Archaea are prominent members of the prokaryotic communities colonizing common forest mushrooms

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Abstract

In this study the abundance and composition of prokaryotic communities associated with the inner tissue of fruiting bodies of *Suillus bovinus, Boletus pinophilus, Cantharellus cibarius, Agaricus arvensis Lycoperdon perlatum* and *Piptoporus betulinus* were analyzed using culture-independent methods. Our findings indicate that archaea and bacteria colonize the internal tissues of all investigated specimens and that archaea are prominent members of the prokaryotic community. The ratio of archaeal 16S rRNA gene copy numbers to those of bacteria was >1 in the fruiting bodies of four out of six fungal species included in the study. The largest proportion of archaeal 16S rRNA gene sequences belonged to thaumarchaeotal classes Terrestrial group and Miscellaneous Crenarchaeotic Group (MCG) and Thermoplasmata. Bacterial communities showed characteristic compositions in each fungal species. Bacterial classes Gammaproteobacteria, Actinobacteria, Bacilli and Clostridia were prominent among communities in fruiting body tissues. Bacterial populations in each fungal species had different characteristics. The results of this study imply that fruiting body tissues are an important habitat for abundant and diverse populations of archaea and bacteria.

Keywords: bacteria, archaea, mushroom, qPCR, sequencing

Introduction

Bacteria colonize the tissues of fruiting bodies of basidiomycetes (Swartz 1929, Danell *et al.* 1993, Dahm *et al.* 2005, Timonen and Hurek 2006, Pent *et al.* 2017) and ascomycetes (Barbieri *et al.* 2007, Quandt *et al.* 2015). Bacteria and fungi have a partnership throughout the fungal life cycle; bacteria may even be necessary for the formation of fruiting bodies (Cho *et al.* 2003) and they may supplement the fruiting body with nutrients, such as fixed nitrogen (Barbieri *et al.* 2010). Association between archaea and ectomycorrhizal fungal hyphae has been observed in boreal forest soil environment (Bomberg *et al.* 2003, Bomberg and Timonen 2007). Archaea are detected more frequently and their populations are more diverse on tree roots colonized by ectomycorrhizal fungi than on uncolonized roots or humus (Bomberg and Timonen 2009). However, currently there is no information available on whether the association of archaea with fungal hyphae extends from the mycorrhiza to the fruiting bodies of the fungi.

Bacteria colonizing the fruiting body tissues of basidiomycetes have been studied mainly using culture-based techniques and microscopy (Li and Castellano 1987, Danell *et al.* 1993, Dahm *et al.* 2005, Timonen and Hurek 2006). Recently, Pent et al (2017) performed the first comprehensive study of fruiting body bacteriomes using high throughput sequencing in parallel with culture-based approach. Most of the culturable bacteria recovered from fruiting bodies have been *Pseudomonas* spp. (Danell *et al.* 1993, Rangel-Castro *et al.* 2002, Pent *et al.* 2017), while other groups, such as *Burkholderia* (Pent *et al.* 2017), *Paenibacillus* (Timonen and Hurek 2006), *Xanthomonas* spp., *Streptomyces* spp., *Bacillus* spp. (Danell *et al.* 1993) and *Azospirillum* (Li and Castellano 1987) have been found less consistently. Recent molecular studies have elucidated the internal microbiomes of some ascomycetes indicating that Alphaproteobacteria are predominant members in microbial communities (Barbieri *et al.* 2007, Barbieri *et al.* 2010, Antony-Babu *et al.* 2014, Quandt *et al.* 2015).

Archaea from temperate environments are notoriously hard to grow in cultures, therefore previous culture-based studies of fruiting body-associated prokaryotes have not been able to touch upon the diversity and abundance of them. Despite the obvious evidence of bacterial colonization of fruiting bodies, not much is known yet about the fruiting body tissue as a habitat for archaea. Quantitative estimates of bacterial abundance have been based on the recovery of culturable bacteria from tissues of fruiting bodies of basidiomycetes. In some cases no or a very low number of culturable bacteria have been recovered (Dahm *et al.* 2005, Timonen and Hurek 2006).

We hypothesized that archaea colonize the internal tissue of the fruiting body, not just mycorrhizas or hyphae in forest soils. The purpose of this study was to quantify and characterize archaeal communities in the internal tissue of fruiting bodies of six different species of common forest mushrooms, using culture-independent techniques, quantitative PCR and 16S rRNA gene sequencing. In parallel, we used the same methods to determine the abundance and community composition bacteria colonizing the internal tissue of fruiting body.

Materials and methods

Sample collection

Sample materials were obtained from fruiting bodies of three species of mychorrhizal fungi: *Boletus pinophilus, Suillus bovinus and Cantharellus cibarius* and three species of saprophytic fungi: *Agrarius arvensis*, *Lycoperdon perlatum* and *Piptoporus betulinus*. Six specimens of each species were collected. All specimens were young (ca. 4-8 days old) and without larvae. All specimens were collected from southern Finland from locations specified

in Table 1. After collection, the fruiting bodies were stored at +4°C (1-2 days) until further processing in the laboratory. Fruiting body tissue for DNA-based analysis was collected from the interior of each specimen by first splitting the fruiting body in two halves without touching the exposed tissue and checking for any traces or damage by burrowing animals. Then two flawless, approximately 0.05 g tissue pieces were cut from the exposed interior at the base of the cap of the fruiting body using a sterile scalpel. The tissue samples were placed in a sterile microcentrifuge tube. Samples were immediately frozen at -20°C until DNA extraction.

DNA extraction

Tissue samples were defrosted in room temperature and homogenized in a 1.5 ml microcentrifuge tube with sterile glass beads or silicic acid (Sigma Aldrich) and 100-200 µl of bead beating buffer solution (Ultra Clean Soil DNA Isolation Kit, MoBio Laboratories) using a sterile acid-washed pestle. DNA was extracted from the homogenized fruiting body tissue with Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories) following the manufacturer's protocol. Two replicate DNA samples originating from the same specimen were pooled before further analyses. Concentration of extracted DNA was determined with Nanodrop spectrophotometer (NanoDrop Spectrophotometer ND-1000, V3.5.2).

