Graz University of Technology

Doctoral Thesis

Structural and functional patterns of *Sphagnum*-associated microbial communities: biodiversity and ecology

Dissertation zur Erlangung des akademischen Grades Doktor der Naturwissenschaften (Dr. rer. nat.) an der Fakultät für Technische Chemie, Verfahrenstechnik und Biotechnologie an der Technischen Universität Graz

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Table of contents

Abstract

Mosses of the genus *Sphagnum* are very common and abundant bryophytes in the Northern Hemisphere, they greatly contribute to global carbon turnover and affect global climate. Despite the importance of the peat mosses for the biosphere, its microbiome remains undiscovered. Using a multiphasic approach, bacterial diversity of the cosmopolitan *Sphagnum* species (*S. magellanicum*, *S. fallax* and *S. angustifolium*) from Austrian Alpine bogs was investigated with special focus on ecological specialisation and functional traits of the associated bacteria within an ecosystem-host-microbial framework.

A high specificity of *S. magellanicum*- and *S. fallax*-associated microbiomes was detected with a complex of molecular approaches. With the application of multivariate statistical analysis, pH value and nutrient richness were defined as the main ecological drivers for the microbial communities. *In-situ* detection showed dense and well-structured colonisation patterns in *Sphagnum* phyllosphere. Stems and leaves, especially hyalocytes, of plants were occupied by various taxonomic groups of bacteria. Notably, we demonstrated that associated microbial communities are maintained during the whole lifecycle of the host plants. According to the results of high-throughput sequencing and *in-situ* detection, the core microbiome was shared between the sporophyte and gametophyte of mosses and transferred within the spore capsules.

To examine the extent of host specificity for the associated microbial communities, we investigated *S. fallax* and *S. angustifolium*, two phylogenetically closely related species with overlapping micro-niches but varying trophic specialisation. Profiling of secondary metabolites yielded similar spectra for both *Sphagnum* species and confirmed a common chemical basis for the establishment of the similar microbial communities in/on the compared mosses. In accordance with the resolved community structure, a significant overlap and slight differentiation between *S. fallax*- and *S. angustifolium*-associated bacteria were interpreted as an early state of specification of bacterial communities corresponding to an early state of host species differentiation.

For elucidation of an ecosystem role of *Sphagnum*-associated microbial communities, we analysed abundance, diversity and distribution patterns of diazotrophic and methanotrophic bacteria, essential for nutrient supply and carbon turnover in peatlands. The key genes encoding microbial nitrogen fixation (*nifH*) and methane oxidation (*pmoA*) were present in statistically significantly higher abundances in *S. fallax*, specialised for more nutrient-rich niches, than for ombrotrophically growing *S. magellanicum*. Network community analysis determined discriminative distribution patterns: diazotrophic specialists (specific for one moss species) and methanotrophic generalists (common for both species). In addition, the occurrence of nitrogen-fixing but absence of methane-oxidising bacteria was detected in the sporophyte-associated microbiome of *S. fallax.* The distribution pattern obtained was elucidated in terms of the ecosystem function of the examined bacterial groups.

Overall, it was demonstrated that bryophytes of the genus *Sphagnum* are colonised by well-structured, highly specific microbial communities driven by a-biotic factors and maintained during the whole lifecycle of the hosts. The state of the hosts' differentiation determines the effect of the host-specificity for diversification of the microbial communities. The ecosystem function designates the distribution patterns of the functional microbial groups.

Zusammenfassung

Die in der nördlichen Hemisphäre weit verbreiteten Moose der Gattung *Sphagnum* tragen wesentlich zum globalen Kohlenstoffkreislauf und zur Klimaentwicklung bei. Trotz der Bedeutung der Torfmoose für die Biosphäre, blieb dessen Mikrobiom bislang unerforscht. Mittels eines ganzheitlichen Forschungsansatzes wurden die assoziierten Bakterien der kosmopolitischen *Sphagnum*-Arten *S. magellanicum*, *S. fallax* und *S. angustifolium* aus österreichischen alpinen Mooren untersucht. Im Fokus standen die ökologische Spezialisierung und funktionalen Eigenschaften der Bakteriengemeinschaften in der Wechselwirkung zwischen Umwelt, Wirtpflanze und Mikroorganismus.

Unter Anwendung molekularer kultivierungs-unabhängiger Methoden konnte eine hohe Spezifität des *S. magellanicum*- und *S. fallax*-assoziierten Mikrobioms nachgewiesen werden. Auf Basis einer multivariablen statistischen Analyse wurden pH-Wert und Nährstoffkonzentration als die wichtigsten ökologischen Determinanten für die mikrobiellen Gemeinschaften definiert. Die *in-situ* Visualisierung zeigte eine dichte und gut strukturierte mikrobielle Kolonisierung in der Phyllosphäre von *Sphagnum*. Sowohl innere Bereiche der Stengel als auch innere und äußere Bereiche der Blätter, insbesondere der Hyalocyten, wurden von Bakterien aus verschiedenen taxonomischen Gruppen besetzt. Bemerkenswerterweise bleibt die Assoziation mit der mikrobiellen Gemeinschaft während des gesamten Lebenszyklus der Wirtspflanze erhalten. Pyrosequenzierung sowie mikroskopische Analysen deuten auf ein gemeinsames Kernmikrobiom von Gametophyt und Sporophyt sowie die Übertragung über die Sporenkapseln hin.

Um das Ausmaß der Wirtsspezifität für die assoziierten mikrobiellen Gemeinschaften zu testen, wurden *S. fallax* und *S. angustifolium* untersucht, zwei phylogenetisch eng verwandten Spezies mit überlappenden Mikro-Nischen, jedoch mit unterschiedlicher trophischer Spezialisierung. Die Analyse der sekundären Metaboliten ergab ähnliche Spektren für beide *Sphagnum*-Arten und weist auf eine gemeinsame chemische Basis für die Etablierung ähnlicher mikrobieller Gemeinschaften in den verglichenen Moosen hin. Geringe, aber spezifische Unterschiede bei gleichzeitig signifikanten Überlappungen des bakteriellen Besiedlungsmusters von *S. fallax*- und *S. angustifolium* wurden als Spezialisierung der Bakteriengemeinschaften in einer frühen Phase der Differenzierung der Wirtsspezies interpretiert.

Zur Analyse der Rolle der *Sphagnum*-assoziierten mikrobiellen Gemeinschaften im Ökosystem, untersuchten wir Abundanz, Diversität und Verteilung der diazotrophen und metanotrophen Bakterien, die an der Nährstoffversorgung und den Umsatz von Kohlenstoff in Mooren beteiligt sind. Die für die mikrobielle Stickstofffixierung und Methanoxidation kodierenden Schlüsselgene *nifH* bzw. *pmoA* wurden in statistisch signifikant höheren Abundanzen in *S. fallax*, welches im Vergleich zum ombrotroph wachsenden *S. magellanicum* auf nährstoffreichere Nischen spezialisiert ist, nachgewiesen. Netzwerk-Analysen der Gemeinschaften ergaben eine unterschiedliche Verteilung der funktionellen Gruppen: diazotrophe Spezialisten (spezifisch für eine Moos Art) und methanotrophe Generalisten (gemeinsam für beide Moos Arten). Außerdem wurden im Sporophyt-assoziierten Mikrobiom von *S. fallax* zwar Stickstoff-fixierende, aber keine Methan-oxidierende Bakterien detektiert. Die Verteilung der untersuchten Bakteriengruppen wurde in Bezug auf die Funktion im Ökosystem aufgeklärt.

Zusammenfassend konnte festgestellt werden, dass Moose der Gattung *Sphagnum* während des gesamten Lebenszyklus mit von abiotische Faktoren beeinflussten, gut strukturierten und sehr spezifischen mikrobiellen Gemeinschaften assoziiert sind. Das Ausmaß der Differenzierung des Wirtes bestimmt das Niveau der Spezifität und Diversifizierung der begleitenden mikrobiellen Gemeinschaften. Die Verteilung der funktionellen mikrobiellen Gruppen wird durch deren Ökosystemfunktion bestimmt.

Abbreviations

BLAST	basic local alignment search tool
CLSM	confocal laser scanning microscopy
FISH	fluorescent *in-situ* hybridisation
fw	fresh weight
NCBI	National Center for Biotechnology Information
HPLC	high performance liquid chromatography
nifH	nitrogenase reductase gene
PCR	polymerase chain reaction
PDA	photodiode array
pmoA	particulate methane monooxygenase gene
qPCR	quantitative polymerase chain reaction
SSCP	single-strand conformation polymorphism
UPGMA	unweighted pair group method with average linkages

Introduction

Sphagnum-dominated peatland ecosystems: definition, distribution and role in carbon global turnover

Sphagnum-dominated peatlands are widely distributed but unique terrestrial ecosystems important for the whole biosphere (Succow and Joosten, 2001) (Fig. 1). They are characterised by temporal or constant coverage with water and hydrophilic vegetation, dominated by peat-forming bryophytes of the genus *Sphagnum* (Zavarzin, 2004). The process of the peat formation is caused by an imbalance of primary production and mineralisation of organic matter under the peculiar environmental conditions of peatlands (low average temperature, low pH and high water saturation). The peat formation has a considerable effect on vegetation cover, which undergoes separation from the ground-water and is exposed to the nutrient deficiency. Dependent on the intensity of peat deposition and mineral nutrition type, peatlands are classified as ombrotrophic (precipitation-derived nutrition) or minerotrophic (supplied by ground-waters) without a distinctive boundary (Rydin and Jeglum, 2006).

Figure 1. Typical bog complex Pürgschachen Moor, Liezen, Austria.

Peatland ecosystems are widely distributed in the Northern Hemisphere and cover approximately 4 million km^2 equivalent to 3% of the Earth's land surface (Lappalainen, 1996). Due to the broad distribution and particular features, northern peatlands fulfil important biospheric functions: accumulation and deposition of freshwater, preservation of biodiversity and sequestration of organic carbon. According to the detailed estimates, northern peatlands contain 400 − 500 gigatonnes of sequestered carbon, which greatly contribute to the

global carbon budget (Gorham, 1991; Clymo *et al.*, 1998). Climate change and anthropogenic disturbance of peatlands lead to intensified mineralisation of the organic matter and emission of the greenhouse gases (Strack, 2008; Fenner and Freeman, 2011). Therefore, an understanding of the processes that occur in peatland ecosystems is important for maintaining their sustainability and biodiversity and could be useful in terms of climate change.

Sphagnum mosses as typomorphic vegetation of peatlands

Bryophytes of the genus *Sphagnum* represent a dominant component of peatland vegetation. They possess a unique morphology and specific ecological attributes, which significantly influence the whole ecosystem. Rydin and Jeglum (2006) state that particular features of the boreal peatlands are formed due to the singularity of *Sphagnum* mosses.

The lifecycle of Sphagna comprises haploid (gametophyte) and diploid (sporophyte) generations, presented by leafy plants and capsules containing spores, respectively (Fig. 2). Morphologically, moss shoots are formed by stems with spreading and hanging branches and apical capitula (Frahm, 2001). Sphagna lack root structures, and as the plants grow upwards, the lower parts of the shoots become buried eventually forming peat. Moss leaves possess a single-layer cell net of photosynthetically active chlorocytes connected with dead and empty hyalocytes. The hyalocytes contain a high number of pores, which explains the enormous capacity of mosses to retain water. At the same time, the cell walls of hyalocytes are strengthened by rigid fibrils important for draught resistance.

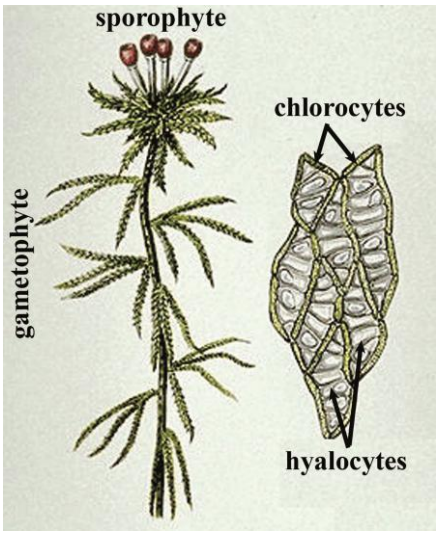

Figure 2. Morphological structure of *Sphagnum* mosses (modified artwork of Claire Tremblay).

Sphagnum mosses actively engineer peatland ecosystems. They have developed efficient mechanisms which increase their competitiveness and fitness. In particular, Sphagna are specialised for all niches along environmental gradients in peatlands (Rydin and Legjum, 2006). In the resided niches, peat mosses outcompete other vegetation by effective nutrient uptake, strong acidification of their environment and resistance to decay and herbivores (Clymo, 1964; van Breemen, 1995; Laine *et al*., 2009). Especially, the process of habitat acidification proves a great typomorphic potential of Sphagna. Intensive release of protons from moss cells and humic acids from plant residues, combined with shielding of the groundwater, leads to a considerable pH drop in *Sphagnum*-dominated ecosystems (Soudzilovskaia *et al*., 2010). Due to the peat formation, Sphagna define not only vegetation structure, but also the vertical stratification of the peatlands. Therefore, *Sphagnum* mosses are the main eukaryotic organisms driving peatland ecosystems.

Microbiome of peatland ecosystems − state of the art

In contrast to macroscopic bryophytes, microorganisms serve as hidden drivers of the peatland ecosystems (Pester *et al*., 2010; Lamers *et al*, 2012). The first studies on peatland microbiome addressed the role of bacteria in peat formation, thus setting the course for the whole century (Waksman and Stevens, 1929; Waksman and Purvis, 1932). For a long period, only cultivation-dependent techniques were available for estimating and describing the bacterial diversity (Ishizawa and Araragi, 1970; Dickinson *et al*., 1974; Williams and Crawford, 1983). Despite methodological limitations, these efforts revealed cultivable fraction of the microbial communities and were further expanded to isolate not-yet cultivated bacteria of certain taxonomical and physiological groups (Kulichevskaya *et al*., 2007; Pankratov *et al*., 2008; Dedysh, 2011). Cultivation-independent surveys comprised clone libraries, molecular fingerprinting and *in-situ* detection of peat communities, and revealed a high abundance of Proteobacteria, Actino- and Acidobacteria, Verrucomicrobia, Planctomycetes and Bacteroidetes, but also a high proportion of unidentified microorganisms (Reims *et al*., 1996; Dedysh *et al*., 2006; Hunter *et al*., 2006; Morales *et al*., 2006). With respect to the carbon turnover driving peatlands, important physiological groups were detected and described − for instance, hydrolytic bacteria degrading complex organic compounds, methanogens generating methane as a final step of mineralisation, but also methanotophic bacteria able to convert emitted greenhouse gas (Juottonen *et al*., 2005; Pankratov *et al*., 2011; Dedysh and Dunfield, 2011).

Less is known about microbial communities associated with living *Sphagnum* plants. The early studies reported an appearance of cyanobacteria on the surface and in the internal compartment of Sphagna (Granhall and Selander, 1973; Baselier *et al*., 1978). Later it was shown that cyanobacterial nitrogen fixation increases biomass production of mosses (Berg *et al*., 2012). Furthermore, Opelt and colleagues (2007a) demonstrated colonisation of peat mosses by highly diverse bacterial communities with antagonistic and plant-growth promoting activities. Moreover, Sphagna were detected as a source of opportunistic pathogens (Opelt *et al*., 2007b). In addition, methanotrophic bacteria that are highly abundant in peat were detected and analysed in moss tissues (Raghoebarsing *et al*., 2005; Kip *et al*., 2011). Despite the obtained results, an understanding of moss-microbial associations remains incomplete and has to be expanded.

Plant-associated microbial communities

With the exception of sterile cultures maintained in laboratory conditions, all natural plants are to different extent colonised by microbial communities. Plants and associated microbes establish various harmful, neutral or beneficial interactions within each group and between each other (Lidicker, 1979). The spectrum of scientific questions within the field varies significantly: from detecting molecular bases of interactions and ecological studies to the implication of the knowledge for environmentally friendly agriculture and assessment of the plant-microbe response to anthropogenic disturbance and climate change (Pieterse and Dicke, 2007; Berg, 2009; Gomes *et al*. 2010; Compant *et al*., 2010).

Beneficial interactions established between microorganisms and plants result in their successful adaption to environmental conditions, the suppression of pathogenic agents, and the enhancement of vital activity (Partida-Martinez and Heil, 2011). Underlying strategies comprise nutrient acquisition, antibiosis of pathogens, production of plant growth hormones from the bacterial site, effective exchange of secondary metabolites between plants and microbes, and the provision of colonisation niches by plants (Berg, 2009). Although the above-mentioned phenomena are thoroughly described, the interactions are never restricted to pairwise relationships; they possess a multiple character and are allocated within the specific habitat (Faust and Raes, 2012; Chapin *et al*., 2012). To this end, plant-microbial interactions *in-situ* should be addressed and elucidated.

Investigated peat mosses

The present project focused on microbial communities associated with three bryophyte species of the genus *Sphagnum*: *Sphagnum magellanicum* Brid., *Sphagnum angustifolium* Jens. and *Sphagnum fallax* Klinngr. (Fig. 3) (Daniels and Eddy, 1990). These peat mosses are very common and abundant in the Northern Hemisphere, but are characterised by different ecological specialisation and certain morphological distinctions (Savitsch-Lubitskaya and Smirnova, 1968; Laine *et al*., 2009). In contrast to *S. magellanicum, S. fallax* and *S. anustifolium* are phylogenetically closely related species which are difficult to discriminate (Smith, 1978).

S. magellanicum (section *Sphagnum*) grows in ombrotrophic to weakly minerotrophic, acidic and relatively dry sites, forming broad carpets or hummocks. Depending on light exposure, the colour of gametophytes varies from pale green to red. The plants are robust and stout. Fascicles are usually composed of two spreading and two hanging branches, which are rather blunt. Rectangular stem leaves have rounded apexes; broadly ovate branch leaves are deeply concave and contain fully enclosed photosynthetic cells. Hyalocytes expose large, ringed pores on the convex surface of the branch leaves.

S. angustifolium (section *Cuspidata*) possesses a wide tolerance from ombrotrophic to intermediately minerotrophic and less acidic conditions and occurs from hummocks to lawns. It tolerates moderate shading and appears in green, yellowish or brownish colours. The gametophytes are small to medium in size and compact. In each fascicle, four branches are densely positioned, but otherwise well-spaced. The spreading branches are tapering and short; attenuated hanging branches completely cover the stem. Equilateral stem leaves are obtuse at the apex; the branch leaves are narrow and slightly sub-erected. Chlorocytes are more exposed on the convex than on the concave surface of the leaves. Hyalocytes of the branch leaves possess large, unringed pores from the concave side and less abundant, small pores from the convex side.

S. fallax (section *Cuspidata*) inhabits preferably oligotrophic to mesotrophic, slightly acidic and wet niches, and occurs in lawns or carpets with moderate shading. The plants are light green, yellow-green or brownish, and medium sized to large. Fascicles possess four branches: acutely decurved and obtuse spreading branches and hanging branches covering the stem. Appressed stem leaves are triangular and broadest at the base, while branch leaves are

lanceolate with chlorocytes open on the concave side. Hyalocytes have apically-located pores from the convex side and wall-thinnings from the concave side of the branch leaves.

Figure 3. *Sphagnum magellanicum* (A), *Sphagnum angustifolium* (B) and *Sphagnum fallax* (C).

Methodical approach

Due to the emergence of high-throughput omic-technologies, the last decade became a milestone for environmental microbiology. Developed metagenomic, metatranscriptomic, metabolomic approaches enabled uncovering of a so-called "uncultured majority" at genetic, transcriptional and metabolic levels. At the same time new methodological and fundamental questions arose (Gilbert *et al*., 2011; Jansson *et al*., 2012). For instance, the problem of low resolution and recovery of dominant microbial groups − the tragedy of uncommon − achieved by molecular fingerprinting techniques (Bent and Forney, 2008) was substituted by the problem of handling and analyzing immense datasets (Gonzalez and Knight, 2012). New fundamental questions concerning rare biosphere and/or multiple interactions did not eliminate the question of how to cultivate the not-yet cultivated microorganisms (Reid and Buckley, 2011; Faust and Raes, 2012, Dedysh, 2011). A necessity to expand our understanding of the microbial ecology at the different levels of complexity leads us to combine conventional and high-throughput methods with environmental data and cultivation surveys and to be critical about the working hypothesis stated at the beginning of each research.

In this study we applied a complex approach to gain new insights into the plant-associated microbial communities. Profiling of the community structure was achieved by single-strand conformation polymorphism (SSCP) (Schwieger and Tebbe, 1998), which provided primary information about the diversification of communities and allowed us to later implement environmental data in multivariate analysis. Moreover, high throughput liquid chromatography (HPLC) and photodiode array (PDA) were used to characterise plant

secondary metabolites, which might contribute to the specificity of the associated microbiome (Opelt *et al*., 2007a). Knowledge on community composition was obtained by the construction of the clone libraries and was deepened by several targeted pyrosequencing surveys. Subsequently, the dominant bacterial taxa were visualised in/on intact plant cells by fluorescent *in-situ* hybridisation (FISH) coupled with confocal laser scanning microscopy (CLSM) and computer-assisted reconstructing (Amann *et al*., 1995; Grube *et al.*, 2009). Furthermore, the diversity of selected functional bacterial groups was investigated using pyrosequencing followed by network analysis (Grube *et al*., 2012), and the abundance of the key genes was recorded using quantitative polymerase chain reaction (qPCR) (Hai *et al*., 2009; Tuomivirta *et al*., 2009).

Objectives of the work

The purpose of the study was to investigate the bacterial community composition and colonisation pattern of different *Sphagnum* species with special focus on their ecological specialisation, and to analyse functional traits of the moss-associated bacteria within an ecosystem-host-microbial framework. Several working hypotheses were advanced to address the research issue.

1. *Sphagnum*-associated bacterial communities are well-structured on/in *Sphagnum* gametophytes**.** In order to reveal colonisation patterns, different compartments of the mature plants, namely leaves and stems, were examined by FISH with group-specific and universal bacterial probes. A fine dimensional resolution of the plant tissues was achieved by CLSM. The observed patterns were further analysed using computer-assisted reconstructions.
2. Microbiome of Sphagna is host-specific and driven by environmental conditions. To study microbial community composition, cosmopolitan but ecologically different *Sphagnum* species, *S. magellanicum* and *S. fallax*, were investigated using a combination of the cultivation-independent approaches (PCR-based SSCP fingerprinting, clone libraries and pyrosequencing amplicon libraries of the microbial 16S rRNA genes). UPGMA and multivariate analyses were applied to compare the community fingerprints and to test distribution of the microbial communities along a-biotic gradients. Moreover, the influence of the host-specificity for diversification of the microbial communities was tested for sister-species, *S. fallax* and *S. angustifolium*, characterised by overlapping micro-niches but different ecological specialisation. Secondary metabolites of Sphagna, assumed as a chemical basis for establishing highly specific plant-microbial associations, were analysed by HPLC. Community composition was resolved by applying PCR-SSCP fingerprinting and pyrosequencing of the microbial 16S rRNA and *nifH* genes.
3. Bacterial communities are maintained during the whole lifecycle of the host plants. The first proof was provided by FISH-CLSM visualisation of the internal compartment of the spore capsules. An expanded analysis was achieved by pyrosequencing of the microbial 16S rRNA genes obtained from the surface-sterilised sporophyte. Finally, sporophyte- and gametophyte-originated amplicon libraries were compared to reveal a core microbiome of the mosses.

4. Bryophyte-associated bacteria possess functional traits important for the host nutrition and sustainability of the peatlands. To understand the ecosystem role of the *Sphagnum*-associated communities, diazotrophic and methanotrophic bacteria were selected for high-throughput sequencing analysis. Diversity and abundance of the functional groups were resolved by pyrosequencing and qPCR of the key genes for nitrogen fixation (*nifH*) and methane oxidation (*pmoA*). Distribution patterns among different Sphagna were unveiled by profile clustering network analysis of the amplicon libraries.

Results

1. Analysis of the bacterial communities associated with *S. magellanicum* and *S. fallax*

Host-specific Sphagnum-associated microbiomes are driven by a-biotic factors and maintained during the whole lifecycle of the hosts

Peat mosses *S. magellanicum* and *S. fallax* are cosmopolitan bryophytes differently specialised along ecogradients (pH, moisture, nutrient concentrations, etc.). *S. magellanicum* grows in strongly acidic, dry and ombrotrophic habitats, while *S. fallax* inhabits weakly acidic, more water-saturated and mesotrophic habitats. Gametophytes and sporophytes of Sphagna were sampled in three Alpine bogs in Austria and analysed by the multifaceted approach.

I. High host-specificity of *Sphagnum*-associated microbial communities was detected by PCR-SSCP fingerprinting of 16S rRNA genes with universal bacterial primers. According to UPGMA and permutation analyses, the eubacterial community patterns of *S. magellanicum* and *S. fallax* clustered separately at 9% similarity level and formed two statistically significantly different groups. Patterns of samples of the same *Sphagnum* species from different geographical sites grouped together. Subsequently, community composition clearly differed between moss species as revealed by analysis of 16S rRNA gene clone libraries. Thus, *S. magellanicum* was dominated by Alphaproteobacteria (29.1%), followed by Gammaproteobacteria (11.8%) and Bacteroidetes (8.2%), in contrast to *S. fallax*, where Verrucomicrobia (35.2%), Planctomycetes (22.5%) and Alphaproteobacteria (18.3%) prevailed. Furthermore, the composition of Alphaproteobacteria resolved by pyrosequencing of 16S rRNA genes with taxon-specific primers was similar in the spectra of dominant families and genera but considerably differed by sub-dominant and minor groups. Altogether, a high specificity of microbial communities associated with different *Sphagnum* species was confirmed by all techniques employed.

II. Dense bacterial colonisation of *Sphagnum* gametophytes was detected by applying the FISH-CLSM approach. Alphaproteobacteria, Planctomycetes and unidentified bacteria of different morphology occupied the phyllosphere (above-ground plant parts) and formed close inter-group associations. As determined by three-dimensional computer reconstructions, bacteria were attached to the external or internal surface of the *Sphagnum* cell walls. Moreover, an internal space of hyalocytes was shown as a main

compartment inhabited by bacteria. *S. fallax* and *S. magellanicum* possessed different colonisation patterns. For instance, the outer cortex of *S. fallax* stems was occupied by Alphaproteobacteria, while *S. magellanicum* stems remained uncolonised.

III. *S. fallax*- and *S. magellanicum*-dominated niches were characterised by different vegetation and a-biotic conditions. In contrast to *S. fallax*, *S. magellanicum* habitats were characterised by lower values of soil reaction (corresponding to pH), moisture, temperature, lower amount of nutrients and more intensive exposure to sunlight according to the Ellenberg's indicator values for bryophytes and vascular plants (Ellenberg *et al*., 1991). Tested by multivariate statistical analysis, eubacterial SSCP profiles were statistically significantly oriented along nutrient concentration and soil reaction gradients. Thus, *Sphagnum*-associated microbial communities are driven by a-biotic factors.

IV. The first proof of similarity (55%) between sporophyte- and gametophyte-associated microbiomes of *S. fallax* was obtained by PCR-SSCP fingerprinting followed by UPGMA analysis. Comparison of Alphaproteobacteria-specific pyrosequencing libraries demonstrated that core microbiome was shared between sporophyte and gametophyte: the common dominant taxa were classified to Acetobacteraceae, Rhodospirillaceae and Caulobacteraceae families; communities shared eight out of 14 classified genera. Moreover, FISH-CLSM observations detected embedded bacterial cells in the matrix within the spore capsules. To conclude, *Sphagnum*-associated microbiome is maintained over the whole lifecycle of the host plants.

