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EXPERIMENTAL ARTICLES

Microbial Communities of Ancient Seeds Derived from Permanently Frozen Pleistocene Deposits

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Abstract—Microbial communities from the surface of ancient seeds of higher plants and embedding frozen material dated to the late Pleistocene (formed about 30 thousand years ago) were studied by various methods: scanning electron microscopy, epifluorescence microscopy, and inoculation of nutrient media, followed by identification of isolated cultures. Both prokaryotic and eukaryotic microorganisms were found on the surface of ancient seeds. The total quantity of bacterial cells determined by direct counting and dilution plating (CFU) for the samples of ancient seeds exceeded the value in the embedding frozen material by one to two orders of magnitude. This pattern was not maintained for mycelial fungi; their quantity in the embedding material was also rather high. A significant difference was revealed between the microbial communities of ancient seeds and embedding frozen material. These findings suggest that ancient plant seeds are a particular ecological niche for microorganisms existing in permafrost and require individual detailed study.

Key words: permafrost, ancient seeds, microbial communities.

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Experimental data have given evidence in recent years that permafrost deposits serve as a natural cryodepository. It has been demonstrated that under these conditions microorganisms of different systematic groups can remain viable for many millennia. Permafrost has been shown to contain various bacterial communities [1, 2] including anaerobic ones [3]. Cyanobacteria [2, 4], green algae [4], and fungi and yeasts [2] have been more than once isolated from permafrost. In addition to microorganisms, permafrost deposits of north-eastern Eurasia, which has not thawed out for tens of thousands of years, contain other paleobiological objects, including the buried seeds of higher plants. Large agglomerations (up to 600–800 thousand units of seed material) have been found in ancient rodent burrows preserved in permafrost. The best preservation was characteristic of the paleoecological material extracted from fossil burrows of ground squirrels referred to as one of the species of subgenus *Urocitellus* [5, 6].

The age of fossil burrows determined by radiocarbon analysis is 28–32 thousand years. They have not thawed out from the moment of burial and freezing up to now. Many of the several dozen revealed burrows contained significant amounts of well-preserved paleoecological material: seeds, residues of vegetative organs of plants, hair, residues of small animals and insects, feces, etc. [6]. Most often, the burrows were shown to contain the fruitcases and seeds of narrow-leaved campion (*Silene stenophylla*), seeds of snow cinquefoil (*Potentilla nivea*), seeds of bluegrasses (*Poa botryoides, P. attenuate*), seeds and fruitcases of grayish plantain (*Plantago canescens*), seeds and pods of variable sisymbrium (*Sisymbrium polymorphum*), the cloves of viviparous bistort (*Bistorta vivipara*), seeds of arctic sorrel (*Rumex arcticus*), seeds of creeping buttercup (*Ranunculus repens*), and fruit of sedges (*Carex* spp.) [7]. Positive results of germination in tissue cultures of paleoseeds from fossil burrows [8] indicate that not all of them have lost their vital functions even after storage in permafrost for several millennia.

A plant surface is actively colonized by microorganisms. During this process, different plant organs, including viable seeds, form specific microbial communities on their surface due to excretion of different

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substances into the environment. The structure of these communities is conditioned by physiological and genetic characteristics of plants and microorganisms [9]. Consequently, the set of microorganisms associated with the surface of healthy viable seeds is not random and is relatively constant. Previously, this has been confirmed for the seeds of different cultivated and wild plants [10].

In our opinion, preservation of viability and functional specificity of microbial communities formed on the surface of seeds is of primary significance for maintenance of the viability of the latter. In view of assessment of stability of natural ecosystems, we are interested whether long-term exposure to negative temperatures affects the species diversity and specificity of local minor ecological niches and subsystems, which characterize biological heterogeneity as the most important quality of natural ecosystems (soils and rocks) that is irreproducible in models.

The goal of the present work was to analyze the structural–taxonomic peculiarities of microbial communities immobilized on the surface of ancient seeds from permafrost.

MATERIALS AND METHODS

Our subjects of research were fossil seeds of higher plants excavated from the ancient burrows of ground squirrels preserved in permafrost deposits. The samples were taken from late Pleistocene deposits in the region of the Kolyma lowland on Duvannyi Yar outcrops located in the lower reach of the Kolyma River and Stanchikovsky Yar outcrops located on the Maly Anyui River, tributary of the Kolyma River.

