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EXPERIMENTAL ARTICLES

Analysis of the Diversity of Diazotrophic Bacteria in Peat Soil by Cloning of the *nifH* Gene

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Abstract—The diversity of nitrogen-fixing microorganisms in the soil of an oligotrophic *Sphagnum* peat bog was studied by molecular cloning of fragments of the *nifH* gene encoding one of the main components of the nitrogenase complex. The fragments were amplified from the DNA isolated from the peat samples collected at the same site in January (library I) and November (library II), 2005. Analysis of the *nifH* sequence libraries revealed high diversity of diazotrophic bacteria in peat soil: the first library consisted of 237 clones and 55 unique sequence types, the second one included 171 clones and 52 sequence types. Comparison of the two clone libraries showed that the composition and population structure of the nitrogen-fixing community depended greatly on the sampling time; they shared only 11 phylotypes. The sequences of representatives of the class *Alphaproteobacteria* prevailed in both libraries (27% and 57% of clones in libraries I and II, respectively). Representatives of the classes *Deltaproteobacteria* and *Chlorobea* were minor components of library I (6% and 7% of clones, respectively), whereas they prevailed in library II (18% and 24% of clones, respectively). Members of the class *Chloroflexi* were present only in library I, while members of the classes *Bacilli, Clostridia*, and *Methanomicrobia* were present only in library II. Our studies demonstrated that, for complete evaluation of the diversity of natural nitrogen-fixing communities, *nifH* libraries should consist of at least 200–300 clones.

Key words: peat soil, cloning, *nifH* gene.
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The role of soil microbial communities in the biogeochemical cycles of elements, preservation of nutrient resources within the ecosystem, as well as in the maintenance of soil fertility, have aroused considerable scientific interest in these communities [1]. For a better understanding of the functioning of soil as a system, we need to study both the quantitative and qualitative (i.e., species composition) characteristics of the soil microbial community [2]. Since soil microbial communities contain a number of microorganisms incapable of growth on nutrient media, traditional cultural methods do not permit evaluation of their true biodiversity [3]. The application of molecular techniques to assess this biodiversity allowed us to solve many problems related to evaluation of the genotypic diversity [3]; however, a number of issues concerning the efficiency of these methods remain unresolved.

Analysis of the clone libraries of the total 16S rRNA gene amplificate is one of the most frequently used molecular biological techniques for studying natural microbial communities [4]. This approach is presently widely used for analyzing various functional genes, including the nitrogen fixation genes [5, 6]. In spite of its undeniable advantages, this approach presents some problems to researchers investigating natural microbial communities. One of them is the selection of a representative number of clones which is sufficient to assess the biodiversity but does not make the investigation too labor-consuming and expensive [7, 8]. In most of the studies on the diversity of nitrogen-fixing microorganisms, small clone libraries have been used. For instance, the clone libraries used to study the diversity of diazotrophic microorganisms in soil and plant rhizosphere consisted of 50 and 77 clones, respectively [9, 10]. Only recently, a number of studies describing large *nifH* fragment libraries have appeared. For instance, a clone library consisting of 257 clones was used in the analysis of the rhizosphere soil [11]. Whether this amount of clones can adequately represent the diversity of microorganisms in a particular ecosystem is an important issue of molecular ecology. The answer to this problem will result in a more accurate and efficient interpretation of the results obtained by qualitative evaluation of microbial communities.

Northern *Sphagnum* peat bogs are characterized by high acidity (pH 3.5–5.0), low temperatures, and

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extremely low concentrations of mineral nutrients; they are, therefore, extreme habitats [12]. Thus, the microbial diversity in these environments may be assumed to be low, which, undoubtedly, makes these ecosystems unique models for studies of the diversity of a particular microbial community performing a certain geochemical function (e.g., dinitrogen fixation).

The goal of this work was to study the diversity of soil diazotrophic bacteria in the samples from the peat deposit of the acidic ombrotrophic Sosvyatskoe bog by molecular cloning of the *nifH* gene fragments.

MATERIALS AND METHODS

Soil sampling. Peat soil samples were collected in winter (January) and autumn (November), 2005 from the same site of the central part of the Sosvyatskoe oligotrophic bog (Zapadnaya Dvina Field Station of the Institute of Forestry, Russian Academy of Sciences, Tver oblast). The vegetation of the bog consisted of *Sphagnum* sp., *Oxycoccus quadripetalus* and *Carex rostrata*. Soil samples were aseptically collected as 200-cm^3 blocks and stored at 4°C until analysis was performed.

