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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.

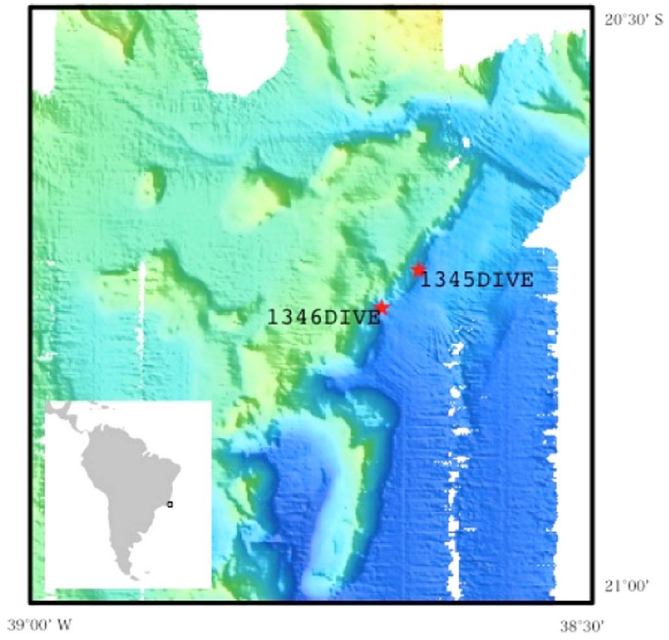

Fig. 1. Location of the deep-sea sediment sampling sites in the Sao Paulo Plateau.

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

Table 1
Details of the sediment samples, obtained sequence reads, fungal OTU richness, coverage, and diversity indices.

Sample Name	Presence of asphalt seep	Depth below the seafloor (cm)	Sequence data filtering			Diversity			
			No. of reads		% of high quality reads	Number of observed OTUs (97%)	Good's coverage (%)	Shannon (H)	Simpson (1/ *D*)
			Before filtering	After filtering					
1345 3–1	–	0–1	155,195	49,543	31.92	25	99.95	1.47	3.42
1345 3–2	–	1–4	119,866	38,730	32.31	30	99.98	1.52	3.43
1345 3–3	–	4–7	133,907	48,036	35.87	12	100.00	1.04	1.99
1345 3–4	–	7–10	230,909	79,748	34.54	10	100.00	1.67	4.35
1345 3–5	–	10–13	129,255	44,588	34.50	15	99.99	1.54	3.69
1345 3–6	–	13–16	109,285	32,879	30.09	32	99.96	1.73	4.53
1345 5–1	○	0–1	219,173	76,895	35.08	11	100.00	1.57	3.82
1345 5–2	○	1–4	143,212	53,063	37.05	12	100.00	1.45	3.40
1345 5–3	○	4–7	162,682	58,634	36.04	10	100.00	1.48	3.68
1345 5–4	○	7–10	145,858	52,160	35.76	13	99.99	1.59	4.02
1345 5–5	○	10–13	85,469	27,744	32.46	33	99.93	1.69	4.27
1345 5–6	○	13–16	73,048	25,616	35.07	35	99.94	1.72	4.20
1346 3–1	○	0–1	100,951	33,498	33.18	33	99.94	1.56	3.76
1346 3–2	○	1–4	77,688	26,275	33.82	33	99.97	1.62	3.85
1346 3–3	○	4–7	68,072	23,550	34.60	47	99.92	1.73	4.13
1346 3–4	○	7–10	109,907	40,498	36.85	18	99.97	1.43	3.42
1346 3–5	○	10–13	133,752	41,361	30.92	18	100.00	1.55	3.83
1346 4–1	○	0–1	179,663	60,896	33.89	33	99.96	1.64	4.13
1346 4–2	○	1–4	170,981	60,240	35.23	54	99.91	1.76	4.35
1346 4–3	○	4–7	75,410	25,041	33.21	35	99.95	1.73	4.38

OTUs (n = 1582) were removed as most pyrosequencing singletons are considered to be artifacts (Tedersoo et al., 2010). Classification of the sequences was performed using the UNITE + International Nucleotide Sequence Databases (INSD: NCBI, EMBL, DDBJ) ITS reference database (ver. 6; released on September 10, 2014) with the BLASTn algorithm (Abarenkov et al., 2010). Results were then confirmed by using the top-100 best BLASTn analyses, which were performed manually (https://blast.ncbi.nlm.nih.gov/). Some results were modified when confirmed by manual analysis. The diversity of fungal communities in each sample was compared using multiple metrics for rarefaction, observed OTU richness, Good's coverage (complement of the ratio between local singleton OTUs and the total sequence count) (Good and Toulmin, 1956), Simpson diversity index (1/D) (Simpson, 1949), Shannon diversity index (H) (Shannon, 1948), calculated in Mothur software package. Community similarities across the samples were visualized using non-metric multidimensional scaling (NMDS) based on the Bray-Curtis metrics in R (R Core Team, 2015). NMDS was selected as a preferred ordination procedure because it makes few assumptions about the distribution of the species. Bray-Curtis metrics was chosen because it is not affected by the number of null values between samples and these methods are commonly used in microbial ecology studies (Ramette, 2007). Ordination plots were created using the "metaMDS" function in the R vegan package (Oksanen et al., 2013), which incorporated relative abundance (transformation first square-root then Wisconsin double standardization) or presence–absence of OTU data. The effect of the presence of asphalt seep, sampling sites and depth from the sea floor on fungal community structure was assessed by permutational multivariate analyses of variance (PERMANOVA) (Anderson, 2001). PERMANOVA was selected as it is suggested to be much more robust to correlations and heterogeneous variances and generally more powerful in detecting changes in community structure, also it is widely used in ecology (Anderson and Walsh, 2013). These analyses were conducted using the "Adonis" function of the R vegan package (Oksanen et al., 2013), with 999 permutations. The effect of the presence of asphalt seep and sampling sites on individual OTUs and mean values of number of OTUs, Shannon diversity index and Simpson diversity index was also analyzed by using Student's or Welch's *t*-test. *F*-test (ref) was performed prior to *t*-test and Student's *t*-test (ref) was applied for samples with homogeneity of variance and Welch's *t*-test (ref) (was applied for samples with heterogeneity of variance) in Excel.