Quantitative PCR

The abundances of bacterial and archaeal 16S rRNA genes in fruiting bodies were determined using quantitative PCR (qPCR). All qPCR reactions were run in triplicate and no-template-control reactions, where DNA template was replaced with an equal volume of

ultrapure water, were run in duplicate. Each 20 µl reaction mixture for archaeal 16S rRNA gene quantification consisted of 1x Dynamo Flash SYBR Green mastermix (Thermo), 0.5 µM (final concentration) of primers Arch349F 5'-GYGCASCAGKCGMGAAW-3' and 539R 5'-GCBGGTDTTACCGCGGCGGCTGRCA-3' (Takai and Horikoshi 2000), 5 µL of diluted template DNA and nuclease-free water up to 20 µL. A standard curve was generated using a dilution series of a commercially prepared plasmid consisting of a vector pUC57 (length 2710 bp) and a 894 bp insert (GenScript), which was synthetized according to DNA sequence of a 16S rRNA gene fragment belonging to an uncultivated 1.1c-group Thaumarchaeota (NCBI accession number **AM903348.1**). The concentrations of standards ranged from $3x10^6$ copies per reaction to $3x10^2$ copies per reaction. For eubacterial 16S rRNA gene quantification, 25 µl PCR reactions consisted of 1x Maxima SYBR green mastermix (Thermo), 0.3 µM (final concentration) of each primer Eub338 5'-ACTCCTACGGGAGGCAGCAG-3' and Eub518 5'-ATTACCGCGGCTGCTGG-3' (Fierer *et al.* 2005), 5 µL of diluted template DNA and ultrapure water up to 25 µL. Template DNA was substituted with nuclease free water in negative controls. A standard curve was generated using a 10-fold dilution series ranging from $3x10^6$ to 30 copies per reaction of a plasmid containing a 16S rRNA gene fragment from *Burkholderia glathei*. The plasmid was prepared by amplifying a 16S rRNA gene fragment from DNA extracted from a pure culture of *Burkholderia glathei* by PCR, using primers 25f and 1492R (Hurek *et al.* 1997) as described above. The fragment was ligated into a pJet 2.1 cloning vector and cloned using GeneJet cloning kit (Thermo Scientific). Plasmid DNA from a culture of transformed cells was purified with GeneJet Plasmid Miniprep Kit (Thermo Scientific) and quantified with Nanodrop spectrophotometer (Thermo Scientific). All qPCR products were verified by melt curve analysis and by running one of the triplicate reactions on an ethidium bromide (0.2 µg/ml) stained 1.2 % agarose gel.

Sequencing

DNA samples from three specimens of each fungal species were selected for sequencing archaeal and bacterial 16S rRNA gene amplicons. *L. perlatum* was left out due to an insufficient amount of sequencing template.

To prepare the archaeal 16S rRNA gene amplicons for sequencing, the original qPCR products were run on 2 % agarose gel prepared with 1x SB buffer and stained with ethidium bromide (0.2 µg/ml). DNA bands were excised from the gel and purified using GeneJET gel extraction kit (Thermo Scientific). The purified DNA fragments were additionally cleaned using Agencourt AMPure XP magnetic particles (Beckman Coulter) according to the manufacturer's protocol. Sequencing libraries were generated by ligating Illumina flowcell adapters and 9-base barcode sequences using a 2-step protocol adapted from Spencer *et al*. (2016): adapters were ligated into original PCR products by amplification with miseq_A349_F1 and miseq_A539_R1 primers (Supplementary Table 1). The first ligation PCR reaction consisted of 1x Dynamo Flash SYBR Green mastermix (Thermo), 0.5 µM of each primer F1 and R1, 2 µL of original PCR product and ultrapure water up to 20 µL. Thermal cycling was done at 95°C for 7 min., 15 cycles at 95° 10 s. 56° 30 s., then 72° for 5 min. The products with adapters and barcodes were run on a gel, excised, extracted from the gel and purified with Agencourt AMPure XP magnetic particles (Beckman Coulter) following the manufacturer's protocol. The second part of the of the adapters and barcode sequences were ligated in a subsequent PCR reaction, that consisted of 1x Dynamo Flash SYBR Green masterimix (Thermo), 0.25 µM of each primer miseq_uni_F2 and miseq_uni_R2_bcxxx (where xxx stands for a code corresponding to a unique 9 nucleotide barcode) (Supplementary Table 1), 2 µL of original PCR product and ultrapure water up to

20 µL. Thermal cycling was done at 95°C for 7 min., 8 cycles at 95° for 10 s. 56° for 30 s., then 72° for 5 min. PCR products were held at +4°C after completion of thermal cycling. The products were cleaned as described after the first ligation reaction and quantified using Qubit 2.0 fluorometer (Life Technologies, Thermo Fisher Scientific Inc.). Amplicons were pooled in equimolar quantities into one amplicon library. Sequencing using Illumina MiSeq was done at Macrogen Inc. in Seoul, South Korea.

V1 – V3 regions of bacterial 16S rRNA genes were sequenced using Illumina MiSeq at the Institute of Biotechnology at the University of Helsinki. Prior to sequencing, a two-step PCR was used to amplify V1-V3 regions of 16S rRNA genes, using the primers F27 (Chung *et al.* 2004) and pD´ (Edwards *et al.* 1989), amended with partial TruSeq adapter sequences at their 5' ends. Sterile water instead of template DNA was added into PCR control samples.

Bioinformatics

Archaeal 16S rRNA gene sequences were analyzed using QIIME software package, version 1.8.0 (Caporaso *et al.* 2010). Paired-end reads of archaeal 16S rRNA gene amplicons from Illumina MiSeq sequencing were joined with SeqPrep program (URL: https://github.com/jstjohn/SeqPrep). Reads were subsequently quality filtered with split_libraries_fastq.py command using default settings, except that the maximum unacceptable Phred quality score was set at 19. Reads passing quality filtering were clustered into OTUs using pick_open_reference_otus.py workflow command. OTUs were clustered at 97% similarity level. Representative OTU sequences were aligned and checked for presence of chimeras using Chimera Slayer. Taxonomic classification of OTUs was done using BLAST algorithm (Altschul *et al.* 1990) and Silva database, release 111 as a reference database (Pruesse *et al.* 2007).

Bacterial 16S rRNA gene sequences were joined using Pear 0.9.10 (Zhang *et al.* 2014). Reads were subjected to quality filtering and phiX removal using bbduk.sh script provided by BBTools 37.02. The reads were subsequently subjected to the UPARSE pipeline for OTU calling implemented in usearch version 9.2.64 using the standard parameter minsize 2 with the cluster_otu functionality (Edgar 2013). OTU taxonomic classification was performed using assign_taxonomy.py script with standard parameters provided by Qiime version 1.9.1 (Caporaso *et al.* 2010), using Silva database release 128 as a reference database (Quast *et al.* 2013). OTU sequences were aligned using Sina version 1.2.11 (Pruesse *et al.* 2012) and Silva database release 128 as a reference database. The processed sequence data was normalized using cumulative-sum scaling (CSS) (Paulson *et al.* 2013) in metagenomeSeq R package (Paulson *et al.*).

An additional analysis was performed for the terrestrial group Thaumarchaeota from this study to investigate their similarity to 1.1c thaumarchaeotal sequences retrieved from fungal samples by (Bomberg *et al.* 2010). To investigate the pairwise similarity (%), the selected sequence fragments from our study were aligned with 16S rRNA gene sequences from 1.1c Thaumarchaeota from the previous study using Geneious software version 6.1.5 (Kearse *et al.* 2012).

