

Taxonomic diversity of bacterial populations inhabiting gametophytes of *Sphagnum* mosses from different geographic regions of Russia

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Abstract. In this study we have analyzed the diversity of the endophytic bacterial community associated with *Sphagnum* mosses from North-West Region and Khanty-Mansiysk Autonomous District of Russia during the years 2009–2012. We isolated a more than 400 strains which were identified by means of phenotypic tests and by 16S rRNA sequences. The ribosomal data showed that the isolates belonged to genera *Pseudomonas* (20–57%), *Colimonas* (7–10%), *Flavobacterium* (6–8%), *Burkholderia* (5–6%), *Serratia* (3%). The data reported in this work are consistent with the results of research performed by the Berg group with samples of mosses of the Austrian Alps. It was found that *Sphagnum* mosses are a promising source for the isolation of beneficial microorganisms.

Key words: *Sphagnum* mosses, endophytic bacteria, microbial community, biodiversity.

INTRODUCTION

Colonization of *Sphagnum* hyaline cells by heterotrophic bacteria was first mentioned in the works of Swedish researchers (Granhall & Hofsten, 1976). Their electron microscopic studies revealed, alongside with cyanobacteria, also heterotrophic ones. The possibility that internal tissues of *Sphagnum* plants may be colonized by methanotrophic microorganisms was first discussed in detail by Raghoebarsing and colleagues (Raghoebarsing et al., 2005; Raghoebarsing et al., 2006). These studies dealt with the symbiosis between *Sphagnum* mosses and methanotrophic bacteria ensuring carbon production for the plant construction. Dedysh (Dedysh et al., 1998; Dedysh et al., 2000; Dedysh, 2009) suggested that methanotrophic bacteria were not simply present in the bog water or peat deposits but inhabited *Sphagnum* mosses, including their internal parts. A recently coined term ‘*Sphagnum*-associated methanotrophy’ (Larmola et al., 2010; Kip et al., 2011) is applied to the stable symbiosis between *Sphagnum* mosses and

methanotrophic bacteria colonizing their inner parts. The latter assimilate methane and provide mosses with carbon.

The most complete body of information on the heterotrophic bacteria associated with *Sphagnum* mosses, including the data obtained by molecular-genetic methods, can be found in Opelt and Berg (2004); Opelt et al. (2007b); Vandamme et al. (2007) and Bragina et al. (2012). Detailed molecular-genetic studies of the total DNA extracted from the same moss species (*S. fallax* or *S. magellanicum*) and the analysis of clone libraries showed that non-culturable species of *Alphaproteobacteria* and *Gammaproteobacteria* were dominant in the microbial communities of *S. magellanicum* (Bragina et al., 2012). Microbial community of *S. fallax* was very different, the dominant species belonging to *Verrucomicrobia* and *Planctomyces*. The authors analyse the dependence of the composition of specific microbial communities of *Sphagnum* mosses on abiotic factors such as nutrient availability and water pH. They suggest that microbial community composition may change with the changing environmental conditions.

The aim of this work is study of the taxonomic composition of heterotrophic bacterial populations, associated with *Sphagnum* mosses from two geographically remote regions of Russia (Leningrad region (North-West of Russia) and the Khanty-Mansiysk Autonomous District (Western Siberia) and as well as some of their physiological properties to explain possible role in the functioning of plant-microbial symbiosis.

MATERIALS AND METHODS

Experimental Site and Sample Collection

In this study endophytic bacteria were isolate from *Sphagnum* moss gametophytes of two species: *Sphagnum fallax* (H. Klinggr.) H. Klinggr and *S. magellanicum* Brid. Samples of *Sphagnum* moss collected during expeditions in the two geographically distant regions of Russia: Leningrad Region (Northwest region) and the Khanty-Mansiysk Autonomous District (Western Siberia). In each region were selected 3 geographically distant points located at a distance of several tens of kilometers. One geographical point is not associated with other wetland ecosystems, often having its geographical name. List of sampling points is shown (Fig. 1). Identification of *Sphagnum* species was carried out in the field on the anatomical and morphological characteristics in accordance with the determinant of *Sphagnum* moss (Ignatov et al., 2006).

