelation. We also assessed the direction of the effect of the individual predictors (positive or negative), represented with the sign (+/−) of each  $R^2$  score (e.g., −0.1 means that the factor explains 10% of the variability and the effect is negative). The generated  $p$ -values were corrected for the importance score of the individual predictors using a Bonferroni correction (Nicodemus et al., 2010). The hyper-parameters of the algorithm were set as follows:  $m\text{try} = 4.5$ ,  $n\text{tree} = 500$ , and  $\text{permutation} = 999$ .

## Structural equation modeling

We built an SEM to partition the direct versus indirect effects of abiotic factors in explaining the first principal coordinates (PCo1) of Cercozoa, using the function *lavaan* from the *lavaan* package (version 0.6.9). The model was built based on the output of the RF analysis. We considered the direct effects of the abiotic factors on the composition of Cercozoa. The first principal component of the PCoA analysis (see above) was used to describe the compositions of Cercozoa and fungi. All data were scaled prior to the analysis.

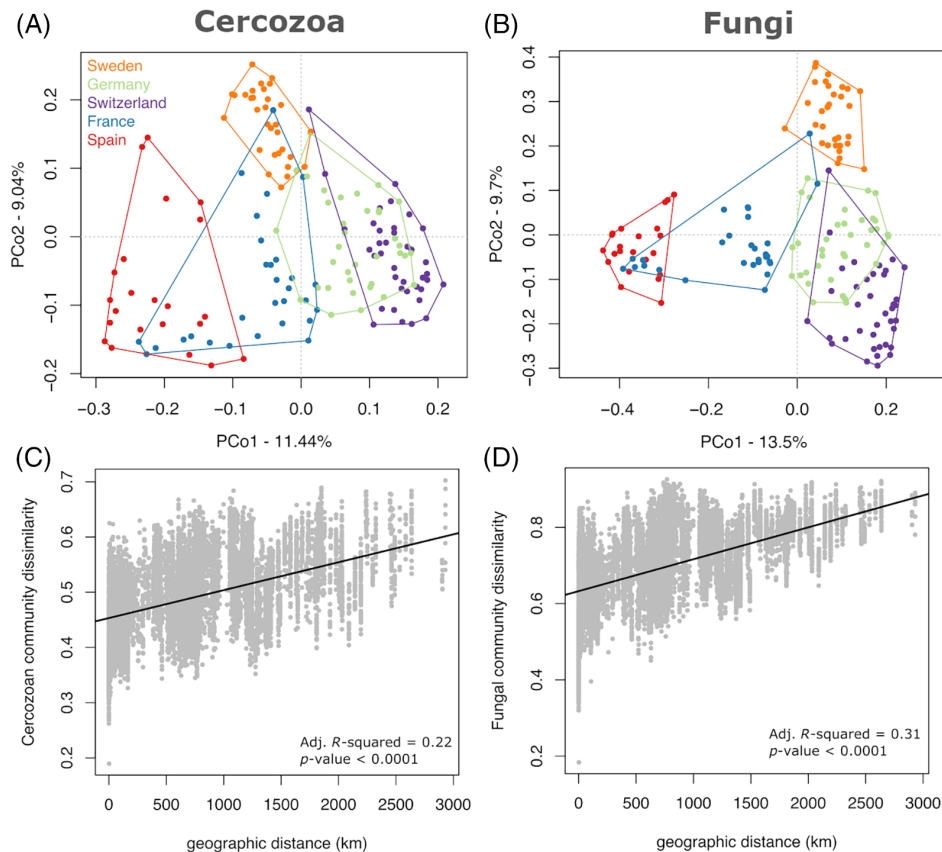
## Sparse partial least square regression analysis

To examine multiple correlations between different taxonomic groups (here fungal phyla and cercozoan families for which omnivory is known or suspected), we used the sparse partial least square (spls) regression method—a multivariate regression for performing simultaneous variable selection in two data sets—using the *spls* function (mode = “regression”,  $n\text{comp} = 2$ ) as implemented in the package *MixOmics* version 6.10.9 (Haynes, 2013). We used the function *perf* (validation = “Mfold”, folds = 10,  $n\text{repeat} = 50$ ) to evaluate the performance of the fitted spls and determined the  $n\text{comp}$  value from the *spls* function. All statistical analyses were performed using R version 4.3.3.

