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Fungal diversity in deep-sea sediments associated with asphalt seeps at the Sao Paulo Plateau

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ABSTRACT

We investigated the fungal diversity in a total of 20 deep-sea sediment samples (of which 14 samples were associated with natural asphalt seeps and 6 samples were not associated) collected from two different sites at the Sao Paulo Plateau off Brazil by Ion Torrent PGM targeting ITS region of ribosomal RNA. Our results suggest that diverse fungi (113 operational taxonomic units (OTUs) based on clustering at 97% sequence similarity assigned into 9 classes and 31 genus) are present in deep-sea sediment samples collected at the Sao Paulo Plateau, dominated by Ascomycota (74.3%), followed by Basidiomycota (11.5%), unidentified fungi (7.1%), and sequences with no affiliation to any organisms in the public database (7.1%). However, it was revealed that only three species, namely *Penicillium* sp., *Cadophora malorum* and *Rhodosporidium diobovatum,* were dominant, with the majority of OTUs remaining a minor community. Unexpectedly, there was no significant difference in major fungal community structure between the asphalt seep and non-asphalt seep sites, despite the presence of mass hydrocarbon deposits and the high amount of macro organisms surrounding the asphalt seeps. However, there were some differences in the minor fungal communities, with possible asphalt degrading fungi present specifically in the asphalt seep sites. In contrast, some differences were found between the two different sampling sites. Classification of OTUs revealed that only 47 (41.6%) fungal OTUs exhibited > 97% sequence similarity, in comparison with pre-existing ITS sequences in public databases, indicating that a majority of deep-sea inhabiting fungal taxa still remain undescribed. Although our knowledge on fungi and their role in deep-sea environments is still limited and scarce, this study increases our understanding of fungal diversity and community structure in deep-sea environments.

1. Introduction

Fungi are one of the most important components in ecosystems and they occupy a wide variety of environments by virtue of their highly versatile physiology function (Gostincar et al., 2010; Tedersoo et al., 2014). It is now evident that deep-sea environments, where extreme conditions are present, are also an ecological niche for fungi. Since the first report of fungal isolation from deep-sea (Roth et al., 1964), many fungi have been isolated from various deep-sea environments, with the majority showing similarity to terrestrial species but also including some novel fungal species (Nagahama et al., 2001a; Nagahama, 03,2003; Raghlukumar et al., 2004; Damare et al., 2006; Burgaud et al., 2009, 2011; Singh et al., 2010; Manohar et al., 2015). In the last decade, fungal diversity in deep-sea environments has been investigated more intensively by culture-independent environmental DNA-based techniques, which have revealed diverse fungal phylotypes, including novel lineages (Bass et al., 2007; Lai et al., 2007; Nagano et al., 2010; Singh et al., 2011, 2012; Xu et al., 2014; Zhang et al., 2014). Highly novel lineages have been revealed, especially in unique deep-sea chemosynthetic ecosystems, such as hydrothermal vents and methane cold-seeps (Le Calvez et al., 2009; Nagahama et al., 2011).

Natural asphalt seeps formed by seabed accumulations of heavy hydrocarbons, are another feature of deep-sea ecosystems which can be associated with chemosynthetic communities (MacDonald et al., 2004; Jones et al., 2014). During the *lata-piuna cruise* 2013, natural asphalt seeps were newly discovered at the deep seafloor in the Sao Paulo Plateau off Brazil. There was no chemosynthetic community observed in this site, but more extensive and diverse epifauna were found in the

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presence of asphalt seeps. Fungi are known to be capable of degrading petroleum hydrocarbons, including heavy hydrocarbons, such as asphalt (Uribe-Alvarez et al., 2011; Nasrawi, 2012; Xue et al., 2015). The presence of fungi in oil-contaminated marine sediments is often reported and the dramatic increase of fungal communities in post-oil spill sediments has been observed in the Gulf of Mexico (Sadaba and Sarinas, 2010; Bik et al., 2012; Fasanella et al., 2012). However, fungal diversity associated with asphalt seeps in deep-sea environments has not been studied and remains unclear.

The aim of this study was to investigate fungal diversity in deep-sea sediments associated with asphalt seeps found in the Sao Paulo Plateau, in order to increase our knowledge of fungal communities in deep-sea ecosystems. To the best of our knowledge, this is the first report describing fungal diversity associated with asphalt seeps in deep-sea environments.

2. Materials and methods

In the present study, a total of 20 sediment samples (of which 14 samples were associated with asphalt seeps and 6 samples were not associated) originated from four different cores collected in two different sampling sites and were investigated using Ion Torrent PGM, targeting ITS region of ribosomal RNA.

2.1. Site description and sediment sampling

Four sediment core samples were collected using the human-occupied vehicle (HOV) *Shinkai 6500* at the Sao Paulo Plateau during the 2nd leg of the *Iata-piuna* cruise. Core 1345_3 and Core 1345_5 were sampled during dive no. 1345 operated on 17 May 2013 (20° 41. 14482' S, 38° 38. 1529' W; water depth = 2720 m). Core 1346_3 and Core 1346_4 were sampled during dive no. 1346 operated on 19 May 2013 (20° 43. 14482' S, 38° 38. 1529' W; water depth = 2651 m) (Fig. 1). Core 1345_5, Core 1346_3 and Core 1346_4 were sampled at asphalt seep sites. Core 1345_3 was sampled at the non-asphalt seep site. Each sediment core sample was cut into layers at different depths from the surface of the sea floor. A total of 20 sediment samples (1345_3_1~6, 1345_5_1~6, 1346_3_1~5, 1346_4_1~3) were obtained, of which 14 samples were from asphalt seep sites and 6 samples were from the non-asphalt seep site. The details of the sampling sites and the collected sediment samples are described in Table 1.