Isolation of endophytic moss-associated bacteria

Endophytic moss-associated bacteria were isolated using original method of surface sterilization of plant samples. *Sphagnum* gametophytes 10–15 cm in an amount of 4–5 plants were weighed, placed in sterile 500 mL flask and washed three times in sterile water. The plant samples were sterilized for 10 min in 10% hydrogen peroxide, and then washed five times in sterile water. The surface sterilized plant fragments were crushed in sterile conditions, suspended with 10 ml sterile phosphate buffer, serially diluted in sterile water and plated onto R2A medium (Difco, USA). Plates were incubated for 5 days at 20°C, after which CFU were counted to calculate the mean number of colonies (\log_{10} CFU) based on fresh weight. Isolates obtained by plating were purified and stored at -80 °C in sterile broth containing 20% glycerol.

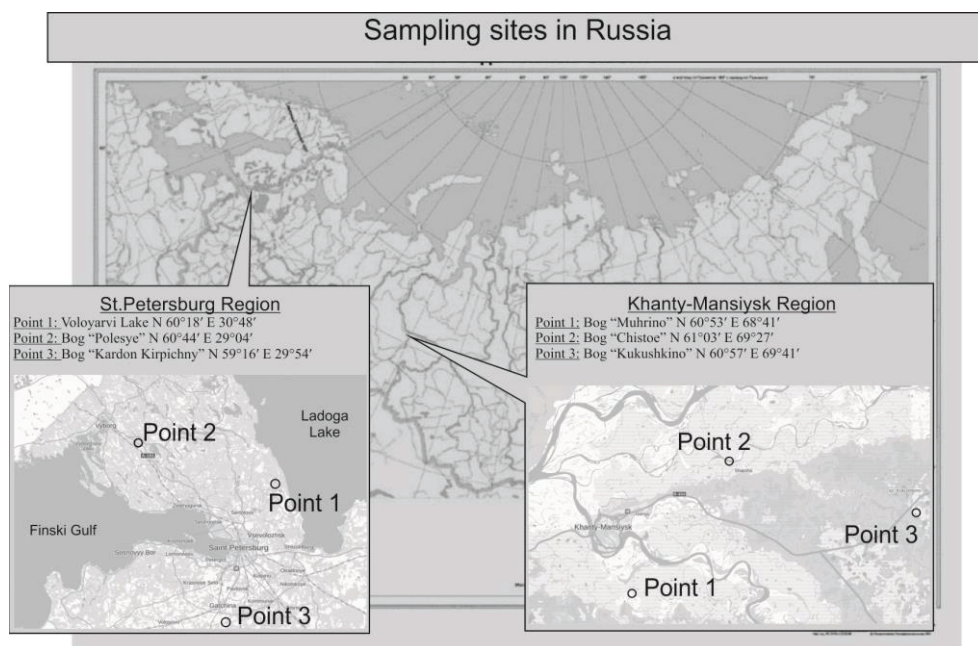


Figure 1. Geographic regions of *Sphagnum* mosses sampling.

RFLP-analysis and identification of bacterial isolates

Bacterial DNA from isolated bacterial strains was extracted using lysis by lysozyme and SDS, protein precipitation by 3M sodium acetate, purification by phenol:chloroform:isoamyl (24:24:1) and DNA precipitation by isopropanol. Briefly, portions of the 16S rRNA genes were obtained via PCR amplification with primers 27 fm (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3') (Weisburget al., 1991). The amplified DNA fragments were subsequently digested with the two nucleases *Msp* I and *Hae* III. The resulting fragments were subsequently separated on a 2% agarose gel and the profiles of the endophytic strains were compared. For nucleotide sequence determination, PCR products were separated on a 1% agarose gel, recovered and purified from agarose using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Sequencing was performed by manufacturer's recommendations for GS Junior (Roshe, The Switzerland). Similarity searches in GenBank were performed using BLAST (<http://www.ncbi.nlm.nih.gov/blast/>; Altschulet al., 1997).

