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Ciliates from ancient permafrost: Assessment of cold resistance of the resting cysts

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Abstract

There is evidence that resting cysts of soil ciliates and numerous taxa of other protists can survive in permafrost for thousands of years at subzero temperatures; however, our knowledge about mechanisms of long term cryobiosis remains incomplete. In order to better understand the means by which ancient cysts survive, we investigated resistance to cyclical supercooling stress of resting cysts of the soil ciliate *Colpoda steinii* (Colpodida, Ciliophora). Three clonal strains were used for comparison, isolated from Siberian tundra soil, ancient Holocene (5–7,000 y) and late Pleistocene (32–35,000 y) permafrost sediments. To determine the viability of the ancient and contemporary ciliate cysts we improved and validated a cultivation-independent method of vital fluorescent staining with a combination of two nucleic acid binding dyes, acridine orange and propidium iodide. The viability of *Colpoda steinii* cysts during low-temperature experiments was measured using both the proposed vital fluorescent staining method and standard germination test. Our results indicate that the dual-fluorescence technique is a more accurate, rapid, and efficient method for estimating cyst viability. We found that cysts of ancient ciliates display lower tolerance to the impact of cyclical cold compared to cysts of contemporary ciliates from Siberian permafrost affected soils.

Keywords: *Colpoda steinii*; Permafrost; Viability; Fluorogenic dyes; Excystation

Introduction

A vast variety of microorganisms are preserved in subsurface permafrost layers at subzero temperatures for a geologically significant period of time (Gilichinsky and Rivkina 2011; Gilichinsky et al. 1992). "Viable fossils" isolated from permafrost include both anaerobes and aerobes, spore-forming and non-spore forming bacteria, actino- and micromycetes, cyanobacteria, green algae, yeast and protozoa. Our understanding of the adaptive and protective mechanisms enabling long-term cryptobiosis of eukaryotic microorganisms remains limited. Possible mechanism for the survival of protists in deep permafrost sediments is the formation of a dormant stage (Kochkina et al. 2012; Shatilovich et al. 2009; Stoupin et al. 2012; Vishnivetskaya et al. 2001). It is well known that heterotrophic protist dormant stages, named resting cysts, are extremely resistant to inhospitable conditions and can remain viable for a long time (Ekelund et al. 2002; Foissner 1987; Gutiérrez et al. 2001; Marquardt et al. 1966). This remarkable capability ensures

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the long-term survival and dispersal of numerous representatives of protists in water and soil environments (Finlay et al. 2000; Foissner 2011). Studies of the resting cysts of heterotrophic protists isolated from permanently frozen deposits in Siberian Arctic different in their genesis, lithology and age require the development of cyst viability assays that are not based on the excystation rate. One difficulty connected to using the excystation rate method is uncertainties about the temporal characteristics of ancient ciliates clonal life cycles. As was shown earlier, microorganisms revived from permafrost may require a long incubation period, which is likely related to reparation processes necessary to deal with reversible metabolic damage that the cells accumulate through long-term cryobiosis (Gilichinsky and Rivkina 2011; Vishnivetskaya et al. 2003).

The methods that can be applied to assessing the viability of protist resting cysts include excystation *in vitro* and dye exclusion. Excystation *in vitro* is a widely employed approach based on estimation of the excystation rate (Ekelund et al. 2002; Jarroll et al. 1981; Müller et al. 2008, 2010). However, the main drawback of the technique is that the triggering mechanisms of excystation are not well explored (Ekelund and Ronn 1994). Even under the most favourable conditions, a portion of the population remains encysted. This phenomenon has been termed "ciliatostasis" (Ekelund et al. 2002; Foissner 1987, 2011) and leads to underestimating the total number of viable cysts in a studied sample or culture. In addition, the rate of excystation can be affected by the growth medium, differences between strains, frequency of observation, the age of the cysts, and environmental or cultivation conditions of cyst formation (Chambers and Thompson 1974; Foissner 2006, 2011; Müller et al. 2006; Nakamura and Matsusaka 1991).

Dye exclusion is an alternative approach to assessing cyst viability that is based on the integrity of the cellular plasma membrane. One of the most promising dye exclusion methods is live/dead fluorescent staining based on different viability characteristics such as membrane integrity and metabolic activity (*e.g.*, esterase enzyme activity, respiration, *etc.*). Numerous fluorophores, *e.g.* propidium iodide (PI), fluorescein diacetate (FDA), DAPI, Sytox Green, Syto-9, Syto-59, Congo Red and Calcofluor White are routinely used in assessing the viability of parasitic protist oocytes (Bukhari et al. 2000; Campbell et al. 1992; Conell et al. 2001; Connelly et al. 2007; Neumann et al. 2000; Sauch et al. 1991; Smith and Smith 1989) and dinoflagellate cysts (Binet and Stauber 2006; Gracia et al. 2013; Gregg and Hallegraeff 2007). Although this assay is simple to perform, to the best of our knowledge, it has not been tested on cysts of soil heterotrophic protists.

Thus, one goal of this work was to develop and to validate live fluorescence staining techniques to estimate the viability of ancient and contemporary soil ciliate resting cysts. Another goal was to assess cold resistance of studied cysts under conditions of an environmental experiment. In this experiment we attempted to simulate environmental stress typical for polar soil habitats – frequent cycles of supercooling–heating in the spring and autumn seasons. The application of the fluorophores propidium iodide (PI) and acridine orange (AO) has the potential to indicate cyst membrane integrity and viability (Taylor and Wang 1989). In order to explore the efficiency of the staining method we compared the results obtained by the proposed culture-independent dual-fluorescence assay with those of the *in vitro* excystation method.

In the present study we tested the proposed approach on resting cysts of the Siberian soil ciliate *Colpoda steinii* Maupas, 1883, isolated from permafrost affected soil and Holocene and late Pleistocene permafrost sediments.

The purpose of the present study was, thus, twofold: (1) to develop a reliable fluorescent staining protocol for cyst viability assessment in soil ciliates and (2) to estimate the impact of stressful cyclical supercooling challenges on the cyst viability of ciliates isolated from permafrost sediments and modern tundra soils.

Material and Methods

Sampling and cultivation

The study sites were located on the Kolyma lowlands in the tundra zone of the northeast sector of Siberia (67–70° N, 152–162° E). Permafrost temperatures at the sites varied from −10 to −12 °C and its thickness reached 800 m (Gilichinsky and Rivkina 2011). Samples of modern soil, including peat, litter, and mosses, were taken from the upper 5-cm layer in wet polygonal tundra of the Khomus-Yuryakh river valley. Samples of the frozen buried Pleistocene soil were collected from outcrops of the Malyi Anyui River at depths of 20–40 m below the surface. This paleosol was formed under tundra-steppe conditions during the MIS stage 3 (26–50 ka BP). Samples of Holocene permafrost deposits were collected from intact frozen cores from a borehole drilled near Lake Oler. Sampling under sterile conditions was performed using procedures that have been described in a number of previous publications (Gilichinsky et al. 2007; Shi et al. 1997). Methods of processing the collected material and isolating and cultivating ancient protists that survived as resting cysts in the permanently frozen deposits have also been previously described in detail (Stoupin et al. 2012; Shatilovich et al. 2009). The age estimate of the deposits was based on stratigraphy and radiocarbon dating (Gubin and Lupachev 2012; Zanina et al. 2011).

Cultivable ciliates were isolated from the soil and permafrost samples during enrichment cultivation without nutritional supplementation by the non-flooded plate method (Foissner 1993). The cultivation took place under two temperature regimes (8 °C and 20 °C). Strains of *Colpoda steinii* were established as clonal cultures from the enrichment culture and maintained at 22 °C in NCL medium in the presence of sterile wheat grain or *Escherichia coli* cells as a food source. The NCL medium was prepared by adding 1 g of Cerophyll (cereal grass leaves) to 1 l of Prescott and James's