ORIGINAL ARTICLE

Molecular characterization of the spatial diversity and novel lineages of mycoplankton in Hawaiian coastal waters

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Microbial community diversity and composition have critical biogeochemical roles in the functioning of marine ecosystems. Large populations of planktonic fungi exist in coastal ocean waters, yet their diversity and role in carbon and nutrient cycling remain largely unknown. Lack of information on critical functional microbial groups limits our understanding of their ecological roles in coastal oceans and hence our understanding of its functioning in the ocean's carbon and nutrient cycles. To address this gap, this study applied the molecular approach denaturing gradient gel electrophoresis (DGGE) coupled with clone library construction to investigate mycoplankton communities in Hawaiian coastal waters. Mycoplankton communities displayed distinct lateral and vertical variations in diversity and composition. Compared with the open ocean, surface (<100 m) near-shore waters had the greatest diversity and species richness of mycoplankton, whereas no differences were found among stations at depths below 150 m. Vertical diversity profiles in the coastal waters suggested that diversity and species richness were positively correlated to phytoplankton biomass in the coastal waters, but not in offshore waters. A total of 46 species were identified and belonging to two phyla *Basidiomycota* and *Ascomycota*, with the basidiomycetes as the dominant group ($n=42$). The majority ($n=27$) of the basidiomycetes are novel phylotypes showing less than 98% identity in the 18S rRNA gene with any sequence in GenBank. This study provides insight into mycoplankton ecology and is the first molecular analysis of planktonic fungi in the oceans.

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Introduction

Microbial community diversity and composition have critical biogeochemical roles in the functioning of marine ecosystems. Recent advances in molecular techniques and ecological genomics have greatly advanced our understanding of the processes mediated by bacteria and archaea in the oceans, particularly in carbon and nutrient cycles. As one of the most variable marine habitats, coastal waters are generally characterized by a high biodiversity and high primary production (Jickells, 1998; Danovaro and Pusceddu, 2007). The primary production of the

coastal waters often exceeds the consumption of herbivores, and therefore, a large fraction of primary organic matter becomes available to consumers as detritus (Newell, 1982). A large fraction of this detritus is degraded by heterotrophic microbes before entering higher trophic levels (Manini *et al.*, 2003; Pusceddu *et al.*, 2003). Heterotrophic bacteria and archaea are known to have a large role in this degradation, export and storage of organic matter in the coastal oceans (Moran and Miller, 2007; Mou *et al.*, 2008). Although large populations of heterotrophic eukaryotes are well-documented in coastal waters, their diversity and function in carbon and nutrient cycles remain largely unexplored (Giovannoni and Stingl, 2005; Hallam *et al.*, 2006; Fenchel, 2008; Strom, 2008).

Fungi have long been known to exist in the world's oceans. Although the populations of planktonic fungi in coastal waters can be quite large, their diversity and function in biogeochemical cycling remain largely unknown. Nevertheless, they fulfill a wide range of important ecological functions, particularly those associated with the decomposition of organic matter, nutrient and carbon cycling and biodegradation of hydrocarbons in both marine and

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terrestrial habitats (Christensen, 1989; Pang and Mitchell, 2005). Much of what is known regarding the fungal diversity and functioning in marine environments is based on the data derived from marine substrata (for example, wood, mangroves and sands) using culture-based techniques (Kis-Papo, 2005; Pang and Mitchell, 2005; Vogel *et al.*, 2007). These methods detect only a small fraction of fungal communities, and hence, their results provide only a selective and invariably biased window on diversity (Wang *et al.*, 2008b). Of an estimated 10 000 marine fungal species, only about 5% of them have been described (Kis-Papo, 2005). Recently, substantial advances have been made in our understanding of the fungal community ecology in natural environments through the application of molecular techniques, including clone library construction (Borneman and Hartin, 2000), automated rRNA intergenic space analysis (Ranjard *et al.*, 2001), terminal restriction fragment length polymorphism (Lord *et al.*, 2002) and denaturing gradient gel electrophoresis (DGGE) (May *et al.*, 2001; Smit *et al.*, 2003). DGGE coupled with clone library construction has been shown to be an efficient molecular approach to study fungal communities in diverse terrestrial environments (May *et al.*, 2001; Anderson *et al.*, 2003; Smit *et al.*, 2003; Anderson and Cairney, 2004; Arenz *et al.*, 2006; Artz *et al.*, 2007; Wang *et al.*, 2008b). However, none of these molecular methods has been used to explore mycoplankton (that is, planktonic aquatic fungi) communities.