3. Results

3.1. Sequence analysis

A total of 2,624,283 sequences with an average read length of 192 bp were generated by an Ion Torrent PGM for 20 sediment samples collected from the Sao Paulo Plateau (of which, 14 samples were associated with asphalt seeps and 6 samples were not). After quality control, a total of 898,995 sequences (34%) were used for analysis. The numbers of sequences for each sample are listed in Table 1. Clustering at 97% identity produced 1695 unique OTUs across the 20 sediment samples, of which 1582 OTUs were singletons. The remaining 113 OTUs were used in further analyses. Rarefaction curve showed a plateau or a decline in the slope (Fig. 2) and Good's coverage was higher than 99.9% (Table 1) throughout the samples. This data indicates excellent OTU coverage afforded by the deep sequencing.

3.2. Fungal diversity

The highest number of OTUs (54 OTUs) was obtained in the sample

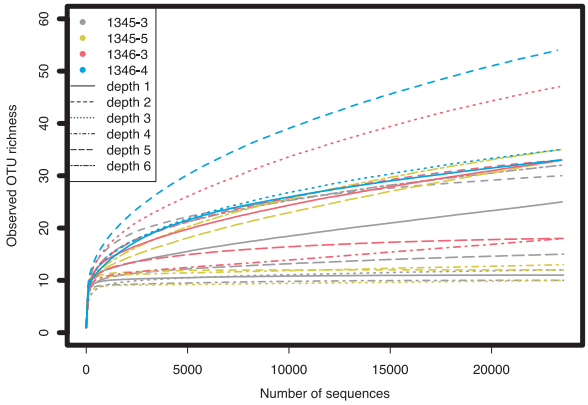

Fig. 2. Rarefaction curves of the observed fungal OTU richness at 97% sequence similarity in each sample.

Table 2
The effect of the presence of asphalt seeps and sampling sites on fungal diversity. ns: not significant.

Diversity index	Asphalt seeps			Sampling site		
	Absent	Present	*P* value	1345	1346	*P* value
OTUs	21	19	ns	20	34	*
Shannon (H)	1.5	1.58	ns	1.54	1.63	ns
Simpson (1/*D*)	3.57	3.90	ns	3.73	3.98	ns

*$p < 0.05$.

1346_4_2 and the lowest number of OTUs (10 OTUs) was observed in 1345_3_4 and 1345_5_3 (Table 1). The effect of the presence of asphalt seeps and different sampling sites on fungal diversity was assessed by comparing the value of multiple diversity indices (Table 2). Observed OTUs number was slightly lower in samples from asphalt seeps, and both Shannon and Simpson diversity indices showed slightly higher values in samples from asphalt seeps. Overall, it was suggested through *t*-tests that there was no significant difference in fungal diversity between the samples with the presence and the absence of the asphalt seeps. However, significant difference ($p = 0.013$) was observed in OTU richness between the samples collected from two different dive points. Higher number of OTUs were observed in samples from dive point 1346 (mean: 34 OTUs) than 1345 (mean: 20 OTUs). Both Shannon and Simpson diversity indices value were also higher in the samples from dive point 1346, however not at a statistically significant level (Table 2). Unique and shared OTU counts between the asphalt seep site and non-asphalt seep site (OTUs only from #1345 sampling site were used to match the number of samples), and between the #1345 and #1346 sampling sites are shown in Fig. 3. 27 OTUs were shared in both asphalt and non-asphalt seep sites. 23 OTUs were unique to the asphalt seep sites and 25 OTUs were unique to the non-asphalt site (Fig. 3A). 65 OTUs were shared in both #1345 and #1346 dive sites. 36 OTUs were unique to the #1346 dive site and 12 OTUs were unique to the #1345 dive site (Fig. 3B).

3.3. Fungal communities

Classification of the observed 113 OTUs are shown in Table 3. Composition of the OTUs at the phylum level among the 20 samples from the Sao Paulo Plateau were 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%) (Fig. 4A). Fungal communities were dominated by Eurotiomycetes (23 OTUs), followed by Sordariomycetes (16), Unclassified (16), Leotiomycetes (15), Saccharomycetes (15), Dothideomycetes (14), Microbotryomycetes (7), Tremellomycetes (4), Cystobasidiomycetes (2), and Pezizomycetes (1) (Fig. 4B). The most frequently detected OTU genus was *Penicillium* (16 OTUs) followed by *Cadophora* (12), *Galactomyces* (10), *Preussia* (6), *Rhodosporidium* (5), *Stachybotrys* (5), *Trichoderma* (5), *Aspergillus* (4), and *Pseudeurotium* (4). The three most abundant OTUs were *Penicillium* sp. (33% of the sequences), *Cadophora malorum* (28%) and *Rhodosporidium diobovatum* (23%). These OTUs

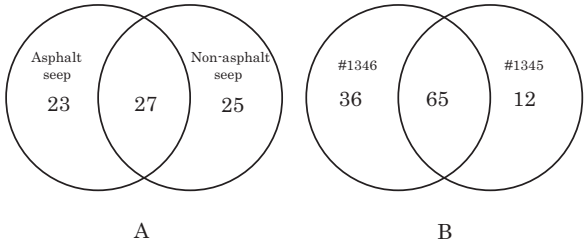

Fig. 3. Venn diagram showing the number of OTUs that were shared or unique (A) in asphalt seep site and non-asphalt seep site (B) in sampling site #1345 and #1346.

