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Mycelial fungi in cryopegs

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Abstract: Mycelial fungi from cryopegs (tundra, Kolyma lowland, Russia) have been studied. The use of media with different amounts of salt and cultivation at 4 and 25 °C allowed us to isolate filamentous fungi assigned to 11 species. The micromycetes of genus *Geomyces* were found most often. The total amount of fungi reached $1–4 \times 10^2$ CFU ml^{-1} of water. The extreme conditions of the cryopegs – the high salinity of the water (150–200 g l^{-1}) and the constant low temperatures (average annual temperature is from −9 to −11 °C) – might serve as a model for the conditions of interplanetary environments.

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Introduction

Cryopegs are mineralized headwater lenses below the Arctic massive ground ice bodies. The results of investigations into the biodiversity of microorganisms in cryopegs are only available in a few works (Bakermans *et al*. 2003; Zalar *et al*. 2003) due to the low availability of these samples. Until now, researchers' attention has mainly been focused on the investigation of the influence of the separate ecological factors describing the extreme conditions of life on microorganisms. There are numerous data about the content and the qualitative composition of mycelial fungi in fresh (Dudka 1985) and sea (Ellis-Evans 1985) water, saline soils (Domsch *et al*. 1980), ice of the Antarctic continent (Abyzov *et al*. 1998) and permafrost of the Antarctic and Arctic regions (Kochkina *et al*. 2001). The study of cryopegs enables us to work simultaneously with a set of the factors such as a high concentration of salts, a water environment, low temperatures, and sandy and loamy ancient grounds. The high salinity of the water (sometimes up to 300 g l^{-1}) of the underground lenses of cryopegs prevents them from freezing at the rather low temperatures (−15 to −20 °C). Similar free-water conditions are assumed possible for the permafrost regions of Mars and Europa (Davis *et al*. 2002; Gilichinsky *et al*. 2003b). There has been a recent growth in research in the field of astrobiology with the use of terrestrial extreme conditions as a model for exobiology. Some similar projects unite groups of scientists in different countries around the world (LLTP 2002).

The assumption that the most elementary forms of life on some planets are similar to terrestrial forms has already been expressed. Russian scientist L.K. Lozina-Lozinsky stated that it is impossible to study life in the conditions other than terrestrial, if we do not first model or imitate organisms or cells under these conditions in environments on Earth (Lozina-Lozinsky 1966). Concerning the above-mentioned statement, a study of the biodiversity of mycelial fungi in cryopegs modelling the possible conditions of life in interplanetary ecosystems was carried out.

Materials and methods

Cryopegs were exposed in two sites from closely situated boreholes at Cape Chukochii (70° 05′ N, 159° 55′ E) and the Lake Yakutskoe area (69° 50′ N, 159° 30′ E) on the tundra zone of the Kolyma Lowland near the coast of the East Siberian Sea. The permafrost here extends to a depth of 800 m with an annual temperature variation from −9 to −11 °C. The cryopegs are confined to a 20-m-thick marine horizon ($m\mathbf{Q_{II}}^{2–3}$), sandwiched between non-saline terrigenous layers at depths of 40–50 m below the surface. After regression of the polar ocean, the water-saturated bottom sediments were exposed subaerially and frozen. This was accompanied by the freezing out of salts in the water to form lenses of supercooled sodium chloride brines with salinities of 170–300 g l^{-1} (Gilichinsky *et al*. 2003a).

The samples were collected in 1999 from holes 14/99 and 15/99 aseptically and with suitable microbial control as described earlier (Gilichinsky *et al*. 1989; Shi *et al*. 1997). The salinity of the water was 150–200 g l^{-1} NaCl.

Before inoculation to nutrient media, samples were stored at 4 °C. Water samples for inoculation were concentrated by filtration: 50 ml of each sample was filtrated through a

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sterilized filter (Synpor, diameter of pores 0.3 μm). Afterwards the filters were cut and transferred in a tube with 4 ml of sterilized water. For better CFU (colonies forming units) separation, the tube was shaken on Vortex (V-3, Elmi, 3400 rev/min) for 2 min.