Phylogenetic analysis

Computer-assisted evaluation of bacterial community profiles obtained by RFLP was performed by using the TotalLab program (version TL120; TotalLab Ltd, UK). Cluster analysis was performed with the UPGMA (unweighted pair group method with arithmetic averages) algorithm.

RESULTS AND DISCUSSION

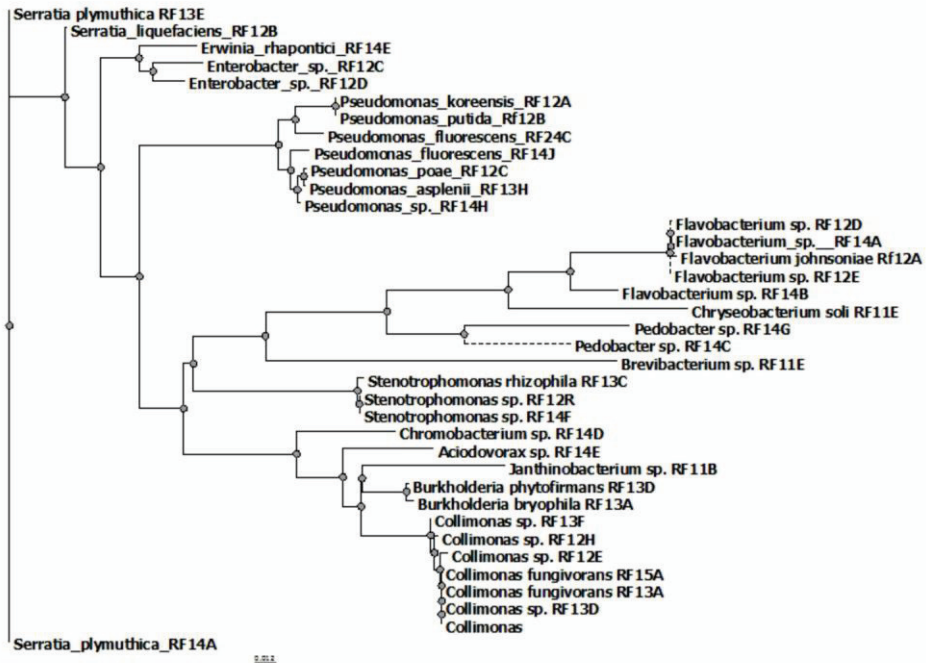
Altogether, more than 400 culturable bacterial strains were isolated from the tissues of *S. fallax* and *S. magellanicum* sampled in the St-Petersburg Region and Western Siberia. Their cultural and morphological properties were characterized. Most isolated bacteria (> 98%) were Gram-negative. They were represented by very small (< 1.5 µm) rounded or oval cells or short rods. On the R2A medium they formed fast- or slowly growing colonies, flattened and creeping, transparent or semi-transparent, brightly coloured (red, violet, pink, yellow, orange) or colourless (beige or milky white). The total abundance of microorganisms isolated on culture media varied in the range 10⁵–10⁶ CFU g⁻¹ of plant tissue. Nitrogen-fixing and oligonitrophilic bacteria grew rather abundantly on the nitrogen-free medium but formed few morphotypes (not more than 5). They formed transparent colourless slimy colonies of middle size, either creeping on the agar surface or rounded and convex.

