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EXPERIMENTAL ARTICLES

Prokaryotic Ultramicroforms in a *Sphagnum* Peat Bog of Upper Volga Catchment

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Abstract—The waters of small lakes located in swampy catchment areas of upper Volga contain considerable amounts of ultrasmall microbial cells that pass through 0.22-μm-pore-size filters. As shown in our previous study [1], most of these cells represent the bacterial genera *Herbaspirillum, Herminiimonas, Curvibacter*, and *Burkholderia* of the class *Betaproteobacteria*, as well as euryarchaea of the uncharacterized clade LDS. The aim of the present study was to investigate the possible effect of the waters draining swampy areas on the composition of the filterable microbial fraction in lakes fed by swampy catchments. To address this question molecular identification was performed of prokaryotic ultramicroforms in the peat of the ombrotrophic *Sphagnum* bog Obukhovskoe, located, like the lakes studied previously [1], in the Mologa–Sheksna catchment area. The number of filterable microorganisms in 1 g wet peat was 3.8×10^6 cells, or 0.5% of total microbial cell number in the peat. From the DNA of the filterable cell fraction, 100 clones of bacterial and 77 clones of archaeal 16S rRNA genes were obtained. The bacterial clone library contained 16S rRNA gene sequences representing the classes *Beta-* and *Gammaproteobacteria* (the genera *Janthinobacterium* and *Pseudomonas,* respectively) and the phylum *Bacteroidetes* (the genera *Chryseobacterium* and *Epilithonimonas*) and differed significantly from the clone library of bacterial ultramicroforms of lake water. By contrast, the pools of filterable archaea in bogs and lakes were essentially similar. They were represented by the euryarchaeal clade LDS and methanogens of the orders *Methanobacteriales* and *Methanosarcinales*. Additionally, the pool of filterable archaea of the bog included methanogens of the order *Methanomicrobiales* and representatives of the uncharacterized euryarchaeal clade RC-V (Rice Cluster V) and of the phylum *Thaumarchaeota*.

Keywords: filterable microbial forms, *Sphagnum* peat bog, 16S rRNA gene clone libraries

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Numerous lakes and rivers of the boreal zone of Russia and other countries of the northern hemisphere are fed by humic acidic waters formed in swampy catchment areas [2]. In our previous study, we found that one milliliter of water of small acidic lakes located in the swampy Mologa–Sheksna catchment area of upper Volga contains up to 10^4 ultrasmall microbial cells that pass through "bacterial" filters with a pore size of 0.22 μm [1]. Molecular identification of these cells showed that there were both bacteria and archaea among them. The filterable bacteria of the lake water primarily represented the genera *Herbaspirillum, Herminiimonas, Curvibacter*, and *Burkholderia* within the class *Betaproteobacteria*. A minor bacterial fraction was composed of representatives of the *Alpha-* and *Gammaproteobacteria*, as well as *Actinobacteria*. In the pool of filterable archaea, only *Euryarchaeota* representatives were revealed, most of them belonging to the new yet undescribed clade of archaea, conventionally designated by several researchers by the LDS abbreviation (Lake Dagow Sediment) [3, 4]. The 16S rRNA sequences of this archaeal clade have been detected primarily in aerobic freshwater habitats [4]; however, these organisms remain uncultured so far, and their physiology and metabolic potentials are unknown.

It is noteworthy that for wetland ecosystems located in river catchments on lowland territories, predominance of small microbial cells is typical [5–7]. Cells measuring less than 0.5 μm comprised up to 80% of the total microbial population revealed by DAPI staining in the peat of Bakcharskoe bog in western Siberia [5]. Because of the low fluorescence intensity, these cells could hardly be identified by fluorescent in situ hybridization (FISH) with the conventional set of probes specific to the major groups of the *Bacteria* domain. Interestingly, a polymerase chain reaction with template DNA from the fraction of cells smaller than 0.2 μm resulted in amplification of only archaeal but not bacterial 16S rRNA genes [8]. The nucleotide sequences determined in that work showed the affiliation of bog ultramicroarchaea with undescribed groups of the *Archaea* domain, which lack cultured representatives. According to Lysak et al. [6], the number of microbial cells that passed through 0.2 μm-pore-size filters varied in the ombrotrophic peat bog

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from 1×10^8 to 5×10^8 cells per gram dry peat. Less than a half of this filterable cell population could be identified by FISH, and these were representatives of *Archaea* and of the bacterial phyla *Proteobacteria, Bacteroidetes* and *Actinobacteria* [6]. There have been no studies aimed at more detailed identification of microbial ultramicroforms in wetland ecosystems.

