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1 Mining for NRPS and PKS genes revealed a high diversity in the *Sphagnum* bog  
2 metagenome

3

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10

11 Running title: NRPS and PKS screening in the *Sphagnum* microbiome

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14

15 **Abstract**

16 *Sphagnum* bog ecosystems are one of the oldest vegetation forms harbouring a specific  
17 microbial community, which is known to produce an exceptionally wide variety of  
18 bioactive substances. Although the *Sphagnum* metagenome indicate a rich secondary  
19 metabolism, the genes are not yet explored. To analyse non-ribosomal peptide synthetases  
20 (NRPS) and polyketide synthases (PKS) the diversity of NRPS and PKS genes in *Sphagnum*-  
21 associated metagenome was investigated by *in silico* data mining and sequence-based  
22 screening (PCR-amplification of 9500 fosmid clones). The *in silico* Illumina-based  
23 metagenomic approach resulted in the identification of 279 NRPS, 346 PKS, as well as 40  
24 PKS-NRPS hybrid gene sequences. Occurrence of NRPS sequences was strongly dominated  
25 by the phyla *Proteobacteria*, especially by the genus *Burkholderia*, while PKS sequences were  
26 mainly affiliated to *Actinobacteria*. Thirteen novel NRPS-related sequences were identified  
27 by PCR-amplification screening, displaying amino acid sequence identities of 48 to 91% to  
28 annotated sequences of the phyla *Proteobacteria*, *Actinobacteria* and *Cyanobacteria*. Some of  
29 the identified metagenomic clones showed closest similarity to peptide synthetases from  
30 *Burkholderia* or *Lysobacter*, which are emerging bacterial sources of yet undescribed  
31 bioactive metabolites. This study highlights the role of the extreme natural ecosystems as a  
32 promising source for detection of secondary compounds and enzymes, serving as a source for  
33 biotechnological applications.

34

35 **Keywords:** *Sphagnum* moss, NRPS/PKS, metagenome, fosmid library, *in silico* data mining

36

## 37 Introduction

38 The plant microbiome has established itself in recent years as an important player in the field  
39 of plant health and agricultural productivity (1). Mosses, especially *Sphagnum* species, are a  
40 phylogenetically old group of land plants in bog ecosystems, which are unique extreme  
41 habitats displaying high acidity, low temperature and water saturation, together with  
42 extremely low concentrations of mineral nutrients (2). *Sphagnum* bogs in particular reflect an  
43 enormous importance because of their approved role in the global carbon cycle and have  
44 therefore been used globally as an indicator of climate change (3). The role of *Sphagnum*  
45 mosses as an important model for examining the plant-microbe interactions as well as the  
46 ecology of plant-associated bacteria has been reported (4). *Sphagnum* mosses are in particular  
47 characterized by a specific but diverse microbial community (5–7, 4), which fulfil important  
48 functions in cooperation with the host, promoting plant growth by enhancing nutrient supply  
49 and showing antagonistic activity against plant pathogens (4, 8). In fact, high abundance of  
50 functional systems that are responsible for oxidative and drought stress, repair, resistance and  
51 genetic exchange were detected recently by metagenomic analysis of the *Sphagnum*  
52 microbiome (4). The biological activity of bryophytes and their traditional use in medicine  
53 and agriculture are well known (9). It has been shown, that *Sphagnum* species produce  
54 bioactive secondary metabolites, which influence their microbial colonisation (8). Bryophytes  
55 have been traditionally used in China, India and among Native Americans for their antifungal  
56 properties, and *Sphagnum* moss was employed as natural disinfectant for natural nappies or  
57 wound dressings in Europe (9). There are more than 300 natural compounds that have been  
58 isolated from bryophytes, mainly from liverworts (*Marchantiophyta*) but also from mosses  
59 (*Bryophyta*) (10). Some of the reported natural products in mosses are highly unsaturated fatty  
60 acids, alkanones, triterpenoids and flavonoids (10). Biological effects observed for extracts of  
61 mosses include antimicrobial, antifungal, cytotoxic, and antitumor activities (11, 12). The  
62 analysis of endo- and ectophytic bacterial strains revealed that *Sphagnum* moss harbours an

63 extraordinary high proportion of antifungal isolates, as well as a lower proportion of  
64 antibacterial isolates, which can partly explain the medicinal use (8). However, the major part  
65 (97%) of microbial communities associated with *Sphagnum* mosses belong to non-cultivable  
66 forms (7). Therefore, the antimicrobial potential of the moss microbiome remains mostly  
67 unexplored.

68         Prominent classes of active compounds from microbial and plant origin (antibiotics,  
69 antifungals or antitumor agents) are synthesized by large multi-modular enzymes, the non-  
70 ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) or hybrids thereof  
71 (13). The widespread occurrence of the NRPS and PKS genetic machinery across all three  
72 domains of life (bacteria, archaea and eukarya) has been reported (14). Bacteria host the  
73 majority of the described NRPS/PKS gene clusters, which are especially common in the phyla  
74 *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Cyanobacteria* (14). Both NRPSs and PKSs  
75 are regarded as multi-enzymatic mega-synthases (200-2000 kDa) organized in a modular  
76 assembly line fashion, that contains catalytic modules for single rounds of chain elongation  
77 and variable modifications of the intermediate product (13). In non-ribosomal peptides  
78 (NRPs) defined monomers, amino acids or non-proteinogenic monomers, are incorporated by  
79 specific modules consisting of three essential catalytic domains. The adenylation (A) domain  
80 catalyse the activation of the amino acid, which is then transferred to the peptidyl carrier  
81 protein (PCP), followed by condensation of the bound amino acid (condensation (C) domain)  
82 (15). In a similar way, PKS mega-enzymes consist of an acyltransferase (AT) domain for  
83 selection of the monomer substrate, usually malonyl- or methyl-malonyl-CoA, priming it to  
84 the acyl carrier protein (ACP), followed by chain elongation and condensation (C-C-bond  
85 formation) by a ketosynthase (KS) domain (16). In addition to the core domains, a variable  
86 set of domains for further modifications of the peptide chain (epimerization,  
87 heterocyclization) (15) or the polyketide chain (ketoreduction, dehydration) are available  
88 (16). Termination of the chain is catalysed in both NRPS and PKS by a thioester (TE)

89 domain. Because of structural and functional similarities between elements of each class,  
90 NRPS and PKS can form mixed assembly lines (hybrid gene clusters) (14). Rational design  
91 of combinatorial PKS and NRPS modules is an emerging strategy to design tailor-made  
92 antibiotics or therapeutic compounds (17).

