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EXPERIMENTAL ARTICLES

Evaluation of the Phylogenetic Diversity of Prokaryotic Microorganisms in *Sphagnum* Peat Bogs by Means of Fluorescence In Situ Hybridization (FISH)

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Abstract—The microbial population of sphagnum peat bogs of northern Russia was analyzed with respect to the presence and cell numbers of representatives of particular phylogenetic groups of prokaryotes by means of in situ hybridization with fluorescently labeled group-specific rRNA-targeted oligonucleotide probes with broad detection spectra. The total number of cells that hybridized with universal *Archaea*- and *Bacteria*-specific probes varied, in peat samples of different bogs, from 45 to 83% of the number of cells revealed by DAPI staining. Down the bog profiles, the total number of prokaryotes and the fraction of archaea among them increased. Application of a set of oligonucleotide probes showed that the number of microorganisms belonging to such phylogenetic lineages of the domain *Bacteria* as the phyla *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteria*, and *Planctomycetes* constituted, in total, 14.0–26.5% of the number of eubacteria detected in the samples. Among the bacteria identified in the peat samples, the most abundant were representatives of the classes *Alphaproteobacteria* and *Betaproteobacteria* and the phyla *Acidobacteria, Bacteroidetes*, and *Actinobacteria.*

Key words: *Sphagnum* peat bogs, prokaryotic microorganisms, in situ hybridization, fluorescently labeled rRNA-targeted oligonucleotide probes.

Sphagnum peat bogs are among the dominant terrestrial ecosystems of the boreal and tundra zones of the Northern Hemisphere. They occupy large areas in northern Europe, Canada, and the north of the United States; however, the most extensive peat lands (161 million hectares) are located in Russia. Peat bogs contain about 30% of the global reserves of soil organic carbon [1]. In these acidic, cold ecosystems, the process of organic matter degradation is very slow and limits the recycling of mineral compounds. Therefore, bogs efficiently accumulate biogenic elements, thus protecting the outflowing waters from eutrophication. As a result of these features of the processes occurring in them, northern bogs are the formation center, and one the largest reservoirs of freshwater on Earth. Microorganisms play the major role in the processes of transformation of organic carbon in these ecosystems and in the formation of the chemical composition of bog waters.

The composition of the microbial communities of acidic sphagnum peat bogs is poorly studied. It has been shown that sphagnum peat bogs harbor a huge microbial pool which exceeds that occurring in chernozem soils [2, 3]. However, no more than 0.01% of the total number of microbial cells revealed in peat by luminescence microscopy can be cultivated on nutrient media conventionally used in microbiological practice [3]. Therefore, attempts to analyze peat samples by inoculating nutrient media are doomed to reveal only culturable forms and to thus yield a distorted image of the composition of the microbial community of sphagnum peat bogs. Incomparably greater opportunities to identify the microorganisms of these ecosystems are provided by cultivation-independent molecular techniques. Among these methods is in situ hybridization with rRNA-targeted, fluorescently labeled oligonucleotide probes (fluorescence in situ hybridization, FISH), which combines the possibilities of identification and enumeration of individual phylogenetic groups of microorganisms in samples of different nature [4]. The FISH method has been widely used to study microbial communities in marine and freshwater ecosystems, activated sludge, bioreactors, soils, plant rhizosphere, and many other natural and anthropogenic environments. The application of this method to investigation of sphagnum peat bogs has so far been restricted to the study of microorganisms involved in the methane cycle—methanotrophs and methanogens [5, 6].