Statistical analyses

Differences in archaeal and bacterial 16S rRNA gene copy abundances determined by qPCR in different fruiting bodies were analyzed using the nonparametric Kruskal-Wallis test, and Wilcoxon signed rank sum test for post hoc comparisons. Tests were performed using the R package Stats (R Core Team 2015), with functions Kruskal.test and wilcox.test for non-paired samples. A regression analysis was used to model the effect of fungal species (n=6) on

the ratio of archaeal to bacterial 16S rRNA gene copies (R). *L. perlatum* was used as reference group in the analysis. The ratio R was modelled as: $R=\beta_0+ \beta_i X_i + \varepsilon$, where β_0 = reference group, X_1 = *A. arvensis* , X_2 = *B. pinophilus,* X_3 = *C. cibarius,* X_4 = *P. betulinus,* X_5 = *S. bovinus* and ε is the error term. The model was constructed in R environment using the function lm in the package Stats (R Core Team 2015). Differences in bacterial communities in the fruiting bodies of fungal species were determined by distance-based Redundancy Analysis (db-RDA) using the function capscale in R package vegan (Oksanen et al. 2017). In the db-RDA, fungal species were used as explanatory variable to constrain the normalized 16S rRNA gene sequence data. The Bray-Curtis dissimilarity index was used to measure between-sample dissimilarity. The significance of differences between bacterial communities in each fungal species was calculated by the function adonis in R package vegan (Oksanen *et al.* 2017), with 999 permutations.

Nucleotide sequence accession number

Raw sequence data have been deposited to the National Center of Biotechnology Information's Sequence Read Archive under study accession number **SRP073783**.

Results

Quantities of archaeal and bacterial 16S rRNA gene copies

The quantity of archaeal rRNA gene copies ranged from 3.0 $x10^6$ (in *L. perlatum*) to 3.2 $x10^8$ (in *S. bovinus*) copies per gram (fw) of fruiting body tissue. Copy numbers varied

significantly between different species (Kruskal-Wallis chi-squared = 22.638, df = 5, p = 0.0004) (Figure 1a). Bacterial 16S rRNA gene copy numbers ranged from 5.9 $x10^6$ (in *B. pinophilus*) to 1.9 $x10^8$ copies per gram (in *P. betulinus*). Variations in bacterial copy numbers between species were also significant (Kruskal-Wallis chi-squared = 21.988, df = 5, p-value = 0.0005) (Figure 1b). Archaeal 16S rRNA gene copy abundance exceeded that of bacterial in all six specimens of *S. bovinus* and *B. pinophilus*. In *C. cibarius*, archaeal and bacterial 16S copy abundance were roughly equal in half of the specimens (3), while in the other half of the specimens, bacterial 16S rRNA gene copy abundance clearly exceeded archaeal copy abundance (Supplementary Figure 1). Fungal species had a significant effect on the ratio of archaeal to bacterial 16S rRNA gene copy abundance (regression analysis, $p < 0.001$, Supplementary Table 2); in *S. bovinus* the ratio was is 15.5 times higher and in *B. pinophilus* 22.7 times higher than in *L. perlatum*, which was chosen as a reference group in the analysis because it had the lowest ratio of 0.4 (Figure 2).

Sequences of archaeal 16S amplicons

Sequencing of PCR amplicons amplified with Archaea-specific primers yielded a total of 12737 good quality archaeal 16S sequences, which clustered into 57 OTUs at 97% similarity level. The quality of sequences and thus, sequencing depth varied considerably between samples. Taxonomically classifiable archaeal sequences were distributed in 4-6 archaeal classes depending on the fungal species (Figure 3a). Archaeal communities in fruiting bodies of all fungal species were clearly dominated by thaumarchaeotal classes Terrestrial group, *Thermoplasmata*, and Miscellaneous Crenarchaeotal Group (MCG) while archaea of Marine group I, Soil Crenarchaeotic group (SCG) and Sc-EA05 Thaumarchaeota represented smaller proportions of the communities.

Sequences classified in this study as Terrestrial group Thaumarchaeota had highest (78-99 %) similarities to 1.1c thaumarchaeotal sequences, which were retrieved from mycorrhizosphere samples by Bomberg *et al.* (2010). The highest match (99 % identity) to the sequences from our study originated from a pine mycorrhiza. In comparison, Terrestrial group thaumarchaeotal 16S rRNA gene sequences from this study had 63-75 % similarities with representatives of common soil thaumarchaeotal groups: *Nitrosotalea devanaterra* (group 1.1a) and *Nitrososphaera viennensis* (group 1.1b) (Supplementary Table 3).

Sequences of bacterial 16S amplicons

Sequencing yielded 1647881 sequences that passed quality filtering and they clustered into 177 bacterial OTUs at 97% similarity level. Bacterial communities of all fungal species formed loose groups showing that they had characteristic bacterial populations. Fungal species explained 30 % of the total variation in the bacterial communities (Figure 4). The populations of fruiting bodies of mycorrhizal fungi did not cluster together apart from those of saprophytic fungi. Bacterial orders with highest relative abundances in the entire data set (Pseudomonadales and Bacillales) were present in all fruiting bodies, but their relative abundances showed considerable variation between fungal species (Figure 3b), and sometimes even between the specimens of the same species. Bacterial community compositions of fruiting bodies of *S. bovinus* differed significantly (adonis, p=0.036) from the compositions of other fungal species. Compared to other fruiting bodies, *S. bovinus* had higher relative abundance of Enterobacteriales, Clostridiales and Dehalococcoidia. Orders Pseudomonadales and Bacillales together formed a major proportion of bacterial communities in *A. arvensis* (87 %), *B. pinophilus* (50 %) and *P. betulinus* (46 %). Lactobacillales were particularly abundant in *B. pinophilus*, contributing to the high relative

abundance of the class Bacilli in this species, while *A. arvensis* was heavily dominated by Bacillales. *P. betulinus* had particularly high proportion of Corynebacteria. In *C. cibarius* bacterial community had higher relative abundance of Sphingobacteriales (24 %), Rhizobiales (13 %), Caulobacterales (11 %) and Burkholderiales (10 %) than other fungal species.

Discussion

The results of this study indicate that both archaea and bacteria are abundant in the internal tissues of fruiting bodies, based on enumeration by qPCR. We observed significant variations in the abundance of archaeal and bacterial 16S rRNA gene copies between different fungal species. To our knowledge our data represent the first estimates of archaeal and bacterial abundance in fruiting bodies of fungi obtained using culture-independent approach. The quantities of 16S rRNA gene copies do not correspond to cell numbers as such; according to the ribosomal RNA operon copy number database (rrnDB) version 4.4.4 (Stoddard *et al.* 2015) the number of 16S rRNA gene copies in sequenced archaeal genomes varies from 1 to 4 and 1 to 15 in bacteria. Here, the archaea:bacteria 16S rRNA gene copy number ratios ≥ 1 still indicate that archaea form a significant proportion of prokaryotic biomass in fruiting body tissues of some fungi. Such high ratios of archaeal versus bacterial 16S rRNA gene copy abundances are not common in terrestrial habitats, although in archaea-rich marine sediments archaeal abundances exceeding that of bacteria have been observed (Lipp *et al.* 2008, Lloyd *et al.* 2013). In contrast to previous culture-based studies, our new data show that bacteria are abundant in the internal tissues of fruiting bodies, such as in *S. bovinus*, where the numbers of culturable bacteria were very low (Timonen and Hurek 2006).