Thus, it remained unclear whether the waters draining peatlands can influence the composition of filterable microbial cells in aquatic ecosystems fed by swampy catchments. The present work was initiated in order to find an answer to this question by identifying the prokaryotic ultramicroforms in the *Sphagnum* peat of Obukhovskoe bog, located, like the lakes Motykino and Dubrovskoe studied previously [1], in the Mologa–Sheksna catchment area.

MATERIALS AND METHODS

Study site. This work used *Sphagnum* peat collected from a depth of 5–15 cm in Obukhovskoe bog (Yaroslavl' oblast, 58°14′N, 38°12′E) in May 2010. Obukhovskoe bog is a typical component of the swampy landscape in the Mologa–Sheksna catchment area of upper Volga. The vegetation cover is composed of *Sphagnum angustifolium, Sph. fuscum, Carex* spp., *Oxicoccus* sp., and *Vaccinium* sp. Peat thickness reaches 3 m. The bog water has a pH value of 4.2. The water table at the time of sampling was at a depth of 5 cm.

Determination of filterable cell numbers by microscopy. For this analysis, 1 g wet peat (97% water content) was used. Microbial cells were desorbed with MiniMix homogenizer (model 100, Interscience, France) and the BagFilter sterile bags supplied together with it. Each bag has two compartments separated by a filtering insert, which makes it possible to separate the peat suspension from large (≥1 mm) particles of undecomposed plant debris. Wet peat (1 g) was placed in one of the two compartments, suspended in 20 mL of sterile water, and homogenized for 10 min. After this, 1 mL of the suspension enriched with microbial cells was taken from the other compartment, fixed with ethanol added at 1 : 1 ratio, and stored at –20°C. For determination of the total number of microbial cells, the fixed sample was diluted with water to a volume of 15 mL, and a 0.3-mL aliquot was filtered through a 0.22-μm-pore-size Millipore filter with the use of a vacuum pump. For enumeration of filterable cells, the remaining peat suspension was passed through membrane filters with a pore size of 0.22 μm; the cells that passed through this filter were trapped on filters with a pore size of 0.09 μm. The filters with trapped cells were cut into four sectors, which were stained with a 0.5 μM solution of the DNA-specific fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) for 10 min, washed with distilled water, and dried. The number of trapped cells was determined using a Zeiss Axioplan 2 microscope (Carl Zeiss, Germany) with a Zeiss 02 light filter specific for DAPI staining. Cells were counted in 100 microscope fields with subsequent calculation of their number in 1 g of wet peat. The total microbial cell number and the number of cells that passed through 0.22-μm-pore-size filter were determined in three replicates.

Molecular identification of filterable forms of prokaryotes. For molecular identification of filterable prokaryotes, 2 g of peat was suspended in 20 mL of sterile water, treated in a MiniMix homogenizer as described above, and filtered through membrane filters with a pore size of 5 μm with the use of a vacuum pump to get rid of large particles. The fraction of ultrasmall cells was collected by passing this filtrate through 0.22-μm-pore-size nitrocellulose filters and subsequent trapping of filterable cells on 0.1-μm-pore-size filters. Two filters with trapped cells were used to isolate total DNA with the use of a FastDNA SPIN kit for soil (Biol 101, United States) according to manufacturer's recommendations. The obtained total DNA was used as template in polymerase chain reaction (PCR). Fragments (~1490 bp) of bacterial 16S rRNA genes were amplified in PCR with universal bacterial primers 9f and 1492r [9], and fragments (~800 bp) of 16S rRNA genes of archaea, with the primers 109f and 915r [10]. PCR was run in a PE GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer Applied Biosystems, United States). PCR products were checked by electrophoresis in a 1.2% agarose gel with subsequent staining with ethidium bromide and visualization in a UV transilluminator. The amplicons were cloned using a pGem-T Easy Vector SystemII kit (Promega, United States). Recombinant clones were selected by amplification of the cloned fragments with the vector-specific primers T7 and SP6. The clones were sorted by restriction analysis using *Msp*I and *Rsa*I endonucleases for bacterial amplicons and *Hha*I and *Bsu*RI (*Hae*III) endonucleases for archaeal amplicons. Electrophoresis of the restriction products was performed in a 2.5% agarose gel.