The aim of the present study was to apply in situ hybridization with rRNA-targeted, fluorescently labeled oligonucleotide probes with a broad specificity spectrum in order to investigate the phylogenetic

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Table 1. rRNA-targeted oligonucleotide probes used in this study

Probe	Specificity	Target site of 16S rRNA	Probe sequence, 5'–3'	Formamide, %[a]	NaCl, mM[b]	Reference
EUB338	Bacteria	338–355	G CTGCCTCCCGTAGGAGT	20	225	[7]
ARCH915	Archaea	915–934	G TGCTCCCCCGCCAATTCCT	30	112	[8]
ARC344	Archaea	344–363	T CGCGCCTGCTGC I CCCCGT	30	112	[9]
ALF1b	Alphaproteobacteria	19–35	C GTTCGYTCTGAGCCAG[c]	20	225	[10]
BET42a	Betaproteobacteria	1027–1043[d]	G CCTTCCCACTTCGTTT	35	80	[10]
GAM42a	Gammaproteobacteria	1027–1043[d]	G CCTTCCCACATCGTTT	35	80	[10]
SRB385Db	Deltaproteobacteria	385–402	C GGCGTTGCTGCGTCAGG	20	225	[11]
CF319a	Cytophaga-Flavobacterium	319–336	T GGTCCGTGTCTCAGTAC	35	80	[12]
CFB560	Cytophaga-Flavobacterium	560–575	WCCCTTTAAACCCART	30	112	[13]
PLA46	Planctomycetes	46–63	G ACTTGCATGCCTAATCC	30	112	[14]
HGC69a	Actinobacteria	1901–1918[d]	T ATAGTTACCACCGCCGT[e]	25	159	[15]
LGC354A,	Firmicutes	354–371	T GGAAGATTCCCTACTGC ,	35	80	[16]
LGC354B,			C GGAAGATTCCCTACTGC ,			
LGC354C[f]			C CGAAGATTCCCTACTGC			
HoAc1402	Acidobacteria	1402–1419	C TTTCGTGATGTGACGGG[g]	10	450	[17]
IRog2	Acidobacteria (group a)	728–744	G CAGTGGGGAATTGTTC	35	80	[18]

[a] Formamide concentration in the hybridization buffer.
[b] NaCl concentration in the washing buffer.
[c] Y = C or T.
[d] The target molecule is 23S rRNA.
[e] The probe is used in a combination with an unlabeled competitor probe 5'-TATAGTTACGGCCGCCGT-3'.
[f] Equimolar mixture of three labeled oligonucleotides.
[g] The probe is used in a combination with an unlabeled competitor probe 5'-CTTTCGTGACGTGACGGG-3'.

diversity of prokaryotic microorganisms in sphagnum peat bogs.

MATERIALS AND METHODS

The objects of study were peat samples collected along the vertical profiles of four sphagnum peat bogs of the boreal (Moscow, Bryansk, and Yaroslavl oblasts) and tundra (Vorkuta oblast) zones of Russia:

(1) The oligo-mesotrophic ombrotrophic Kurovskoe bog located on the territory of Losinoostrovskoe forestry in Pushkino raion of the Moscow oblast (55°N, 38°E). The vegetation cover consists of *Sphagnum medium, S. parvifolium, Eriophorum vaginatum, Vaccinium myrtillus*, and *Oxicoccus* sp. The thickness of peat is 1.8–4.3 m. The pH of the bog water is 4.7–5.2.

(2) The oligotrophic ombrotrophic bog of the Bryanskii Les national nature preserve (54° N, 32 °E). The vegetation cover consists of *Sphagnum* sp., *E. vaginatum, Oxalis quadripetalus, Betula pendula, V. myrtillus, Calluna* sp., and *Ledum palustre.* The thickness of peat is 1.0–1.5 m. The pH of the bog water is 4.5–4.7.

(3) The oligotrophic ombrotrophic Obukhovskoe bog located on the territory of the Yaroslavl oblast near the village of Obukhovtsevo (57° N, 39° E). The vegetation cover consists of *S. angustifolium, S. fuscum, Carex* spp., *Oxicoccus* sp., and *Vaccinium* sp. The thickness of peat reaches 3 m. The pH of the bog water is 4.2.

(4) A tundra oligotrophic sedge–*Sphagnum* ombrotrophic bog located 20 km south of Vorkuta near the Tal'nik station (67° N, 63° E). The vegetation cover consists of *S. fuscum, Carex stans* and *E. angustifolium.* The thickness of peat reaches 0.5–0.8 m. The pH of the bog water is 4.5–4.8.