Inoculation was conducted in triplicate on organic medium MEA (Malt Extract Agar) and synthetic media Czapek in two variants (with different concentrations of sucrose, 2.0% (Cz 2.0) and 0.1% (Cz 0.1)). NaCl in concentrations of 1, 5 and 20% was used to reveal halophilic fungi.

Lactic acid was added at a concentration of 4 ml l^{-1} to nutrient media to suppress the undesirable growth of bacterial cells. The inoculated plates were incubated at 4 and 25 °C. The grown colonies were examined and enumerated on the 30th day. The isolates obtained were reinoculated on MEA and stored at 4 °C. Micromycetes were identified based on their cultural and morphological properties using the respective manuals (von Arx 1981 etc; Carmichael 1980).

To control the sterility of the air in the microbiological box, open Petri dishes with agar medium were exposed to air for 10 min and then were incubated at 4 and 25 °C. To control the sterility of the filters, these were also inoculated on agar plates and incubated at the same conditions. In the control samples, no fungi were found on the Petri dishes.

To determine the effect of NaCl on the growth of isolated strains, we had cultivations on Cz 2.0 and Cz 0.1 with the addition of 1% NaCl at two temperatures (10 and 25 °C).

The influence of temperature on growth of *Geomyces* strains was tested on MEA after 7 days of incubation at a range of temperatures (from 2 to 30 °C).

Results

Viable mycelial fungi were found in cryopegs at incubation temperatures of both 4 and 25 °C. Their number was small, from units up to hundreds CFU in 1 ml of water (Table 1). Higher quantitative parameters for both of the holes investigated were revealed on MEA in the 25 °C cultivation. At low temperature (4 °C), virtually no fungi were isolated from hole 14/99, as opposed to the small amount of fungi isolated from hole 15/99 in almost all of the experimental variants. The addition of NaCl to the nutrient media also completely suppressed the growth of fungi in water from hole 14/99, whereas micromycetes from hole 15/99 even grew at concentrations of salt accounting for 20%.

A total of 40 fungal strains were isolated as a result of the research. They represented 12 different taxa, mainly Anamorfic fungi (Table 2). The total number of fungal genera recovered using MEA (hole 14/99, six taxa; hole 15/99, eight taxa) was higher than the number of genera recovered on Cz (two and three taxa for Cz 2.0 and Cz 0.1, respectively). Some species were present in both samples (*Cladosporium herbarum*, *Geomyces pannorum* var. *pannorum*, *Penicillium minioluteum*, *Alternaria alternata*).

As our research has shown, all of the isolated organisms were capable of developing on nutrient media with the addition of 1% NaCl, which could serve as a confirmation of the fact that they were actually isolated from cryopegs, instead of being introduced at the time of selection of samples (Gilichinsky *et al.* 2003a). The essential decrease of fungal growth rate on media with the addition of salt in comparison with control media was not observed (Table 2).

Table 1. *Number of fungi in cryopegs*

Conditions		Number of fungal CFU ml^{-1} water			
		Temperature of cultivation 4 °C		Temperature of cultivation 25 °C	
Media	Concentration of NaCl in media (%)	Hole 14/99	Hole 15/99	Hole 14/99	Hole 15/99
MEA	0	0.0	18.0	110.0	400.0
	1–5	0.0	2.0*	0.0	1.4
	20	0.0	0.0	0.0	0.0
Cz 0.1	0	0.0	30.0	2.0	9.0
	1–5	0.0	12.0*	0.0	23.0*
	20	0.0	0.0	0.0	14.0*
Cz 2.0	0	4.0	16.0	0.0	10.0
	1–5	0.0	8.0*	0.0	0.0
	20	0.0	0.0	0.0	0.0

* Micromycetes are represented only by *Geomyces pannorum* var. *pannorum*.