were dominated throughout all the samples and account for 84% of all the sequences. Other relatively abundant 6 OTUs [*Galactomyces pseudocandidum* (4.2%), *Guehomyces pullulans* (3.7%), *Acrostalagmus luteoalbus* (3.2%), *Stachybotrys chartarum* (1.5%), *Leucosporidiella yakutica* (0.8%), *Preussia typharum* (0.6%)] were also observed throughout the samples. The majority of OTUs (n = 104) accounts for less than 0.5% of the sequences (Table 3). Classification of OTUs revealed that 42 out of 113 (37.2%) fungal OTUs exhibited > 98% sequence similarity, and 47 (41.6%) exhibited > 97% similarity, to pre-existing ITS sequences in public databases.

Distribution and abundance of fungal OTUs at the class level in each sample is shown in Fig. 5. Although in each sample there is some difference in the abundance of each fungal class, the composition of fungal communities throughout all the samples showed a similar pattern.

Analysis on individual OTUs, OTU18, 28, 29, 42, 49 and 52 showed statistical significance in their (increased) distribution pattern, when in the presence of asphalt seeps. On the other hand, OTU13, 14, 17, 18, 23, 27, 29, 32, 39, 42 were distributed more abundantly and specifically in the samples from dive point 1346, with statistically significant levels ($p < 0.05$, Table 3). PERMANOVA analysis showed significant difference in fungal community structure between samples from dive point 1345 and 1346 ($p = 0.01$, Table S1). However, there was no significant difference suggested by either the presence of asphalt seep or different depth from the seafloor. NMDS ordination of the fungal community structure (both abundance-based and presence/absence based metrics) did not show prominent difference but the ellipses represent with 95% confidence interval indicate that there is some trend between the sampling sites and presence of asphalt seep but not with the depth from the sea floor (Fig. 6).

4. Discussion

The presence of fungi in deep-sea environments, including deep marine subsurface and their ecological importance in the ecosystems, is recognized with much interest recently. However, our current knowledge about fungal diversity in deep-sea environments is only available within a limited number of researched regions and environments. The aim of this study was to investigate the fungal diversity in one of the unique deep-sea environments, asphalt seeps (without chemosynthetic fauna) found in the Sao Paulo Plateau during the *lata-piuna* cruise in 2013.

4.1. Fungal communities in deep-sea sediments at the Sao Paulo Plateau

A total of 113 OTUs were recovered from deep-sea sediment samples collected at the Sao Paulo Plateau. Although the data from different study designs cannot be easily compared, this number is higher than those previously reported from several deep-sea environments by cloning methods (24 OTUs, restriction analysis: Lai et al., 2007; 9 OTUs, 98% similarity: Le Calvez et al., 2009; 43 OTUs, 99% similarity: Nagano et al., 2010; 35 OTUs, 99% similarity: Nagahama et al., 2011; 46 OTUs, 98% similarity: Singh et al., 2012; 45 OTUs, 98% similarity: Zhang et al., 2014), but relatively low in comparison with similar studies in terrestrial soil (1987 OTUs, 97% similarity: Brown et al., 2013; 704 OTUs, 97% similarity: Miura et al., 2015). It is likely that deep sequencing by Ion Torrent PGM could successfully recover more species and provide qualitative fungal community structure in deep-sea sediments. In fact, except for the first 11 most abundant OTUs, fungal OTUs detected in this study accounts for less than 0.1% of all the sequences. It is expected that these OTUs are difficult to detect by conventional cloning methods. The most OTU-rich phylum was Ascomycota (74.3%), followed by Basidiomycota (11.5%), which was consistent with previous studies on fungal diversity in deep-sea sediments (Singh et al., 2011; Xu et al., 2014). Zygomycota and Chytridiomycota did not appear in the samples investigated, however they may be included in unclassified fungal sequences or unaffiliated sequences to any

Table 3
Classification of obtained 113 OTUs with overall abundance and *t*-test results testing the effect of the presence of asphalt seeps and the different sampling sites on individual OTUs.