Microbial cells were extracted from 2-g peat samples according to the procedure developed for the analysis of sphagnum peat [5]. The extracts were fixed with a 4% formaldehyde solution in phosphate buffer (NaCl, 8.0 g; KCl, 0.2 g; Na_2HPO_4, 1.44 g; NaH_2PO_4, 0.2 g; H_2O, 1 l; pH 7.0) and mixed (1 : 1) with 100% ethanol to be stored at –20°C.

Suspensions of the fixed samples were applied, in an amount of 1–2 μl, to hybridization slides with wells separated by Teflon covering. The specimens obtained were allowed to stand for 12–24 h and then treated with

Table 2. Total cell number of the bacterial population determined in *Sphagnum* peat samples by DAPI staining and the population densities of representatives of the domains *Archaea* and *Bacteria* identified by hybridization with the universal probes ARCH915 + ARC344 and EUB338

Sample*	Number of cells ($N \times 10^8$/g wet peat) revealed by DAPI staining	Number of cells ($N \times 10^7$/g wet peat) revealed by hybridization with domain-specific probes	
		Archaea	Bacteria
Oligotrophic Obukhovskoe bog (Yaroslavl oblast), 10 to 20 cm layer	12.53 ± 1.43	2.28 ± 0.55	58.70 ± 8.10
		1.8%**	46.9%
Oligo-mesotrophic Kurovskoe bog (Moscow oblast), 10 to 20 cm layer	1.30 ± 0.24	0.95 ± 0.18	6.22 ± 0.83
		7.3%	47.9%
Oligotrophic bog of the Bryanskii Les nature preserve (Bryansk oblast), 10 to 20 cm layer	2.08 ± 0.30	0.75 ± 0.18	10.39 ± 1.48
		3.6%	49.9%
Tundra oligotrophic bog near Vorkuta, 13 to 19 cm layer	2.26 ± 0.57	3.44 ± 0.41	10.02 ± 2.76
		15.2%	44.3%

* Samples were taken from the peat layer occurring at the level of the water table.
** Percent of the total number of cells stained with DAPI.

a series of ethanol solutions (50%, 80%, and 100%). For hybridization, we used a set of rRNA-targeted oligonucleotide probes developed earlier for the detection of representatives of the domains *Bacteria* and *Archaea*, as well as for detection of certain phylogenetic groups within *Bacteria* (Table 1). The probes, labeled with Cy3 fluorescent dye, were synthesized by "Syntol" (Moscow, Russia). Hybridization with the fluorescently labeled probes was performed according the technique developed by Stahl and Amann [8] at 46°C. The hybridization conditions used for different probes (formamide concentration in the hybridization buffer and NaCl concentration in the washing buffer) are presented in Table 1. On completion of hybridization, the specimens were additionally stained for 5–7 min with a 1 μM solution of the universal DNA-specific fluorescent dye 4',6'-diamidino-2-phenylindole (DAPI) and then rinsed with distilled water and dried.

The specimens were examined under a Zeis Axioplan 2 (Jena, Germany) epifluorescence microscope using Zeiss 20 and Zeiss 02 light filters to reveal staining with the Cy3-labeled probes and DAPI, respectively. The density of the populations of target microorganisms in the samples was determined by counting cells hybridizing with the probes in 100 fields of view with subsequent calculation of the population density per gram of wet peat. Statistical treatment of the results was performed with the use of Microsoft Excel 2000.

