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Research Article

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RESEARCH ARTICLE

Multitrophic interactions in the rhizosphere microbiome of wheat: from bacteria and fungi to protists

Maike Rossmann[1], Juan E. Pérez-Jaramillo[2], Vanessa N. Kavamura[3], Josiane B. Chiaramonte[1], Kenneth Dumack[4], Anna Maria Fiore-Donno[4,†], Lucas W. Mendes[5,‡], Márcia M. C. Ferreira[6], Michael Bonkowski[4], Jos M. Raaijmakers[2], Tim H. Mauchline[3] and Rodrigo Mendes[1,*,§]

[1]Laboratory of Environmental Microbiology, Embrapa Environment, Rodovia SP 340 km 125.5, 13918-110, Jaguariúna SP, Brazil, [2]Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW); Institute of Biology, Leiden University, Droevendaalsesteeg 10, 6708 PB, Wageningen, The Netherlands, [3]Sustainable Agriculture Sciences, West Common, AL5 2JQ, Harpenden, Rothamsted Research, UK, [4]Institute of Zoology & Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, Zülpicher Straße 47b, 50674 Köln, Germany, [5]Cell and Molecular Biology Laboratory, Center for Nuclear Energy in Agriculture CENA, University of Sao Paulo, Av. Centenário, 303, 13416-000, Piracicaba SP, Brazil and [6]Laboratory of Theoretical and Applied Chemometrics, Department of Chemistry, State University of Campinas (UNICAMP), Rua Josué de Castro, s/n, 13083-970, Campinas SP, Brazil

*Corresponding author: Laboratory of Environmental Microbiology, Embrapa Environment, Rodovia SP 340 km 125.5, 13918-110, Jaguariúna SP, Brazil. Tel: +55 (19) 3311 2755; Fax: +55 (19) 3311 2640; E-mail: rodrigo.mendes@embrapa.br

One sentence summary: Protists as a key factor in rhizosphere microbiome assembly was demonstrated in landraces and modern cultivars of wheat.

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†Anna Maria Fiore-Donno, http://orcid.org/0000-0001-6265-1907
‡Lucas W. Mendes, http://orcid.org/0000-0003-0980-7006
§Rodrigo Mendes, http://orcid.org/0000-0002-9817-4118

ABSTRACT

Plants modulate the soil microbiota by root exudation assembling a complex rhizosphere microbiome with organisms spanning different trophic levels. Here, we assessed the diversity of bacterial, fungal and cercozoan communities in landraces and modern varieties of wheat. The dominant taxa within each group were the bacterial phyla Proteobacteria, Actinobacteria and Acidobacteria; the fungi phyla Ascomycota, Chytridiomycota and Basidiomycota; and the Cercozoa classes Sarcomonadea, Thecofilosea and Imbricatea. We showed that microbial networks of the wheat landraces formed a more intricate network topology than that of modern wheat cultivars, suggesting that breeding selection resulted in a reduced ability to recruit specific microbes in the rhizosphere. The high connectedness of certain cercozoan taxa to bacteria and fungi indicated trophic network hierarchies where certain predators gain predominance over others. Positive correlations between protists and bacteria in landraces were preserved as a subset in cultivars as was the case for the

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Sarcomonadea class with Actinobacteria. The correlations between the microbiome structure and plant genotype observed in our results suggest the importance of top-down control by organisms of higher trophic levels as a key factor for understanding the drivers of microbiome community assembly in the rhizosphere.

Keywords: rhizosphere microbiome; microbiome assembly; plant-microbe interactions; 16S rRNA amplicon sequencing; 18S rRNA amplicon sequencing; ITS amplicon sequencing; protists

INTRODUCTION

Wheat cultivation occupies more land area than any other commercial crop and is the second most produced grain after maize, with more than 215 million hectares planted and 735 million tons of grains harvested annually (FAO 2020). More than 10 000 years ago, wheat (*Triticum* spp.) was one of the first domesticated food crops and played an important role in the transition from hunter-gatherers to farmers (Faris 2014). The earliest records of hexaploid wheat, *Triticum aestivum*, date from 8800 to 8400 years ago and originate from several areas including Can Hassan III in southern Turkey and Abu Hureyra in Syria (de Moulins 2000; Fairbairn *et al.* 2012).

Since then, domestication and subsequent improvement (diversification) have resulted in increased productivity of wheat and other crops (Preece *et al.* 2017). These processes dramatically changed the plant shape (Moose and Mumm 2008) and were accompanied with progressive alterations in the environment (i.e. habitat expansion) and in the crop management practices, with production systems highly dependent on the addition of external inputs (Matson *et al.* 1997; Milla *et al.* 2014). Studies have already shown that domestication and breeding influenced root architecture. Gioia *et al.* (2015) described the impact of domestication on shoot and root phenotypic architecture in tetraploid wheat. Pérez-Jaramillo *et al.* (2017) observed higher specific root length and reduced root density in wild common bean when compared to modern cultivars, suggesting that wild ancestors with their thinner roots could have been more efficient in the foraging and uptake of water (Comas *et al.* 2013).

However, plant domestication did not only affect root architecture, and, just recently, root-associated microbial populations has been considered. As example, Szoboszlay *et al.* (2015) confirmed a small but significant effect of plant genotype between rhizosphere communities of ancestral and domesticated varieties of corn. In barley, plant genotype exerted a strong effect on the root microbial communities when rhizosphere microbiomes of a modern variety, a landrace, and a wild genotype were compared (Bulgarelli *et al.* 2015). Similarly, bacterial populations associated with the rhizosphere of wild rice species differed from those associated with cultivars (Shenton *et al.* 2016). A recent study, recapitulating the breeding history of wheat, suggested that the effect of genotypes on the composition of their associated microbiota is an inherent factor to selection process (Tkacz *et al.* 2020). While most plant microbiome studies focus on bacterial and fungal communities (Franke-Whittle *et al.* 2015; Souza *et al.* 2016; Leff *et al.* 2017; Hartman *et al.* 2018), the rhizosphere microbiome also supports whole microbial food webs with organisms spanning different trophic levels (Mendes, Garbeva and Raaijmakers 2013). In this study, we included the Cercozoa, as an example of a higher trophic level. Cercozoa, along with Amoebozoa, are dominant soil protists (Urich *et al.* 2008; Bates *et al.* 2013; Burki and Keeling 2014; Geisen *et al.* 2015; Grossmann *et al.* 2016) with affinities for the rhizosphere and phyllosphere (Flues *et al.* 2018; Sapp *et al.* 2018). Environmental sequencing studies (Burki and Keeling 2014) have revealed thousands of lineages of which only a fraction, about 600, is yet described (Pawlowski *et al.* 2012). The diversity of this phylum is extensive, including nearly all trophic levels and thus ecological roles and a vast array of morphologies. Feeding strategies comprise heterotrophic free-living flagellates and amoebae (feeding on bacteria, fungi and smaller protists), autotrophic and parasites of plants, fungi, algae and oomycetes (Burki and Keeling 2014).

In this study, we investigated correlations between the microbiome structure and plant genotype to begin to understand the contribution of higher trophic groups, as exemplified by Cercozoa, to microbiome assembly in the wheat rhizosphere. For this purpose, we adopted the experimental strategy based on the ‘Back to the roots’ approach, proposed by Pérez-Jaramillo, Mendes and Raaijmakers (2016) and selected eight genotypes of wheat following the trajectory from ancient wild relatives to modern cultivars. As modern cultivars are genetically less diverse than their wild relatives, it has been hypothesized that modern cultivars may have lost some of the traits needed to recruit host-specific root microbes (Bulgarelli et al. 2013, 2015; Pérez-Jaramillo et al. 2017, 2018; Tkacz *et al.* 2020). This experimental framework associated with amplicon sequencing to determine the composition of bacterial, fungal and cercozoan communities allowed us to shed light on how plant breeding impacted microbial networks in the rhizosphere and on the role of Cercozoa on structuring the rhizosphere microbiome.