Two types of food chains, namely the classical grazing food chain and the microbial food chain, dominate the flow of elements and energy in ocean ecosystems (Wang, 2002). Microbes salvage organic

materials such as phytoplankton exudates and phyto-/zoo-detritus from the grazing food chain (Pomeroy and Wiebe, 1993). The large pool of dissolved organic carbon at the base of the microbial food chain has been thought to be primarily used by bacterioplankton (Sherr and Sherr, 1987). However, several lines of evidence suggest that mycoplankton can also use dissolved organic carbon and contribute to secondary production in the microbial food web as well (Kimura and Naganuma, 2001; Kimura *et al.*, 2001). A typical milliliter of seawater contains from 10^3 to 10^4 fungal cells (Kubanek *et al.*, 2003). Coastal waters may contain much larger mycoplankton populations because these waters contain significant fungal and nutritional inputs from terrestrial sources (Kimura *et al.*, 1999, 2001; Vogel *et al.*, 2007). However, mycoplankton diversity in coastal oceans remains largely uncharacterized. In this study, we used molecular methods to explore planktonic fungal communities and present the first molecular analysis of the diversity of fungal communities in the Hawaiian coastal waters.

Materials and methods

Oceanographic sampling and environmental data

To evaluate spatial variability in mycoplankton structure in the Hawaiian coastal waters, samples were collected along a shipboard sampling transect on the northeast side of the island of Oahu (Hawaii) (Figure 1) on a cruise aboard the R/V Kilo Moana in January 2007. Water samples were collected from six depths (5, 15, 50, 100, 150 and 200 m) with 12-l polyvinyl chloride bottles attached to a Sea Bird CTD rosette sampler. Vertical profiles of water

Figure 1 CTD sampling sites at the windward of the island of Oahu during the cruise of R/V Kilo Moana in January 2007.

temperature, salinity, dissolved oxygen and fluorescence were monitored at each sampling site using the corresponding sensors, meters or probes equipped on the CTD sampler. Chlorophyll-*a* concentration was measured using the method described by Holm-Hansen and Riemann (1978) and Welschmeyer (1994).

Nucleic acid extraction and DGGE analysis

Seawater samples were filtered through 0.2- μ m of polycarbonate filters. Filters for DNA extraction were stored at -80°C until further processing. Total genomic DNA was extracted from the filters using the FastDNA kit (Qbiogene, Irvine, CA, USA), according to the manufacturer's instructions. All PCRs for DGGE analysis were performed using the Expand High Fidelity PCR System (Roche, Indianapolis, IN, USA) according to the manufacturer's instruction. Genomic DNA extracted from water samples was used as a PCR template for the amplification of the small subunit rRNA gene using the nested PCR approach following the procedure described by Gao *et al.* (2008), based on Kowalchuk *et al.* (1997). All PCR applications were performed in 50 μ l reactions for 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min. PCR products were separated on a 1% agarose gel, stained with ethidium bromide and visualized under UV transillumination. PCR products from three separate amplification reactions were precipitated using ethanol to clean the product and to increase DNA concentrations.

DGGE analysis was performed using a model DGGE-2001 electrophoresis system (CBS Scientific Company Inc., CA, USA) with a denaturing gradient of 30–70% in a 7.5% polyacrylamide gel, following the manufacturer's instructions. The gel was made from 12 ml of 0% denaturing solution (2 ml of $50\times$ TAE, 18.8 ml of 40% Acrylamide/Bis-acrylamide (37.5:1) and 79.2 ml of water) and 12 ml of 100% denaturing solution (2 ml of $50\times$ TAE, 18.8 ml of 40% polyacrylamide solution, 40 ml of formamide, and 42 g of urea) using a GM-40 Gradient Maker according to the manufacturer's instructions. Before casting, 80 μ l of ammonium persulfate (APS) and 6 μ l of TEMED were added to each solution. PCR products were mixed with 1/3 volume of $10\times$ sucrose loading buffer, and DNA fragments were separated for 16 h at 100 V and 60°C . The gel was stained for 20 min with ethidium bromide and documented using a UV transillumination and VisiDoc-It imaging systems (UVP).