RESULTS

Table 2 presents the results of determination of the total cell number of microorganisms in sphagnum peat by DAPI staining. These analyses were performed for samples taken from a peat layer occurring at the interface of the aerobic and anaerobic parts of the bog profile, i.e., at the level of the water table. The microbial cell numbers varied in the range of 1.3–12.5 × 10^8 cells/g wet peat. These values are close to those determined earlier for raised *Sphagnum* bogs [3, 5]. The number of cells revealed in the same samples by hybridization with 16S rRNA–targeted universal bacterial probe (EUB338) was 6.2–58.7 × 10^7 cells/g wet peat, or 45–49% of the total cell number determined by DAPI staining (Table 2). The cell number of the representatives of the domain *Archaea* revealed with the probes ARCH915 and ARC344 was lower by an order of magnitude and varied in the range of 0.8–3.4 × 10^7 cells/g wet peat. On the whole, the fraction of cells that could be detected with the universal probes specific for representatives of the domains *Bacteria* and *Archaea* made up 49–60% of the total number of cells revealed by DAPI staining (Table 2). The cells that could not be detected with the aforementioned probes may either represent resting microbial forms, which are not revealed by FISH or belong to the domain *Eukaryota* or to yet unknown, phylogenetically separated lineages of eubacteria or archaea, which are not targeted with the currently used "universal" probes. Nevertheless, the fraction of cells that could be identified with the probes used appeared to be rather high in *Sphagnum* peat: the values that we obtained are not inferior to the highest values obtained by other researchers who studied various freshwater habitats [19].

The profile distribution of the total number of cells detectable by DAPI staining and variations of the ratio between eubacteria and archaea in the microbial complex were analyzed for an oligo-mesotrophic bog of the boreal zone (Kurovskoe bog) and a tundra oligotrophic bog as examples (Fig. 1). Downward the bog profiles, an increase in the cell numbers of the representatives of *Bacteria* and *Archaea* was observed. However, the pro-

portion of eubacteria decreased considerably in the lower anaerobic layers, and the proportion of archaea increased. Thus, in the peat of Kurovskoe bog, the proportion of eubacteria decreased from 59% in the 0–10 cm layer to 33% in the 30–40 cm layer, whereas the proportion of archaea increased from 5 to 22% (Fig. 1a). Both for the boreal bog and the tundra bog, the proportion of prokaryotic microorganisms that could not be identified with the probes used increased markedly with depth. In the lower layers of the bogs, their fraction was as large as 45–55%.

For identification of phylogenetic groups of eubacteria and estimation of their population densities in the sphagnum peat bogs, we used a set of 12 group-specific oligonucleotide probes that are most commonly used in modern molecular-ecological studies (Table 1). The presumable detection spectrum of these probes includes representatives of several phylogenetic lineages of the domain *Bacteria*, namely, the phyla *Proteobacteria, Bacteroidetes, Planctomycetes, Actinobacteria, Firmicutes*, and *Acidobacteria.* The results of hybridization of the peat samples taken from different *Sphagnum* bogs with these probes are presented in Table 3.

Representatives of *Proteobacteria* were the most numerous among the organisms identified in peat. The probes applied in the present work targeted four of the five recognized classes of this phylum: *Alpha-, Beta, Gamma-*, and *Deltaproteobacteria.* In total, representatives of these classes comprised up to 13.5% of the total number of eubacteria in the samples. The highest number of cells, up to 1.5×10^7/g peat, was revealed upon hybridization with probes targeting *Alpha-* and *Betaproteobacteria*; the lowest was the cell number of *Deltaproteobacteria*.

Representatives of the phylum *Bacteroidetes* comprised up to 3.5% of the eubacteria revealed in peat. Some organisms of this phylogenetic group, primarily *Cytophaga* representatives, can decompose polymeric organic compounds. The application of two probes—CFB319a and CFB560—was necessitated by the fact that neither of them is universal enough to detect all representatives of this phylogenetic group [13]. Cell enumeration with probe CFB319a, which had been suggested for the detection of organisms of the *Cytophaga–Flavobacterium* group [12], revealed only 0.6–2.9×10^6 cells/g peat, whereas application of the alternative probe CFB560 allowed us to detect about twice as many representatives of this group (Table 3).