MATERIAL AND METHODS

Selection of wheat genotypes

In order to study a range of wheat genotypes belonging to the *Triticum aestivum* species, we selected three landraces, Karakilcik, Iran 1-29-11 334 and Pakintan 81 and five modern materials. Among the cultivars, three are cultivars of historical importance for Brazil, Frontana (released in 1940 and recommended for cultivation in Brazil for 48 years), BH 1146 (released in 1955 and recommended for 43 years) and IAC 5-Maringá (released in 1969 and characterized by high productivity) and two recently obtained cultivars, BRS Guamirim (released in 2005) and Quartzo (released in 2008) (Table S1, Supporting Information).

Soil used in the experiment

Soil samples were collected in the region of Assis municipality, São Paulo, Brazil from an agricultural field (22°55’45.36’S–50°07’22.33’W) and from a forest (22°55’35.50’S–50°07’38.59’W). Agricultural soil samples were collected in an area with a history of wheat cultivation, rotated with soybean, for more than 10 years. We also included in the study a soil covered by native forest collected in an adjacent area that shares the same physical properties with the agricultural soil. We used the forest soil as a control treatment to understand the impact of a soil with a contrasting microbial diversity on the rhizosphere microbiome

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assembly. Physical and chemical analyses of the soil was performed in the Soil Fertility Laboratory at the 'Luiz de Queiroz' College of Agriculture, University of Sao Paulo (ESALQ/USP), Piracicaba SP, Brazil (Table S2, Supporting Information). The soil was collected at a depth up to 20 cm, air dried and passed through a 2 mm mesh screen before use.

Plant experiment

Seeds of wheat were sterilized according to Robinson *et al.* (2016) and germinated on filter paper moistened with sterile deionized water in the dark for approximately 4–6 days (Page and Feller 2013). The seedlings were transferred to pots of 250 ml capacity and filled with approximately 220 g of soil. A completely randomized experimental design was used and included the control treatment (bulk soil), eight plant genotypes cultivated in agricultural and forest soils and four replicates. Each replicate was composed of a pot with two plants and pots without plants were used as control (bulk soil samples). The rhizosphere sampling was performed during the flowering stage (Zadok's scale) to synchronize microbiome analysis for all accessions with plant development. The experiment was conducted in a room under photoperiod of 16 h daylight and 8 h darkness. The minimum and maximum temperatures of the room during the experiment ranged from 20.7°C to 26.1°C.

For rhizosphere sampling, the whole root system was harvested by carefully removing the plants from pots and gently shaken to remove excess soil adhered to the root system. The rhizosphere soil was collected and stored in tubes of 2 ml capacity, immediately frozen in liquid nitrogen and kept at −80°C until the total DNA extraction. Samples of 1 g were collected to determine soil moisture.

DNA isolation and sequencing

DNA extractions were performed with the RNA PowerSoil® Total RNA Isolation Kit and the RNA PowerSoil® DNA Elution Accessory Kit (MO BIO – Carlsbad, California, USA), according to the manufacturer's instructions. DNA samples were further purified using the PowerClean® Pro DNA Clean-Up Kit (MO BIO), according to the manufacturer's instructions, in order to improve the quality of the downstream analyses.

Approximately 400 ng of DNA for each sample was sent to Novogene Bioinformatics Technology Co., Ltd (Leading Edge Genomic Services & Solutions) for amplification, library preparation and sequencing. The amplification of the V3-V4 hypervariable region of the 16S rRNA gene was performed using Phusion® High Fidelity PCR Master Mix (New England Biolabs – Ipswich, Massachusetts, USA) and the primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) (Yu *et al.* 2005), resulting in amplicons of 466 bp. The PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen—Hilden, Germany). The sequencing libraries were generated with the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina – San Diego, California, USA) following manufacturer's recommendations, followed by binding of adapters containing the indices. The library was sequenced on an Illumina HiSeq2500 platform (Illumina) and 250 bp paired-end reads were generated. The amplification of ITS1/ITS2 was performed using the same polymerase as above. Primers ITS5–1737F (GGAAGTAAAAGTCGTAACAAGG) and ITS2–2043R (GCTGCGTTCTTCATCGATGC) were used to amplify the ITS1 and primers ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) for ITS2 (White *et al.* 1990), resulting in amplicons of 307 bp and 386 bp, respectively. The PCR products were purified using the Qiagen Gel Extraction Kit, the libraries were generated with the TruSeq® DNA PCR-Free Sample Preparation Kit, followed by binding of adapters containing the indices, and sequenced on an Illumina HiSeq2500 platform as described for 16S rRNA sequencing.

For the cercozoan sequences, the primers design, barcoding primers, amplification, library preparation and Illumina sequencing were performed according to Fiore-Donno *et al.* (2018).

Sequencing data processing

For the ribosomal 16S rRNA gene, the RDP extension to PANDASeq (Masella *et al.* 2012), named Assembler (Cole *et al.* 2014), was used to merge paired-end reads with a minimum overlap of 10 bp and a Phred score of at least 30. Forward and reverse primer sequences were removed using Flexbar version 2.5 (Dodt *et al.* 2012). Next, sequences were trimmed based on sequence quality by running the Sickle tool (Joshi and Fass 2011) (minimum length is 25 and maximum length is 150) and converted to FASTA format and concatenated into a single file. All reads were clustered into OTUs using the UPARSE strategy of dereplication, sorting by abundance with at least two sequences and clustering using the UCLUST smallmem algorithm (Edgar 2010). These steps were performed with VSEARCH version 1.0.10 (Rognes *et al.* 2015). Next, chimeric sequences were detected using the UCHIME algorithm (Edgar *et al.* 2011) implemented in VSEARCH. Before the dereplication step all reads were mapped to OTUs using the usearch_global method implemented in VSEARCH to create an OTU table and converted to BIOM-Format 1.3.1 (McDonald *et al.* 2012). Finally, taxonomic information for each OTU was added to the BIOM file by using the RDP Classifier version 2.10 (Cole *et al.* 2014). All steps were implemented in a Snakemake workflow (Köster and Rahmann 2012). The OTU table was filtered using QIIME (1.9.1) custom scripts (Kuczynski *et al.* 2012). The bacteria reads were extracted using the command *split_otu_table_by_taxonomy.py* and singletons, doubletons and chloroplast sequences were discarded with the command *filter_otus_from_otu_table.py*, resulting in a filtered OTU table for further analysis.

The ITS sequence reads were classified using the UNITE database (Nilsson *et al.* 2018). A FASTA file was obtained containing the sequences of all species hypothesis (SH), including singletons. These referenced sequences were trimmed with ITSx to contain only the ITS2 region. Each OTU representative sequence was aligned with VSEARCH 1.11.1 using the usearch_global algorithm against the re-formatted UNITE database using only the top hits with at least 0.5 identity to the reference sequence (top_hits_only flag) and reporting also non-matching query sequences (output_no_hits flag). When multiple best hits were reported, the lowest common ancestor was determined using STAMPA (Mahé 2016). Finally, taxonomic information for each OTU was added to the BIOM file.

For the cercozoan sequences, paired reads were assembled using mothur v.3.7 (Schloss *et al.* 2009). This programme was also used in the following steps, allowing one difference in the primers, no differences in the barcodes, no ambiguities and removing assembled sequences < 100 nt and with an overlap < 100 bp. Reads were sorted by samples via detection of the barcodes. The quality check and removal/cutting of low-quality reads was conducted with the default parameters. Sequences were blasted using BLAST+ with an e-value of 1e-50 and sequences were identified in the PR2 database (Guillou *et al.* 2013) and noncercozoan sequences were removed. Chimeras were identified using UCHIME (Edgar *et al.* 2011) as implemented

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in mothur with a penalty for opening gaps of −5 and a template for aligning OTUs as previously described (Fiore-Donno *et al.* 2018). Sequences were clustered using vsearch v.1 (Rognes *et al.* 2016), with abundance-based greedy clustering (agc).