DNA extraction and amplification of the *nifH* gene fragments using universal primers. DNA was extracted from the mixed averaged sample of peat soil taken from a depth of 10–20 cm and exhibiting high nitrogenase activity. One gram of soil was used for each DNA extraction (five replicates). The total DNA from the bacterial communities of these soil samples was isolated according to the previously described technique [13].

Amplification of the *nifH* gene fragments (about 450 bp) was carried out on a Gradient Mastercycler (Eppendorf, Germany) and Tetrad2 (Bio-Rad, United States) devices, using the primers F1 (5'-TAY GGI AAR GGI GGI ATY GGI AAR TC-3') and R6 (5'-GCC-ATC-ATY-TCI-CCI-GA-3'). The temperature–time PCR profile used is described in [14]. The reactions were performed separately for each of the ten individual DNA preparations obtained (two peat soil samples, five replicates).

Analysis of the PCR products was carried out by electrophoresis in 1% agarose gel, which was then stained with ethidium bromide. The results of electrophoresis were documented using a BioDoc Analyze System (Biometra, Germany). PCR fragments were isolated and purified using the Wizard PCR Preps kit (Promega, United States) according to the manufacturer's recommendations.

Amplification of the *nifH* gene fragments using the primers specific to the genus *Oscillochloris*. Amplification was performed on a Gradient Mastercycler (Eppendorf, Germany) using the direct F73 (5'-AATAAGCTGATGGTGGTGG-3') and reverse R412 (5'-CGGAGCAGACGATGTAAATCTC-3') primers. The PCR fragments obtained as a result of amplification of the *nifH* gene fragments with the universal F1–R6 primers were used as a template. The PCR reaction mixture (25 μl) contained the following: 1× buffer (67 mM of Tris–HCl, pH 8.8; $(NH_4)_2SO_4$, 17 mM; $MgCl_2$, 2 mM); 5 nmol of each of the deoxy nucleoside triphosphates; 6.25 pmol of direct and reverse primer each; 1 μl of the PCR fragment, and 1.5 U of Smart*Taq* DNA polymerase (Dialat Ltd., Russia). The temperature–time PCR profile was as follows. The first six cycles: 95°C, 3 min; 53°C, 2 min; and 72°C, 30 s; 25 subsequent cycles: 95°C, 30 s; 53°C, 30 s; and 72°C, 30 s; the final polymerization: 72°C, 7 min. The analysis of PCR products was performed as described above.

Cloning and sequencing. Purified amplificates obtained as a result of five independent (for each peat sample) PCR reactions with primers F1 and R6, were cloned and transformed into competent *E. coli* DH10β cells using the pGEM-T easy vector system (Promega, United States) and GeneJET™ (Fermentas, Lithuania) kits. Sequencing of the clones was carried out using the universal plasmid primers SP6 and 141FpJET and a Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, United States). Sequencing of amplification products was performed on a DNA Analyzer 3730 automatic sequencer (Applied Biosystems Inc., United States). Nucleotide sequences with a similarity level of more than 95% were clustered into one group (sequence type). In order to determine whether the size of the clone library was sufficient for analysis of the diazotrophic community structure, the curves were analyzed describing the dependence of mathematical expectation of the number of sequence types in a random sample from the library size using the Analytic Rarefaction software package (http://www.uga.edu/~strata/software/Software.html).

Phylogenetic analysis. Preliminary analysis of the obtained nucleotide sequences of *nifH* fragments was performed using the NCBI BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST). The on-line Chimera Check program (http://rdp8.cme.msu.edu) was used to detect chimeric sequences. The ORF Finder software package (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used for translation of the sequences. The nucleotide sequences and the deduced amino acid sequences of the studied genes were edited and aligned with the appropriate sequences from the closest relatives using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html).

RESULTS AND DISCUSSION

Analysis of clones from the *nifH* clone library I. The clone library obtained from the peat sample collected in January, 2005 consisted of 257 randomly selected clones containing inserts of the expected size. Analysis of deduced *nifH* sequences showed that approximately 8% of the sequences were chimeric; they were excluded from further analyses. Thus, the first library consisted of 237 clones.