For two species with low growth rate, *Cladosporium herbarum* and *Penicillium verrucosum*, distinctions between development at 10 and 25 °C were not observed; the representatives of genera *Alternaria, Aureobasidium, Verticillium* and other species of *Penicillium*, on the other hand, showed a rather sharply reduced growth rate at 10 °C (Table 2).

However, the greatest interest there is represented by species *Geomyces pannorum* var. *pannorum* and *Geomyces vinaceus*. As reported by Finotti *et al.* (1996), strains of *Geomyces vinaceus* isolated from Antarctica have optimum growth near a temperature of 4 °C. Our results also indicate that *Geomyces* strains have optimum growth at 10 °C (Fig. 1, Table 2), forming germ tubes at a temperature of 2 °C. However, according to the data from the taxonomic description for *Geomyces pannorum* var. *pannorum*, the optimum temperature for extension rate ranges from 20 to 25 °C (van Oorschot 1980). Macro characteristics of *Geomyces pannorum* var. *pannorum* colonies were also considerably changed at low temperatures (Fig. 2). A decrease in temperature promotes a more active growth of colonies. At the same time, sporulation of fungi is more active at 20 °C, where the conditions are more unfavourable for growth.

Only representatives of *Geomyces pannorum* var. *pannorum* were isolated on nutrient media with 5–20% salt content at all temperatures (Table 1).

Discussion

It is known that in relation to various external conditions, all microorganisms can be classified as preferring or capable of survival under those or other conditions. In relation to low temperatures, microorganisms can be classified as

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Table 2. *Species of micromycetes in cryopegs*

Taxa	Media				Ratio of colonies diameter at different salinity and temperature	
	Hole 14/99		Hole 15/99			
	MEA	Cz	MEA	Cz	Control/ test*	Control/ test**
Alternaria alternata (Fries: Fries) von Keissler		Y		Y	1.07	3.22
Aureobasidium pullulans (de Bary) Arnaud var. *pullulans*			Y		1.03	6.67
Cladosporium herbarum (Persoon: Fries) Link	Y	Y	Y		1.20	0.87
Geomyces pannorum (Link) Sigler et Carmichael var. *pannorum*	Y		Y	Y	1.05	0.48
Geomyces vinaceus Dal Vesco				Y	0.97	0.41
Basidiomycetes spp.	Y		Y		***	***
Penicillium aurantiogriseum Dierckx	Y				0.97	2.24
Penicillium verrucosum Dierckx			Y		0.91	1.06
Penicillium minioluteum Dierckx	Y		Y		1.01	4.0
Ulocladium botrytis Preuss			Y		1.08	***
Valsa sordida Nitschke	Y				***	***
Verticillium sp.			Y		1.04	2.02

* Average diameter of fungal colonies on media without NaCl to the same on media with 1% NaCl.
** Average diameter of fungal colonies incubated at 25 °C to the same at 10 °C.
*** No data.

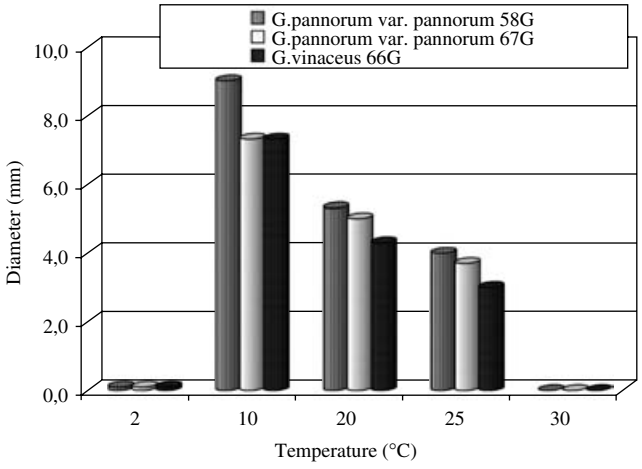

Fig. 1. Temperature ranges of growth for *Geomyces* strains from cryopegs.

