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Short communication

Ascomycete phylotypes recovered from a Gulf of Mexico methane seep are identical to an uncultured deep-sea fungal clade from the Pacific

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ABSTRACT

Deep-sea endemic fungi are one component of an under-sampled invisible biosphere whose contribution to benthic ecosystems is not yet understood. In the last decade, molecular techniques have facilitated the discovery of several new deep-sea fungal groups, especially in habitats such as hydrothermal vents and methane seeps. We assessed fungal diversity at a methane seep in the Gulf of Mexico by sequencing partial *ITS* and *LSU* gene regions from environmental DNA recovered from microoxic and anoxic sediment. While most phylotypes were closely allied with common fungal species, the dominant phylotype did not match any known terrestrial species and aligned with an uncultured deep-sea fungus found in oxygen-depleted sediment at multiple sites in the Pacific Ocean. Despite its apparently broad distribution and frequent occurrence in oxygen-depleted sediment, the ecological role of this phylotype is not yet known.

The deep sea is home to a rich and largely unexplored microbial biosphere. Culture-independent studies have produced an ever-growing body of knowledge regarding the diversity, distribution and ecology of deep-sea microbes. Despite many large scale investigations into microbial diversity (i.e. Sogin *et al.* 2006), there have been relatively few studies of deep-sea fungi. *Marine Mycology*, the first exhaustive review of marine-occurring fungi, listed only five species endemic to the deep sea (Kohlmeyer & Kohlmeyer 1979) and that number did not increase until the 21st century (Hawksworth 2001). Fungal species isolated from the deep sea presently number in the low hundreds (Raghukumar *et al.* 2010).

Using culture-based methods, fungi were first identified from the Atlantic abyssal plane (4 450 m) in 1964 (Roth *et al.* 1964). Since then, fungi have been reported from many deep ocean habitats including deep-sea sediment (Damare *et al.* 2006), hydrothermal vents (Gadanho & Sampaio 2005), and methane seeps (Takishita *et al.* 2006). Culture-independent molecular techniques have greatly increased the rate of discovery of new fungal taxa from the oceans. While most fungi recovered from the deep sea are closely related to known terrestrial groups, several novel clades, known only from the deep sea, have recently been discovered (e.g., DSF-group1 – Nagano *et al.* 2010; *Candida oceani* – Burgaud *et al.* 2011;

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DSGM-64 and TAGIRI-23 – Jones et al. 2011). The ecology of these deep-sea fungal taxa remains largely unexplored.

The goal of this study was to establish a baseline assessment of fungal diversity at deep-sea methane seeps in the Gulf of Mexico. Methane seeps form when steep pressure gradients force hydrocarbon-enriched water upwards through the sediment (Levin 2005). Seep sediments are characterized by steep chemical gradients and shallow (<10 cm) anoxic layers and support microbial and animal communities that are dependent on chemosynthetic primary production (Levin 2005). Many fungi can thrive under conditions similar to those observed in deep sea (Lorenz & Molitoris 1997; Raghukumar et al. 2004) and a variety of fungi are able to grow under anaerobic conditions, including fermentative yeasts such as *Saccharomyces cerevisiae* and other members of Saccharomycotina (Ascomycota), members of Neocallimastigomycotina (Chytridiomycota) growing in cattle rumen, and litter decomposers in the early diverging lineage Blastocladiomycota (Held et al. 1969; Liggenstoffer et al. 2010). Evidence for any ecological roles played by potentially anaerobic fungi from deep-sea environments is not clear.

To investigate the distribution of fungi associated with deep-sea methane seeps, we collected sediment cores from the Alaminos Canyon 601 methane seep in the Gulf of Mexico (26 23.938 N, 94 30.589 W; 2 400 m) using an Ocean Instruments Mark III box corer outfitted with a transponder to monitor its location. Only one sediment core was successfully recovered from this location. Reduction/oxidation potential (redox) was recorded at 1 cm intervals for the first 15 cm of the core. Twenty-two sub-cores, ranging from 10 to 30 cm sediment depth, were taken from the core using serological pipettes (tip removed; 1 cm diameter, 33 cm length). Two sub-cores were preserved in 10 % formalin for microscopy and twenty sub-cores were preserved in 95 % ethanol for molecular analysis. All sub-cores were frozen at −20 °C and remained frozen until processed.

Sub-cores were sectioned at 1 cm intervals for microscopy and molecular analyses. Formalin preserved sub-cores were stained with BactiDrop Calcofluor (Remel: Lenexa, KS) and examined under a fluorescent microscope. No hyphal elements were observed. Environmental DNA from ethanol preserved sub-cores was extracted using a PowerSoil DNA extraction kit (MO BIO Laboratories: Carlsbad, CA) following manufacturer's protocols. An approximately 900-bp region containing portions of the internal transcribed spacer (*ITS*) and large subunit rDNA (*LSU*) gene was amplified using the primers ITS1 (5′–TCC GTA GGT GAA CCT GCG G), ITS4 (5′–TCC TCC GCT TAT TGA TAT GC), LROR (5′–ACC CGC TGA ACT TAA GC), and LR5 (5′–TCC TGA GGG AAA CTT CG). Environmental DNA was amplified according to the methods outlined in O'Brien et al. (2005), ligated into plasmids, transformed into *Escherichia coli* cells, and cloned, using a Topo-TA 5-min DNA cloning kit (Invitrogen: Carlsbad, CA) following manufacturer's protocols. Manufacturer's maximum recommended incubation times were used for each step. Colonies were screened, amplified, and sequenced on an ABI 3730xl DNA Analyzer following methods reported in O'Brien et al. (2005). Alaminos Canyon sequences were compared against the NCBI GenBank database. Neighbour-joining trees were constructed in Mega (version 4.0, 1 000 replications, Tamura et al. 2007) using highly similar matches and a representative subset of major fungal taxa (Fig 1). Sequences were deposited on GenBank (accession # **JF821197–JF821214**).

A total of 39 fungal sequences were recovered from Alaminos Canyon sediment. Ascomycota accounted for the majority of recovered sequences (27), followed by Basidiomycota (6), and Chytridiomycota (6). Number of recovered sequences was highest at the redox boundary in the sediment core (23 of 39 sequences). Transition from oxidized to reduced sediment occurred at approximately 4 cm below the surface. A phylotype within Ascomycota was found to occur almost exclusively within 1 cm of the redox boundary (18 out of 23

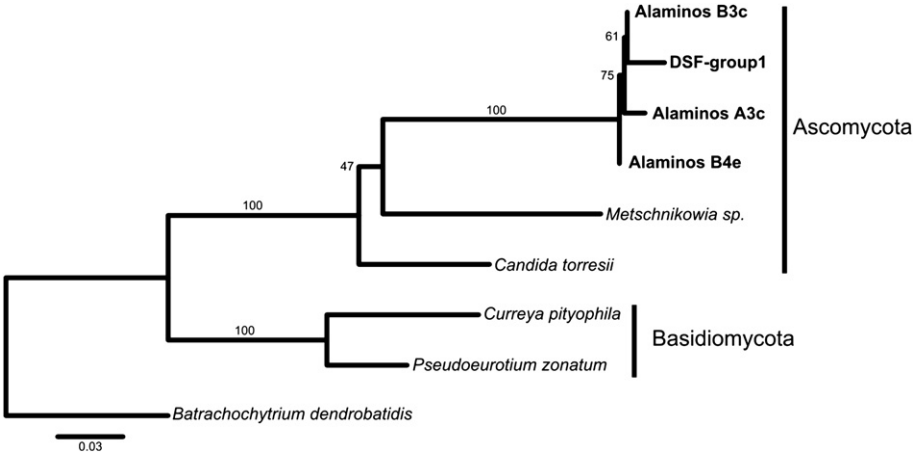

Fig 1 – Neighbour-joining tree of representative sequences from Alaminos Canyon phylotype: Alaminos Canyon B3c (accession # JF821209), Alaminos Canyon A3c (accession # JF821201), Alaminos Canyon B4e (accession # JF821213), DSF-group1 (accession # AB507846), closest NCBI GenBank match (*Metschnikowia* sp. – accession # FJ794943), and representatives of major fungal groups (Ascomycota: *Candida torresii* – accession # U45731; Basidiomycota: *Curreya pityophila* – accession # DQ384102 and *Pseudoeurotium zonatum* – accession # DQ470988; Chytridiomycota: *Batrachochytrium dendrobatidis* – accession # NG027619). Bootstrap values (1 000 replicates) are reported on or adjacent to each branch. Scale is nucleotide substitutions per base.

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sequences). This phylotype also comprised the majority of all sequences recovered from Alaminos Canyon sediment (25 out of 39 sequences).

Phylogenetic analysis revealed that the dominant phylotype recovered from Alaminos Canyon had no known cognates in terrestrial systems. This phylotype did align with other uncultured fungi from deep-sea sediments and clustered within clade DSF-group1 (Nagano *et al.* 2010; Fig 1). Nagano *et al.* (2010) reported the occurrence of this group in deep-sea sediments in the western Pacific (1 200–9 800 m). It has also been isolated from methane seeps near Japan (640 m; Takishita *et al.* 2007) and the Gulf of California in the eastern Pacific (1 600 m; Bass *et al.* 2007). DSF-group1 has been detected in several microoxic deep-sea environments, including bacterial mats and methane seep sediment (Takishita *et al.* 2007). This group's closest known relative is *Metschnikowia bicuspidata*, a parasitic fungus that occurs on freshwater Daphnia species (Nagano *et al.* 2010). Other fungal parasites are known from the deep sea. For instance, black yeasts were reported parasitizing *Bathymodiolus brevior* mussels from hydrothermal vents in North Fiji Basin (Van Dover *et al.* 2007).

Without morphological or ecological data, it is difficult to determine if uncultured fungi identified through molecular methods are members of the deep-sea community or contaminants with no significant ecologic contribution. Recent phylogenetic studies all reject a marine origin for Kingdom Fungi, and instead support the view that fungi from marine systems are derived from terrestrial cognates (James *et al.* 2006; Zuluaga-Montero *et al.* 2010). Even the most notorious marine fungus, *Aspergillus sydowii*, which caused massive coral die-offs in the Caribbean, is descended from a widespread lineage of terrestrial fungi (Weir-Brush *et al.* 2004). Several lines of evidence suggest that DSF-group1, thus far only identified through molecular methods, is endemic to the deep sea and not a contaminant. It is broadly distributed throughout the Pacific Ocean and found in the Gulf of Mexico, but it is not found in any terrestrial system; it diverges from its closest genetic match, with well supported phylogenetic trees (bootstrap = 100; Fig 1); and it has been consistently associated with oxygen-depleted environments, suggesting that it may take advantage of these environments either as a facultative anaerobe or by consuming other organisms that thrive there. This strongly supports the conclusion of Nagano *et al.* (2010) that there is a globally distributed, deep-sea endemic, fungal group within Ascomycota.

The discovery of a novel fungal group that is broadly distributed in deep-sea ecosystems suggests that further molecular studies could reveal a reservoir of previously unknown fungal biodiversity. Deep-sea communities, especially those restricted to chemoautotrophic ecosystems, tend to segregate by well-defined biogeographic provinces (Sibuet & Olu 1998; Van Dover *et al.* 2002). Fungal biogeography in the deep sea is poorly understood, but DSF-group1 appears to contradict this general trend. The larger question may be whether fungi endemic to the deep sea are rare colonists with a few well-distributed exceptions, such as DSF-group1, or cosmopolitan in low abundance throughout deep-sea ecosystems.

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