Isolation and purification of plasmid DNA was carried out using Wizard® Plus Minipreps DNA Purification System (Promega). Nucleotide sequences were determined on an ABI 377A sequencer (Perkin-Elmer Applied Biosystems, United States) and edited using the SeqMan (Laser Gene 7.0; DNA Star Package) software. The comparison of sequences with those available in the GenBank database was performed using the Blast2 software (http://www.ebi.ac.uk/Tools/blast2/). The clone libraries were checked for the presence of chimeras with the use of the Bellerophon 3.0 software (http://compbio.anu.edu.au/bellerophon/bellerophon.pl). Phylogenetic dendrograms were constructed using the ARB software package (http://www.arb-home.de). The statistical significance of the dendrograms was estimated using Phylip software package by constructing 1000 alternative trees and bootstrap analysis.

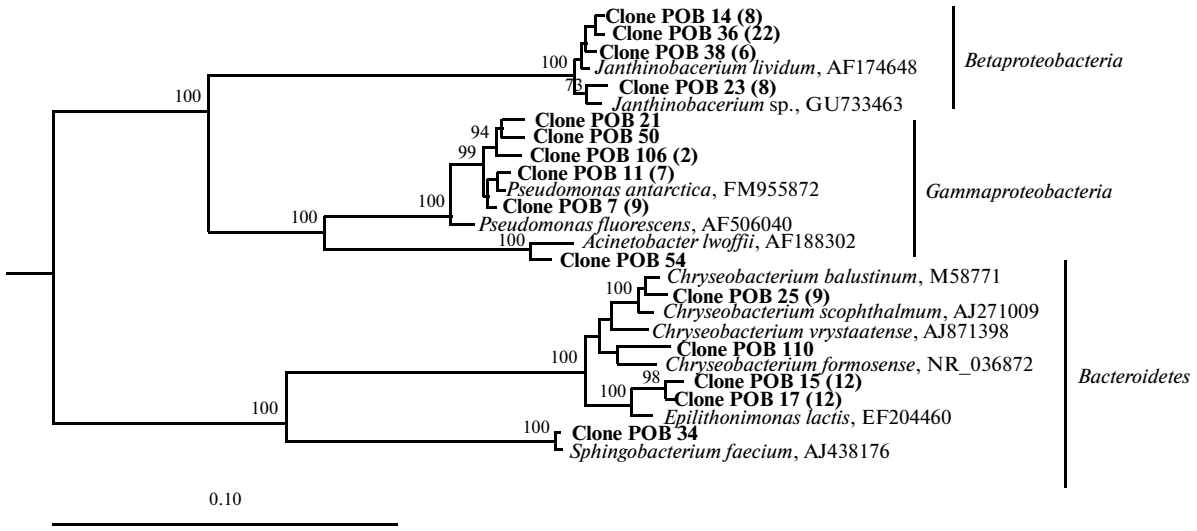

Fig. 1. Phylogenetic dendrogram constructed based on comparative analysis of the 16S rRNA gene sequences of filterable bacteria from *Sphagnum* peat and the 16S rRNA genes of some representatives of the *Proteobacteria* and *Bacteroidetes*. The clones obtained from the peat of Obukhovskoe bog are designated by the letters POB. As outgroups, the 16S rRNA genes of *Gemmata obscuriglobus* (X54522), *Isophaera pallida* (AJ231195), *Schlesneria paludicola* (AM162407), and *Planctomyces limnophilus* (X62911) were used. Bar, 0.1 substitutions per nucleotide position.

The newly determined nucleotide sequences of the 16S rRNA genes of filterable peat-inhabiting prokaryotes have been deposited in GenBank under accession numbers JQ697092–JQ697150.

RESULTS AND DISCUSSION

Filterable cell number. The total number of microorganisms revealed by DAPI staining in 1 g of wet peat of Obukhovskoe bog was $(8.06 \pm 0.86) \times 10^8$ cells. About 0.5% of these cells, $(3.75 \pm 0.42) \times 10^6$ L^{-1}, passed through 0.22 μm-pore-size filters. The values obtained by Lysak et al. [6] are of the same order of magnitude if recalculated per 1 g wet peat.

