

Original citation:

Müller, Christina A., Oberauner-Wappis, Lisa, Peyman, Armin, Amos, Gregory C. A., Wellington, E. M. H. (Elizabeth M. H.), 1954-, Berg, Gabriele and Müller, V.. (2015) Mining for nonribosomal peptide synthetase and polyketide synthase genes revealed a high level of diversity in the sphagnum bog metagenome. *Applied and Environmental Microbiology*, 81 (15). pp. 5064-5072.

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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 synthases 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 ≤ 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^R cells. The fosmid library was spread onto LB agar plates containing
153 12.5 µg ml⁻¹ 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⁻¹) 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⁻¹
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 ddH₂O) 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), ddH₂O
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 µg 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 *α-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 *α*-, *β*-, and *γ*-
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* (*γ-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 α -, β -, and γ -*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, β -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

454 **Acknowledgements**

455 We thank Gerhard G. Thallinger (Graz), Christin Zachow (Graz), Henry Müller (Graz),
456 Cornelia Rainer (Graz) and Barbara Fetz (Graz) for helpful discussions and valuable technical
457 support. We also would like to acknowledge Timothy G. Mark for English revision of the
458 manuscript. This work has been supported by a grant to G.B. from the Federal Ministry of
459 Economy, Family and Youth (BMWFJ), the Federal Ministry of Traffic, Innovation and
460 Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol
461 and ZIT - Technology Agency of the City of Vienna through the COMET-Funding Program
462 managed by the Austrian Research Promotion Agency FFG.

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

469 **References**

- 470 1. **Berg G, Zachow C, Müller H, Philipps J, Tilcher R.** 2013. Next-generation bio-
471 products sowing the seeds of success for sustainable agriculture. *Agronomy* **3**:648-
472 656.
- 473 2. **Daniels R, Eddy A.** 1985. Handbook of Europaen *Sphagna*. Institute of Terrestrial
474 Ecology, Natural Environment Research Council, Cambrian News, Aberystwyth, UK.
- 475 3. **Whinam J, Copson G.** 2006. *Sphagnum* moss: an indicator of climate change in the
476 sub-Antarctic. *Polar Rec* **42**:43-49.
- 477 4. **Bragina A, Oberauner-Wappis L, Zachow C, Halwachs B, Thallinger GG, Müller**
478 **H, Berg G.** 2014. The *Sphagnum* microbiome supports bog ecosystem functioning
479 under extreme conditions. *Mol Ecol* **23**:4498-4510.

- 480 5. **Opelt K, Berg G.** 2004. Diversity and antagonistic potential of bacteria associated
481 with Bryophytes from nutrient-poor habitats of the Baltic Sea Coast. *Appl Environ*
482 *Microbiol* **70**:6569–6579.
- 483 6. **Opelt K, Chobot V, Hadacek F, Schönmann S, Eberl L, Berg G.** 2007.
484 Investigations of the structure and function of bacterial communities associated
485 with *Sphagnum* mosses. *Environ Microbiol* **9**:2795–2809.
- 486 7. **Bragina A, Berg C, Cardinale M, Shcherbakov A, Chebotar V, Berg G.** 2012.
487 *Sphagnum* mosses harbour highly specific bacterial diversity during their whole
488 lifecycle. *ISME J* **6**:802–813.
- 489 8. **Opelt K, Berg C, Berg G.** 2007. The bryophyte genus *Sphagnum* is a reservoir for
490 powerful and extraordinary antagonists and potentially facultative human
491 pathogens. *FEMS Microbiol Ecol* **61**:38–53.
- 492 9. **Frahm J-P.** 2004. Recent developments of commercial products from Bryophytes.
493 *The Bryologist* **107**:277–283.
- 494 10. **Asakawa Y, Ludwiczuk A, Nagashima F.** 2013. Phytochemical and biological
495 studies of bryophytes. *Phytochemistry* **91**:52–80.
- 496 11. **Banerjee RD, Sen SP.** 1979. Antibiotic Activity of Bryophytes. *The Bryologist*
497 **82**:141–153.
- 498 12. **Basile A, Giordano S, López-Sáez JA, Cobianchi RC.** 1999. Antibacterial activity of
499 pure flavonoids isolated from mosses. *Phytochemistry* **52**:1479–1482.
- 500 13. **Walsh CT.** 2007. The chemical versatility of natural-product assembly lines. *Acc*
501 *Chem Res* **41**:4–10.
- 502 14. **Wang H, Fewer DP, Holm L, Rouhiainen L, Sivonen K.** 2014. Atlas of
503 nonribosomal peptide and polyketide biosynthetic pathways reveals common
504 occurrence of nonmodular enzymes. *Proc Natl Acad Sci* **111**:9259–9264.