Diversity indices analyses

The alpha diversity was calculated using QIIME customs scripts. The command *alpha_rarefaction.py* was used to rarefy the OTU tables to counts up to 29 600, 19 500 and 6200 reads to bacterial, fungal and cercozoan gene sequences, respectively. This was the lowest sequencing depth obtained from a sample and therefore used as a threshold for rarefaction and alpha diversity calculations (Gotelli and Colwell 2001). The *alpha_diversity.py* command was applied to rarefied data and observed OTUs and Shannon and Chao1 metrics were obtained (Fig. S1, Supporting Information). One-way ANOVA and Tukey HSD were performed in R (R Core Team 2015). For the Beta-diversity calculations, the entire filtered OTU tables was used and normalized using the function cumNorm from the R package metagenomeSeq (v.1.12) (Paulson *et al.* 2016). For bacterial and fungal sequences, we used a cumulative-sum scaling method, which calculates the scaling factors equal to the sum of counts up to a particular quantile to normalize the read counts in order to avoid the biases generated with current sequencing technologies due to uneven sequencing depth (Paulson *et al.* 2013). For cercozoan sequences, the rarefaction was the most adequate normalization technique.

Ordination analyses

A Constrained Principal Coordinate Analysis (CAP) using phylogenetic groups, landraces (L1, L2 and L3) and cultivars (C1 to C5) as environmental factors, was calculated based on Bray–Curtis dissimilarity using the function capscale in Vegan package v.2.3–2 (Oksanen *et al.* 2016) implemented in the Phyloseq package v.1.10 (McMurdie and Holmes 2013) in R (R Core Team 2015).

Microbiome taxonomical composition analyses

In order to visualize the differential microbial community composition among treatments, we used the Statistical Analysis of Metagenome Profile software (STAMP) (Park, Hochholdinger and Gierl 2014). Comparison was based on *P*-values calculated using the two-sided Welch's t-test and correction was made using Benjamini–Hochberg false discovery rate (Benjamini and Hochberg 1995). For visualization, heatmaps were constructed based on z-score transformed Phylum/Class abundance, i.e. the deviation from row mean in units of standard deviations above or below the mean, to improve normality and homogeneity of the variances.

Core microbiome analyses

Venn diagrams were constructed in order to verify the proportion of OTUs that were exclusive or shared between samples using the webtool Venny 2.0.2 (Oliveros 2007). For core microbiome analyses, rarefied OTU tables (35 000 reads each sample) were used for both soils. The QIIME command *compute_core_microbiome.py* was applied in order to obtain a list of OTUs observed in all wheat rhizosphere samples. Only core OTUs with a relative abundance > 0.5% were included for graphical purposes. Pie and donut charts were built in R. The core microbiome was analyzed using QIIME with the minimum fraction of samples set at 100%.

Partial least squares discriminant analysis

We used the classification at the family level to compare microbiomes associated with landraces and cultivars of wheat using PLS-DA (Barker and Rayens 2003). PLS-DA models were performed using the PLS Toolbox® (version 6.5) from Eigenvector Technologies and Matlab.

Network analyses

Network analyses were performed to assess the complexity of the correlations among microbial taxa in the wheat rhizosphere. Non-random co-occurrence analyses were performed using SparCC, a tool capable of estimating correlation values from compositional data (Friedman and Alm 2012). For this, quality reads were clustered at 97% identity and an OTU table affiliated at family level was used for analysis. As we included Bacteria, Fungi and Cercozoa, in order to make classification comparable, the nodes in the reconstructed network represent taxa at family level, whereas the edges represent significantly positive or negative correlations between nodes. Considering the use of a high number of samples and data sets, the use of family level in our networks reduced the complexity and allowed us to obtain a clearer pattern (Faust and Raes 2012). For each network, *P*-values were obtained by 99 permutations of random selections of the data table, subjected to the same analytical pipeline. Statistically significant ($P < 0.01$) SparCC correlations with a magnitude of > 0.7 or < -0.7 were included into the network analyses.

The network graphs were made based on a set of measures, as number of nodes, number of edges, modularity, number of communities, average node connectivity, average path length, diameter and cumulative degree distribution. Co-occurrence analyses were carried out using the Python module 'SparCC', properties measurements were calculated with the interactive platform Gephi and networks visualization with Cytoscape (Bastian and Jacomy 2009). In addition, in order to test the correlation between the different communities, i.e. Bacteria, Fungi and Cercozoa, we conducted a Mantel test using R.

Bioinformatic analyses and data access

Statistical and bioinformatic analyses were performed according to Pérez-Jaramillo *et al.* (2017). Raw sequences of the bacterial 16S rRNA gene and fungal ITS have been deposited in MG-RAST (https://www.mg-rast.org/linkin.cgi?project = mgp88200), and the cercozoan 18S rRNA gene sequences have been deposited in GenBank BioProject PRJNA360862 under accession SRR5189947.

RESULTS

Rhizosphere microbiome assembly in agricultural and forest soils

There were clear differences in alpha diversity (Chao1, Shannon Index) of microbial communities between different soil types, with higher diversity of bacteria and (less pronounced) Cercozoa in agricultural soil, but no differences of alpha diversity between plant genotypes (Fig. S2, Supporting Information). Alpha diversity of fungi did not differ between treatments (Fig. S2, Supporting Information).

As for beta diversity, we found differences in the rhizosphere microbiome assembly between agricultural and forest soils (Fig.

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S3, Supporting Information). While soil type had a major influence on the composition of bacterial and cercozoan rhizosphere communities (R2 = 0.69 and 0.20, $p < 0.001$), it was less important for fungal community composition (R2 = 0.05, $p < 0.001$; Tables S3-S5, Supporting Information). Differential abundance analysis revealed which taxa are significantly more/less abundant in the rhizosphere of wheat cultivated in agricultural or forest soil (Fig. S4, Supporting Information). Eight bacterial phyla, three fungal phyla and four cercozoan classes were responsible for the differences between the microbiomes of the studied agricultural and forest soils (Fig. S4, Supporting Information). Acidobacteria, Bacteroidetes, Chloroflexi, Nitrospirae and Gemmatimonadetes were more abundant in agricultural soil while Actinobacteria, Verrucomicrobia and Firmicutes were more abundant in rhizosphere of plants cultivated in forest soil (Welch's two-sided test; $p< 0.05$; FDR corrected). Among fungal phyla, Ascomycota was more abundant in agricultural soil and Chytridiomycota and Basidiomycota in forest soil (Welch's two-sided test; $p < 0.05$; FDR corrected). Imbricatea, Thecofilosea, Sarcomonadea and Granofilosea were the most abundant cercozoan classes, with Imbricatea being more numerous in agricultural soil (Welch's two-sided test; $p < 0.05$; FDR corrected).

Genotype effect on rhizosphere microbiome assembly

Regardless of soil type or wheat genotypes, bacteria, fungi and cercozoa formed clearly distinct rhizosphere communities, which were always significantly differed from bulk soil communites ($P = 0.001$) (Fig. 1). In addition, the composition of rhizosphere microbiomes differed significantly between landraces and modern cultivars, especially considering bacterial (agricultural soil F = 2.2697, $P = 0.001$; forest soil F = 1.9874, $P = 0.001$) (Fig. 1A and B) and fungal communities (agricultural soil F = 1.5950, $P = 0.001$; forest soil F = 1.6785, $P = 0.001$) (Fig. 1C and D). For the cercozoan community, this genotype-dependent effect was more evident in agricultural soil (F = 2.4914, $P = 0.001$) than in the forest soil (F = 2.0958, $P = 0.003$), where protist communities of some modern cultivars clustered with landraces (Fig. 1E and F).