- A detailed representation of the results is given in **publication I** "*Sphagnum* mosses harbor highly specific bacterial diversity during their whole lifecycle".

Contributions: Christian Berg (Karl-Franzens University of Graz. Austria) recorded Ellenberg's indicator values and performed a multivariate analysis; Henry Müller (Graz University of Technology, Austria) contributed to the scientific discussion; Massimiliano Cardinale (Graz University of Technology, Austria) generated three-dimensional computer reconstructions; all collaborators contributed to the scientific discussion.

2. Analysis of the bacterial communities associated with sister-species *S. fallax* and *S. angustifolium*

Sister-species of Sphagnum are characterised by similar spectra of secondary metabolites and establish highly similar microbial communities

The previous study addressed the microbial communities associated with ecologically different and phylogenetically distinct *Sphagnum* spp., which harboured highly specific microbiomes driven by a-biotic factors. To understand the extent of host specificity for the associated microbial communities, we investigated two widely distributed and phylogenetically closely related species with overlapping micro-niches but varying trophic specialisation: *S. fallax* and *S. angustifolium*.

I. HPLC-PDA profiling of plant secondary metabolites resulted in notably similar chromatograms for both *Sphagnum* species. The similarity was explained by spectra of phenols and indol derivatives, while a slight difference was detected in the accumulation of flavonoid conjugates by *S. angustifolium*. The obtained results confirmed a common chemical basis for the establishment of the similar microbial communities in/on the compared mosses.

II. A high similarity of the microbial communities associated with *S. fallax* and *S. angustifolium* was revealed by PCR-SSCP fingerprinting of 16S rRNA and *nifH* genes. SSCP profiles of 16S rRNA genes obtained with universal and Alphaproteobacteria-specific primers showed more than 95 and 87% similarity between *Sphagnum* spp., respectively. On the UPGMA tree, the *nifH* gene SSCP profiles formed two clusters of 41% similarity, and samples of both species were grouped together within each cluster. Moreover, this overlap was statistically approved by detrended correspondence analysis.

III. The analysed Sphagna carried substantially similar alphaproteobacterial and nitrogen-fixing communities explored by pyrosequencing of 16S rRNA and *nifH* microbial genes. The diversity of Alphaproteobacteria comprised dominant Acetobacteraceae, Sphingomonadaceae, Rhodospirillaceae and Caulobacteraceae families. According to the NCBI database, detected alphaproteobacterial genera contained diazotrophic bacteria: *Bradyrhizobium*, *Acetobacter*, *Beijerinckia*, *Gluconacetobacter*, *Methylocystis*, *Methylosinus* and *Rhizobium* sp. Furthermore, the examined *nifH* gene amplicon libraries of *Sphagnum* spp. featured a 53% identity as shown by the Sørensen index (NifH clusters of 96% similarity). Total phylogenetic diversity of nitrogen-fixing communities was distributed among canonical Clusters I, III and Sub-cluster IA (Zehr *et*

al., 2003; Gaby and Buckley, 2011). The most abundant Cluster I, containing sequences of Proteobacteria and Cyanobacteria, was dominated by Alphaproteobacteria.

IV. Alphaproteobacteria presented up to 50% of the microbial communities visualised using the FISH-CLSM approach. Three-dimensional reconstructions revealed that bacteria predominantly colonised hyalocytes of *Sphagnum* gametophytes. Microbiomes associated with *S. fallax* and *S. angustifolium* were characterised by similar colonisation patterns.

- A detailed representation of the results is given in **publication II** "Similar diversity of Alphaproteobacteria and nitrogenase gene amplicons on two related *Sphagnum* mosses".

Contributions: Franz Hadacek and Vladimir Chobot conducted the HPLC-PDA assay (University of Vienna, Austria); Stefanie Maier carried out molecular fingerprinting and *in-situ* detection (Graz University of Technology, Austria); Massimiliano Cardinale (Graz University of Technology, Austria) performed three-dimensional computer reconstructions; Christian Berg (Karl-Franzens University of Graz. Austria) contributed to multivariate analysis and scientific discussion.

3. Analysis of the functional bacterial communities of *S. fallax* and *S. magellanicum*

Ecosystem function defines distribution patterns of the nitrogen-fixing and methane-oxidising bacteria

Strong nutrient limitation and specific carbon turnover dramatically influence microbiome of peatlands. For effective growth and proliferation, bacteria establish multiple interactions with pro- and eukaryotic organisms. To reveal functional traits of *Sphagnum*-associated microbial communities, we analysed the diversity of diazotrophic and methanotrophic bacteria which could fulfil important functions for the host plants and peatland ecosystems.

I. High copy numbers of the key genes for the bacterial nitrogen fixation (*nifH*) and methane oxidation (*pmoA*) processes were detected by qPCR technique. Over all sampling sites, *nifH* gene abundance was statistically significantly higher ($P = 0.0006$) for *S. fallax* ($\log_{10}$ 7.0 ± 0.2 to $\log_{10}$ 7.4 ± 0.3 copies g^{-1} fresh weight), than for *S. magellanicum* ($\log_{10}$ 6.8 ± 0.1 to $\log_{10}$ 7.0 ± 0.2 copies g^{-1} fw). Likewise, *pmoA* gene

copy numbers were statistically significantly higher (P = 0.002) for *S. fallax* ($\log_{10}$ 6.3 ± 0.2 to $\log_{10}$ 6.6 ± 0.3 copies g^{-1} fw) than for *S. magellanicum* ($\log_{10}$ 6.0 ± 0.3 to $\log_{10}$ 6.1 ± 0.2 copies g^{-1} fw) over all sampling sites.

II. Nitrogen-fixing communities were characterised by specific distribution among *Sphagnum* spp. Thus, *S. fallax*- and *S. magellanicum*-originated *nifH* pyrosequencing libraries formed two distinct groups with all cut-offs applied for the calculation of beta-diversity. BLAST analysis of the NifH composition revealed a high diversity of proteobacterial clusters, dominated by Alphaproteobacteria. With respect to the statistical analysis, 23 out of 118 NifH clusters (92% similarity) were statistically significantly different between Sphagna. Profile clustering network analysis resolved distribution of specific NifH patterns among investigated bryophytes. *S. magellanicum*-specific NifH clusters were related to *Methylobacterium* spp., Beijerinckiaceae, *Methylocapsa* sp., *Beijerinckia* spp. and *Azorhizobium* spp., while *S. fallax*-specific NifH clusters were assigned to *Burkholderia* spp., *Rhodoferax* sp., *Cupriavidus* sp. and *Bradyrhizobium* spp.

III. The diversity of the recovered methane-oxidising bacteria was highly similar for both *Sphagnum* species. A beta-diversity analysis of *pmoA* pyrosequencing libraries yielded up to 99% similarity between different mosses. PmoA composition examined by BLAST comprised type Ia (*Methylomonas* spp.) and type II (*Methylocystis* spp.) methanotrophs. Network analysis revealed general distribution of methanotrophs in/on bryophytes among all sampling sites.

IV. The occurrence of nitrogen-fixing but absence of methane oxidising bacteria in sporophyte-associated microbiome of *S. fallax* was demonstrated by an analysis of the 16S rRNA gene pyrosequencing library. According to the NCBI database, bacteria known for nitrogen fixation were distributed among 12 classified genera, which accounted for 45% of the total diversity. The *Burkholderia*, *Herbaspirillum*, *Pseudomonas*, *Sphingobacterium*, *Leptothrix* and *Rhizobium* genera were identified as the most dominant diazotrophic taxa.

- A detailed representation of the results is given in **manuscript I** "Ecosystem function designates functional patterns: diazotrophic specialists but methanotrophic generalists on *Sphagnum* mosses".

Contributions: Daniel Moser (Graz University of Technology, Austria) wrote a normalisation script; Christian Berg (Karl-Franzens University of Graz, Austria) contributed to the scientific discussion.

Discussion

*Ecological factors driving Sphagnum-associated microbial communities: interplay of a-biotic factors and host-specificity (**publication I and II**)*

Comparing *Sphagnum* species with different ecological specialisation, we posed the question: which factors drive the high specificity of *Sphagnum*-associated microbiome? Multivariate statistical analysis revealed nutrient richness and soil reaction (synonymous for pH) as the main a-biotic parameters which effected microbial distribution. Although the microbiome of peatlands is adapted to the nutrient-poor conditions, nutrient gradient structures the microbial communities on the local scale (Dedysh, 2011). The pH, detected in the current study as a key a-biotic parameter, was reported as a main driver for soil microbial communities at the continental scale (Lauber *et al*., 2009). Diversification of the microbial communities detected by multivariate analysis was further resolved by clone and amplicon libraries and resulted in greatly varied community composition supported by a difference of colonisation patterns.

The question whether the a-biotic factors modulate the composition of host-specific bacterial communities or serve as primary drivers of bacterial community composition was discussed. There are several facts supporting a modulatory effect of the a-biotic drivers. In a previous study of Opelt *et al*. (2007a), *S. fallax* and *S. magellanicum* were characterised by different spectra of secondary metabolites, which could be a reason for the host specificity. In addition, microbial colonisation of the sporophyte detected hereby indicates a direct transfer of the microbiome over the whole lifecycle of the bryophytes and contributes to the host specificity of associated bacteria. Moreover, the evolutionary history of Sphagna and long co-exposure with associated bacteria in the peatland ecosystems can be suggested as a reason for the high correlation between a-biotic parameters, peat mosses and their microbiome.

To test an influence of the host specificity for diversification of the microbial communities, we examined the closely related species *S. angustifolium* and *S. fallax* with overlapping micro-niches but different in trophic specialisation (Smith, 1978; Daniels and Eddy, 1985). In this study, *S. angustifolium* and *S. fallax* were characterised by similar secondary metabolites. The community structure resolved by SSCP fingerprinting and pyrosequencing was highly similar for both species. A large overlap and slight differentiation in more *S. fallax*- and more *S. angustifolium*-preferring bacteria shown by multivariate analysis could be interpreted as an early state of specification of bacterial communities corresponding to an early state of host species differentiation. This conclusion also explains a

difference between a theory of the "host specificity" stated by Opelt *et al*. (2007c) and proved in our previous study (see **publication I**), and results of Larmola *et al*. (2010), who identified only a-biotic drivers. Ecological consequences of the detected specificity are elucidated in **manuscript I**; here we demonstrated that specificity of the microbial community is essential for life under the extreme and highly varying environmental conditions within the peatland ecosystems and for fulfilling important ecological functions.

*Transmission of the Sphagnum-associated microbiome (**publication I**)*

Sexual reproduction of *Sphagnum* mosses is determined by the formation of spores. After meiosis and maturation, spores that are released from the spore capsules and exposed to the airflows could be transferred over relatively large distances (Sundberg, 2005). Spores transferred to another location form a persistent spore bank and germinate under appropriate conditions (Fenton and Bergeron, 2006). We hypothesised that bacterial communities associated with Sphagna are transmitted by spores. Using the FISH-CLSM approach, this hypothesis was approved; we detected a high abundance of bacteria inside the spore capsules. Moreover, by pyrosequencing and bioinformatic analysis we revealed a high degree of qualitative similarity between the microbial communities of the sporophyte and the gametophyte bearing genera well-known for their beneficial interactions with plants. From microbial ecology studies of higher plants, we know that plants which represent the diploid sporophyte acquire microbial populations mainly from soil (Berg and Smalla, 2009). However, it was recently shown that seeds also harbour microorganisms originating from the mother plants (van Overbeek *et al*., 2011). The maintenance of the microbial communities during the whole lifecycle confirms the importance of associated bacteria for the host plants.

*Ecology of the functional microbial patterns on Sphagna (**manuscript I**)*

Harsh conditions of the peatlands force the *Sphagnum* mosses to adapt and establish multiple interactions to achieve environmental sustainability. Our hypothesis assumed different distribution patterns (specific or ubiquitous) of the *Sphagnum*-associated microbial communities dependent on their function for the host plants and ecosystem.

An investigation of the functional microbial groups by pyrosequencing and network analysis revealed distinct ecological patterns: diazotrophic specialists and methanotrophic generalists. Microbial nitrogen fixation is essential for the plant germination and growth

(Tauro *et al*., 2009; Moir, 2011). Recently it was demonstrated that microbially-fixed nitrogen is assimilated by Sphagna and enhances their growth (Berg *et al*., 2012). In a previous study we were able to show that a core microbiome is transferred from the moss sporophyte to the gametophyte (see **publication I**). Moreover, bacteria known for nitrogen fixation account for 45% of the total microbial diversity of sporophyte. These facts underline the importance of nitrogen fixation for the host plants and explain distribution of the nitrogen-fixing bacteria specific for analysed *Sphagnum* species.

In spite of fixed nitrogen being provided to plants by microorganisms, carbon fixation is achieved by plants during photosynthesis. Although moss-associated methanotrophs can supply the host plants with the carbon dioxide, carbon uptake is not restricted to microbial activity (Raghoebarsing *et al.,* 2005). No methane-oxidising bacteria were detected in sporophyte-associated microbiome. Moreover, Putkinen *et al.* (2012) showed that methanotrophs are actively dispersed by water within the peatland ecosystems. All above-mentioned considerations explain ubiquitous distribution of detected methanotrophic bacteria independently of moss species.

The applied high-throughput molecular approach involved certain technical challenges – primer selection and limitation of reference libraries. Considering applied primers, the *nifH* gene-specific primers recovered the broadest spectrum of diazotrophic bacteria (Gaby and Buckley, 2012), while *pmoA*-targeting primers missed one of the habitat-specific methanotrophic patterns (Siljanen *et al*., 2011). Bioinformatic analysis of the pyrosequencing libraries was performed for deduced amino acid PmoA and NifH sequences. For frame-corrected translation, we used reference libraries available in the Functional Gene Pipeline and Repository of the Ribosomal Database Project (http://fungene.cme.msu.edu/FunGenePipeline/). These reference libraries are technically restricted to a certain size and could be incomplete due to the unimplemented, newly described methanotrophic bacteria. Elucidated functional traits reflected genetic diversity of *Sphagnum*-associated microbiome, which could be different from real microbial activity. Taking together, it is necessary to assess the obtained results and conclusions critically and to expand the survey by a metatranscriptomic approach and analysis of the microbial activity *in situ* (stable isotope probing, etc.).

Quantification of the functional microbial genes (*nifH* and *pmoA*) showed statistically significantly higher gene copy numbers for *S. fallax* than for *S. magellanicum*. The detected difference coincides with the effect of a-biotic factors driving microbiome (see **publication I**). In contrast to *S. fallax, S. magellanicum* habitats are characterised by more harsh a-biotic

conditions, which limit the total microbial abundance and activity, as shown by Opelt and Berg (2004).

Sphagna as an environmental model for investigation of plant-microbe interactions

For a better understanding of the plant biology, various bryophytes are explored as model systems in order to study environmental stress tolerance, plant-microbe interactions as well as genetic organisation and physiology of the plants (Oliver *et al*., 2000; Wood *et al*., 2000; Ponce de León, 2011). Although bryophytes will never substitute seed plants in model studies, successful implementation of the *Physcomitrella patens* model validates the previous argument (Ponce de León *et al*., 2007; Rensing *et al*., 2008; Reski, 2005). Our findings combined with the ecological concept of peatland ecosystems suggest *Sphagnum* mosses as a unique environmental model for investigation of plant–microbe interactions (Succow and Joosten, 2001):

I. peat mosses are the first land plants and were co-exposed with microbial communities of the bogs for a long evolutionary time;
II. Sphagna establish highly specific associations with microorganisms maintained during the whole lifecycle of the mosses;
III. as a dominant vegetation component of water-saturated ecosystems not influenced by mineral soil, they link terrestrial and aquatic habitats;
IV. due to the morphologic simplicity of the phyllosphere, peat mosses are appropriate for microscopic observations;
V. they can be cultivated *in vitro* and investigated *in-situ* (in peatlands);
VI. *Sphagnum* mosses are relevant for the global climate and could be used as ecological indicators.

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Sphagnum mosses harbour highly specific bacterial diversity during their whole lifecycle

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Sphagnum mosses harbour highly specific bacterial diversity during their whole lifecycle

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Knowledge about *Sphagnum*-associated microbial communities, their structure and their origin is important to understand and maintain climate-relevant *Sphagnum*-dominated bog ecosystems. We studied bacterial communities of two cosmopolitan *Sphagnum* species, which are well adapted to different abiotic parameters (*Sphagnum magellanicum*, which are strongly acidic and ombrotrophic, and *Sphagnum fallax*, which are weakly acidic and mesotrophic), in three Alpine bogs in Austria by a multifaceted approach. Great differences between bacterial fingerprints of both Sphagna were found independently from the site. This remarkable specificity was confirmed by a cloning and a deep sequencing approach. Besides the common Alphaproteobacteria, we found a discriminative spectrum of bacteria; although Gammaproteobacteria dominated *S. magellanicum*, *S. fallax* was mainly colonised by Verrucomicrobia and Planctomycetes. Using this information for fluorescent *in situ* hybridisation analyses, corresponding colonisation patterns for Alphaproteobacteria and Planctomycetes were detected. Bacterial colonies were found in high abundances inside the dead big hyalocytes, but they were always connected with the living chlorocytes. Using multivariate statistical analysis, the abiotic factors nutrient richness and pH were identified to modulate the composition of *Sphagnum*-specific bacterial communities. Interestingly, we found that the immense bacterial diversity was transferred via the sporophyte to the gametophyte, which can explain the high specificity of *Sphagnum*-associated bacteria over long distances. In contrast to higher plants, which acquire their bacteria mainly from the environment, mosses as the phylogenetically oldest land plants maintain their bacterial diversity within the whole lifecycle.

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Introduction

Bog ecosystems belong to the oldest vegetation forms, with more or less constant conditions for thousands of years. It covers 4 million km^2, approximately 3% of the earth's surface, and have a high value for biodiversity conservation, as reservoir of fresh water, for human welfare and our world climate due to its extraordinary role in carbon sequestration (Raghoebarsing *et al.*, 2005). The latter resulted in a net cooling effect on the global radiation balance (Dise, 2009). On the other side, these long-existing ecosystems are extremely sensitive to changing abiotic factors connected with climate change (Belyea and Malmer, 2004; Dise, 2009). When peatlands degrade, the stored carbon will be released. For example, drainage of peat soils results in CO_2 and N_2O emissions of globally 2–3 Gt CO_2-eq per year (Joosten and Couwenberg, 2009). For Alpine bog ecosystems, profound changes are expected due to global change (Theurillat and Guisan, 2001). The bryophyte genus *Sphagnum*, consisting of approximately 300 different species, is distributed world wide and forms the dominant component of bog vegetation (Daniels and Eddy, 1985). Therefore, *Sphagnum* moss has been used globally as an indicator of climate change (Whinam and Copson, 2006; Granath *et al.,* 2009), and microbial communities living in *Sphagnum* were shown early indicators of ecosystem disturbances in a microcosm experiment (Jassey *et al.*, 2011). Hence, knowledge concerning microbial ecology is important to protect, maintain and manage *Sphagnum* bog ecosystems.

Sphagnum mosses form a unique habitat for microorganisms such as high acidity and low temperature; water saturation, together with extremely low concentrations of mineral nutrients, are

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characteristic abiotic factors. Furthermore, *Sphagnum* leaves are highly specialised; they form a special tissue of living, chlorophyll-containing chlorocytes and dead cell content-free hyalocytes, which are responsible for their huge potential to store water. *Sphagnum* species also produce bioactive secondary metabolites influencing microbial colonisation (Opelt *et al.*, 2007a). So far, mainly the microbial populations involved in CH_4 cycling living on dead Sphagna (Dedysh *et al.*, 1998; Dedysh *et al.*, 2001; Horn *et al.*, 2003; Pankratov *et al.*, 2008; Rahman *et al.*, 2010) have attracted research interest. Recently, we could show that living *Sphagnum* mosses are colonised in high abundances with specific microorganisms, which fulfil important functions like nutrient supply and pathogen defence for moss growth and health (Opelt *et al.*, 2007a; Opelt *et al.*, 2007b). New questions thus arose: (i) do new molecular and microscopic techniques allow deeper insights into *Sphagnum*-associated bacterial diversity?; (ii) what are the main drivers of this diversity?; and (iii) how is this specific bacterial diversity acquired? Regarding the latter, from higher plants, we know that bacterial communities have a certain degree of plant specificity, but the majority of bacteria is environment-acquired and only a few bacterial strains are transferred within the lifecycle (reviewed in Berg and Smalla, 2009). Although for higher plants, the sporophyte generation makes up almost their whole life cycle, bryophytes have a dominant photosynthetically active gametophyte stage. Bryophytes represent the phylogenetically oldest group of land plants, and due to the specific communities (Opelt *et al.*, 2007c), our hypothesis was that this diversity is transferred directly from the sporophyte to the gametophyte and vice versa.

The objective of this work was to study the structure and origin of *Sphagnum*-associated bacteria, which were detected in three different Alpine bogs in Austria. To analyse differences between different *Sphagnum* species, two dominant and cosmopolitan species were selected: *Sphagnum magellanicum* and *Sphagnum fallax*. *S. magellanicum* BRID (section *Sphagnum*) is typical for strong acidic, oligotrophic and ombrotrophic habitats, whereas *S. fallax* H. KLINGGR (section *Cuspidata*) grows in weakly acidic, more mesotrophic situations influenced by minerotrophic groundwater (Daniels and Eddy, 1985). A polyphasic approach was applied to study bacterial communities on gametophytes and sporophytes: (i) microbial fingerprints by PCR–SSCP (single-strand conformation polymorphism) of 16S rRNA genes, (ii) clone libraries and phylogenetic analysis of clones, (iii) deep-sequencing of Alphaproteobacteria and (iv) fluorescent *in situ* hybridisation with universal and group-specific probes, coupled with confocal laser scanning microscopy (FISH–CLSM) and image analysis. Results taken together showed that *Sphagnum* gametophytes, as well as sporophytes, have a similar intimate and highly specific interaction with their associated bacteria.

Materials and methods

Experimental design and sampling procedure

To analyse differences between two *Sphagnum* species, *S. magellanicum* BRID (section *Sphagnum*) and *S. fallax* H. KLINGGR (section *Cuspidata*) were selected. Both bryophytes belong to the typical and cosmopolitan vegetation in peat bogs (Daniels and Eddy, 1985). Adult gametophytes of moss species were sampled in three different natural habitats in Alpine bogs in Austria in September 2009 (Table 1). Ecological characters of the habitats were described by composition of plant communities and average Ellenberg's indicator values for vascular plants and bryophytes (Ellenberg *et al.*, 1991). From each of three investigated bogs, four single replicates per *Sphagnum* species consisting of 15–20 plantlets were collected and stored separately. The living green parts of the plantlets were placed into sterile plastic bags and transported to the laboratory. *S. fallax* plants forming sporophytes were solely detected in the Rotmoos bog. Sporophyte samples of *S. fallax* consisting of enclosed spore capsules were collected and processed separately. In general, sporophytes of *S. magellanicum* are uncommonly found.

Total community DNA isolation

Before DNA isolation, the bacterial fraction associated with gametophytes was extracted according to the slightly modified protocol of Opelt and Berg, 2004. Briefly, 5 g of plant material were physically disrupted with sterile pestle and mortar, and resuspended in 10 ml of 0.85% NaCl. A volume of 2 ml of suspension were centrifuged at 13000 r.p.m. for 20 min at 4 °C, and the pellet was used for isolation of the total community DNA as described before (Martin-Laurent *et al.*, 2001). For mechanical lysis, the cells were homogenised twice in a FastPrep FP120 Instrument (QBiogene, BIO101, Carlsbad, CA, USA) for 30 s at speed 5.0 m sec^{-1}. Extraction of bacteria associated with sporophyte of *S. fallax* was carried out by grinding of 10 closed-spore capsules with the FastPrep FP120 cell disrupter (30 s, speed 5.0 m sec^{-1}). Before grinding, the capsules were surface sterilised as described previously (Opelt *et al.*, 2007a). Cell lysis and isolation of DNA was performed similarly to the cell pellets of gametophytes. The obtained DNA was purified using the FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH, USA) according to the manufacturer's protocol. Final aliquots of the total community DNA were further used for PCR-based approaches.

Microbial fingerprinting by PCR–SSCP of 16S rRNA genes

PCR-based SSCP analysis of the microbial 16S rRNA genes was carried out with universal bacterial primers Com1, Unibac-II-927rP (Schwieger and Tebbe, 1998). PCR and preparation of the single-stranded DNA

Table 1 Sampling sites and ecological parameters of the habitats

Bog	*Moss species*	*Sample number*[a]	*Geographical parameters*		*Abiotic parameters*[c]				
			Coordinates	*Altitude (m)*	*Soil reaction value*	*Nutrient value*	*Light value*	*Moisture value*	*Temperature value*
Rotmoos (Styria)	*S. magellanicum*	RM1	N47 41.030 E15 09.276	699	1.4	1.4	7.8	7.5	3.4
		RM2	N47 41.021 E15 09.245	699	—	—	—	—	—
		RM3	N47 40.971 E15 09.270	698	—	—	—	—	—
		RM4	N47 41.017 E15 09.319	693	—	—	—	—	—
	S. fallax	RF1	N47 40.908 E15 09.244	693	3.0	2.8	7.0	8.2	3.9
		RF2	N47 40.958 E15 09.175	691	—	—	—	—	—
		RF3, RFS[b]	N47 41.041 E15 09.232	690	—	—	—	—	—
		RF4	N47 41.055 E15 09.264	689	—	—	—	—	—
Wasenmoos (Salzburg)	*S. magellanicum*	WM1	N47 18.373 E12 24.927	1216	1.5	1.4	7.6	7.2	3.2
		WM2	N47 18.363 E12 24.944	1216	—	—	—	—	—
		WM3	N47 18.337 E12 25.119	1214	—	—	—	—	—
		WM4	N47 18.315 E12 25.126	1208	—	—	—	—	—
	S. fallax	WF1	N47 18.387 E12 24.871	1211	2.6	2.7	6.9	7.8	3.6
		WF2	N47 18.391 E12 24.866	1215	—	—	—	—	—
		WF3	N47 18.385 E12 24.882	1217	—	—	—	—	—
		WF4	N47 18.347 E12 24.980	1213	—	—	—	—	—
Pürgschachen Moor (Styria)	*S. magellanicum*	PM1	N47 34.905 E14 20.402	637	1.6	1.4	7.7	7.8	3.6
		PM2	N47 34.910 E14 20.454	639	—	—	—	—	—
		PM3	N47 34.839 E14 20.497	640	—	—	—	—	—
		PM4	N47 34.805 E14 20.493	639	—	—	—	—	—
	S. fallax	PF1	N47 34.789 E14 20.398	638	2.5	2.3	6.8	7.3	3.8
		PF2	N47 34.814 E14 20.356	636	—	—	—	—	—
		PF3	N47 34.824 E14 20.346	635	—	—	—	—	—
		PF4	N47 34.848 E14 20.344	634	—	—	—	—	—

Abbreviations: F, *Sphagnum fallax*; M, *Sphagnum magellanicum*; P, Pürgschachen Moor; R, Rotmoos; RFS, sporophyte sample of *S. fallax*; S, sporophyte sample of *S.fallax*; W, Wasenmoos.
[a]Letters indicate bogs and *Sphagnum* species: R, W, P, F, M and S. Arabic numerals specify replicates.
[b]RFS was collected at the same sampling point as sample RF3.
[c]Abiotic parameters were expressed by average Ellenberg's indicator values for vascular plant and bryophyte species. Numbers indicate properties of the habitat along ther ecological gradients (soil reaction: 1 = extremely acidic, 9 = calcareous; nutrient richness: 1 = extremely nutrient poor, 9 = extremely nutrient rich; light exposure: 1 = deep shadowed, 9 = full light exposed; moisture: 1 = extremely dry, 9 = extremely wet; temperature: 1 = extremely cold, 9 = extremely warm).

were performed according to Schwieger and Tebbe (1998). The amplicons were separated using the TGGE Maxi system (Biometra, Göttingen, Germany) at 400 V and 26 °C for 26 h in 8% (wt/vol) acrylamid gel followed by silver staining. Gel profiles were digitalised by transmissive scanning (Epson

perfection 4990 Photo, Long Beach, CA, USA) and further analysed with the GelCompare II version 5.1 software package (Applied Maths, Kortrijk, Belgium). A similarity matrix was constructed using Pearson's correlation coefficients (r) and cluster analysis was done by the unweighted pair group method with average linkages. The resulting clusters of samples were examined for statistical significance by applying permutation test with 10 000 random permutations of the single samples (Kropf *et al.*, 2004).

