Differential sharing and distinct co-occurrence networks among spatially close bacterial microbiota of bark, mosses and lichens

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Abstract

Knowledge of bacterial community host-specificity has increased greatly in recent years. However, the intermicrobiome relationships of unrelated but spatially close organisms remain little understood. Trunks of trees covered by epiphytes represent complex habitats with a mosaic of ecological niches. In this context, we investigated the structure, diversity and interactions of microbiota associated with lichens, mosses and the bare tree bark. Comparative analysis revealed significant differences in the habitat-associated community structures. Corresponding co-occurrence analysis indicated that the lichen microbial network is less complex and less densely interconnected than the moss- and bark-associated networks. Several potential generalists and specialists were identified for the selected habitats. Generalists belonged mainly to *Proteobacteria*, with *Sphingomonas* as the most abundant genus. The generalists comprise microorganisms with generally beneficial features, such as nitrogen fixation or other supporting functions, according to a metagenomic analysis. We argue that beneficial strains shared among hosts contribute to ecological stability of the host biocoenoses.

Keywords: amplicon sequencing, co-occurrence patterns, host microbe associations, metagenome, microbial ecology

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Introduction

Microbes are ubiquitous associates of all multicellular host organisms. Recent research showed that microbial communities can modulate fitness traits of their hosts, and because this capacity is of great ecological significance, the role of microbiota for plants has received much interest in environmental microbiology and biotechnology (Frey-Klett *et al.* 2007; Berg *et al.* 2014), most of the research therefore focused on economically important crops. In comparison, the diversity of microbiomes in naturally abundant and ecologically important plant phenotypes has been far less studied. However, with an estimated total number of around

3.04 trillion trees (Crowther *et al.* 2015), they could be particularly important reservoirs of microbial communities worldwide. Trees also develop long living phenotypes that provide varied surfaces for microbial colonization.

Focusing on the Ginkgo tree, Leff *et al.* (2014) analysed the structure of branch and trunk microbiota. They detected less diversity in leaf communities, and these were distinct from communities of branches and barks. Laforest-Lapointe *et al.* (2016) compared the phyllosphere of various tree species. According to their results, the substantial intraspecific variation is not significantly different from variation among species, whereas the location of the leaves contributed only 6% of the variation. Surface and other host-specific characteristics in the heterogeneous tree habitats might account for clear differences in the associated

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microbiomes. In addition, tree barks are rarely bare in their natural environment. Their persistent surfaces provide stable substrates for other organisms, known as epiphytes. The most common types of tree epiphytes are mosses and lichens.

Lichens produce macroscopic fungal morphs, which shelter internalized photosynthetic partners and host previously poorly recognized bacteria and fungi in their surfaces layers (Bjelland *et al.* 2011; Erlacher *et al.* 2015; Grube *et al.* 2015; Spribille *et al.* 2016). The colonizing bacteria form host-specific communities (Grube *et al.* 2009), commonly with *Alphaproteobacteria* (mainly the order *Rhizobiales*) as the predominant group followed by other lineages such as *Acidobacteria*, *Actinobacteria*, *Betaproteobacteria*, *Spartobacteria* and *Sphingobacteria* (Cardinale *et al.* 2008; Bates *et al.* 2011; Mushegian *et al.* 2011; Hodkinson *et al.* 2012; Aschenbrenner *et al.* 2014). The potential metabolic contribution by bacteria for the lichen holobiont was recently studied by metagenomics and metaproteomics (Grube *et al.* 2015). Bacterial functions included phosphate solubilization, nitrogen fixation and release of amino acids, besides provision of vitamins, detoxification and stress resistance mechanisms. Microbiota of mosses are also specific for their host species (e.g. Opelt & Berg 2004; Bragina *et al.* 2012, 2014). Some of the culturable strains have growth-promoting effects on mosses (Tani *et al.* 2011), and *Cyanobacteria* associated with mosses can contribute to forest ecosystems by nitrogen fixation (Lindo & Whiteley 2011).

Recent work of Lymperopoulou *et al.* (2016) suggested that vegetation contributes to the microbial composition of nearby outdoor air, and it is thus likely that neighbouring members of vegetation may have a rich exchange of their microbiota. As both mosses and lichens are significant niches coexisting in close neighbourhood on tree barks, they are exposed to the same set of airborne microbiota, but might selectively enrich bacteria, which represent a specific community of their hosts. In this study, we address the question of specificity and intermicrobiome relationship using high-throughput sequencing. We characterize the host-associated microbiomes of the lung lichen *Lobaria pulmonaria* (L.) Hoffm., the predominant co-occurring moss *Pterygynandrum filiforme* Hedw., and the bark of the maple tree, *Acer pseudoplatanus* L., on which the two epiphytes occur. The lung lichen develops in unpolluted, old-growth forests. The observed moss species is frequent in cool montane environments. Comparative metagenomics based on 16S rDNA were used to test to what extent the three habitats support distinct microbiota and whether differences exist in the community structures. The metagenomic analysis was applied more specifically to reveal differences in the functional

potential assignable to a habitat generalist and a lichen specialist.

Materials and methods

Sampling procedure, DNA extraction and sequencing

To explore bacterial community composition and diversity in adjacent habitats [moss: *Pterigynandrum filiforme* (Hedw.); lichen: *Lobaria pulmonaria* (L.) Hoffm.; bark: from *Acer pseudoplatanus* (L.)] in Johnsbach (Styria, Austria, N 47°32′35″, E 14°37′38″, 1175 m above sea level; October/2014) 24 moss, lichen, and bark samples (in total 72) from six trees (*A. pseudoplatanus*) were collected in an open patch of a montane forest dominated by maple in an area of about 500 sqm. Samples were collected from the tree trunk below the primary branching in a height between 1 and 2 metres above ground using sterile tweezers. Specimens were stored separately in sterile polyethylene bags and cooled on dry ice until further processing in the laboratory. Moss and lichen samples were ground with sterilized mortar and pestle, while bark samples were ground with a sterilized hand mill. DNA was extracted using the MoBio Power Soil DNA isolation kit according to manufacturer's protocol. Each DNA extract was supplemented with peptide nucleic acid PCR clamps (0.75 μM of each PNA in the final reaction in a ratio of pPNA:mPNA 1:1) as described by Lundberg *et al.* (2013) and then used as template for PCR amplifications (triplicates with subsequent pooling) with the universal bacterial primer set 515f/806r to target the 16S rDNA hypervariable region 4 (Caporaso *et al.* 2011). Barcoded samples were pooled equimolarly and sent for paired-end Illumina MiSeq sequencing (MWG Eurofins, Germany).