Individual bands were stabbed with pipette tips and each stab was placed into 0.5-ml microcentrifuge tubes with 20 μ l sterile water. The tubes were vortexed vigorously and incubated overnight at 4°C . PCR amplifications were carried out using 1 μ l of the DNA eluted from the bands and the corresponding regular (non-GC-clamped) primers of these bands for 30 cycles as described previously. Again, PCR

products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV transillumination. The correctly sized products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) before being transformed into *E. coli* cells. Clones derived from individual bands were treated as a mini clone library. The plasmid carrying inserts of correct size were sequenced using the T7 or SP6 primers. Sequences obtained in this study were available at the GenBank under accession numbers FJ889078–FJ889125.

Sequence, phylogenetic and statistical analysis

Plasmids were sequenced at the University of Hawaii DNA Core Sequencing Facility on an Applied Biosystems 3730 automated DNA sequencer. Sequences were edited with Chromas Lite, version 2 (Technelysium). Sequence identifications were determined using the BLAST (National Center for Biotechnology Information (NCBI); <http://www.ncbi.nlm.nih.gov/> on 15 January 2009). All clone sequences with $\geq 99\%$ sequence identity were assigned into operational taxonomic units (OTUs) using the software package DOTUR (Schloss and Handelsman, 2005) and were considered to represent the same species, based on the definitions used in other studies of ITS-rRNA in fungi (Kurtzman and Robnett, 1998; Landeweert *et al.*, 2003; Lynch and Thorn, 2006). Each of the fungal 18S rRNA sequences from each species and the matched sequences from GenBank were aligned with BioEdit, version 7.0.5.3 (Hall, 1999). The aligned sequences were imported into PAUP* 4.0b10 (Swofford, 2002). Neighbor-joining trees were estimated based on pairwise genetic distances on the basis of all substitutions with the Jukes–Cantor distance parameter. The robustness of the branching patterns was assessed by bootstrap resampling of the data sets with 1000 replications. Pearson's correlation coefficients (*r*) and probability values (*P*) were calculated to check for colinearity between band number and the measured environmental variables using CORR program in the SAS software package.

Results

DGGE band pattern analysis

DGGE analysis of PCR products amplified from water samples collected at two sites (1 and 4) indicated that band patterns of mycoplankton communities varied at different depths of the same site and the same depth of two different sites (Figures 2a–b). More bands were detected in samples from surface waters (0 to 100 m) at site 1 than those from corresponding depths of site 4, suggesting that more diverse fungal communities are present at these depths in the coastal waters than those in the open ocean. No difference in band numbers was observed in deeper samples (greater than 150 m) at

either site. As the depth increased, DGGE band numbers of water samples from site 1 increased and reached the maximum at a depth of 50 m (16 bands)

Figure 2 DGGE analysis of planktonic fungi derived from the coastal waters of the island of Oahu (a). DGGE bands cloned are side-labeled with dot and number. UPMA tree based on matrix of distance calculated from the difference in DGGE band patterns among water samples from sites 1 and 4 (b).

and then declined. However, for the site 4, the highest band number was detected in the 150-m sample (11 bands) and the second highest from 5 m (10 bands). Therefore, band pattern analysis suggested that the vertical profiles of mycoplankton communities in the coastal water (site 1) and open-ocean water (site 4) differed dramatically, with more diverse mycoplankton in the coastal water than those in open-ocean waters at depths of ≥ 100 m. No diversity difference was observed in waters from depths > 150 m at both sites. Two common bands (A and B) were found in samples from all depths of site 1 whereas three bands (C, D and E) were detected only in top 3 depths. In contrast, although 4 bands (F, G, H and I) were detected in samples from more than one sample, no common bands were detected in samples collected from site 4. This difference may result from differential mixing and/or nutrients at the two sites. Finally, composition of mycoplankton communities at site 1 displayed less variation across the vertical profile; on the other hand, those at site 4 were more heterogeneous (Figure 2b).