This study shows that archaeal communities in fruiting body tissues are diverse based on sequencing of 16S rRNA genes. Fruiting bodies included in this study were colonized by archaeal classes that are commonly found in both aquatic and terrestrial environments. The metabolic potential and roles of these organisms in the prokaryotic community inside the fruiting bodies remains unknown at this point due to lack of cultured representatives or genomic information. A metagenomic assembly of representatives from the "Soil Crenarchaeotic Group" (SCG) suggested, that these archaea might participate in both nitrification and denitrification (Butterfield *et al.* 2016). Some of the dominant groups, such as the ubiquitous MCG group, are diverse both phylogenetically and metabolically (Kubo *et al.* 2012, Meng *et al.* 2014). In marine sediments the MCG group archaea may derive energy from mineralization of proteins (Lloyd *et al.* 2013), degradation of aromatic compounds (Meng *et al.* 2014), and possibly also from physically and chemically recalcitrant organic matter, such as membrane lipids (Takano 2010). Marine group I thaumarchaeota are mostly pelagic mixotrophs also with versatile metabolic potential, including aerobic ammonia oxidation and hydrolysis of urea (Swan *et al.* 2014). Thermoplasmata were the only euryarchaeal class present in the fruiting bodies. Sequences belonging to archaea of this class (order Thermoplasmatales) have been recovered from forest soil (Burke *et al.* 2012) as well as from freshwater habitats (Jurgens *et al.* 2000, Fillol *et al.* 2015). Thermoplasmatales may have methanogenic potential (Paul *et al.* 2012), but their activities are still mostly unknown. In this study, sequencing depth within replicates of same species as well as between different specimens varied considerably and this also likely affected strongly the observed numbers of archaeal OTUs. For this reason statistical assessment of differences between archaeal communities was not performed, as the results would not represent accurately the natural variation between the communities.

Although our results give the first glimpse of the diversity of archaea colonizing internal tissues of fruiting bodies, the short length (< 200 bp) of the 16S rRNA gene fragments set limits to taxonomic resolution and comparisons with uncultivated archaea found in specific habitats. Nevertheless, the short 16S rRNA gene sequences of the terrestrial group Thaumarchaeota from this study had high % identities with sections of longer sequences of mycorrhizosphere associated 1.1c Thaumarchaeota previously found by Bomberg *et al.* (2003, 2010). This implies that archaea from mycorrhizal roots and external hyphae might effectively colonize the fruiting bodies as well. It has been hypothesized previously that the group 1.1c Thaumarchaeota are involved in carbon cycling through uptake and turnover of single-carbon compounds (such as methane, methanol or carbon dioxide) and they may carry out this role also in fruiting bodies as well (Timonen and Bomberg 2009, Bomberg *et al.* 2010).

Bacterial communities between different fungal species showed species-specific characteristics, although only the bacterial community of *S. bovinus* was statistically significantly different from the others in this study. Fungal genus was a significant factor affecting the composition of bacterial community in a study comprising fruiting bodies of eight genera within the class Agaricomycetes (Pent *et al.* 2017). There were large variations in relative abundances of certain bacterial taxa within biological replicates, such as in the case of Corynebacteriales. Because of this, we have focused the discussion of the results on bacterial groups that appeared evenly in biological replicates to avoid spurious conclusions. The variation between biological replicates may be caused by uneven distribution of bacteria within the fungal tissue or variation between individual fruiting bodies. Soil properties may also have an effect on the composition of bacterial community in fruiting body tissue (Pent *et al.* 2017). In this study replicates for each species originated from the same general area and therefore there should be no major differences in soil properties that could have an effect on

the composition of bacterial communities, although we can not exclude the possible effect of minor differences within the sampling locations.

Class Enterobacteriaceae (orders Pseudomonadales and Enterobacteriales) was a predominant bacterial group in all fruiting bodies. They were also predominant groups among bacteria recovered through cultivation from fruiting bodies of *C. cibarius* and *S. bovinus* by Pent et al (2017). Enterobacteria and Pseudomonads can act as mycorrhiza helper bacteria facilitating interaction between plant roots and mycorrhizal fungi (Frey-Klett *et al.* 2007). In this study, we also found a high relative abundance of Bacilli in fruiting bodies of *A. arvensis, P. betulinus* and *B. pinophilus*, whereas they formed only < 2% of the community in *C. cibarius* and *S. bovinus*. Bacilli have been recovered from inner tissues of fruiting bodies through cultivation (Danell *et al.* 1993, Zagriadskaia *et al.* 2014). In line with our findings, Pent *et al.* (2017) found a low relative abundance of Bacilli in *S. bovinus* and none in *C. cibarius* by sequencing bacterial 16S rRNA genes. Orders Clostridiales and and Dehalococcoides had particularly high relative abundances in *S. bovinus*. There are no previous reports of finding Dehalococcoides in fungal fruiting bodies while Pent *et al.* (2017) had detected Clostridiales in some of their fruiting body material. Clostridiales are obligate anaerobes and their role may be related to cellulose degradation (de Boer *et al.* 2005). Dehalococcoides are obligate organohalide respiring bacteria (Loffler *et al.* 2013) and their presence is likely linked to degradation of organohalogens produced by the host. Basidiomycetes fungi are capable of *de novo* synthesis of halogenated organic compounds making them a major source of organohalogens in terrestrial environments (deJong and Field 1997). In our study, *C. cibarius* had higher relative abundance of Sphingobacteriales than in other fungal species. Pent *et al.* (2017) found sequences of these bacteria from *C. cibarius* tissue, but were not able to culture them, which may explain why these bacteria have not been recovered from fruiting body tissues by cultivation in earlier culture-based studies. Also Alphaproteobacterial orders

Rhizobia and Caulobacteria and Betaproteobacterial order Burkholderiales had higher relative abundances in *C. cibarius* than in other fruiting bodies. Alphaproteobacteria were prominent groups in ascomycete *Elaphomyces granulatus* based on relative abundance of 16S rRNA gene sequences (Quandt *et al.* 2015). Rhizobia and Burkholderiales were also found in bacterial 16S rRNA gene sequence libraries from *C. cibarius* and *S. bovinus* in the study by Pent *et al.* (2017). To our knowledge, Caulobacteria have not been detected by sequencing or cultivation in fruiting bodies yet. These three orders may have a role in glucan degradation, as suggested by Eichorst and Kuske (2012). Bacteria belonging to these classes are adapted in low-nutrient environments and they may have a role in supplementing nutritional demands of the host by fixing nitrogen, (Li and Castellano 1987, Barbieri *et al.* 2010, Sellstedt and Richau 2013), or solubilizing phosphate for the use of the fungus (Pavic *et al.* 2013).