Molecular identification of filterable bacterial forms. The clone library of 16S rRNA genes of filterable bacterial forms from peat included 100 clones, of which 65 represented the phylum *Proteobacteria* and 35 represented the phylum *Bacteroidetes* (Fig. 1). Proteobacteria were represented by two classes, *Betaproteobacteria* (44 clones) and *Gammaproteobacteria* (21 clones), and the phylum *Bacteroidetes* was represented by the classes *Flavobacteria* (34 clones) and *Sphingobacteria* (1 clone).

All cloned 16S rRNA gene sequences affiliated with *Betaproteobacteria* exhibited highest similarity (98–99%) to those of the bacteria from the genus *Janthinobacterium*, particularly to *J. lividum* and to taxonomically uncharacterized psychrophilic representatives of this genus isolated from sediment of an Antarctic lake [11].

The class *Gammaproteobacteria* was represented by 21 cloned sequences, of which 20 exhibited high (99%) similarity to 16S rRNA genes of psychrophilic representatives of the genus *Pseudomonas: Ps. psychrophila, Ps. antarctica*, and Antarctic isolates. One of the cloned genes belonged to a representative of the genus *Acinetobacter* and was closest (99%) to the gene of an isolate of this genus from a melt water stream of an Arctic glacier [12].

A large group of the cloned 16S rRNA sequences of filterable peat-inhabiting bacteria belonged to the class *Flavobacteria* (Fig. 1). Ten of such clones represented the genus *Chryseobacterium*, and 24 clones represented the genus *Epilithonimonas*. One clone exhibited high (99%) similarity to the 16S rRNA gene of *Sphingobacterium faecium*, isolated from an Alpine glacier.

Thus, the diversity of filterable bacterial forms revealed in the bog was lower than that in acidic lakes. In both bogs and lakes, betaproteobacteria made up a considerable fraction of filterable cells. However, in the bog they were represented exclusively by members of the genus *Janthinobacterium*, whereas in lakes, by members of the genera *Herbaspirillum*, *Herminiimonas*, *Curvibacter*, and *Burkholderia* [1]. Ultrasmall pseudomonads were present in both lakes and the bog. The major distinction of the pool of filterable prokaryotes of the bog was the presence of *Bacteroidetes* representatives: flavobacteria and sphingobacteria. According to Lysak et al. [6], representatives of this group of bacteria made up 25% of all ultramicrobacteria identified in peat soil by FISH.

We failed to reveal filterable forms of actinobacteria in the bog. Here, our results differ from FISH data [6], according to which actinobacteria made up one-third of all ultramicrobacteria identified in peat soil. Ultrasmall forms of actinobacteria are known to be a typical component of the bacterioplankton of oligotrophic and oligomesotrophic lakes [13–15]; however, no analogous taxa of *Actinobacteria* have so far been revealed in peatlands.

All of the bacteria that we identified in the fraction of filterable cells of the bog belonged to well-studied taxa. However, it is worth noting that the taxonomic descriptions of most of these bacteria (of the genera *Janthinobacterium, Pseudomonas* and *Epilithonimonas*) lack indications of the possible formation of ultrasmall cells. At the same time, filtered water has been reported to contain pseudomonads along with other microorganisms [16–18]. Notably, before establishment of phylogenetic systematics, bacteria of the genus *Janthinobacterium* were also considered to be pseudomonads. Under substrate limitation and temperatures unfavorable for growth, these bacteria most probably form smaller cells than when grown on laboratory media. As for our detection in the bog of filterable representatives of *Chryseobacterium*, it is worth noting that one of the reports concerning the formation of ultrasmall cells (with and average volume of ~0.08 μm^2) by bacteria described three representatives of this genus obtained from deep Greenland ice core. These isolates were subsequently characterized as representing a new species, *Chryseobacterium greenlandense* [19]. One more communication was devoted to ultrasmall *Chryseobacterium* representatives isolated from Lake Baikal sediments and old oil sludge [20].