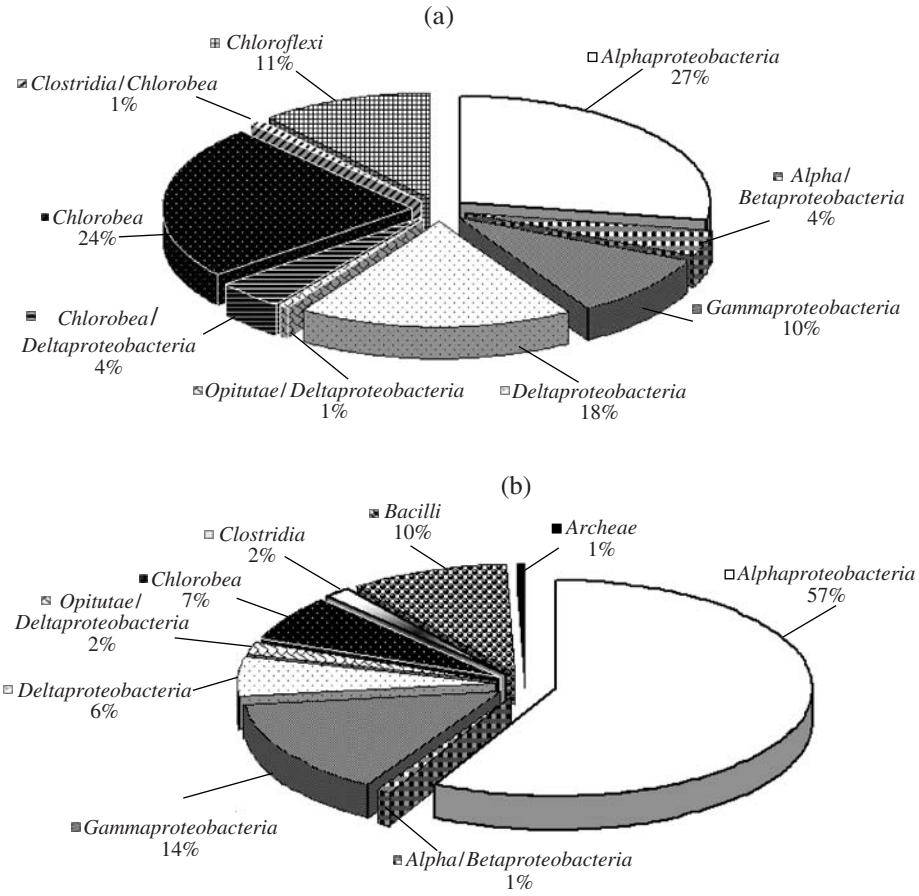

Fig. 1. The percent ratio of the phylogenetic group of diazotrophs based on the number of clones obtained from the samples collected from the peat deposit of the ombrotrophic Sosvyatskoe bog : on the basis of analysis of the clone library I (a); on the basis of analysis of the clone library II (b).

On the basis of similarity (95%) between their sequences, these clones were grouped into 55 different sequence types of diazotrophs isolated from peat soil. The deduced sequences of these sequence types showed an 83–100% similarity with the known *nifH* sequences (table). Phylogenetic analysis of the obtained sequence types revealed their similarity with the sequences from the representatives of various groups of microorganisms, including *Alphaproteobacteria* (18 sequence types), *Alpha/Betaproteobacteria* (2 sequence types), *Gammaproteobacteria* (4 sequence types) and *Deltaproteobacteria* (8 sequence types), *Opitutae/Deltaproteobacteria* (1 sequence type), *Chlorobea/Deltaproteobacteria* (2 sequence types), *Chlorobea* (16 sequence types), *Clostridia/Chlorobea* (1 sequence type), and *Chloroflexi* (3 sequence types).

Figure 1a shows the percent ratio between the phylogenetic groups in the *nifH* clone library I. The diagram shows that representatives of the classes *Alphaproteobacteria* (approximately 27% of all the clones within the library), *Deltaproteobacteria* (18% of the clones), and *Chlorobea* (24% of the clones) prevailed. Other groups, which made up less than 11% of all the clones, may be considered minor components. The predominance of representatives of the *Alphaproteobacteria* in the bacterial community from the *Sphagnum* peat bog was demonstrated by Dedysh et al. [12] by means of FISH and 16S rRNA clone library analysis, as well as by Morris et al. [15] who studied the functionally active methanotrophic population from a peat microcosm.

The results of our statistical evaluation of the data obtained (Fig. 2) suggest that a clone library consisting of 237 clones is not large enough to represent all the *nifH* sequence types in the peat sample under consideration. Hence, even in such extreme habitats as an acidic oligotrophic peat bog the diversity of nitrogen-fixing bacteria is greater than the 55 sequence types obtained by us. For a more detailed description of the structure of this community, analysis of large clone libraries consisting of at least 200–300 clones is required.