OTU No.	Best BLASTn match (UNITE + INSD database)							Overall abundance (%)	*t*-test	
	Phylum	Class	Species	Identity (%)	Coverage (%)	E-value	Accession No.		Asphalt seep	Sites
1	A	Eurotiomycetes	*Penicillium* sp.	100	100	1E − 121	KJ458976	33.2054	ns	ns
2	A	Leotiomycetes	*Cadophora malorum*	99.3	100	1E − 70	KF053539	28.3302	ns	ns
3	B	Microbotryomycetes	*Rhodosporidium diobovatum*	100	100	4E − 110	JQ993385	23.3796	ns	ns
4	A	Saccharomycetes	*Galactomyces pseudocandidum*	98.5	100	4E − 60	JN974291	4.2208	ns	ns
5	B	Tremellomycetes	*Guehomyces pullulans*	99.5	100	1E − 107	JX171177	3.7033	ns	ns
6	A	Sordariomycetes	*Acrostalagmus luteoalbus*	100	100	3E − 103	KC800578	3.2421	ns	ns
7	A	Sordariomycetes	*Stachybotrys chartarum*	99.6	100	2E − 110	KM231858	1.5319	ns	ns
8	B	Microbotryomycetes	*Leucosporidiella yakutica*	99.0	100	8E − 104	AY212989	0.8197	ns	ns
9	A	Dothideomycetes	*Preussia typharum*	99.5	100	6E − 100	JX143871	0.6233	ns	ns
10	A	Dothideomycetes	*Phoma eupyrena*	100	100	1E − 101	JX537965	0.4484	ns	ns
11	A	Dothideomycetes	*Paraphaeosphaeria sporulosa*	100	100	9E − 134	JX496014	0.1129	ns	ns
12	B	Cystobasidiomycetes	*Cystobasidium slooffiae*	99.5	100	7E − 98	FJ515215	0.0850	ns	ns
13	A	Saccharomycetes	*Kluyveromyces nonfermentans*	100	100	2E − 151	AB011507	0.0439	ns	*
14	A	Leotiomycetes	*Pseudeurotium ovale*	96.6	100	1E − 46	KC800595	0.0407	ns	*
15	A	Saccharomycetes	*Meyerozyma guilliermondii*	100	100	1E − 117	KJ502664	0.0343	ns	ns
16	A	Dothideomycetes	*Pseudogymnoascus* sp.	100	100	7E − 120	KF039892	0.0236	ns	ns
17	A	Sordariomycetes	*Trichoderma saturnisporum*	96.4	100	1E − 127	KC874898	0.0145	ns	*
18	A	Eurotiomycetes	*Aspergillus niger*	99.0	100	1E − 44	KJ544770	0.0102	*	*
19	B	Tremellomycetes	*Cryptococcus curvatus*	99.4	100	5E − 85	KF472136	0.0096	ns	ns
20	A	Saccharomycetes	*Cyberlindnera* sp.	98.3	99	3E − 112	HM461656	0.0094	ns	ns
21	A	Saccharomycetes	*Candida parapsilosis*	100	100	4E − 107	KF619554	0.0083	ns	ns
22	–	–	–	–	–	–	–	0.0079	ns	ns
23	A	Sordariomycetes	*Trichoderma erinaceum*	98.3	99	1E − 80	KF313116	0.0068	ns	**
24	U	unidentified	uncultured fungus	92.8	42	2E − 19	JN905298	0.0053	ns	ns
25	U	unidentified	uncultured fungus	94.1	22	2E − 21	JN905298	0.0045	ns	ns
26	A	Dothideomycetes	*Pseudeurotium* sp.	98.4	84	3E − 88	AB520862	0.0040	ns	ns
27	A	Eurotiomycetes	*Penicillium simplicissimum*	100	58	3E − 37	FR670313	0.0040	ns	*
28	A	Eurotiomycetes	*Aspergillus niger*	91.5	100	8E − 63	FJ960825	0.0038	*	ns
29	A	Saccharomycetes	*Galactomyces pseudocandidum*	94.7	91	1E − 30	JN974291	0.0036	*	*
30	U	unidentified	uncultured fungus	93.8	25	1E − 18	JX974809	0.0030	ns	ns
31	U	unidentified	uncultured fungus_clone G57	99.2	100	3E − 61	DQ279844	0.0021	ns	ns
32	A	Eurotiomycetes	*Penicillium* sp.	100	51	7E − 33	KJ458976	0.0019	ns	*
33	A	Sordariomycetes	*Trichoderma harzianum*	98.1	100	2E − 126	KC555182	0.0019	ns	ns
34	U	unidentified	fungal endophyte	98.1	19	5E − 17	KF435157	0.0017	ns	ns
35	A	Eurotiomycetes	*Penicillium* sp.	97.9	100	1E − 111	KJ458977	0.0017	ns	ns
36	A	Pezizomycetes	*Sarcosomataceae* sp.	90.0	29	3E − 20	KF128875	0.0017	ns	ns
37	A	Leotiomycetes	*Cadophora malorum*	100	76	2E − 80	KJ417832	0.0017	ns	ns
38	–	–	–	–	–	–	–	0.0015	ns	ns
39	A	Eurotiomycetes	*Penicillium* sp.	98.8	74	3E − 35	AF261663	0.0015	ns	*
40	U	unidentified	uncultured fungus	81.4	100	2E − 34	KC965876	0.0015	ns	ns
41	A	Eurotiomycetes	*Neosartorya fischeri*	87.3	100	8E − 59	GU966493	0.0015	ns	ns
42	U	unidentified	uncultured fungus	97.7	78	4E − 34	KF512546	0.0015	*	*
43	A	Eurotiomycetes	*Talaromyces* sp.	92.2	100	2E − 43	KC871022	0.0013	ns	ns
44	A	Dothideomycetes	*Preussia typharum*	92.5	100	4E − 49	JX143871	0.0013	ns	ns
45	B	Microbotryomycetes	*Rhodosporidium diobovatum*	94.1	100	8E − 89	JQ993385	0.0013	ns	ns
46	A	Saccharomycetes	*Debaryomyces hansenii*	97.3	100	1E − 120	KJ816874	0.0013	ns	ns
47	–	–	–	–	–	–	–	0.0011	ns	ns
48	A	Eurotiomycetes	*Penicillium oxalicum*	99.2	91	1E − 54	KJ619622	0.0009	ns	ns
49	A	Sordariomycetes	*Pestalotiopsis vismiae*	98.2	98	1E − 48	KF574897	0.0009	*	ns
50	A	Dothideomycetes	*Preussia funiculata*	99.4	90	3E − 87	KC427070	0.0009	ns	ns
51	A	Dothideomycetes	*Cladosporium sphaerospermum*	98.6	99	8E − 104	KJ546144	0.0009	ns	ns
52	A	Sordariomycetes	*Trichoderma paraviridescens*	93.3	100	1E − 55	KJ528987	0.0009	*	ns
53	A	Leotiomycetes	*Cadophora malorum*	91.2	100	3E − 30	KF053539	0.0009	ns	ns
54	A	Eurotiomycetes	*Penicillium* sp.	89.5	90	7E − 38	JX140804	0.0006	ns	ns
55	A	Saccharomycetes	*Galactomyces pseudocandidum*	93.5	98	2E − 33	JN974291	0.0006	ns	ns
56	A	Eurotiomycetes	*Talaromyces* sp.	99.2	100	2E − 119	KC871022	0.0006	ns	ns
57	A	Sordariomycetes	*Arthrinium* sp.	93.8	99	2E − 89	KJ767257	0.0006	ns	ns
58	A	Leotiomycetes	*Cadophora malorum*	98.8	64	6E − 33	KF053539	0.0006	ns	ns
59	A	Dothideomycetes	*Pseudeurotium bakeri*	88.6	100	3E − 30	JX967103	0.0006	ns	ns
60	A	Leotiomycetes	*Cadophora malorum*	97.1	98	1E − 24	KC292887	0.0006	ns	ns
61	A	Eurotiomycetes	*Penicillium* sp.	89.3	100	5E − 33	AF261663	0.0006	ns	ns
62	B	Microbotryomycetes	*Rhodosporidium diobovatum*	87.0	100	1E − 23	JQ993385	0.0006	ns	ns
63	B	Microbotryomycetes	*Rhodosporidium diobovatum*	91.7	100	1E − 82	JQ993385	0.0006	ns	ns
64	A	Sordariomycetes	*Stachybotrys chartarum*	98.5	86	2E − 94	KM231858	0.0006	ns	ns
65	A	Dothideomycetes	*Preussia flanaganii*	100	70	1E − 33	NR077168	0.0006	ns	ns
66	B	Tremellomycetes	*Guehomyces pullulans*	92.5	100	4E − 32	KF057530	0.0006	ns	ns
67	A	Eurotiomycetes	*Penicillium* sp.	91.3	100	3E − 40	JX140804	0.0004	ns	ns
68	A	Leotiomycetes	*Cadophora malorum*	94.8	100	1E − 60	KF053539	0.0004	ns	ns
69	A	Saccharomycetes	*Galactomyces pseudocandidum*	90.4	98	3E − 41	JN974291	0.0004	ns	ns
70	A	Leotiomycetes	*Cadophora malorum*	95.7	94	3E − 46	KF053539	0.0004	ns	ns
71	A	Leotiomycetes	*Geomyces* sp.	96.0	89	5E − 86	AB752246	0.0004	ns	ns
72	A	Eurotiomycetes	*Penicillium vulpinum*	91.1	87	1E − 40	L14535	0.0004	ns	ns