The internal environment in fruiting bodies reshapes the bacterial communities compared to communities found e.g. in *Pinus sylvestris* mycosphere and in the surrounding uncolonized soil. These environments are dominated by bacteria belonging to classes Alphaproteobacteria, Actinobacteria and Acidobacteria (Timonen *et al.* 2017). Factors affecting the composition of the prokaryotic community in the fruiting body tissue include the presence of antimicrobial compounds excreted by the fungi (de Carvalho *et al.* 2015). Also carbohydrate, crude protein, sugar and lipid contents between *Boletus edulis, A. arvensis, C. cibarius and L. perlatum* can vary greatly (Barros *et al.* 2007, Barros *et al.* 2008, Kalac 2009, Heleno *et al.* 2011), which could be a selecting factor for prokaryotic community composition. The availability of different carbon sources inside the fruiting bodies as well as the ability of colonizing prokaryotes to utilize the fungal storage sugars (such as trehalose and mannitol) could explain at least some proportion of the variation seen in prokaryotic community compositions. Also, the physical composition of the fruiting body, such as

403 porosity and moisture, may play a role in shaping the prokaryotic abundance and community
404 composition and distribution within the fruiting body. It is likely that the increased moisture
405 of degrading fruiting bodies with larval infestation and increased leakage of substrates from
406 fungal tissues could support more bacteria than young fruiting bodies. All fruiting bodies
407 analyzed in this study were relatively young and showed no signs of decay. However, even
408 small variations in fruiting body age may cause differences in the archaeal and bacterial 16S
409 copy abundances and community composition as the biochemical composition of the fruiting
410 body tissue changes over time (Citterio *et al.* 2001, Barbieri *et al.* 2010).

411 The view of archaeal biomass in ecosystems and their contribution to biogeochemical cycles
412 has changed radically in recent years - however, our understanding of their distribution in
413 different habitats is still developing. The mixture of both aquatic and terrestrial archaeal
414 classes in the communities colonizing the tissues of fruiting bodies suggests that present
415 habitat-based broad classification will likely be subject to change in the future, as archaeal
416 diversity in different habitats is further explored. The data from this study do not explain the
417 success of archaea in fruiting body tissues. The apparent enrichment of archaea in fruiting
418 body tissues of *B. pinophilus* and *S. bovinus* suggests that fungi-archaea associations must be
419 important in ecosystems to the extent that archaea remain associated with the fungi even
420 outside the soil environment to accompany fruiting bodies during their short life cycle
421 (approximately 2 weeks). It is not yet known whether the composition of the archaeal
422 population changes over the life cycle of the host and whether the archaeal activity affects the
423 host somehow. Such an abundance of archaea in this (or any) natural habitat shows that the
424 environment is important in shaping the composition of the microbial community associated
425 with it. Differences in bacterial abundance and in community composition between different
426 fungal species suggest that bacterial populations form a network of interactions between
427 themselves and the host. The composition of the community is likely a result of protagonistic

and antagonistic interactions between the host and microbes as well as between the microbes themselves. A recent study by Schulz-Bohm *et al.* demonstrated the pervasive effect of microbes to a life style of a saprotrophic fungus *Mucor hiemalis* (Schulz-Bohm *et al.* 2017). An antibiotic-induced shift in microbial community composition altered the morphology, secondary metabolite production and morphology of the fungus. These results suggest that the network of interactions between fungi and bacteria may be more complex than is previously thought and bacteria are important cohabitants for fungi.

Our findings transform our view of prokaryotic populations in fruiting bodies. We identify fruiting bodies as a previously unknown habitat for temperate archaeal populations, where in some cases archaeal abundance may exceed that of bacteria. We also show that fruiting bodies of different fungal species harbor characteristic bacterial communities.

Acknowledgements

This work was funded by Finnish Academy grant no. 131819. We would also like to acknowledge Sarah Preheim for designing and generously providing the adapter and barcode sequences for our use in Illumina sequencing and Daniel Piotrowski for proofreading the manuscript.

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632 Tables

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634 Table 1. Fungal species included in the study and characterization of sample collection sites located in

635 Southern Finland.

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Species	**Sample code**	**Coordinates of sample collection sites**	**Site characteristics (no. of specimens collected)**
Suillus bovinus (Fr.) Roussel	Sb	60°01´ N, 23°34´ E	Dry pine forest (6).
Boletus pinophilus Pilát & Dermek (Bp)	Bp	60°38´ N, 25°20´ E, 59°54´ N, 23°43´ E	Dry pine forest (5), mixed forest (1)
Cantharellus cibarius Fr.	Cc	60°01´ N, 23°34´ E 59°54´ N, 23°43´ E,	Dry pine forest (2), mixed forest (4)
Agaricus arvensis Schaeff.	Aa	60°11´ N, 24°53´ E	Grassy field (6)
Lycoperdon perlatum Pers.	Lp	60°13´ N, 25°01´ E	Mixed forest (6)
Piptoporus betulinus (Bull.) P. Karst.	Pb	59°54´ N, 23°43´ E	Mixed forest (6)

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642 Figure captions

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644 Figure 1. Abundance of archaeal (A) and bacterial (B) 16S rRNA gene sequences in fruiting

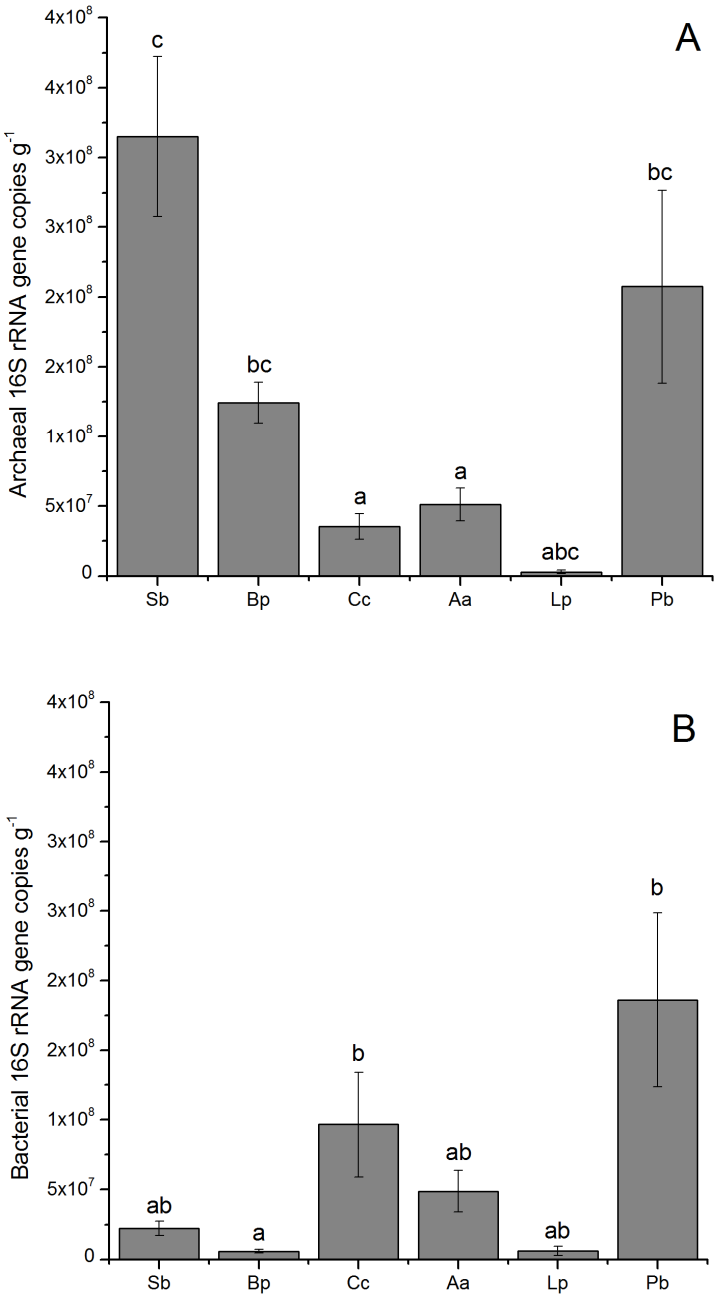
645 bodies. Solid bars represent means (n=6, except for Lp n=3), and error bars standard errors.