Analysis of clones from the *nifH* clone library II. The peat sample collected from the same site of the Sosvyatskoe bog in November, 2005, almost a year after the first sample was collected, was used for an additional analysis of the composition of the nitrogen-

Primary identification of clones with the BLAST analysis

Phylogenetic group	Library I		Library II	
	Sequence type (number of clones)	Similarity with the closest bacterial species (%)	Sequence type (number of clones)	Similarity with the closest bacterial species (%)
Alphaproteobacteria	SB-1 (3)	**99%** *Methylocella palustris*		
	SB-3 (6)	95% *Bradyrhizobium elkanii* 95% *Methylocystis minimus*	SB-56* (2) ~96% SB–3**	95% *Bradyrhizobium japonicum* 95% *Bradyrhizobium elkanii* 95% *Rhizobium* sp.
	SB-4 (5)	95% *Methylocystis minimus*	SB-57 (3) ~99% SB-56	95% *Bradyrhizobium japonicum* 95% *Bradyrhizobium elkanii* 95% *Rhizobium* sp.
	SB-5 (6)	96% *Rhizobium* sp. 96% *Azorhizobium caulinodans* 96% *Methylocystis minimus*		
	SB-6 (2)	96% *Azorhizobium caulinodans* 95% *Xanthobacter autotrophicus*	**SB-6** (33)	96% *Azorhizobium caulinodans* 95% *Xanthobacter autotrophicus*
			SB-58 (5) =100% SB-6	96% *Azorhizobium caulinodans* 95% *Xanthobacter autotrophicus*
			SB-59 (3) ~97% SB-6	95% *Azorhizobium caulinodans* 95% *Bradyrhizobium elkanii*
	SB-7 (6)	**99%** *Bradyrhizobium elkanii* 97% *Bradyrhizobium japonicum*	SB-60 (1) ~97% SB-6	96% *Azorhizobium caulinodans* 95% *Bradyrhizobium* sp.
	SB-8 (4)	95% *Azospirillum brasilense* 95% *Azospirillum lipoferum* 94% *Sinorhizobium fredii*	SB-61 (5) ~97% SB-8	95% *Azospirillum brasilense* 95% *Gluconacetobacter diazotrophicus* 95% *Rhizobium* sp.
			SB-62 (1) ~96% SB-8	94% *Azospirillum brasilense* 94% *Azospirillum lipoferum* 94% *Gluconacetobacter diazotrophicus*
			SB-63 (3) ~96% SB-8	95% *Zymomonas mobilis* 95% *Gluconacetobacter diazotrophicus*
	SB-9 (4)	92% *Bradyrhizobium japonicum* 92% *Rhizobium* sp. 92% *Sinorhizobium fredii*	SB-64 (1)	98% *Methylocystis parvus* 95% *Bradyrhizobium japonicum*
	SB-10 (4)	94% *Xanthobacter autotrophicus* 93% *Azorhizobium caulinodans*	SB-65 (5)	97% *Methylocystis parvus* 96% *Bradyrhizobium* sp.
			SB-66 (1)	97% *Methylocystis parvus* 96% *Bradyrhizobium elkanii*
	SB-11 (5)	96% *Methylocystis minimus* 95% *Methylocystis echinoids* 95% *Azorhizobium caulinodans*	SB-68 (5) ~97% SB-11	98% *Azorhizobium caulinodans* 98% *Bradyrhizobium* sp. 97% *Methylocystis minimus*
			SB-69 (1) ~97% SB-11	97% *Bradyrhizobium* sp. 96% *Azorhizobium caulinodans*
			SB-70 (2) ~96% SB-11	98% *Bradyrhizobium* sp. 97% *Azorhizobium caulinodans*
			SB-71 (1) ~95% SB-11	97% *Azorhizobium caulinodans* 96% *Methylocystis minimus*
			SB-72 (1) ~95% SB-11	95% *Azorhizobium caulinodans* 94% *Methylocystis minimus*
	SB-12 (5)	95% *Methylocystis echinoids* 94% *Methylocystis minimus* 94% *Azorhizobium caulinodans*		
	SB-13 (4)	96% *Bradyrhizobium* sp. 96% *Methylocystis echinoids*	SB-73 (1) ~97% SB-13	95% *Bradyrhizobium* sp. 95% *Methylocystis echinoids*
	SB-14 (2)	96% *Azorhizobium caulinodans* 96% *Bradyrhizobium* sp.	SB-74 (2)	95% *Azospirillum brasilense* 95% *Rhizobium etli*
	SB-15 (2)	93% *Azorhizobium caulinodans* 93% *Methylocystis minimus*	SB-75 (1)	98% *Methylocapsa acidiphila* 98% *Beijerinckia indica*
	SB-41 (3)	96% *Bradyrhizobium elkanii* 95% *Sinorhizobium fredii*	SB-76 (2)	98% *Methylocapsa acidiphila* 98% *Beijerinckia indica*

Table. (Contd.)