SSCP is based on the differences in the conformation of single-stranded DNA fragments. The electrophoretic mobility of the single-stranded DNA fragments depends on their three-dimensional conformation. Each of the amplification products was identified by its electrophoretic distance on SSCP gel and the number of DNA fragments. According to the distance of the bands, the SSCP gels were theoretically divided into operational taxonomic units. The presence or absence of individual amplified product DNA bands in each group was scored. The obtained matrix was used to compare statistically (see statistics).

Construction and analysis of 16S rRNA gene clone libraries

Total community DNA samples of the mosses from the site of the highest diversity determined by fingerprints (*S. magellanicum:* Pürgschachen Moor and *S. fallax:* Wasenmoos) were pooled together and used as template. 16S rRNA gene fragments were amplified with 799f/1492r primers, which avoid plant-derived amplicons substantially (Lane, 1991; Chelius and Triplett, 2001). The clone libraries were constructed as previously described (Sun *et al.*, 2008). PCR fragments for sequencing were generated with USP (5′-GTAAAACGACAACCAGT-3′) and RSP (5′-CAGGAAACAGCTATGACC-3′) vector-specific primers (Sigma-Aldrich, Taufkirchen, Germany). To exclude transformants with chloroplast-derived DNA, we applied restriction test with *Sph*I-HF (New England Biolabs, Frankfurt am Main, Germany). Restriction profiles of chloroplast sequences were calculated *in silico* (data not shown). PCR products were sequenced with the Applied Biosystems 3130 Genetic Analyser (Foster City, CA, USA). The sequences were submitted to the EMBL Nucleotide Sequence Database under accession numbers FR832168–FR832348.

Taxonomic affiliation of the partial 16S rRNA gene sequences was defined by alignment with reference sequences from GenBank, using the BLASTn algorithm. A Bellerophon programme was applied to screen the clone libraries for the presence of chimeric sequences (Huber *et al.*, 2004). Multialignments of the selected sequences were produced by ClustalX software version 2.0.12 (Larkin *et al.*, 2007). Neighbour-joining phylogenetic trees were reconstructed using PHYLIP software package version 3.69 (Felsenstein, 1989). Confidence levels for the internal branches were assessed by bootstrap analysis with 100 resamplings.

FISH and CLSM

Single gametophytes of *S. magellanicum* and *S. fallax* were fixed with 4% paraformaldehyde/phosphate-buffered salt (3:1, v/v). Separated leaves and stems, sectioned with a razor blade, were stained by in-tube FISH (Grube *et al.*, 2009). Sporophytes of *S. fallax* were fixed likewise gametophytes. For fixation, surface-sterilised capsules containing spores were disclosed by vortexing in phosphate buffer. Staining of spores was carried out on glass slides. The samples were hybridised with rRNA-targeting probes (genXpress, Wiener Neudorf, Austria) specific for Alphaproteobacteria and Planctomycetes, as dominant phylogenetic groups revealed by the clone libraries and with a set of universal bacterial probes. Hybridisation was carried out at 41 °C. The probes and corresponding stringency conditions are listed in Supplementary Table S1.

CLSM was performed with a Leica TCS SPE confocal microscope (Leica Microsystems, Mannheim, Germany). Fluorescent dyes Cy3 and Cy5 labelled to the FISH probes were sequentially excited with 532 and 635 nm laser beams, respectively; the emitted light was detected in the range of 556–607 and 657–709 nm, respectively. An additional channel (excitation at 488 nm; emission range 508–556 nm) was applied for acquiring the autofluorescence of the moss cells. Photomultiplier gain and offset were individually optimised for every channel and every field of view to improve the signal/noise ratio. Confocal stacks were acquired with a Leica ACS APO ×40 OIL CS objective (NA: 1.15) and a Leica ACS APO ×63 OIL CS objective (NA: 1.30) by applying a Z-step of 0.4–0.8 μm. Three-dimensional reconstructions were created with the software Imaris7.0 (Bitplane, Zurich, Switzerland).

Deep sequencing and bioinformatic analysis of Alphaproteobacteria

Pooled samples of the total-community DNA of *S. fallax* and *S. magellanicum* gametophytes were investigated by barcoded pyrosequencing approach (Binladen *et al.*, 2007) with the Alphaproteobacteria-specific primers ADF 681f/1492r (Blackwood *et al.*, 2005). Total-community DNA of *S. fallax* sporophyte was explored with universal bacterial primers 799f/1492r (Lane, 1991; Chelius and Triplett, 2001). Pyrosequencing libraries were generated by LGC Genomics, Berlin, Germany, using the Roche/454 GS FLX Titanium platform (454 Life Science Corporation, Brandford, CT, USA).

Raw-sequencing reads were quality filtered and trimmed by length ($\geqslant$150 bp). Rarefaction analysis was performed for phylotype clusters at 0.03, 0.05 and 0.1 genetic distance, corresponding to the levels

of the species, genera and families, respectively (Schloss and Handelsman, 2006; Hur and Chun, 2004). Due to the different number of sequences among samples, the data were normalised considering the same number of sequences to all samples. Richness estimates and diversity indices were calculated for normalised data sets, using default settings in the open source software package QIIME (http://qiime.sourceforge.net/), which allows analysis of high-throughput community sequencing data (Caporaso *et al.*, 2010).

Compositional analysis was performed using the BLAT pipeline integrated into the web interface SnoWMAn version 1.8 (https://epona.genome.tugraz.at/snowman/). Greengenes database was used as a reference database. Taxonomic assignment to family and genus levels was accomplished by the integrated Ribosomal Database Project classifier with 50% confidence threshold. For reads corresponding to *S. fallax* sporophyte, relative abundances of taxonomic groups within Alphaproteobacteria were recalculated to the total number of reads affiliated to the class.

Multivariate statistics: ecological analysis of bacterial and plant communities

Correspondence analysis was used to answer the question whether a correlation exists (1) between the independently sampled bacterial communities (defined as operational taxonomic units) of the different sampling points and (2) between bacterial communities and environmental data. For the latter, unweighted average indicator values by Ellenberg *et al.*, 1991 for the vascular plants and bryophytes were applied as independent variables (Table 1). We used the canonical correspondence analysis for unimodal data of the software package Canoco 4.5 (Lepš and Smilauer, 2003). Significance of the environmental variables for the microbial communities was tested by Monte-Carlo test with 1000 permutations.

Results

Insights into bacterial diversity by fingerprints and clone libraries

SSCP profiling of 16S rRNA genes amplified with universal primers showed highly diverse and specific bacterial communities on both *Sphagnum* species. Statistical analysis resulted in two distinct clusters of *S. fallax*- and *S. magellanicum*-originated profiles at similarity level of 9% (Figure 1). Within each species-specific cluster, only several of the samples from the different geographical sites were grouped together. Microbial fingerprints obtained from *Sphagnum* species of different bogs showed also a high similarity to each other, for example, *S. magellanicum* from Rotmoos and Wasenmoos (RM4, WM1).

A first insight into the specific biodiversity was achieved by construction of two individual clone libraries: total community DNA isolated from

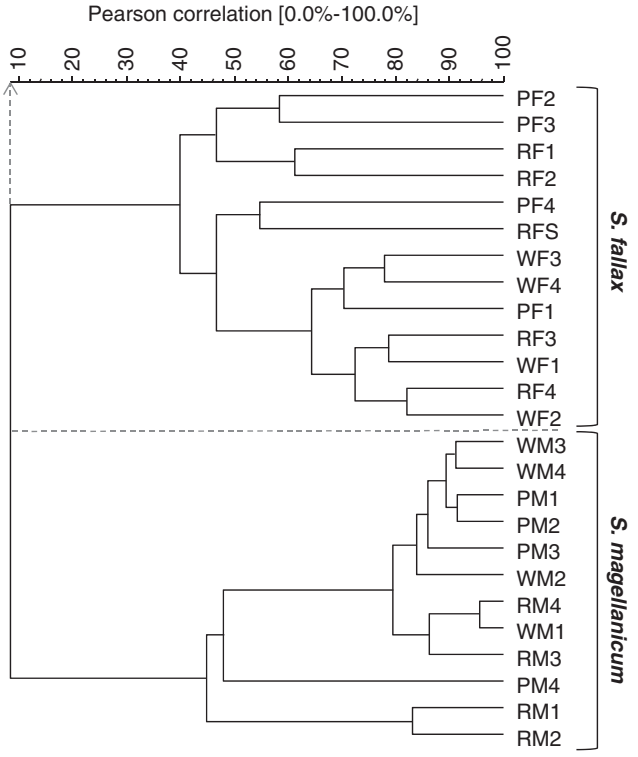

Figure 1 Dendrogram based on amplified 16S rRNA gene fragments of bacterial communities associated with *S. magellanicum* (M) and *S. fallax* (F; FS = sporophyte) from different sites in Austria (R, Rotmoos; W, Wasenmoos; P, Pürgschachen Moor) obtained by using eubacterial primers and separated by single-strand conformation polymorphism (SSCP). The patterns obtained were grouped by unweighted pair group method with average linkages.

S. magellanicum and *S. fallax* from one site was used as template. First, we solved the methodological problem to avoid the analysis of a high proportion of clones with plant-derived DNA. Introduction of a restriction assay with SphI before sequencing allowed to recognise and to eliminate plant-derived clones. Composition of microbial communities of *S. fallax* and *S. magellanicum* was clearly different (Figure 2). Altogether, *S. magellanicum* clone library consisted of 110 clones representing 12 taxonomic groups. The dominant bacterial fraction belonged to Alphaproteobacteria (29.1%), followed by sub-dominant Gammaproteobacteria (11.8%). *S. fallax*-associated community was recreated from 71 clones affiliated to eight taxa. Dominant groups belonged to Verrucomicrobia (35.2%), Planctomycetes (22.5%) and again Alphaproteobacteria (18.3%).

BLAST analysis resulted in a high proportion of not-yet-cultivated bacteria. To perform the phylogenetic analysis of clones, we constructed a phylogenetic tree for each community. Sequences of *Sphagnum*-associated bacterial clones and reference strains clustered together within defined taxonomic groups with high bootstrap values ($\geqslant$50; Supplementary Figure S1). Only three clones were closely related ($\geqslant$97% similarity) to taxonomically described bacterial species.

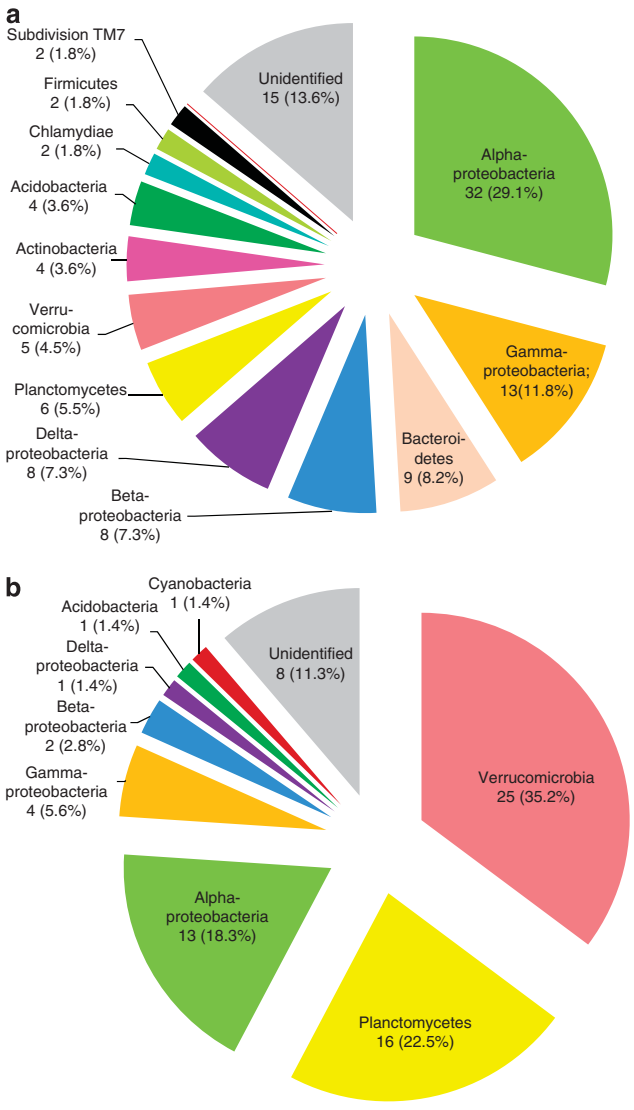

Figure 2 Bacterial community composition revealed by 16S rRNA gene clone libraries. Absolute and relative abundances of the taxonomic groups are shown for the *S. magellanicum*- (**a**) and *S. fallax*-associated (**b**) bacterial communities.

A certain number of clones possessed sequence similarities $\geqslant$97% with clones from northern terrestrial habitats and acidic environments of different geographical regions. Interestingly, several clusters of Verrucomicrobia and Planctomycetes were solely formed by *Sphagnum* clones. BLASTn alignment of the sequences within these *Sphagnum*-specific clusters resulted in a sequence identity $\leqslant$95% with database sequences. Thus, microbial diversity of *Sphagnum* comprised bacteria occurring also in other habitats, as well as *Sphagnum*-specific bacteria never found elsewhere, yet.

Deep insight into Alphaproteobacteria by pyrosequencing

As a dominant component of both *Sphagnum* communities, Alphaproteobacteria group was selected for a deep sequencing study. The rarefaction analysis of the amplicon libraries is shown in Supplementary Figure S2. Comparison of the rarefaction analyses with the number of phylotype clusters estimated by Chao1 richness estimator revealed that pyrosequencing effort reached 57.7–68.9% of estimated richness at the taxonomic level of families (Supplementary Table S2). Richness estimates of the genera and species showed that 45.9–50.3% and 31.9–32.5% of estimated richness, respectively, was recovered. The deepest classification was obtained at the ranks of families and genera (Figure 3).

Comparison of the classified reads revealed that the two investigated bryophyte species shared dominant bacterial groups and considerably differed in spectrum of sub-dominant and minor groups. Gametophytes of both moss species were dominated by members of the Acetobacteraceae family, followed by Caulobacteraceae and Rhodospirillaceae. Abundances of sub-dominant Bradyrhizobiaceae, Sphingomonadaceae, Methylocystaceae and Rhizobiaceae were clearly higher among *S. fallax*-associated Alphaproteobacteria than on *S. magellanicum*. A low number of Kordiimonadaceae and Phyllobacteriaceae were uniquely detected in *S. magellanicum*, whereas Rhodobacteraceae and Methylobacteraceae were detected only associated with *S. fallax*.

At genus level, more differences of the communities were revealed. In total, amplicon library of the gametophytes included sequences of 54 genera. *S. magellanicum* harboured 41 of them and *S. fallax* harboured 37. The *Sphagnum* species shared 24 bacterial genera. Both bryophytes were dominated by three alphaproteobacterial genera (*Acidocella*, *Magnetospirillum* and *Acidisphaera*), whereas subdominant genera (*Rhodopila*, *Phenylobacterium*, *Bradyrhizobium*, *Novosphingobium* and *Caulobacter*) occurred in different abundances.

Diversity of bacterial species was explored by Shannon diversity index (H') calculated at the genetic distance of 3%. Shannon values indicated higher diversity of Alphaproteobacteria for *S. magellanicum* (7.92) than for *S. fallax* (7.67).

Spatial structure of the bacterial communities

Due to the unique morphology of *Sphagnum* plantlets, the next step was to analyse the colonisation pattern of bacteria on/in *Sphagnum*. Especially the cell structure of leaves forms regular and peculiar microenvironments for the microbial communities; one-layer net of photosynthetically active cells (chlorocytes) is alternated with dead hyaline cells (hyalocytes). The latter possess large pores and are temporally filled with water. Combination of FISH, CLSM and computer-assisted three-dimensional reconstructions revealed colonisation patterns of *Sphagnum* gametophytes by two specific groups of microorganisms: Alphaproteobacteria

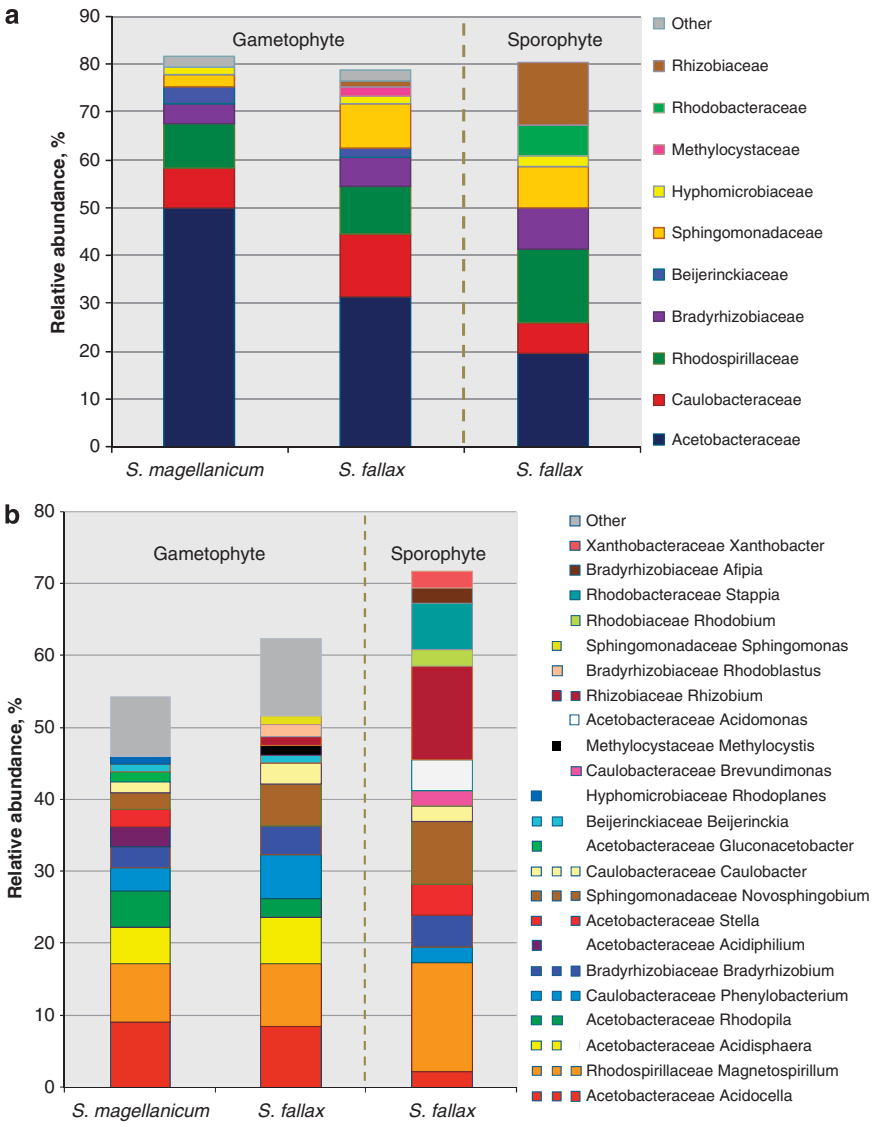

Figure 3 Taxonomic classification of Alphaproteobacteria associated with *S. fallax* and *S. magellanicum*. Pyrosequencing reads are classified at family (**a**) and genus level (**b**) with a confidence threshold of 50%. Unclassified reads are not shown. Groups not reaching 1% of relative abundance are included in 'Other'. Multi-coloured charts at the legend are shown for each genus and sample correspondingly.

and Planctomycetes. Bacterial micro-colonies were observed on the outer surface, as well as in the inner space of the gametophytes of both *Sphagnum* species (Figure 4). Three-dimensional reconstructions showed attachment of the bacteria to the cell wall of the *Sphagnum* cells. Internal spaces of the hyalocytes were densely colonised by micro-colonies closely associated to each other (Figure 5).

In general, using different probes (Supplementary Table S2), both *Sphagnum* species were characterised by similar colonisation patterns. Analyses using group-specific probes showed differences for the colonisation of *Sphagnum* leaves by Alphaproteobacteria and Planctomycetes. On both moss gametophytes, Alphaproteobacteria occurred in various morphological forms: coccoid, rod-shaped and vibroid cells, tetrads and sarcina-like aggregates (Figure 5a). Regarding the occurrence of Planctomycetes, we observed more colonies associated with *S. fallax* than *S. magellanicum*. Usually they formed less abundant colonies of coccoid cells (Figure 5d). Noteworthy, the outer cortex of the *S. fallax* stem tissues was occupied by Alphaproteobacteria in contrast to the non-colonised stems of *S. magellanicum* (Supplementary Figure S3).

Ecological factors driving the bacterial communities

Primary characterisation of the niches occupied by *Sphagnum* revealed differences in the composition of plant communities and abiotic conditions. *S. magellanicum* frequently grew together with *S. fuscum, Eriophorum vaginatum, Vaccinium oxycoccos, Andromeda polifolia, Calluna vulgaris* and *Drosera rotundifolia,* whereas *S. fallax* was

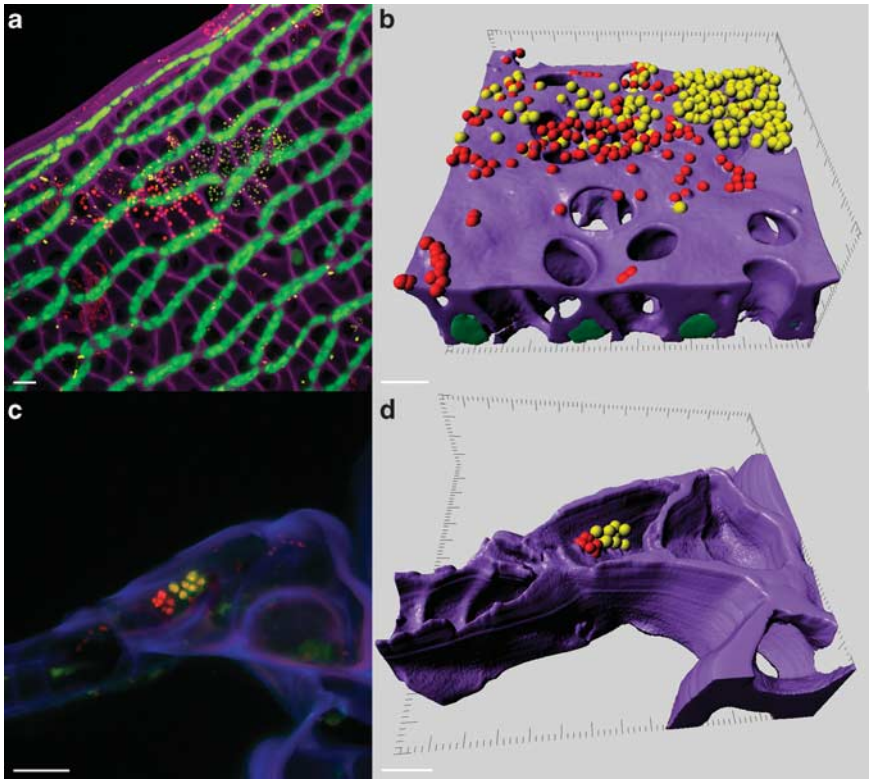

Figure 4 Localisation of bacteria in moss gametophytes. Fluorescent *in situ* hybridisation (FISH) of *S. fallax* leaves showed colonisation of the outer surface (**a** and **b**) and hyaline cells (**c** and **d**). Violet: cell walls of *Sphagnum* cells; green: chlorophyll-containing *Sphagnum* chlorocytes; yellow: Alphaproteobacteria; red: other bacteria. Images acquired by confocal laser scanning microscopy (CLSM; panels **a** and **c**) and processed by 3D computer reconstruction using Imaris7.0 (**b** and **d**). Scale bar = 10 μm.

mainly accompanied by *S. angustifolium, S. palustre, Carex rostrata, Dryopteris carthusiana, Frangula alnus, Molinia caerulea* and *Vaccinium myrtillus.* On the basis of Ellenberg's indicator values of bryophytes and vascular plants, both *Sphagnum* species showed distinctive preferences along ecological gradients (Table 1). In contrast to *S. fallax*, *S. magellanicum* habitats were characterised by lower values of soil reaction, moisture and temperature, lower amount of nutrients and more intensive exposure to the sun light.

The influence of the environmental conditions on the microbial communities of bryophytes was examined using multivariate statistical analysis (Figure 6). By the Monte-Carlo permutation test, a statistical significance was proved for nutrient richness ($P<0.001$, correlation with first axis 0.8992) and for soil reaction ($P<0.002$, correlation with first axis 0.8876). Both have the character of co-variables.

Comparison of sporophyte- and gametophyte-associated communities

High similarity of microbial communities of *S. fallax* sporophyte and gametophyte was initially detected by molecular fingerprinting of 16S rRNA genes with universal bacterial primers (Figure 1). DNA pattern of the *S. fallax* sporophyte community shared up to 55% similarity with gametophyte samples. The microbial diversity of the sporophyte- and gametophyte-associated microbial communities was investigated in more detail by a deep sequencing approach for Alphaproteobacteria and by FISH–CLSM. For the amplicon library of Alphaproteobacteria associated with the sporophyte, the saturation at the family level reached 77.0% (Supplementary Figure S3). Sporophyte library remained unsaturated at the genera and species levels: 55.0% and 50.8% of the estimated richness was uncovered.