On the outcrops of the permafrost sediments, fossil burrows of ground squirrels occurred in the buried layers of pulverescent loams, 15 to 40 m deep. There is reason to believe that the temperature, gas, and humidification regimes stabilized a short time after burial and conversion of the burrows into permafrost state and remained practically constant up to the present moment [7]. All these factors contributed to good morphological preservation of the paleoecological material, including paleoseeds taken for analysis:

Burrow no. P1300: seeds of different species of bluegrass (*Poa* spp.). The age of soil is 26–50 thousand years.

Burrow no. P1311: seeds of snow cinquefoil (*Potentilla nivea*). The age of soil is 26–50 thousand years.

Burrow no. P1075: seeds and full fruitcases of narrow-leaved campion (*Silene stenophylla*). The age of the burrow determined by the radiocarbon method is 32 thousand years (Beta 157195).

For research purposes, the seeds were collected under sterile conditions and stored in a refrigerator within the temperature range of –7 to –15°C before the analysis; these values are close to the temperature of permafrost soils of the Kolyma lowland (–7 to –11°C). The samples of embedding material, which was a layer of pulverescent loam (intermediate between the burrow bedding and frozen rock) with an abundant admixture of heavily fragmented residues of plant tissues, animal hair, bird feathers, and seeds were collected, as well as the modern analogs of the seeds.

The existence of microorganisms associated with the surface of native ancient seeds was confirmed by the method of scanning electron focused-beam microscopy (SEM). The studies were performed in a Hitachi S-520 SEM scanning electron microscope (Hitachi, Japan) at a maximal magnification of up to 10000–15000 and a voltage of 20 kV. The FemtoScan-001 software package was used for additional statistical processing of the parametric data from digital images [11].

The total quantity of bacterial cells for the seeds and embedding material was determined using direct epifluorescence microscopy [12].

The bacteria were enumerated by plating different dilutions of the suspension on TSA (trypticase–soy agar). The temperature preferences of bacterial communities from the seeds were determined by cultivation at three temperatures: 4, 20, and 35°C. Inoculation was made in five repeats for each dilution and temperature.

The taxonomic structure of associative bacterial communities was studied for the best preserved seeds of narrow-leaved campion (*Silene stenophylla*) from burrow P1075 and also for embedding material from the same burrow. Prepared suspensions were spread on TSA medium and cultivated at 20°C. The isolated pure cultures were defined by the sequencing of 16S rRNA amplicons (ca. 250 nucleotides in length) followed by comparison with the sequences from nr Database NCBI [13]. PCR was performed with one of the three group-specific primers (16s-s1, 16s-s2, and 16s-s3) in combination with the universal primer (16s-as1):

16s-s1: GGTGGGGATGACGTCAAATCATCATGC

16s-s2: GGTGGGGATGACGTCAAGTCATCATGG

16s-s3: GGTGGGGATGACGTCAAATCAGCACGG

16s-as1: CCCTTACGGCTACCTTGTTACGACTT

PCR was performed in a Tertsik device (DNA-Technology, Russia). The amplification program was as follows: 94°C, 2 s ⟶ 55°C, 2 s ⟶ 72°C, 2 s (5 cycles); 94°C, 2 s ⟶ 58°C, 2 s ⟶ 72°C, 2 s (25 cycles). PCR product was detected by electrophoresis in 1% agarose gel by staining with ethidium bromide. The sequences of PCR products were determined by the two chains for two independent PCR products, using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., United States) and original reagents. The DNASTAR software package (DNASTAR Inc., United States) was used for the sequence analysis. The processed sequences were compared with the sequences from the nr Database NCBI. Sequencing of a 250-bp fragment was sufficient for reliable genus affiliation of only some of the cultures. To define the remaining samples, additional sequencing of a larger 16S rRNA fragment