93         Rapid development of new metagenomic approaches permit the assessment and  
94 exploitation of the taxonomic as well as the functional diversity of microbial communities  
95 (18, 19). The discovery of novel biocatalysts for production of natural active compounds can  
96 be accomplished through screening of metagenomic libraries, for example by PCR-based  
97 screening techniques. Metagenomic applications were recently used for detection of  
98 NRPS/PKS genes of bacterial communities in soil (20) and marine environments (21), but  
99 not for plant-associated microhabitats or extreme bog ecosystems known to be rich in  
100 antimicrobial activity (22).

101         In this study, our aim was to explore the diversity of sequences assigned to NRPS and  
102 PKS genes in the *Sphagnum*-associated bacteria, allowing us greater insight into potentially  
103 novel synthetic pathways and biocatalysts. We combined two sequence-based screening  
104 methods to search for NRPS and PKS related sequences: *in silico* mining in the moss  
105 metagenomic database and PCR-amplification screening of a metagenomic fosmid library in  
106 *E. coli*. Origin and abundance of the identified metagenomic sequences were investigated.  
107 Our results demonstrate how sequence-based screenings can be used to detect NRPS and PKS  
108 genes involved in the biosynthesis of secondary metabolites within the *Sphagnum* moss  
109 microbiome.  
110

111 **Methods**

112 *In silico analysis of the Sphagnum moss dataset*

113 The recently published metagenomic dataset of the *Sphagnum* microbiome (4) was employed  
114 for data mining of NRPS and PKS gene sequences. The generated paired-end reads from  
115 untreated and normalized sequences from a previous study by Bragina *et al.* (4) were quality  
116 filtered (trimming of read ends with an average <Q30 and polyN nucleotides) using the  
117 PRINSEQ software according to the manual (<http://prinseq.sourceforge.net>). The normalized  
118 dataset consisted of ssDNA sequences after treatment and separation by hydroxyapatite  
119 chromatography (4). Untreated and normalized datasets were pooled and the generated mixed  
120 dataset was used for *de novo* assembly with the SOAPdenovo2 software  
121 (<http://soap.genomics.org.cn/soapdenovo.html>) using default parameters for metagenomic  
122 datasets (23). Briefly, the SOAPec correction tool was used first to filter short reads (kmer  
123 size  $\leq 17$ , quality value of 33, thread of 12), using a low frequency cutoff value of 5. For  
124 contig assembly (de Bruijn graph) a kmer size of 23 was employed, using an average insert  
125 size of 200 bp, a read length cut-off of 100 bp, a paired end cutoff value of 3 and a minimum  
126 alignment length of 32. The resulting fasta file from *de novo* assembly, including the  
127 assembled scaffolds and contigs, was employed as query for blastx analysis using a self-  
128 developed bioinformatics workflow (fasta-file splitting, blastx, and generation of the blastx  
129 database). Then, the resulting moss metagenomic blastx database was mined for NRPS and  
130 PKS gene sequences employing a self-developed script, which works on the basis of a search  
131 term. The terms “non ribosomal peptide synthetase” and “polyketide synthase” were used for  
132 the search. Additionally, to compare the abundance of NRPS and PKS with other commonly  
133 found microbial genes a search was performed for monooxygenases and rpoD RNA  
134 polymerases.

135

136 *Sampling and total community DNA isolation*

137 Sampling of *S. magellanicum* gametophytes and preparation of the microbiome for total  
138 community DNA isolation (enrichment of microbial fraction and removal of plant debris) was  
139 performed as reported in Bragina *et al.* (4). To construct the fosmid library for PCR-based  
140 screening, total community DNA was extracted using the Meta-G-Nome™ DNA Isolation Kit  
141 (Epicentre, Madison, WI, USA) according to the manufacturer's protocol. Metagenomic DNA  
142 was randomly sheared to fragment sizes of approximately 40 kb that were used for  
143 construction of the fosmid library.

144

#### 145 *Metagenomic fosmid library generation and PCR screening*

146 A metagenomic fosmid library from the *Sphagnum* moos microbiome was constructed using  
147 the CopyControl Fosmid Library Production Kit (Epicentre) as described by the  
148 manufacturer's instructions. In short, the isolated metagenomic DNA (1 µg) of approximately  
149 40 kb was directly used for blunt-end repair and was ligated into the CopyControl  
150 pCC2FOS™ vector (1.1 µg vector, 0.62 µg insert DNA). The ligated DNA was packaged  
151 with MaxPlax Lambda Packaging Extracts. The packaged phage particles were employed to  
152 infect *E. coli* EPI300-T1<sup>R</sup> cells. The fosmid library was spread onto LB agar plates containing  
153 12.5 µg ml<sup>-1</sup> chloramphenicol and incubated at 37 °C overnight. In total, 9500 clones were  
154 randomly transferred to 96-well microtiter plates (MTP) containing 150 µl of LB medium  
155 with chloramphenicol (12.5 µg ml<sup>-1</sup>) using sterile tooth picks, each plate consisting of ninety-  
156 five different clones and one negative control (only medium). MTP cultures were grown at 37  
157 °C overnight by shaking at 225 rpm, and finally stored at -70 °C after addition of glycerol to a  
158 final concentration of 25% (v/v) to each well. To estimate the average insert size in the  
159 fosmid clones, restriction digestion was performed with BamHI. For the PCR screening, 10  
160 clones were pooled together for a total of 10 MTP-pools in LB medium (12.5 µg ml<sup>-1</sup>  
161 chloramphenicol). The pooled MTPs were cultivated under the conditions described above,  
162 upon addition of 1x Fosmid Autoinduction Solution (Epicentre) to induce high copy number.