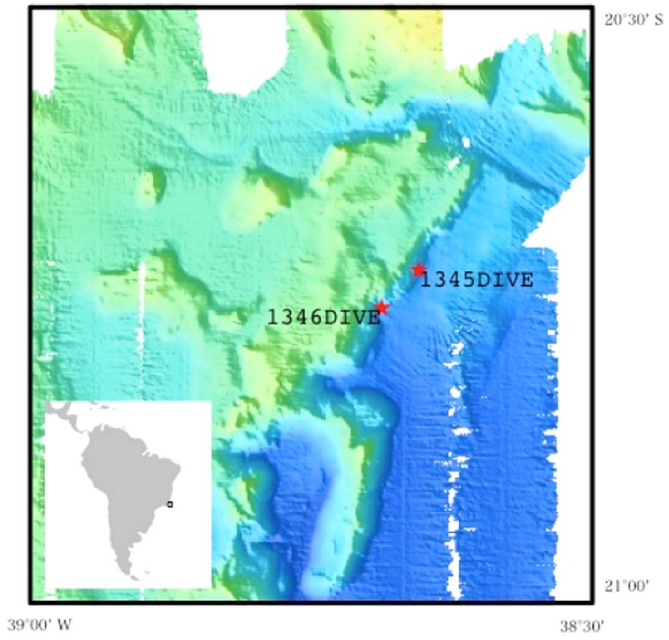

Fig. 1. Location of the deep-sea sediment sampling sites in the Sao Paulo Plateau.

2.2. DNA extractions, PCR amplifications and sequencing

DNA was extracted from 0.5 g of each sediment sample by the employment of ISOIL for beads beating kit (Nippon Gene, Japan), in accordance with the manufacturer's instructions. Extracted DNA was stored at −20 °C, prior to PCR amplification. For extractions, a negative extraction control containing all reagents minus sediment was performed. Fungal DNA was amplified with primer set ITS-1FS (5′-CTTGGTCATTTAGAGGAAGTAA-3′) / ITS4 (5′-TCCTCCGCTT˙ATTGATATGC-3′) as a primary primer set and ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) / ITS2 (5′-GCTCCGTTCTTCATCGATGC-3′) as a nested primer set (White et al., 1990; Gardes and Bruns, 1993). PCR reaction mixes (20 μl) contained: 10 μl of SYBR Premix Ex Taq (TaKaRa, Japan), 0.4 μM (each) of a pair of primers and 1–2 μl of DNA template (10–100 ng). For the nested PCR, 0.5 μl of primary PCR product was used as a DNA template. The 7500 Real Time PCR System (Applied Biosystems) was used to determine the optimal cycle number by reference to cycle threshold (Ct) values for Ion Torrent PGM analysis. The real- time PCR conditions used were 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 34 s, and 95 °C for 15 s, followed by 60 °C for 60 s. Ct values were defined as the number of cycles required for normalized fluorescence to reach a manually set threshold of 20% total fluorescence. PCR amplification was performed in a GeneAmp® PCR system 9700 (Applied Biosystems) with calculated Ct value, which was 19 for all samples, with the same conditions as the real-time PCR. The PCR products were purified using the Agencourt AMPure XP Reagent (Beckman Coulter, Brea, CA, USA). The purified PCR amplicons were end-repaired using the Ion Plus Fragment Library Kit (Life Technologies Inc., Grand Island, NY, USA), following the manufacturer's protocol. The end-repaired amplicons were purified using the Agencourt AMPure XP Reagent. Sequencing adapters with the sample identification barcoding key were ligated using an Ion Xpress Fragment Library Kit, following the manufacturer's protocol. The adapter-ligated and nick-translated amplicons were purified using the Agencourt AMPure XP Reagent. The concentrations of the prepared libraries were determined by quantitative PCR using the Ion Library Quantitation Kit (Life Technologies Inc.). The amount of library required for template preparation was calculated using the template dilution factor calculation described in the protocol. Diluted libraries were pooled for library amplification using the Ion One Touch and ES systems (Life Technologies Inc.). Emulsion PCR to incorporate the library to the sequencing beads was performed using the Ion OneTouch instrument with an Ion OneTouch OT2 400 Kit (Life Technologies Inc.). Finally, the library sample was sequenced on an Ion Torrent Personal Genome Machine using an Ion 318 chip and the Ion PGM 400 sequencing Kit (Life Technologies Inc.), following the manufacturer's protocols. The raw sequence data (.fastq file) are available in the DNA Data Bank of Japan (DDBJ) under accession number DRA004208-004227.

2.3. Data processing and analyses

The sequence data was analyzed using the Mothur pipeline (v. 1.32.1) following a modified standard operating procedure (Schloss et al., 2011). In brief, data was subjected to quality control, whereby each sequence was screened for a match to the sequencing primer and thresholds for average Phred quality score ($Q \geq 20$), ambiguous bases (count = 0), and homopolymers (length ≤ 8). Sequences shorter than 100 bp after quality trimming were not considered. All potentially chimeric sequences were identified using Mothur-embedded UCHIME (chimera.uchime) (Edgar et al., 2011) and were removed. The sequence dataset was normalized to 23,550 sequences per sample (the smallest sample size) to reduce bias associated with different numbers of reads in the different samples (Gihring et al., 2012). Unique sequences were pairwise aligned (Needleman and Wunsch, 1970) and the resultant distance matrix clustered into operational taxonomic units (OTUs) using the nearest neighbor algorithm at > 97% similarity. Singleton