## RESULTS

The community composition (beta diversity) of both fungi and Cercozoa exhibited predictable spatial structure in the cereal fields across the 3000-km latitudinal gradient (Figure 1A,B). Increasing geographic distance was associated with increasing changes in the community composition of fungi and Cercozoa (Cercozoa: adjusted  $R^2 = 0.22$ ; Fungi: adjusted  $R^2 = 0.31$ , Figure 1C,D). More than 90% of the community composition of fungi and Cercozoa (represented by the two first principal coordinates PCo1 and PCo2) was determined by the abiotic and biotic environmental factors (RF analysis:  $R^2$ -fitting<sub>(cercozoa, PCo1)</sub> = 0.948;  $R^2$ -fitting<sub>(cercozoa, PCo2)</sub> = 0.963, Table S2).

The RF analysis showed that bacteria, fungi, soil pH and MAP were the major determinants of the community composition of Cercozoa (Figure 2A). About 50% of the first principal coordinate (PCo1) of Cercozoa was explained by bacteria (PCo2 bacteria:  $R^2 = 0.304$ ,  $p < 0.0001$ ) and fungi (PCo1 fungi:  $R^2 = 0.242$ ,  $p < 0.0001$ ), while 50% of the second principal coordinates (PCo2) of Cercozoa was explained by bacteria (PCo1 bacteria:  $R^2 = 0.33$ ,  $p < 0.0001$ ), soil pH ( $R^2 = -0.216$ ,  $p < 0.0001$ ) and MAP ( $R^2 = 0.116$ ,



**FIGURE 1** Beta diversity of Cercozoa and fungi across the 156 cereal cropping sites in Europe represented by the principal coordinate analysis (PCoA), showing the dissimilarity in the taxonomic community composition of (A) Cercozoa and (B) fungi across the North–South gradient. Distance decay analysis of the linear interaction between geographic distance and the community dissimilarity of (C) Cercozoa and (D) fungi. The values represent the adjusted  $R^2$  and  $p$ -value of the linear regression.

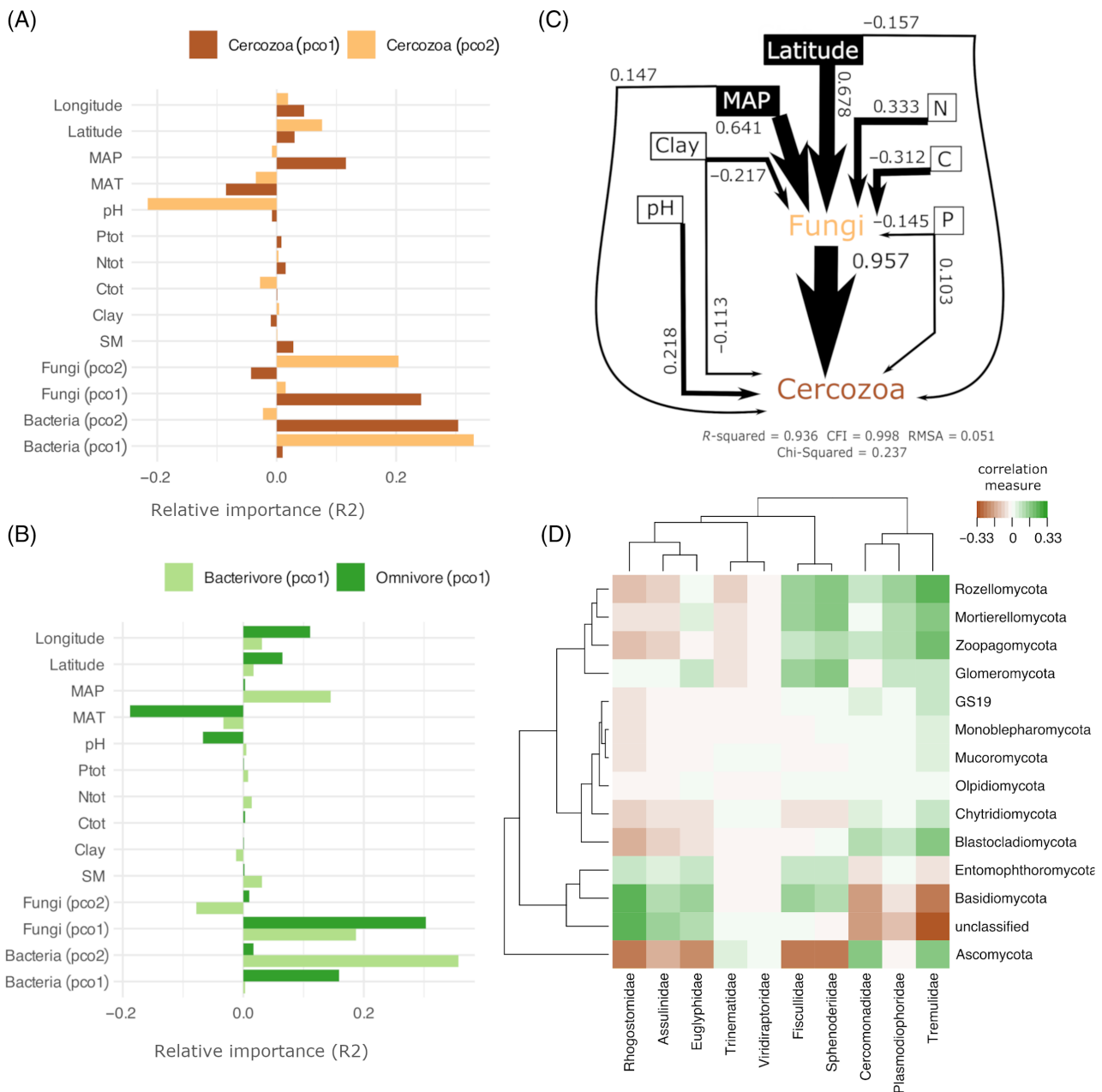
$p < 0.0001$ ). The other soil abiotic parameters were less important and explained only ca. 10% of the cercozoan PCo1 and PCo2 (Table S2). Altogether, our main finding supports our hypothesis that fungi are more determinant than thought in structuring the community composition of Cercozoa in cropland soils.