Differences in rhizosphere bacterial community assembly

The bacterial communities were dominated by representatives of Proteobacteria (37.5%), Actinobacteria (29.1%) and Acidobacteria (12.0%) (Fig. S5, Supporting Information). Overall, considering the phylum level, the abundance patterns bacteria, fungi and Cercozoa differed between agricultural and forest soils and also between landraces and cultivars (Fig. 2). The difference between wheat genotypes was further confirmed by the higher percentage of unique OTUs found in the microbiomes of landraces (21%) compared to microbiomes of modern cultivars (1%) (Fig. S6, Supporting Information). For example, considering relative abundance, in forest soil eight bacterial families discriminated landraces and cultivars and seven of them (Bdellovibrionaceae, Comamonadaceae, Peptostreptococcaceae, Sterptococcaceae, Xanthomonadaceae and unclassified Rickettsiales and Xanthomonadales) were more abundant or exclusively found in the rhizosphere of landraces (Table 1). Peptostreptococcaceae was the only bacterial family differentiating landraces and cultivars in both soil types.

The bacterial core microbiome of landrace genotypes included seven characteristic genera, *Acidibacter* (relative abundance = 0.52%), *Bryobacter* (0.57%), *Candidatus Solibacter* (0.55%), *Haliangium* (0.50%), *Mesorhizobium* (0.57%), *Phenylobacterium* (0.53%) and *Shinella* (0.67%), not occurring in the core microbiome of the wheat cultivars. On the other hand, only the genus *Pseudonocardia* (0.53%) was exclusively found in the core microbiome of wheat cultivars (Fig. 3A).

Differences in rhizosphere fungal community assembly

The fungal communities were dominated by representatives of Ascomycota (91.0%), Chytridiomycota (3.7%) and Basidiomycota (2.1%) (Fig. S5, Supporting Information). The phylum Ascomycota was more abundant in the rhizosphere of wheat cultivars (Welch's t-test; $P = 0.033$; Benjamini–Hochberg corrected) and Chytridiomycota in landraces (Welch's t-test; $P = 0.049$; Benjamini–Hochberg corrected) of agricultural soil (Fig. 2). Also in fungi, the microbiome of landraces supported a higher percentage of unique OTUs (17%) compared to cultivars (3%) (Fig. S6, Supporting Information). For example, five of six fungal families in total that differentiated rhizosphere communities of landraces and cultivars in agricultural soil are more abundant or exclusively found in landraces (Ajellomycetaceae, Bolbitiaceae, Gigasporaceae, Inocybaceae and Mortierellaceae) (Table 1). In forest soil, the differences between landraces and cultivars were explained by the relative abundance of three fungal families in cultivars (Hydnodontaceae, Leptosphaeriaceae and unclassified Diaporthales) and two families in landraces (Pleomassariaceae and unclassified Mortierellales) (Table 1). Members of the order Mortirellales were more abundant or exclusively found in association with landraces regardless of soil type. For the fungal core microbiome, the genus *Metarhizium* (relative abundance = 0.53%) was found only in the cultivar microbiome (Fig. 3B).

Differences in rhizosphere cercozoan community assembly

Overall, cercozoan communities were dominated by representatives of Sarcomonadea (42.3%), followed by Thecofilosea (27.1%) and Imbricatea (19.2%) (Fig. S5, Supporting Information). In forest soil, Sarcomonadea was more abundant in the rhizosphere of cultivars than in landraces (Welch's t-test; $P = 8.5E\text{-}05$; Benjamini–Hochberg corrected), while Skiomonadea was more abundant in landraces (Welch's t-test; $P = 0.018$; Benjamini–Hochberg corrected) (Fig. 2). Interestingly, in agricultural soil, although Imbricatea (Welch's t-test; $P = 0.021$; Benjamini-Hochberg corrected), a group of unclassified Filosa.I (Welch's t-test; $P = 0.028$; Benjamini-Hochberg corrected) and a group of unclassified Cercozoa (Welch's t-test; $P = 0.005$; Benjamini–Hochberg corrected) were significantly enriched in the rhizosphere as compared to bulk soil, no difference was observed between communities of landraces and cultivars (Fig. 2).

Strikingly, microbiome communities of cultivars consisted only of common OTUs that were found in all treatments, while in the landraces a high percentage of unique OTUs occurred (53%) (Fig. S6, Supporting Information). Cercozoan taxa that differentiated landraces and cultivars in agricultural soil included four cercozoan families out of five in total (Limnofilidae, Protaspididae, Thaumatomonadidae and unclassified Cryomonadida) (Table 1), and in forest soil five out of seven families (Rhogostomidae, Mesofilidae, unclassified Cercozoa, unclassified Imbricatea and unclassified Tectofilosida) (Table 1). At the genus level, *Eocercomonas* (relative abundance = 10.1%) was the only cercozoan genus exclusively found in the rhizosphere of cultivars (Fig. 3C).

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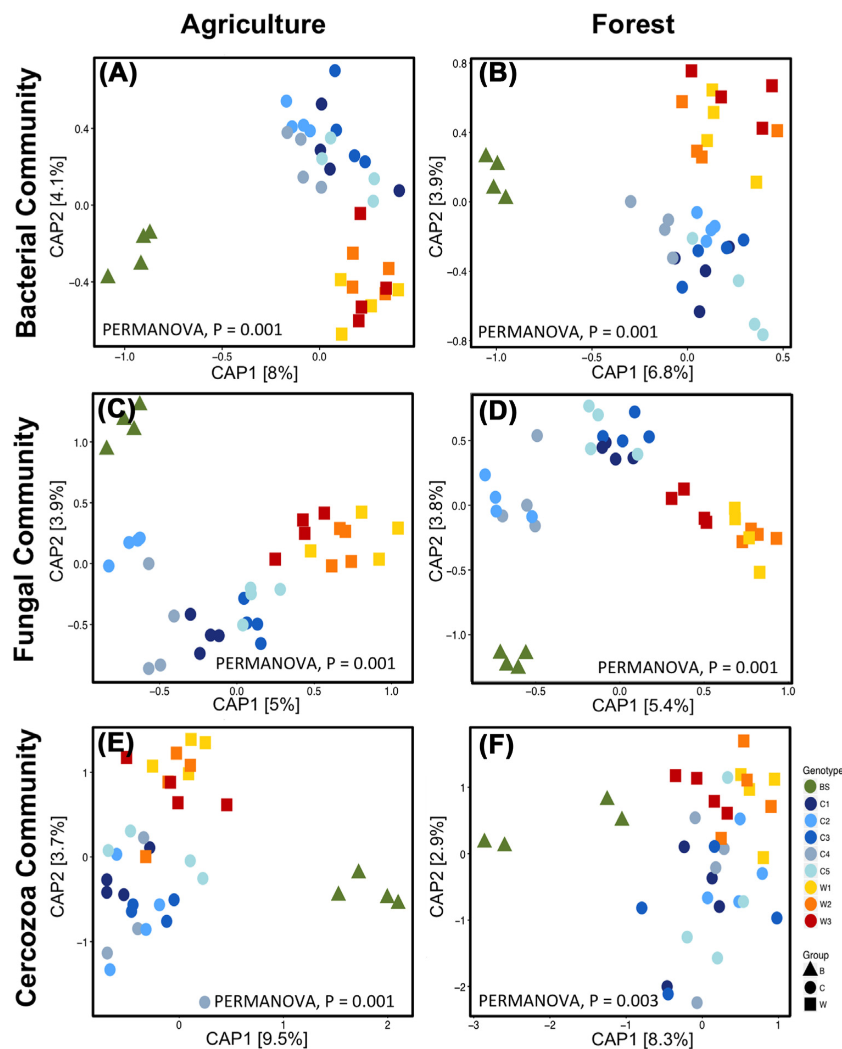