Molecular genetic identification based on analysis of the 16S rRNA gene fragments had allowed to study the taxonomic diversity of microorganisms isolated from two independent geographic regions (Figs 2, 3). Based on the presented results (more than 150 isolates were sequenced after RFLP-analysis) the major taxonomic branches in the composition of bacterial communities associated with *Sphagnum* mosses had been established. Class *Gammaproteobacteria* order *Enterobacteriales* included *Serratia*, *Enterobacter* and several others. A number of strains had classified as opportunistic forms of animals and plants pathogens (for example *Klebsiella* sp. – the opportunistic forms of animals and humans, *Erwinia rhapontici* – opportunistic bacterial plant pathogen), but some species can act as growth-stimulating and biocontrol agents (genera *Serratia*, *Klebsiella*, *Rahnella*) (Opelt et al., 2007c; Mehnaz et al., 2010; Liu et al., 2014; Neupane et al., 2015) *Serratia plymuthica* described as dominant specie isolated from *S. fallax*, and *S. magellanicum* gametophytes. Another equally dominant taxonomic branch *Burkholderiales* divided into two main families: the family *Oxalobacteraceae*, presented by *Collimonas* spp., and the family *Burkholderiaceae* presented by *Burkholderia* spp. The minor genera included *Janthinobacterium* sp., *Pandorea* sp. et al.

Phylum *Bacteroidetes* was presented by genera *Flavobacterium*, *Pedobacter*, *Chryseobacterium*. A characteristic feature here was the presence of *Flavobacterium* sp. in bacterial populations of *Sphagnum* mosses from different geographical origin, which formed on media characteristic bright–yellow colonies. It is possible that this specie of flavobacteria is specific for the *Sphagnum* mosses bacterial community. The largest taxonomic branch included the family *Pseudomonadaceae*, the number of which species reached 30–40% of all isolated bacteria. Among the characteristic species of *Pseudomonas* should be noted *P. poae*, *P. fluorescens*, *P. asplenii*.

The taxonomic composition of natural populations of heterotrophic bacteria associated with *Sphagnum* moss had the number of general patterns from different groups of bacteria (Figs 2, 3). One of the general observed trends was variation in the number of *Pseudomonas* and *Collimonas* for different types of *Sphagnum* moss. The genera *Pseudomonas* was the most numerous group of endophytic bacteria *S. magellanicum*, while *Pseudomonas* population for *S. fallax* was not so dominance, and the *Collimonas* and *Flavobacterium* here had large number of species (Table 1, Figs 2, 3).

S. fallax



S. magellanicum

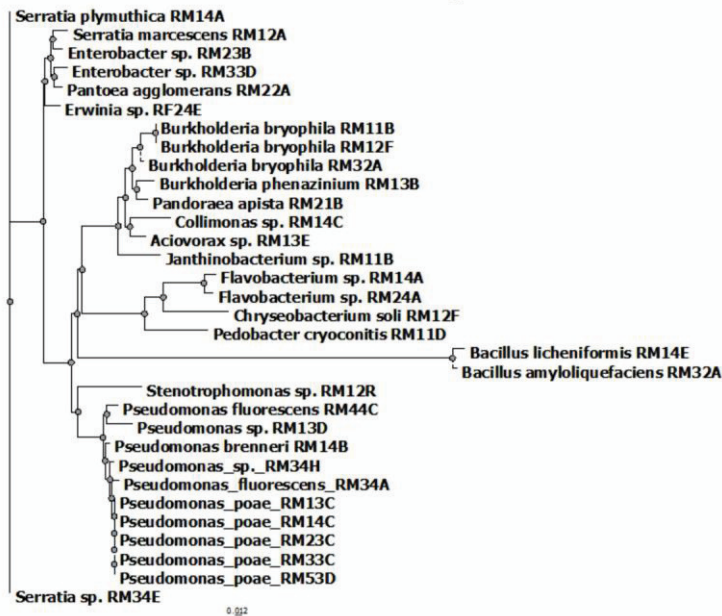


Figure 2. Phylogenetic trees obtained by maximum-likelihood analysis, reflecting the relationships of 16S rRNA gene sequences amplified from RNA isolated from *S. fallax* and *S. magellanicum*, sampled in the Leningrad region (Lake Voloyarvi, Vsevolozhsk District, 2010). Cluster analysis was performed with the UPGMA (unweighted pair group method with arithmetic averages) algorithm.