Alphaproteobacteria associated with sporophyte and gametophyte showed substantial similarities (Figure 3). Like in the gametophyte, dominant clusters in the sporophyte were affiliated with Acetobacteraceae, Rhodospirillaceae and Caulobacteraceae families. Bradyrhizobiaceae and Sphingomonadaceae were present at similar abundances in both gametophyte and sporophyte. Certain differences in contrast to gametophyte were found; Rhizobiaceae and Rhodobacteraceae presented a dominant fraction only in the sporophyte. Bejerinckiaceae, Methylocystaceae, Brucellaceae, Aurantimonadaceae and Methylobacteriaceae were detected only in the gametophyte. Alphaproteobacterial sequences from the sporophyte of *S. fallax* were classified into 14 genera. Sporophyte- and gametophyte-associated communities shared eight genera (*Acidocella*, *Magnetospirillum*,

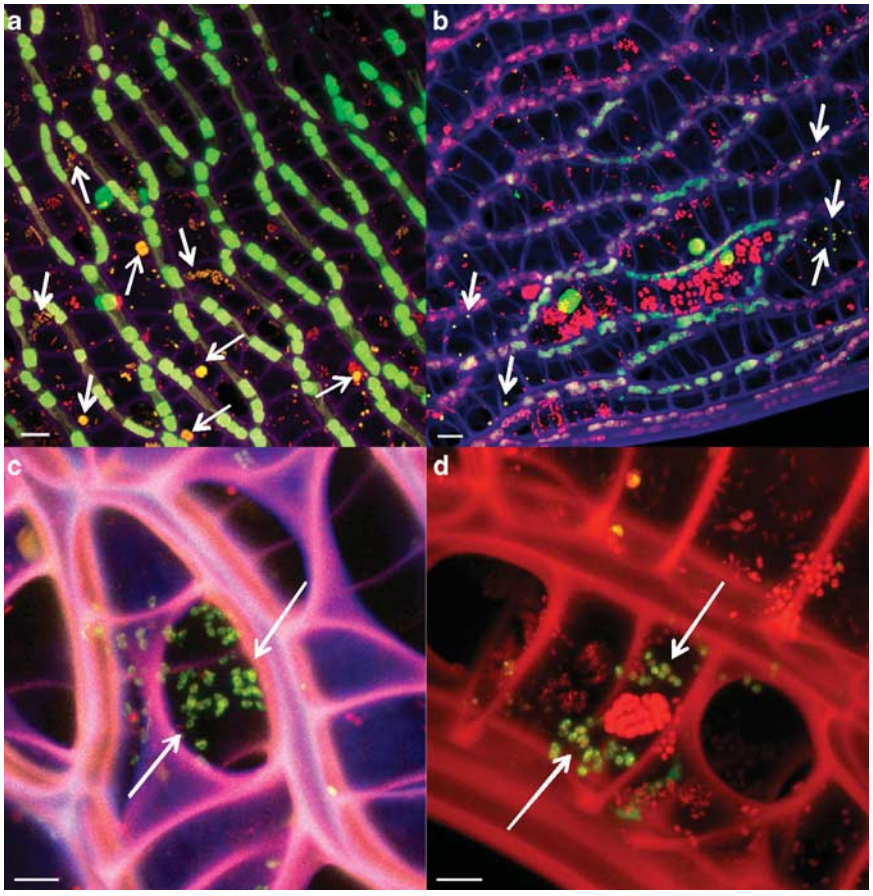

Figure 5 Localisation of bacteria in hyalocytes of *Sphagnum*. Internal space of hyalocytes of *S. fallax* (**a** and **b**) and *S. magellanicum* (**c** and **d**) hybridised with Alphaproteobacteria- and Planctomycetes-specific probes. Yellow: Alphaproteobacteria (**a** and **c**) or Planctomycetes (**b** and **d**) indicated by arrows; red: other bacteria; green: algae. Scale bar = 10 μm (**a** and **b**) or 5 μm (**c** and **d**).

Phenylobacterium, *Bradyrhizobium*, *Stella*, *Novosphingobacterium*, *Caulobacter*, *Rhizobium*), but their abundances was different. *Magnetospirillum*, *Rhizobium* and *Novosphingobium* were detected in higher abundances in sporophyte than in the gametophyte. Six sub-dominant genera *Stappia*, *Stella*, *Acidomonas*, *Rhodobium*, *Afipia* and *Xanthobacter* were specific for the sporophyte. The genera *Bejerinckia, Methylocystis, Rhodoblastus* and *Sphingomonas* were exclusively found in the gametophyte.

Using FISH–CLSM, inside the sporophyte capsules, directly connected with spores, but embedded in a matrix, bacterial cells in high amount were observed (Supplementary Figure S4).

Discussion

The main aim of this study was to analyse both the structure and the origin of *Sphagnum*-associated bacterial communities. Remarkable differences in structural diversity between the *S. fallax* and *S. magellanicum* communities were found. Abiotic parameters, which also determine the occurrence of *Sphagnum* species inside the bog, were identified as drivers of the *Sphagnum*-specific community composition. To find out the origin of this specific bacterial diversity, we compared the gametophyte-associated communities with those of the sporophyte, and found a high similarity. This led to the conclusion that a high portion of bacterial populations is transferred during the whole life cycle from the gametophyte to the sporophyte and vice versa. In the following, we will answer our questions/hypotheses.

New molecular and microscopic techniques allowed deeper insights into the structure of *Sphagnum*-associated bacterial diversity. For example, using FISH, combined with CLSM and three-dimensional modelling. In comparison with Opelt and Berg (2004), we gained new insights into the spatial structure of *Sphagnum*-associated bacteria. Although the whole *Sphagnum* gametophytes were densely colonised by bacterial micro-colonies, especially the dead big hyalocytes of the branch, leaves were occupied by bacteria. Interestingly, all colonies and single cells were attached to cell walls connected with the living cells. This connection supports the symbiotic character of the moss–microbe interaction, which was also confirmed by first functional studies. For example, methanotrophic bacteria provide approximately 10–30% of *Sphagnum*

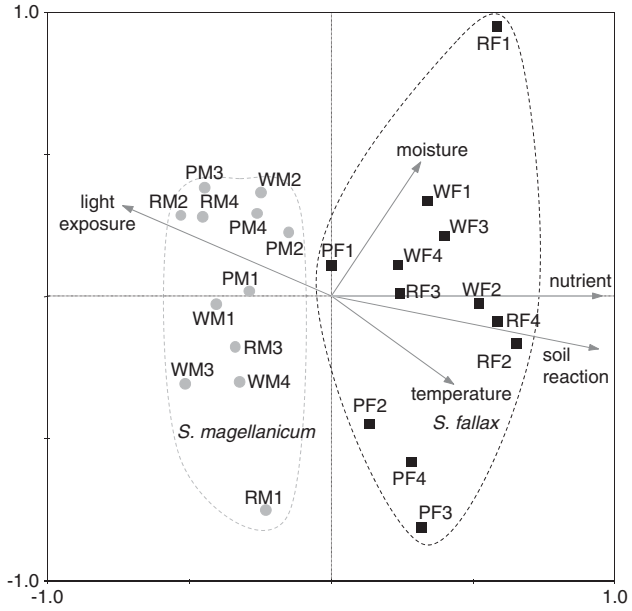

Figure 6 Canonical correspondence analysis biplot of operational taxonomic units identified by SSCP community fingerprints. Independent ecological gradients are given as unweighted average of indicator values by Ellenberg for vascular plants and bryophytes. Single fingerprints of *S. fallax*- and *S. magellanicum*-associated communities are depicted with black squares and gray circles, respectively. Names of the single SSCP patterns correspond to the names of the sample. Dashed ovals were drawn around samples of the same *Sphagnum* species. Species–environment correlations of the first and the second axes are 0.942 and 0.902, respectively. Sum of all Eigenvalues = 0.563; significance for the first axis: *P*-value = 0.002, F-ratio = 2.795 tested by Monte-Carlo permutation test (1000 permutations).

carbon (Larmola *et al.*, 2010). Moreover, *Sphagnum* harboured a high diversity of nitrogen-fixing bacteria and bacterial populations responsible for pathogen defense (Opelt *et al.*, 2007a; Opelt *et al.*, 2007b). In the CLSM images, differences in the occurrence of Alphaproteobacteria and Planctomycetes were found between both moss species. Although Alphaproteobacteria dominated colonisation of *S. magellanicum*, in microscopic pictures of *S. fallax*, more colonies of Planctomycetes were found. This was in accordance with our other results obtained by the analysis of microbial fingerprints and clone libraries. Besides of common Alphaproteobacteria, we found a discriminative spectrum of bacteria in clone libraries; although Gammaproteobacteria dominated *S. magellanicum*, *S. fallax* was mainly colonised by Verrucomicrobia and Planctomycetes. Unfortunately, representatives of Verrucomicrobia could not be detected by CLSM, although appropriate probes were applied. In contrast to the leaves, the stems were less colonised by bacteria. For stem tissues of *S. fallax,* similar colonisation patterns than for methanotrophs observed in *S. cuspidatum* were detected (Raghoebarsing *et al.*, 2005). In contrast, the stem of *S. magellanicum* was not colonised by bacteria. A different morphology of the stem can explain this difference; the stem of *S. magellanicum* is more sclerotised than the stem of *S. fallax*. Furthermore, analysis of the bacterial communities by 16S rRNA gene clone libraries led to a highly discriminative community composition. Deep sequencing of the common Alphaproteobacteria showed more similarities especially for the dominant taxa, whereas differences for sub-dominant and minor taxa were calculated. Nevertheless, we reported the highest differences ever found between species in the plant kingdom (reviewed in Berg and Smalla, 2009). Altogether, our applied multifaceted approach led to matching results and to a comprehensive picture of specific *Sphagnum*-associated bacterial communities. Not to forget that results from the clone libraries, which yielded in a high amount of yet-not-described species, and the deep sequencing approach, where only up to half of the estimated richness was recovered, indicate a hidden, still unknown bacterial diversity, which has to be discovered.

Our research also addressed the question which factors drive the high specificity of *Sphagnum*-associated microbial communities? Using multivariate statistical analysis, abiotic factors, especially nutrient richness and pH, were identified to significantly influence the microbial communities. We used Ellenberg's indicator values, which are long-term indicator values established for plants to assess moisture, nitrogen and soil reaction (represents pH) in soil (Schaffers and Sỳkora, 2000). Interestingly, nutrient richness was identified as main influencing parameter in the nutrient-poor bog environment. The content of nutrients is important for both plant and microbial communities, although in the opposite way (Opelt *et al.*, 2007c). pH expressed as soil reaction factor also significantly influenced *Sphagnum*-associated communities. This factor was often reported as the main driver, for example, in a global study of microbial communities in soil (Lauber *et al.*, 2009). In microcosm experiments under controlled conditions, Jassey *et al.* (2011) identified similar abiotic drivers of the *S. fallax*-associated microbial community, such as pH, conductivity and temperature. Especially, due to the latter, they suggest microbial communities living with *Sphagnum* as early indicators for ecosystem disturbance, especially climate change. The key question is whether the abiotic factors modulate the composition of host-specific bacterial communities, or whether these factors are primary drivers of bacterial community composition. There are several facts supporting the first point. In Opelt *et al.* (2007a), the profile of secondary metabolites including antimicrobial substances was found to be different for *S. fallax* and *S. magellanicum*, which can be one reason for host specificity. A second hint is the specific colonisation of the sporophyte, which indicates a direct transfer of the host-specific bacteria. Furthermore, the high degree of specificity was not only shown at community level, but also at clone level. The same clones were found in

Norwegian, Dutch, German and Sibirian bogs (NCBI database). On the other side, for methanotrophs, Larmola *et al.* (2010) found for transplanted *Sphagnum* species bacterial pattern and activity typical for the abiotic parameters of the destination site. However, this was an artificial experiment; the majority of approximately 300 *Sphagnum* species have very narrow ecological amplitudes (Daniels and Eddy, 1985). Moreover, *Sphagnum* mosses are not only able to adapt to their environment, but to change it; living Sphagna have extraordinarily high cation-exchange capacity and therefore acidify their environment by exchanging tissue-bound protons for basic cations in surrounding water (Soudzilovskaia *et al.*, 2010). In conclusion, the highly specific core microbiome of *Sphagnum*, which is maintained during the whole life cycle, can be subsequently modified by abiotic factors.

To find out how this specific bacterial diversity is acquired, we decided to include also the sporophyte generation into our sampling design. The sporophyte develops from the zygote within the female sex organ or archegonium, and in its early development, is therefore nurtured by the gametophyte. Inside the sporophyte capsules, embedded within thousands of spores, we observed bacteria in high abundance and diversity. Although the proportion differed, we found a high degree of qualitative similarity between the microbial communities of the sporophyte and the gametophyte, including genera well known for their beneficial plant interaction, for example, *Rhizobium*, *Bradyrhizobium* and *Caulobacter*. The direct transfer of bacteria within the whole life cycle is an interesting observation. From microbial ecology studies of higher plants, we know that plants, which represent the diploid sporophyte, acquire their populations mainly from soil (Garbeva *et al.*, 2004; Berg and Smalla, 2009), which was also found for endophytes (Hallmann *et al.*, 1997; Berg *et al.*, 2005). However, recently, it was shown that seeds also harbour microorganisms, which originate from the mother plants (Van Overbeek *et al.*, 2011).

There are several reasons that suggest *Sphagnum* mosses as unique models to study plant-associated microbial diversity, as well as plant–microbe interaction: (i) they were the first land plants and had a long time of co-evolution, (ii) they harbour a highly specific microbial diversity, (iii) they are not influenced by soil due to their ombrotrophic lifestyle, (iv) they present a bridge between terrestrial and aquatic habitats, (v) they are easy to investigate microscopically, because they consist only of a one cell-layer network, (vi) they can be cultivated *in vitro* to study specific interactions, and (vii) they are relevant for our climate on earth. In our study, we found a remarkable bacterial diversity on *Sphagnum* mosses. This is, besides the impact on climate change, one more reason to protect this hidden beautiful biodiversity inside bogs.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)

Supplementary information

Table S1 Probes and stringency conditions at 41°C used for the FISH

Name	*Sequence (5'-3')*	*Specificity*	*Formamide concentration %*	*Reference*
EUB338[a]	GCTGCCTCCCGTAGGAGT	Most bacteria	15	Amann *et al*., 1990
EUB338II[a]	GCAGCCACCCGTAGGTGT	Planctomycetales	15	Daims *et al*., 1999
EUB338III[a]	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	15	Daims *et al*., 1999
ALF968	GGTAAGGTTCTGCGCGTT	Alphaproteobacteria	45	Loy *et al*., 2007
PLA46	GACTTGCATGCCTAATCC	Planctomycetales	40	Neff *et al*., 1998
PLA886[b]	GCCTTGCGACCATACTCCC	Planctomycetes	45	Neff *et al*., 1998
NONEUB	ACTCCTACGGGAGGCAGC	–	–[c]	Amann *et al*., 1990

[a] Used together in equimolar ratio.

[b] Hybridisation with equimolar amount of competitor probe cPLA886 (5' GCCTTGCGACCGTACTCCC 3').

[c] Used for negative control at the same stringency conditions applied for positive FISH probe.

Table S2 Richness estimates and diversity indices for 16S rRNA gene amplicon libraries of *Sphagnum* samples[a].

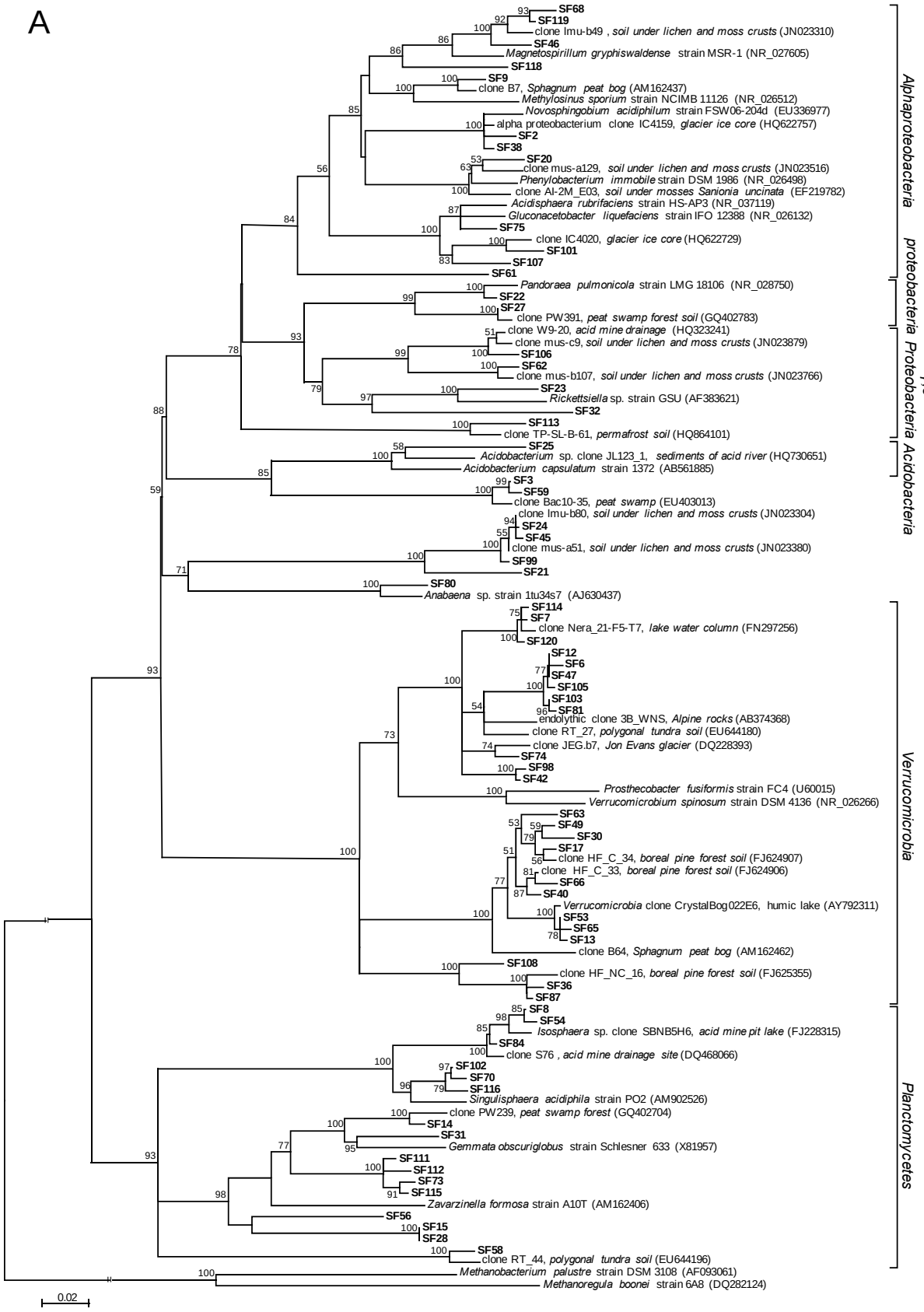
Sample[b]	Genetic distance[c]	Phylotype clusters	Chao1	Coverage (%)	H'[d]
MG	0.03	321	1006	31.9	7.92
	0.05	217	474	45.9	7.07
	0.10	78	114	68.9	4.96
FG	0.03	299	919	32.5	7.67
	0.05	195	388	50.3	6.65
	0.10	78	135	57.7	4.75
FS	0.03	97	191	50.8	4.41
	0.05	63	114	55.0	3.83
	0.10	31	40	77.0	3.02

[a]The number of sequences of each sample was normalised to 495.

[b]Abbreviations correspond to the samples of *S. magellanicum* gametophytes (MG), *S. fallax* gametophytes (FG) and *S. fallax* sporophytes (FS).

[c]Genetic distances represent the taxonomic levels of species (0.03), genera (0.05) and families (0.1).

[d]Shannon diversity indices.


A
SF68
SF119
clone lmu-b49 , soil under lichen and moss crusts (JN023310)
SF46
Magnetospirillum gryphiswaldense strain MSR-1 (NR_027605)
SF118
SF9
clone B7, Sphagnum peat bog (AM162437)
Methylosinus sporium strain NCIMB 11126 (NR_026512)
Novosphingobium acidiphilum strain FSW06-204d (EU336977)
alpha proteobacterium clone IC4159, glacier ice core (HQ622757)
SF2
SF38
SF20
clone mus-a129, soil under lichen and moss crusts (JN023516)
Phenylobacterium immobile strain DSM 1986 (NR_026498)
clone Al-2M_E03, soil under mosses Sanionia uncinata (EF219782)
Acidisphaera rubrifaciens strain HS-AP3 (NR_037119)
Gluconacetobacter liquefaciens strain IFO 12388 (NR_026132)
SF75
clone IC4020, glacier ice core (HQ622729)
SF101
SF107
SF61
Pandoraea pulmonicola strain LMG 18106 (NR_028750)
SF22
SF27
clone PW391, peat swamp forest soil (GQ402783)
clone W9-20, acid mine drainage (HQ323241)
clone mus-c9, soil under lichen and moss crusts (JN023879)
SF106
SF62
clone mus-b107, soil under lichen and moss crusts (JN023766)
SF23
Rickettsiella sp. strain GSU (AF383621)
SF32
SF113
clone TP-SL-B-61, permafrost soil (HQ864101)
SF25
Acidobacterium sp. clone JL123_1, sediments of acid river (HQ730651)
Acidobacterium capsulatum strain 1372 (AB561885)
SF3
SF59
clone Bac10-35, peat swamp (EU403013)
clone lmu-b80, soil under lichen and moss crusts (JN023304)
SF24
SF45
clone mus-a51, soil under lichen and moss crusts (JN023380)
SF99
SF21
SF80
Anabaena sp. strain 1tu34s7 (AJ630437)
SF114
SF7
clone Nera_21-F5-T7, lake water column (FN297256)
SF120
SF12
SF6
SF47
SF105
SF103
SF81
endolythic clone 3B_WNS, Alpine rocks (AB374368)
clone RT_27, polygonal tundra soil (EU644180)
clone JEG.b7, Jon Evans glacier (DQ228393)
SF74
SF98
SF42
Prosthecobacter fusiformis strain FC4 (U60015)
Verrucomicrobium spinosum strain DSM 4136 (NR_026266)
SF63
SF49
SF30
SF17
clone HF_C_34, boreal pine forest soil (FJ624907)
clone HF_C_33, boreal pine forest soil (FJ624906)
SF66
SF40
Verrucomicrobia clone CrystalBog022E6, humic lake (AY792311)
SF53
SF65
SF13
clone B64, Sphagnum peat bog (AM162462)
SF108
clone HF_NC_16, boreal pine forest soil (FJ625355)
SF36
SF87
SF8
SF54
Isosphaera sp. clone SBNB5H6, acid mine pit lake (FJ228315)
SF84
clone S76 , acid mine drainage site (DQ468066)
SF102
SF70
SF116
Singulisphaera acidiphila strain PO2 (AM902526)
clone PW239, peat swamp forest (GQ402704)
SF14
SF31
Gemmata obscuriglobus strain Schlesner 633 (X81957)
SF111
SF112
SF73
SF115
Zavarzinella formosa strain A10T (AM162406)
SF56
SF15
SF28
SF58
clone RT_44, polygonal tundra soil (EU644196)
Methanobacterium palustre strain DSM 3108 (AF093061)
Methanoregula boonei strain 6A8 (DQ282124)
0.02
Alphaproteobacteria
Beta-proteobacteria
γ,δ-Proteobacteria
Acidobacteria
Verrucomicrobia
Planctomycetes

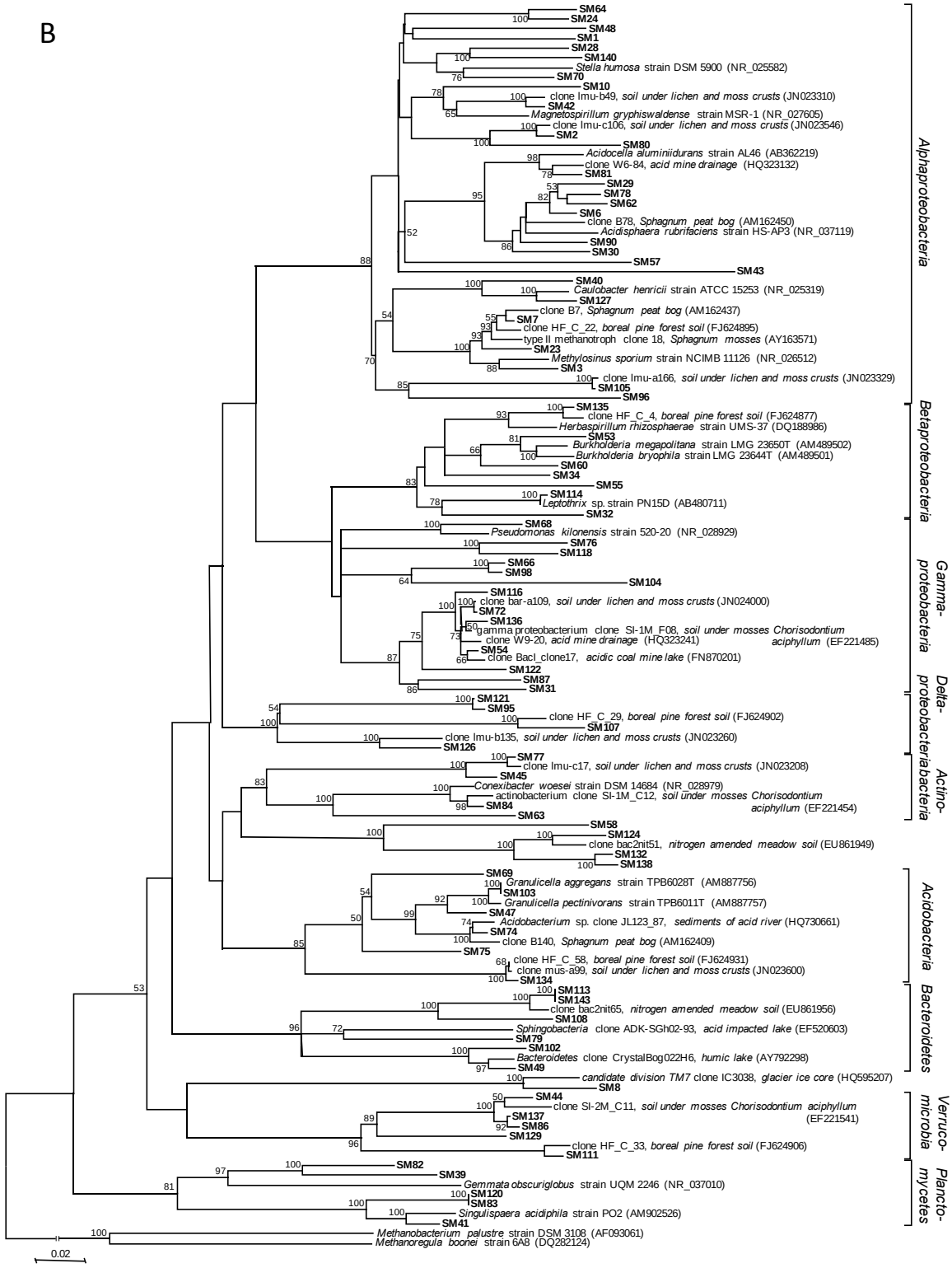


Figure S1 Neighbor-joining trees of *Sphagnum fallax*- (A) and *S. magellanicum* (B)-associated microbial communities, based on 757 and 787 nt 16S rRNA gene multialignment, respectively. Phylogenetic relationships are shown for 67 (A) and 82 (B) bacterial clones obtained from *Sphagnum* mosses in this work (bold) and ecologically related reference

sequences. The isolation sources of environmental sequences are shown (italic) and the accession numbers are indicated in brackets. Numbers at nodes indicate bootstrap values out of 100 re-samplings of the data exceeding 50%. Scale bar: 0.02 substitutions per site.