Effects of physical, biological and geochemical features of seawater

Concentrations of chlorophyll-a (Chl-a) and fluorescence increased with depth, reaching the maximum at 50 m, and then declined at site 1 as the depth increased (Figure 3). They displayed the same general vertical patterns in site 4, but with the maximum found deeper at the depth of 100 m (Figure 3). Concentrations of Chl-a and fluorescence of water samples at depths of ≥ 50 m at site 1 were much higher (about 1.2–2 and 3–4.5 folds for Chl-a and fluorescence, respectively) than those depths at site 4. However, these two parameters were lower at 100 m or below at site 1 compared with those at site

Figure 3 Vertical profile of DGGE band numbers, fluorescence and chlorophyll-a of Hawaiian waters.

4 (Figure 3). Overall the peak in photosynthetic biomass was comparable between the sites, but the biomass peak within the water column was deeper in the open ocean station, due to the increase in water transparency. Integrated photosynthetic biomass was higher in the coastal waters than in the open-ocean site. Salinity of seawater did not display any correlation with band patterns and band numbers at both sites (data not shown). However, seawater temperature ($r=0.91$, $P=0.01$) and Chl-*a* ($r=0.85$, $P=0.04$) displayed significant correlations with band number at site 1, but not site 4. This further indicated that mechanisms regulating mycoplankton diversity in the coastal and open-ocean waters might vary.

Diversity and phylogenetic analysis

Major bands were excised from DGGE gel and were cloned for sequencing analysis. Of 124 clone sequences, a total of 46 fungal operational taxonomic units, or 'species,' were identified using the method described by Gao *et al.* (2008). These unique fungal species belonged to two phyla: *Basidiomycota* ($n=42$) and *Ascomycota* ($n=4$). All the ascomycete species were members of the class *Dothideomycetes*. However, the vast majority ($n=39$, 93%) of basidiomycete species were not affiliated with any member of the known fungal taxa and, hence, are likely new fungal phylotypes (Table 1 and Figure 4). Of the four ascomycete species, MF4.6.2.5 was a member of the order *Pleosporales*, most of which are saprobes or parasites on terrestrial plants. The species MF4.1.1.1 was closely matched to the marine yeast *Aureobasidium pullulans*, a complex commonly found in marine environments, and was a member of the order *Dothideales*. The other two phylotypes did not have immediate phylogenetic neighbors and were new fungal phylotypes. Furthermore, ascomycete species were only detected in water samples from three depths (5, 150 and 200 m) at site 4 and absent at depths of 15, 50 and 100 m at the same site.

Basidiomycete species were found in all depths at the two sites. Among 42 basidiomycete species, 19 of them were from site 1 and the rest from site 4. The majority ($n=29$, 69%) of the basidiomycete species were clustered into four clades (A, B, C and D), which did not include the known taxa from GenBank (Figure 4). Therefore, these sequences are new fungal phylotypes. Five species were included in clade A and present at depths 100 m or below at both sampling sites. Clade B contained 12 species from both sites and further branched into two subclades (B1 and B2). Members of subclade B1 included phylotypes derived from samples collected at both sites at several depths whereas subclade B2 only contained those derived from depths 100 to 150 m. Phylotypes of clade C were present at both sites and not depth-specific. Members of clade D were unique to the depth of 150 m at

site 4. Finally, members of clades C and D belonged to unknown basidiomycetes because they did not have phylogenetic neighbors in GenBank. Overall, our results suggest that some basidiomycete phylotypes are depth-specific and others belong to 'cosmopolitan phylotypes'.

Two basidiomycete species (MF4.3.2.7, and MF1.1.2.7) did not cluster with any fungal sequences, with unknown phylogenetic affiliations (Figure 4). Still, an additional three basidiomycete species belonged to members of *Agaricomycetes* and further branched with members of *Boletales* (MF4.1.3.5) and *Polyporales* (MF1.1.8.1 and MF4.3.2.8). Species MF4.3.1.5 and M1.5.1.7 were affiliated with pucciniomycete fungal clone sequences derived from sediment of a contaminated aquifer. Finally, along with fungal sequence derived from marine sponges, marine sediments of aquifer, hypersaline basin and other natural habitats, four species derived from site 1 and 4 branched into clade E. Particularly, three sequences (MF1.1.2.3, MF4.2.1.1 and MF4.3.2.2) belonged to the same phylotype group and were closely affiliated with the fungal clone sequence 8-1p-11 derived from the Hawaiian sponge *Suberites zeteki*.