Gram-positive bacteria with a high DNA G+C content, which belong to the phylum *Actinobacteria*, were present in all of the samples investigated; their population density reached 1.2×10^7 cells/g peat. In contrast, representatives of *Firmicutes* (gram-positive bacteria with a low DNA G+C content) were a minor component of the prokaryotic complex of sphagnum peat.

Acidobacteria and *Planctomycetes* are widespread but poorly studied phylogenetic groups of eubacteria,

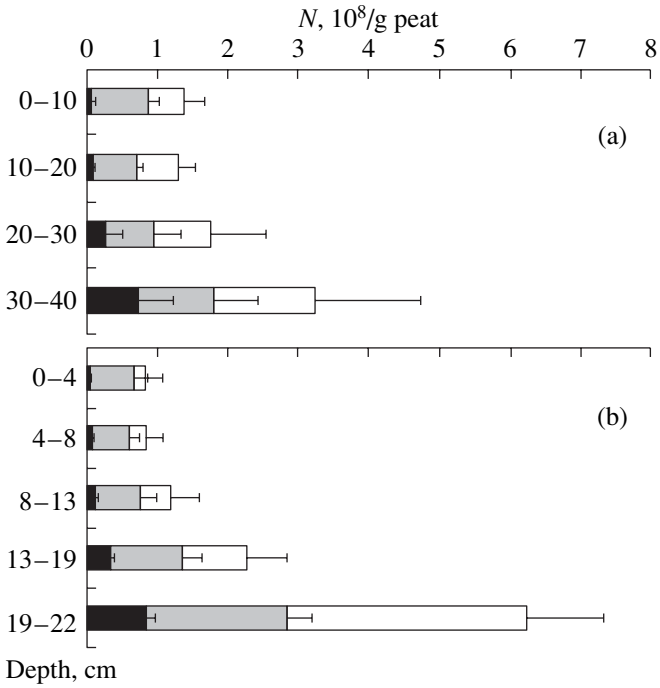

Fig. 1. Cell numbers of eubacteria (gray color), archaea (black color), and microorganisms that could not be identified with oligonucleotide probes (white color) in *Sphagnum* peat samples taken along the profiles of (a) the oligomesotrophic Kurovskoe bog and (b) a tundra oligotrophic bog.

represented by a very few cultured strains [14, 18, 20, 21]. These microorganisms were also detected in the sphagnum peat samples studied, *Acidobacteria* representatives being more numerous. The two probes used in our work are complementary (their detection spectra do not overlap). Consequently, the total cell number of *Acidobacteria* representatives is a total of cells revealed by the probes HoAc1402 and Igor2. Thus, the cell number of acidobacteria in sphagnum peat was up to 2.7×10^7 cells/g wet peat.

DISCUSSION

This study is the first attempt to elucidate the phylogenetic diversity of eubacteria in acidic sphagnum peat bogs by means of FISH. Earlier, this method was only used for the analysis of methanotrophic [5] and methanogenic [6] populations of these ecosystems. Thus, we cannot compare the results of the present study with data of other researchers by virtue of complete lack of similar investigations.

Among the bacteria that we identified in peat, the most numerous were representatives of the classes *Alpha-* and *Betaproteobacteria* and of the phyla *Acidobacteria, Bacteroidetes*, and *Actinobacteria.* It is noteworthy that, according to data from several molecular-ecological investigations, precisely these groups of eubacteria are the most abundantly represented in freshwater ecosystems [20].