- 505 15. **Strieker M, Tanović A, Marahiel MA.** 2010. Nonribosomal peptide synthetases:
506 structures and dynamics. *Curr Opin Struct Biol* **20**:234–240.
- 507 16. **Staunton J, Weissman KJ.** 2001. Polyketide biosynthesis: a millennium review. *Nat*
508 *Prod Rep* **18**:380–416.
- 509 17. **Walsh CT.** 2002. Combinatorial biosynthesis of antibiotics: challenges and
510 opportunities. *ChemBioChem* **3**:124–134.
- 511 18. **Simon C, Daniel R.** 2011. Metagenomic analyses: past and future trends. *Appl*
512 *Environ Microbiol* **77**:1153–1161.
- 513 19. **Mendes R, Garbeva P, Raaijmakers JM.** 2013. The rhizosphere microbiome:
514 significance of plant beneficial, plant pathogenic, and human pathogenic
515 microorganisms. *FEMS Microbiol Rev* **37**:634–663.
- 516 20. **Parsley LC, Consuegra EJ, Kakirde KS, Land AM, Harper WF, Liles MR.** 2010.
517 Identification of diverse antimicrobial resistance determinants carried on bacterial,
518 plasmid, or viral metagenomes from an activated sludge microbial assemblage.
519 *Appl Environ Microbiol* **76**:3753–3757.
- 520 21. **Hodges TW, Slattery M, Olson JB.** 2012. Unique Actinomycetes from marine caves
521 and coral reef sediments provide novel PKS and NRPS biosynthetic gene clusters.
522 *Mar Biotechnol* **14**:270–280.
- 523 22. **Opelt K, Berg C, Schönmann S, Eberl L, Berg G.** 2007. High specificity but
524 contrasting biodiversity of *Sphagnum*-associated bacterial and plant communities
525 in bog ecosystems independent of the geographical region. *ISME J* **1**:502–516.
- 526 23. **Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J, He G, Chen Y, Pan Q, Liu Y, Tang J, Wu**
527 **G, Zhang H, Shi Y, Liu Y, Yu C, Wang B, Lu Y, Han C, Cheung DW, Yiu S-M, Peng S,**
528 **Xiaoqian Z, Liu G, Liao X, Li Y, Yang H, Wang J, Lam T-W, Wang J.** 2012.

- 529 SOAPdenovo2: an empirically improved memory-efficient short-read *de novo*
530 assembler. *GigaScience* **1**:18.
- 531 24. **Laskaris P.** 2009. Evolution of the streptomycin and viomycin biosynthetic clusters
532 and resistance genes. Ph.D. thesis, University of Warwick.
- 533 25. **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.**
534 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search
535 programs. *Nucleic Acids Res* **25**:3389–3402.
- 536 26. **Bultreys A, Gheysen I, Hoffmann E de.** 2006. Yersiniabactin production by
537 *Pseudomonas syringae* and *Escherichia coli*, and description of a second
538 Yersiniabactin locus evolutionary group. *Appl Environ Microbiol* **72**:3814–3825.
- 539 27. **Amos GCA, Borsetto C, Laskaris P, Krsek M, Berry AE, Newsham KK, Calvo-**
540 **Bado L, Pearce DA, Wellington EMH.** 2015. Diversity among natural product
541 synthetic genes in Antarctic and European soils. *Plos One* **in revision**.
- 542 28. **Vandamme P, Opelt K, Knöchel N, Berg C, Schönmann S, Brandt ED, Eberl L,**
543 **Falsen E, Berg G.** 2007. *Burkholderia bryophila* sp. nov. and *Burkholderia*
544 *megapolitana* sp. nov., moss-associated species with antifungal and plant-growth-
545 promoting properties. *Int J Syst Evol Microbiol* **57**:2228–2235.
- 546 29. **Shcherbakov AV, Bragina AV, Kuzmina EY, Berg C, Muntyan AN, Makarova NM,**
547 **Malfanova NV, Cardinale M, Berg G, Chebotar VK, Tikhonovich IA.** 2013.
548 Endophytic bacteria of *Sphagnum* mosses as promising objects of agricultural.
549 *Microbiology* **82**:306–315.
- 550 30. **Donadio S, Monciardini P, Sosio M.** 2007. Polyketide synthases and nonribosomal
551 peptide synthetases: the emerging view from bacterial genomics. *Nat Prod Rep*
552 **24**:1073.