Initial data analysis

Data analysis was performed using the pipeline QIIME 1.9.0 (Caporaso *et al.* 2010). Initially, raw Illumina MiSeq forward and reverse reads were joined (default method: fastq-join) for each of the 72 samples. The fastq sequence data demultiplexing was performed with a minimal per nucleotide phred quality threshold of Q = 20 and a maximum number of errors in the barcodes of 1.5 (QIIME default setting). Additionally, the sequences were quality-checked for chimeric sequences (usearch61; Edgar 2010). Subsequently, the OTU table was generated with the script 'pick_open_reference_otus.py' using default settings except for the reference database. The more comprehensive SILVA database (release 119; Pruesse *et al.* 2007) was employed for reference sequences and for taxonomy

assignment instead of the Greengenes database. OTU clustering was performed with uclust as OTU picking method and a sequence similarity threshold of 97% representing theoretical taxonomic units at species level. Chloroplast, mitochondrial and other nonbacterial sequences were excluded from the data set before further analyses.

Structure and diversity of bacterial communities across the habitats

For a general description of the bacterial community structure and diversity the initial OTU tables were rarefied to 5100 sequences at a genetic distance of 3%. Samples comprising less than this threshold were discarded (more precisely: three samples in total – one lichen and two bark samples).

Beta-diversity significance between sample groupings (grouped per habitat type) was calculated with Adonis based on weighted and unweighted UniFrac distances (Lozupone & Knight 2005). Additionally, a nonmetric multidimensional scaling (NMDS) analysis was conducted using the R (R Core Team, 2013) packages vegan (Oksanen *et al.* 2016) and labdsv (Roberts 2016) based on a Bray–Curtis dissimilarity matrix of pairwise distances between samples. Based on a nonparametric two-sample *t*-test using the default number of Monte Carlo permutations (999), a comparative analysis of the habitat-specific alpha diversity indices, richness (Shannon diversity index) and evenness (Simpson's equability) was performed. Comparative analysis on OTU frequencies to identify statistically significant differences between OTU abundances in the three habitats was performed with the QIIME script 'group_significance.py' using the Kruskal–Wallis test based on the rarified OTU table.

Identification of bacterial generalists and habitat specialists

In order to determine generalists and specialists at OTU level, the initial biome table was reduced to OTUs, which were present in at least three of four replicates per habitat per tree to create a more well-founded data set for further analysis. Then, the reduced OTU table was rarified (5290 sequences per sample; consequently five samples were discarded). Subsequently, OTUs, which occurred in more than 90% of all remaining samples regardless of their habitat affiliation, were termed as bacterial generalists. Further all OTUs (with a minimal count of at least 20 sequences), which occurred exclusively in one of the habitats and were detectable in at least one third of the habitat-specific samples, were categorized as specialists. All other OTUs, which did

not match these criteria, were not taken into consideration. For further descriptions, these OTUs were taxonomically summarized at genus level. To identify the lichen-specific lineage LAR1 ('Lichen-associated-*Rhizobiales* 1') in our data set, we utilized 19 LAR1 sequences from GenBank (Accession nos.: GU191849.1, GU191851.1–GU191857.1, GU191859.1, GU191860.1, GU191864.1-GU191872.1) and aligned them to all representative sequences of our *Rhizobiales*-affiliated OTUs (regardless of the habitat affiliation) with ClustalX2 (Larkin *et al.* 2007) to create a bootstrapped neighbour-joining tree. Additional BLASTn searches using the NCBI standard nucleotide BLAST analysis tool were performed with the representative sequences of cyanobacterial OTUs (min. 20 sequences; detectable in at least one third of the samples) for a more detailed characterization (Zhang *et al.* 2000).

Habitat-specific bacterial association networks

The 16S rDNA data described above were processed as three subsets, one for each habitat (bark, moss, lichen) and then normalized to a total count of 7360 sequences per sample. Subsequently, the core OTUs, here defined as the OTUs which were present in at least 75% of the samples, were identified for each data subset and then encoded as a matrix in which each row represented a bacterial OTU and each column the habitat-specific samples including the taxonomic assignment metadata as the last column. Finally, the bark, moss and lichen subsets comprised 214 OTUs (21 samples), 278 OTUs (24 samples), and 330 OTUs (22 samples), respectively. The construction of a correlative co-occurrence network was performed with the Cytoscape plugin CONET 1.1.0.-beta (http://psbweb05.psb.ugent.be/conet/) based on an ensemble approach by combining four different measures: Pearson, and Spearman (pairwise correlation measures), and Bray–Curtis, and Kullback–Leibler (dissimilarity measures) as described by Faust *et al.* (2012). Instead of setting measure-specific thresholds manually, we requested the 1000 top- and bottom-ranking edges for each method to retrieve positive as well as negative correlations. Additionally, edge scores were calculated only between clade pairs without parent–child relationship to prevent correlations between higher and lower level taxa of the same lineage. Finally, *P*-values were computed from method- and edge-specific permutation and bootstrap score distributions with 1000 iterations each. Before the removal of unstable edges which scores were outside the 0.95 range of their bootstrap distribution, measure-specific *P*-values were merged using Brown's method and corrected for multiple testing by the Benjamini–Hochberg method. Co-occurrence patterns were visualized as networks using CYTOSCAPE

version 3.2.1 with the implemented organic layout (Shannon *et al.* 2003). Corresponding statistical analysis was conducted with the implemented tool NETWORKANA-LYZER (release 2.7; Assenov *et al.* 2008).