Table 3. The numbers of cells revealed in sphagnum peat by hybridization with oligonucleotide probes specific to certain phylogenetic groups within the domain *Bacteria*

Sample	Numbers of cells ($N \times 10^7$/g wet peat), revealed by hybridization with group-specific oligonucleotide probes and their fractions in the total number of eubacteria (%)										
	ALF1b	BET42a	GAM42a	SRB385	CFB319a	CFB560	PLA46	HGC69a	LGC354	HoAc1402	IRog2
Oligotrophic Obukhovskoe bog (Yaroslavl oblast), 10 to 20 cm layer	1.47 ± 0.16 (2.50)	1.15 ± 0.33 (1.96)	0.57 ± 0.19 (0.97)	0.94 ± 0.37 (1.61)	0.23 ± 0.20 (0.39)	0.56 ± 0.20 (0.95)	0.73 ± 0.25 (1.25)	1.20 ± 0.25 (2.04)	0.32 ± 0.23 (0.55)	1.20 ± 0.25 (2.52)	1.48 ± 0.39 (2.04)
Oligo-mesotrophic Kurovskoe bog (Moscow oblast), 10 to 20 cm layer	0.08 ± 0.02 (1.36)	0.06 ± 0.02 (0.94)	0.08 ± 0.02 (1.28)	0.04 ± 0.02 (0.66)	0.06 ± 0.03 (1.03)	0.14 ± 0.06 (2.24)	0.04 ± 0.01 (0.70)	0.02 ± 0.02 (0.33)	0.08 ± 0.06 (1.27)	0.11 ± 0.04 (1.76)	0.10 ± 0.05 (1.54)
Oligotrophic bog of the Bryanskii Les nature preserve (Bryansk oblast), 10 to 20 cm layer	0.35 ± 0.06 (3.36)	0.24 ± 0.06 (2.36)	0.36 ± 0.09 (3.43)	0.08 ± 0.03 (0.72)	0.29 ± 0.06 (2.83)	0.06 ± 0.03 (0.55)	0.14 ± 0.04 (1.30)	0.28 ± 0.08 (2.68)	0.13 ± 0.06 (1.23)	0.19 ± 0.08 (1.83)	0.16 ± 0.06 (1.50)
Tundra oligotrophic bog near Vorkuta, 13 to 19 cm layer	0.47 ± 0.06 (4.66)	0.48 ± 8.56 (4.82)	0.32 ± 0.10 (3.17)	0.08 ± 0.03 (0.76)	0.25 ± 0.08 (2.51)	0.34 ± 0.08 (3.45)	0.10 ± 0.02 (0.98)	0.15 ± 0.06 (1.53)	0.06 ± 0.02 (0.62)	0.15 ± 0.04 (1.53)	0.16 ± 0.06 (1.61)

The total fraction of the representatives of various phylogenetic groups that we detected in peat by hybridization with a set of group-specific probes (Table 1) made up 14.0–26.5% of the number of the eubacterial cells revealed by hybridization with the universal bacterial probe EUB338. This is a rather low value, given that in other habitats, similar sets of probes allow identification of no less than 45–50% and sometimes of up to 90% of eubacteria [17]. The low proportion of organisms that could be identified in sphagnum peat may be due to several reasons. First of all, in the present work we estimated the abundance of representatives of only six out of the fifty-two currently recognized phylogenetic lineages of the domain *Bacteria* [21], and a number of important phylogenetic groups of eubacteria, such as *Verrucomicrobiales*, were left outside the scope of our study. It is also quite possible that the specificity of many of the probes that we applied is not broad enough to cover all the actual phylogenetic diversity of the target groups. The latter supposition is confirmed by the fact that a number of *Bacteroidetes* strains that we isolated from sphagnum peats belonged to new taxa and could not be detected by any of the probes currently used for the detection of this group (Pankratov *et al.*, unpublished data).

The situation with the detection of other bacterial groups may be similar. For example, none of the probes suggested so far for the detection of *Acidobacteria* and *Planctomycetes* [14, 17, 18], including the probes used in the present work, is universal for these phylogenetic groups; therefore, the actual abundance of their representatives in sphagnum peat may be considerably higher than the values determined in the present work. Thus, the main reason for the low efficiency of the set of probes that we used is the unique prokaryotic population of these acidic, cold, oligotrophic ecosystems; a considerable part of this population apparently belongs to poorly studied bacterial groups. More complete identification of the prokaryotes of sphagnum peat bogs requires the design of new probes that would more completely encompass the microbial diversity in these ecosystems.

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