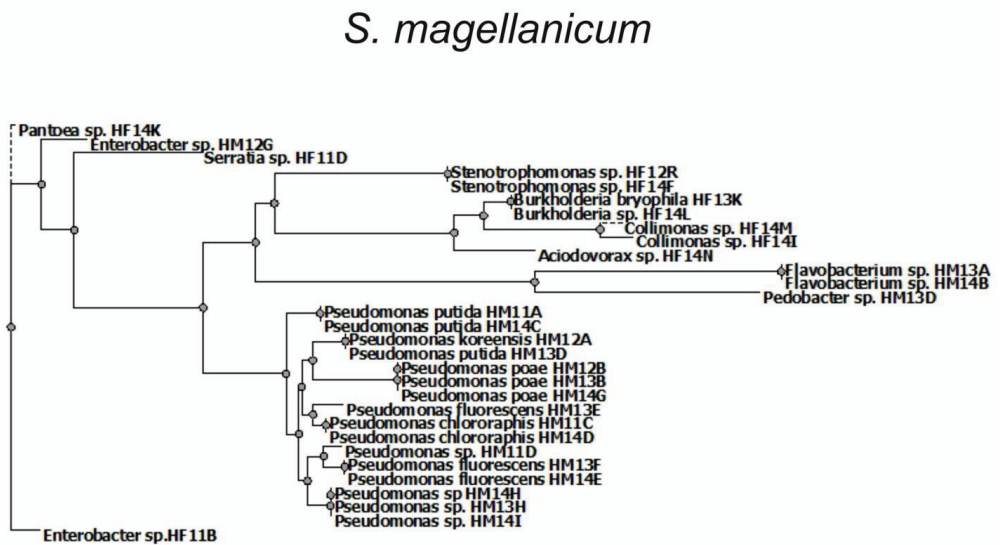
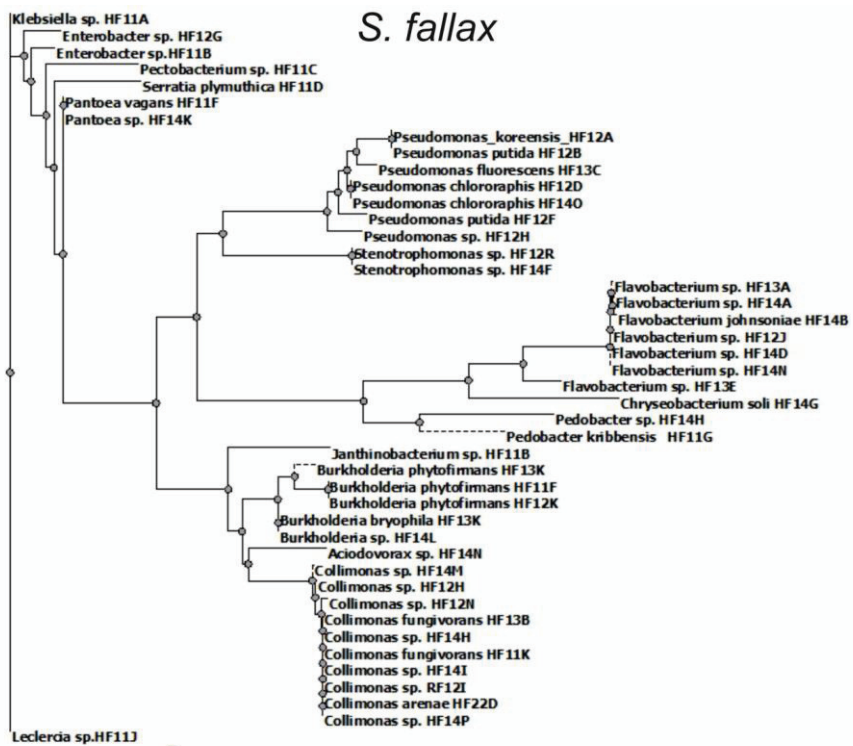


Figure 3. Phylogenetic trees obtained by maximum-likelihood analysis, reflecting the relationships of 16S rRNA gene sequences amplified from RNA isolated from *S. fallax* and *S. magellanicum*, sampled in the Khanty-Mansiysk region (Bog ‘Muchrino’, 2012). Cluster analysis was performed with the UPGMA (unweighted pair group method with arithmetic averages) algorithm.