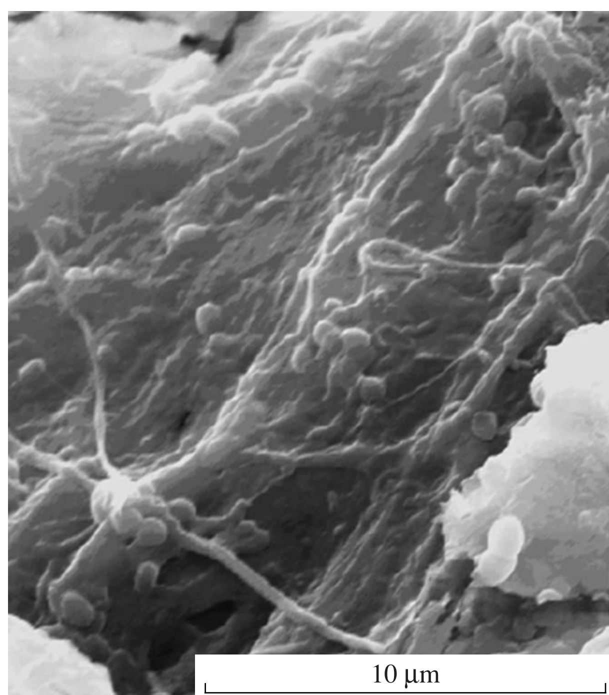

Fig. 1. Biomorphic objects on the surface of ancient seeds defrosted immediately before the experiment (scale bar, 10 μm).

(about 600 nucleotides in length) was carried out. In this case, PCR fragments were obtained using one of the two group-specific primers (16s-s4 and 16s-s5) in combination with another primer (16s-as2):

16s-s4: GTG CCA GCA GCC GCG GTA AT

16s-s5: CGT GGG GAG CAA ACA GGA TTA GA

16s-as2: CCC TTA CGG CTA CCT TGT TAC GAC TT

Mycelial fungi were studied in four samples: fruitcases and seeds of narrow-leaved campion from burrow P1075, embedding material from the same sample, seeds of snow cinquefoil from burrow P1311, and cereal seeds from burrow P1300. The quantitative and qualitative analysis was performed by plating of washouts from the seeds and suspensions of embedding material on organic media: Maltz agar (MEA) and potato–glucose agar (PDA), with the addition of 0.4% lactic acid for bacterial growth inhibition. The experiments on separation of the surface and internal fungal contamination of seeds were performed with the ancient seeds of narrow-leaved campion from sample P1075 by surface sterilization of the seeds with 3% hydrogen peroxide solution [14]. Inoculated media were concurrently cultivated at 4 and 25°C. Taxonomic affiliation of the fungal isolates was determined phenotypically using modern manuals [15].

RESULTS AND DISCUSSION

Microorganisms on the surface of paleoseeds. The examination of ancient seeds by scanning electron microscopy confirmed a complex structure of their surface, with different surface morphology for the seeds of different plant species. The seeds were covered with a gel-like substance comprising numerous microbial cells (Fig. 1). Mycelium of different thickness (1.0 to 8.0 μm), morphologically corresponding to actinomycete and fungal hyphae, fungal spores, and other eukaryotic cells, presumably microalgae, were found on the surface of all tested ancient seeds. The size of eukaryotic cells varied from 4 to 10 μm. Bacterial cells of 0.5–1.0 μm and microcolonies were spread everywhere on the surface of ancient seeds. The above objects had no signs of damage and their morphology corresponded to the morphology of soil microorganisms, suggesting their potential viability. After incubation of the samples in a moist chamber, the quantity of eukaryotic cells, mycelium length and frequency of occurrence, as well as the area occupied by the biogel, increased. These features confirm the possibility of activation of the communities in situ in the absence of additional nutrient sources.

Total bacterial quantity (epifluorescence analysis). The total bacterial quantity per gram of air-dry weighed sample of paleoseeds was about 10^9 cells. For comparison, the analogous value for the samples of embedding permanently frozen matter was lower by one to two orders of magnitude (Fig. 2a). This may be the result of colonization of seeds before freezing or of the propagation of microorganisms directly in permafrost. In the latter case, such divergence of the quantity of prokaryotes may be indirect evidence of more favorable living conditions for microorganisms close to the seed surface as compared with the rest of permafrost soil. It is known that on nutrient media with cryoprotectors microorganisms can grow even at negative temperatures [16].

Enumeration of bacterial cells by direct plating. The bacteria ready for proliferation immediately after thawing were counted on TSA medium. This medium was selected due to abundance of available nutrients, which allows obtaining colonies with well-marked morphology. Thus, on the one hand, TSA medium was used because the excretions of germinating seeds are also characterized by great diversity of nutrients available to microorganisms. On the other hand, some researchers have noted that low-nutrient media were in many cases unfavorable for microbial enumeration in permafrost deposits immediately after thawing out. Microorganisms were better activated in rich media.

The content of active bacterial cells associated with ancient seeds varied from 10^6 to 10^8 CFU/g. In the embedding permafrost material, the number of heterotrophic bacteria capable of growth after defrosting was within the range of 10^3–10^6 CFU/g, with an average value of 10^5 CFU/g (Fig. 2b), i.e., two orders of magnitude less than on the seeds. According to the previously published data, the quantity of bacteria in the same pul-

verescent deposits of yedoma suite (muck series) was 3×10^3–2×10^4 CFU/g [1].