The RF analysis (Figure 2B) further showed that the community composition of Cercozoa classified as bacterivores was not only explained by bacteria (PCo2 bacteria:  $R^2 = 0.357$ ,  $p < 0.0001$ ) and abiotic factors such as MAP ( $R^2 = 0.145$ ,  $p < 0.0001$ ) but also well explained by the community composition of fungi (PCo1 fungi:  $R^2 = 0.187$ ,  $p < 0.0001$ ). For the community composition of Cercozoa classified as omnivores, the community composition of fungi was a major determinant (PCo1 fungi:  $R^2 = 0.303$ ,  $p < 0.0001$ ) followed by MAT ( $R^2 = -0.188$ ,  $p < 0.0001$ ) and bacteria (PCo1 bacteria:  $R^2 = 0.159$ ,  $p < 0.0001$ ). Here, we show the RF output for the PC1 of the two trophic groups. The RF analysis conducted on the PCo2 of bacterivores and omnivores is provided in Table S2.

We explored the variance partitioning between the first principal coordinate PCo1 of fungi and Cercozoa using an SEM analysis. The SEM analyses (Figure 2C

and Table S3) showed that a large proportion of the PCo1 of Cercozoa ( $R^2 = 0.936$ ) was explained by the model. The main outcomes were: (1) the path from fungi to Cercozoa showed the largest parameter value, and (2) fungal community composition mediated a large part of the abiotic effects on the community composition of Cercozoa. This is shown by the partitioning between direct and indirect paths of the abiotic factors in the SEM. Total N (model estimate = 0.333) and total C (model estimate = -0.312) were fully mediated by the compositional change in fungal communities, while MAP, clay content and total P were partly mediated by the community composition of fungi (Figure 2C and Table S3).