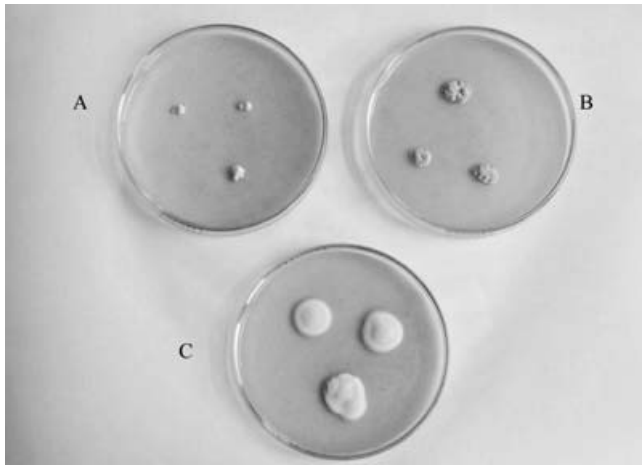

Fig. 2. Growth of *Geomyces pannorum* var. *pannorum* at different temperatures (21st day): A 25 °C; B 20 °C; C 10 °C.

psychrophiles or psychrotrophic (Morita 1975), which usually have an optimum temperature of 10–15 °C.

Over 75% of the Earth's biosphere is permanently cold. Representatives of all major taxa inhabit temperatures just below 5 °C. A novel habitat for psychrophiles was recently described at depths of nearly 3600 m, close to the surface of the large subglacial Lake Vostok (Johnson 2003), where liquid veins contain dissolved ions and these have been proposed to support at least one cell per cm^3.

It has been shown that chloride salts, apart from KCl, raise the survival rate of living organisms at low temperature. Microorganisms live within a great range of salinities, from essentially distilled water to saturated salt solutions (Rothschild & Mancinelli 2001). Hypersaline waters are defined as having salt concentrations greater than that of seawater (3.5% w/v). Extreme halophiles require NaCl concentrations above 15.6% (w/v) for growth. The salinity of water samples in our experiment was near to 15–20%.

The ability of fungi for growth in medium at NaCl concentrations near to 27% was already known (Sterflinger 1998). Recently, it was found that salterns form a natural ecological niche for osmophilic micromycetes of genus *Wallemia* (Zalar *et al*. 2003) and halophilic black yeasts (Gunde-Cimerman 2000). Obligatory halophilic fungi from order *Thraustochytriales* – *Thraustochytrium pachydermum* and *Schizochytrium aggregatum* were found in the winter pools on the roads and sidewalks of countries that apply dry salt of various origins to combat ice formation (Kuznetsov 2001).

All strains isolated in our research showed the same growth rate in the medium with 1% NaCl as in the medium without NaCl (Table 2). However, only strains of three of the species exhibited an increased growth rate at low temperature (ratio ** in Table 2, less 1.0). The most frequently found strains of *Geomyces* are among them. They are not true halophilic micromycetes, but they have adapted to the conditions of

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cryopegs (a combination of high salinity and low temperature) better than other fungi. Other micromycetes may not be considered as active in these adverse conditions, but do survive as spores.

The area of distribution of *Geomyces* is wide. It can be isolated from different kinds of soils with pH from 3.5 up to 8.0, from plants and vegetative residue, and it is capable of infecting the nails and skin of humans. Moreover, the representatives of this species can be found in various low-temperature niches, such as ground high-mountainous Alpine biocenosis (Sogonov 2003), in the ice and soils of Antarctica (Tosi *et al*. 2002) and in the permafrost of the Arctic region (Ivanushkina *et al*. 2003). The abundance of *Geomyces pannorum* in Antarctica is often connected to the ability of these fungi to utilize keratin, which is present on the skin of animals and the feathers of birds living there. There is an assumption that birds may act as vectors for the transport of microorganisms between Antarctica and more northern landmasses (Marshall 1998).