Table 1. Number and distribution of genera *Sphagnum*-associated bacteria

Genera	Nord-West Region						Khanty-Mansiysk Region					
	Point 1 ^a		Point 2 ^a		Point 3 ^a		Point 1 ^b		Point 2 ^b		Point 3 ^b	
	SM	SF	SM	SF	SM	SF	SM	SF	SM	SF	SM	SF
<i>Bacillus</i>	2	0	1	1	0	3	0	0	2	1	2	0
<i>Delftia</i>	0	0	2	3	1	1	0	0	1	1	1	0
<i>Rhodococcus</i>	0	0	1	0	1	0	0	0	0	1	1	0
<i>Chryseobacterium</i>	1	1	3	1	1	0	0	1	2	0	0	3
<i>Acidovorax</i>	1	1	2	0	0	0	1	1	1	0	0	2
<i>Erwinia</i>	1	1	0	1	1	3	0	1	0	0	1	1
<i>Enterobacter</i>	2	1	0	3	3	5	1	2	1	0	5	3
<i>Pedobacter</i>	1	2	2	2	4	1	1	2	2	3	3	2
<i>Serratia</i>	3	3	1	3	2	5	1	1	3	5	2	1
<i>Burkholderia</i>	4	2	1	4	2	2	2	5	1	2	2	3
<i>Stenotrophomonas</i>	1	3	3	1	1	7	2	2	2	3	2	2
<i>Flavobacterium</i>	2	5	1	3	3	8	2	7	3	3	1	5
<i>Collimonas</i>	1	7	4	5	3	7	0	10	2	5	3	3
<i>Pseudomonas</i>	10	7	6	2	10	5	16	7	5	2	8	3
<i>Other</i>	3	4	5	6	8	7	4	3	4	1	6	7
Σ isolates	32	37	32	44	40	60	30	44	29	27	37	35

^aPoint 1 – Voloyarvi Lake; Point 2 – Bog ‘Polesye’; Point 3 – Bog ‘Kardon Kirpichny’

^bPoint 1 – Bog ‘Muchrino’; Point 2 – Bog ‘Chistoe’; Point 3 – Bog ‘Kukushkino’

A characteristic feature was the low quantitative of gram-positive spore-forming bacteria from genus *Bacillus*: it was isolated and identified 2 species from the genus *Bacillus*: *B. licheniformis* and *B. amyloliquefaciens*. The reasons of this phenomenon may be the waterlogging in the tall marsh ecosystems and habitat conditions *Sphagnum* or evolutionarily later appearance of spore-forming bacteria, when the foundations of the symbiosis between *Sphagnum* and other microorganisms have already been established. Since sporulation was formed in bacteria as an adaptation to adverse environmental conditions, especially a lack of moisture and the associated drying cells, it is logical to assume in the environment where the plant is only transferred to the terrestrial life, and much of it is in the water, plant-microbe symbiosis will occur with microorganisms that do not suffer from drying out and being in the aquatic environment. Such active microorganisms colonized the internal tissues and form a symbiotic relationship with *Sphagnum*, were the gram-negative bacteria.

Discussion

Sphagnum mosses are more dependent than other plants from the symbiosis with microorganisms, since this lack of root system and the ability to absorb nutrients from the soil substrate. The ability of mosses to the rate of photosynthesis, as well as high absorption activity and surface area are compensated these limitations of interstitial *Sphagnum* gametophytes. Products of photosynthesis thus can be used to support a variety of microorganisms providing food to their host, protection against phytopathogens, adaptation to abiotic stress and regulation of development.

Colonization of *Sphagnum* hyaline cells by heterotrophic bacteria was first mentioned in the works of Swedish researchers (Granhall & Hofsten, 1976). Their electron microscopic studies revealed, alongside with cyanobacteria, also heterotrophic ones. The most complete body of information on the heterotrophic bacteria associated with *Sphagnum* mosses, including the data obtained by molecular-genetic methods, can be found in Opelt and Berg (2004), Vandamme et al. (2007), Opelt et al. (2007) and Bragina et al. (2012).