Figure 1. Microbiome community structure in wheat rhizosphere. Constrained Analysis of Principal Coordinates (CAP) of bacterial diversity (16S rRNA), fungal diversity (ITS1/ITS2) and cercozoan diversity (18S rRNA) in the rhizosphere of the three landraces (L1–3), five modern cultivars (C1–5) and bulk soil (BS), which was used as control. Statistical significance of the constrained analysis was assessed by Permanova, P = 0.001 for all presented data. Bacterial communities in agricultural land **(A)**; Bacterial communities in forest soil **(B)**; Fungal communities in agricultural soil **(C)**; Fungal communities in forest soil **(D)**; Cercozoan communities in agricultural soil **(E)**; Cercozoan communities in forest soil **(F)**; Iran 1-29-11 334 (L1); Karakilcik (L2); Pakintan 81 (L3); BH1146 (C1); Frontana (C2); IAC 5-Maringá (C3); BRS Guamirim (C4); Quartzo (C5). Triangles correspond to bulk soil, squares to landraces (warm colors) and circles to modern cultivars (cool colors).

Network structure of the rhizosphere microbiome

The rhizosphere microbiome of the landrace genotypes showed the highest level of complexity in both soil types (Fig. 4). The network of landraces consisted of 223 nodes and 1869 correlations (edges) in agricultural soil, while networks of forest soil contained 221 nodes and 1162 edges. The networks of landraces had the highest number of connections per node (average degree = 16.762 and 10.516 in agricultural and forest soils, respectively), average network distance between all pairs of nodes (average path length = 2.617 and 2.698 in agricultural and forest soils, respectively) and average clustering coefficient (0.212 and 0.167 in agricultural and forest soils, respectively) (Table S6, Supporting Information). Networks of landraces had the lowest number of modules in agricultural soil but highest in forest soil when compared to modern cultivars. In order to avoid potential biases due to a comparison of unequal numbers of accessions, we tested the robustness of network topology by reconstructing networks by using five subsets of three randomly selected cultivars and compared these with landraces. The results obtained confirm our results obtained with all five cultivars, i.e. microbial networks of landraces show a significantly higher level of complexity than the microbial networks of modern cultivars (Table S7, Supporting Information).

Centrality indices are based on shortest paths distance within graphs and indicate the most important nodes, which may be interpreted as key taxa inside a connected community (Borgatti 2005). The three nodes with highest betweenness centrality in networks of landraces in agricultural soil were a bacterium in the phylum Gemmatimonadetes, and two fungi in the families Cordycipitaceae (Ascomycota), and Pleosporaceae (Ascomycota), respectively. In landraces of the forest soil, the three nodes with highest betweenness centrality were two bacteria in the orders Xanthomonadales (Proteobacteria) and Solirubrobacterales (Actinobacteria), respectively and a fungus belonging to the family Coniochaetaceae (Ascomycota).

In modern cultivars, the three nodes with highest betweenness centrality in networks of agricultural soil were represented by an arbuscular mycorrhizal fungus in Glomeraceae

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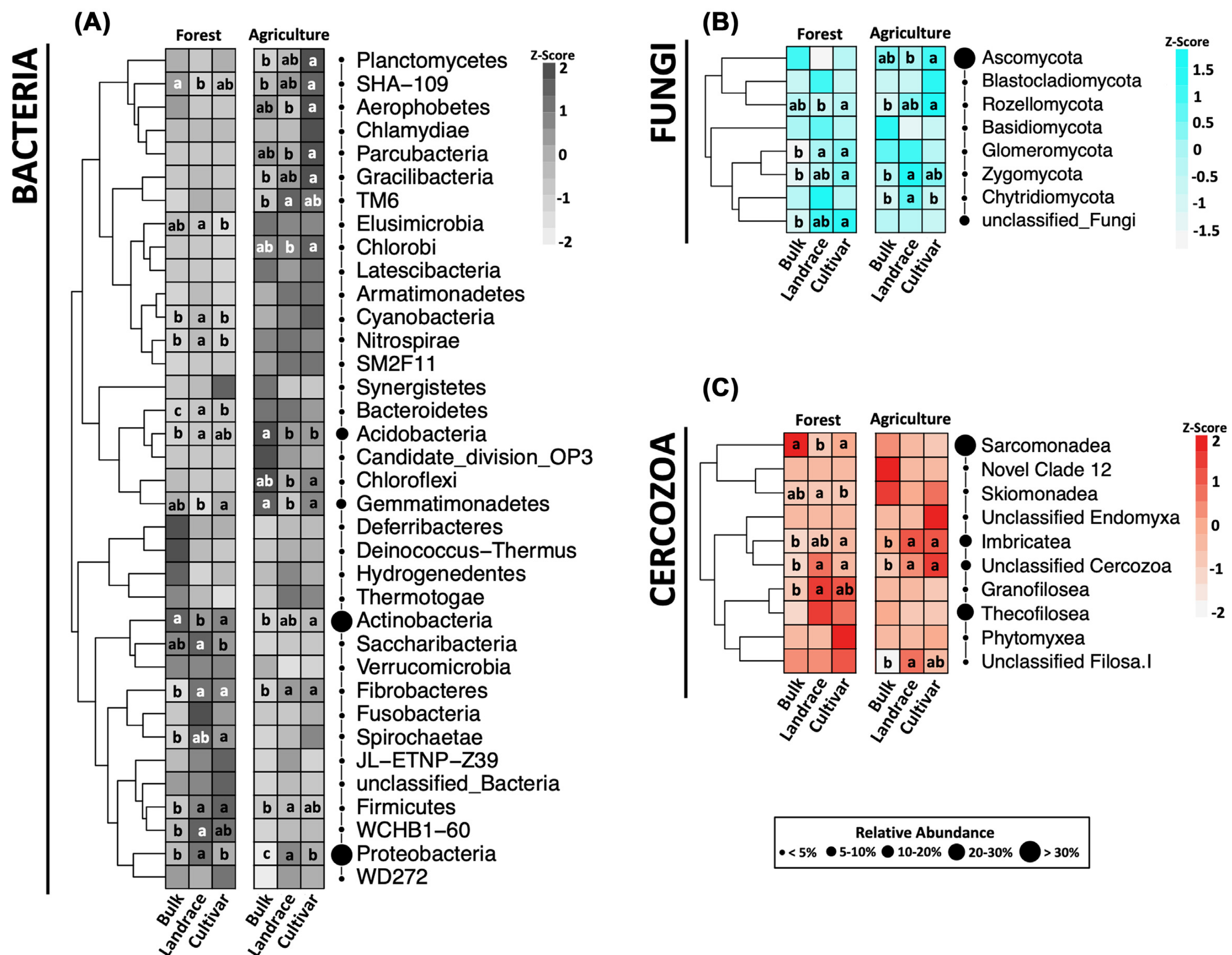

Figure 2. Heatmaps showing the differential abundance of **(A)** bacterial phyla, **(B)** fungal phyla and **(C)** cercozoan classes from bulk soil and rhizosphere of wheat landraces and cultivars grown in agricultural and forest soil. The color key relates the heatmap colors to the standard score (z-score), i.e. the deviation from row mean in units of standard deviations above or below the mean. Different lower case letters refer to significant differences between the treatments in the same soil based on Welch's t-test with Benjamini-Hochberg correction ($P < 0.05$). Relative abundance of microbial taxa is indicated by the size of the black circles.