FISH/CLSM

Fluorescence in situ hybridization (FISH) was performed to visualize the colonization pattern of *Alphaproteobacteria* among other eubacteria on the lichen thallus. For this purpose, thalli were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) with a ratio of 3:1 at 4 °C for at least 4 h. Prior to FISH application (according to Cardinale *et al.* 2008), cross sections of the thalli were prepared. For the detection of *Alphaproteobacteria,* the Cy5-labelled probe ALF968 (42 °C, 35% formamide; Neef 1997) and an equimolar mixture of the Cy3-labelled probes EUB338, EUB338II, EUB338III (42 °C, 15% formamide) for eubacteria was used (Amann *et al.* 1990; Daims *et al.* 1999). Additionally, NONEUB probes (Wallner *et al.* 1993) labelled with the respective fluorochromes, were used as negative controls. Details about oligonucleotide probes are available on probeBase (Loy *et al.* 2007). SlowFade Gold antifade reagent (Molecular Probes, Eugene, OR, USA) was used to suppress photobleaching of the fluorescent-labelled probes. Visualization was performed with a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany). Confocal stacks were processed with IMARIS 7.3 (Bitplane, Zurich, Switzerland). Overlapping micrographs were processed with Microsoft Image Composite Editor to create stitched panorama images.

Functional analysis of a generalist and a specialist

The potential functional contributions of a generalist and a lichen-associated specialist were examined based on the metagenome of the *Lobaria*-associated microbial community (ID 4530091.3, Grube *et al.* 2015) deposited on the metagenomics analysis server MG-RAST (Meyer *et al.* 2008). Meta-genomic sequences were compared to GenBank using the implemented analysis tools on MG-RAST with a maximum e-value of 1e-5 and a minimum identity cut-off of 70%. Subsequently, all reads assigned to the genera *Sphingomonas* (*Proteobacteria*) and *Mucilaginibacter* (*Bacteroidetes*) were aligned to the protein reference database NCBI-NR using DIAMOND with default settings (version 0.7.9; Buchfink *et al.* 2014). Functional assignments were performed with MEGAN5 (version 5.10.5; Huson *et al.* 2011) based on SEED classification (Overbeek *et al.* 2005). The abundances of these function-assigned sequences were normalized for further comparison.

Results

Bacterial community structure and diversity of three neighbouring habitats

The bacterial community composition and diversity of the adjoining habitats *Lobaria pulmonaria* (lichen), *Pterigynandrum filiforme* (moss) and *Acer pseudoplatanus* (maple, tree bark, Fig. 1A) were assessed using Illumina-based high-throughput sequencing of 16S ribosomal RNA gene amplicons. After initial processing, 33 817 operational taxonomic units (OTUs) were identified at species level (97% sequence identity) with a total read count of 1 121 997 sequences (min: 2455; max: 45 032) representing 24 bark, moss and lichen specimens, respectively. The species richness (Shannon's H: bark: 7.94 ± 0.86; moss: 8.58 ± 0.67; lichen: 8.04 ± 0.61) did not reach saturation and covered 48.19% (SD 4.41), 45.98% (SD 3.02), and 44.79% (SD 3.24) of the estimated OTU richness (according to the chao1 estimator; Chao
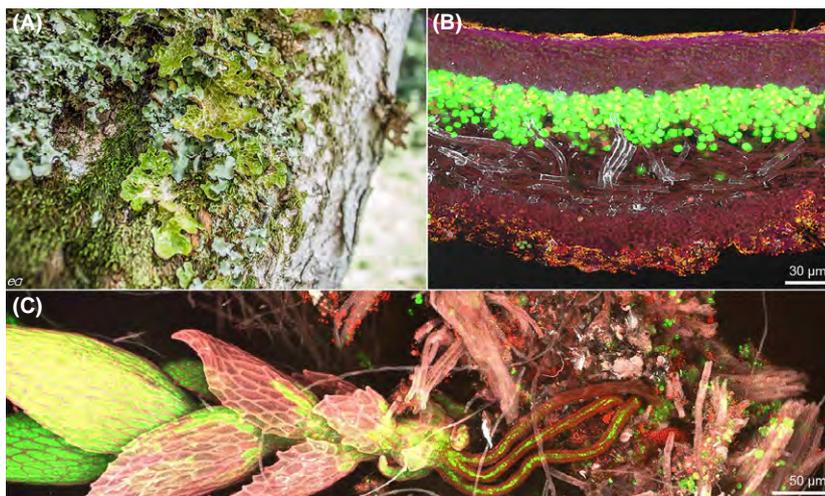

Fig. 1 (A) Macroscopic picture of a maple tree indicating the adjacent microhabitats: *Lobaria pulmonaria* (L.) Hoffm. (lichen), *Pterygynandrum filiforme* Hedw. (moss) and *Acer pseudoplatanus* L. (tree bark); (B) Confocal laser-scanning microscopy of a lichen cross section. *Alphaproteobacteria* (yellow) and other eubacteria (red) colonize the upper and lower cortex of the lichen thallus, and the algae are green dots forming an internal layer. (C) Stitched panorama micrograph of moss plant and lichen fragments with associated bacteria.

1984) at species level for bark, moss and lichen samples, respectively (Figs S1 and S2, Table S1, Supporting information). Comparative analysis of habitat-specific alpha diversity indices (Shannon diversity index and Simpson's equability), based on a nonparametric two-sample *t*-test, revealed no significant difference between the bark and the lichen-associated microbiota in terms of their richness ($P = 1.0$), but a significant difference in their evenness (Simpson's D: bark: 0.091 ± 0.040, lichen: 0.046 ± 0.031, $P = 0.003$). However, moss-associated microbiota showed the highest species richness and were significantly different to both bark ($P = 0.015$) and lichen-associated ($P = 0.048$) bacterial communities. According to Simpson's evenness moss (0.098 ± 0.061) and bark-associated microbiota were both equally distributed ($P = 1.0$), while the evenness for moss and lichens was significantly different ($P = 0.006$). Additionally, Shannon diversity indices calculated for the habitat-associated communities of each maple tree separately revealed that the species richness of bark and moss communities varied more across the trees than the lichen-associated communities (Fig. S3, Supporting information). The phylogenetic beta-diversity, however, revealed both quantitatively and qualitatively significant differences (weighted and unweighted Uni-Frac distance matrices, respectively) in the habitat-specific bacterial community structures across the three habitats, according to nonparametric Adonis ($P \leq 0.001$; Fig. 2).