163 Denaturation (15 min at 99°C) of diluted MTP cultures (1:2 in ddH<sub>2</sub>O) and centrifugation  
164 (4000 rpm, 5 min) were performed in order to make the fosmid DNA accessible to PCR  
165 screening with the two previously reported degenerated primer pairs NRPS1 and NRPS2 (24)  
166 which are given in Supplementary Material, Table S1. A standard PCR reaction (25 µl)  
167 contained 1x Taq 2xMaster Mix (12.5 µl, New England Biolabs, Ipswich, UK), 0.4 µM of  
168 each primer (1 µl degenerated primer, Table 1; Sigma-Aldrich, Wien, Austria), ddH<sub>2</sub>O  
169 (4.25 µl), 5% (v/v) DMSO (1.25 µl) and 5 µl of pooled template DNA. Following PCR  
170 program was used: 95 °C, 5 min; 35 cycles of 95 °C, 1 min; 57 °C, 1 min; 68 °C, 1 min; and  
171 elongation at 68 °C, 10 min. PCR products were analysed by 2% agarose/TAE gel  
172 electrophoresis. Localization of positive clones was achieved by repetition of the PCR as  
173 described above, employing in this case the 10 single clones from the previously identified  
174 positive MTP-pool.

175

#### 176 *Phylogenetic analysis of identified fosmid clones*

177 Diluted PCR products (1:1000) from single fosmid clones identified as positive hits during  
178 rescreening of the library were amplified with shorter non-degenerated primer pairs  
179 NRPS1ndeg and NRPS2ndeg (nested PCR), employing the above mentioned PCR program.  
180 These primers resemble those used for library screening but lacking the degenerated  
181 nucleotides in the 3'-region (Supplementary Material, Table S1). The resulting PCR products  
182 were purified using a Wizard® SV 96 PCR Clean-Up System (Promega, Mannheim,  
183 Germany) and sent for Sanger sequencing at LGC Genomics (Berlin, Germany). Based on the  
184 first sequencing results, selected fosmids (3-F3, 3-H3, 2-D4, 2-F4, 7- B9, 6-H4) were partially  
185 sequenced by primer walking using for each a sequence-specific primer (Supplementary  
186 Material, Table S1). This allowed retrieval of longer DNA sequences contiguous to the  
187 previously identified NRPS gene region (up to 1100 bp) directly from the fosmid clones.  
188 Analysis of the obtained sequences was performed using BLASTx (25) against the non-

189 redundant (nr) protein sequences database at NCBI (<http://www.ncbi.nlm.nih.gov/protein>) or  
190 the KEGG database (<http://www.genome.jp/kegg>). The retrieved amino acid (aa) sequences  
191 of positive fosmid clones were employed for phylogenetic analysis, together with the most  
192 similar gene sequences from the blastx search. Alignment of aa sequences and construction of  
193 the phylogenetic tree were performed with CLC Main Workbench 6.9.1. The phylogenetic  
194 tree was generated using the Unweighted Pair Group Method using Arithmetic averages  
195 (UPMGA), Kimura Protein as distance measure and a bootstrap of 1000 replicates.  
196

197 **Results**

198 *Data mining in the moss metagenomic dataset*

199 The metagenomic dataset of *Sphagnum magellanicum* moss (Illumina HiSeq 2x100 paired-  
200 end sequencing) consist of 17323 Mbp raw (pair-number: 86617475) and 14141 Mbp  
201 normalised metagenomic DNA (pair-number: 70705608) (4). *De novo* assembly of the pooled  
202 metagenome (raw and normalized reads; N50 of 199) yielded 1062181 contig sequences  
203 (168393 scaffolds and 893788 contigs), featuring a total size of 188.2 Mbp with an average  
204 length of 183 bp (Supplementary Material, Table S2).

205 Blastx analysis of the metagenomic dataset revealed that NRPS, PKS and NRPS-PKS  
206 hybrid gene sequences are present in the moss microbiome. Without cutoff settings, the blastx  
207 dataset consist of 279 NRPS, 346 PKS, and 40 hybrid or mixed gene sequences (Table 1).  
208 This translates into a rate of 0.063% contigs containing NRPS/PKS gene sequences in the  
209 assembled *Sphagnum* metagenome (665 out of 1062181 contigs). In comparison, other  
210 common microbial gene sequences such as those coding for monooxygenases (3244 contigs)  
211 or the rpoD RNA polymerase (sigma 70 factor; 160 contigs), contributed to the assembly with  
212 rates of 0.305% and 0.015%, respectively. It has to be considered that the estimated rates rely  
213 on the availability of annotated homolog genes in the employed databases, which can be  
214 subjected to changes along with the discovery of novel sequences. Therefore, our results may  
215 underrepresent the real frequency of these protein families in the microbial community. The  
216 highest abundance of NRPS closest matches belong to the phyla *Proteobacteria*  
217 (*Burkholderia* spp., n=50, 18%; *Pseudomonas* spp., n=40, 14%; *Myxococcus* spp., n=27,  
218 10%) and *Actinobacteria* (*Streptomyces* spp., n=25, 9%; *Rhodococcus* spp., n=10, 4%). In the  
219 case of PKS, closest matches are mainly represented by *Actinobacteria* (*Mycobacterium* spp.,  
220 n=92, 27%; *Streptomyces* spp., n=46, 13%) and uncultured bacteria (n=18, 5%). The  
221 remaining hits from the *in silico* search show a diverse distribution of underrepresented taxa  
222 from *Proteobacteria* and *Actinobacteria*, but also from *Cyanobacteria* (e.g. *Nostoc*,

223 *Anabaena*, *Pseudoanabaena*, *Microcystis*, *Fischerella*). Hybrid-gene matches are mainly  
224 affiliated to the phylum *Proteobacteria* (*Pseudomonas* spp., n=7, 18%; *Lysobacter* spp., n=6,  
225 15%, and *Myxococcus* spp., n=6, 15%).