In our studies, we present data on the diversity of microorganisms that were isolated from two species of sphagnum mosses selected in two geographically distant regions of Russia. The data reported in this work are consistent with the results of research performed by the Berg group with samples of mosses of the Austrian Alps. It was found that Sphagnum mosses are a promising source for the isolation of beneficial microorganisms.

According to Opelt et al. (2007a; 2007b), with the use of SSCP method, the composition of microbial community was shown to vary depending on the moss species (two *Sphagnum* species, *Sphagnum magellanicum* and *S. fallax*, were studied), which were collected in Austrian Alps. Samples of the same moss species from Germany and Norway had a similar composition of microbiota. The total of 1,222 strains of heterotrophic bacteria were isolated from surface-sterilized *S. fallax* and *S. magellanicum* samples by inoculation on solid cultural media.

Our data are consistent with the data on the species diversity of bacteria, isolated from Alps Sphagnum Mosses. Molecular-genetic identification of bacteria from Alps *Sphagnum* mosses revealed species of *Burkholderia*, *Serratia*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Rahnella* and *Dyella* (dominant genera) as well as some representatives of *Moraxella*, *Microbacterium* and *Streptomyces*. According to Opelt et al. (2007a; 2007b), more than 26% of the isolated strains had fungicidal activity against phytopathogenic fungi *V. dahliae* and *Rhizoctonia solani*. *S. fallax* had a higher percentage of strains with fungicidal activity than *S. magellanicum*. A special place among the isolated strains of *Sphagnum*-associated microorganisms was occupied by various *Burkholderia* species, bearing nifH genes and apparently possessing nitrogen-fixing activity.

Apparently, plants mosses form within its own hyalocytes certain conditions that are favorable for development of strictly selected genera of bacteria. These bacteria come into close relationship with the host-plant, supporting its metabolic activity. In this community of microorganisms we can identify key species, occurring in mosses of different geographical origin – for example, the characteristic species *Flavobacterium* sp. very common in the natural endophytic communities described in our work. This species is easily distinguishable morphologically in petri dishes from other colonies of microorganisms. Other abundant species identified during our study was *Pseudomonas poae*, found in large quantities (up to 10^4 – 10^5 CFU g⁻¹ of plant tissue) in the microbial communities of *S. magellanicum*, rarely in the composition of endophytic microbiome of *S. fallax*.

During this work, regardless of Berg et al., from the same species of *Sphagnum* mosses we isolated representatives of *Burkholderia*, later identified as *B. bryophila* (Shcherbakov et al., 2013). This species probably also is a characteristic in the composition of endophytic microbiome. In the composition of the microbial community of *S. fallax* one of the characteristic species were *Collimonas* sp., not identified up to species (degree of genetic similarity of the sequences of the 16S rRNA gene with the

closest species was 98%), forming morphologically homogeneous population, which have been actively developed on the surface of nutrient medium.

These isolates are likely to play an important role in the growth and development of plants *Sphagnum*. Requiring more detailed study of the mechanisms of interaction of these strains with the host-plant, their metabolic activity, chemical components produced by bacteria and the host-plant, we plan to continue the studies in the future.

CONCLUSIONS

The studies of researchers (Raghoebarsing et al., 2005; Bragina et. al., 2012) had shown that tissue of *Sphagnum* mosses colonize diverse microbial communities, and that these bacteria can be used for practical purposes (Shcherbakov et al., 2013). In this work it was shown that the taxonomy composition of heterotrophic bacterial communities of *Sphagnum* mosses from different geographical origin include the genera *Pseudomonas*, *Serratia*, *Burkholderia*, *Flavobacterium*, *Collimonas*, *Stenotrophomonas*.

Our results confirm the validity of our strategy of sampling and high diversity and density of bacteria present in the tissues inside of *Sphagnum* mosses. In addition, we found a number of strains that can also represent a new species, and now require further investigation in our laboratory.

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