The sparse partial least square regression analysis (spls) reported correlations ranging between  $r = 0.248$  and  $r = -0.240$  (Figure 2D and Table S4). We show that almost all 10 selected cercozoan families (the one for which omnivory is known or suspected) were strongly linked with Basidiomycota and Ascomycota (Figure 2D), the two dominant fungal phyla in our dataset (Table S5). Among the cercozoan families, Rhogostomidae (the dominant taxa in our dataset, Table S6) showed a strong relationship with both Basidiomycota



**FIGURE 2** Relative importance ( $R^2$ ) of biotic and abiotic factors for predicting the community composition of Cercozoa (PCo1 and PCo2) (A) and of Cercozoa classified as bacterivores and omnivores (PCo1) (B). The orientation of the bars representing the  $R^2$  values indicates positive or negative variable associations. (C) SEM evaluating the direct and indirect effects of abiotic factors on the community composition of Cercozoa (PCo1). All shown paths are significant at  $p$ -value  $< 0.05$  (Table S3). (D) Output of the spls analysis showing correlations between the relative abundance of fungal phyla (used as predictors in the model) and cercozoan families (used as the response variable in the model). PCo1 and PCo2 are the first and second principal coordinates computed from the Bray–Curtis dissimilarity matrix.

( $r = 0.248$ ) and Ascomycota ( $r = -0.263$ ). Fisculidae ( $r = -0.240$ ), Euglyphidae ( $r = -0.207$ ) and Sphenoderiidae ( $r = -0.238$ ) were all strongly and negatively associated with Ascomycota, while Tremulidae ( $r = -0.238$ ) was strongly and negatively associated with Basidiomycota. The correlation values of the other cercozoan families were below 0.2 or above  $-0.2$  (Table S5).

## DISCUSSION

We provide novel insights into the potential importance of fungal communities as major drivers of the community composition of Cercozoa in cultivated soils and across a large climatic gradient in Europe. Our results indicate that fungi and Cercozoa are more strongly linked than previously assumed, even across large



geographical scales in cereal systems in Europe. Our findings extend our knowledge regarding the recent evidence on the contribution of biotic factors in modulating the community assembly of soil protists, as found by a study conducted in natural areas (Seppey et al., 2020).

Two hypotheses may explain the Cercozoa-Fungi link observed. Firstly, Cercozoa and fungi may share similar environmental preferences across the latitudinal gradient or follow the same spatial structure. Secondly, biotic interactions could be a key force structuring the community composition of Cercozoa in croplands. Our analysis indicates that it is likely not one or the other, but a combination of the two. This is particularly well supported in the SEM results: the Cercozoa-Fungi link is indeed explained by similar environmental responses and especially they both follow the same precipitation pattern. The correlative nature of our data does not allow robust attribution of causality, as is typical of observational studies in ecology. Nonetheless, our data strongly suggest that there is a link to explore between Cercozoa and fungi.

Only a limited number of observations of fungivorous protists have been conducted on obligate fungivores (e.g., 17) by (e.g., Amacker et al., 2022) which probably represents a small percentage of soil protists. The strong link we observed between fungi and omnivorous Cercozoa suggests that fungivory may be more widespread than assumed and that selective feeding on fungal communities may be an important structuring force of Cercozoan communities.

Our data support a recent environmental survey revealing the importance of protistan community composition and their top-down control exerted on the community composition of fungi (Rohart et al., 2017), probably by selective feeding (Dumack et al., 2018; Estermann et al., 2023). It is important to mention that our data based on a large-scale gradient can only suggest a direct link but not prove potential interactions. However, there is increasing experimental evidence of interactions between protists and fungi through facultative fungivory among protists. For example, our spms analysis detected a strong correlation between Rhogostomidae, a dominant family of Cercozoa in the environment (Huang et al., 2021), and the two most dominant fungal phyla of our dataset (Ascomycota and Basidiomycota). Our data also support current knowledge and suggest that their omnivory includes fungi besides bacteria.

Our SEM shows the importance of biotic drivers (represented by the fungal communities) in structuring the cercozoan communities, while the climatic and edaphic factors play a lesser or indirect role. This suggests that biotic interactions occur between Cercozoa and fungi and raises questions on the effects of climate change on the structure of the soil food web. Our capacity to model the response of ecosystems to climate change has to consider not only how the

communities will respond individually, but also how their interactions will respond to climate change (Öztoprak et al., 2020; Pescador et al., 2022). Here we show that not only bacteria but also fungi must be taken into account when designing more resilient agroecosystems supported by diverse microbial communities.

In conclusion, our results indicate that the community composition of Cercozoa in cereal systems across European croplands is more strongly determined by the community composition of fungi than by differences in the MAP and MAT. This indicates that protist-fungal interactions may be more important than previously thought in shaping the community composition of protists in agricultural systems. Experimental evidence using molecular approaches such as metatranscriptomics is needed to explore deeply how common facultative fungivory is among Cercozoa. The role of such microbial interactions within the soil food web in magnifying or buffering the effects of climate change on communities and their associated functions needs to be targeted in future research, especially now that food production in Europe is severely impacted by climatic change.