Earlier this species was known as *Crysosporium verrucosum* (Tubaki 1961) and then was transferred in a synonym of the name *Geomyces pannorum* var. *pannorum*. Some knowledge of the taxonomy of investigated species allows us to detect their presence in various lists of isolated species by different authors. For example, we have found *Geomyces pannorum* var. *pannorum* in the great review of Vishniak (1993) under the out-of-date name. As to the other species mentioned in this work, it should be noted that nearly all have been isolated in our earlier study of Arctic and Antarctic permafrost (Ivanushkina *et al*. 2003).

Conclusion

The ability of mycelial fungi to exist in cryopegs has been established. Their number reaches $1–4 \times 10^2$ CFU ml^{-1} of water and biodiversity can be more than 10 species. The received results do not contradict the available data on the presence of living organisms in permafrost and expand our knowledge in this area. So, the study of a hypersaline lake in the Vestfold Hills of Eastern Antarctica has shown that among the microorganisms that can be cultivated there were one species of extremely halophilic archaea and one species of algae. However, these make up less than 10% of the total inhabitants of this ecotope. Several other species are present, as indicated by molecular analysis, but could not be isolated with the methods used (Bowman *et al*. 2000). Thus, the application of updated methods for isolation and cultivation of mycelial fungi could essentially expand the list of fungal species found in extreme inhabitation.

The presence of micromycetes *Geomyces pannorum* var. *pannorum* and the temperature parameters of growth of this species allow us to assume that representatives of this species are not only capable of surviving at high salinity and low temperature, but are also capable of active life in the conditions of cryopegs. This fact can form the basis for assumptions that similar organisms could be found in samples of water from other planets.

There are assumptions (Andersen *et al*. 2002) and even indirect proofs (Mallin & Edgett 2000) of the existence of mineralized water on Mars. Therefore, there are bases to assume that eucariotic microorganisms similar to those isolated from cryopegs could be present in this water.

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References

Abyzov, S.S., Mitskevich, I.N. & Poglazova, M.N. (1998). *Mikrobiologiya* **67(4)**, 547–555. (English transl. *Microbiology* **67(4)**, 451–458.)

Andersen, D.T., Pollard, W.Y., McKay, C.P. & Heldman, J. (2002). *J. Geophys. Res.* **107(3)**, 4/1–4/7.

Bakermans, C., Tsapin, A.I., Souza-Egipsy, V., Gilichinsky, D.A. & Nealson, K.H. (2003). *Environ. Microbiol.* **5(4)**, 321–326.

Bowman, J.B., McCammon, S.A., Rea, S.M. & McMeekin, T.A. (2000). *FEMS Microbiol. Lett.* **183**, 81–88.

Carmichael, J.W., Kendrick, W.B., Conners, I.L. & Sigler, L. (1980). *Genera of Hyphomycetes*. University of Alberta Press, Edmonton.

Davis, W.L., McKay, C.P. & Navarro-Gonzalez, R. (2002). Earth analogs for life on Europa. In *Proc. Europe Focus Group Workshop*, pp. 9–11. Arizona State University/Ames Research Center.

Domsch, K.H., Gams, W. & Anderson, T. (1980). *Compendium of Soil Fungi*, vol. 2. Academic Press, London.

Dudka, I.A. (1985). *Vodnye Nesovershennye Griby* (Water Anamorphic Fungi). Naukova dumka, Kiev (in Russian).

Ellis-Evans, J.C. (1985). *British Antarctic Survey Bull.* **68**, 37–45.

Finotti, E., Paolino, C., Lancia, B. & Mercantini, R. (1996). *Current Microbiol.* **32**, 7–10.

Gilichinsky, D.A., Rivkina, E.M., Laurinavichius, K.S., Shcherbakova, V.A., Komarov, I.A. & Volkov, N.G. (2003a). *The Earth Cryosphere* **3**, 75–86 (in Russian).