Yedoma suite is a complex of yedoma deposits.

Yedoma is a form of frozen rocks. These are ice veins forming a complicated system, up to several tens of meters in height along with embedding (i.e., where they are located) sand and dust grounds. Hypothetically, yedoma rocks were formed 45–10 thousand years ago under conditions of tundra, tundra–steppe, and forest–tundra, when air temperatures were much lower than presently. Only about 8500 years ago did warming set in and the formation of yedoma stop [26].

A statistically significant maximum of the quantity and morphological diversity of bacterial colonies in the media inoculated with cell suspensions desorbed from the seeds was registered at 20°C; 30–50% of the maximal CFU yield was maintained at 4°C; at 35°C, the quantity of colonies was 1–5% of the maximum. Consequently, bacterial communities on the surface of paleoseeds may be characterized as mesophilic and psychrotolerant. This result confirms the previously published data demonstrating that most of the microorganisms isolated from frozen deposits are psychrophils: they grow at 4°C with the growth optimum at 20°C; 95% of isolates do not grow at temperatures above 30–35°C [16].

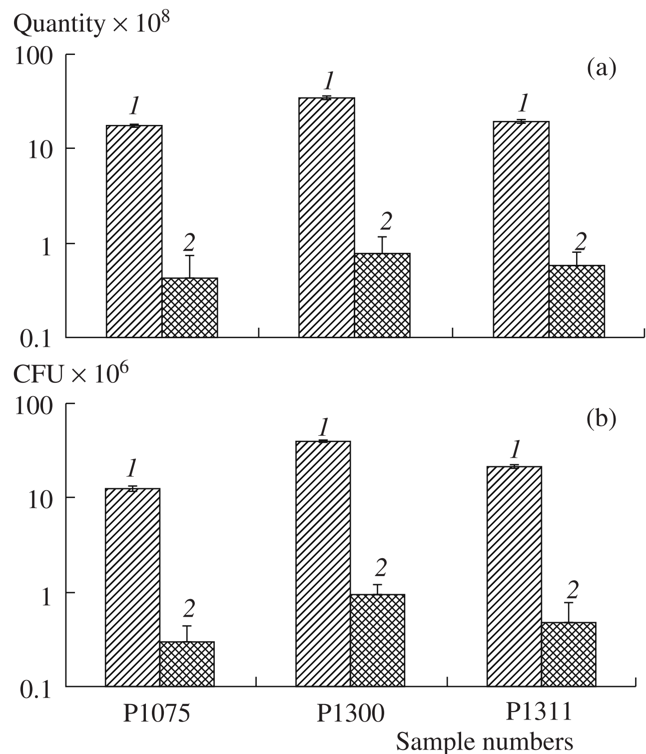

Fig. 2. Total quantity of bacteria in the samples of ancient seeds (*1*) and embedding material (*2*): (a) direct count by epifluorescence analysis (U/g); (b) count of colony-forming units on a nutrient medium (CFU/g).

Identification of bacteria isolated from the samples of ancient seeds and embedding material. The isolated bacterial strains (31) were subjected to molecular genetic analysis in the course of this work. Among them, 15 strains were isolated from ancient campion seeds and the other 16 strains were isolated from the frozen embedding material (Table 1).

In general, the taxonomic structure of the bacterial community from the frozen matter embedding the seeds exhibited rather low diversity. Gram-positive organisms were predominant (up to 80% of the total quantity). Gram-positive bacteria with high G+C content were an absolute majority. Among them, members of the genus *Arthrobacter* predominated. Besides this, members of the genera *Sphingomonas, Variovorax, Micrococcus, Pseudomonas, Subtercola*, and *Agreia* were isolated.

At the same time, in spite of the long period of close contact with the embedding frozen material, the microbial communities of ancient seeds were significantly different. Two of the three cases demonstrated a distinct predominance of gram-negative bacteria. Among them, the leading positions were held by the members of the genus *Devosia.* Basing on NCBI data, one may come to a conclusion that this bacterium is affiliated with eurybionts. However, the recent data indicate that some representatives of the genus *Devosia* can have mutualistic relations (mutually beneficial symbioses) with legumes and form nitrogen-fixing root nodules [17]. Besides *Devosia*, bacteria of the genera *Rhodoglobus, Arthrobacter, Paenibacillus, Roseomonas, Sanguibacter, Sinorhizobium*, and *Clavibacter* were isolated from the surface of ancient seeds. The strains of *Paenibacillus, Clavibacter*, and *Sinorhizobium* isolated in this work were genetically close to plant-associated strains of the same genera [13, 18]. All gram-negative bacteria isolated from the surface of ancient seeds belong to α-proteobacteria. It is well known that α-proteobacteria are usually among the typical plant associants and can make close contacts with plants.