Phylogenetic group	Library I		Library II	
	Sequence type (number of clones)	Similarity with the closest bacterial species (%)	Sequence type (number of clones)	Similarity with the closest bacterial species (%)
	SB-42 (1)	95% *Azospirillum brasilense* 95% *Azospirillum lipoferum* 94% *Sinorhizobium fredii*	**SB-42** (14)	95% *Azospirillum brasilense* 95% *Azospirillum lipoferum* 94% *Sinorhizobium fredii*
	SB-43 (2)	91% *Bradyrhizobium japonicum* 91% *Rhizobium* sp. 91% *Sinorhizobium fredii*		
	SB-44 (2)	**100%** *Methylocystis echinoids* 99% *Methylocystis minimus*	SB-67 (6) ~98% SB-44	97% *Methylocystis minimus* 97% *Methylocystis echinoids*
Alpha/Beta-proteobacteria	SB-2 (4)	94% *Azospirillum brasilense* 93% *Burkholderia vietnamiensis*	SB-77 (1)	98% *Polaromonas naphtalenivorans* 98% *Burkholderia vietnamiensis*
	SB-16 (5)	92% *Polaromonas naphtalenivorans* 92% *Burkholderia vietnamiensis* 92% *Sinorhizobium meliloti*		
Gammaproteobacteria	**SB-17** (4)	**100%** *Methylobacter bovis* 99% *Methylobacter luteus*	**SB-17** (2)	**100%** *Methylobacter bovis* 99% *Methylobacter luteus*
	SB-18 (15)	89% *Halorhodospira halophila* 87% *Azotobacter vinelandii*	**SB-18** (5)	89% *Halorhodospira halophila* 87% *Azotobacter vinelandii*
			SB-78 (6) ~99% SB-18	88% *Halorhodospira halophila* 85% *Halorhodospira halochloris*
			SB-79 (1) ~97% SB-18	89% *Halorhodospira halophila* 87% *Methylomonas methanica*
			SB-80 (2) ~94% SB-18	87% *Halorhodospira halophila* 87% *Methylomonas methanica*
	SB-19 (2)	85% *Methylobacter bovis* 85% *Methylomonas methanica*	SB-81 (1)	87% *Azotobacter vinelandii* 87% *Thiorhodospira sibirica*
	SB-20 (2)	85% *Methylomonas methanica* 84% *Halorhodospira halophila*	SB-82 (2)	88% *Halorhodospira halophila* 87% *Azotobacter vinelandii*
			SB-83 (1)	89% *Rhodopseudomonas palustris*
			SB-84 (1)	91% *Rhodospirillum rubrum* 88% *Azotobacter vinelandii*
			SB-85 (4)	93% *Rhodospirillum rubrum* 91% *Azotobacter vinelandii*
Deltaproteobacteria	SB-21 (8)	95% *Pelobacter propionicus* 94% *Geobacter uraniumreducens*		
	SB-22 (2)	88% *Desulfovibrio gigas* 86% *Syntrophobacter fumaroxidans*	SB-88 (1)	93% *Syntrophobacter fumaroxidans* 87% *Desulfatibacillum alkenivorans*
	SB-23 (6)	88% *Desulfovibrio gigas* 87% *Syntrophobacter fumaroxidans*	**SB-23** (1)	88% *Desulfovibrio gigas* 87% *Syntrophobacter fumaroxidans*
	SB-24 (11)	89% *Desulfovibrio gigas* 85% *Syntrophobacter fumaroxidans*		
	SB-25 (7)	87% *Desulfovibrio vulgaris* 85% *Syntrophobacter fumaroxidans*	**SB-25** (3)	87% *Desulfovibrio vulgaris* 85% *Syntrophobacter fumaroxidans*
	SB-26 (2)	88% *Desulfovibrio vulgaris* 86% *Desulfovibrio gigas*	**SB-26** (4)	88% *Desulfovibrio vulgaris* 86% *Desulfovibrio gigas*
	SB-27 (2)	86% *Syntrophobacter fumaroxidans* 86% *Desulfovibrio vulgaris*		
	SB-28 (4)	86% *Desulfovibrio vulgaris* 85% *Desulfovibrio gigas* 85% *Syntrophobacter fumaroxidans*	**SB-28** (1)	86% *Desulfovibrio vulgaris* 85% *Desulfovibrio gigas* 85% *Syntrophobacter fumaroxidans*
Opitutae/Deltaproteobacteria	SB-45 (2)	89% *Opitutaceae bacterium* 87% *Desulfatibacillum alkenivorans*	SB-86 (2) ~99% SB-26	93% *Opitutaceae bacterium* 90% *Desulfovibrio vulgaris*
			SB-87 (1) ~98% SB-26	91% *Opitutaceae bacterium* 89% *Desulfovibrio vulgaris*

Table. (Contd.)