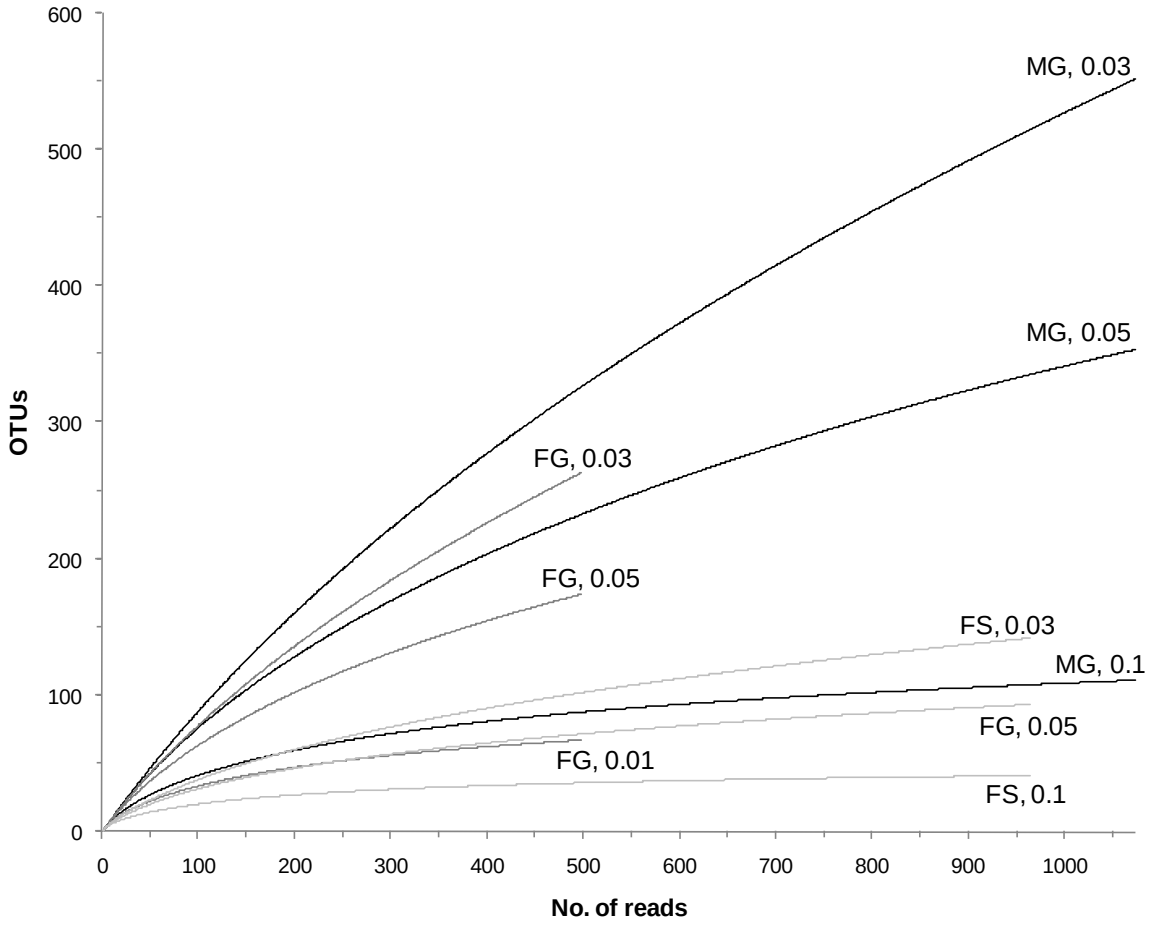


Figure S2 Rarefaction curves for 16S rRNA gene amplicon libraries of *Sphagnum* samples. Saturation curves are presented for *Alphaproteobacteria* associated with *S. fallax* (FG) and *S. magellanicum* gametophytes (MG) and for total bacteria associated with *S. fallax* sporophytes (FS). Phylotype clusters are identified at the genetic distance of 0.03, 0.05 and 0.1, corresponding to the taxonomic levels of species, genera and families, respectively.

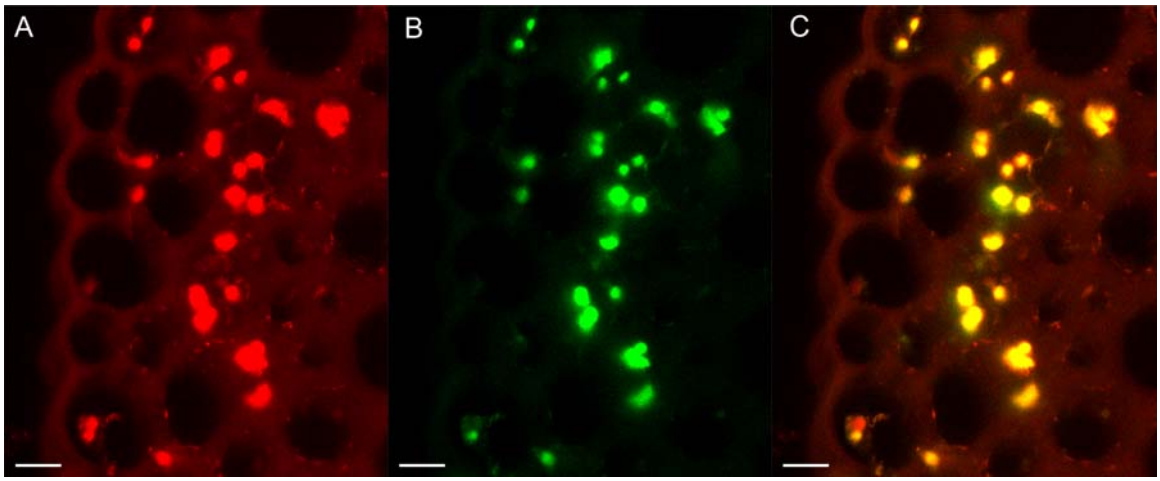


Figure S3 Stem of *S. fallax* colonised by *Alphaproteobacteria*. Signal from cross-section of stem hybridised with EUB338, II, III probes (A), ALF968 probe (B) and overlapping of both signals (C). *Alphaproteobacteria* appear as yellow in panel C. Scale bar = 10 µm.

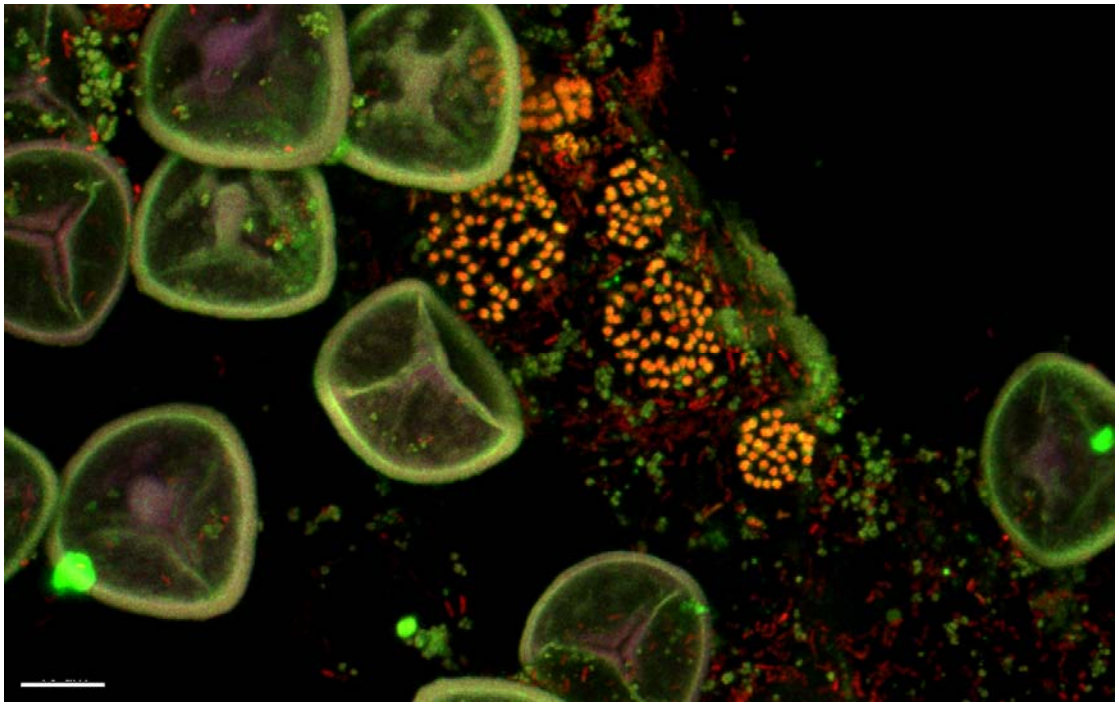


Figure S4 Fluorescent *in situ* hybridisation (FISH) of *S. fallax* sporophytes. Image is acquired by Confocal Laser Scanning Microscopy (CLSM): spores of *S. fallax* (green) embedded into the matrix (green) containing perine grains (orange) and bacteria (red, stained with set of universal EUB338, II, III probes). Scale bar = 10 µm.

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Daims H, Brühl A, Amann R, Schleifer KH, Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

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Publication II

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Similar diversity of Alphaproteobacteria and nitrogenase gene amplicons on two related *Sphagnum* mosses

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Similar diversity of Alphaproteobacteria and nitrogenase gene amplicons on two related *Sphagnum* mosses

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Sphagnum mosses represent a main vegetation component in ombrotrophic wetlands. They harbor a specific and diverse microbial community with essential functions for the host. To understand the extend of host specificity and impact of environment, *Sphagnum fallax* and *Sphagnum angustifolium*, two phylogenetically closely related species, which show distinct habitat preference with respect to the nutrient level, were analyzed by a multifaceted approach. Microbial fingerprints obtained by PCR-single-strand conformation polymorphism of 16S rRNA and nitrogenase-encoding (*nifH*) genes were highly similar for both *Sphagnum* species. Similarity was confirmed for colonization patterns obtained by fluorescence *in situ* hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM): Alphaproteobacteria were the main colonizers inside the hyaline cells of *Sphagnum* leaves. A deeper survey of Alphaproteobacteria by 16S rRNA gene amplicon sequencing reveals a high diversity with *Acidocella*, *Acidisphaera*, *Rhodopila*, and *Phenylobacterium* as major genera for both mosses. Nitrogen fixation is an important function of *Sphagnum*-associated bacteria, which is fulfilled by microbial communities of Sphagna in a similar way. *NifH* libraries of *Sphagnum*-associated microbial communities were characterized by high diversity and abundance of Alphaproteobacteria but contained also diverse amplicons of other taxa, e.g., Cyanobacteria and Deltaproteobacteria. Statistically significant differences between the microbial communities of both *Sphagnum* species could not be discovered in any of the experimental approach. Our results show that the same close relationship, which exists between the physical, morphological, and chemical characteristics of *Sphagnum* mosses and the ecology and function of bog ecosystems, also connects moss plantlets with their associated bacterial communities.

Keywords: *Sphagnum fallax*, *Sphagnum angustifolium*, SSCP fingerprints, FISH–CLSM, amplicon library, Alphaproteobacteria, nitrogenase

INTRODUCTION

Northern wetlands belong to the oldest vegetation forms with more or less constant conditions for thousands of years. *Sphagnum*-dominated peatlands represent one of the most extensive types of Northern wetlands (Dedysh, 2011). They cover with 4 million km^2 approx. 3% of the Earth surface and have a high value for biodiversity–conservation, as reservoir of fresh water, for human welfare and our world climate due to its extraordinary role in carbon sequestration (Gorham, 1991; Clymo et al., 1998). Despite their age, these long-existing ecosystems are extremely sensitive to changing a-biotic factors connected with climate change (Belyea and Malmer, 2004; Dise, 2009). As the dominant vegetation component of the peatlands, *Sphagnum* moss has been used globally as an indicator of climate change (Gignac and Vitt, 1994; Whinam and Copson, 2006; Granath et al., 2009). The ecological significance of bogs is directly related to the physical, morphological, and chemical characteristics of *Sphagnum* peat mosses; which set *Sphagnum* apart from other mosses to practically every stage of the life cycle (Shaw et al., 2003). Moreover, *Sphagnum* mosses are able to change their environments: living Sphagna have extraordinarily high cation exchange capacity and therefore acidify their environment by exchanging tissue-bound protons for basic cations in surrounding water (Soudzilovskaia et al., 2010). Interestingly, *Sphagnum* leaves are highly specialized: they form a network of living, chlorophyll-containing chlorophytes and dead, cell content-free hyalocytes, which are responsible for their high water holding capacity. *Sphagnum* species also produce species specific bioactive secondary metabolites influencing microbial colonization (Opelt et al., 2007a).

Sphagnum mosses are colonized by diverse bacterial communities. Microbial populations involved in CH_4 cycling, i.e., methanotrophic bacteria (Dedysh et al., 1998; Dedysh, 2002; Raghoebarsing et al., 2005; Larmola et al., 2010; rev. in Dedysh, 2011) as well as methanogens including archaea (Horn et al., 2003; Freitag et al., 2010) have attracted research interest due to their important function for methane emission. Recently, we could show that living *Sphagnum* mosses are colonized in high abundances with specific microorganisms, which fulfill other important functions like

nutrient supply and pathogen defense for moss growth and health (Opelt et al., 2007a,b). An extremely high impact of the *Sphagnum* species was found on the structure of the microbial diversity, and this diversity is transferred directly from the sporophyte (within the sporangium capsule) to the gametophyte and *vice versa* (Bragina et al., 2011). In the latter, we analyzed bacterial communities of two ubiquitous *Sphagnum* species, *S. magellanicum*, and *Sphagnum fallax*, in three Alpine bogs in Austria. Extremely high differences between bacterial communities of both Sphagna were found by a combination of methods independently from the site. For example, a discriminative spectrum of bacteria was identified: while Alpha- and Gamma-proteobacteria dominated *S. magellanicum*, *S. fallax* was mainly colonized by Verrucomicrobia, Planctomycetes, and Alphaproteobacteria. In addition, bacterial communities were strongly driven by a-biotic factors (nutrient richness and pH), and correlated strongly with the composition of higher plant communities. The specific microbial diversity associated with the highly diverse *Sphagnum* genus (Daniels and Eddy, 1985) is largely unknown but important to understand and protect *Sphagnum* in bog ecosystems.

The objective of this work was to study the structure and function of *Sphagnum*-associated bacteria to understand extend and degree of host specificity. Therefore, two phylogenetically closely related and widely distributed species with overlapping micro-niches but varying trophic specialization were selected: *Sphagnum angustifolium* (Warnst.) C. E. O. Jensen and *S. fallax* H. Klinggr. (Daniels and Eddy, 1985; Flatberg, 1992; Sastad et al., 1999). Both *Sphagnum* species were first characterized by their secondary metabolite profile. A polyphasic approach was applied to study bacterial communities with a special focus on Alphaproteobacteria and nitrogen-fixing bacteria: (i) microbial fingerprints by PCR-single-strand-conformation polymorphism (SSCP) applying universal and group-specific 16S rRNA gene-targeting primers and nitrogenase (*nifH*) gene-specific primers, (ii) fluorescent *in situ* hybridization with universal and group-specific probes coupled with fluorescence *in situ* hybridization confocal laser scanning microscopy (FISH–CLSM) and image analysis, (iii) deep-sequencing of Alphaproteobacteria, and (iv) a functional approach to analyses the potential for nitrogen fixation by nitrogenase (*nifH*) genes in amplicon libraries.

MATERIALS AND METHODS

SAMPLING PROCEDURE

Adult gametophytes of *S. angustifolium* (section *Cuspidata*) and *S. fallax* (section *Cuspidata*) were sampled from the bog "Pürgschachen Moor" (Liezen, 1.7 km SW Ardning, N47° 34.789′E14°2017′) in Austria in July and November 2010. In this bog, *S. angustifolium* has broader ecological amplitude and grow in mesotrophic wet hollows with some ground water influence as well as in more ombrotrophic hummocks over ground water level. *S. fallax* grow only in wet mesotrophic conditions in which we collected the samples of both species in comparable ecological situations. The pH of the surrounding peat water was measured at all sampling points and showed mean values of 4.00 (SD, 0.15) for *S. fallax* and 4.04 (SD, 0.31) for *S. angustifolium*. Altogether eight independent replicates per *Sphagnum* species consisting of composite samplings of 15–20 plantlets were collected and stored separately. The approximate length of the sampled plants was 14.5 cm (SD, 2.3 cm) for *S. fallax* and 15.1 cm (SD, 2.5 cm) for *S. angustifolium*. The eight sampling points were situated in a distance of at least 10 m, while both species had a distance of not more than 1 m at each single point. The living green parts of the plantlets were placed into sterile plastic bags and transported cooled to the laboratory.

CHEMICAL ANALYSIS OF *SPHAGNUM* SECONDARY METABOLITES

Spectra of secondary metabolites of *Sphagnum* samples were analyzed using high-performance liquid chromatography with UV photodiode array detection (HPLC-PDA) as described previously (Opelt et al., 2007a).

TOTAL-COMMUNITY DNA ISOLATION

The bacterial fraction associated with gametophytes was extracted according to the modified protocol of Opelt and Berg (2004). Briefly, 5 g of plant material were physically disrupted with sterile pestle and mortar and re-suspended in 10 ml of 0.85% NaCl. Two milliliter of the suspension was centrifuged at 13000 rpm for 20 min at 4°C and the pellet was used for isolation of the total-community DNA. For mechanical lysis, the cells were homogenized twice in a FastPrep® FP120 Instrument (MP Biomedicals) for 30 s at speed 5.0. The obtained DNA was purified using the FastDNA® SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's protocol. Final aliquots of the total-community DNA were further applied in PCR-based approaches.

MICROBIAL FINGERPRINTING BY PCR-SSCP

Fingerprinting of the moss-associated bacterial communities was carried out by PCR-based SSCP described by Schwieger and Tebbe (1998). 16S rRNA genes of *Bacteria* were amplified with universal bacterial primers Com1/Unibac-II-927r^{P} (Schwieger and Tebbe, 1998; Zachow et al., 2008). A set of Alphaproteobacteria-specific primers ADF681F/1492r, followed by ADF681F/927r^{P}, was applied using a semi-nested protocol (Blackwood et al., 2005). Bacterial nitrogenase gene (*nifH*) fragments were amplified in a nested approach with nifH3/19F, nifH11/nifH22^{P} primers (Yeager et al., 2004). The amplicons were separated using the TGGE Maxi system (Biometra) at 400 V and 26°C in acrylamide gel followed by silver staining.

Strand conformation polymorphism is based on the differences in the conformation of single-stranded DNA fragments. The electrophoretic mobility of the single-stranded DNA fragments depends on their three-dimensional conformation. Each of the amplification products was identified by its electrophoretic distance on SSCP gel and the number of DNA fragments. According to the distance of the bands, the SSCP gels were virtually divided into operational taxonomic units (OTUs). The presence or absence of individual amplified product DNA bands in each group was scored. The obtained matrix was used to compare statistically (see statistics).

FLUORESCENT *IN SITU* HYBRIDIZATION AND CONFOCAL LASER SCANNING MICROSCOPY

Single gametophytes of *S. angustifolium* and *S. fallax* were fixed with 4% paraformaldehyde/phosphate buffered salt (3:1, v/v).

Separated leaves were stained by in-tube FISH (Grube et al., 2009). Fluorescently labeled rRNA-targeting probe ALF968 specific for Alphaproteobacteria (Loy et al., 2007) was applied in combination with equimolar mixture of universal bacterial probes EUB338, EUB338II, EUB338III (Amann et al., 1990; Daims et al., 1999). *Sphagnum* samples were consequently hybridized with ALF968 (41˚C, 45% formamide) followed by EUB338/EUB338II/EUB338III (41˚C, 15% formamide). Negative control was hybridized with non-target NON-EUB probe (Amann et al., 1990) at the same stringency conditions applied for the positive FISH probes.

Confocal laser scanning microscopy was performed using a confocal microscope Leica TCS SPE (Leica Microsystems). Fluorescent dyes Cy3 and Cy5 labeled to the FISH probes were sequentially excited with 532 and 635 nm laser beams, respectively; the emitted light was detected in the range of 556–607 and 657–709 nm, respectively. An additional channel (excitation at 488 nm; emission range 508–556 nm) was used for acquiring the autofluorescence of the moss cells. Photomultiplier gain and offset were individually optimized for every channel and every field of view, in order to improve the signal/noise ratio. Confocal stacks were acquired with a Leica ACS APO 40X OIL CS objective (NA: 1.15) and a Leica ACS APO 63X OIL CS objective (NA: 1.30) by applying a Z-step of 0.4–0.8 μm. Three-dimensional reconstructions were created with the software Imaris 7.0 (Bitplane).

DEEP-SEQUENCING AND BIOINFORMATIC ANALYSIS

Diversity of Alphaproteobacteria and nitrogen-fixing bacteria associated with *Sphagnum* species was deeply investigated by barcoded pyrosequencing approach. The total-community DNA was amplified with the set of Alphaproteobacteria-specific primers ADF681F/Unibac-II-927r (Blackwood et al., 2005; Zachow et al., 2008) and *nifH* gene-specific primers nifH3/nifH4, nifH1/nifH2 in the nested approach (Zehr and Turner, 2001) using Taq-&Go™Ready-to-use PCR Mix (MP Biomedicals). Duplicate PCR products from all templates were purified with Wizard® SV Gel and PCR Clean-Up System (Promega). Amplicons of each *Sphagnum* sp. were pooled together and subjected to the pyrosequencing using the Roche/454 GS FLX+ Titanium platform executed by GATC Biotech (Konstanz, Germany).

The 16S rRNA gene amplicon libraries specific for Alphaproteobacteria were analyzed as specified by Bragina et al. (2011). Shortly, raw sequencing reads were quality and length filtered (≥150 bp). Rarefaction analysis was performed for phylotype clusters of 97, 95, and 90% similarity by using the tools of the RDP's Pyrosequencing Pipeline (Cole et al., 2009). Datasets were normalized to the same number of sequences. Richness estimates and diversity indices were calculated in the open source software package QIIME (Caporaso et al., 2010). Classification of the reads was performed using the BLAT pipeline within the web interface SnoWMAn version 1.11[1] with 80% confidence threshold.

Amplicon libraries of the nitrogenase gene (*nifH*) were explored using the FunGene Pipeline of RDP server[2] with parameters stated by Farnelid et al. (2011). Primer sequences were trimmed and reads of a low quality and shorter 200 bp were removed. Filtered reads were translated into amino acid sequences and clipped at 60 aa. Further analyses were carried out on amino acid sequences. For the rarefaction, datasets were clustered with 100, 96, and 92% similarity cut-offs. Richness estimates and diversity indices were calculated for the subsets normalized to the same number of sequences by QIIME software. Compositional diversity was compared by Sørensen (C_s) and Shannon (H') indices at 96% similarity level. Phylogenetic analysis was performed for the clusters of 92% similarity with ≥10 sequences. Reference sequences were obtained using the NCBI algorithm TBLASTN and a phylogenetic tree was constructed as described previously (Bragina et al., 2011).

STATISTICS

Computer-assisted analysis of SSCP profiles was performed using the GelCompare II version 5.1 software package (Applied Maths). Similarity matrices were constructed based on Pearson's correlation coefficients (r) and cluster analyses were done by the unweighted pair group method with average linkages (UPGMA). SSCP profiles of the microbial communities generated with universal bacterial and *nifH* gene-specific primers were further applied for the multivariate analysis. Single DNA bands, characterized by the relative position and abundance on the gel, were defined as *OTUs* and used as response variables for detrended correspondence analyses (DCA) by Canoco 4.5 for Windows (Lepš and Smilauer, 2003).

RESULTS

PROFILING OF THE *SPHAGNUM* SECONDARY METABOLITES

High-performance liquid chromatography-PDA profiling of plant extracts yielded notably similar chromatograms and spectra of the prevailing peaks of the secondary metabolites for both *Sphagnum* species. The UV spectra suggested various phenols and indol derivatives including sphagnic acid (peak 7; **Figure 1**). The only noteworthy difference was the more pronounced accumulation of flavonoid conjugates (naringenin and apigenin) in *S. angustifolium*. The late retention time precluded glycosides but more lipophilic molecules. The result testified common chemical basis for establishment of the similar microbial diversity of the compared moss species.

MOLECULAR FINGERPRINTING OF MICROBIAL COMMUNITIES

In the molecular fingerprinting approach we used a specific set of primers: universal primers to get an overview about the whole bacterial community associated with *Sphagnum*, primers for Alphaproteobacteria because this is a ubiquitous and cosmopolitan phylogenetic class associated with *Sphagnum* (Bragina et al., 2011), and *nifH* primers to detect nitrogen-fixing bacteria as important functional group (Opelt et al., 2007a). All SSCP fingerprints obtained with universal and group-specific primers revealed high similarity of the microbial communities associated with *S. fallax* and *S. angustifolium* (**Figure 2**). UPGMA analysis of the Bacteria profiles resulted in a minimum similarity level of 95.5% of bryophyte-associated communities. Alphaproteobacteria patterns formed a common cluster at 87.1% similarity. Fingerprints of the *nifH* gene clustered into two groups at 41.2% similarity. Within

[1]https://epona.genome.tugraz.at/snowman/
[2]http://fungene.cme.msu.edu/FunGenePipeline/

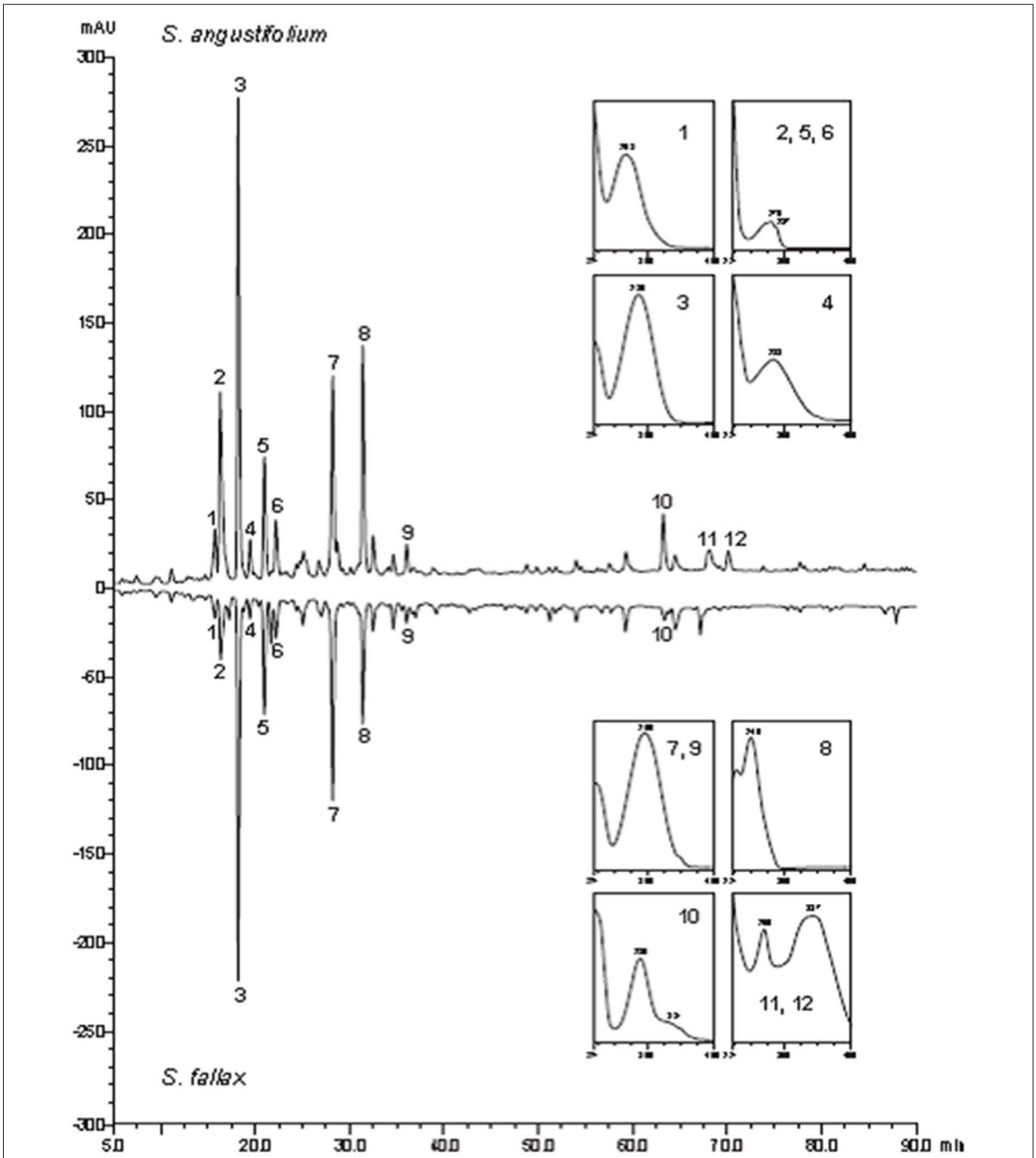

FIGURE 1 | High-performance liquid chromatography -UV secondary metabolite profiles of *S. angustifolium* and *S. fallax* at 229 nm (UV spectra (200–400 nm; 1, phenol; 2, 5, 6, indole derivative; 3, 4, phenols; 7, 9, sphagnic acid; 8, phenol; 10, naringenin type flavonoid; 11, 12, apigenin flavonoids.

each cluster, samples of both Sphagna grouped together. This overlap of the microbial communities was confirmed statistically by a detrended correspondence analysis (**Figure 3**).