An interesting finding stemming from the analysis of the phylogenetic affiliation of the filterable bacterial forms found in the bog was that virtually all of their closest relatives were isolated from low-temperature ecosystems, particularly Antarctic lakes and waters and sediments of melt water streams of Arctic and high-altitude glaciers. Unfortunately, relevant publications do not provide information of on the cell size ranges of these microorganisms.

Molecular identification of filterable archaeal forms. The clone library of 16S rRNA genes of filterable archaea comprised 77 clones, of which 73 represented the *Euryarchaeota* phylum, and 4, the phylum *Thaumarchaeota*.

About half of the clones of 16S rRNA genes of euryarchaea represented methanogens of the orders *Methanobacteriales, Methanomicrobiales*, and *Methanosarcinales*. A group that was particularly large (41 clones) was comprised by 16S rRNA gene sequences belonging to the order *Methanomicrobiales* and exhibiting 97–98% identity with the 16S rRNA gene of the acidophilic peat-inhabiting methanogen *Methanoregula boonei* [21, 22], a typical inhabitant of acidic wetland ecosystems, characterized by very small cells with a diameter of 0.2–0.3 μm. The only clone that represented the order *Methanobacteriales* belonged to the phylogenetic lineage of *Methanobacterium subterraneum*, which has very thin (0.10–0.15 μm) rod-shaped cells [23]. Representatives of the order *Methanosarcinales* were detected by us both in the water of acidic lakes [1] and in the peat of the bog.

The remaining half of the cloned 16S rRNA gene sequences of filterable peat-inhabiting archaea belonged to the as yet uncharacterized clades of mesophilic *Euryarchaeota*, conventionally named LDS (Lake Dagow Sediment) (22 clones) and RC-V (Rice Cluster V) (5 clones) (Fig. 2). The similarity of these cloned sequences with the 16S rRNA genes of taxonomically characterized archaea did not exceed 75%. The first clones belonging to the RC-V phylogenetic group were obtained in 1998 from soil of flooded rice microcosms [24]. The LDS phylogenetic clade was formed later (in 2004) on the basis of a group of clones retrieved from lake sediments [3]. In 2008, the LDS and RC-V clades comprised about 370 and 500 nucleotide sequences, respectively [4]. Detailed analysis of the parameters of ecosystems from which these clones had been retrieved showed that the main habitats of these uncultured groups of euryarchaea are oxic fresh waters and freshwater sediments [4]. No data are presently available on the physiology and metabolism of the representatives of these groups. Our studies show that cells of LDS representatives have ultramicro dimensions. The LDS-affiliated 16S rRNA gene sequences retrieved from peat were highly similar to those retrieved in our previous study from the waters of acidic lakes Dubrovskoe and Motykino [1] and formed common clusters with them (Fig. 2). These clusters also included 16S rRNA gene sequences earlier retrieved from waters of Arctic rivers, lakes, and bogs [25–27], as well as from boreal peatlands [28].

Four of the retrieved 16S rRNA gene sequences of filterable peat-inhabiting archaea represented the *Thaumarchaeota* [29, 30]. This group of archaea includes only a limited number of cultured and characterized representatives; nevertheless, organisms possessing ultrasmall cells are already known among them. The typical inhabitant of seawater *Candidatus* Nitrosopumilus maritimus has rod-shaped cells that are only 0.17–0.22 μm thick [31]. However, the nucleotide sequences of thaumarchaeota obtained in our work were closest (up to 99%) to clones earlier retrieved from various wetland ecosystems [28, 32, 33]. Most probably, particular groups of *Thaumarchaeota* are typical inhabitants of northern wetlands.