Although the six predominant phyla (more than 4% relative sequence abundance in at least one habitat) were shared by all three habitats, differences in their relative abundances were detectable (Fig. S4, Supporting information). The majority of sequences in each habitat was assigned to the phylum *Proteobacteria* (bark:

54.7%, moss: 37.0%, lichen: 42.6%), with *Alphaproteobacteria* as the predominant class. Additionally, *Cyanobacteria* and *Bacteroidetes* were overrepresented in at least one of the habitats by accounting about 20% of all sequences (*Cyanobacteria*: 1.0%, 13.0%, 20.2%; *Bacteroidetes*: 11.0%, 21.3%, and 10.6% on bark, moss and lichen, respectively). Other highly abundant phyla, in particular on bark, were *Actinobacteria* (12.6%, 3.5%, 5.6%) and *Acidobacteria* (10.7%, 7.1%, 3.9%), whereas both cryptogams showed higher relative abundances of *Verrucomicrobia* (4.0%, 8.4%, 9.6%).

Also, the analysis at lower taxonomic levels indicated habitat preferences as indicated by the heatmap in Fig. 3. Comparative analysis of OTU frequencies across sample groups (habitats) using the Kruskal–Wallis test revealed 149 OTUs with statistically significant differences in their abundance (Bonferroni corrected *P*-values < 0.01) across the three habitats. The majority of all differently represented OTUs belonged to *Proteobacteria* (bark: 59%, moss: 53%, lichen: 66%), followed by *Actinobacteria* (22% bark), *Bacteroidetes* (33% moss) and *Verrucomicrobia* (18% lichen). Bark and the lichen-associated *Proteobacteria* were mainly predominated by the alphaproteobacterial families *Sphingomonadaceae*, *Acetobactericeae* and various *Rhizobiales* ones, whereas moss samples were dominated by beta- and deltaproteobacterial families such as *Comamonadaceae* or *Haliangiaceae* (Table 1; Fig. S5, Supporting information). The bare bark also had higher relative contents of the families *Acidobacteriaceae* and *Microbacteriaceae* (*Acidobacteria*, *Actinobacteria*), while moss samples were also dominated by *Chitinophagaceae* (*Bacteroidetes*) and lichens by various verrucomicrobial families and the cyanobacterial Family I (equivalent to *Nostocaceae* in the Greengenes database).
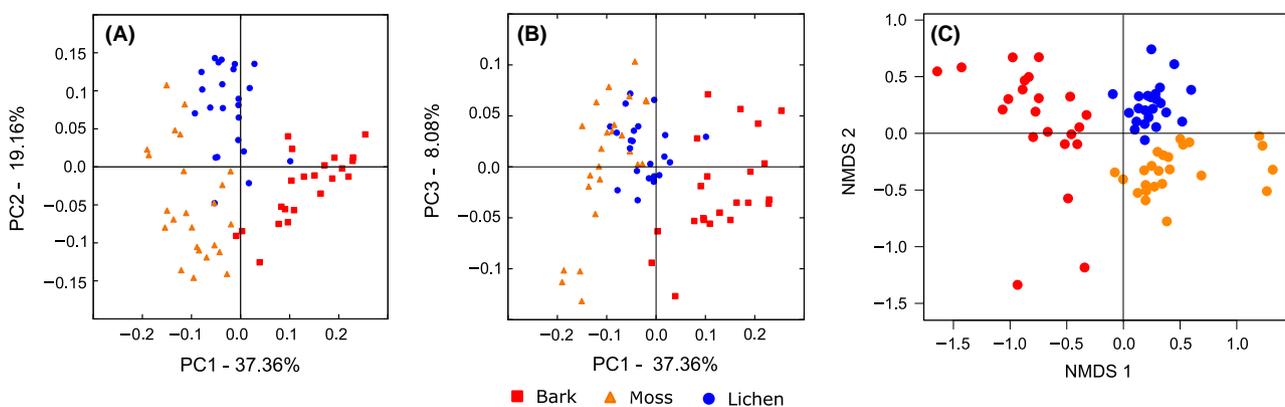

Fig. 2 Bark, moss and lichen samples indicated in two-dimensional PCoA plots (A, B) and a nonmetric multidimensional scaling (NMDS) plot (C) at a genetic distance of 3%. The PCoA plots were calculated based on weighted Unifrac distance matrix using QIIME. Each axis represents the per cent variation explained by each principal coordinate. The significance of sample groups (bark, moss and lichen) was calculated with Adonis $P \leq 0.001$. The NMDS plot was created on a Bray–Curtis dissimilarity matrix using R. The colour code indicates bark samples in red, moss in orange and lichen samples in blue.
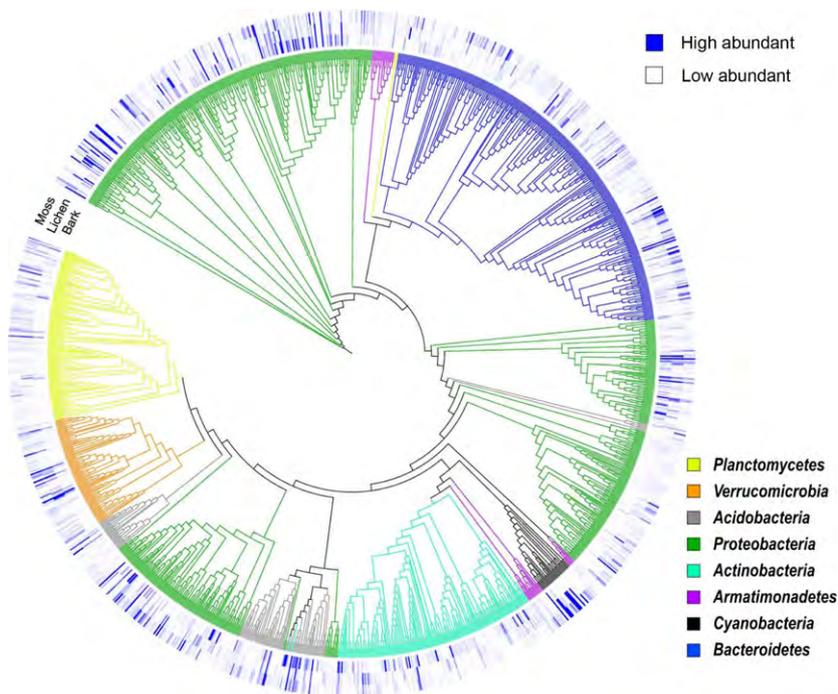
Fig. 3 Predominant bacterial phyla of the microhabitats visualized in a bootstrapped neighbour-joining tree based on clustalX alignment. The tree is surrounded by an additional heatmap representing the relative sequence abundances of each taxon for each habitat (blue: high abundant – max. 35 780 sequences; white: low abundant –min. 20 sequences).