Phylogenetic group	Library I		Library II	
	Sequence type (number of clones)	Similarity with the closest bacterial species (%)	Sequence type (number of clones)	Similarity with the closest bacterial species (%)
Chlorobea/Deltaproteobacteria	SB-29 (3)	86% *Syntrophobacter fumaroxidans* 85% *Chlorobium phaeobacteroides* 85% *Prosthecochloris vibrioformis*		
	SB-33 (7)	87% *Desulfovibrio gigas* 87% *Pelodictyon luteolum* 87% *Prosthecochloris vibrioformis*		
Chlorobea	SB-30 (2)	88% *Chlorobium phaeobacteroides* 88% *Prosthecochloris vibrioformis*		
	SB-31 (2)	84% *Prosthecochloris vibrioformis* 83% *Pelodictyon luteolum*		
	SB-32 (4)	87% *Prosthecochloris vibrioformis* 87% *Pelodictyon luteolum* 87% *Chlorobium limicola*		
	SB-34 (10)	87% *Chlorobium phaeobacteroides* 85% *Pelodictyon luteolum*		
	SB-35 (2)	87% *Prosthecochloris vibrioformis* 86% *Pelodictyon luteolum*		
	SB-36 (15)	87% *Prosthecochloris vibrioformis* 86% *Pelodictyon luteolum*	**SB-36** (1)	87% *Prosthecochloris vibrioformis* 86% *Pelodictyon luteolum*
	SB-38 (6)	88% *Prosthecochloris vibrioformis* 88% *Pelodictyon luteolum* 88% *Chlorobium limicola*	SB-89 (4) ~99% SB-38	87% *Prosthecochloris vibrioformis* 87% *Pelodictyon luteolum* 87% *Chlorobium limicola*
	SB-46 (1)	87% *Pelodictyon luteolum* 87% *Chlorobium limicola* 87% *Prosthecochloris vibrioformis*		
	SB-47 (1)	87% *Prosthecochloris vibrioformis* 86% *Pelodictyon luteolum* 86% *Chlorobium limicola*		
	SB-48 (2)	86% *Prosthecochloris vibrioformis* 85% *Pelodictyon luteolum* 85% *Chlorobium limicola*		
	SB-49 (1)	85% *Prosthecochloris vibrioformis* 85% *Pelodictyon luteolum* 85% *Chlorobium limicola*	**SB-49** (2)	85% *Prosthecochloris vibrioformis* 85% *Pelodictyon luteolum* 85% *Chlorobium limicola*
	SB-50 (1)	86% *Pelodictyon luteolum* 86% *Chlorobium limicola* 86% *Chlorobium phaeobacteroides*		
	SB-51 (1)	86% *Prosthecochloris vibrioformis* 85% *Chlorobaculum macestae* 84% *Pelodictyon luteolum*	**SB-51** (1)	86% *Prosthecochloris vibrioformis* 85% *Chlorobaculum macestae* 84% *Pelodictyon luteolum*
	SB-52 (6)	86% *Prosthecochloris vibrioformis* 84% *Chlorobaculum macestae* 84% *Pelodictyon luteolum*	SB-90 (1) ~97% SB-51	85% *Prosthecochloris vibrioformis* 84% *Chlorobium limicola* 84% *Pelodictyon luteolum*
	SB-53 (1)	87% *Pelodictyon luteolum* 87% *Chlorobium limicola* 87% *Prosthecochloris vibrioformis*	SB-91 (3) ~90% SB-51	80% *Prosthecochloris vibrioformis* 80% *Chlorobium limicola* 80% *Pelodictyon luteolum*
	SB-54 (2)	88% *Pelodictyon luteolum* 88% *Chlorobium limicola* 88% *Prosthecochloris vibrioformis*		
Clostridia/Chlorobea	SB-37 (2)	85% *Desulfotomaculum reducens* 84% *Prosthecochloris vibrioformis* 84% *Pelodictyon luteolum*		

Table. (Contd.)