Representatives of many of the established bacterial genera have been previously revealed in cold biotopes, such as soils of the polar regions, glaciers of the Greenland and Antarctic, mountain glaciers, deep water of different reservoirs, etc. For example, *Subtercola boreus* has been previously isolated from permanently cold groundwater [19]. The genus *Rhodoglobus* is presented in the interactive NCBI database by only three members, all of which have been isolated from permafrost [20].

Mycelial fungi isolated from the samples of ancients seeds and embedding material. The quantity of mycelial fungi in the samples varied from $1–4 \times 10^4$ to $2–3 \times 10^6$ (Table 2). The highest values were revealed in the samples of litter (from feeding chamber) and on the fruitcases of narrow-leaved campion. The temperature of cultivation had no effect on the total level of quantity of micromycetes.

Table 1. Results of molecular genetic anlaysis

Sample	Tentative genus or species	Sequenced region (nucleotides)	Similarity with the database sequence, %	Colonies on plates, %	Group
Community from ancient seeds of narrow-leaved campion (*Silene stenophylla*). Burrow P1075	*Devosia* sp. R-21940	622	97	30–80	α-*Proteobacteria*
	Rhodoglobus sp. GICR18	646	93	5–30	High G+C content
	Paenibacillus validus PR-B2	212	97	5–27	Low G+C content
	Clavibacter michiganensis subsp. *michiganensis*	281	97	5–15	High G+C content
	Phenanthrene-degrading bacterium L31B	506	96	3–15	High G+C content
	Sanguibacter marinus 1-19T	249	97	3–10	High G+C content
	Sinorhizobium sp. TB2-10	662	98	2–9	α-*Proteobacteria*
	Arthrobacter sp. J64	733	96	1–5	High G+C content
	Paenibacillus sp. DF5US1	279	98	1–5	Low G+C content
	Roseomonas gilardii subsp. *rosea*	713	94	1–5	α-*Proteobacteria*
Community from the frozen material embedding the seeds. Burrow P1075	*Arthrobacter* sp. J64	731	100	20–47	High G+C content
	Subtercola boreus DSM 13056	285	98	19–38	High G+C content
	Arthrobacter sp. Tibet-ITa1	712	99	15–26	High G+C content
	Pseudomonas sp. JR1	732	97	10–15	γ-*Proteobacteria*
	Arthrobacter sp. 18/4	556	95	6–12	High G+C content
	Arthrobacter sp. SIB M20(5)	530	97	3–9	High G+C content
	Arthrobacter sp. CAB1	730	98	3–9	High G+C content
	Sphingomonas sp. JFJ-Iso-Bact01	669	97	3–6	α-*Proteobacteria*
	Arthrobacter sp. NJ3	721	98	2–6	High G+C content
	Arthrobacter sp. MSCB-17	257	99	2–5	High G+C content
	Variovorax koreensis GH 9-3	253	96	1–5	β-*Proteobacteria*
	Micrococcus lylae	288	99	1–5	High G+C content
	Pseudomonas sp. JR1	732	95	1–5	γ-*Proteobacteria*
	Pseudomonas sp. M9-3	276	96	1–3	γ-*Proteobacteria*
	Agreia pratensis DSM 14226T	563	98	1–3	High G+C content

The species composition of fungi isolated from the tested samples is presented in Table 3. In the sample of embedding material from burrow P1075, *Phoma nebulosa* and other representatives of pycnidium fungi were predominant among the fungal species revealed at cultivation temperature 25°C. Previously it has been noted that soil samples from the Antarctic were one of the possible sources for *P. nebulosa* isolation [21]. No data have been found concerning their occurrence in the Arctic region. At 4°C, the situation was quite different. Fungi of the species *Geomyces pannorum* were predominant.