(continued on next page)

Table 3 *(continued)*

OTU No.	Best BLASTn match (UNITE + INSD database)							Overall abundance (%)	*t*-test	
	Phylum	Class	Species	Identity (%)	Coverage (%)	E-value	Accession No.		Asphalt seep	Sites
73	A	Leotiomycetes	*Cadophora* sp.	93.5	96	2E − 64	HQ267070	0.0004	ns	ns
74	A	Leotiomycetes	*Cadophora* sp.	93.6	100	9E − 73	HQ267070	0.0004	ns	ns
75	–	–	–	–	–	–	–	0.0004	ns	ns
76	A	Sordariomycetes	*Arthrinium* sp.	96.4	98	4E − 71	KJ767257	0.0004	ns	ns
77	A	Saccharomycetes	*Galactomyces pseudocandidum*	95.5	75	2E − 29	JN974291	0.0004	ns	ns
78	B	Tremellomycetes	*Guehomyces* sp.	96.2	99	8E − 41	JX675151	0.0004	ns	ns
79	A	Eurotiomycetes	*Penicillium chrysogenum*	93.5	100	9E − 99	GQ458038	0.0004	ns	ns
80	A	Saccharomycetes	*Galactomyces pseudocandidum*	90.7	100	6E − 32	JN974291	0.0004	ns	ns
81	B	Microbotryomycetes	*Rhodotorula graminis*	97.9	69	4E − 40	FR717632	0.0004	ns	ns
82	B	Microbotryomycetes	*Rhodosporidium diobovatum*	91.2	100	3E − 53	JQ993385	0.0004	ns	ns
83	A	Saccharomycetes	*Galactomyces pseudocandidum*	98.7	72	2E − 30	JN974291	0.0004	ns	ns
84	–	–	–	–	–	–	–	0.0004	ns	ns
85	A	Leotiomycetes	*Cadophora* sp.	92.7	100	4E − 66	HQ267070	0.0004	ns	ns
86	A	Dothideomycetes	*Preussia typharum*	93.7	100	4E − 57	JX143871	0.0004	ns	ns
87	A	Leotiomycetes	*Cadophora malorum*	91.5	99	2E − 42	KF053539	0.0004	ns	ns
88	A	Eurotiomycetes	*Penicillium* sp.	90.2	96	4E − 40	JX140804	0.0004	ns	ns
89	A	Sordariomycetes	*Acrostalagmus luteoalbus*	98.8	79	2E −80	KC461515	0.0004	ns	ns
90	–	–	–	–	–	–	–	0.0004	ns	ns
91	A	Eurotiomycetes	*Penicillium* sp.	100	64	1E − 34	KJ458976	0.0004	ns	ns
92	A	Sordariomycetes	*Trichoderma inhamatum*	96.6	100	1E − 118	Z68188	0.0004	ns	ns
93	A	Eurotiomycetes	*Penicillium* sp.	89.6	100	3E − 79	KJ619589	0.0004	ns	ns
94	A	Dothideomycetes	*Pseudeurotium* sp.	87.9	86	8E − 38	HM589261	0.0004	ns	ns
95	A	Saccharomycetes	*Galactomyces pseudocandidum*	89.6	100	1E − 39	JN974291	0.0004	ns	ns
96	A	Sordariomycetes	*Stachybotrys chartarum*	96.1	97	1E − 37	KM231858	0.0004	ns	ns
97	A	Eurotiomycetes	*Penicillium* sp.	98.8	61	4E − 35	AF261663	0.0004	ns	ns
98	A	Sordariomycetes	*Stachybotrys* sp.	96.0	100	1E − 38	JN545814	0.0004	ns	ns
99	–	–	–	–	–	–	–	0.0004	ns	ns
100	B	Cystobasidiomycetes	*Cystobasidium slooffiae*	94.1	100	3E − 78	FJ515215	0.0004	ns	ns
101	A	Eurotiomycetes	*Aspergillus niger*	95.2	100	2E − 105	KJ544770	0.0004	ns	ns
102	A	Leotiomycetes	*Cadophora malorum*	94.2	100	2E − 36	KF053539	0.0004	ns	ns
103	A	Sordariomycetes	*Tolypocladium cylindrosporum*	100	100	8E −114	KJ028796	0.0004	ns	ns
104	A	Sordariomycetes	*Stachybotrys chartarum*	94.4	86	1E − 52	KM231858	0.0004	ns	ns
105	A	Saccharomycetes	*Galactomyces pseudocandidum*	92.5	100	8E − 47	JN974291	0.0004	ns	ns
106	A	Dothideomycetes	*Phoma herbarum*	98.2	98	1E − 46	LN808945	0.0004	ns	ns
107	A	Eurotiomycetes	*Penicillium* sp.	93.3	100	2E − 48	KJ458976	0.0004	ns	ns
108	A	Saccharomycetes	*Galactomyces pseudocandidum*	92.5	100	3E − 46	JN974291	0.0004	ns	ns
109	A	Dothideomycetes	*Preussia flanaganii*	94.5	88	1E − 39	NR077168	0.0004	ns	ns
110	U	unidentified	uncultured fungus	94.7	100	2E − 42	GQ524004	0.0004	ns	ns
111	–	–	–	–	–	–	–	0.0004	ns	ns
112	A	Leotiomycetes	*Geomyces pannorum* var. *asperulatus*	96.0	97	1E − 39	DQ117449	0.0004	ns	ns
113	A	Eurotiomycetes	*Aspergillus tubingensis*	93.9	100	2E − 41	AY585545	0.0004	ns	ns

Phylum rank: (A: Ascomycetes, B: Basidiomycetes, U: Unidentified, -: No match) ns: not significant, $*p < 0.05$; $**p < 0.01$.

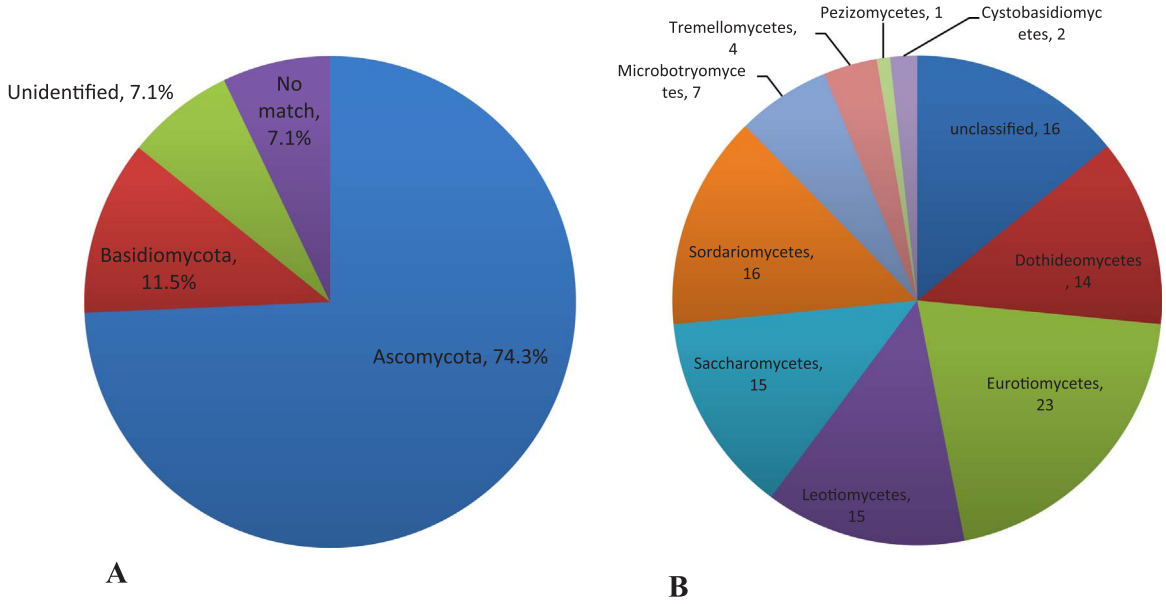

Fig. 4. Proportion of obtained 113 OTUs assigned to (A) phylum level (B) class level.

Fig. 5. Abundance of detected fungal sequences at the class level in each sample. Sample ID in bold indicating the samples associated with asphalt seeps. (Unidentified, Cystobasidiomycetes and Pezizomycetes OTUs were not included, as the number is too low to display in the charts).

organisms in the public database. For example, OTU 40 can be speculated to affiliate into the phylum Chytridiomycota from having some Chytridiomycota sequences in Blast search results. Due to the short length of sequence reads by Ion Torrent PGM and variability of ITS region, it was difficult to perform reliable phylogenic analysis for those unclassified sequences. Further investigation analyzing longer and more conservative regions will be needed to reveal the phylogenic position of cryptic diversity found in deep-sea sediments at the Sao Paulo Plateau. All nine fungal classes recovered in the present study were commonly reported in other deep-sea environments, except Pezizomycetes (Nagano and Nagahama, 2012). Pezizomycetes seems to be rarely reported or to have not been reported previously from deep-sea environments. Only one OTU (36) was classified as Pezizomycetes and this OTU was recovered only from the sample 1346_3_2. Three classes (Microbotryomycetes, Tremellomycetes, Cystobasidiomycetes) within the phylum Basidiomycota were recovered in this study and all 13 OTUs affiliated within Basidiomycota were known as yeast formed species (*Leucosporidiella*, *Cystobasidium*, *Cryptococcus*, *Rhodosporidium*, *Guehomyces*, *Rhodotorula*). Consistent with previous studies, basidiomycetous yeasts are commonly found in deep-sea environments (Nagano and Nagahama, 2012). However, one of the most commonly detected basidiomycetous yeast from deep-sea environments by culture-independent methods, the genus *Malassezia* within the class Exobasidiomycetes, was not found in this study.

There were a considerable amount of unclassified OTUs (16 OTUs, 14%) detected in this study. Also, only 42 out of 113 (37.2%) fungal OTUs exhibited > 98% sequence similarity, and 47 (41.6%) exhibited > 97% similarity, to pre-existing ITS sequences in public databases. These results suggest that a large amount of deep-sea sediment-inhabiting fungal taxa remain undescribed. Nevertheless, we need to note that a considerable amount of described fungal taxa still do not have their sequences recorded in public databases. In addition, although singleton OTUs were removed to avoid the risk of including spurious reads generated by sequencing errors in our data processing procedure, some rare species may be included in the discarded data, underestimating the diversity (Brown et al., 2015).

The three most abundant OTUs appeared in deep-sea sediments sampled from the Sao Paulo Plateau, namely *Penicillium* sp., *Cadophora malorum* and *Rhodosporidium diobovatum, which* were previously reported in other deep-sea environments (Burgaud et al., 2009; Nagano

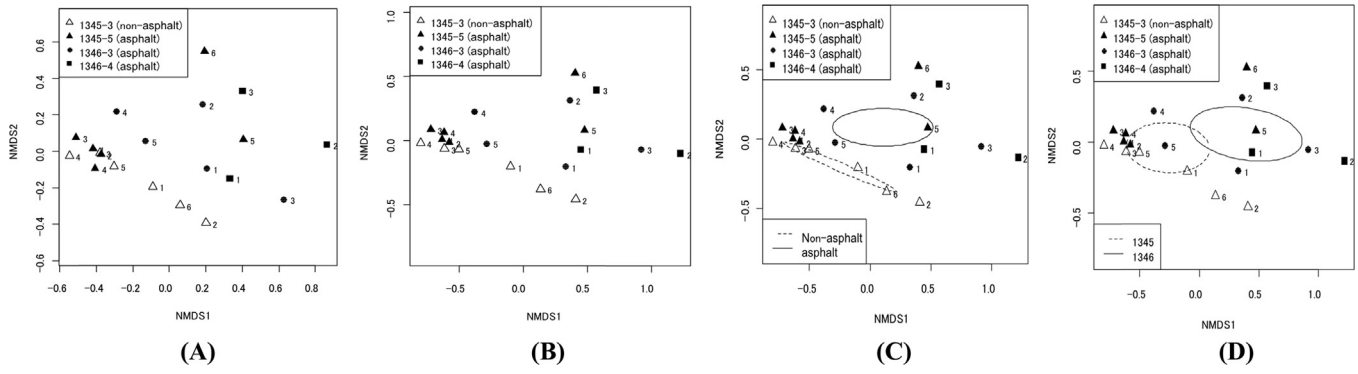

Fig. 6. NMDS ordination of the fungal community structure. Open symbols represent samples from the non-asphalt seep site and filled symbols represent samples from the asphalt seep sites. The numbers beside the symbols represent the sample depths from the seafloor (1: 0-1cmbsf, 2: 1-4cmbsf, 3: 4-7cmbsf, 4: 7-10cmbsf, 5: 10-13cmbsf, 6: 13-16cmbsf). (A) Presence-absence based, 2D stress was 0.13. (B) Relative abundance based, 2D stress was 0.10. (C) Relative abundance based, 2D stress was 0.10. Ellipses represent the 95% confidence interval on standard errors of means: Non-asphalt (dash line), Asphalt (solid line). (D) Relative abundance based, 2D stress was 0.10. Ellipses represent the 95% confidence interval on standard errors of means: 1345 (dash line), 1346 (solid line).

and Nagahama, 2012; Nagano et al., 2014). It has been suggested that the genus of *Aspergillus* and *Penicillium* within the family Eurotiomycetes are ubiquitous in deep-sea environments (Nagano and Nagahama, 2012) and this was also consistent with this study on samples in the Sao Paulo Plateau. *Cadophora malorum* has been isolated from mussels and shrimp in deep-sea hydrothermal vents (Burgaud et al., 2009) and also from mosses in the Antarctic (Tosi et al., 2002). These isolates were suggested as psychrotrophs. The abundant detection of *Cadophora malorum* in this study suggests an adaptation and a wide distribution of this species in cold deep-sea environments. Furthermore, *Cadophora* spp. have also been reported from deep marine sediments at 12–765 m below the seafloor (Ciobanu et al., 2014; Nagano et al., 2016). These species may be well adapted to extreme environments. *Rhodosporidium diobovatum* is one of the most frequently reported yeast from deep-sea environments and its ability to assimilate nitrate is recognized (Nagahama et al., 2001b; Gadanho and Sampaio, 2005; Burgaud et al., 2010; Manohar et al., 2015). This yeast species was distributed abundantly in all 20 samples tested and may have an important role in nitrogen cycles in deep-sea sediments. Although it appeared to be in low abundance, DSF-group1 (OTU 31) was detected in deep-sea sediments from the Sao Paulo Plateau. This group has been recognized as uncultured taxa related to *Metschnikowia/Candida*, frequently detected from deep-sea environments (Nagano and Nagahama, 2012). This study has provided further evidence to support the wide distribution of this group in deep-sea environments from across the Pacific Ocean to the Atlantic Ocean.

4.2. Fungal community structure in asphalt seep and non-asphalt seep sites

PERMANOVA statistical analysis suggested that there is no significant difference in fungal diversity and fungal community between asphalt seep and non-asphalt seep sites, despite the presence of mass hydrocarbon deposits and the high amount of macro organisms surrounding asphalt seeps, which are not present at non-asphalt seep sites. However, some difference was observed while analyzing individual OTUs. OTU18 (*Aspergillus niger*, 99.01%), OTU28 (*Aspergillus niger*, 91.49%), OTU29 (*Galactomyces pseudocandidum*, 94.68%), OTU42 (uncultured fungus, 97.65%), OTU49 (*Pestalotiopsis vismiaeonly*, 98.2%) and OTU52 (*Trichoderma paraviridescens*, 93.29%) were distributed specifically in asphalt seep sites (Table 3). *Aspergillus*, *Pestalotiopsis* and *Trichoderma* have been reported for their ability to degrade petroleum hydrocarbons, including asphalt (Uribe-Alvarez et al., 2011; Nasrawi, 2012; Xue et al., 2015). These 6 OTUs may also be degrading asphalt extrusion on the deep-sea floor and play an important role in the environment. For OTUs distribution, asphalt and non-asphalt seep sites shared 27 OTUs but 23 OTUs were unique to the asphalt seep site and 25 OTUs were unique to the non-asphalt site (Fig. 3A). This result suggests the presence of different fungal communities in asphalt and non-asphalt seep sites. However, the abundance of these unique OTUs in each environment accounts for less than 0.1% of whole community. Therefore, it is inferred that these OTUs did not have much influence on PERMANOVA statistical analysis on community structure. Similarly, NMDS ordination did not show a distinct difference, but the ellipses represent with 95% confidence interval that there is some trend between asphalt and non-asphalt sites. In addition, it needs to be stated that the uneven sampling size (Asphalt seep site: N = 3, Non-asphalt seep site: N = 1) may have affected statistical analysis. Overall, it is suggested that there is no significant difference in major fungal communities between asphalt and non-asphalt seep sites. This is presumptively because, the majority of fungi can not utilize asphalt, as asphaltene, the main component of asphalt, is known to have one of the most complex, high molecular weights in nature (Badre et al., 2006).

4.3. Fungal community difference in dive point 1345 and 1346

Statistically significant difference was observed in fungal community structure between the samples collected from dive points 1345 and 1346 by PERMANOVA analysis (Table S1). NMDS ordination did not show clear separation between the two dive points but some trends can be observed. Although there was a little overlap, the ellipses represent 95% confidence interval, indicating some difference between the two dive points (Fig. 6). For fungal diversity, a much higher number of OTUs was observed in samples from dive point 1346, with the diversity index value also showing a slightly higher value in dive point 1346 (Table 2). However, we could not find any obvious environmental factors available for the samples (geological feature, water depths, temperature, type of sediment particles, pH, Si, NH^{4+}, Na, K, Mg, Ca, Cl, Br, $SO_{4)}$ to explain the presence of higher fungal diversity in dive point 1346 and the different community in these two locations. Some environmental factors, such as total organic carbon (TOC) and Ca have been reported for their strong correlation with the richness of fungal taxonomic previously (Orsi et al., 2013; Tedersoo et al., 2014). Further investigation on a larger scale is needed to understand the relationship between geochemical settings and the fungal taxonomic richness in deep-sea environments.

5. Conclusion

This study suggests that diverse fungi are present in deep-sea sediment samples collected at the Sao Paulo Plateau, Brazil. The detected fungal community structure was similar to those previously reported in other deep-sea environments, with the dominant presence of *Aspergillus*, *Penicillium*, psychrotrophic fungi, red-pigmented basideomycetous yeasts and the presence of uncultured deep-sea taxa. There was also some distinct features, such as the presence of Pezizomycetes and the absence of the genus *Malassezia.* To the best of our knowledge, this is the first report describing the fungal community in the deep-sea of Brazilian coast. Unexpectedly, there was no significant difference in major fungal community structure between the asphalt seep and the non-asphalt seep sites, despite the presence of mass hydrocarbon deposits and the high amount of macro organisms surrounding asphalt seeps. However, there are some differences in minor fungal community and possible asphalt degrading fungi are present specifically in asphalt seep sites. In contrast, statistically significant difference was observed in fungal community structure between the samples collected from dive points 1345 and 1346. However, the cause of the difference remains unclear. Furthermore, a high number of previously undescribed fungal OTUs were detected, indicating that deep-sea sediments are inhabited by a large amount of unknown fungal taxa. Although our knowledge on fungal diversity in deep-sea environments has significantly increased in the last decade, it is still fragmentary and limited within the vast expanse of deep-sea. Further comprehensive investigation on deep-sea fungi will be needed to provide better understanding on the global distribution of fungi, ecological role of fungi and evolutionary history of fungi in deep-sea environments. Finally, high-throughput approaches allowed us to reveal the new insights into deep-sea fungal community ecology, however, we need to aware that there are invariable methodological biases (e.g. sample handling, primer selection in PCR amplification, data processing, data analyzing) and the obtained results may not reflect the true diversity. It is known that the primer bias in PCR amplification is especially severe and using different primer sets may provide different results. Although more sophisticated methods are now available, comprehension of ecological community remains a major technical challenge.

Conflict of interest

The authors declare that they have no conflict of interest, financial or otherwise, to report in relation to the publication of this manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr2.2017.05.012.

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