(Ascomycota), a fungus of the family Ophiocordycipitaceae (Ascomycota), and a bacterium in the order Sphingomonadales (Proteobacteria). Considering the forest soil, the first three nodes with highest betweenness centrality in modern cultivars were occupied by two Proteobacteria, in the orders Sphingomonadales and Rhodospirillales, and a fungus in the family Clavicipitaceae (Ascomycota).

Considering the number of potential trophic interactions between cercozoans and bacteria, cercozoans and fungi, and correlations among cercozoans, landraces showed the greatest number of correlations, especially between fungi and cercozoa. However, modern cultivars displayed a greater number of correlations between bacteria and cercozoans (Tables S8-S11, Supporting Information).

DISCUSSION

Landraces and cultivars assemble different rhizosphere microbiomes

As demonstrated in previous studies, our results confirmed that the composition of bacterial and fungal communities strongly differed between landraces and cultivars. Pérez-Jaramillo *et al.* (2018) and other studies (Aleklett *et al.* 2015; Shi *et al.* 2015), showed that plant domestication resulted in a similar overall taxonomic shift in the prokaryotic root microbiome with a reduced abundance of the Bacteroidetes phylum on modern accessions and a increase in members of the Actinobacteria. Furthermore, we demonstrate for the first time that communities of heterotrophic unicellular eukaryotes resident at higher trophic levels in the root microbiome, as exemplified by the Cercozoa, are similarly affected by the crop breeding process. This is surprising, as most studies to date have emphasized the influence of protists on the composition of plant-associated bacterial and fungal communities (Rosenberg *et al.* 2009; Jousset *et al.* 2010; Müller, Scheu and Jousset 2013; Geisen *et al.* 2016; Weidner *et al.* 2016; Flues, Bass and Bonkowski 2017; Thakur and Geisen 2019). Our data in contrast suggest a structuring effect of the rhizosphere prey on their protistan consumers as shown for the model plant *Arabidopsis thaliana* (Sapp *et al.* 2018). In extension of the results by Sapp *et al.* (2017), our data show that rhizosphere-associated communities of cercozoa were not only plant species-specific but even genotype specific in wheat. Although the data obtained (observed correlations) point to

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Table 1. Identification of bacterial, fungal and cercozoan families responsible by discrimination between landraces and cultivars revealed by using PLS-DA permutation tests.

Agricultural soil			Forest soil		
	Taxa	r (x-y)		Taxa	r (x-y)
Bacterial families*					
L	Clostridiaceae	– 0.59	L	Bdellovibrionaceae	– 0.52
L	Coxiellaceae	– 0.50	L	Comamonadaceae	– 0.61
L	Peptostreptococcaceae	– 0.53	L	Peptostreptococcaceae	– 0.50
C	Kallotenuaceae	0.41	L	Sterptococcaceae	– 0.51
C	unclas_Thermomicrobia	0.59	L	unclas_Rickettsiales	– 0.56
			L	unclas_Xanthomonadales	– 0.51
			L	Xanthomonadaceae	– 0.61
			C	Gemmatimonadaceae	0.58
Fungal families*					
L	Ajellomycetaceae	– 0.57	L	Pleomassariaceae	– 0.37
L	Bolbitiaceae	– 0.42	L	unclas_Mortierellales	– 0.36
L	Gigasporaceae	– 0.46	C	Hydnodontaceae	0.33
L	Inocybaceae	– 0.42	C	Leptosphaeriaceae	0.43
L	Mortierellaceae	– 0.46	C	unclas_Diaporthales	0.32
C	unclas_Saccharomycetales	0.41			
Cercozoan families**					
L	Limnofilidae	– 0.30	L	Rhogostomidae	– 0.24
L	Protaspididae	– 0.37	L	Mesofilidae	– 0.46
L	Thaumatomonadidae	– 0.28	L	unclas_Cercozoa	– 0.39
L	unclas_Cryomonadida	– 0.25	L	unclas_Imbricatea	– 0.26
C	unclas_Cercozoa	0.30	L	unclas_Tectofilosida	– 0.32
			C	Bodomorphidae	0.26
			C	unclas_Euglyphida	0.21

*The variables were selected considering the correlation coefficient 'y' greater than 0.6 and, on average, higher than 0.7.
**The variables were selected considering the correlation coefficient 'y' greater than 0.4 and, on average, higher than 0.65.
L: families differentially more abundant or exclusive in landraces.
C: families differentially more abundant or exclusive in modern cultivars.

the described patterns, experimental validations are needed for conclusive evidence in this aspect.

In general, the microbiome of landraces harbored a core microbiome with a higher number of exclusive genera and most of the families responsible for the rhizosphere microbiome differentiation between landraces and modern cultivars.

Based on these correlations, the results suggest that modern cultivars have an altered ability to recruit specific microbes than their wild relatives. Considering that over time these changes in the host were accompanied by transitions to new environments and alterations in management practices, it is interesting to note that despite the land-use change of soils from forest to agriculture, landraces and cultivars were still able to recruit rhizosphere microbiomes over 80% identical at the OTU level. This stability in recruiting the core microbiome was observed when synthetic bacterial communities were used to recolonize *Arabidopsis*, as the bacterial assembly resembles the plant natural microbiota (Bai *et al.* 2015). Interestingly, *Fusarium*, which is a fungal genus comprising several plant pathogens (Beckman 1987), represented an important portion of the wheat core microbiome in landraces and modern cultivars. Nevertheless, we did not observe disease symptoms caused by *Fusarium*, which can be explained by the presence of weakly virulent or non-pathogenic species of *Fusarium* on wheat (Smiley and Patterson 1996; Gebremariam *et al.* 2017).

As expected, a clear separation between rhizosphere soil and bulk soil microbiomes was observed. This result reinforces that microbiome composition in the rhizosphere is defined by the selective pressure exerted mainly by root exudates and rhizodeposition (Badri *et al.* 2013; Lakshmanan, Selvaraj and Bais 2014). Members of the microbiome, inhabit the rhizosphere, being attracted by and feeding on rhizodeposits of their chosen host plants (Philippot *et al.* 2013). Beside that, different growth rates, substrate utilization spectra and competitive abilities of the different microbial groups further differentiate rhizosphere community assembly processes (Pérez-Jaramillo *et al.* 2017). Recent studies have described the rhizosphere effect on protists. Asiloglu and Murase (2016, 2017) described how newly developed rice roots were rapidly and densely populated by specific protist taxa, with a clear boundary between rhizosphere and non-rhizosphere areas, but it was still unclear whether this was linked to rhizosphere gradients of oxygen availability in the paddy soils or resource supply from roots. Their results support a metatranscriptomics study of Turner *et al.* (2013) who identified plant species-specific protists in Amoebozoa and Alveolata in the rhizospheres of pea, wheat and oat. Sapp *et al.* (2017) finally confirmed specific cercozoan rhizosphere communities of *Arabidopsis thaliana*.

It is well known that plants can modulate the microbiome assembly in the rhizosphere (Park, Hochholdinger and Gierl 2004; Hartmann *et al.* 2009; Cesco *et al.* 2010). Besides functioning as substrates for microbial growth, root exudates contain signaling molecules, microbial attractants, stimulants and also inhibitors or repellents (Baetz and Martinoia 2014). In addition, bacterivorous protists have a significant impact on the availability of mineral N and P in the rhizosphere of plants (Trap *et al.* 2016) and their presence drastically changes the composition of the soil microbial community (Rosenberg *et al.* 2009; Bonkowski, Koller and Jousset 2011; Koller *et al.* 2013).