- 553 31. **Berg G.** 2009. Plant–microbe interactions promoting plant growth and health:
554 perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol*
555 *Biotechnol* **84**:11–18.
- 556 32. **Jin CW, Li GX, Yu XH, Zheng SJ.** 2010. Plant Fe status affects the composition of
557 siderophore-secreting microbes in the rhizosphere. *Ann Bot* **105**:835–841.
- 558 33. **Chen Y, Ntai I, Ju K-S, Unger M, Zamdborg L, Robinson SJ, Doroghazi JR, Labeda**
559 **DP, Metcalf WW, Kelleher NL.** 2011. A proteomic survey of nonribosomal peptide
560 and polyketide biosynthesis in Actinobacteria. *J Proteome Res* **11**:85–94.
- 561 34. **Schröder J.** 1999. The chalcone/stilbene synthase-type family of condensing
562 enzymes, p. 749–771. *In* Meth-Cohn, SDBN (ed.), *Comprehensive Natural Products*
563 *Chemistry*. Pergamon, Oxford.
- 564 35. **Moore BS, Hopke JN.** 2001. Discovery of a new bacterial polyketide biosynthetic
565 pathway. *ChemBioChem* **2**:35–38.
- 566 36. **Pidot SJ, Coyne S, Kloss F, Hertweck C.** 2014. Antibiotics from neglected bacterial
567 sources. *Int J Med Microbiol* **304**:14–22.
- 568 37. **Partida-Martinez LP, Hertweck C.** 2007. A gene cluster encoding rhizoxin
569 biosynthesis in “*Burkholderia rhizoxina*”, the bacterial endosymbiont of the fungus
570 *Rhizopus microsporus*. *ChemBioChem* **8**:41–45.
- 571 38. **Bragina A, Cardinale M, Berg C, Berg G.** 2013. Vertical transmission explains the
572 specific *Burkholderia* pattern in *Sphagnum* mosses at multi-geographic scale. *Front*
573 *Microbiol* **4**.
- 574 39. **Welker M, Von Döhren H.** 2006. Cyanobacterial peptides – Nature’s own
575 combinatorial biosynthesis. *FEMS Microbiol Rev* **30**:530–563.
- 576 40. **Xie Y, Wright S, Shen Y, Du L.** 2012. Bioactive natural products from *Lysobacter*.
577 *Nat Prod Rep* **29**:1277.

- 578 41. **Islam MT, Hashidoko Y, Deora A, Ito T, Tahara S.** 2005. Suppression of damping-
579 off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88
580 is linked to plant colonization and antibiosis against soilborne
581 Peronosporomycetes. *Appl Environ Microbiol* **71**:3786–3796.
- 582 42. **Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A,**
583 **Schäberle TF, Hughes DE, Epstein S, Jones M, Lazarides L, Steadman VA, Cohen**
584 **DR, Felix CR, Fetterman KA, Millett WP, Nitti AG, Zullo AM, Chen C, Lewis K.**
585 2015. A new antibiotic kills pathogens without detectable resistance. *Nature*
586 **517**:455–459.
- 587 43. **Scholz MB, Lo C-C, Chain PS.** 2012. Next generation sequencing and bioinformatic
588 bottlenecks: the current state of metagenomic data analysis. *Curr Opin Biotechnol*
589 **23**:9–15.
- 590 44. **Rokni-Zadeh H, Mangas-Losada A, Mot RD.** 2011. PCR detection of novel non-
591 ribosomal peptide synthetase genes in lipopeptide-producing *Pseudomonas*. *Microb*
592 *Ecol* **62**:941–947.
- 593 45. **Meyer JM, Neely A, Stintzi A, Georges C, Holder IA.** 1996. Pyoverdinin is essential
594 for virulence of *Pseudomonas aeruginosa*. *Infect Immun* **64**:518–523.
- 595 46. **Guenzi E, Galli G, Grgurina I, Gross DC, Grandi G.** 1998. Characterization of the
596 Syringomycin synthetase gene cluster. A link between prokaryotic and eukaryotic
597 peptide synthetases. *J Biol Chem* **273**:32857–32863.
- 598 47. **Hegemann JD, Zimmermann M, Xie X, Marahiel MA.** 2013. Caulosegnins I–III: a
599 highly diverse group of Lasso peptides derived from a single biosynthetic gene
600 cluster. *J Am Chem Soc* **135**:210–222.