Gilichinsky, D., Rivkina, E., Shcherbakova, V., Laurinavichuis, K. & Tiedje, J. (2003b). *Astrobiology* **3(2)**, 331–341.

Gilichinsky, D.A., Zvyagintsev, D.G., Khlebnikova, G.M., Fyodorov-Davydov, D.G., Kudryavtseva, N.N., Vorob'eva, E.A. & Chaikovskaya, N.R. (1989). *Izv. Akad. Nauk SSSR, Ser. Geol.* **6**, 103–115 (in Russian).

Gunde-Cimerman, N., Zalar, P. & de Hoog, G.S. (2000). *FEMS Microbiol. Ecol.* **32**, 235–240.

Ivanushkina, N.E., Kochkina, G.A. & Ozerskaya, S.M. (2005). *Fungi in ancient permafrost sediments of the Arctic and Antarctic Regions*, In *Life in ancient ice*, eds. Castello, J.D. and Rogers, S.O., Chapter 9, pp. 127–139. Princeton University Press. (ISBN: 0-691-074, 336pp)

Johnson, R. (2003). *The X-philes: Microorganisms at the Extremes*. Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, http://rucus.ru.ac.za/~wolfman/Essays/philes.html

Kochkina, G.A., Ivanushkina, N.E., Karasev, S.G., Gavrish, E.Yu., Gurina, L.V., Evtushenko, L.I., Spirina, E.V., Vorob'eva, E.A., Gilichinskii, D.A. & Ozerskaya, S.M. (2001). *Mikrobiologiya* **70(3)**, 412–420 (English transl. *Microbiology* **70**(3), 356–364.)

Kuznetsov, E.A. (2001). *Vodnie ecosystemi i organismi* **3(5)**, 69 (in Russian).

Lozina-Lozinsky, L.K. (1966). Reactions of cells and their protein components on the action of extremal factors. In *Proc. Academy of Sciences of the USSR*, pp. 3–10. Nauka, Moscow (in Russian).

Mallin, M.C. & Edgett, K.S. (2000). *Science* **288(5475)**, 2330–2335.

Marshall, W.A. (1998). *Microbial Ecol.* **36**, 212–219.

Morita, R.Y. (1975). *Bacteriol. Rev.* **39**, 144–167.

Rothschild, L.J. & Mancinelli, R.L. (2001). *Nature* **409**, 1092–1101.
Shi, T., Reevs, R.H., Gilichinsky, D.A. & Friedmann, E.I. (1997). *Microbial Ecol*. **33**, 169–179.
Sogonov, M.V. (2003). Biodiversity and spread of soil micromycetes in alpine biogeocenosis of Teberda nature reserve. *PhD Thesis*, Moscow State University, Moscow (in Russian).
Sterflinger, K. (1998). *Antonie van Leeuwenhoek* **74**, 271–281.
The Life at Low Temperatures Project (LLTP). (2002). *Research Findings First Year of Project* (*July 2001–June 2002*). http://astrobiology.msu.edu/project3.html
Tosi, S., Casado, B., Gerdol, R. & Caretta, G. (2002). *Polar Biol*. **25**, 262–268.
Tubaki, K. (1961). *The Antarctic Materials* (*Special Publication*, vol. 14), pp. 1–14. Seto Marine Biological Laboratory.
van Oorschot, C.A.N. (1980). *Stud. Mycol*. **27**, 89.
Vishniak, H.S. (1993). *Antarctic Microbiology*, ed. Freedman, E.I., pp. 297–342. Wiley–Liss, New York.
Vreeland, R.H., Rosenzweig, W.D. & Powers, D.W. (2000). *Nature* **407**, 897–900.
von Arx, J.A. (1981). *The Genera of Fungi Sporulating in Pure Culture*, 3rd edn. J. Cramer, Vaduz.
Zalar, P., de Hoog, G.S. & Gunde-Cimerman, N. (2003). *Proc. First Congress of European Microbiologists*, Lujbljana, Slovenia, 29 June–3 July, 2003, abstract P 4–71.