Bacterial generalists and specialists for each habitat

To identify generalists and habitat related specialists at OTU level (97% similarity), the data set was reduced to OTUs, which were present in at least three of four replicates per habitat per tree. Subsequently, OTUs occurring in more than 90% of all samples were here termed generalists. Conversely, OTUs detected uniquely in one habitat were classified as specialists. Applying these criteria, about 1.2% of these OTUs (52 OTUs; 19.9% of the sequences) were identified as generalists, while 2.5% (109 OTUs; 2.7% of all sequences) were categorized as specialists. About 50% of the generalists belonged to *Alphaproteobacteria* and were represented by four taxonomic orders (*Rhizobiales, Sphingomonadales, Rhodospirillales, Caulobacterales*) and *Sphingomonas* as the most abundant genus. The remaining OTUs were assigned to various genera affiliated to *Bacteroidetes, Actinobacteria* and *Verrucomicrobia* (Table 2). Bark, moss and lichen specimens revealed 49, 31 and 29 OTUs, respectively, as putative specialists. Specialists on bark were mainly assigned to the phyla *Proteobacteria* (mostly *Alphaproteobacteria), Actinobacteria* and *Acidobacteria* with high abundant genera such as *Asticcacaulis,* or *Bryocella* (*Acidobacteria*). Contrary, moss-specific OTUs were mainly assigned to *Bacteroidetes* such as *Spirosoma,* followed by *Proteobacteria* (*Beta-,* and *Deltaproteobacteria*) and genera within *Planctomycetes*. *Lobaria* specialists belonged mainly to *Proteobacteria,* especially *Alphaproteobacteria,* represented by genera such as *Sphingomonas* and *Acidiphilium.* Sequences representing LAR1

(Lichen-associated *Rhizobiales* 1) were not classified as lichen-specific *Rhizobiales*. The most abundant specialist on lichens was *Mucilaginibacter* (*Bacteroidetes*).

Approximately 68% of the cyanobacterial OTUs were assigned to the genus *Nostoc* and 30% of these were exclusively shared between mosses and lichens, but in accordance with the functional role in cephalodia, *Nostoc* was proportionally higher abundant in lichens. *Leptolyngbya* sp. and *Gloeotrichia* sp. were also shared by mosses and lichens, but these genera were more abundant on mosses. Representative sequences of cyanobacterial OTUs subjected to BLASTn analyses (NCBI nt database) for a more detailed taxonomic characterization revealed their similarity (95–99%) with *Nostoc punctiforme* (including genome-sequenced strain PCC73102). Yet, this assignment is tempered with caution, as sequences from symbiotic *Nostoc* genotypes and other lichen symbiotic cyanobacteria cannot be reliably classified at species level (Rikkinen 2013).

Co-occurrence networks of bark, moss and lichen-associated microbial communities

Microbial co-occurrence relationships were constructed for each habitat based on a combination of four different measurements including Spearman and Pearson correlations, as well as Bray–Curtis and Kullback–Leibler dissimilarities. Robust and statistically significant correlations were visualized as microbial association networks (Fig. 4). Habitat-specific networks differed

Table 1 Differently represented OTUs based on Kruskal–Wallis test summarized at family level

Phylum	Order	Bark	Moss	Lichen
Acidobacteria	*Acidobacteriales*	*Acidobacteriaceae*		
Actinobacteria	*Frankiales*	*Frankiaceae*		
	Micrococcales	*Dermacoccaceae*		
		Microbacteriaceae		
	Propionibacteriales	*Nocardioidaceae*		
	Kineosporiales		*Kineosporiaceae*	*Kineosporiaceae*
Bacteroidetes	*Sphingobacteriales*	*Sphingobacteriaceae*	*Sphingobacteriaceae*	*Sphingobacteriaceae*
			Chitinophagaceae	
	Cytophagales		*Cytophagaceae*	
Cyanobacteria	SubsectionIV			*Family I*
Proteobacteria	*Caulobacterales*	*Caulobacteraceae*		*Caulobacteraceae*
	Rhizobiales	1174-901-12		1174-901-12
		Beijerinckiaceae		*Beijerinckiaceae*
		Methylocystaceae		*Methylobacteriaceae*
	Rhodospirillales	*Acetobacteraceae*		*Acetobacteraceae*
	Sphingomonadales	*Sphingomonadaceae*		*Sphingomonadaceae*
				JG34-KF-161
	Burkholderiales	*Alcaligenaceae*	*Comamonadaceae*	
		Burkholderiaceae		
	Enterobacteriales	*Enterobacteriaceae*		
	Myxococcales		*Polyangiaceae*	*Polyangiaceae*
			Haliangiaceae	
Verrucomicrobia	*Chthoniobacterales*	*Chthoniobacteraceae*	*Chthoniobacteraceae*	*Chthoniobacteraceae*
			DA101 soil group	LD29
				Xiphinematobacteraceae
				Opitutaceae

profoundly between the habitats: the lichen network was less complex and less coherent than the bark and moss networks. According to statistical analysis, the network diameter, reflecting the maximal distance between two nodes, was lower for the bark and moss networks than for the lichen one indicating that these networks are more compact and the their nodes are in closer proximity to each other (Table 3). Also the network density (density of node connections) was lower for the lichen network compared to the two others. Additionally, network centralization measurements indicate a more decentralized network topology for the lichen habitat (Table 3).

Habitat-specific networks varied also in the average number of neighbours (number of nodes directly connected to a given node). While in the bark and moss network nodes with the highest number of neighbours were connected to 31 and 63 other nodes, respectively, the maximal node degree in the lichen network was 7. This was also reflected by the network heterogeneity for each network describing the tendency of a network to contain hub nodes. In case of the lichen network, this hub was assigned to the alphaproteobacterial genus *Rhizobium* which had exclusively positive correlations mainly to other *Alphaproteobacteria* including genera such as *Rhizomicrobium*, *Bradyrhizobium* or *Sphingomonas*. In the bark network, the node with the highest connectivity was assigned to an uncultured bacterium of the phylum WD272 and showed the strongest co-occurrence with a member of the family *Caulobacteraceae*. Interestingly, in terms of the moss network, the hub, which was identified as an uncultured bacterium of *Acidobacteria*, had exclusively negative correlations mainly (31 of 63) to *Proteobacteria*, especially *Alphaproteobacteria*. Nevertheless, 48.6% (bark), 32.1% (moss) and 41.0% (lichen) of the nodes of each network belonged to *Alphaproteobacteria,* which were mainly represented by the orders *Rhizobiales*, *Rhodospirillales*, *Caulobacterales* and *Sphingomonadales*. The majority of the correlations (bark: 91.0%, moss: 81.5% and lichen: 91.0%) was identified as co-occurrences (positive correlations). However, the average per-node clustering coefficient for each network was low indicating that the neighbours of each node were hardly connected among themselves.

