Fungus-bacterium associations are widespread in fungal cultures isolated from a semi-arid natural grassland in Germany

L.A.H. Muller[1,2,*], M.-B. Ballhausen[1,2,*], D.R. Andrade-Linares[3], L. Pinek[1,2], P. Golubeva[1,2] and M.C. Rillig[1,2].

[1] Institut für Biologie - Ökologie der Pflanzen, Freie Universität Berlin, Altensteinstr. 6, 14195 Berlin, Germany.

[2] Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), Altensteinstr. 6, 14195 Berlin, Germany.

[3] Research Unit Comparative Microbiome Analysis, Helmholtz Zentrum München, Ingolstaedter Landstraße 1, 85764 Neuherberg, Germany.

[*] These authors contributed equally

Corresponding author:

Matthias C. Rillig

Freie Universität Berlin - Institute of Biology

Altensteinstraße 6

14195 Berlin

Germany

E-mail: rillig@zedat.fu-berlin.de

Tel.: +49 30 838 53165

Fax: +49 30 838 53886

Abstract

We report on a study that aimed at establishing a large soil-fungal culture collection spanning a wide taxonomic diversity and systematically screening the collection for bacterial associations. Fungal cultures were isolated from soil samples obtained from a natural grassland in eastern Germany and bacterial associations were assessed by PCR-amplification and sequencing of bacterial 16S rRNA. In addition, intraspecies genetic diversities of a subset of the isolated species were estimated by double-digest restriction associated DNA sequencing. A total of 688 fungal cultures, representing at least 106 fungal species from 36 different families, were obtained and even though clonal isolates were identified in almost all fungal species subjected to ddRAD-seq, relatively high genetic diversities could be observed in some of the isolated species. Sixty-nine percent of the fungal isolates in our collection were found to be associated with bacteria and the most commonly identified bacterial genera were *Pelomonas*, *Enterobacter* and *Burkholderia*. Our results indicate that bacterial associations commonly occur in soil fungi, even if antibiotics are being applied during the isolation process, and provide a basis for the use of our culture collection in ecological experiments that want to acknowledge the importance of intraspecies genetic diversity.

Keywords: Soil fungi, Bacteria, Fungal-bacterial interaction, ddRAD sequencing

Introduction

Fungi and bacteria frequently interact beyond exhibiting a mere physical proximity, and their interactions can be considered from different perspectives. From a physical perspective, fungi and bacteria can associate more or less intimately in seemingly disordered polymicrobial communities, in mixed biofilms or as highly specific symbionts. Functionally, on the other hand, their interactions vary from antagonistic to obligately symbiotic through a range of molecular mechanisms (e.g. antibiosis, signaling and chemotaxis).

Bacteria can associate with fungi as hyphal surface colonizers or as endohyphal symbionts. Hyphal surface colonizers form multispecies biofilm communities that either feed on fungus-derived exudates (Kaiser *et al*. 2015) or on fungal cell wall components/cytoplasmic nutrients (Ballhausen *et al*. 2015). These bacteria are sustained by fungal nutrients and are therefore to a certain extent adapted to the colonization of hyphal surfaces. They can usually persist without their fungal host but are not able to compete with bacterial taxa that are adapted to oligotrophic environments (Leveau and Preston 2008). In contrast, endohyphal bacteria colonize the inside of fungal hyphae. It is not well understood how they enter the hyphae, but one way seems to be through the production of chitinases that break down chitin oligomers in the fungal cell wall and thereby facilitate bacterial entry (Moebius *et al*. 2014). Experimental work also suggests that the nutrient status of the fungus, environmental temperature and the concentration of ions such as magnesium might play a role in paving the way for a successful bacterial invasion into the fungus (Arendt *et al*. 2016).

Araldi-Brondolo *et al*. (2017) consider three different classes of endohyphal bacteria based on various factors such as fungal and bacterial taxonomy, transmission mode, obligate or facultative nature of the bacterial association and metabolic reliance on the host. Class 1 endohyphal bacteria, proposed to be classified under the genus *Candidatus* Moeniiplasma by Naito *et al*. (2017), have limited biosynthetic capacities and have so far not been isolated

in culture. Curing the fungal host from class 1 endohyphal bacteria through the use of antibiotics can result in adverse physiological reactions like lower sporulation (Mondo *et al*. 2017) or growth rates (Guo *et al*. 2017). Class 2 (e.g. *Candidatus* Glomeribacter gigasporum in various Glomeromycotina) and class 3 (e.g. *Paenibacillus* in *Laccaria*) endobacteria are facultative colonizers and can be isolated and cultured, while the host fungus can usually be cured from the bacteria through the usage of antibiotics without its traits being affected (Baltrus *et al*. 2017).

Endohyphal bacteria have been described in a taxonomically and ecologically diverse group of fungi, but most research has been done on plant-associated fungi. Already in 1996, hyphae of the arbuscular mycorrhizal fungus *Gigaspora margarita* were shown to be colonized by "bacteria-like organisms" (Bianciotto *et al*. 1996) which were later described as β-proteobacteria and named *Candidatus* Glomeribacter gigasporarum (Bianciotto 2003). Curing *G. margarita* from the endobacteria turned out to be possible, but lead to a change in fungal traits such as branching, hyphal growth and spore germination (Lumini *et al*. 2007).

Endohyphal bacteria have also been shown to influence the interaction between pathogenic fungi and plants. The fungus *Rhizopus microsporum*, the causative agent of rice seedling blight, harbors the endobacterium *Burkholderia rhizoxinica* that is responsible for the production of rhizoxin, a mycotoxin that induces the disease symptoms. Upon curing the fungus from the endobacterium with the antibiotic ciprofloxacin, it was no longer able to produce the mycotoxin (Lackner *et al*. 2009).

Leaf endophytic fungi have been systematically screened for endohyphal bacteria and about 20% of all isolated fungal strains were found to be associated with endohyphal bacteria (Hoffman and Arnold 2010). Interestingly, most associations were found to be facultative. Curing did not result in impaired fungal traits and the genomes of the isolated endohyphal

bacteria were neither reduced in size, nor did the bacteria show signs of adaptions to an endohyphal lifestyle in most cases (Baltrus *et al*. 2017).

Although bacteria and fungi co-occur in soil environments and fungus-bacterium interactions in this habitat have recently received increased attention (Ballhausen and de Boer 2016; Bonfante and Desirò 2017; Worrich *et al*. 2017), a systematic screening of endohyphal bacterial associations with saprotrophic fungi has, to our knowledge, never been performed. Functional consequences of colonization by endohyphal bacteria could, however, be substantial for saprotrophic fungi. This was illustrated by a recent study in *Mortierella elongata* where an endobacterium was found to provide access to metabolic pathways that would not be accessible to the fungus alone, hinting at a long-shared evolutionary history (Uehling *et al*. 2017).

In the present study, we aimed for a systematic screening of the association between bacteria and soil fungi in a natural grassland in eastern Germany. Fungal cultures were isolated and cultivated on growth media supplemented with antibiotics in order to limit the interference from loosely-associated hyphal surface colonizers, and associated bacteria were identified by PCR-amplifying and sequencing bacterial 16S rRNA. In addition, a subset of the fungal isolates was genetically characterized by double-digest restriction associated DNA sequencing (ddRAD-seq) in order to identify clonal isolates and to assist in future studies that aim at characterizing intraspecies diversity of fungal traits using our culture collection.

Material and methods

Site description and soil sampling

Soil samples were taken in a protected area close to Mallnow, Lebus, Germany (52°27.778' N, 14°29.349' E). The nature reserve is characterized by a hilly landscape with a calciferous boulder clay soil that is very sandy and predominantly hosts a species-rich dry-grassland vegetation (annual precipitation of approximately 500 mm and mean annual temperature of 8.7 °C). The main tree species are *Robinia pseudoacacia*, *Pinus sylvestris* and *Betula* spec., and the open areas are dominated by different grass species such as *Arrhenaterum elatius*, *Festuca brevipila* and *Corynephorus canescens*. Twenty soil samples were collected along an elevational gradient at each of three locations in the reserve (Figure 1). After removing the vegetation along the surface, samples of soil from 10 to 15 cm depth were taken with a soil corer and subsamples of ca. 2.5 g were transferred to 50 mL centrifuge tubes.

Isolation of fungal cultures

Soil samples were resuspended in a wash solution containing 0.1 % sodium pyrophosphate and 0.05 % Tween 80 (Thorn *et al*. 1996). After shaking the resuspended soil on an oscillatory shaker for 30 min, the samples were centrifuged at 1,000 rpm for 5 min and the supernatant was discarded. The soil washing procedure was repeated once with a solution containing 0.1 % sodium pyrophosphate to remove free spores and serial dilutions of washed soil in sterile distilled water were plated on to Petri dishes with malt extract agar (MEA) medium in addition to 48-well microplates containing MEA and Rose Bengal agar (RBA) medium. Bacterial growth was inhibited by adding streptomycin sulfate, penicillin G and chlortetracycline-HCl to the MEA and RBA media at respective concentrations of 30, 30 and 60 mg/L. Besides the fact that usage of the above-mentioned antibiotics is common practice for selective fungal isolations, we want to stress that bacterial presence despite the

usage of antibiotics hints at a resilient and tight fungus-bacterium association. Petri dishes and microplates were initially incubated at 22 °C for 24 h and then at 12 °C to slow down fungal growth. Fungal growth was monitored daily for up to 30 days, and single microcolonies were transferred to potato dextrose agar (PDA) supplemented with the abovementioned antibiotics and selectively cultured until contamination-free.

Taxonomic identification of fungi and associated bacteria

DNA was isolated from fungal cultures grown on PDA medium using the Soil DNA Isolation 96-Well Kit (Norgen Biotek Corp., Thorold, Canada) and the DNeasy PowerSoil HTP 96 Kit (Qiagen, Hilden, Germany) according to the respective protocols provided by the manufacturers with a final elution of the extracted DNA using 100 μL sterile water.

The fungal internal transcribed spacer (ITS) region was polymerase-chain-reaction (PCR) amplified using the oligonucleotide primers ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes & Bruns 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al*. 1990) in 20 μL reactions containing 0.5 μM of each oligonucleotide primer, 1 μL extracted DNA and 4 μL 5× FIREPol Master Mix (Solis Biodyne, Tartu, Estonia). The PCR temperature profile consisted of an initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were visualized after electrophoresis using 1.5% agarose gels and validated before unidirectional Sanger sequencing using the ITS1F oligonucleotide primer at LGC Genomics (Berlin, Germany). If the PCR did not yield a product, a second PCR was performed with 100× diluted template DNA.

Bacterial 16S rRNA was PCR amplified using the oligonucleotide primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3'; Weisburg *et al*. 1991) and 1492r (5'-GGTTACCTTGTTACGACTT-3'; Weisburg *et al*. 1991) in three independent 40 μL reactions containing 0.25 μM of each oligonucleotide primer, 2 μL extracted DNA and 8 μL 5× FIREPol

Master Mix using the following temperature profile: initial denaturation at 94 °C for 2 min, 34 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 90 s with a 1 s increment per cycle, and a final extension at 72 °C for 10 min. Proper amplification was assessed for the individual PCRs by agarose gel electrophoresis and recorded, and pooled PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany) following the manufacturer's instructions before unidirectional sequencing using 27f primers at LGC Genomics (Berlin, Germany).

Fungal cultures were identified by comparing the obtained ITS DNA sequences with the sequences in the UNITE database (Kõljalg *et al*. 2013) and associated bacteria were identified by comparing the 16S rRNA sequences with the sequences stored in the GenBank database using the BLAST Network Service (NCBI, National Center for Biotechnology Service; http://www.ncbi.nlm.nih.gov/BLAST). A taxonomic tree summarizing our fungal culture collection at the genus level along with the bacterial association data was constructed using the NCBI Taxonomy Database (Schoch *et al*. 2020) and the ggtree v2.4.1 package (Yu *et al*. 2017) in R v4.0.2 (R Core Team 2020). All sequence data were deposited at NCBI GenBank under accession numbers MW268772-MW269399/MW486432-MW486491 (fungal ITS) and MW519526-MW519615 (bacterial 16S).

Double digest restriction associated DNA sequencing

The ddRAD sequencing protocol developed by Peterson *et al*. (2012) was used with slight modifications to generate reduced representation libraries for 96 fungal isolates representing 11 different species (five to ten isolates per species; see Table S2). In brief, genomic DNA was extracted from mycelia grown on PDA using the DNeasy Powersoil kit (Qiagen, Hilden, Germany) and 250 ng of each DNA sample was digested by 16 U *Eco*RI and 4 U *Mse*I (New England Biolabs GmbH, Frankfurt am Main, Germany) in 20 μL 1× Cutsmart

buffer at 37 °C for 8 hours. Stock solutions (100 μM) of eight double-stranded barcoded P5 and one P7 adaptor (see Table S3a for adaptor sequences) were created by incubating equimolar amounts of forward and reverse strands in 1× hybridization buffer (10 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM EDTA) for 2.5 minutes at 97.5 °C and allowing the mixtures to slowly cool down to room temperature. P5 and P7 adaptors were ligated to the restriction fragments by adding 20 μL 2× T4 DNA ligase buffer containing 400 U T4 DNA ligase (New England Biolabs GmbH, Frankfurt am Main, Germany), 1 pmol of one of the P5 adaptors (see Table S2) and 14 pmol P7 adaptor to each restriction digest and incubating the reaction mixtures at 16 °C for eight hours. T4 DNA ligase was subsequently heat-inactivated by incubation at 65 °C for 10 minutes. Following adaptor ligation, the restriction fragments were combined into 12 pools (each pool consisted of eight samples labelled with different P5 barcodes) and the fragment pools were purified using AMPure XP beads (Beckman Coulter, Krefeld, Germany) with a bead-to-sample ratio of 1.5. The restriction fragments in each pool were PCR-amplified (temperature profile: 98 °C for 30 sec, followed by ten cycles of 98 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec) using the Q5® Hot Start High-Fidelity 2× Master Mix (New England Biolabs GmbH, Frankfurt am Main, Germany) with a common P5 oligonucleotide primer and a P7 oligonucleotide primer carrying a unique index sequence (see Table S2 for primer assignment and Table S3b for primer sequences). Ten independent PCR amplifications with 20 ng template DNA, 40 pmol of each oligonucleotide primer and a reaction volume of 20 μL were performed for each pool of restriction fragments and combined prior to size selection. Fragment size selection was carried out using the BluePippin system (Sage Science, Beverly, MA, USA) with a target size range of 450 to 550 basepairs. Equimolar amounts of the 12 pools were combined into the final sequencing library and the library was purified using AMPure XP beads (bead-to-sample ratio of 1.5) before being analyzed on a Bioanalyzer (Agilent, Santa Clara, CA, USA). Paired-end

sequencing of the library was performed using a full Illumina (Berlin, Germany) MiSeq lane at the Berlin Center for Genomics in Biodiversity Research (Berlin, Germany). All sequencing read data were deposited at the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) and are available under study accession number PRJEB40896.

Analysis of ddRAD-seq data

The raw sequencing reads were processed using Stacks v2.53 (Catchen *et al*. 2013), following the guidelines proposed by Rochette and Catchen (2017) and Paris *et al*. (2017) for the *de novo* assembly of ddRAD loci and the derivation of genotypes. After assessing the overall data quality using FastQC (Andrews 2010), the raw sequencing reads were demultiplexed according to the P5 barcodes, trimmed to a uniform length of 200 basepairs and cleared of low-quality reads (reads with a Phred score < 10 and reads without valid restriction sites) using the process_radtags program of Stacks. The Stacks pipeline to generate SNPs and haplotypes was executed separately for each species using the denovo_map.pl program. The values of the parameters *M* (distance allowed between stacks) and *n* (distance between catalog loci) were optimized according to the "80% rule" suggested by Paris *et al*. (2017), whereas *m* (minimum stack depth of coverage) was kept at its default value (= 3). The populations module of Stacks was used to restrict the output to the first SNP per locus and to those loci that were present in at least 80% of the individuals, and VCFtools v0.1.13 (Danecek *et al*. 2011) was used to further restrict the data to those loci with exactly two alleles and a site depth between 10 and 100. Pairwise genetic distances between the isolates were calculated as allelic sharing distances (Gao and Martin 2009) using the dist.asd function of the pegas v0.14 library in R v4.0.2.

Results

We isolated 628 cultures of filamentous fungi and 60 cultures of yeasts with a large taxonomic variety that spans at least 17 fungal orders, 36 families, 57 genera and 106 species (see Table S1 and Figure S1). The majority of the fungal cultures (87 %) were classified as ascomycetes and they were most frequently assigned to the families Aspergillaceae (41 % of all cultures), Hypocreaceae (10 % of all cultures) and Nectriaceae (6 % of all cultures). The most common genera in our collection are *Penicillium* (41 % of all cultures), *Trichoderma* (10 % of all cultures) and *Fusarium* (5 % of all cultures), with the most common species being *Penicillium jensenii* (17 % of all cultures), *Penicillium roseopurpureum* (11 % of all cultures) and *Cryptococcus aerium* (4 % of all cultures).

After filtering out low-quality ddRAD-seq reads, a total of 37,256,840 paired-end sequence reads was obtained. The number of paired-end reads per isolate varied between 136,876 and 934,877 (average = 411,430, SD = 156,749; see Table S2), with six isolates (MI # 39, 123, 385, 386, 442 and 446) being left out from the analysis due to low numbers of reads. Optimal values of the main parameters used by Stacks v2.53 to build the loci and call the variants are listed in Table S4. The depth of coverage per isolate varied from 16x to 223x, with an average of 52x (SD = 33x; see Table S2), and the final number of SNPs per species ranged between 212 (*Purpureocillium lilacinum*) and 3,345 (*Fusarium oxysporum*; average = 1,375, SD = 888; see Table S4) after quality filtering.

The pairwise allelic sharing distances among conspecific isolates are listed in Table S5. Distances varied extensively and likely clonal isolates were identified in all fungal species except for *Oidiodendron cereale*. In some cases (e.g. *P. jensenii*), the same genet was represented by isolates from different sampling plots. Rescaling all sample sizes to 10 isolates, the number of genets represented by our samples varied between four (*P. jensenii*) and 10 (*O. cereale*). Retaining only one isolate per genet, the average allelic sharing distance

within species varied between 0.65 (*Mucor moelleri*) and 1.65 (*P. jensenii*; average = 1.16, SD = 0.35; see Table S5).

Four hundred and seventy-two out of 688 (69 %) fungal cultures were found to be associated with bacteria. Because the reproducibility of the bacteria-specific PCR was relatively low, we performed three independent PCRs for each fungal DNA extract. All three PCRs generated a bacteria-specific fragment for 15 % of the fungal isolates, whereas for 25 % and 29 % of the isolates a bacteria-specific fragment was obtained for respectively two and one out of the three independent PCRs. None of the three independent PCRs generated a bacteria-specific fragment for 31 % of the isolates. Disregarding *Trichoderma asperellum* and *T. neokoningii*, there was a tendency for higher numbers of isolates per fungal species with a bacterial association than without such association (see Figure 2 and Table S6).

Bacterial 16S rRNA sequences that showed significant homology to sequences in the public repositories were obtained for 135 fungal isolates (see Figure S2 for a taxonomic overview of associated bacteria and Figure S3 for a heatmap illustrating the association between fungal and bacterial species in our culture collection). The majority of the bacterial rRNA sequences (83 %) showed the highest homology with sequences from the phylum Proteobacteria and the most common families that showed significant homology were Comamonadaceae (40 % of all 16S sequences), Burkholderiaceae (12 % of all 16S sequences), and Enterobacteriaceae (12 % of all 16S sequences). The most common bacterial genera that were retrieved in the homology search were *Pelomonas* (11 % of all 16S sequences), *Enterobacter* (11 % of all 16S sequences), and *Burkholderia* (10 % of all 16S sequences), and the most common species were *Pelomonas puraquae* (10 % of all 16S sequences), *Comamonas testosteroni* (7 % of all 16S sequences), and *Enterobacter cloacae* (7 % of all 16S sequences). The bacterial genera that were associated with the highest numbers of fungal genera were *Enterobacter* (8 genera), *Burkholderia* (6 genera), and *Diaphorobacter* (5 genera).

Discussion

The set of soil fungi that we isolated and brought into culture spans a wide taxonomic range that is typical for natural grasslands, with a dominance of ascomycetes (Tedersoo *et al*. 2014) and a common occurrence of heavily sporulating genera such as *Penicillium*, *Trichoderma*, *Mortierella*, *Fusarium* and *Mucor* (Warcup 1951). The genetic diversity among our isolates and the number of potential clones varied considerably across species. Although clonal isolates sometimes originated from the same soil sample, most often they were brought into culture from different soil samples obtained from the same or even different sampling plots. The frequent occurrence of common genotypes of *P. jensenii* and *M. carneum* in different soil samples may reflect a dominance of those genotypes in either the local spore bank and/or the local mycelial community.

The majority of our fungal isolates were found to be associated with bacteria. A genus for which this observation does not hold, however, is *Trichoderma*. For several *Trichoderma* species, most notably *T. neokoningii* and *T. asperellum*, the number of isolates found to be associated with bacteria was much lower than the number of isolates not associated with bacteria. A possible explanation for the apparently rare occurrence of bacterial symbionts in these species is the common production of antimicrobial peptides by *Trichoderma* spp. (Daniel and Filho 2007) which might prevent facultative interactions with bacteria.

If fungi were observed to associate with bacteria, we did not observe a clear pattern of co-occurrence. On the level of fungal genus or species, we found isolates to be associated with multiple, taxonomically different bacterial species. Several of the bacterial genera that were most dominant and/or associated with the highest number of fungal isolates are well known for their co-occurrence with fungi (genus *Burkholderia*; Stopnisek *et al*. 2016; Warmink *et al*. 2009) or with other eukaryotic hosts such as plant seeds (genus *Enterobacter*; Nelson 2018). Interestingly, other associated bacteria shared the trait of being able to degrade/withstand

toxic compounds (e.g. genera *Pelomonas* (Wang *et al*. 2020), *Comamonas* (Nesvera *et al*. 2015), *Delftia* (Shetty *et al*. 2015) and *Diaphorobacter* (Qiu *et al*. 2015)).

Bacteria of the genus *Burkholderia* have been isolated from hyphal surfaces (Coenye and Vandamme 2003), and were shown to be able to move along and feed on fungal hyphae (Rudnick *et al*. 2015; Warmink *et al*. 2009). It is no surprise that they were associated with fungi from our collection. It is, however, interesting that *Burkholderia* seems to be tolerant to the antibiotics which we applied as part of our fungal isolation protocol. The fact that we found toxic compound tolerators/degraders together with the notion that some fungal genera that are known to produce antibiotics seem to be less colonized by bacteria might indicate that a fraction of bacterial hyphosphere colonizers may be able to tolerate antibacterial secondary metabolites.

This study clearly shows that genetic diversity is present among fungal isolates in natural grasslands, even at low spatial scales (up to several 100 meters), and that bacteria are frequently associated with cultured soil fungi. However, more research is required to investigate to what extent fungal intraspecies diversity affects isolate specificity of bacterial associations. There is no doubt that intraspecies diversity affects fungal traits and there is the necessity to include it into ecological experiments if one wants to truly generalize results at the fungal species level. With this collection of isolates, we hope to provide a resource for such experiments for fungal experimental ecologists.

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Figure 1 - Locations of our sampling plots (P1, P2 and P3) in a protected natural grassland area close to Mallnow, Lebus, Germany.

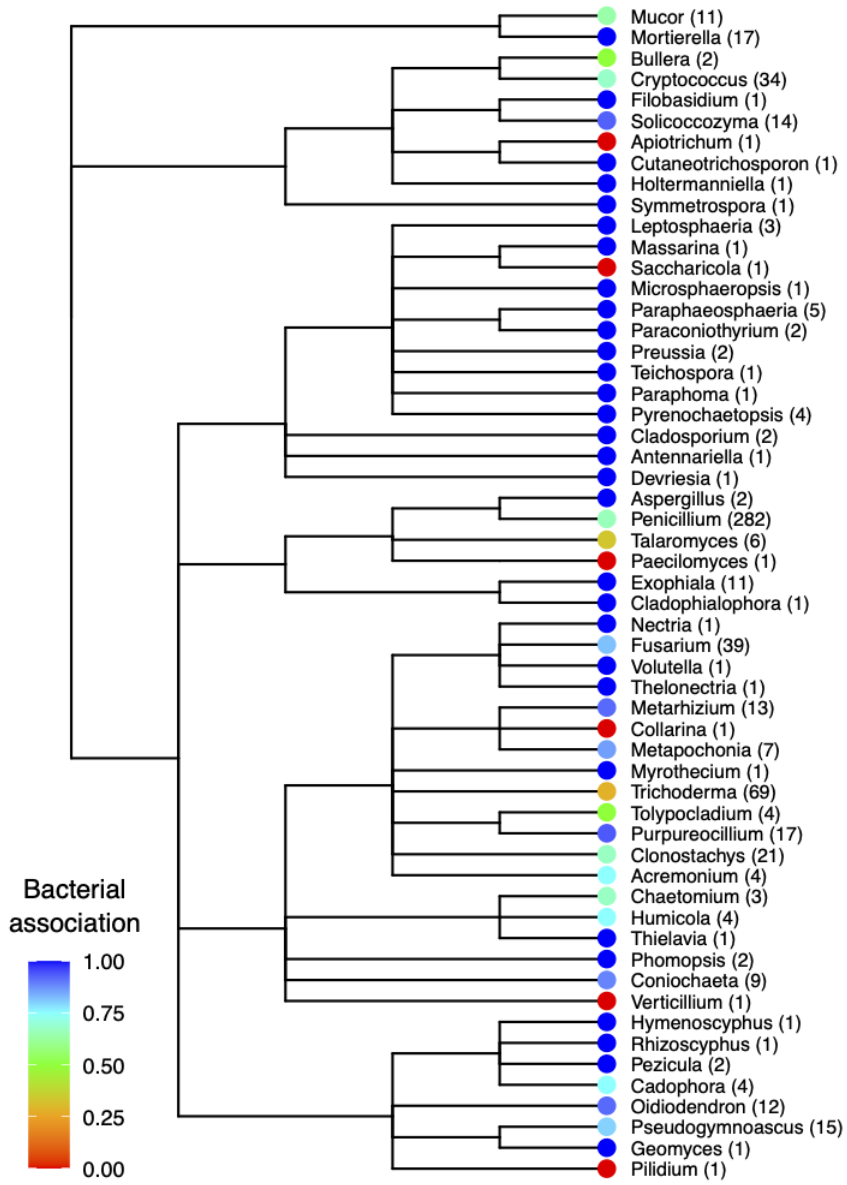

Figure 2 - Taxonomic tree at the genus level according to the NCBI Taxonomy Database (Schoch et al. 2020) summarizing our fungal culture collection obtained from a natural grassland area in Germany; number of fungal cultures per genus is provided in parenthesis and tip color indicates the fraction of the cultures that were shown to be associated with bacteria based on a PCR assay.