MICROBIAL COLONIZATION PATTERNS

Sphagnum gametophytes characterized by the unique morphology were studied for microbial colonization patterns by FISH. Again,

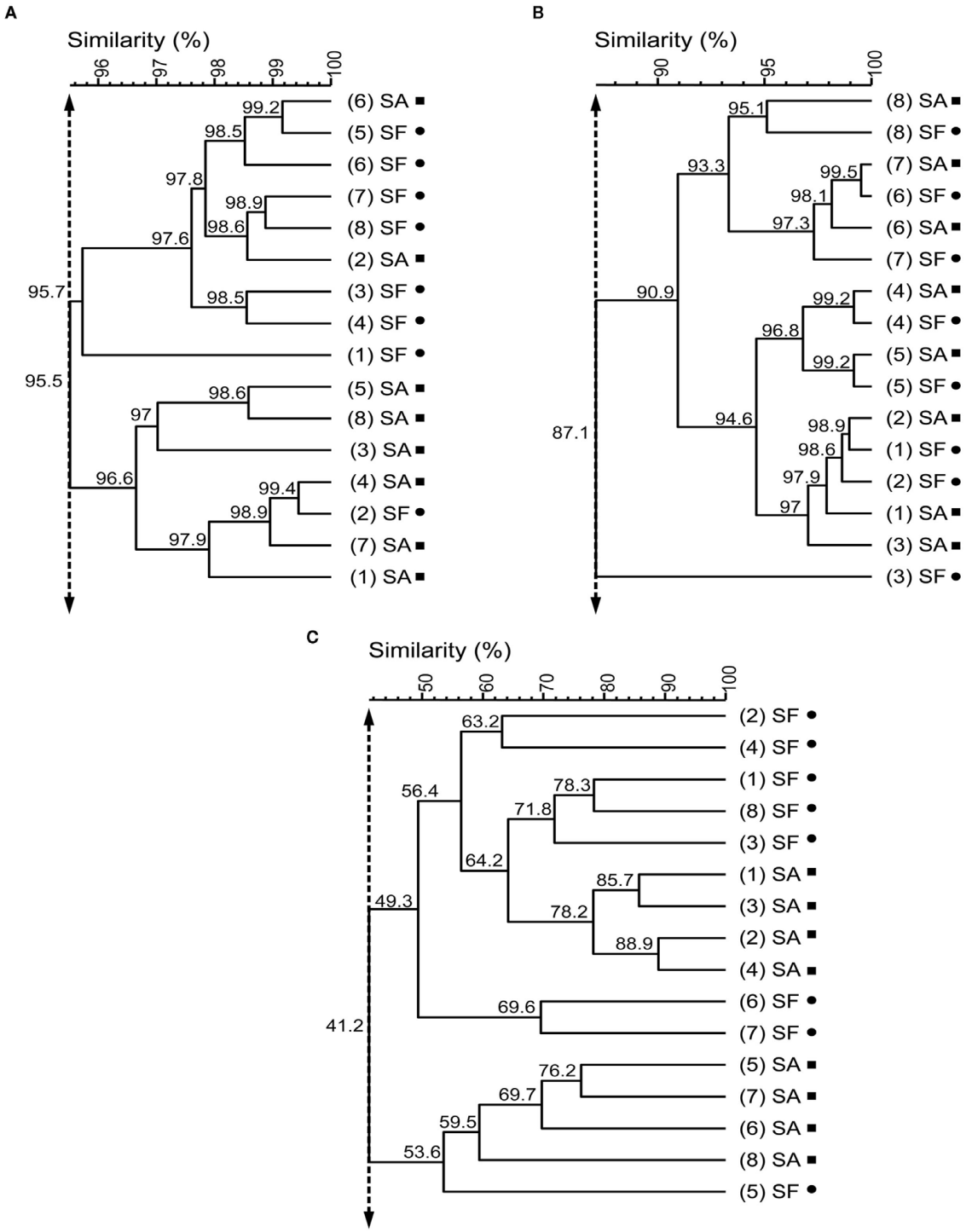

FIGURE 2 | Unweighted pair group method with average linkages dendrograms of bacterial communities associated with *S. angustifolium* (SA, squares) and *S. fallax* (SF, circles). The dendrograms were generated from the PCR-SSCP profiles of *Bacteria* **(A)**, Alphaproteobacteria **(B)**, and bacterial *nifH* genes **(C)** using unweighted pair group method with average linkages (UPGMA). Numbers in round brackets indicate replicates. Double-headed vertical arrows indicate the similarity for the groupings.

we applied universal and Alphaproteobacteria-specific probes according to the above mentioned reasons. The stem and branch leaves are usually differentiated in size and shape, but equally are formed by dimorphic leaf cells in which large, empty hyaline cells perforated by pores are enclosed in a network of narrower, chlorophyllose cells (**Figures 4A,D**). CLSM observation of both

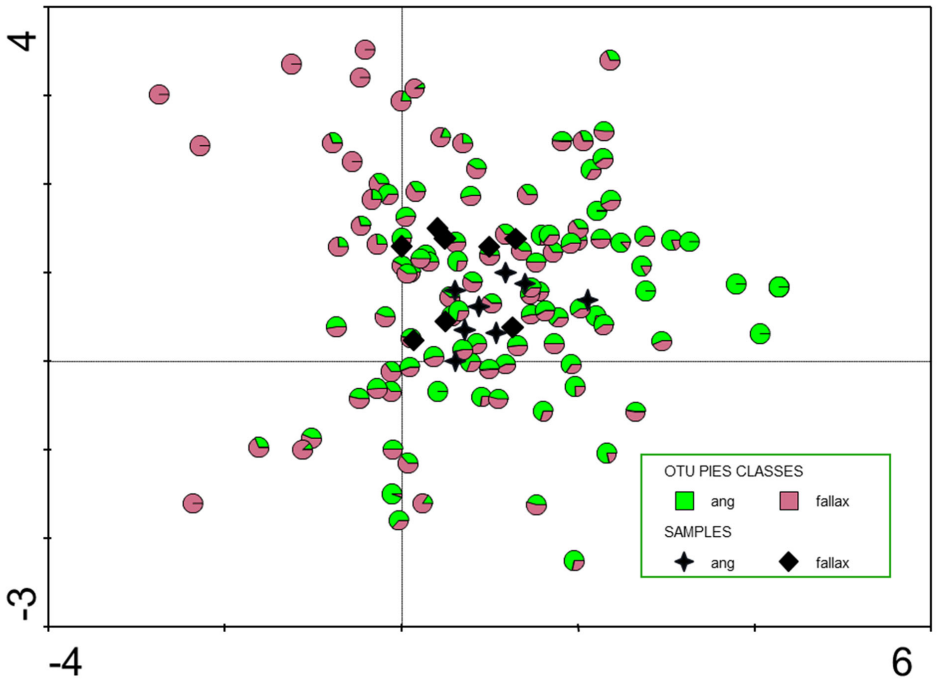

FIGURE 3 | Detrended correspondence analysis (DCA, indirect unimodal gradient analysis) of operational taxonomic units (OTUs) identified by SSCP community fingerprints. Eigenvalues of first and second axis are 0.244 and 0.141, respectively; sum of all eigenvalues 1.715. Black stars and diamonds show the location of the 16 samples, colored circles the location of the 111 *OTUs* in the biplot. The colors indicate its preference to *Sphagnum angustifolium* (ang) or *S. fallax*, respectively.

leave types showed dense colonization by bacterial colonies of the internal space of the gametophytes as shown for branch leaves in **Figures 4B,E**. Alphaproteobacterial cells presented up to 50% of the detected bacterial colonies. Three-dimensional reconstruction of the acquired images supported that bacteria primarily occupied dead hyaline cells **Figures 4C,F**. In conclusion, FISH–CLSM approach displayed similar colonization patterns for *S. angustifolium* and *S. fallax* by the bacterial communities.

DEEP-SEQUENCING OF ALPHAPROTEOBACTERIA AND *NIFH* GENES

Alphaproteobacteria and nitrogen-fixing bacteria were selected to get a deeper insight by a pyrosequencing approach. The 16S rRNA gene amplicon libraries specific for Alphaproteobacteria were rarefied as shown in **Figure 5**. Richness estimation of the normalized datasets revealed that pyrosequencing effort reached 66.0–74.2% of estimated richness for the clusters of 90% similarity (**Table 1**). The clusters of 95 and 97% similarity reflected 49.3–51.2 and 45.4–46.2% of estimated richness, respectively.

Taxonomic composition of alphaproteobacterial populations, compared at the ranks of families and genera, was substantially similar among *Sphagnum* spp. (**Figure 6**). Dominant *Acetobacteraceae* family was prevailed by genera *Acidocella*, *Acidisphaera*, and *Rhodopila*. Within families *Sphingomonadaceae* and *Rhodospirillaceae*, the most members belonged to *Novosphingobium* spp. and *Magnetospirillum* spp., correspondingly. Withal, composition and ratio of subdominant *Caulobacteraceae* varied between mosses. The family was more abundant in *S. fallax* sample and consisted of genus *Phenylobacterium* (detected all over) and genus *Caulobacter* (unique for *S. fallax*). Diversity of species was accessed by Shannon diversity index (H') for clusters of 97% similarity. Comparison of the index values revealed a slightly higher diversity of Alphaproteobacteria for *S. fallax* (4.60) than for *S. angustifolium* (4.18).

According to the NCBI database, identified alphaproteobacterial genera comprise bacteria known for the nitrogen fixation. Particularly, genera *Bradyrhizobium*, *Acetobacter*, and *Beijerinckia* were found in both libraries, while genera *Gluconacetobacter*, *Methylocystis*, *Methylosinus*, and *Rhizobium* were solely detected in the *S. fallax* library.

Rarefaction analysis of the nitrogenase gene libraries resulted in similar saturation profiles of the *Sphagnum* samples (**Figure 5**). Normalized datasets represented 61.0–62.7% of estimated richness at 92% similarity (**Table 1**). The clusters of 96% similarity covered 55.2–66.6%, while unique clusters reflected 19.8–22.1% of estimated richness, correspondingly. Compositional diversity was assessed applying 96% similarity cut-off to confine the clusters. The Sørensen similarity index indicated that samples featured 53% identity. Nitrogenase diversity estimated by Shannon was again slightly higher for *S. fallax* (7.59), than for *S. angustifolium* (7.20; **Table 1**).

Phylogenetic analysis of the NifH composition revealed that retrieved sequences, 60 amino acids in length, were distributed among canonical Clusters I, III, and Sub-cluster IA (**Figure 7**). The most abundant Cluster I, comprising sequences of *Proteobacteria* and Cyanobacteria, was dominated by Alphaproteobacteria. According to the BLAST analysis, the most prevalent alphaproteobacterial amplicons were ≥95% similar to

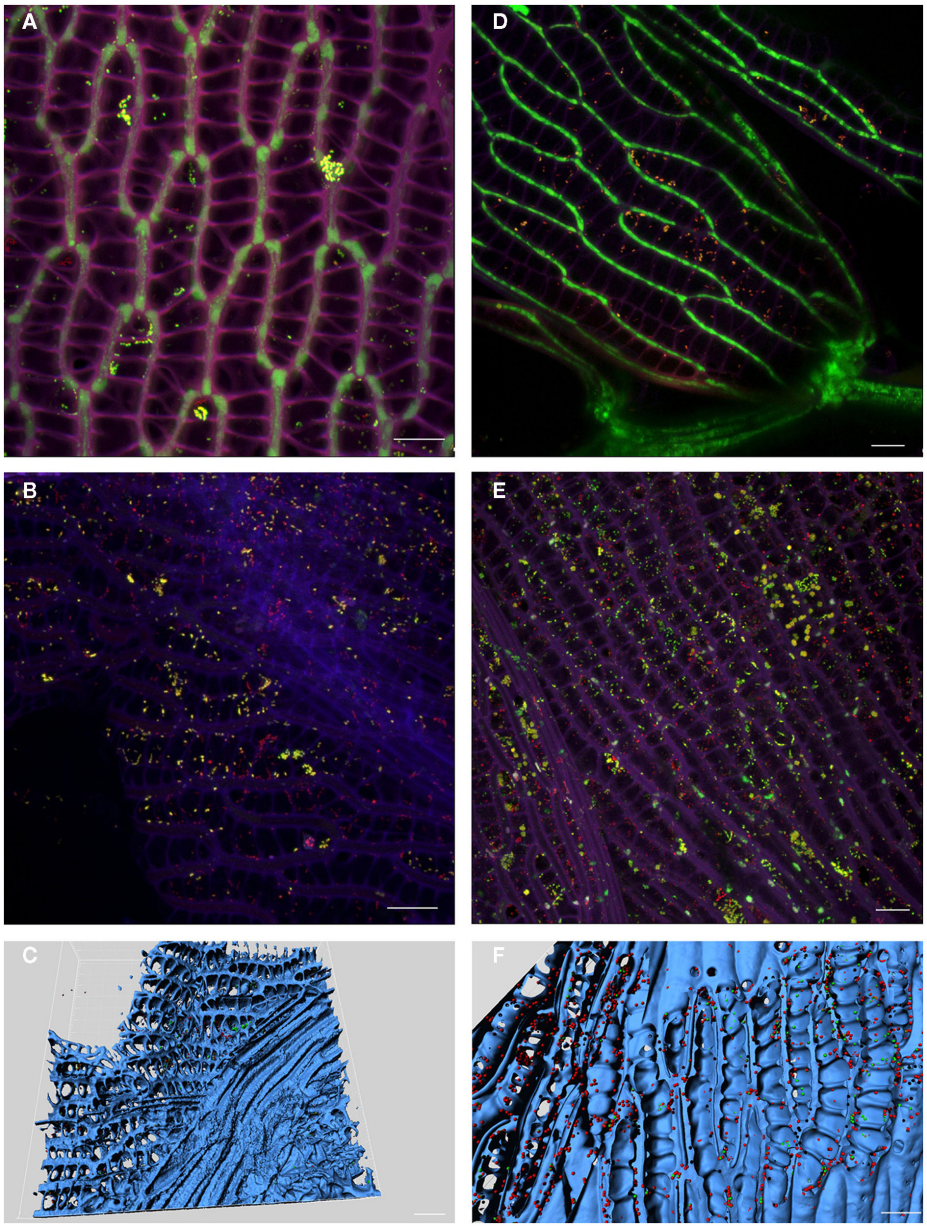

FIGURE 4 | Localization of bacteria in moss gametophytes. Fluorescent *in situ* hybridization of *S. angustifolium* **(A–C)** and *S. fallax* **(D–F)** leaves showed colonization of hyaline cells by Alphaproteobacteria. Images acquired by confocal laser scanning microscopy (CLSM) **(A,B,D,E)**: violet – cell walls of *Sphagnum* cells; green – chlorophyll-containing *Sphagnum* chlorocytes; yellow – Alphaproteobacteria; red – other bacteria. 3D computer reconstructions of CLSM images using Imaris7.0 **(C,F)**: blue – moss tissue; red: Alphaproteobacteria; green: other bacteria. Scale bar = 20 μm.

Bradyrhizobium, *Azorhizobium*, *Rhizobium*, *Methylobacterium*, *Rhodocista*, and *Acetobacter* species. Considerable proportion of amplicons showed 96–100% identity with *Methylocella*, *Methylocapsa*, and *Beijerinckia* reference sequences. Detected Betaproteobacteria were prevailed by *Burkholderia* spp. (≥95% similarity). Minor portion of Cluster I amplicons was affiliated with cyanobacterial genera *Anabaena* and *Tolypotrix* (100% similarity). Sub-cluster IA contained sequences 96–100% similar to *Geobacter* sp. Within the Cluster III amplicons grouped with reference *Spirochaeta* and *Thermincola* species (82–93% similarity).

In conclusion, *nifH* amplicon libraries of *Sphagnum*-associated microbial communities were characterized by high diversity and abundance of alphaproteobacterial amplicons.

DISCUSSION

Sphagnum mosses form an outstanding group of *Bryophyta*; they are unique in their morphological and developmental features at every stage of the life cycle (Shaw et al., 2003). More than for other plants, physical and chemical characteristics of *Sphagnum* mosses are related to the ecology and function of Northern

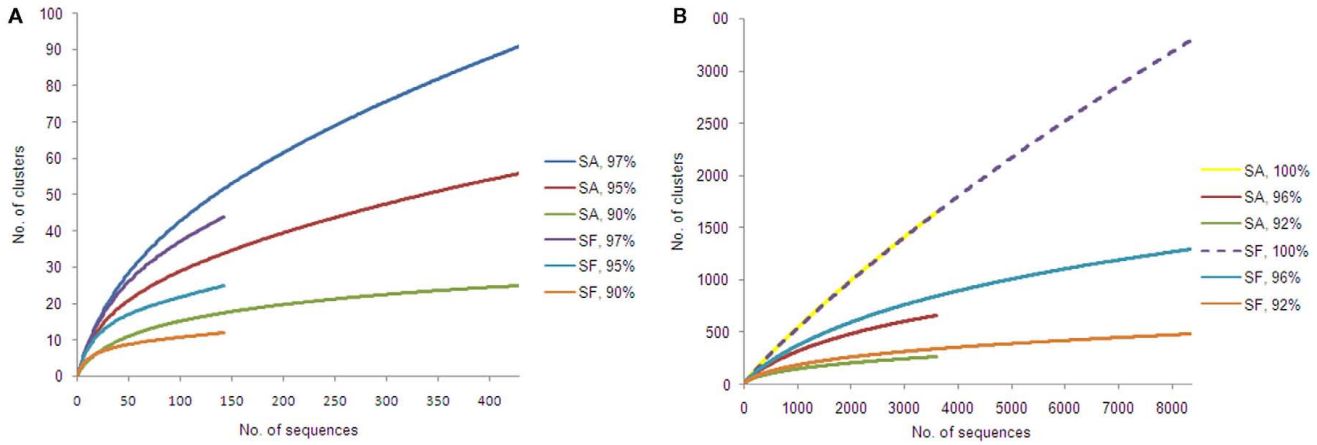

FIGURE 5 | Rarefaction curves for amplicon libraries of *Sphagnum* samples. Saturation curves are presented for samples of *S. angustifolium* (SA) and *S. fallax* (SF). Alphaproteobacteria **(A)** and *nifH* datasets **(B)** were clustered with similarity cut-offs defined.

Table 1 | Richness estimates and diversity indices for amplicon libraries of *Sphagnum* samples[a].

***Sphagnum* species**	**Indices**											
Index	**Clusters**			**Chao1**			**Coverage (%)**			**Shannon (*H'*)**		
ALPHAPROTEOBACTERIA												
Similarity cut-offs[b]	97%	95%	90%	97%	95%	90%	97%	95%	90%	97%	95%	90%
S. angustifolium	41	26	14	91	52	22	45.4	49.3	66.0	4.18	3.23	2.08
S. fallax	42	27	13	91	53	17	46.2	51.2	74.2	4.60	3.72	2.55
NIFH												
Similarity cut-offs[c]	100%	96%	92%	100%	96%	92%	100%	96%	92%	100%	96%	92%
S. angustifolium	1644	655	263	7447	983	420	22.1	66.6	62.7	8.76	7.20	5.72
S. fallax	1646	848	343	8301	1536	562	19.8	55.2	61.0	8.68	7.59	6.15

[a]*The number of sequences of each sample was normalized to 131 (Alphaproteobacteria) and 3601 (NifH).*

[b]*Similarity cut-offs applied for clustering of the nucleotide sequences.*

[c]*Similarity cut-offs applied for clustering of the amino acid sequences.*

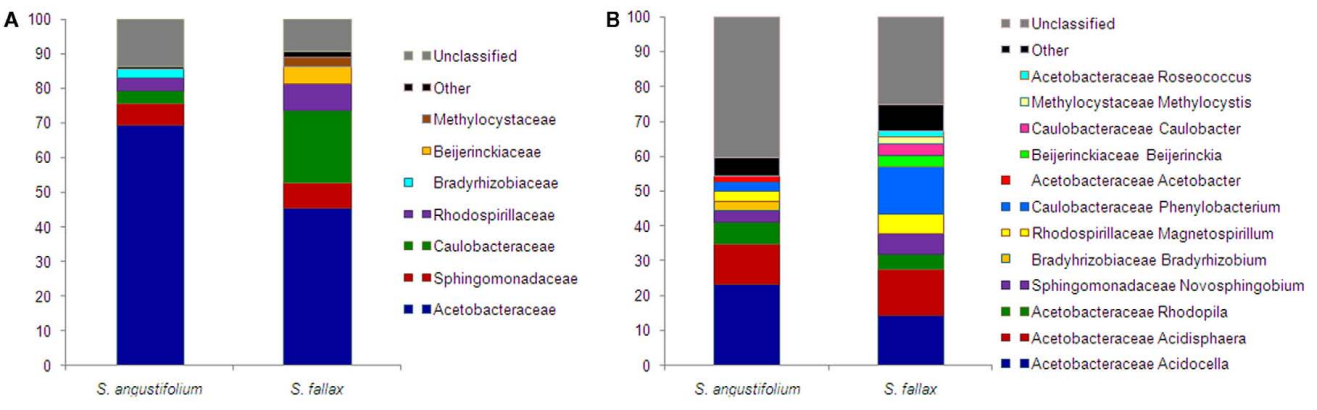

FIGURE 6 | Taxonomic classification of Alphaproteobacteria associated with *S. angustifolium* and *S. fallax*. Pyrosequencing reads are classified at family **(A)** and genus level **(B)** with a confidence threshold of 80%. Groups below 1% of relative abundance are included in *Other*. Multi-colored charts at the legend are shown for each sample correspondingly.

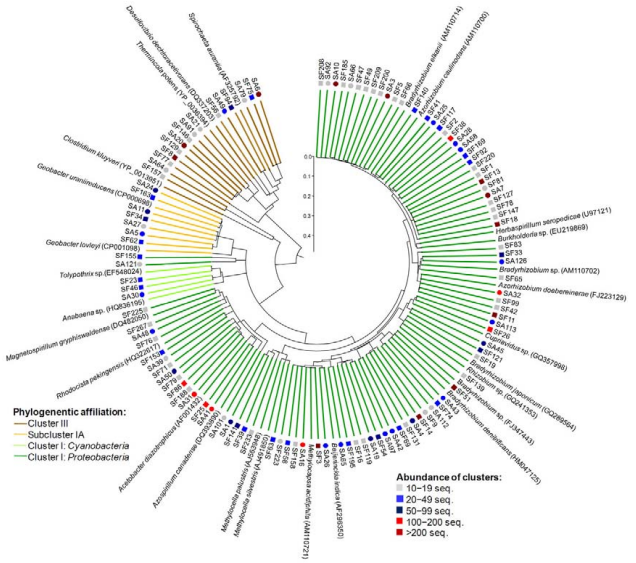

FIGURE 7 | Phylogenetic composition of the nitrogenase gene (*nifH*) amplicon libraries of *S. angustifolium* (SA, circles) and *S. fallax* (SF, squares). Neighbor-joining phylogenetic tree was constructed with both one representative sequence per NifH cluster (92% similarity) and the nearest reference sequences (accession numbers in brackets). A partial sequence of the light-independent photochlorophyllide reductase subunit L (BchL) from *Chlorobaculum tepidum* (Acc. Nr. AAG12203) was used as out-group. Reliability of the tree topology was evaluated by 100 bootstrap resamplings (bootstrap values not shown). Abundance and phylogenetic affiliation of the clusters are indicated at the legend. Scale bar = 0.1 substitutions per site.

peatlands. In this study we have shown the same deep relationship between moss plantlets and their associated bacterial communities. In the multifaceted approach applied to study the structure and function of bacteria, only minor but not statistically significant differences were found between *S. angustifolium* and *S. fallax*, two peat mosses which shared similar ecological conditions inside the bog ecosystem.

This high similarity is in contrast to former studies of *Sphagnum*-associated bacteria of different ecological amplitudes (Opelt et al., 2007c; Bragina et al., 2011). The main difference between the former studies and the present study is the close taxonomic and ecological relationship of the investigated *S. angustifolium* and *S. fallax* unlike *S. magellanicum*. *S. magellanicum* belongs to another section within the genus *Sphagnum* (section *Sphagnum*) and is typical for strong acidic, oligotrophic, and ombrotrophic habitats, whereas *S. angustifolium* and *S. fallax* (section *Cuspidata*) grow in weakly acid, more mesotrophic situations influenced by minerotrophic groundwater (Daniels and Eddy, 1985). Also from the morphological point of view, *S. angustifolium* and *S. fallax* are difficult to distinguish, and in former times both taxa were considered as varieties of one species *S. recurvum* P. Beauv (Smith, 1978). In contrast to *S. magellanicum* (Opelt et al., 2007a), *S. angustifolium*, and *S. fallax* are characterized by similar secondary metabolites. The overlap of common properties for the mosses was also found for the bacterial community. **Figure 3** not only shows a high degree of similarity between the microbial communities also a differentiation in more *S. fallax* (negative along the first axis) and more *S. angustifolium* (positive along the first axis) preferring bacteria. So, the situation shown exemplarily in **Figure 3** could be interpreted as an early state of specification of bacterial communities correspondent with an early state of host species differentiation. Interestingly, our results also explain differences between the theory of *Sphagnum* species specific communities established by Opelt et al. (2007c) and Bragina et al. (2011) and results obtained by Larmola et al. (2010) who identified only a-biotic drivers. For methanotrophs, they found for transplanted *Sphagnum* species bacterial pattern and activity typical for the a-biotic parameters of the destination site. However, this was an artificial experiment; the majority of the approx. 300 *Sphagnum* species has very narrow ecological amplitudes and would not grow in nature under different conditions (Daniels and Eddy, 1985). In Northern wetlands, which belong to an old vegetation type with more or less extreme but constant conditions for thousands of years, *Sphagnum* mosses have established a highly specific and adapted symbiosis with their associated microbes.