Functional analysis of a generalist and a lichen specialist

To characterize the potential symbiotic functions (Fig. 5) of the generalist *Sphingomonas* and the most abundant

Table 2 Habitat-specific specialists and generalists summarized at genus level

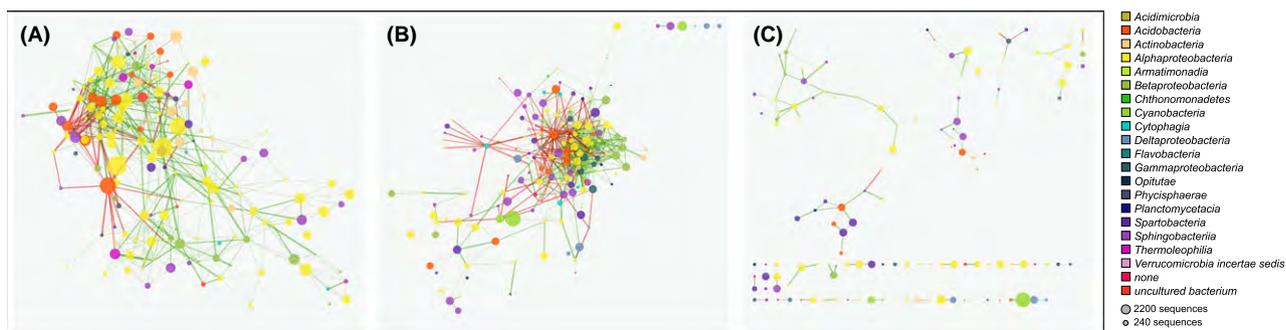| Phylum	Bark Specialists	Moss	Lichen	Generalists
Acidobacteria	*Bryocella* *Terriglobus*	*Solibacter*		
Actinobacteria	*Actinomycetospora* *Jatrophihabitans*	*Pseudonocardia*		*Jatrophihabitans* *Marmoricola*
Bacteroidetes	*Mucilaginibacter*	*Ferruginibacter* *Mucilaginibacter* *Pedobacter* *Sediminibacterium* *Segetibacter* *Spirosoma*	*Mucilaginibacter*	*Ferruginibacter* *Mucilaginibacter* *Pedobacter*
Cyanobacteria			*Nostoc*	
Planctomycetes	*Singulisphaera*		*Singulisphaera*	
Proteobacteria	*Asticcacaulis* *Achromobacter* *Agaricicola* *Burkholderia* *Hansschlegelia* *Pseudomonas* *Rhodobium* *Rhodovastum* *Sphingomonas*	*Albibacter* *Haliangium* *Sorangium*	*Acidiphilium* *Methylobacterium* *Sphingomonas*	*Acidiphilium* *Aquincola* *Bradyrhizobium* *Limnobacter* *Massilia* *Methylobacterium* *Piscinibacter* *Rhizobacter* *Sandarakinorhabdus* *Sphingomonas*
Verrucomicrobia		*Chthoniobacter*		*Chthoniobacter*

Fig. 4 Co-occurrence networks for each habitat – bark (A), moss (B) and lichen (C). Networks were constructed with the Cytoscape plugin CoNet based on an ensemble approach of various measurements (Pearson, Spearman, Bray–Curtis, Kullback–Leibler). OTUs are represented as nodes and correlations as edges (positive = co-occurrence: green; negative = co-exclusion: red). The node sizes are correlated to the OTU abundances, and node colour indicates the corresponding taxonomic assignment at class level. The edge width is represented by the q-values (Benjamini–Hochberg corrected *P*-values) and is proportional to the significance of supporting evidence. *P*-values were computed from method- and edge-specific permutation and bootstrap score distributions with 1000 iterations each.

specialist on lichens, *Mucilaginibacter*, we utilized a metagenome of the *L. pulmonaria*-associated microbiota. Genes attributed to both nitrogen (ammonia assimilation) and phosphate metabolic pathways were exclusively found for the generalist. *Sphingomonas* was involved in various pathways of aromatic compound metabolisms, such as transport and degradation of benzoate, catabolism of salicylate and gentisate or the degradation of phenylpropanoid compounds. The specialist

Mucilaginibacter was involved in sulphur and iron metabolism (in particular, a TonB-dependent receptor for iron acquisition was present), and potentially degrades n-phenylalkanoic acid. Both genera also differed in the detected secondary metabolites. While the generalist was exclusively capable to produce the plant hormone auxin, the specialist was found to synthesize quinolinic acids and its derivates as biologically active compounds in metazoan cell defence and differentiation. The functional

Table 3 Habitat-specific network parameters

Parameters	Bark	Moss	Lichen
Number of nodes	175	168	134
Number of edges	737	915	122
Average number of neighbours	8.42	10.89	1.82
Clustering coefficient	0.42	0.38	0.14
Network diameter	9.00	9.00	13.00
Network radius	5.00	1.00	1.00
Network centralization	0.13	0.32	0.04
Network density	0.05	0.07	0.01
Network heterogeneity	0.77	1.13	0.70

category of virulence, disease and defence was represented by two main resistance mechanisms to antibiotics and toxic compounds. While the specialist only encoded for beta-lactamases, the generalist additionally had multidrug resistance efflux pumps. Additionally, integrated gene transfer elements (*Bacteroides* conjugative transposons) were detected for *Mucilaginibacter*. Genes attributed to oxidative stress response, such as catalases, glutathiones or transcriptional regulators, were found only in the generalist.