- 601 48. **Rondon MR, Ballering KS, Thomas MG.** 2004. Identification and analysis of a
602 siderophore biosynthetic gene cluster from *Agrobacterium tumefaciens* C58.
603 Microbiology **150**:3857–3866.
- 604 49. **Lackner G, Moebius N, Partida-Martinez L, Hertweck C.** 2011. Complete genome
605 sequence of *Burkholderia rhizoxinica*, an endosymbiont of *Rhizopus microsporus*. J
606 Bacteriol **193**:783–784.
- 607 50. **Clarke DJ.** 2008. *Photorhabdus*: a model for the analysis of pathogenicity and
608 mutualism. Cell Microbiol **10**:2159–2167.
- 609 51. **Bender CL, Alarcón-Chaidez F, Gross DC.** 1999. *Pseudomonas syringae*
610 phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide
611 synthetases. Microbiol Mol Biol Rev **63**:266–292.
- 612 52. **Manavalan B, Murugapiran SK, Lee G, Choi S.** 2010. Molecular modeling of the
613 reductase domain to elucidate the reaction mechanism of reduction of peptidyl
614 thioester into its corresponding alcohol in non-ribosomal peptide synthetases. BMC
615 Struct Biol **10**:1.
- 616 53. **Forseth RR, Amaike S, Schwenk D, Affeldt KJ, Hoffmeister D, Schroeder FC,**
617 **Keller NP.** 2013. Homologous NRPS-like gene clusters mediate redundant small-
618 molecule biosynthesis in *Aspergillus flavus*. Angew Chem Int Ed **52**:1590–1594.
- 619 54. **Wallace BA.** 1990. Gramicidin channels and pores. Annu Rev Biophys Biophys
620 Chem **19**:127–157.
- 621

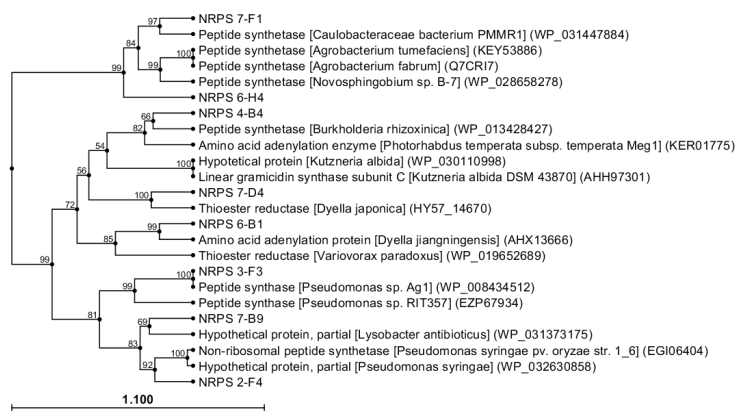
622 **Figures and Tables**

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>	25	46	2
Actinobacteria	<i>Rhodococcus</i>	10	1	0
Actinobacteria	<i>Brachybacterium</i>	0	0	3
α -Proteobacteria	<i>Bradyrhizobium</i>	10	4	3
β -Proteobacteria	<i>Burkholderia</i>	<u>50</u>	5	0
β -Proteobacteria	<i>Rubrivivax</i>	0	6	0
β -Proteobacteria	<i>Bordetella</i>	0	6	0
β -Proteobacteria	<i>Ralstonia</i>	2	0	4
γ -Proteobacteria	<i>Pseudomonas</i>	40	2	<u>7</u>
γ -Proteobacteria	<i>Lysobacter</i>	0	0	6
γ -Proteobacteria	<i>Pectobacterium</i>	12	0	0
γ -Proteobacteria	<i>Xanthomonas</i>	9	0	0
γ -Proteobacteria	<i>Xenorhabdus</i>	6	1	0
δ -Proteobacteria	<i>Myxococcus</i>	27	5	6
δ -Proteobacteria	<i>Candidatus</i>	4	2	2
Firmicutes	<i>Paenibacillus</i>	1	6	1
uncultured bacterium		12	18	1
Remaining strains		66	152	5
Total count		279	346	40

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



629

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.