Phylogenetic group	Library I		Library II	
	Sequence type (number of clones)	Similarity with the closest bacterial species (%)	Sequence type (number of clones)	Similarity with the closest bacterial species (%)
Clostridia			SB-92 (1)	87% *Clostridium kluyveri* 85% *Clostridium acetobutylicum*
			SB-93 (2) ~95% SB-92	87% *Desulfotomaculum reducens* 86% *Clostridium kluyveri*
Bacilli			SB-94 (16)	89% *Bacillus arseniciselenatis* 87% *Paenibacillus durus* 86% *Bacillus alkalidiazotrophicus*
			SB-95 (1) ~82% SB-94	87% *Bacillus alkalidiazotrophicus* 85% *Bacillus arseniciselenatis* 83% *Paenibacillus durus*
Chloroflexi	SB-39 (16)	**97%** *Oscillochloris trichoides* DG-6		
	SB-40 (8)	**95%** *Oscillochloris trichoides* DG-6		
	SB-55 (2)	93% *Oscillochloris trichoides* DG-6		
Archaea			SB-96 (1)	93% *Methanosarcina acetivorans* 92% *Methanosarcina barkeri*

Notes: * The sequence types in bold were present in both clone libraries.

** Similarity between the amino acid sequence of this sequence type and the sequence of the most closely related sequence type.

fixing community. The construction of library II was performed in two stages: at first, the library consisted of 115 clones and then it was extended to 214 clones. Analysis of the samples revealed that chimeric sequences constituted 16 and 24% in the first and second sample, respectively; they were excluded from further analyses. Thus, a total of 96 clones, plus another 75 clones afterwards, were selected for further investigation; that is, the library consisted of a total of 171 clones.

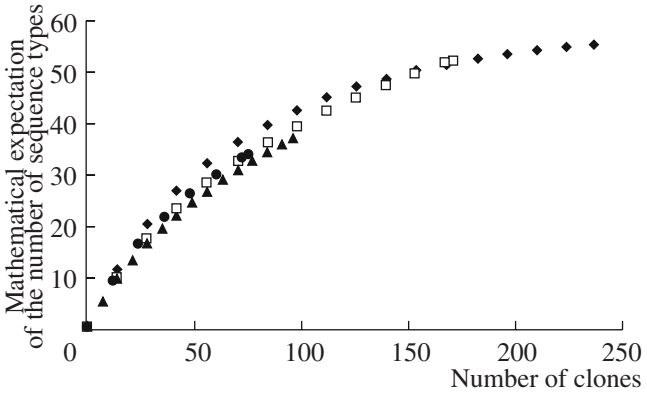

Fig. 2. Curves describing the dependence of the number of sequence types of diazotrophs isolated from the Sosvyatskoe bog on the size of the clone sample. The *nifH* fragment library consisted of 237 (◆), 171 (□), 96 (▲), and 75 (●) clones.

The first 96 clones were grouped into 37 sequence types. The phylogenetic analysis of the obtained sequence types revealed their similarity with the sequences from the representatives of various groups of microorganisms, including the *Alphaproteobacteria* (17 sequence types), *Gammaproteobacteria* (8 sequence types), *Deltaproteobacteria* (4 sequence types), *Opitutae/Deltaproteobacteria* (1 sequence type), *Chlorobea* (4 sequence types), *Clostridia* (1 sequence type), and *Bacilli* (2 sequence types).

The subsequent extension of the clone library allowed us to add 34 sequence types to it. Only 19 of these sequence types coincided with the sequences obtained as a result of the analysis of the first 96 clones from library II. The new 15 sequence types were classified into various phylogenetic groups, including the *Alphaproteobacteria* (6 sequence types), *Alpha/Betaproteobacteria* (1 sequence type), *Gammaproteobacteria* (2 sequence types), *Deltaproteobacteria* (1 sequence type), *Opitutae/Deltaproteobacetria* (1 sequence type), *Chlorobea* (2 sequence types), *Clostridia* (1 sequence type), and *Methanomicrobia* (1 sequence type).

Figure 1b shows the percent ratio between the phylogenetic groups in the *nifH* clone library II (171 clones). The diagram shows that representatives of the class *Alphaproteobacteria* (approximately 57% of all the clones within the library) prevailed.