FISH-CLSM

A typical example for the biocoenosis of adjoining lichens and mosses on a maple tree bark is illustrated

in Fig. 1A. The bacterial colonization pattern on the upper and lower surface of the lung lichen with focus on *Alphaproteobacteria* was visualized on the basis of a thallus cross section (Fig. 1B). While *Alphaproteobacteria* formed larger colony patches, other eubacteria appeared rather dispersed. A microscopic view on the lichen–moss interaction was depicted in Fig. 1C. Hairlike protuberances of moss rhizoids are anchored in the hyphal tissue of a disrupted lichen thallus fragment. Associated eubacteria as well as green algae were detectable on their surfaces.

Discussion

While previous works suggested specificity of lichen- and moss-associated bacterial communities, their interrelationships were never studied (Grube *et al.* 2009; Bragina *et al.* 2012). Our present results revealed the overlaps in the microbial community structures at all taxonomic ranks, with complex patterns at finer taxonomic resolution. Several potential generalists and specialists were identified for the selected environmental niches. The majority of the bacterial generalists belonged to *Alpha-* and *Betaproteobacteria*, mainly represented by the orders *Rhizobiales*, *Sphingomonadales* and *Burkholderiales*. *Sphingomonas* is a ubiquitously occurring and facultatively photosynthetic genus, which was recently also isolated from the endosphere of another maple tree species, *Acer*
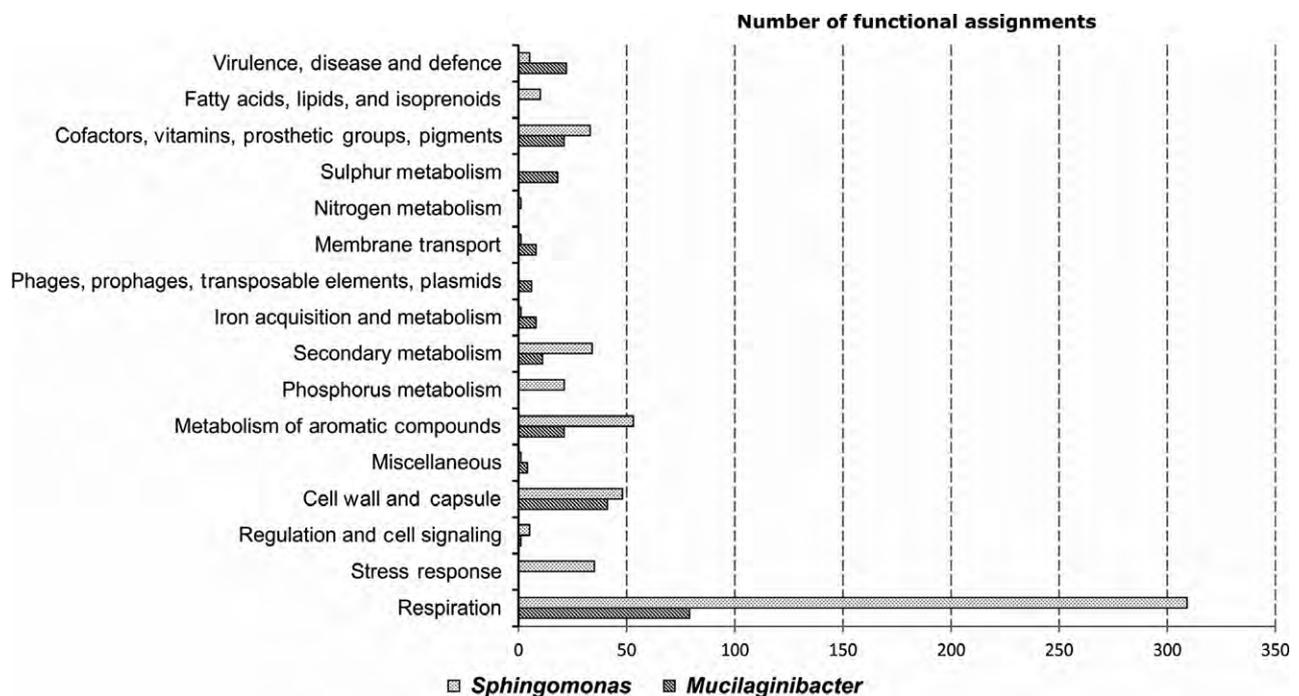

Fig. 5 Functional assignments to the generalist *Mucilaginibacter* (*Bacteroidetes*) and a lichen specialist *Sphingomonas* (*Proteobacteria*) based on SEED classification performed with MEGAN5. The genus-specific sequences were derived from the metagenome of the *Lobaria*-associated microbial community (Grube *et al.* 2015).

negundo (Shen & Fulthorpe 2015), and it is known as a generally abundant genus in the phyllosphere (Vorholt 2012). While the quantitative distribution of generalists was relatively uniform in all habitats, potential specialists differed in both their taxonomic affiliation and relative abundances for each habitat. Bark and lichen specialists belonged mainly to *Proteobacteria* (especially *Alphaproteobacteria*). Members of *Rhizobiales* were shown to be particularly abundant in lichens. Previously, this order was linked to potential symbiotic functions, including provision of specific secondary metabolites and nutrient cycling in lichens (Erlacher *et al.* 2015; Grube *et al.* 2015). They are also well known for their nitrogen fixing symbiosis with plants (Long 1989). *Burkholderia* and *Pseudomonas* (*Beta-* and *Gammaproteobacteria,* respectively*)* were generally detected in each habitat, but specialist OTUs in these genera were only identified on bark. *Burkholderia* species comprise beneficial plant associated bacteria, which fix nitrogen or degrade aromatic compounds (Suárez-Moreno *et al.* 2012). More recently, *Burkholderia* was also identified as member of the moss core microbiome with beneficial, but also with potentially pathogenic features (Bragina *et al.* 2013). *Burkholderia* and *Pseudomonas* isolated from lichens exhibit highly effective antagonists of certain bacterial and fungal pathogens (Cernava *et al.* 2015a,b). Thus, an essential role of this bacterial fraction in pathogen defence and microbial community stabilization is envisioned. However, while moss was associated with multiple low abundant specialists assigned to *Bacteroidetes*, lichen-associated specialists within this phylum were represented only by the genus *Mucilaginibacter,* which was also the most abundant specialist on lichens. *Mucilaginibacter* potentially contributes to nutrient cycling and supply for other members within the microbial community or the host itself. Bacteria in this genus readily produce and release extracellular polysaccharides, and they degrade various polysaccharides such as pectin and hemicellulose (Pankratov *et al.* 2007; Urai *et al.* 2008), which are typically found in lichens. Specialists assigned to *Actinobacteria* were primarily found on bark, but one member, *Pseudonocardia*, exclusively occurred on moss. *Pseudonocardia* is so far known for antibiotic assistance to stabilize ant symbioses (Zhang *et al.* 2007), but the antagonistic potential of other moss-associated bacteria was already demonstrated in other studies (Opelt *et al.* 2007). The relevance of antagonistic functions in the bark-associated habitats remains still to be studied in greater detail.