Discussion

Small subunit rRNA genes have been recognized as powerful markers for both identifying fungi and resolving taxonomic positions at different levels (Bruns *et al.*, 1991; Sugiyama, 1994). Application of PCR in amplifying 18S rRNA genes has contributed to our better understanding of evolutionary relationship between fungi (White *et al.*, 1990; Willemotte *et al.*, 1993) and also enhanced our ability to describe fungal communities with respect to environmental factors and perturbations (Kowalchuk *et al.*, 1997; Malosso *et al.*, 2006; Oros-Sichler *et al.*, 2006; Jumpponen, 2007). DGGE analysis based on 18S rRNA fragments has been used to reveal fungal communities in many terrestrial natural habitats (Kowalchuk *et al.*, 1997; Borneman and Hartin, 2000; Vainio and Hantula, 2000; May *et al.*, 2001; Brodie *et al.*, 2003; Nikolcheva *et al.*, 2003), but its application for investigating fungal communities in marine environments remains rare (Pang and Mitchell, 2005; Gao *et al.*, 2008). This report describes the first use of a DGGE-based strategy targeting this gene to characterize planktonic fungal communities in the Hawaiian coastal waters.

Marine fungi have been defined as those that can complete their entire life cycles within the sea, that is, those that can grow and sporulate exclusively in a marine or estuarine habitat (Kohlmeyer and Volkmann-Kohlmeyer, 2003). Several lines of evidence suggest the existence of 'marine fungal phylotypes' (Gao *et al.*, 2008; Wang *et al.*, 2008a; Li and Wang, 2009), but marine fungi are still considered

Table 1 Taxonomic distribution of 18S rRNA clone sequences derived from DGGE analysis of planktonic fungi