Different letter above the bar indicates statistically significant difference (Wilcoxon signed rank sum test, $p < 0.05$).

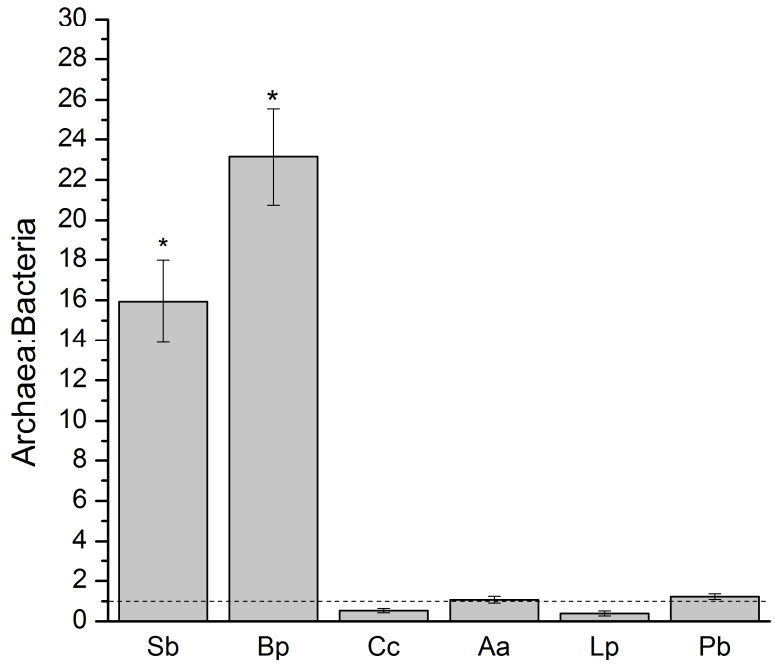
Figure 2. Ratio of archaeal to bacterial 16S rRNA gene copy abundance. The copy numbers were determined using qPCR with domain-specific primers. Regression analysis determined that *S. bovinus* and *B. pinophilus* (marked with an asterisk) have statistically significant ($p < 0.001$), increasing effect on the ratio of archaeal to bacterial 16S rRNA gene copy numbers. Dashed line indicates a ratio of 1:1.

Figure 3. Taxonomic distribution of archaeal (A) and bacterial (B) 16S sequences in different fungal species. Relative abundances are calculated from pooled sequences of three biological replicate samples.

Figure 4. Distance-based Redundancy Analysis (db-RDA) of bacterial populations in fruiting body tissues. The ordination is based on Bray-Curtis dissimilarity using fungal groups as explanatory variables. The ellipses represent variation around the group centroids at 0.75 confidence interval.

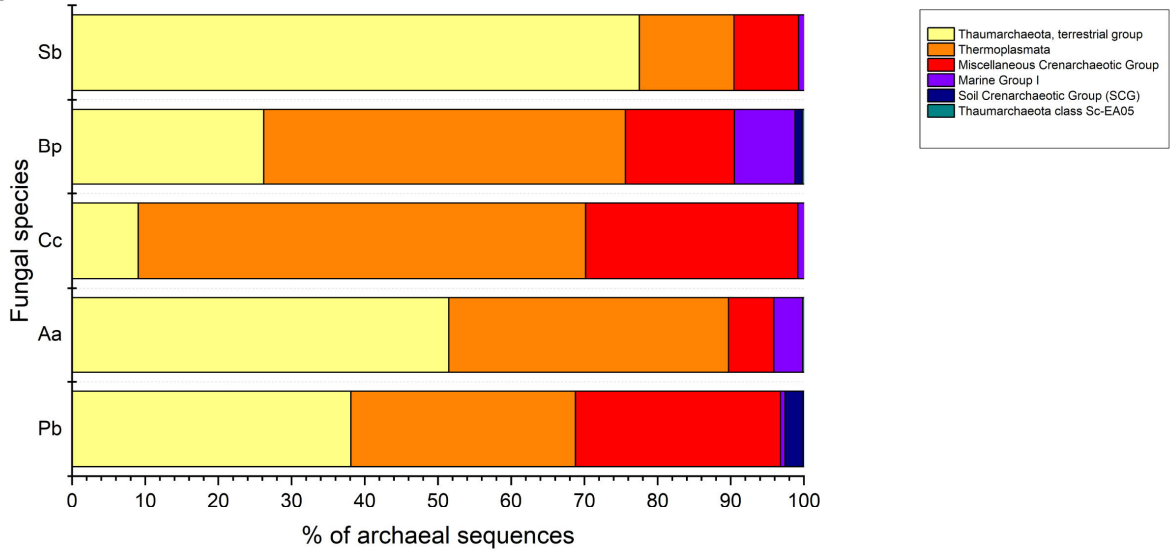

A
Archaeal 16S rRNA gene copies g-1
4x10^8
4x10^8
3x10^8
3x10^8
2x10^8
2x10^8
1x10^8
5x10^7
0
c
bc
a
a
abc
bc
Sb
Bp
Cc
Aa
Lp
Pb
B
Bacterial 16S rRNA gene copies g-1
4x10^8
4x10^8
3x10^8
3x10^8
2x10^8
2x10^8
1x10^8
5x10^7
0
ab
a
b
ab
ab
b
Sb
Bp
Cc
Aa
Lp
Pb