## AUTHOR CONTRIBUTIONS

**Florine Degruene:** Data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Kenneth Dumack:** Writing – review and editing (supporting). **Masahiro Ryo:** Formal analysis (supporting); methodology (supporting); writing – review and editing (supporting). **Gina Garland:** Data curation (equal); methodology (supporting); writing – review and editing (supporting). **Aurélien Saghai:** Data curation (equal); methodology (supporting); writing – review and editing (supporting). **Sana Romdhane:** Data curation (equal); writing – review and editing (supporting). **Samiran Banerjee:** Data curation (equal); writing – review and editing (supporting). **Anna Edlinger:** Writing – review and editing (supporting). **Chantal Herzog:** Writing – review and editing (supporting). **David S. Pescador:** Methodology (supporting); writing – review and editing (supporting). **Pablo García-Palacios:** Data curation (supporting); formal analysis (supporting); methodology (supporting); writing – review and editing (supporting). **Anna Maria Fiore-Donno:** Formal analysis (supporting); methodology (supporting); writing – review and editing (supporting). **Michael Bonkowski:** Writing – review and editing (supporting). **Sara Hallin:** Conceptualization (lead); funding acquisition (lead); writing – review and editing (supporting). **Marcel G. A. van der Heijden:** Conceptualization (lead); funding acquisition (lead); project administration (lead); writing – review and editing (supporting). **Fernando T. Maestre:** Conceptualization (lead); funding acquisition (lead); writing – review and



editing (supporting). **Laurent Philippot**: Conceptualization (lead); funding acquisition (lead); writing – review and editing (supporting). **Michael Glemnitz**: Writing – review and editing (supporting). **Klaus Sieling**: Writing – review and editing (supporting). **Matthias C. Rillig**: Conceptualization (lead); funding acquisition (lead); writing – review and editing (supporting).

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## CONFLICT OF INTEREST STATEMENT

None declared.

## DATA AVAILABILITY STATEMENT

Sequences are openly available at the European Nucleotide Archive (ENA) and can be obtained under the accession number PRJEB35080: <https://www.ebi.ac.uk/ena/browser/view/PRJEB35080>. All data and scripts are available at: [https://github.com/flopy007/Degrune\\_et\\_al\\_2024.git](https://github.com/flopy007/Degrune_et_al_2024.git).

## ORCID

**Florine Degrune** <https://orcid.org/0000-0002-3409-5859>

**Kenneth Dumack** <https://orcid.org/0000-0001-8798-0483>

**Masahiro Ryo** <https://orcid.org/0000-0002-5271-3446>

**Gina Garland** <https://orcid.org/0000-0002-1657-3669>

**Sana Romdhane** <https://orcid.org/0000-0003-0295-2278>

**Aurélien Saghai** <https://orcid.org/0000-0002-7069-2159>

**Samiran Banerjee** <https://orcid.org/0000-0002-1402-0171>

**Anna Edlinger** <https://orcid.org/0000-0003-3538-2371>

**Chantal Herzog** <https://orcid.org/0000-0002-3085-2015>

**David S. Pescador** <https://orcid.org/0000-0003-0395-9543>

**Pablo García-Palacios** <https://orcid.org/0000-0002-6367-4761>

**Anna Maria Fiore-Donno** <https://orcid.org/0000-0001-6265-1907>

**Michael Bonkowski** <https://orcid.org/0000-0003-2656-1183>

<https://orcid.org/0000-0002-9069-9024>

**Sara Hallin** <https://orcid.org/0000-0002-9069-9024>  
**Marcel G. A. van der Heijden** <https://orcid.org/0000-0001-7040-1924>

**Fernando T. Maestre** <https://orcid.org/0000-0002-7434-4856>

**Laurent Philippot** <https://orcid.org/0000-0003-3461-4492>

**Michael Glemnitz** <https://orcid.org/0000-0002-6506-1889>

**Klaus Sieling** <https://orcid.org/0000-0002-2151-2297>

**Matthias C. Rillig** <https://orcid.org/0000-0003-3541-7853>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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