'Species' ID ^a	Taxon		Closest relative (accession no.)	Percentage of identity	Clone no. in libraries
	Phylum	Class			
MF1.1.2.1	Basidiomycota	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	95	1
MF1.1.2.3			Basidiomycete clone 8-2-5 (EU085016)	99	9
MF1.1.2.7			Basidiomycete clone 8-2-5 (EU085016)	98	1
MF1.1.8.1		Agaricomycetes	<i>Corioloopsis gallica</i> strain 7630-SP (AY336772)	99	1
MF1.1.9.3		unknown	Basidiomycete clone 8-2-5 (EU085016)	96	3
MF1.2.1.1		Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	100	41
MF1.2.4.3			Basidiomycete clone 8-2-5 (EU085016)	96	1
MF1.3.2.2		unknown	Pucciniomycotina clone D0735_34_S (EU646990)	96	2
MF1.4.2.1		Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	97	1
MF1.4.2.2			Basidiomycete clone 8-2-5 (EU085016)	99	3
MF1.4.2.3	unknown	Pucciniomycetes	Basidiomycete clone 8-1p-11 (EU085018)	96	3
MF1.4.2.4			Basidiomycete clone 8-2-5 (EU085016)	98	1
MF1.4.3.4	unknown	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	96	1
MF1.5.1.1			Basidiomycete clone 8-1p-5 (EU085018)	97	2
MF1.5.1.4	Pucciniomycetes	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	96	3
MF1.5.1.5			Basidiomycete clone 8-2-5 (EU085016)	99	3
MF1.5.1.7	Pucciniomycetes	Pucciniomycetes	<i>Termitomyces</i> sp. Mc (AB051894)	93	1
MF1.6.1.3			Basidiomycete clone 8-2-5 (EU085016)	99	5
MF1.6.1.8	Agaricomycetes	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	95	1
MF4.1.3.5			<i>Phaseoleae</i> clone Elev_18S_724 (EF024316)	94	1
MF4.1.1.7	Pucciniomycetes	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	99	2
MF4.2.1.1			Basidiomycete clone 8-2-5 (EU085016)	99	4
MF4.2.1.4	unknown	Pucciniomycetes	fungus clone KT2-C 18S (DQ177358)	96	1
MF4.3.1.5			<i>Pucciniomycotina</i> clone D0735_42_M (EU647044)	99	2
MF4.3.2.2	unknown	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	99	4
MF4.3.2.5			<i>Pucciniomycotina</i> clone D0735_21_M (EU647025)	96	1
MF4.3.2.7	unknown	Agaricomycetes	<i>Fomitopsis rosea</i> strain 6954-T (AY336764)	97	1
MF4.3.2.8			<i>Corioloopsis gallica</i> strain 7630-SP (AY336772)	99	1
MF4.4.1.1	unknown	Pucciniomycetes	<i>Pucciniomycotina</i> clone D0735_42_M (EU647044)	96	1
MF4.4.1.3			Basidiomycete clone 8-2-5 (EU085016)	98	1
MF4.4.1.4	Pucciniomycetes	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	98	2
MF4.4.1.6			Basidiomycete clone 8-2-5 (EU085016)	98	1
MF4.4.1.7	Pucciniomycetes	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	99	1
MF4.5.1.2			Basidiomycete clone 8-2-5 (EU085016)	99	4
MF4.5.1.5	unknown	Pucciniomycetes	fungus clone WIM108 (AM114819)	95	2
MF4.5.3.1			Basidiomycete clone 8-2-5 (EU085016)	99	2
MF4.5.3.3	Pucciniomycetes	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	93	1
MF4.5.3.7			Basidiomycete clone 8-2-5 (EU085016)	93	1
MF4.6.1.2	Pucciniomycetes	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	99	2
MF4.6.1.4			fungus clone WIM108 (AM114819)	98	4
MF4.6.2.7	unknown	Pucciniomycetes	Basidiomycete clone 8-2-5 (EU085016)	96	1
MF4.6.5.2			<i>Pucciniomycotina</i> clone D0735_34_S (EU646990)	95	2
MF4.1.1.1	Ascomycota	Dothideomycetes	<i>Aureobasidium pullulans</i> strain NG (EU009480)	99	2
MF4.5.2.2			<i>Aureobasidium pullulans</i> strain NG (EU009480)	99	1
MF4.6.2.5			<i>Lepidosphaeria nicotiae</i> strain 559.71 (DQ384068)	99	3
MF4.6.4.1			<i>Aureobasidium pullulans</i> strain NG (EU009480)	99	4

^a“species” derived from clone libraries are labeled with MF (marine fungi), followed by four numbers. The first, second, third and the fourth digit stands for site number, depth (1 = 5 m, 2 = 15 m, 3 = 50 m, 4 = 100 m, 5 = 150 m, and 6 = 200 m), band numbers and clone number, respectively.

as an ecologically, but not taxonomically, defined group (Kohlmeyer and Volkmann-Kohlmeyer, 2003). Marine fungi primarily include the *Ascomycetes*, the *Basidiomycetes*, and the *Chytridiomycetes* (Kohlmeyer and Volkmann-Kohlmeyer, 2003; Fell and Newell, 1998; Pang and Mitchell, 2005). In addition, the *Oomycetes* and *Labyrinthulomycetes*, formerly known as pseudofungi or fungal-like organisms were originally classified as fungi and have now been reclassified under the new Kingdom of Straminipila (Raghukumar, 2004; Wang and Johnson, 2009). As they are still commonly studied by marine

mycologists and mycoplankton ecologists (Fell and Newell, 1998; Kimura and Naganuma, 2001; Raghukumar, 2004), marine mycoplankton can be broadly defined to include marine filamentous fungi, yeasts and fungal-like protists in water column (Wang and Johnson, 2009). Results of this study revealed two phyla of marine fungi in the Hawaiian coastal water, with basidiomycetes as the dominant phylotypes (Table 1 and Figure 4). Several factors could contribute to the absence of other mycoplankton groups in the coastal waters. First, the abundance of the other mycoplankton groups may be low in the analyzed

Figure 4 Neighbor-joining tree based on fungal 18S rRNA gene sequences derived from Hawaiian waters.

samples, and it can particularly be the case for the *Chytridiomycetes* and the *Oomycetes*. