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Short communication

Retrieval of deeply buried culturable fungi in marine subsurface sediments, Suruga-Bay, Japan

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ABSTRACT

Culturable fungal communities were investigated in marine subsurface sediment cores obtained at three different sites in Suruga Bay. Sediment core samples were examined at five different depths, namely 3 m, 12 m, 21 m, 31 m and 40 m below the seafloor (mbsf) at each site. Although the occurrence and diversity of culturable fungi in subsurface sediments appeared extremely low, fungi were successfully cultured from 5 out of 15 samples, including a sample taken from 40 m below the seafloor. The most frequently detected fungal species were *Aspergillus* spp. followed by *Pichia* and *Cadophora*. Two possible novel spp. belonging to the phylum Ascomycota were also isolated.

Although the presence of fungi in deep subsurface environments has been suggested in some recent reports, there has been only limited information available describing the identification and diversity of culturable fungi from deep subsurface sediments. Our results provide further evidence to support that fungi persist in deep marine subsurface biospheres. Also, it was suggested that marine subsurface biospheres can be a new fungal resource. Further investigation on cultured deep subsurface fungi will provide a better understanding of the ecology and physiology of extremotolerant fungi and their potential for biotechnological application.

Since the discovery of the subseafloor biosphere, life is shown to extend deeper into the Earth's subsurface. Initially, the deep subsurface biosphere was considered to be the realm of prokaryotic organisms, because of the constraints of temperature, energy, dioxygen and space, which seemed to preclude the possibility of more complex, multicellular eukaryotic organisms from surviving at deep depths. However, the more we study the deep biosphere, the more it becomes apparent that prokaryotes are not the only organisms adapted to these extreme conditions. Edgcomb et al. (2011) investigated the eukaryotic microbial communities in deep-sea subsurface sediments up to 37 m below the seafloor (mbsf) by DNA and RNA based clone library analyses and revealed the molecular signatures of diverse eukaryotes such as, Fungi, Animalia, Chlorophta, Rhodophyta, Apicomplexa, Rhizaria and unclassified eukaryotes. Furthermore, the presence of a bacteriophagous nematode in fracture water retrieved from the terrestrial deep subsurface (0.9–3.6-km below the surface) was reported by Borgonie et al. (2011). The discovery of multicellular life in these reports implicates the presence of diverse eukaryotic life in the subsurface biosphere and also suggested that deep ecosystems are more complex than previously accepted. In the last decade, evidence has accumulated to support the existence of fungi in deep subsurface sediments. The very first recovery of culturable fungi from deep-sea sediments more than a few metres below the seafloor was reported by Raghlukumar et al. (2004). It was thought that these fungi only persist as vegetative spores. However, Orsi et al. (2013a,b) showed some evidence that fungi are active in deep subseafloor. Furthermore, Redou et al. (2014) and Ciobanu et al. (2014) reported the presence of fungi at record depths,

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1740 mbsf in the subseafloor of the Canterbury Basin. Diversity and physiology of culturable fungal communities from these deep sediments were analysed by Redou et al. (2015) and their adaptation to the environment was revealed. It is now evident that the deep subsurface biosphere is another ecological niche for fungi. However, to date there are still only a limited number of investigations of culturable fungal diversity in deep subsurface sediments.

In the present study, we investigated the diversity of culturable fungi in deep subsurface sediments up to 40 mbsf. Investigation was carried out using sediment samples at five depths, approximately 3, 12, 21, 31 and 40 mbsf collected during the CK09-01 Expedition 903 at three different sites C9006A (B1: 34°52′ N, 138°34′ E, 756 m deep), C9007A (B2: 34°51′ N, 138°34′ E, 767 m deep) and C9008A (B3: 34°51′ N, 138°33′ E, 731 m deep) in Suruga Bay, Japan. The cores were obtained by a hydraulic piston coring system (HPCS), producing the least external chemical and microbiological contamination during the drilling operation, with sediment samples for cultivation analysis taken only from the inner section of each core to avoid contamination. In addition, control plates were always prepared and exposed during culturing procedures to check for laboratory contamination. Approximately 5 ml of 15 subsurface sediments (all very dried silty clay) were suspended in 10 ml of sterile artificial sea water and vortexed for 1 min. 2 ml aliquots were mixed with approximately 50 ml of MYP medium agar (Malt extract 4 g, Yeast extract 2 g, Polypeptone 2 g per litre of artificial sea water) and 1/5 diluted Marine Agar 2216 (Difco) supplemented with antibiotics (Streptomycin 200 mg, Penicillin 200 mg) prior to getting cold and solidifying in a 150 mm Petri dish. The plates were incubated at 4, 15 °C and room temperature for up to 6 months under aerobic and atmospheric pressure. For the identification of cultured fungi, genomic DNA was amplified with primer, ITS-1FS and ITS4 (White et al. 1990; Gardes and Bruns, 1993). PCR amplicons were sequenced and all confirmed sequences were compared with sequences stored in the GenBank database using the BLAST alignment software (http://www.blast.genome.ad.jp/). All sequences were subsequently submitted to DDBJ and accession numbers were issued (AB725389–AB725398). For methane measurement, the 5 cm^3 of sediment samples were taken by putting a syringe into cored sediments twice (to 4 cm deep from the bottom of the section) immediately after the core was cut into sections. The samples were put into 20 cm^3 vials and sealed. After heating in a 70 °C oven, 5 cm^3 of headspace gases were taken and methane was measured by a gas chromatograph with flame ionization detector (FID). For weight percent total organic carbon (TOC) and sulfur measurement, approximately 3 g of the sediments were freeze-dried for 12 h. After pounding in a mortar, 20 mg of the dried sediments were analyzed for quantification of sulfur using a FLASH EA 1112 series (Thermo Electron Corp.).

It appeared that the number of fungi which could be cultured by conventional culturing methods from marine subsurface sediments was very low. Fungi could not be isolated from the majority (10 out of 15) of marine subsurface sediment samples. However, a total of 11 fungal strains were successfully isolated from 5 out of 15 samples (B1_1_3, B2_4_3, B2_5_3, B3_2_3, B3_3_3) (Table 1). The most diverse fungi (7 species) were isolated from sediment collected 11.5–13 mbsf at site B3 (B3_2_3). Culturable fungi were obtained in the deepest sediment samples (40 mbsf) investigated but only from samples taken from site B2. Identification by ITS sequence analyses suggested that all isolated fungal species belonged to the phylum Ascomycota. The most frequently isolated fungal species were *Aspergillus* spp. (from 4 samples: B2_4_3, B2_5_3, B3_2_3, B3_3_3), followed by *Pichia* spp. (from 2 samples: B1_1_3, B3_2_3). 9 out of 11 isolates showed more than 99% sequence similarity to previously cultured and well-described fungi. B3_2_3_SC2 (AB725392) showed only 92% similarity to *Phaeosphaeriopsis musae* (DQ659336) and B3_2_3_SC3 (AB725393) showed 94% similarity to *Phaeoisaria clematidis* (EU552148). The phylogenetic analysis for these possible novel species is shown in Fig. 1.

Aspergillus spp. were detected as the most frequently cultured fungi in marine subsurface sediments and were found in the deepest sediments we examined, as deep as 40 mbsf. *Aspergillus* spp. are known to be globally distributed taxa in marine environments, even in deep-sea extreme environments as well as terrestrial environments. *Aspergillus* species have also previously been isolated from the shallower parts of deep-sea sediment collected from 3.7 mbsf (Raghlukumar et al. 2004), as well as deep subsurface sediments up to 403 mbsf (Redou et al. 2015). It is now suggested

Table 1
Sequence-based identification of cultured fungi from deep subsurface sediment samples. Grey highlighting indicates two possible novel fungal species.

Sample name	Depth (mbsf[a])	Isolate name	Closest relative	Phylum	QC/MI	DDBJ accession no.
C9006A (B1)						
B1_1_3	3	B1_1_3_SC14	*Pichia guilliermondii* (AM160625)	Ascomycota	100%/99%	AB725388
B1_2_3	12	–	–	–	–	–
B1_3_3	21	–	–	–	–	–
B1_4_3	31	–	–	–	–	–
B1_5_3	40	–	–	–	–	–
			C9007A (B2)			
B2_1_3	3	–	–	–	–	–
B2_2_3	12	–	–	–	–	–
B2_3_3	21	–	–	–	–	–
B2_4_3	31	B2_4_3_SC10	*Aspergillus versicolor* (EF652480)	Ascomycota	100%/100%	AB725389
B2_5_3	40	B2_5_3_SC9	*Aspergillus versicolor* (JN093265)	Ascomycota	100%/100%	AB725390
			C9008A (B3)			
B3_1_3	3	–	–	–	–	–
		B3_2_3_SC1	*Cadophora luteo-olivacea* (FJ430742)	Ascomycota	100%/100%	AB725391
		B3_2_3_SC2	*Phaeosphaeriopsis musae* (DQ885894)	Ascomycota	92%/92%	AB725392
		B3_2_3_SC3	*Phaeoisaria clematidis* (EU552148)	Ascomycota	100%/94%	AB725393
B3_2_3	12	B3_2_3_SC4	*Aspergillus versicolor* (JN093265)	Ascomycota	100%/100%	AB725394
		B3_2_3_SC5	*Aspergillus versicolor* (EF652480)	Ascomycota	100%/100%	AB725395
		B3_2_3_SC6	*Paecilomyces* sp. (JN546116)	Ascomycota	100%/99%	AB725396
		B3_2_3_SC12	*Pichia guilliermondii* (AM160625)	Ascomycota	100%/99%	AB725397
B3_3_3	21	B3_3_3_SC8	*Aspergillus sydowii* (EF652473)	Ascomycota	100%/100%	AB725398
B3_4_3	31	–	–	–	–	–
B3_5_3	40	–	–	–	–	–

[a] mbsf = metre below the seafloor.