Noteworthy is the fact that an additional phylogenetic group (*Archaea*, 1 clone), which we also failed to detect in the clone library of 96 clones, was revealed

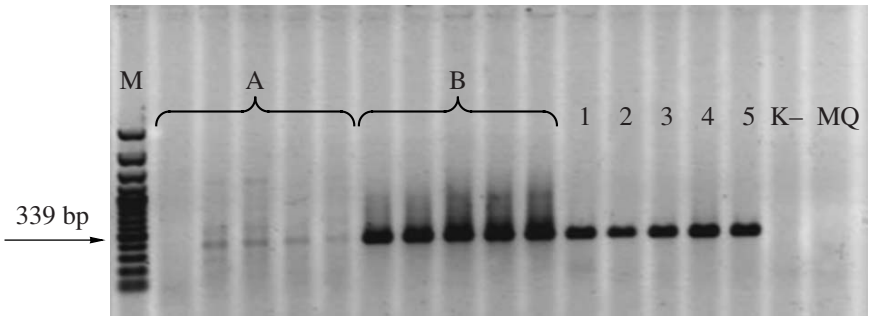

Fig. 3. Amplification of the *nifH* gene fragments using the primers specific to the genus *Oscillochloris*: total DNA extracted from peat (5 replicates) was used as a template (A); PCR fragments of the *nifH* gene obtained with the universal primers F1–R6 was used as a template (B). The arrow points to the target fragment. M, DNA molecular mass marker; K, *Bacillus licheniformis* (negative control); MQ, control in the absence of the DNA template. The numerals show the DNA of various *Oscillochloris* sp. strains (positive control).

after extension of the clone library up to 171 clones. The results obtained confirmed our suggestion that minor phylogenetic groups cannot be detected in the samples consisting of less than 100 clones. It should be noted that this conclusion is true for the studied community and the *nifH* gene. The situation may change with the change of the investigated subject. For instance, the study of the structure of the bacterial community inhabiting the rhizosphere of legumes using the 16S rRNA clone library demonstrated that the library consisting of 90 clones was large enough to estimate the bacterial diversity [8]. The results of our statistical evaluation of the clone libraries demonstrated that, in the case of the nitrogen-fixing community from peat soil, a clone library consisting of 96 or even the one supplemented with 75 more clones is not large enough to represent the diversity of diazotrophic microorganisms (Fig. 2). Figure 2 shows that the master curves $\bar{S}_m$, constructed for the clone libraries consisting of 96 and 75 clones, as well as for the joint library of 171 clones, were parts of the same curve, which may point to the statistical significance of the estimation obtained by the method used.

Hence, the obtained results demonstrated that, as in the case of library I, the diversity of nitrogen-fixing bacteria in the acidic oligotrophic peat bog was higher than the one determined in our experiment; therefore, a library consisting of 171 clones is not large enough.

Comparative analysis of libraries I and II. Comparative analysis of the two libraries revealed that they share 11 common sequence types. In libraries I and II, the proportion of clones of the common sequence types was 24.5 and 39.2% (of the total number of clones in each library), respectively. These results demonstrate that the composition of microbial communities is subject to significant seasonal variations. To gain a better understanding of the composition of the nitrogen-fixing community from peat soil, further investigation is required of peat samples collected both at various sites of the peat bog and in different seasons.

Comparative analysis of libraries I and II demonstrated that the representatives of the class *Alphaproteobacteria* prevailed in both libraries (27 and 57% of the clones in libraries I and II, respectively). According to the results of the analysis of library II, the representatives of the classes *Deltaproteobacteria* and *Chlorobea* were minor components (6 and 7% of clones, respectively); however, they prevailed in library I (18 and 24% of clones, respectively).

Analysis of library I revealed no representatives of the classes *Bacilli, Clostridia*, and *Methanomicrobia*; in library II, they were represented by 10, 2, and 1% of the clones, respectively.

Interestingly, no representatives of the class *Chloroflexi* were detected in the peat sample collected in November (library II); however, they were detected in the peat sample collected in January (library I) and comprised 11% of all the clones. However, with the primers specific to the *nifH* gene fragments of the representatives of the genus *Oscillochloris*, such sequences were detected in the peat sample collected in November (Fig. 3). This is another confirmation of the conclusion that a clone library consisting of more than 100 clones is required for detection of minor components of the community. The representatives of the *Chloroflexi* have been detected in the peat sample by Dedysh et al. [12] by 16S rRNA clone library analysis.

Analysis of two *nifH* clone libraries consisting of 408 clones allowed us to reveal 96 sequence types of diazotrophs in peat soil, which demonstrates the high diversity of dinitrogen-fixing microorganisms. The construction of clone libraries consisting of several hundreds of clones is required for efficient application of the cloning of functional genes in studies of the structure of the soil microbial communities.

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