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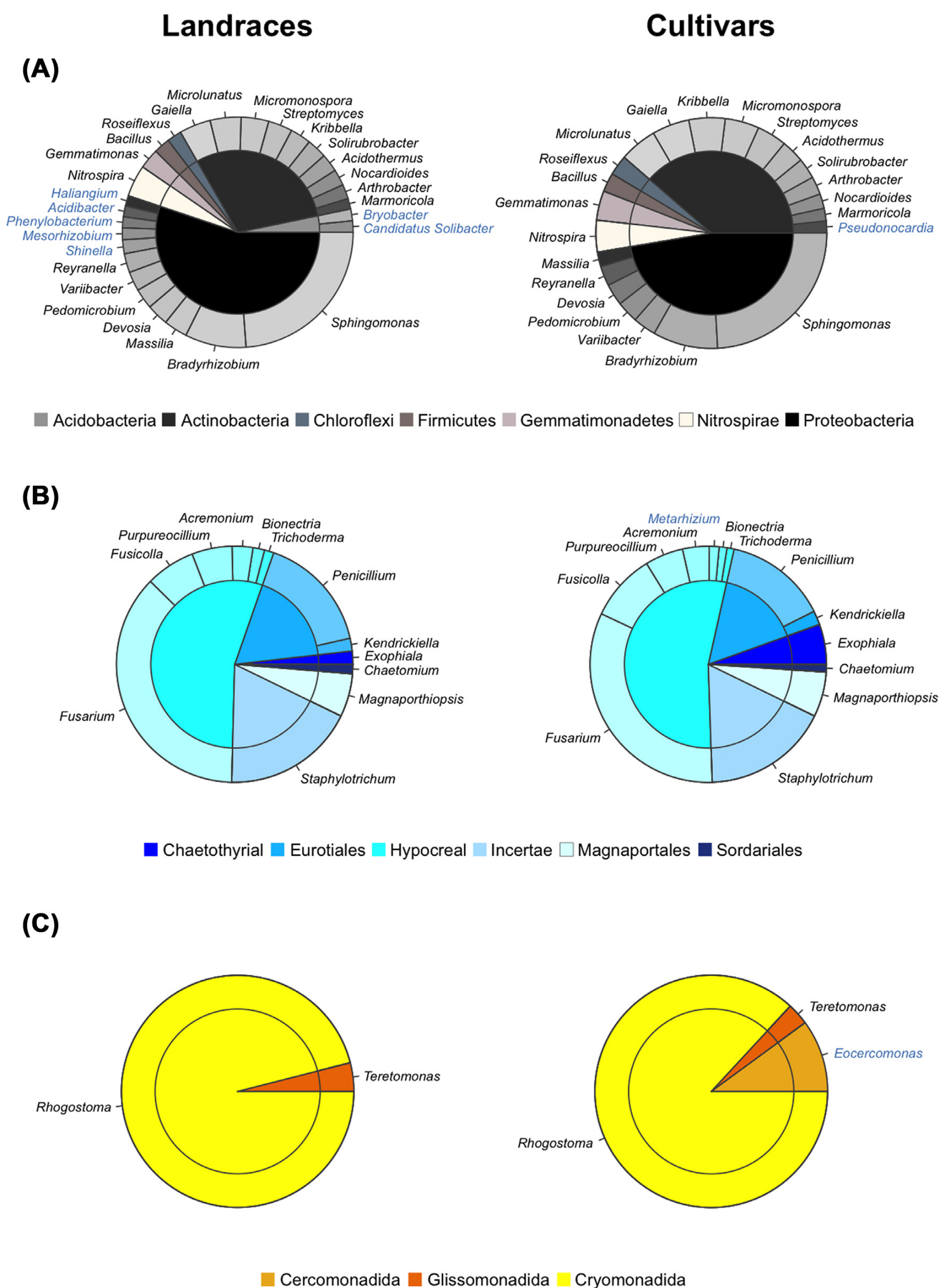

Figure 3. (A), Bacterial core microbiome of landraces and cultivars (723 and 613 OTUs, respectively). Inner circle represents phylum level, outer circle represent genus level and blue names represent genera exclusively present in landraces or cultivars. **(B)**, Fungal core microbiome of wheat landraces and cultivars (169 and 157 OTUs, respectively). Inner circle represents order level, outer circle represent genus level and blue names represent genera exclusively present in landraces or cultivars. **(C)**, Cercozoan core microbiome of landrace and cultivar (5 and 3 OTUs, respectively). Inner circle represents order level, outer circle represent genus level and blue names represent genera exclusively present in landraces or cultivars. OTUs found in all samples were considered part of the core considering 24 samples from landraces (3 genotypes x 4 replicates x 2 land uses) and 40 samples from cultivars (5 genotypes x 4 replicates x 2 land uses).

The higher bacterial and Cercozoa alpha diversity found in agricultural soil when compared to natural systems seems counterintuitive, as one would expect a diversity depletion in agricultural systems. However, as demonstrated by Rodrigues *et al.* (2013), while the conversion of forest to agriculture increased local taxonomic and phylogenetic diversity of soil bacteria (alpha diversity), the bacterial communities become more similar across space resulting in lower beta diversity.

Wheat landraces support a more intricate and complex microbiome connections than cultivars

The rhizosphere is a unique environment in terrestrial ecosystems that integrates complex networks of microbiota and macrobiota networks (Berg and Smalla 2009). For example, certain bacteria promote the formation of ectomycorrhiza, while on the other hand bacteria colonize the surface of fungal hyphae and benefit from fungal exudates (Frey-Klett, Garbaye and Tarkka

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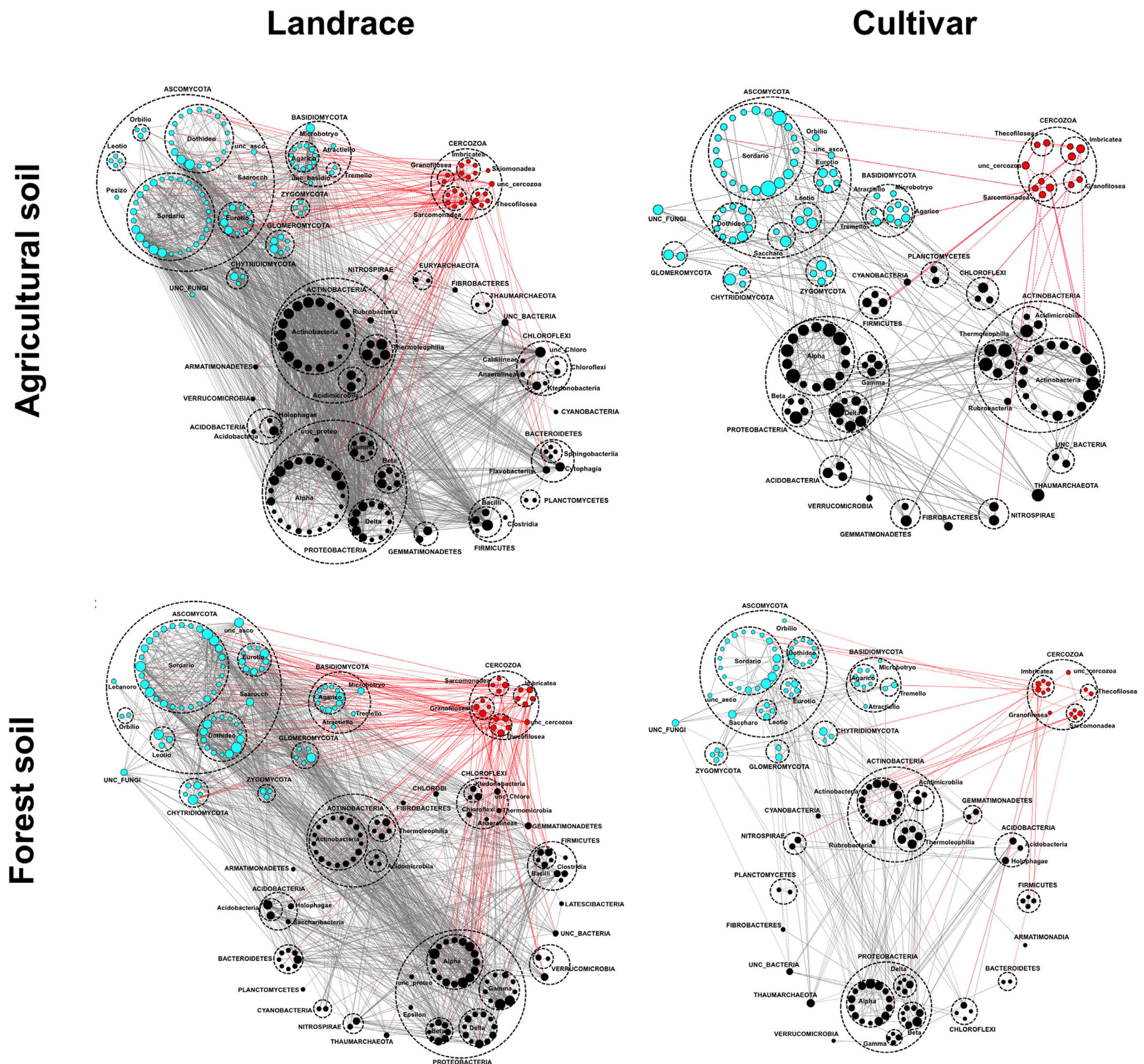