The *Phoma* fungi also dominated on the samples of campion seeds from the same burrow, and it was noted at cultivation of inocula at both temperatures. It is interesting that we have isolated the fungus *Phoma crystallifera* with the frequency of occurrence approaching 70%. This fungus, described recently, is usually iso-

Table 2. The quantity of mycelial fungi in tested samples

Sample description	CFU/g of air-dry sample at cultivation temperatures of	
	4°C	25°C
Embedding frozen material. Burrow P1075	1.88×10^6	2.75×10^6
Ancient fruitcases and seeds of narrow-leaved campion (*Silene stenophylla*). Burrow P1075	1.12×10^6	1.12×10^6
Ancient seeds of snow cinquefoil (*Potentilla nivea*). Burrow P1311	2.94×10^4	3.09×10^4
Ancient seeds of bluegrasses (*Poa* spp.). Burrow P1300	1.96×10^4	1.22×10^4

lated from the surface of contemporary campion plants (the genus *Silene*). Another representative of the genus *Phoma, P. herbarum*, with a 30% frequency of occurrence, has also been isolated from this sample. This latter organism has been previously isolated more than once by other researchers from Antarctic soils near the sites of bird concentration [22]. *Phialophora fastigiata* occurs in the same habitats [23]. In the inner part of ancient campion seeds sterilized from the surface, mycelial fungi were not found (sample P1075). Here, pink yeasts were noticed, especially abundant under cultivation at 4°C.

When studying paleoseeds, one has often to face the absence of an endosperm inside the seeds. This might be due to the fact that the seeds could get into feed chambers of fossil burrows in an immature state. Moreover, during the long period of storage, the endosperm could have been degraded by a fungus that had penetrated to the internal spheres of the seeds. Fungi are known to grow at extremely low levels of water activity and at weakly negative temperatures [24]. Temperature and moisture fluctuations could result in damage to an external seed coat, thus facilitating the penetration of the fungi. Therefore, the absence of mycelial fungi in the internal spheres of the seeds is, in our opinion, indirect evidence of favorable conditions for seed storage without contrast fluctuations of temperature and humidity, at least during the initial, most critical period of burial, before transition to the permafrost state.

The fungi of the genus *G. pannorum* amounted practically to 100% of the fungal complexes of ancient bluegrass seeds (sample P1300) at a cultivation temperature of 25°C. At a lower temperature, 20% of the fungi were represented by dark sterile mycelium with sclerotia and the other 80% belonged to the same species, *G. pannorum.*

Table 3. Species composition of mycelial fungi isolated from tested samples

Sample	Species composition of mycelial fungi	Temperature of isolation (°C)
Embedding frozen material. Burrow P1075	*Phoma nebulosa* (Pers. 1800) Berk. 1860	25
	Coelomycetes sp.	25
	Penicillium aurantiogriseum Dierckx 1901	25
	Geomyces pannorum (Link 1824) Sigler et J.W. Carmich. 1976	4
	Cladosporium sphaerospermum Penz. 1882	4
Ancient fruitcases and seeds of narrow-leaved campion (*Silene stenophylla*). Burrow P1075	*Phoma crystallifera* Gruyter, Noordel. et Boerema 1993	4; 25
	Phoma herbarum Westend. 1852	4; 25
	Aspergillus sclerotiorum G.A. Huber 1933	4; 25
	Penicillium granulatum Bainier 1905	4; 25
	Cladosporium sphaerospermum Penz. 1882	4; 25
	Phialophora fastigiata (Lagerb. et Melin 1928) Conant 1937	4; 25
Ancient seeds of snow cinquefoil (*Potentilla nivea*). Burrow P1311	Light-colored sterile mycelium	25
	Geomyces pannorum (Link 1824) Sigler et J.W. Carmich. 1976	4; 25
Ancient seeds of bluegrasses (*Poa* spp.). Burrow P1300	*Penicillium aurantiogriseum* Dierckx 1901	25
	Dark-colored sterile mycelium with sclerotia	4
	Geomyces pannorum (Link 1824) Sigler et J.W. Carmich. 1976	4; 25

The species *G. pannorum* was predominant in the complex of micromycetes isolated from the ancient seeds of snow cinquefoil (sample P1311) at 4°C. It is well known that they often occur in arctic soil samples and can grow at very low positive and even weakly negative (–2°C) temperatures [25]. The light sterile mycelium typical of arctic permafrost soils appeared at the cultivation temperature 25°C.

Thus, taxonomic analysis of the isolated microorganisms has shown a significant difference between the microbial communities of ancient seeds and embedding frozen material. So far, it is difficult to estimate the time of their appearance. However, we can establish the presence of such differences and state that the ancient seeds of higher plants are a specific habitat for microorganisms in permafrost, which promotes their preservation and requires thorough study.

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