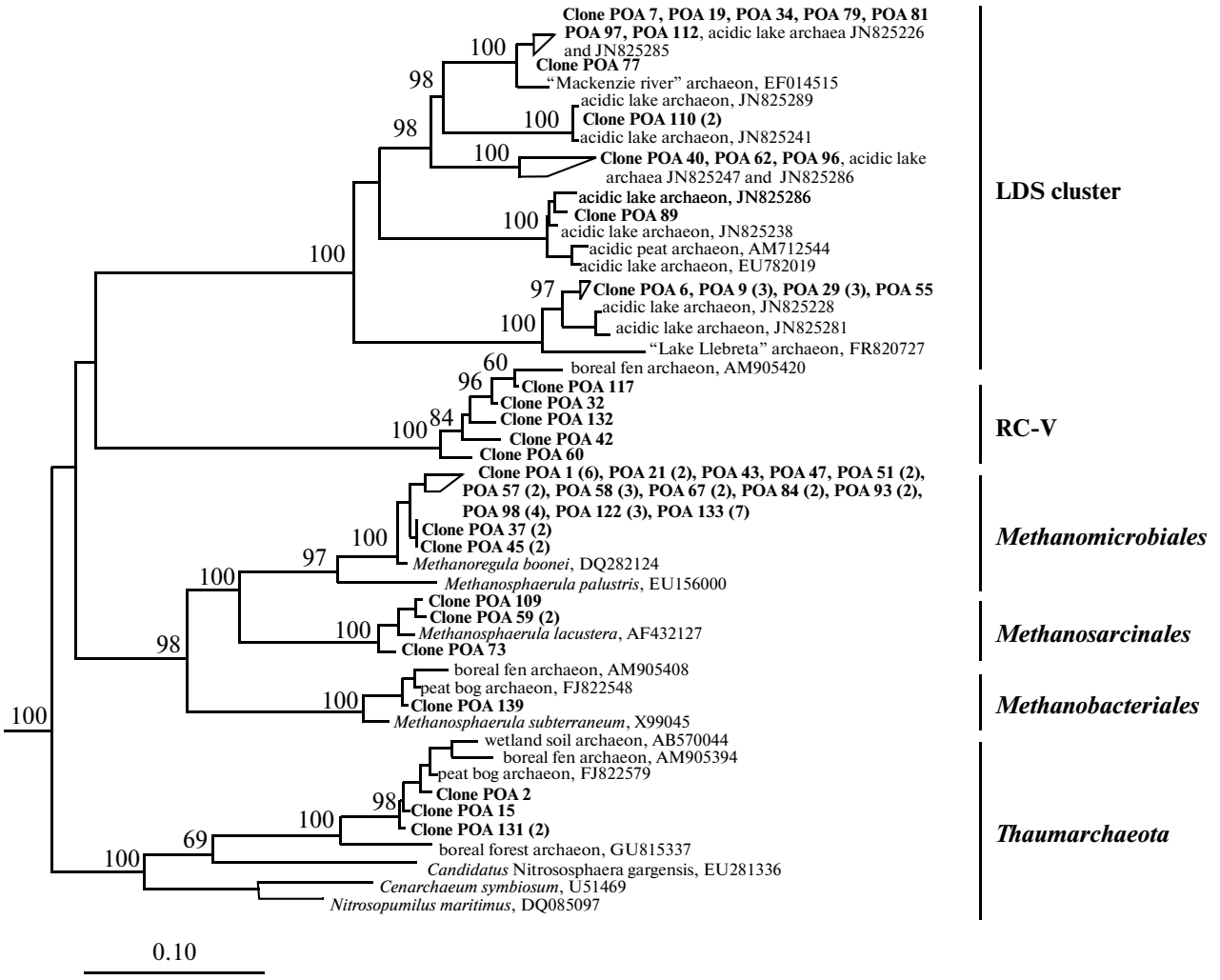

Fig. 2. Phylogenetic dendrogram constructed based on comparative analysis of the 16S rRNA gene sequences of filterable archaea from *Sphagnum* peat, the 16S rRNA genes of some taxonomically characterized archaea, and a number of clones obtained in molecular studies of various ecosystems. The clones obtained from the peat of Obukhovskoe bog are designated by the letters POA. As outgroups, the 16S rRNA genes of *Gemmata obscuriglobus* (X54522), *Isophaera pallida* (AJ231195), *Schlesneria paludicola* (AM162407), and *Planctomyces limnophilus* (X62911) were used. Bar, 0.1 substitutions per nucleotide position.

As demonstrated by the present study, the composition of ultrasmall bacterial forms passing through 0.22-μm-size filters in the waters of lakes does not exhibit considerable similarity to that in the surrounding peatland catchments. . By contrast, the pools of filterable archaea of these two ecosystems are dominated by one and the same group of ultrasmall *Euryarchaeota* representatives belonging to a new, as yet undescribed class of archaea. Investigation of the biology and physiology of these microorganisms is of considerable theoretical and practical interest.

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