What are the ecological consequences of this specificity of the microbial community? Well-adapted to a-biotic parameters to the place where they live, *Sphagnum* mosses together with their microbiome as so called "meta-organisms" fulfill important functions for ecosystem services. These functions can only fulfilled in cooperation with the associated microbial community. The latter is responsible to fix nitrogen for the host plant, to solubilize phosphor but also to provide carbon from peat-delivered methane (Raghoebarsing et al., 2005; Opelt et al., 2007a). Studies have shown that oxidation of CH_4 by methanotrophic microbes residing in the *Sphagnum* layer is controlled by environmental factors, i.e., water table and temperature (Larmola et al., 2010) but beside this the rate was specific for *Sphagnum* species (Gifford et al., 2011). Taken together, the specificity of the microbial community is essential to live under the extreme and highly varying ecological gradients within the bog ecosystem and to fulfill the ecological functions. The bog ecosystem is more complex than previously thought but this is important to know to maintain bog ecosystems in Northern wetlands. The high specificity, narrow ecological amplitude and closed relationship can be one reason that *Sphagnum* is highly sensitive to changing a-biotic parameters connected with climate change.

Little is known about the specific ecology of *Sphagnum*-associated bacteria which are to a high degree still not culturable (Dedysh, 2011). Dominant alphaproteobacterial taxa associated with Sphagna are known as acidophilic or acidotolerant bacteria able to grow chemo-organotrophically or phototrophically and to survive under oligotrophic conditions. *Sphagnum*-associated microbial communities should be included in biodiversity–conservation agenda and used for predictive microbial ecology as requested by Bodelier (2011).

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Manuscript I

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Ecosystem function designates functional patterns: diazotrophic specialists but methanotrophic generalists on *Sphagnum* mosses

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Ecosystem function designates functional patterns: diazotrophic specialists but methanotrophic generalists on *Sphagnum* mosses

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***Running title*:** Functional patterns of *Sphagnum* bacteria

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***Sphagnum*-associated bacteria are important for growth and health of their host but also to maintain climate-relevant *Sphagnum*-dominated bog ecosystems. We studied the two bacterial functions nitrogen fixation and methane oxidation from two cosmopolitan but ecologically different *Sphagnum* species in three Alpine bogs in Austria by qPCR, pyrosequencing and corresponding network analyses. In contrast to methanotrophic generalists, the diazotrophic microbiome was specific for each *Sphagnum* species. The different functional patterns were found independently from the site. For *S. fallax* statistically significantly higher abundances of nitrogenase (*nifH*) genes than for *S. magellanicum* were determined. Furthermore, the majority of NifH clusters (92% similarity) were specific for one species. By network analysis we identified these discriminative diazotrophs: for *S. magellanicum* clusters related to *Methylobacterium* spp., Beijerinckiaceae, *Methylocapsa* sp., *Beijerinckia* spp. and *Azorhizobium* spp. and for *S. fallax* clusters similar to *Burkholderia* spp., *Rhodoferax* sp., *Cupriavidus* sp. and *Bradyrhizobium* spp. Although also different abundances of genes responsible for methane oxidation (*pmoA*) were found, both *Sphagnum*-originated libraries formed 99% common pattern. Network analysis revealed the genera *Methylomonas* (type I) and *Methylocystis* (type II) as generalists. Our conclusion that ecosystem function designates these different functional patterns was confirmed by a comparison with 16S rRNA gene amplicon library from the *Sphagnum* sporophyte. Nitrogen fixers are essential for *Sphagnum* growth; inside spores 45.5% of the total diversity comprises bacteria known for nitrogen fixation. No potential methanotrophic bacteria, which are less important for the *Sphagnum* themselves but significant for the whole ecosystem, were detected within the sporophyte library.**

Key words: nitrogen fixation, methane oxidation**,** bog ecosystem, *S. magellanicum* / *S. fallax*

Introduction

Bog ecosystems have accumulated more atmospheric carbon than any other terrestrial ecosystem today. Due to this extraordinary role in carbon sequestration and net cooling effects on the global radiation balance, bogs, which cover 4 Mill. km^2 on Earth, have a high value for our world climate (Strack, 2008; Dise, 2009). Bacterial communities living in bog ecosystems are involved in these important ecosystem functions and are solely responsible for several of them (Dedysh, 2011). For example, bacterial methane oxidation is crucial for the global carbon budget; here the *Sphagnum*-methanotroph consortium acts as a natural filter for methane (Raghoebarsing *et al.*, 2005; Larmola *et al.*, 2010). On the other side, microbial nitrogen fixation enables plant growth under ombrotrophic, nitrogen-limited conditions in bog ecosystems (Opelt *et al.*, 2007a). The bryophyte genus *Sphagnum* consisting of approx. 300 different species is world-wide distributed and forms the dominant component of bog vegetation (Daniels and Eddy, 1985). Therefore, *Sphagnum* mosses have been used globally as an indicator of climate change (Whinam and Copson, 2006), and microbial communities living in *Sphagnum* were shown early indicators of ecosystem disturbances (Jassey *et al*., 2011). Recently, we could show that living *Sphagnum* mosses are colonised in high abundances with specific microorganisms (Opelt *et al.*, 2007a, b, Bragina *et al.*, 2012a, b). While the composition, spatial distribution and transmission of the bacterial communities are well-known, less is known about the functional diversity within the microbiome.

Plant specificity of the associated bacteria and fungi is a long and well-established phenomenon (rev. in Berg and Smalla, 2009; Bulgarelli *et al.,* 2012). Interestingly, in a study by Germida & Siciliano (2001), the evolutionary relationship in plant–microbe interactions was revealed: old wheat cultivars were colonised by phylogenetically diverse rhizobacteria, whereas the rhizosphere of modern cultivars was dominated by fast-growing Proteobacteria. Bryophytes

were the first land plants. Especially *Sphagnum*-dominated bog ecosystems belong to the oldest vegetation forms with more or less constant conditions for thousands of years. Thereby plant and microbial communities of the bogs were co-exposed for a long evolutionary time and developed multiple interactions. Correspondingly, an extremely high impact of the *Sphagnum* species was found on the structure of the microbial communities, which are transferred directly from the sporophyte (within the sporangium capsule) to the gametophyte and *vice versa* (Bragina *et al.*, 2012a). In contrast to the above reported specificity, methane oxidizing bacteria on different *Sphagnum* species were highly similar (Larmola *et al.* 2010; Putkinen *et al.,* 2012). Our hypothesis for this study was that different microbial groups can show specific or redundant pattern according to their function within the ecosystem.

Here, we designed a new approach combining qPCR, pyrosequencing and network analyses of functional genes to study the diazotrophic and methanotrophic bacterial communities associated with *Sphagnum* mosses from three different Alpine bogs in Austria. While the quantitative and structural analysis of the microbiome is well-established, pyrosequencing and bioinformatic analysis of functional gene amplicon libraries are still restricted to a few, mainly methodical studies (Farnelid *et al*., 2011; Kip *et al*., 2011; Lüke and Frenzel, 2011). We analysed *nifH* and *pmoA* genes to find differences between bacterial communities of two dominant and cosmopolitan *Sphagnum* species, *S. magellanicum* and *S. fallax*. *S. magellanicum* BRID. (section *Sphagnum*) is typical for strong acidic, oligotrophic and ombrotrophic habitats, whereas *S. fallax* H. KLINGGR. (section *Cuspidata*) grows in weakly acid, more mesotrophic situations influenced by minerotrophic groundwater (Daniels and Eddy, 1985). Network analysis using Cytoscape 2.8 software (Smoot *et al.*, 2011) was applied to identify which functional taxa are generalists (broadly distributed across all habitats) or specialists (restricted to certain habitats but locally abundant) according to Barberán *et al.* (2011).

Materials and methods

Sampling design

To analyse differences between two *Sphagnum* species, *S. magellanicum* BRID. (section *Sphagnum*) and *S. fallax* H. KLINGGR. (section *Cuspidata*) were selected. Both bryophytes belong to the typical and cosmopolitan vegetation in peat bogs (Daniels and Eddy, 1985). Adult gametophytes of moss species were sampled in three different natural Alpine bogs in Austria in September 2009 (Supplementary Table S1). From each of three investigated bogs, four single replicates in at least 10m distance per *Sphagnum* species consisting of 15-20 plantlets were collected and stored separately. The living green parts of the plantlets were placed into sterile plastic bags and transported to the laboratory. *S. fallax* plants forming sporophytes were solely detected in the Rotmoos bog. Enclosed spore capsules of *S. fallax* were collected and processed separately. In general, sporophytes of *S. magellanicum* are uncommonly found.

Isolation of total-community DNA

The microbial fraction associated with moss gametophytes was extracted as described previously (Bragina *et al.*, 2012a). In short, 5g of plant material were physically disrupted with sterile pestle and mortar and resuspended in 10 ml of 0.85% NaCl. 2ml of suspension were centrifuged at 13000 rpm for 20 min at 4°C. Obtained pellet was used for isolation of the total-community DNA with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). For mechanical lysis, the cells were homogenised twice in a FastPrep® FP120 Instrument (Qbiogene, BIO101, Carlsbad, CA, USA) for 30 s at speed 5.0 m sec^{-1} and treated according to the manufacturer`s protocol. Extraction of bacteria from surface-sterilised sporophyte of *S. fallax* was carried out as described before (Bragina *et al.*, 2012a). Final aliquots of the total-community DNA were further subjected for PCR-based approaches.

Quantitative real-time PCR

Quantification of the microbial *nifH* and *pmoA* genes was conducted with primer pairs nifH-F/ nifH-R and A189f/A621r as referenced (Hai *et al.*, 2009; Tuomivirta *et al.*, 2009). Standards were generated by cloning of the respective gene fragments from *Erwinia carotovora* subsp. *atroseptica* SCRI1043 and *Methylosinus sporium* ATCC35069 into the pGEM-T® Easy Vector (Promega, Madison, WI, USA). Cloned fragments were re-amplified with vector-specific primers USP (5′-GTAAAACGACAACCAGT-3′) and RSP (5′-CAGGAAACAGCTATGACC-3′) and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). To eliminate inhibitory effects of co-extracted substances, total-community DNA was diluted 1:25 (data of preliminary experiments). Target genes were amplified using KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA) in 10 µl reaction mixtures. Each measurement was performed two times in three independent runs on the Rotor-Gene 6000 (Corbett Research, Mortlake, Australia). Specificity of amplicons was confirmed by melting-curve analyses and gel-electrophoresis of the PCR products. Concentrations were calculated to copy number per g fresh weight and analysed statistically.

Deep-sequencing and bioinformatic processing

Diversity of methane-oxidizing and nitrogen-fixing bacteria associated with Sphagna was deeply investigated by barcoded pyrosequencing approach. The total-community DNA of *Sphagnum* gametophytes was amplified with the set of *nifH* gene-specific primers nifH3/nifH4, nifH1/nifH2 in the nested approach (Zehr and Turner, 2001) and *pmoA* gene-specific primers A189f/A650r (Holmes *et al*., 1995; Bourne *et al*., 2001, Kip *et al*., 2011) using Taq-&Go™ Ready-to-use PCR Mix (MP Biomedicals, Solon, OH, USA). Total-community DNA of *S. fallax* sporophyte was

explored with universal bacterial primers 799f/1492r (Lane, 1991; Chelius *et al*., 2001). Duplicate PCR products from all templates were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Amplicons derived from the same *Sphagnum* sp. and sampling site were pooled together and subjected to the pyrosequencing using the Roche / 454 GS FLX and FLX+ Titanium platforms executed by LGC Genomics (Berlin; Germany) and GATC Biotech (Konstanz, Germany) correspondingly.

Amplicon libraries of the *nifH* and *pmoA* microbial genes were explored with the FunGene Pipeline of RDP server (http://fungene.cme.msu.edu/FunGenePipeline/) using the parameters described previously (Farnelid *et al*., 2011; Lüke and Frenzel, 2011). For the *nifH* amplicon libraries, primer sequences were trimmed and reads of a low quality and shorter 200 bp were removed. Filtered reads were translated into amino acid sequences and clipped at 60 aa. Further analyses were carried out on amino acid sequences. Rarefaction curves were generated for clusters with 100%, 96% and 92% similarity cut-offs. To calculate richness estimates and diversity indices, datasets were normalised to the same number of sequences using an in-house developed Perl script (10-times random re-sampling followed by subset formation). NifH clusters of 92% similarity (≥10 sequences) were further explored by statistical and network analyses. Representative sequences for each cluster were aligned using the NCBI algorithm TBLASTN.

The amplicon libraries of *pmoA* gene were processed in a similar way. Trimmed reads of a high quality and ≥400 bp long were translated into amino acid sequences and clipped at 131 common positions. Rarefaction curves were obtained for clusters of 100%, 93% and 82% similarity. Richness estimates and diversity indices were calculated for the datasets normalised to the identical sequencing depth. The PmoA clusters of 93% similarity (≥10 sequences) were characterised by the alignment of the representative sequences and subjected for further analyses.

The 16S rRNA gene amplicon library of *S. fallax* sporophyte was pre-processed as described previously (Bragina *et al*., 2012a, b). Rarefaction analysis and estimation of richness and diversity were performed using tools of RDP Pipeline (Cole *et al*., 2009). Phylotype clusters were defined with 97%, 95% and 80% similarity cut-offs, corresponding to the levels of the species, genera and phyla, respectively (White *et al*., 2010; Will *et al*., 2010). Classification of the reads was performed by the UCLUST pipeline integrated into the web interface SnoWMAn version 1.11 (https://epona.genome.tugraz.at/snowman/) with 50% confidence threshold.

Statistics

The *nifH* and *pmoA* gene copy numbers were statistically analysed using PASW Statistics 18 software (SPSS, Chicago, IL, USA). The data were tested for normal distribution by Q-Q plots and the Shapiro-Wilk test. Homogeneity of variances was checked by the Levene test. Significance of the difference between *S. magellanicum* and *S. fallax* in each sampling site and over all sampling sites was calculated using t-test with independent samples.

To detect differentially abundant microbial clusters associated with Sphagna, NifH (92%) and PmoA (93%) clusters with ≥10 sequences were explored by Metastats web interface (White *et al*., 2009). Populations of *S. fallax* (MR, MW, MP datasets) and *S. magellanicum* (FR, FW, FP datasets) were examined using a combination of the nonparametric t-test, exact Fisher's test and the false discovery rate with 1000 permutations. P-values were determined for each cluster correspondingly.

Profile clustering network analysis

Network analysis was performed to visualize functional microbial patterns and to compare their abundance between *Sphagnum* species across three sampling sites (Grube *et al*., 2012). NifH

(92%) and PmoA (93%) clusters with cumulative read change ≥5 sequences were examined for abundance ratio between *S. magellanicum* and *S. fallax* in each sampling site. If the ratio of values exceeded 2.0, the clusters were assigned to the specific profile (abundant in *S. magellanicum* or *S. magellanicum*). To refine NifH network analysis, we considered only clusters statistically different between *Sphagnum* species ($p < 0.05$). Visualization of the network was carried out with the open source software Cytoscape 2.8 (Smoot *et al.*, 2011).

Results

Abundances of functional bacterial genes in Sphagnum mosses

Quantification of *nifH* and *pmoA* genes resulted in high copy numbers for both moss species (Fig. 1). Concerning nitrogen fixation, higher *nifH* gene copy numbers were detected for *S. fallax* ($\log_{10}$ 7.0 ± 0.2 to $\log_{10}$ 7.4 ± 0.3 copies g^{-1} fresh weight) compared to the *S. magellanicum* samples ($\log_{10}$ 6.8 ± 0.1 to $\log_{10}$ 7.0 ± 0.2 copies g^{-1} fw). Statistically significant difference was detected between *Sphagnum* spp. in sampling sites Rotmoos and Pürgschachen Moor ($p = 0.004$; 0.01) and comparing copy numbers between species over all sampling sites ($p = 0.0006$). With regard to methane oxidation, *pmoA* gene copy numbers were likewise slightly higher for *S. fallax* ($\log_{10}$ 6.3 ± 0.2 to $\log_{10}$ 6.6 ± 0.3 copies g^{-1} fw) than for *S. magellanicum* samples ($\log_{10}$ 6.0 ± 0.3 to $\log_{10}$ 6.1 ± 0.2 copies g^{-1} fw). Significant difference was statistically shown between Sphagna from Pürgschachen Moor ($p = 0.02$) and testing copy numbers over all sampling sites ($p = 0.002$).

Deep insight into functional microbial diversity by pyrosequencing

For high-throughput characterisation of diazotrophic and methanotrophic bacteria in Sphagna, pyrosequencing libraries of the *nifH* and *pmoA* genes were analysed. The nitrogenase gene libraries were rarefied as shown in Supplementary Fig. S1. Richness estimation of the normalised

datasets revealed that pyrosequencing effort attained 58.2–76.5% of estimated richness for the clusters of 92% similarity (Table 1). The NifH clusters of 96 and 100% reflected 42.0–60.6 and 15.0–24.1% of estimated richness, correspondingly. Nitrogenase diversity estimated by Shannon diversity index (H') was higher for *S. fallax* than for *S. magellanicum* among all sampling sites (Table 1).

BLAST analysis of the NifH composition revealed an immense dominance of the amplicons related to Proteobacteria. The *nifH* deduced amino acid sequences were distributed among different classes and prevailed by Alphaproteobacteria. The most frequent NifH clusters were ≥95% similar to *Bradyrhizobium*, *Azorhizobium*, *Beijerinckia*, *Rhodopseudomonas*, *Rhizobium* and *Methylobacterium* species. Detected Betaproteobacteria were presented by the genus *Burkhoderia* followed by *Cupriavidus* and *Rhodoferax* (≥97% similarity). NifH sequences related to Gammaproteobacteria were assigned to *Stenotrophomonas* and *Pseudomonas* genera (95–98% similarity). The minor NifH clusters contained sequences 95–100% similar to Deltaproteobacteria (*Geobacter* spp.). Sub-dominant portion of amplicons was affiliated to cyanobacterial genera *Anabaena*, *Tolypothrix* and *Aphanizomenon* (≥95% similarity). According to the NCBI database, certain part of the retrieved sequences showed 98-100% identity to methanotrophic bacteria of genera *Methylocapsa*, *Methylocystis*, *Methylocella*, *Methylosinus* and *Methylomonas*.

Rarefaction analysis of the particulate methane monooxygenase gene libraries resulted in similar saturation profiles for all *Sphagnum* samples with the exception of *S. fallax* from Pürgschachen Moor (Supplementary Fig. S1). While normalised datasets reached complete saturation at 82% similarity (Table 1), the PmoA clusters of 92 and 96% covered 17.4–24.7% and 46.3–94.5% of estimated richness, correspondingly. Except for FP library (*S. fallax*,

Pürgschachen Moor), methane monooxygenase diversity estimated by Shannon index was highly similar between *Sphagnum* spp. (Table 1).

PmoA diversity explored by BLAST analysis comprised type I and type II methanotrophs in equal abundance ratio. Considerable portion of PmoA clusters was 89–95% similar to genus *Methylomonas* (type Ia), while the second group of sequences showed 95–98% identity to *Methylocystis* spp. (type II). Peculiarly, *pmoA* gene library of *S. fallax* from Pürgschachen Moor consisted solely from the amplicons related to the type II methanotrophs.

Comparison of the functional microbial patterns

Nitrogenase gene (*nifH*) amplicon libraries were compared using abundance-based Sørensen indices corrected for unseen species (Chao *et al*., 2006). Applying 96% and 92% cut-offs amplicon libraries formed *S. fallax*- and *S. magellanicum*-specific patterns at 55% and 67% similarity levels correspondingly (Fig. 2). According to the statistical analysis, 23 out of 118 examined NifH clusters (92%) showed significant difference between *Sphagnum* spp. Network analysis demonstrated distribution of the specific NifH patterns between *S. fallax* and *S. magellanicum* among all sampling sites (Fig. 3). *S. magellanicum*-abundant NifH pattern consisted of clusters related to *Methylobacterium* spp., Beijerinckiaceae, *Methylocapsa* sp., *Beijerinckia* spp. and *Azorhizobium* spp. In contrast to *S. magellanicum*, *S. fallax*-abundant NifH pattern contained clusters assigned to *Burkholderia* spp., *Rhodoferax* sp., *Cupriavidus* sp. and *Bradyrhizobium* spp.

Comparison of the *pmoA* gene amplicon libraries revealed high similarity between both bryophytes. With applied cut-offs, *S. fallax*- and *S. magellanicum*-originated libraries formed common pattern at 99% similarity level (Fig. 2). Statistical analysis of 23 PmoA clusters (93%) resulted in statistically significant difference between moss species for 2 minor clusters. Network

analysis revealed general distribution of the PmoA pattern among Sphagna in all sampling sites (Fig. 3).

Linking taxonomic and functional microbial patterns of sporophyte and gametophyte

To reveal cross-links between taxonomic and functional diversity of moss microbiome, 16S rRNA gene amplicon library of *S. fallax* sporophyte generated with universal bacterial primers was analysed. Rarefaction analysis revealed complete saturation of the sporophyte library at the phyla level (Supplementary Fig. S2). At the level of species and genera pyrosequencing survey achieved 54.8% and 65.2% of estimated richness, respectively (Table 1).

Classified 16S rRNA gene sequences were distributed among five bacterial phyla: over-dominant Proteobacteria (82.8%) followed by sub-dominant Bacteroidetes (9.4%) and Acidobacteria (7.5%) with only few sequences affiliated to Actinobacteria and OP10. Bacteria associated with sporophyte were assigned to 37 genera (Fig. 4). According to the NCBI database, 12 detected genera (45.5% of the total diversity) comprise bacteria known for nitrogen fixation. The most dominant *nifH*-holding taxa were presented by *Burkholderia* (35.1%), *Herbaspirillum* (2.6%), *Pseudomonas* (2.0%), *Sphingobacterium* (1.9%), *Leptothrix* (1.2%) and *Rhizobium* (1.0%). Obtained results coincide with the network analysis: NifH clusters related to *Burkholderia* spp. formed a specific pattern in *S. fallax* gametophytes. Interestingly, no methanotrophic bacteria were detected within the sporophyte library.

Discussion

Plant life in nutrient-poor or ombrotrophic bog ecosystems depends on their associated bacteria. We investigated two important functional groups of *Sphagnum*-associated bacteria in three

Alpine *Sphagnum*-dominated bog ecosystems and found two distinct ecological patterns: diazotrophic specialists and methanotrophic generalists.

What are the reasons for different patterns detected by pyrosequencing of bacterial *nifH* and *pmoA* genes and corresponding network analyses? Our hypothesis for this study was that different microbial groups can show specific or redundant pattern according to their function within the ecosystem. Stated hypothesis was approved by the obtained results. Nitrogen fixation is an essential process for all forms of life including plant growth (Moir, 2011). Without bacterial nitrogen no plant germination and growth is possible, especially under nutrient-depleted conditions (Tauro *et al*., 2009). On the investigated *Sphagnum* species we found highly specific nitrogen fixers; they are essential for *Sphagnum* growth. In a former study we found out, that a core microbiome is transferred from the moss sporophyte to the gametophyte (Bragina *et al.,* 2012a). This core microbiome contained also a high proportion (45.5%) and diversity of potential nitrogen-fixing bacteria and is responsible for the high specificity. This fact underlines the importance of nitrogen fixation for the *Sphagnum* moss themselves. In contrast, methane oxidation is an important ecosystem function for the whole bog – and terrestrial – ecosystem but not essential for the plant themselves. Carbon is provided to the plant metabolism mainly from air-borne carbon dioxide fixed by photosynthesis of the plants. Although *Sphagnum* mosses form a habitat for methane-oxidizing bacteria, and methanotrophs are more active when they are associated, their composition is not specific (Larmola *et al.,* 2010). Putkinen *et al.* (2012) showed that methanotrophs are dispersed by water within the bog. They are not members of the sporophyte-transferred *Sphagnum* core microbiome.

In our study we applied high-throughput molecular approaches - pyrosequencing and quantitative PCR - to resolve functional diversity of the *Sphagnum* microbiome. However, pyrosequencing and bioinformatic analysis of functional gene amplicon libraries are restricted to

few currently published works (Farnelid *et al*., 2011; Kip *et al*., 2011; Lüke and Frenzel, 2011) and therefore, it is necessary to assess data and conclusions critically. Primer selection played a crucial role for detection and comparison of diazotrophic and methanotrophic microbial patterns by pyrosequencing. A set of applied *nifH*-gene specific primers was shown to cover the brightest spectrum of *nifH* sequences (Gaby and Buckley, 2012). In contrast, existing *pmoA*-targeting primers feature modest coverage, which should be considered during the data analyses. Thus, unveiled particulate methane monooxygenase diversity comprised type II and type Ia methanotrophic bacteria, which were ubiquitously detected under all moisture conditions, while type Ib methanotrophs specific for habitats with high water table remained uncovered (Siljanen *et al*., 2011). In addition, a much higher diversity of methanotrophs is predicted and newly described methanotrophic bacteria have to be implemented for the primer design (Luesken *et al*., 2011).

In our study we demonstrated statistically higher abundances of the diazotrophic specialists as well as methanotrophic generalists for *S. fallax*, than for *S. magellanicum.* This difference underlines influence of a-biotic parameters shaping the microbiome (Opelt *et al*., 2007c; Bragina *et al*., 2012a). *S. magellanicum* lives in typical strong acidic, oligotrophic and ombrotrophic habitats, whereas *S. fallax* grows in weakly acidic, more mesotrophic situations influenced by minerotrophic groundwater. Therefore, *S. magellanicum* would need more nitrogen than *S. fallax.* We assume that life in the ombrotrophic bog bulks is very limited, not only by nutrients also by the extreme pH (2-4.5), which explain these differences.

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Table 1 Richness estimates and diversity indices for amplicon libraries of *Sphagnum* samples[a]

Sphagnum samples[b]	*Indices*											
Index	*Clusters*			*Chao1*			*Coverage (%)*			*Shannon (H')*		
NifH												
Similarity cut-offs[c]	100%	96%	92%	100%	96%	92%	100%	96%	92%	100%	96%	92%
MR	618	342	122	2567	615	169	24.1	55.6	72.2	4.75	4.24	3.34
MW	702	372	145	3098	671	240	22.7	55.4	60.5	5.05	4.34	3.55
MP	636	312	108	2639	515	141	24.1	60.6	76.5	4.76	4.03	3.05
FR	724	376	178	4728	767	306	15.3	49.0	58.2	5.11	4.44	3.55
FW	778	440	216	4326	872	331	18.0	50.5	65.2	5.55	4.89	4.18
FP	846	418	194	5624	995	323	15.0	42.0	60.0	5.87	4.91	4.15
PmoA												
Similarity cut-offs	100%	93%	82%	100%	93%	82%	100%	93%	82%	100%	93%	82%
MR	605	26	6	2689	28	6	22.5	94.5	100.0	5.10	2.02	1.04
MW	673	31	6	3871	67	6	17.4	46.3	100.0	5.39	2.15	1.08
MP	623	24	5	2995	39	5	20.8	61.5	100.0	5.19	2.01	1.01
FR	624	28	6	3517	33	6	17.7	84.8	100.0	5.20	2.11	1.06
FW	611	41	7	2473	62	7	24.7	66.1	100.0	5.22	2.15	1.08
FP	454	20	5	2061	25	5	22.0	80.0	100.0	4.14	1.42	0.78
16S rDNA												
Similarity cut-offs	97%	95%	80%	97%	95%	80%	97%	95%	80%	97%	95%	80%
FSR	161	111	11	294	170	11	54.8	65.2	100	3.37	3.07	1.60

[a] The number of sequences of each sample was normalised to 1658 (NifH) and 1150 (PmoA); the 16S rRNA gene amplicon library consists of 1168 sequences.

[b] Abbreviations specify *Sphagnum* species and the sampling sites: M, *Sphagnum magellanicum*; F, *Sphagnum fallax*; R, Rotmoos; W, Wasenmoos; P, Pürgschachen Moor. FSR corresponds to the sporophyte sample of *S. fallax* collected in Rotmoos.

[c] Similarity cut-offs applied for clustering of the amino acid (NifH, PmoA) and nucleotide sequences (16S rDNA).

Legends

Figure 1 Abundance of the bacterial *nifH* and *pmoA* genes in *Sphagnum* gametophytes detected by qPCR. The log abundances of *nifH* (**a**) and *pmoA* (**b**) gene copies per g fresh weight of *S. magellanicum* (black bars) and *S. fallax* (grey bars) in three sampling sites. Error bars indicate confidence intervals. Asterisks denote significant differences between moss species (*, $p < 0.05$; ** $p < 0.01$).

Figure 2 Unweighted pair group method with average linkages (UPGMA) analysis of *nifH* and *pmoA* gene amplicon libraries. UPGMA dendrograms were constructed using Chao's corrected Sørensen matrices for NifH (**a-c**) and PmoA (**d-f**) datasets of *S. magellanicum* (M) and *S. fallax* (F) from three sampling sites (R=Rotmoos, W=Wasenmoos, P=Pürgschachen Moor). Similarity cut-offs applied for clustering of datasets are indicated for each dendrogram correspondingly.

Figure 3 Profile clustering network analysis of the microbial NifH and PmoA clusters in *Sphagnum* samples. NifH clusters of 92% similarity (**a**) and PmoA clusters of 93% similarity (**b**) were examined for 2-fold abundance increase in *Sphagnum* spp. in three sampling sites. According to the results single clusters were connected to profiles SM (abundant in *S. magellanicum*), SF (abundant in *S. fallax*) and/or COM (no change). The width of the node connections defines the number of sampling sites. The size of each node is proportional to the cluster abundance. The colour and label of the nodes specify phylogenetic and taxonomic affiliation of the clusters correspondingly. NifH network shows only clusters statistically different ($p < 0.05$) between *Sphagnum* species.

Figure 4 Taxonomic classification of bacteria associated with *S. fallax* sporophyte. 16S rRNA gene amplicon library (FSR) was classified at genus level with confidence threshold of 50%. Only classified reads were designated (1003). Pie chart (**a**) represents relative abundance of the dominant genera. Genera not reaching 1% of relative abundance are assembled in the group Other and are shown in detail as a bar chart (**b**). Genera depicted with asterisks comprise bacteria holding *nifH* gene (according to the NCBI database).

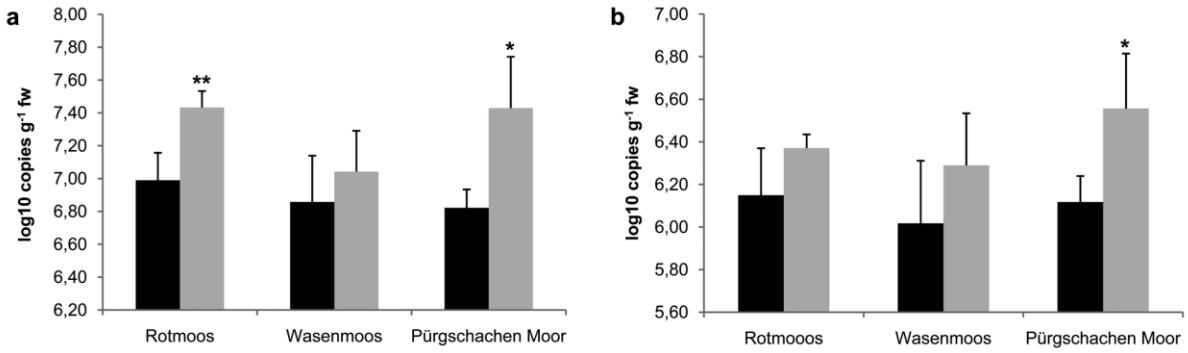
a
8,00
7,80
7,60
7,40
7,20
7,00
6,80
6,60
6,40
6,20
log10 copies g-1 fw
**
*
Rotmoos
Wasenmoos
Pürgschachen Moor
b
7,00
6,80
6,60
6,40
6,20
6,00
5,80
5,60
log10 copies g-1 fw
*
Rotmooos
Wasenmoos
Pürgschachen Moor

Figure 1

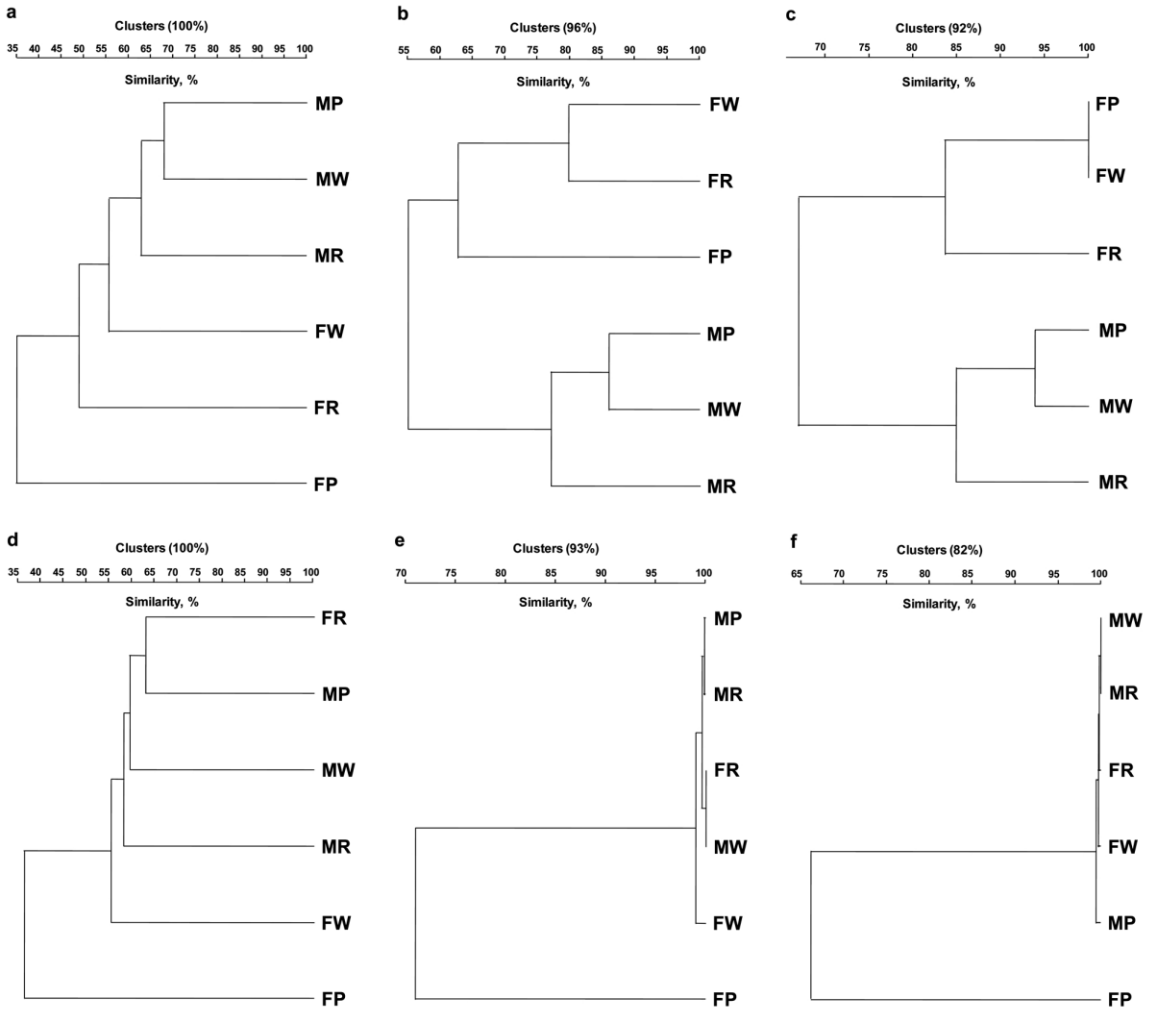
a
Clusters (100%)
35 40 45 50 55 60 65 70 75 80 85 90 95 100
Similarity, %
MP
MW
MR
FW
FR
FP
b
Clusters (96%)
55 60 65 70 75 80 85 90 95 100
Similarity, %
FW
FR
FP
MP
MW
MR
c
Clusters (92%)
70 75 80 85 90 95 100
Similarity, %
FP
FW
FR
MP
MW
MR
d
Clusters (100%)
35 40 45 50 55 60 65 70 75 80 85 90 95 100
Similarity, %
FR
MP
MW
MR
FW
FP
e
Clusters (93%)
70 75 80 85 90 95 100
Similarity, %
MP
MR
FR
MW
FW
FP
f
Clusters (82%)
65 70 75 80 85 90 95 100
Similarity, %
MW
MR
FR
FW
MP
FP

Figure 2

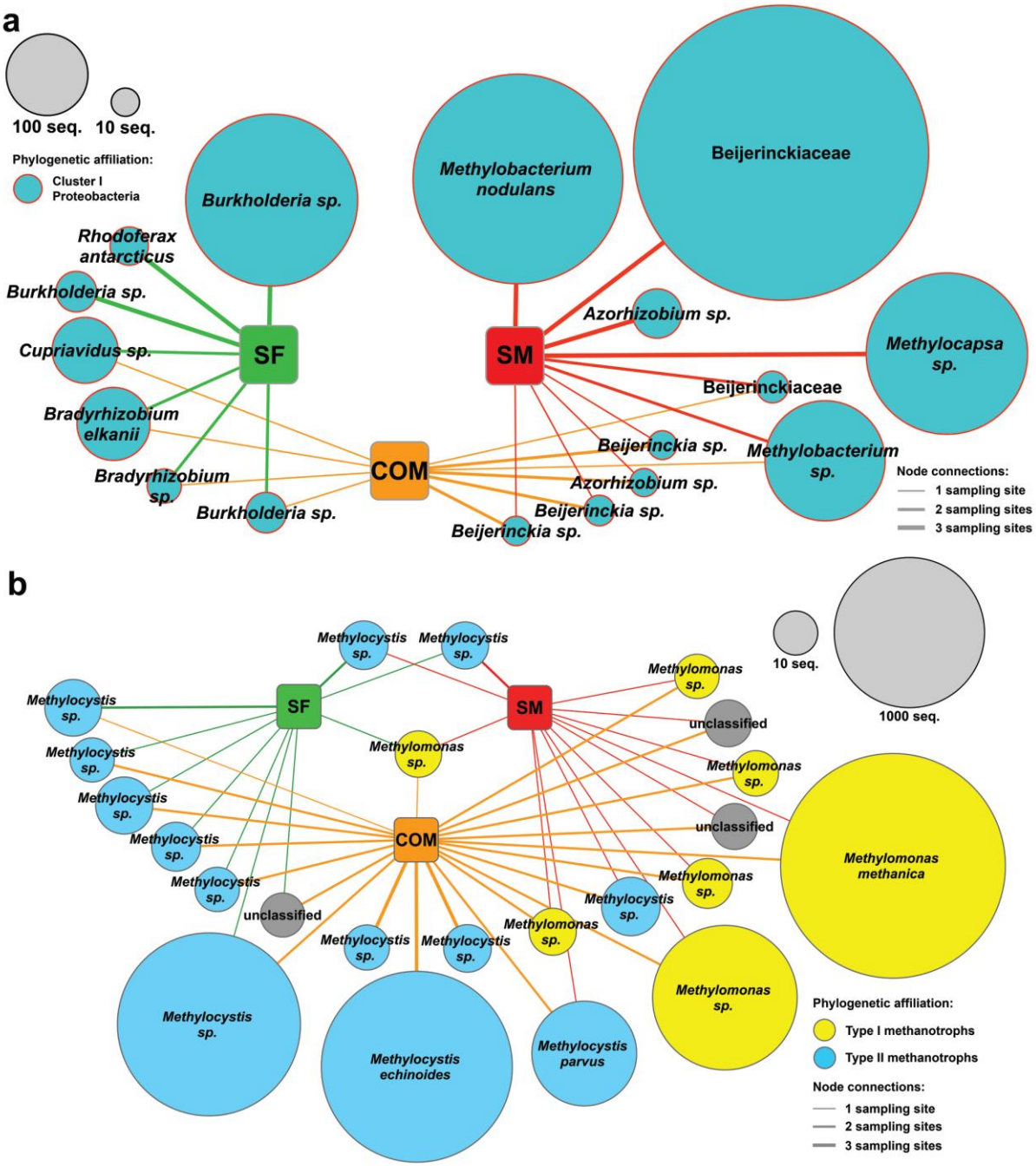
a
100 seq.
10 seq.
Phylogenetic affiliation:
Cluster I Proteobacteria
Burkholderia sp.
Methylobacterium nodulans
Beijerinckiaceae
Rhodoferax antarcticus
Burkholderia sp.
Cupriavidus sp.
Bradyrhizobium elkanii
Bradyrhizobium sp.
Burkholderia sp.
SF
COM
SM
Azorhizobium sp.
Methylocapsa sp.
Beijerinckiaceae
Beijerinckia sp.
Methylobacterium sp.
Azorhizobium sp.
Beijerinckia sp.
Beijerinckia sp.
Node connections:
1 sampling site
2 sampling sites
3 sampling sites
b
10 seq.
1000 seq.
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
SF
COM
SM
Methylomonas sp.
Methylomonas sp.
unclassified
Methylomonas sp.
unclassified
Methylomonas sp.
Methylomonas sp.
Methylocystis sp.
Methylomonas sp.
unclassified
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
Methylocystis sp.
Methylocystis echinoides
Methylocystis parvus
Methylomonas methanica
Methylomonas sp.
Phylogenetic affiliation:
Type I methanotrophs
Type II methanotrophs
Node connections:
1 sampling site
2 sampling sites
3 sampling sites

Figure 3

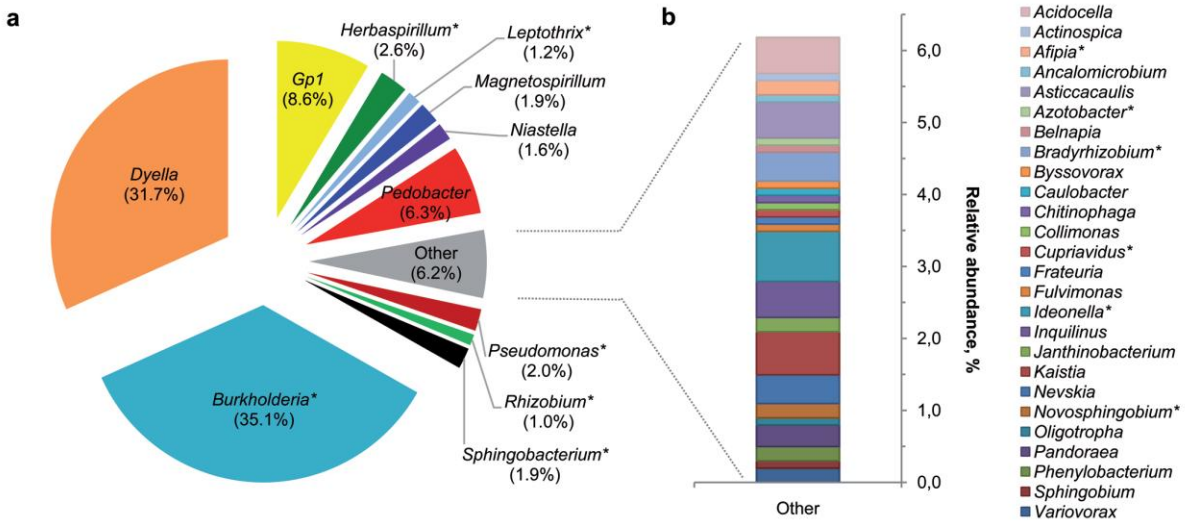
a
Herbaspirillum*
(2.6%)
Leptothrix*
(1.2%)
Gp1
(8.6%)
Magnetospirillum
(1.9%)
Niastella
(1.6%)
Dyella
(31.7%)
Pedobacter
(6.3%)
Other
(6.2%)
Pseudomonas*
(2.0%)
Burkholderia*
(35.1%)
Rhizobium*
(1.0%)
Sphingobacterium*
(1.9%)
b
6,0
5,0
4,0
3,0
2,0
1,0
0,0
Relative abundance, %
Other
Acidocella
Actinospica
Afipia*
Ancalomicrobium
Asticcacaulis
Azotobacter*
Belnapia
Bradyrhizobium*
Byssovorax
Caulobacter
Chitinophaga
Collimonas
Cupriavidus*
Frateuria
Fulvimonas
Ideonella*
Inquilinus
Janthinobacterium
Kaistia
Nevskia
Novosphingobium*
Oligotropha
Pandoraea
Phenylobacterium
Sphingobium
Variovorax

Figure 4

Supplementary information

Table S1 Sampling sites

State, district, locality	*Bog*	*Moss species*	*Sample abbreviation*[a]	*Coordinates*	*Altitude, m*
Styria, Bruck an der Mur, 2 km north-northwest of Weichselboden	Rotmoos	*S. magellanicum*	MR1	N47 41.030 E15 09.276	699
			MR2	N47 41.021 E15 09.245	699
			MR3	N47 40.971 E15 09.270	698
			MR4	N47 41.017 E15 09.319	693
		S. fallax	FR1	N47 40.908 E15 09.244	693
			FR2	N47 40.958 E15 09.175	691
			FR3, FSR[b]	N47 41.041 E15 09.232	690
			FR4	N47 41.055 E15 09.264	689
Salzburg, Zell am See, 0.5 km southeast of Pass Thurn	Wasenmoos	*S. magellanicum*	MW1	N47 18.373 E12 24.927	1216
			MW2	N47 18.363 E12 24.944	1216
			MW3	N47 18.337 E12 25.119	1214
			MW4	N47 18.315 E12 25.126	1208
		S. fallax	FW1	N47 18.387 E12 24.871	1211
			FW2	N47 18.391 E12 24.866	1215
			FW3	N47 18.385 E12 24.882	1217
			FW4	N47 18.347 E12 24.980	1213
Styria, Liezen, 1.7 km southwest of Ardning	Pürgschachen Moor	*S. magellanicum*	MP1	N47 34.905 E14 20.402	637
			MP2	N47 34.910 E14 20.454	639
			MP3	N47 34.839 E14 20.497	640
			MP4	N47 34.805 E14 20.493	639

S. fallax	FP1	N47 34.789 E14 20.398	638
	FP2	N47 34.814 E14 20.356	636
	FP3	N47 34.824 E14 20.346	635
	FP4	N47 34.848 E14 20.344	634

[a] Letters indicate *Sphagnum* species and bogs: M, *Sphagnum magellanicum*; F, *Sphagnum fallax*; R, Rotmoos; W, Wasenmoos; P, Pürgschachen Moor. Arabic numerals specify replicates.

[b] FSR corresponds to the sporophyte sample of *S. fallax* collected at the same sampling point as sample FR3.

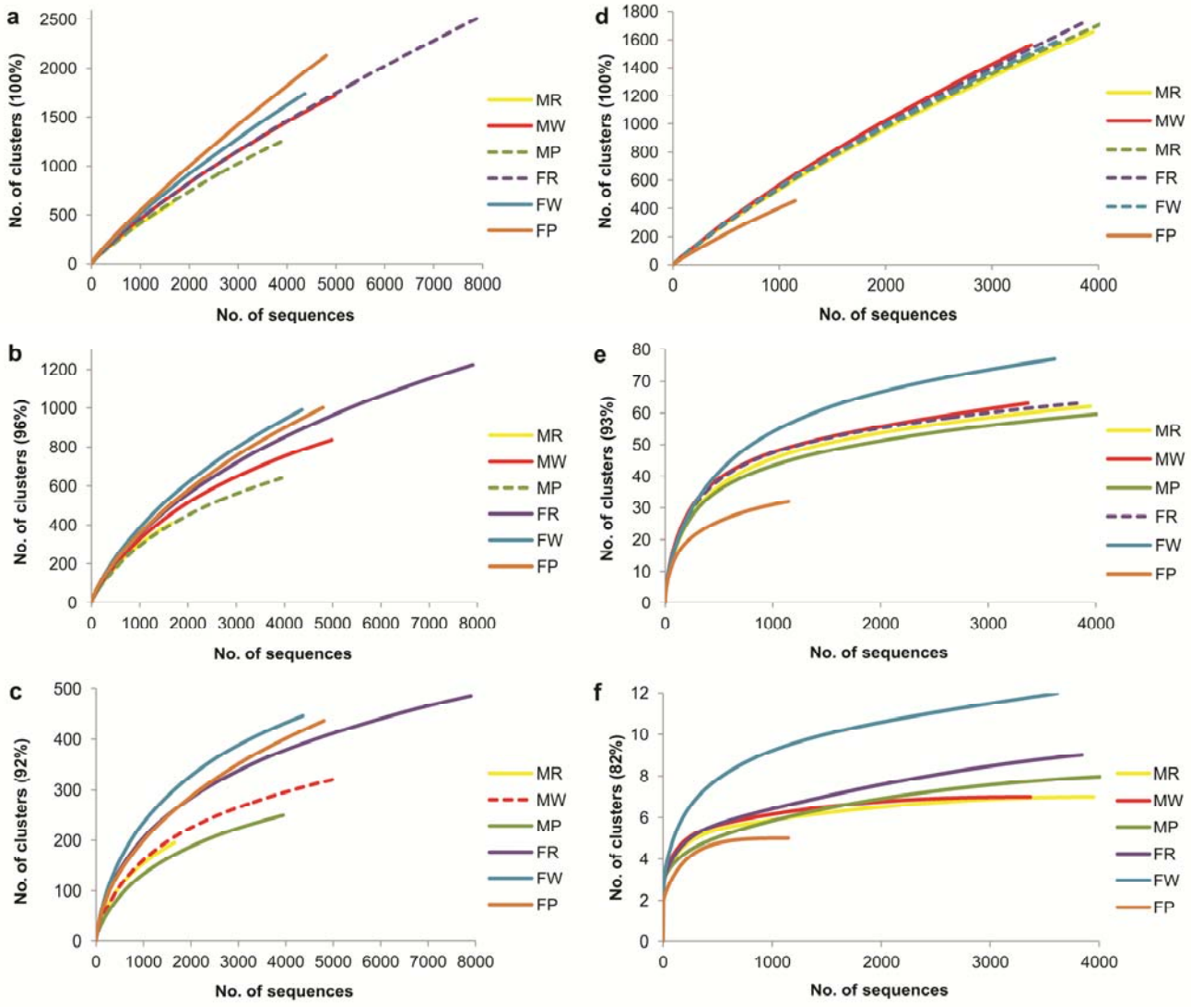

Figure S1 Rarefaction curves for *nifH* and *pmoA* amplicon libraries of *Sphagnum* samples. NifH (**a**, **b**, **c**), PmoA (**d**, **e**, **f**) datasets were clustered with similarity cut-offs defined. Rarefaction was calculated for amplicon libraries of *S. magellanicum* (M) and *S. fallax* (F) from sampling sites Rotmoos (R), Wasenmoos (W) and Pürgschachen Moor (P).

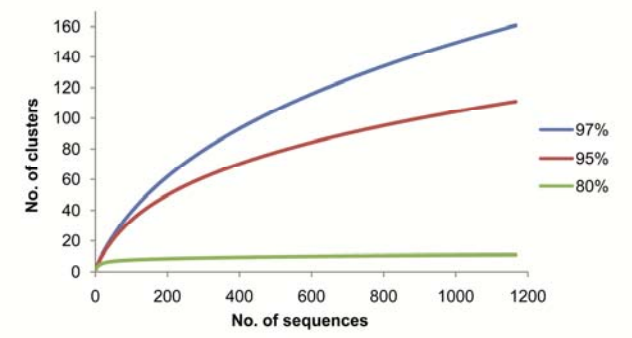

Figure S2 Rarefaction curves for 16S rRNA gene amplicon library of *S. fallax* sporophyte (FSR). Phylotype clusters were identified with 97%, 95% and 80% similarity cut-offs, corresponding to the taxonomic levels of species, genera and phyla, respectively.

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Curriculum vitae

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June 2003	Secondary school certificate in Moscow, Russia

Publication list

Peer-reviewed publications

Bragina, A., Berg, C., Cardinale, M., Shcherbakov, A., Chebotar, V. and Berg G. (2012). *Sphagnum* mosses harbour highly specific bacterial diversity during their whole lifecycle. *ISME J* **6**: 802–813.

Bragina, A., Maier, S., Berg, C., Müller, H., Chobot, V., Hadacek, F. and Berg G. (2012). Similar diversity of Alphaproteobacteria and nitrogenase gene amplicons on two related *Sphagnum* mosses. *Front Microbiol* **2**: 275.

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Non-peer-reviewed publications

Bragina, A., Cardinale, M., Berg, C., Tikhonovich, I., Chebotar, V. and Berg. G. (2010). Bryophytes of the genus *Sphagnum* as an ecological niche for microbial communities. *Proceedings of the international bryological conference devoted to the 110-th birthdays of Zoya Nikolaevna Smirnova and Claudia Ivanovna Ladyzhenskaja*: 36–39.

Berg, G., Bragina, A., Cardinale, M., Zachow, C., Müller, H., Berg, C., Shcherbakov, A., Chebotar, V., and Grube. M. (2011). From metagenome and proteome to biocontrol: analysis of moss- and lichen-associated microbial communities. *IOBC-WPRS Bulletin* **71**: 13–16.

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Bragina, A., Cardinale, M., Berg, C., Tikhonovich, I., Chebotar, W. and Berg G. Bryophytes

of the genus *Sphagnum* as an ecological niche for microbial communities. October 11th, 2010. International bryological conference devoted to the 110-th birthdays of Zoya Nikolaevna Smirnova and Claudia Ivanovna Ladyzhenskaja. St. Petersburg (Russia).

Bragina, A., Maier, S., Berg, C., Cardinale, M., Müller, H., Shcherbakov, A., Chebotar, V. and Berg, G. Highly-specific microbial communities associated with *Sphagnum* mosses: biodiversity and function. February 6th, 2012. 7th DocDay of the Doctoral School of Molecular Biosciences and Biotechnology. Graz (Austria).

Bragina, A., Berg, C., Cardinale, M., Maier, S., Müller, H., Shcherbakov, A., Chebotar, A. and Berg, G. *Sphagnum*-associated bacteria help to feed their hosts and to protect our climate. August 23rd, 2012. 14th International Symposium on Microbial Ecology. Copenhagen (Denmark).

Poster presentations

Krikovtseva, A., Cardinale, M., Berg, C., Tikhonovich, I., Chebotar, V., and Berg, G.. *Sphagnum*-associated microbial communities: biodiversity as a key to the ecological role. July 12th − 17th, 2010. 5th AB-RMS Postgraduate Course and Minisymposium. Mikkeli (Finland).

Bragina, A., Cardinale, M., Berg, C. and Berg, G. Hidden exuberance of bryophytes: biodiversity of moss-associated microbial communities. September 27th − 29th, 2010. 2nd ÖGMBT Annual Meeting. Vienna (Austria).

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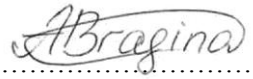
Bragina, A., Berg, C., Cardinale, M., Müller, H., Shcherbakov, A., Chebotar, V. and Berg, G. Endophytes in bryophytes: *Sphagnum* mosses harbour diverse bacteria interesting for biotechnology. March 28th – 30th, 2012. COST Action FA1103 workshop "Current aspects of European endophyte research". Reims (France).

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

12.09.12 ……

Date



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Signature