226       Employing an E value cutoff of  $10^{-20}$  (bitscore >88) it was possible to select the best  
227 matching sequences in the database. This resulted in a confined selection of 34 NRPS and 28  
228 PKS genes, as well as three NRPS-PKS hybrids (Supplementary Material, Table S3). These  
229 sequences display diverse identities to their closest neighbour from the blastx analysis ranging  
230 from 35 to 98%. Many of the selected PKS sequences with a higher similarity (>60%  
231 identity) to annotated genes in the nr database are mainly related to the genus *Mycobacterium*.  
232 The remaining sequences (35-60% identity) show similarity to PKSs from diverse genera,  
233 such as *Streptomyces* or *Rubrivivax*. In the case of NRPS gene sequences, the most abundant  
234 genera from the closest hits are *Burkholderia*, followed by *Bradyrhizobium*, *Pseudomonas*,  
235 *Mycobacterium* and *Pectobacterium*. One hybrid gene sequence is related to the genus  
236 *Lysobacter* and the remaining two resemble the well-studied Yersiniabactin synthase (26)  
237 from *Pseudomonas syringae*. Additionally, five of the NRPS and PKS gene sequences display  
238 the highest similarity to annotated genes from yet uncultured bacteria.

239

#### 240 *Fosmid library screening*

241 The *Sphagnum* moss fosmid library was generated employing 1  $\mu$ g of metagenomic DNA,  
242 obtained from the enriched microbial fraction (1 g) contained in 200 g moss. Based on the  
243 number of clones obtained (96,025) and an average insert size of 27 kb, the library size was  
244 estimated as 2.6 Gb. In total, 9,500 randomly selected clones (0.26 Gb) were employed for the  
245 screening of NRPS genes by PCR amplification in MTP using two different degenerated  
246 oligonucleotide primer pairs (NRPS1 and NRPS2). The primers were previously designed to  
247 target the adenylation domain of NRPS gene clusters in diverse soil samples (27). In total, 25  
248 NRPS1 and 33 NRPS2 wells, each containing a pool of ten clones, gave a positive

249 amplification result. A second round of screening of the corresponding single clones resulted  
250 in 11 NRPS1 and 26 NRPS2 putative positive hits. Positive clones were subjected to  
251 optimization of PCR-conditions with shorter non-degenerated primer pairs in a nested PCR to  
252 avoid the amplification of unspecific products. Amplicons of 21 NRPS positive fosmids that  
253 were obtained as a pure DNA band were sent for sequencing.

254         Based on blastx analysis against the nr protein sequences database (NCBI) and the  
255 KEGG database, 14 sequences showed similarity to genes encoding for peptide synthases  
256 (NRPS, Table 2) and could be therefore assigned to these protein family. Seven clones were  
257 detected with each primer pair (NRPS1 or NRPS2) respectively, resulting in an average hit  
258 rate of one NRPS gene per 37 Mb of screened moss metagenomic DNA.

259         NRPS sequence identities to the closest hits from blastx analysis range from 48 to 99%  
260 (Table 2). Most of the identified closest neighbour sequences belong to the phylum of  
261 *Proteobacteria* (12 hits; 86%), and in particular to the genus of *Pseudomonas* (4 hits, Table  
262 2). The remaining two hits include the phyla *Actinobacteria* (*Kutzneria albida*) and  
263 *Cyanobacteria* (*Rubidibacter lacunae*). Despite of the clone 3-F3, showing a very high aa  
264 sequence homology of 99.4% to a peptide synthase from *Pseudomonas* sp. Ag1, all other 13  
265 clones harbour novel, not yet annotated aa sequences with maximal identities of up to 91%.

266         The obtained aa sequences aligned partially to conserved domains of annotated NRPS  
267 sequences, either to the adenylation domain (A\_NRPS motif; cd05930), the  
268 phosphopantetheine prosthetic group attachment site (pp-binding motif; pfam00550) or the  
269 condensation domain (pfam00668) (see multiple sequence alignment in Supplementary  
270 Material, Figure S1).

271         To gain a better overview of putative NRPS amino acid sequences found in the fosmid  
272 library a phylogenetic tree was generated (Figure 1). Sequences with a hit length of >100 aa  
273 and a gap value <2% were placed on the tree. The phylogenetic analysis exhibits the  
274 distribution of the putative NRPS sequences, which clustered into three main groups. The first

275 group is composed of  $\alpha$ -*Proteobacteria*, containing the sequences NRPS 7-F1 and 6-H4.  
276 These sequences are most related to peptide synthetases from the family of *Caulobacteraceae*  
277 and *Agrobacterium* spp. The product of these peptide synthase gene clusters are, however,  
278 still unknown. The second and more diverse group includes the NRPS sequences 4-B4, 7-D4,  
279 and 6-B1, clustering in closer proximity to peptide synthetases from  $\alpha$ -,  $\beta$ -, and  $\gamma$ -  
280 *Proteobacteria*. Sequences from clones 7-D4 and 6-B1 are close related to thioester-  
281 reductases from *Dyella* and *Variovorax* species. This group is also in close proximity to the  
282 well-studied Gramicidin synthase from *Kutzneria albida* (phylum of *Actinobacteria*). 7-D4  
283 displays furthermore a distant similarity (50%) to the Gramicidin synthetase (LgrC) from  
284 *Streptacidiphilus albus* (Table 2). The closest match of clone 4-B4 to a synthetase coding for  
285 a known product is the Syringopeptin synthetase b from *Photorhabdus asymbiotica* (65% aa  
286 sequence similarity; Table 2). The third group, which includes the NRPS sequences 3-F3, 7-  
287 B9, and 2-F4, comprises species from *Pseudomonas* and *Lysobacter* ( $\gamma$ -*Proteobacteria*). The  
288 sequence of clones 3-F3 and 2-F4 match partially to the biosynthetic pathway genes of the  
289 siderophore Pyoverdin from *Pseudomonas amygdali* and the toxin Syringomycin from *P.*  
290 *syringae*, respectively (61% and 57% similarity, Table 2).