Nostoc lineages were clearly shared between both cryptogams, but occurred in higher abundances on lichens. For its function in nitrogen fixation of the lung lichen, *Nostoc* is of particular importance for the establishment of this lichen. Cornejo & Scheidegger (2013) demonstrated that the cyanobacterial colonies are recurrently recruited from the environment and incorporated via uptake from the thallus surface into the growing thallus. Our results indicate that mosses may serve as rich reservoir of compatible *Nostoc* strains. This could explain why *Lobaria* frequently develops on bark-inhabiting mosses. It remains to be studied more carefully to what extent other shared bacteria of lichen and moss (e.g. photosynthetically active *Sphingomonas*) may also support the establishment of lichens.

Comparison of the bacterial co-occurrence networks revealed structural differences among the niches. Interestingly, over 80% of all correlations were positive, in agreement with a generally self-sustaining assortment of bacteria. In contrast to the large network modules of bark- and moss-associated communities, the lichen network structure was less coherent, with several small network modules comprising only few nodes. The reasons for the different patterns found merit further study. Only *Alphaproteobacteria* form larger colonies on the lichen surfaces according to our previous FISH-CLSM analyses of the lung lichen thalli (Erlacher *et al.* 2015; Grube *et al.* 2015), whereas diverse other eubacteria are present in rather scattered clusters (see also Fig. 1B). These dispersed colonies could account for the loose co-occurrence network structure, and still agrees with a strict control of bacterial colonization on the lichen (favouring *Alphaproteobacteria*). However, some correlative patterns might also be obscured by differences in colonization patterns between the upper and lower surface of the lichen thallus. Although each surface might facilitate distinct bacteria due to dissimilar microclimatic conditions (e.g. moisture), OTUs (bacterial species) originating from either one or the other surface cannot be distinguished in subsequent data analysis. This observation remains to be analysed in more detail by separate sample preparations of both lichen surfaces in further studies.

Also with respect to the information derived from the metagenome we opt for a cautious interpretation. It is hardly possible to assign functional information below the genus level to individual OTUs in a metagenomics data set. Nevertheless, the abundance of the *Mucilaginibacter* OTU as a specialist of the lichen let us hypothesize that the potential functions of *Mucilaginibacter* are of special importance in the lichen symbiosis, but it cannot be excluded that it may also survive the lichen symbiosis better than functionally similar bacteria. The *Sphingomonas* strains found in all habitats may promote their hosts by producing growth-promoting hormones as suggested by the metagenome information. Further potential functions include phosphate solubilization, ammonia assimilation and oxidative stress responses. Both the lichens and mosses studied here are adapted to short-term periodic desiccation in their habitat and

these fluctuations in water availability are also selective conditions for their associated bacteria, which furthermore tolerate production of reactive oxygen species of their hosts (Knief *et al.* 2012). While it is well understood that plants and fungi enrich their specific belowground microbiota from the surrounding substrate (Warmink *et al.* 2009; Bjelland *et al.* 2011; Knief *et al.* 2012; Bragina *et al.* 2014; Schlatter *et al.* 2015), we are only beginning to understand the inter-relationships of spatially close above-ground host communities. In this study, we observed a significant share of species dissimilar but neighbouring hosts.

The shared occurrence of cyanobacteria in mosses and lichens suggests that the presence of cyanobacteria in mosses could facilitate the establishment of lichens on tree trunks. Ecological facilitation is a concept in ecology for positive outcomes of encounters (Bruno *et al.* 2003), for example when one organism provides a more favourable local environment for another. These facilitative conditions may include microclimatic benefits, as assumed by Colesie *et al.* (2012) for the growth of soil lichens adjacent to mosses. Our results suggest a microbiological aspect of ecological facilitation. Because *Nostoc* accounts for nitrogen fixation in cephalodia of *Lobaria*, the presence of suitable strains in mosses can help the lichen to establish near mosses. Cornejo & Scheidegger (2016) recently suggested epiphytic liverworts also as reservoirs of cyanobacterial photobionts of lichens. More examples and data are still needed to understand the role of microbiota in ecological connectivity and stability (e.g. the root-soil connection, or food-human gut connection; Berg & Smalla 2009; David *et al.* 2014). A better knowledge about microbial share and functional implications may also be important to achieve a more holistic ecological view of forests, as the Earth's lungs.

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Conflict of interest

The authors of this study declare no conflict of interest.

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I.A., T.C. and M.G. collected the samples. I.A. and T.C. processed the samples. I.A. did the bioinformatics analyses and corresponding visualizations. I.A., T.C., A.E., G.B. and M.G. wrote the manuscript. G.B. and M.G. complemented the manuscript with profound expertise in the fields of microbiome and lichen research.

Data accessibility

All data represented in this study have been deposited in the NCBI SRA (sequence read archive) as a BioProject, including all 72 raw sequence reads of the *Pterigynandrum filiforme*, *Acer pseudoplatanus* and *Lobaria pulmonaria*-associated microbiomes. It is accessible through the accession number PRJNA290145.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Alpha diversity indices at 3% genetic dissimilarity (normalized to 5100 sequences per sample).

Fig. S1 Bacterial alpha diversity indices indicating species evenness (A) and richness (B). Simpson's equability (evenness) and Shannon diversity (richness) were calculated at a genetic distance of 3% based on a normalized (5100 sequences) OTU table and summarized as boxplots.

Fig. S2 Observed OTUs and Chao1 at a genetic distance of 3% visualized as normalized rarefaction curves.

Fig. S3 Shannon diversity indices for each habitat per tree.

Fig. S4 Relative sequence abundances of the main phyla differed between the three microhabitats.

Fig. S5 Bacterial families with a relative abundance of more than 2% in at least one habitat visualized as heatmap.