Figure 4. Network co-occurrence analysis of microbial communities of soil and rhizosphere samples. A connection stands for SparCC correlation with magnitude > 0.7 (positive correlation = blue edges) or < −0.7 (negative correlation = red edges) and statistically significant ($P < 0.01$). Each node represents taxa affiliated at family level, and the size of node is proportional to the number of connections (degree). Each node was labeled considering the type of community, black color represents the bacterial community (bottom), cyan color represents the fungal community (top-left) and red color (top-right) represents the cercozoan community. Continue lines represent the positive correlations, dashed lines represent the negative correlations and red lines represent the cercozoan interactions with bacterial and fungal communities. **(A)**, landraces in agricultural land; **(B)**, modern cultivars in agricultural land; **(C)**, landraces in forest land; **(D)**, modern cultivars in forest land.

2007). Protists, including Cercozoa, occupy different trophic levels and may consume bacteria, yeasts, filamentous fungi or other protists (Geisen *et al.* 2016). Due to a relatively similar C: N ratio of protists compared to their prey, about one third of the consumed nitrogen is excreted mainly as NH_4^+ (Griffiths 1994), which becomes available to nourish microorganisms and plants (Geisen *et al.* 2018).

The network analysis allowed us an integrated view of the microbial community assembly in the rhizosphere, revealing the complexity of microbial network structure and keystone groups. In this study, all treatments reached modularity values greater than 0.4 and the highest values were found under landraces cultivated in forest soil. According to Newman (2006), such values are indicative of modular structured networks, where high modularity values are positively associated with network stability, improving the resilience of microbial communities to environmental stresses (Krause *et al.* 2003). Most strikingly, microbial networks of the landraces, with their high number of nodes and connections per node (average degree), formed a more intricate network topology of rhizosphere communities than in cultivars.

Keystone taxa have important roles in shaping network structure (Faust and Raes 2012; Lu *et al.* 2013). According to the dependency theory described by Power *et al.* (1996), the predominance of keystone species may be restricted in time and may change with the ecological context. Reinforcing this theory, more recent studies reported a turnover of putative

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keystone species as conditions changed (Power *et al.* 1996; Lu *et al.* 2013; Lupatini *et al.* 2014). Also in these studies, the identity of putative keystone taxa differed between individual networks, indicating distinct differences in the community assembly of landraces and cultivars. It is as yet unclear how much of the variation in the identity of key nodes can be attributed to functional redundancy, i.e. different organisms playing the same functional role in different modules (Shi *et al.* 2016). In addition, Agler *et al.* (2016) demonstrated how host genotypic signatures controls microbial communities by acting directly on hub microbes, this occurs via microbe–microbe interactions and the effects are transmitted to the microbial community. The occurrence of positive correlations was higher than that of negative correlations for all evaluated conditions, which may indicate a dominance of cooperative or syntrophic interactions between bacteria and fungi in wheat rhizosphere microbiomes, as well as a shift to grazing resistant prey organisms in trophic interactions with cercozoa (Jousset, Scheu and Bonkowski 2008). Interestingly, the proportion of negative correlations was higher in landraces than in cultivars, which can be associated with a higher community stability (Thébault and Fontaine 2010).

The results of this study demonstrate that landrace microbiomes are more connected than microbial communities assembled in the rhizosphere of wheat cultivars. This led us to suggest that landraces may have maintained stronger interactions with their environment and with their respective soil microbiomes, while breeding pressure has impaired the capacity of plants to orchestrate microbiome assembly.

Cercozoan role in the rhizosphere microbiome assembly

In the network analyses, we observed a striking difference in the correlations of Cercozoa in rhizospheres of landraces when compared to cultivars. Overall, the number of correlations was much higher in the rhizosphere of landraces suggesting that protists in natural plant communities play more important roles in the microbiome assembly and that this trophic control was impaired during domestication and breeding. When only taking into account correlations between Cercozoa and bacteria, we observed a significant decrease in the number of connections in cultivars. The high connectedness of certain cercozoan taxa to bacteria and fungi indicates trophic network hierarchies where certain predators gain predominance over others. Such trophic loops between bacterivores and fungivores and their basal resources are important for network stability (Neutel, Heesterbeek and de Ruiter 2002).

An inventory of protists from 180 locations across the globe showed that soil protist communities are dominated by consumers, highlighting the role of protists on nutrient turnover and energy transfer across trophic levels (Oliverio *et al.* 2020). The same study revealed that particular soil protists and prokaryotes co-occur globally highlighting the potential importance of specific protist–bacterial interactions in structuring the soil microbiome (Oliverio *et al.* 2020). Therefore, considering the co-evolution between bacteria and protist predators, we assume, as proposed by other authors (Jousset, Scheu and Bonkowski 2008; Bonkowski, Villenave and Griffiths 2009; Jousset 2012), that negative correlations may indicate potential trophic interactions, while positive correlations indicate a community shift to bacterial groups showing grazing resistance. This explanation was supported by the fact that positive correlations between protists and bacteria in landraces were preserved as a subset in cultivars. For example, this was the case for the Sarcomonadea class of protists with Actinobacteria, as positive correlations between these two groups were observed across all treatments. On the other hand, negative correlations between Imbricatea and Actinobacteria were also consistently observed in all treatments. These results reinforce the importance of top-down control by organisms of higher trophic levels, including Cercozoa, as a key factor for understanding the drivers of microbiome community assembly in the rhizosphere.

Although plant breeding has not taken into account the associated microbiome and its functions during plant trait selection, recent techniques have enabled us to access the complexity of correlations taking place in the rhizosphere. Here, we used a comprehensive approach to compare the rhizosphere microbiome assembly in wheat landraces and cultivars by assessing bacterial, fungal and Cercozoa communities and their potential interactions. We demonstrate that landraces and cultivars assemble clearly distinguishable microbiomes. Landraces were able to recruit and sustain more intricate and complex microbiomes when compared to cultivars, reinforcing the hypothesis that modern cultivars lost some of the traits needed to recruit and sustain host-specific root microbiota when compared to their wild relatives. While differences in soil management, plant genotypes and associated rhizodeposition determine the resource basis for microbiome assembly in the rhizosphere, predation of Cercozoa likely plays a key role in structuring of the microbiome by favoring and suppressing specific bacterial populations. A thorough and comprehensive understanding of wheat domestication and breeding, including the changes in rhizosphere microbiome as driven by Cercozoa and other protists, could guide the recent, highly promising approaches involving the preservation or rescue of beneficial interactions between plants and their soil microbiome in order to promote a more sustainable agriculture.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of interest. None declared.

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