291 Additionally, to investigate a possible overlap of NRPS sequences found by both  
292 screening methodologies the fosmid clones sequences were aligned (blastn) against the NRPS  
293 contig dataset from the *in silico* screening. Overlaps with high sequence similarity (up to  
294 100%) were obtained only for very short DNA fragments (<15 bp). The best alignment score  
295 (63 bit, E value of 7.00E-12) was found for the fosmid clone 7-B9 and the scaffold30678 with  
296 a sequence similarity of 91% (alignment length of 47 bp).

297

298 **Discussion**

299 Our strategy to identify NRPS and PKS genes within moss-associated bacteria using two  
300 different approaches resulted in new findings. By *in silico* data mining we gained a valuable  
301 insight into the abundance and origin of NRPS and PKS genes present in the *Sphagnum* moss  
302 microbiome. Our hypothesis that the *Sphagnum* microbiome is a promising sources for novel  
303 NRPS and PKS genes based on ecological knowledge (4, 8) was fulfilled. The biological  
304 activity of bryophytes is well known (9) and several natural compounds have been elucidated  
305 including antibiotics, antifungals or cytotoxic compounds (10). We could show that the  
306 associated microbiota of *Sphagnum* has the biosynthetic potential to synthesize a significant  
307 amount of natural products by NRPS and PKS systems. In fact, the previous functional  
308 analysis of the *Sphagnum* metagenome revealed a high availability of subsystems that are  
309 responsible for the synthesis of bioactive compounds, such as quorum sensing molecules,  
310 toxins-antitoxins, adhesins, and especially siderophores (4). Siderophore production and  
311 antibiotic/antifungal activity has been detected in many bacteria isolated from *Sphagnum* sp.  
312 (5, 6, 28, 29), although none microbial bioactive compounds has been isolated so far. Toxins,  
313 siderophores and antibiotics are commonly synthesized by NRPS/PKS systems (30). This  
314 compounds are often involved in characteristic reactions of microbial antagonisms, where  
315 microbes inhibit each other (antibiotics, toxins) or compete for space, nutrients and minerals  
316 (release of siderophores) in a shared microenvironment (31). A high incidence of  
317 biosynthetic systems for siderophore production was expected since *Sphagnum*-dominated  
318 peat bogs are nutrient deficient environments with low concentrations of bioavailable  
319 minerals like iron (32).

320 The blastx analysis revealed a significant number and also a clear difference in the  
321 bacterial diversity between NRPS and PKS sequences in the *Sphagnum* metagenome (Table  
322 1). The composition of microbial communities derived from PKS-related sequences is  
323 strongly dominated by the genera *Mycobacterium* and *Streptomyces*. These two

324 *Actinobacteria* are well-studied producers of bioactive compounds including both NRPs and  
325 PKs (33). The synthesis of flavonoids in higher plants is nearly ubiquitous and involves the  
326 use of chalcone synthases (CHS), which belongs to the family of type III PKS (34). However,  
327 type III PKSs (CHS-like enzymes) have been also identified in bacteria (e.g. *Streptomyces*  
328 *griseus*, *S. coelicolor*, *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Pseudomonas*  
329 *fluorescens*) and fungi (35). This strongly supports the possible microbial synthesis of  
330 flavonoid and aromatic polyketides (polyphenols such as stilbenes and chalcones) in mosses.

331 In contrast to PKS, NRPS-related sequences showed higher abundance of species  
332 belonging to *Proteobacteria* over those from *Actinobacteria*. A high abundance of protein  
333 coding sequences from *Proteobacteria* and *Actinobacteria* was expected, since our previous  
334 analysis on taxonomic structure and diversity based on 16S rRNA genes of the *Sphagnum*  
335 moss metagenome revealed a dominant role of these two phyla (65.8 % *Proteobacteria* and  
336 5.6% *Actinobacteria*). A similar taxonomic hit distribution of *Proteobacteria* and  
337 *Actinobacteria* (62% and 8% respectively) was estimated on the basis of predicted protein  
338 coding regions and ribosomal RNA genes (4). At the class level the 16S rRNA analysis  
339 revealed high abundance of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -*Proteobacteria*, which correlates well to the high  
340 occurrence of NRPS-related sequences from this taxa (Table 1). A high portion of PKS  
341 sequences (44%, remaining strains in Table 1) is affiliated to diverse bacterial taxa, mainly  
342 *Actinobacteria*, *Cyanobacteria*, *Proteobacteria* or *Firmicutes*. Both *Cyanobacteria* and  
343 *Firmicutes* represent rather subdominant phyla in the *Sphagnum* metagenome (4).

344 Extensively studied bacterial sources for antibiotics are *Streptomyces*, myxobacteria,  
345 cyanobacteria, *Bacillus* and *Pseudomonas* (36). The distribution of PKS and NRPS clusters in  
346 bacterial genomes was also comprehensively reviewed by Donadio *et al.* (30). In the last  
347 years growing knowledge has been gained through sequencing of whole bacterial genomes,  
348 revealing the potential of many unexpected bacterial strains, which harbour the genetic  
349 machinery for production of secondary metabolites. One of the newly discovered groups of

350 secondary metabolites producers is the genus of *Burkholderia*. *Burkholderia* spp. synthesize  
351 toxins (bongkretic acid), antifungal compounds (rhizoxin), and also antibacterial compounds  
352 (enacycloxin) among others (37). Culture-dependent analysis of *Sphagnum magellanicum*-  
353 associated antagonists demonstrated the dominant role of the genus *Burkholderia*, accounting  
354 for 38% of the isolates (6). Besides, new moss-associated *Burkholderia* species (*B. bryophila*  
355 and *B. megapolitana*) displaying antifungal activities and tested positively for the production  
356 of siderophores were also described in this collection (28). Species from the plant-associated  
357 *Burkholderia* cluster were identified as cosmopolitan core members of the *Sphagnum*  
358 microbiome; they were present in the *Sphagnum* sporophyte as well as the gametophyte (38).  
359 By *in silico* screening in the moss database we also detected a significantly high abundance of  
360 NRPS sequences affiliated to *Burkholderia* (18%). Our results undermine the dominant role  
361 of the genus *Burkholderia* in *Sphagnum* mosses, especially in regard to the production of  
362 NRPS-synthesized secondary metabolites.

363 Furthermore, single sequences of both NRPS and PKS were affiliated to  
364 cyanobacteria. Cyanobacteria are a rich source of structurally diverse oligopeptides, mostly  
365 synthesized by NRPSs and NRPS/PKS hybrid pathways (39). Common cyanobacteria genus  
366 like *Nostoc*, *Microcystis*, and *Anabaena*, that were present in the moss metagenome, produce  
367 bioactive peptides (39). Interestingly, mixed/hybrid NRPS-PKS gene clusters were also  
368 present within the moss microbiome dataset. Most of the sequences were assigned to the  
369 phylum *Proteobacteria*, being the most abundant genera *Pseudomonas* and especially  
370 *Lysobacter*. The latter has emerged in the last years, not only as a promising source of new  
371 bioactive natural products, such as antibiotics,  $\beta$ -lactams, cyclic lactams and depsipeptides,  
372 but also as a biocontrol agent for fungal plant infections (36, 40, 41). Furthermore, similar  
373 sequences coding for the siderophore Yersiniabactin from *P. syringae* were detected.  
374 Yersiniabactin acts as a virulence factor, facilitating iron uptake in the host, and is synthesized  
375 by a hybrid PKS/NRPS system located on a transmissible high-pathogenicity island. This

376 pathogenicity island has been encountered in various strains as a result of horizontal transfer  
377 (e.g. in enterobacteria) (26). In the case of Yersiniabactin and similar compounds, mobility  
378 by horizontal transfer to other pathogenic strains (mainly *Proteobacteria*) could account for  
379 the high incidence in metagenomic dataset, as in the *Sphagnum* moss microbiome.

380         Of special interest are those NRPS and PKS aa sequences with a rather low sequence  
381 identity to their next blastx neighbours or displaying similarity to sequences originated from  
382 uncultured bacteria (12 NRPS and 18 PKS sequences; Table 1). Recently, the huge potential  
383 of uncultured bacteria for the screening of novel bioactive compounds was confirmed through  
384 discovery of the novel antibiotic teixobactin, which shows promising properties against multi-  
385 resistant pathogenic strains (42).

386         In addition to *in silico* data mining, PCR-amplification screening of a moss  
387 metagenomic fosmid library led to identification of 13 novel NRPS-related sequences out of  
388 14 detected clones. Only a minimal overlap between the fosmid clone sequences and the  
389 NRPS contigs from the *in silico* analysis was detected. This can be explained by the major  
390 differences between both methodologies. On the one hand, low rates for discovery of positive  
391 hits in metagenomic clone libraries are very common, in our case 0.15% (14 from 9500  
392 screened clones). On the other hand, assemblies of metagenomic DNA usually contain poor  
393 or no coverage of complete genomes or genome portions and are prone to formation of  
394 chimeras (43). The *Sphagnum* assembly employed in this study has a rather low average  
395 contig length of 183 bp, which limits the recovery of complete genes and leads to the low  
396 observed overlap between sequences. Despite of this, the methods complement each other for  
397 studying the biosynthetic capacity of the *Sphagnum* metagenome.

398         In terms of taxonomy, the *in vitro* library screening reflects the findings of the *in silico*  
399 screening. Phylogenetic analysis of the retrieved aa sequences from the *Sphagnum* moss  
400 fosmid library revealed closer proximity of NRPS-related genes to the phylum  
401 *Proteobacteria*. Selected sequences clustered into three main groups, with one representative

402 group being closely related to *Pseudomonas* and *Lysobacter*. The relevance of *Lysobacter* as  
403 an “emerging” producer of bioactive compounds was discussed above. In a similar manner,  
404 novel NRPS systems were recently discovered in *Pseudomonas* spp. by new PCR-screening  
405 methods (44). We encountered partial similarity of some of the sequences (3-F3 and 2-F4) to  
406 the reported peptide synthetase products Pyoverdine and Syringomycin, originated from  
407 opportunistic pathogenic *Pseudomonas* strains. Pyoverdine, a siderophore that facilitates iron-  
408 uptake, and Syringomycin, a cyclic lipodepsipeptide phytotoxin, are both regarded as  
409 important virulence factors secreted by the host cell (45, 46). Production and release of  
410 siderophore has been reported for *Sphagnum*-associated bacteria like *Pseudomonas* sp.,  
411 *Serratia* sp. and *Burkholderia* sp. (5)

412 The second group, containing clones 7-F1 and 6-H4, shows similarity to annotated  
413 peptide synthetases from the *Caulobacteraceae* family and *Agrobacterium* spp. So far, only  
414 ribosomally encoded peptides (so-called Lasso peptides), have been isolated or described in  
415 bacteria belonging to the *Caulobacteraceae* (47). For the *Agrobacterium tumefaciens* strain  
416 C58 only one biosynthetic gene cluster has been characterized, a hybrid NRPS-PKS system  
417 that catalyses the formation of a novel siderophore (48).

418 In the third group, one of the NRPS-sequence (clone 4-B4) is closely related to peptide  
419 synthetases from *Burkholderia rhizoxinica* and *Photorabdus temperate*. Interestingly, both of  
420 these bacteria are symbionts of pathogenic organisms, the fungal pathogen *Rhizopus*  
421 *microspores* and of entomopathogenic nematodes, respectively. Complete genome sequencing  
422 of *Burkholderia rhizoxinica* showed the occurrence of 14 NRPS gene clusters with a yet  
423 unknown function (49). *Photorabdus temperata* is also known to produce a large number of  
424 bioactive compounds, especially stilbenes, where a significant proportion of the genome (6%)  
425 is devoted to the production of secondary metabolites (50). Derivatives of stilbenes have also  
426 been detected in Bryophytes (10), which demonstrates convergence between the metabolic  
427 capacity of the associated bacterium and the plant host. A lower similarity of clone 4-B4 was

428 found for the gene cluster producing Syringopeptin in *Photorhabdus asymbiotica*.  
429 Syringopeptin is like Syringomycin a well-known phytotoxin that is secreted by the host  
430 organism and has been studied in more detail in *Pseudomonas syringae* (51). The last two  
431 sequences in this group, belonging to clones 7-D4 and 6-B1, display similarity to annotated  
432 sequences of thioester-reductases rather than peptide synthetases. The occurrence of reductase  
433 domains in NRPS systems has been reported, for example for the peptaibol synthetase from  
434 *Trichoderma virens* that does not comprise the commonly encountered thioester domain  
435 (TE) for termination of peptide synthesis, but rather a reductase domain (52). Similarly, in  
436 the fungus *Aspergillus flavus* NRPS-like proteins that are involved in the synthesis of  
437 metabolites contain a reductase domain instead of a condensation domain (53). These  
438 sequences, especially 7-D4, show distant homology to a gene cluster coding for Gramicidin, a  
439 linear polypeptide antibiotic (toxin) that forms an ion membrane channel and has been  
440 intensively studied in *Bacillus brevis* (54).

441 By combining two different screening approaches, we gained an excellent overview of  
442 the taxonomic and functional composition of NRPS and PKS gene clusters within the  
443 *Sphagnum* microbiome. The *in silico* data mining approach provided a general survey on the  
444 occurrence and abundance of the NRPS and PKS genetic machinery in *Sphagnum* moss-  
445 associated bacteria. Additionally, single clones containing novel NRPS sequences were  
446 identified by PCR-amplification screening. Analysis of the amplicon sequences suggested the  
447 presence of several novel gene clusters for production of microbial metabolites, such as  
448 siderophores, phytotoxins or antibiotics. These findings are in accordance to the previous  
449 metagenomic analysis and antimicrobial assays that suggested the availability of such  
450 biosynthetic systems in *Sphagnum*. A further characterization of the identified metagenomic  
451 clones will provide a promising basis for the discovery of novel biosynthetic pathways.

452

453

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463

464 **Data accessibility**

465 Query sequences from the *S. magellanicum* metagenome that showed homology to NRPS,  
466 PKS or hybrid NRPS-PKS genes (from *in silico* data mining) were deposited in the DRYAD  
467 repository (<http://datadryad.org/pages/repository>) under the accession number xxxx.

468

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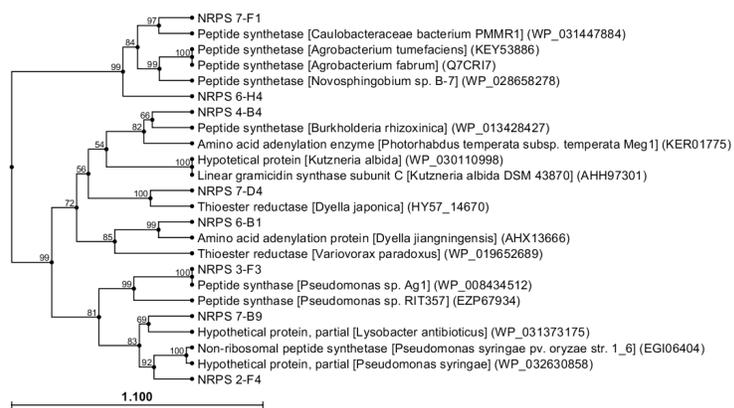
622 **Figures and Tables**

623 **Table 1** Abundance and occurrence of phylas of NRPS, PKS and NRPS-PKS hybrid sequences from *in silico*  
 624 data mining in the *Sphagnum* moss metagenomic database (counts >10 are highlighted in bold, the highest  
 625 values are underlined).

Taxon Phylum/Class	Genus	No. of occurrences		
		NRPS	PKS	Hybrids
Actinobacteria	<i>Mycobacterium</i>	5	<u>92</u>	0
Actinobacteria	<i>Streptomyces</i>	<b>25</b>	<b>46</b>	2
Actinobacteria	<i>Rhodococcus</i>	<b>10</b>	1	0
Actinobacteria	<i>Brachybacterium</i>	0	0	3
$\alpha$ -Proteobacteria	<i>Bradyrhizobium</i>	<b>10</b>	4	3
$\beta$ -Proteobacteria	<i>Burkholderia</i>	<u>50</u>	5	0
$\beta$ -Proteobacteria	<i>Rubrivivax</i>	0	6	0
$\beta$ -Proteobacteria	<i>Bordetella</i>	0	6	0
$\beta$ -Proteobacteria	<i>Ralstonia</i>	2	0	4
$\gamma$ -Proteobacteria	<i>Pseudomonas</i>	<b>40</b>	2	<u>7</u>
$\gamma$ -Proteobacteria	<i>Lysobacter</i>	0	0	6
$\gamma$ -Proteobacteria	<i>Pectobacterium</i>	<b>12</b>	0	0
$\gamma$ -Proteobacteria	<i>Xanthomonas</i>	9	0	0
$\gamma$ -Proteobacteria	<i>Xenorhabdus</i>	6	1	0
$\delta$ -Proteobacteria	<i>Myxococcus</i>	<b>27</b>	5	6
$\delta$ -Proteobacteria	<i>Candidatus</i>	4	2	2
Firmicutes	<i>Paenibacillus</i>	1	6	1
<b>uncultured bacterium</b>		<b>12</b>	<b>18</b>	1
Remaining strains		<b>66</b>	<b>152</b>	5
<b>Total count</b>		<b>279</b>	<b>346</b>	<b>40</b>

626 **Table 2** Blastx analysis of non-ribosomal peptide synthetase (NRPS) putative sequences obtained through PCR-based screening of the *Sphagnum* moss metagenomic fosmid  
 627 library. For fosmid clones 3-F3, 3-H3, 2-D4, 2-F4, 7-B9 and 6-H4 longer sequences (759-1155 bp, 252-384 aa) were retrieved by primer walking. Blastx was performed against  
 628 the non-redundant protein database (<http://www.ncbi.nlm.nih.gov/protein>; 07.04.2015).

Clone ID (Query)	Primer (Screening)	Close hits (Accession no.)	Description [source]	E-value	Score	Bit score	Alignment length [aa]	Identity [%]	Positive [%]	Gaps [%]
3-F3	NRPS1	WP_008434512	Peptide synthase [ <i>Pseudomonas</i> sp. Ag1]	0	1,691	656	345	99.4	99.4	0
		WP_005745216	Pyoverdine sidechain peptide synthetase III, partial [ <i>Pseudomonas amygdali</i> ]	4.76E-131	1,019	397	342	60.6	73.9	0.87
7-B9	NRPS2	WP_031373175	Hypothetical protein, partial [ <i>Lysobacter antibioticus</i> ]	6.26E-116	969	378	320	62.9	73.8	1.25
		WP_032634710	Non-ribosomal peptide synthetase modules, partial [ <i>Pseudomonas syringae</i> ]	8.94E-105	845	330	322	57.2	68.0	3.08
6-H4	NRPS2	WP_031447884	Peptide synthetase [ <i>Caulobacteraceae</i> bacterium PMMR1]	3.68E-114	948	370	301	64.1	76.1	0.66
2-F4	NRPS1	WP_032630858	Hypothetical protein, partial [ <i>Pseudomonas syringae</i> ]	4.78E-88	723	283	254	64.2	76.0	1.18
		WP_004417722	Syringomycin synthetase E, partial [ <i>Pseudomonas syringae</i> ]	2.55E-73	651	255	255	57.3	69.4	1.18
7-F1	NRPS2	WP_031447884	Peptide synthetase [ <i>Caulobacteraceae</i> bacterium PMMR1]	1.47E-54	498	196	147	73.5	81.0	0
4-B4	NRPS2	WP_013428427	Peptide synthetase [ <i>Burkholderia rhizoxinica</i> ]	5.34E-48	451	178	143	70.1	78.5	0.69
		WP_015833634	Syringopeptin synthetase b [ <i>Photorhabdus asymbiotica</i> ]	2.32E-41	400	158	143	64.6	74.3	0.69
7-D4	NRPS2	AHX13665	Thioester reductase [ <i>Dyella jiangningensis</i> ]	1.10E-43	418	166	126	62.7	77.8	0
		WP_042439855	Gramicidin synthetase LgrC, partial [ <i>Streptacidiphilus albus</i> ]	2.08E-29	304	122	122	50	66.4	0.82
2-D4	NRPS1	WP_030110998	Hypothetical protein, partial [ <i>Kutzneria albida</i> ]	2.30E-41	415	165	209	47.9	58.1	3.72
8-C8	NRPS2	WP_011473791	Amino acid adenylation [ <i>Rhodospseudomonas palustris</i> ]	6.93E-34	346	138	156	50.3	61.6	5.03
6-B1	NRPS2	AHX13666	Amino acid adenylation protein [ <i>Dyella jiangningensis</i> ]	5.51E-31	323	129	103	72.8	80.6	0
3-H3	NRPS1	WP_010564295	Peptide synthetase [ <i>Pseudomonas extremotralis</i> ]	1.56E-18	218	89	45	91.1	95.6	0
3-G9	NRPS1	CDG17982	Non-ribosomal peptide synthetase [ <i>Xenorhabdus doucetiae</i> ]	9.97E-10	151	63	39	74.4	76.9	0
2-C8	NRPS1	WP_022609339	Non-ribosomal peptide synthetase module, partial [ <i>Rubidibacter lacunae</i> ]	2.29E-08	138	58	29	86.2	89.7	0
7-C3	NRPS1	WP_032631609	Hypothetical protein, partial [ <i>Pseudomonas syringae</i> ]	0.05	88	39	20	90.0	90.0	0



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630 **Figure 1** Phylogenetic tree of identified NRPS gene metagenomic sequences, obtained from the sequenced-based screening of a *Sphagnum* moss fosmid library. Putative NRPS  
 631 sequences were aligned with reference sequences from the protein database (NCBI; accession no. in parenthesis). The tree was generated using CLC Main Workbench 6.9.1  
 632 software using the UPGMA algorithm, Kimura Protein, and a bootstrap of 1000 replicates. Bootstrap values higher than 50% are indicated at branch points. The bar indicates 1.1  
 633 substitutions per amino acid position.