664

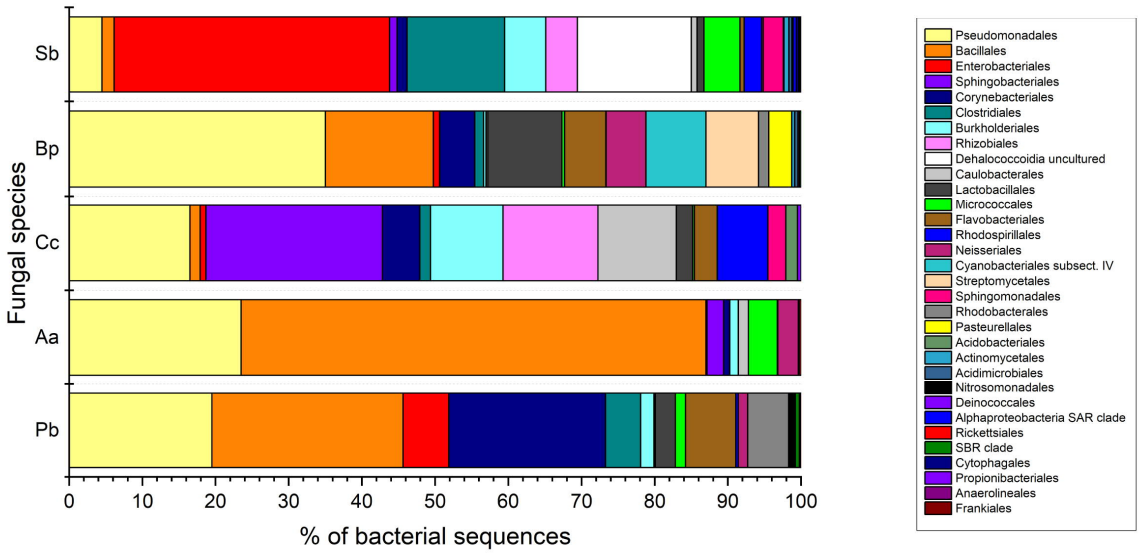

Archaea:Bacteria
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
*
*
Sb
Bp
Cc
Aa
Lp
Pb

665

A


Sb
Bp
Cc
Aa
Pb
Fungal species
0
10
20
30
40
50
60
70
80
90
100
% of archaeal sequences
Thaumarchaeota, terrestrial group
Thermoplasmata
Miscellaneous Crenarchaeotic Group
Marine Group I
Soil Crenarchaeotic Group (SCG)
Thaumarchaeota class Sc-EA05

B


Sb
Bp
Cc
Aa
Pb
Fungal species
0
10
20
30
40
50
60
70
80
90
100
% of bacterial sequences
Pseudomonadales
Bacillales
Enterobacteriales
Sphingobacteriales
Corynebacteriales
Clostridiales
Burkholderiales
Rhizobiales
Dehalococcoidia uncultured
Caulobacterales
Lactobacillales
Micrococcales
Flavobacteriales
Rhodospirillales
Neisseriales
Cyanobacteriales subsect. IV
Streptomycetales
Sphingomonadales
Rhodobacterales
Pasteurellales
Acidobacteriales
Actinomycetales
Acidimicrobiales
Nitrosomonadales
Deinococcales
Alphaproteobacteria SAR clade
Rickettsiales
SBR clade
Cytophagales
Propionibacteriales
Anaerolineales
Frankiales

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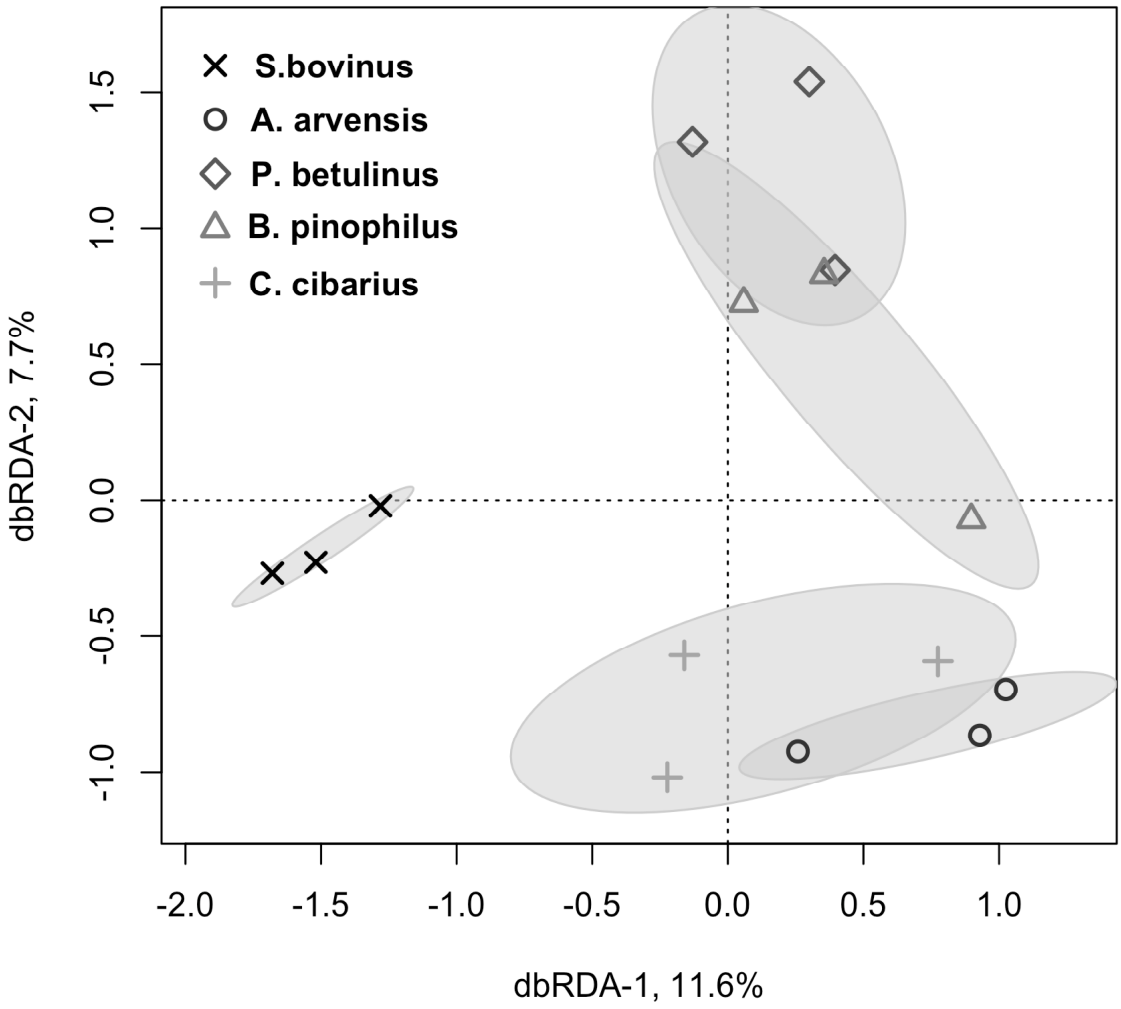

S.bovinus
A. arvensis
P. betulinus
B. pinophilus
C. cibarius
dbRDA-2, 7.7%
dbRDA-1, 11.6%
1.5
1.0
0.5
0.0
-0.5
-1.0
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0

667

Archaea are prominent members of the prokaryotic communities colonizing common forest mushrooms

Rinta-Kanto JM, Pehkonen, K, Sinkko H, Tamminen MV, Timonen S

Supplementary information

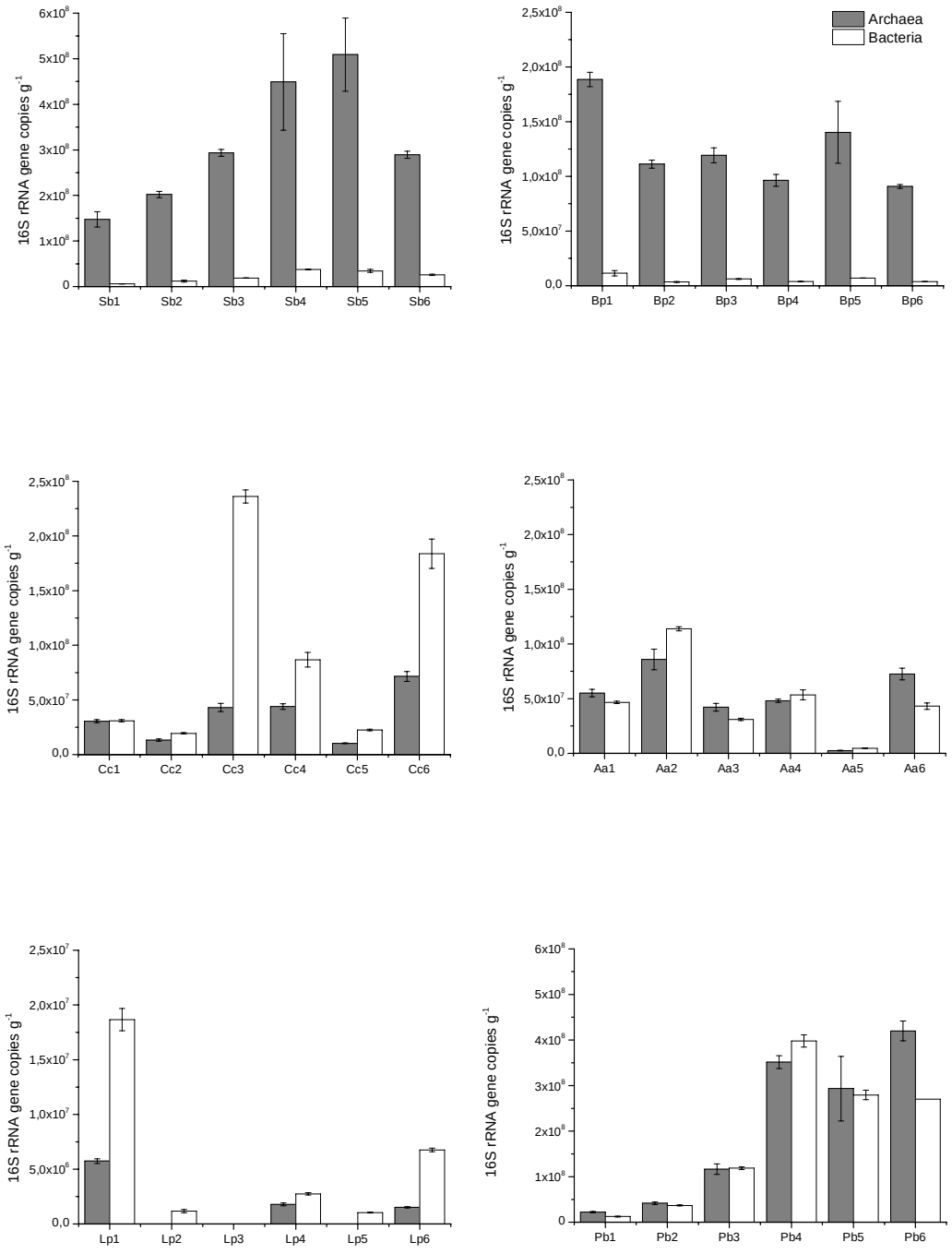
Supplementary Table 1. Primers used for Illumina sequencing library preparation for sequencing of archaeal 16S rRNA gene fragments.

Primer name	Sequence
miseq_A349_F1	ACACGACGCTCTTCCGATCTYRYRGYGCASCAGKCGMGAAW
miseq_uni_F2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
miseq_A539_R1	GGAGTTCAGACGTGTGCTCTTCCGATCTGCBGGTDTTACCGCGGCGGCTGRCA
miseq_uni_R2_bc001	CAAGCAGAAGACGGCATACGAGATTCCGTGCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc002	CAAGCAGAAGACGGCATACGAGATTGTTTCCCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc003	CAAGCAGAAGACGGCATACGAGATGGTAATGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc004	CAAGCAGAAGACGGCATACGAGATGAAACTGGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc005	CAAGCAGAAGACGGCATACGAGATACGGGCTGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc006	CAAGCAGAAGACGGCATACGAGATATGAAGTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc007	CAAGCAGAAGACGGCATACGAGATACTTATTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc008	CAAGCAGAAGACGGCATACGAGATGGCGGGAAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc009	CAAGCAGAAGACGGCATACGAGATACACCTCGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc010	CAAGCAGAAGACGGCATACGAGATCTCATTGGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc011	CAAGCAGAAGACGGCATACGAGATGCTGCCGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc012	CAAGCAGAAGACGGCATACGAGATCGATGGTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc013	CAAGCAGAAGACGGCATACGAGATTCAAAGCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

miseq_uni_R2_bc014	CAAGCAGAAGACGGCATACGAGATCAGCGGCATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc015	CAAGCAGAAGACGGCATACGAGATCCGACAAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc016	CAAGCAGAAGACGGCATACGAGATTAAGGGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc017	CAAGCAGAAGACGGCATACGAGATTTGTGGCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc018	CAAGCAGAAGACGGCATACGAGATAGGTCGGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc019	CAAGCAGAAGACGGCATACGAGATAATGTCAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc020	CAAGCAGAAGACGGCATACGAGATGTTCGCAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc021	CAAGCAGAAGACGGCATACGAGATTATCAATCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc022	CAAGCAGAAGACGGCATACGAGATGTCTAACGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc023	CAAGCAGAAGACGGCATACGAGATTTACTATACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc024	CAAGCAGAAGACGGCATACGAGATTGCACCCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc025	CAAGCAGAAGACGGCATACGAGATTGGGACCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc026	CAAGCAGAAGACGGCATACGAGATGAGTTTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc027	CAAGCAGAAGACGGCATACGAGATAACAGTATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc028	CAAGCAGAAGACGGCATACGAGATATCGCACCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc029	CAAGCAGAAGACGGCATACGAGATCTAGAATCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
miseq_uni_R2_bc030	CAAGCAGAAGACGGCATACGAGATCGCCAAGGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Supplementary Table 2. Regression analysis for the square root-transformed ratio of the mean of archaeal to bacterial rRNA gene copies in different fungal species (n=6, except L. perlatum n=3). In the results, estimate for intercept = mean of *L. perlatum* (reference group).

	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	0.6109	0.2256	2.708	0.0116
A. arvensis	0.4057	0.2763	1.468	0.1536
B. pinophilus	4.1687	0.2763	15.088	1.12E-14
C. cibarius	0.0989	0.2763	0.358	0.7232
P. betulinus	0.4885	0.2763	1.768	0.0883
S. bovinus	3.3445	0.2763	12.104	2.03E-12



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722 Supplementary Figure 1. Abundances of archaeal and bacterial 16S rRNA gene copies in biological
723 replicates of fungal specimens included in this study. Labels on the x-axes correspond to the initial
724 letters of the fungal species names and the number (1-6) identifies the biological replicate.

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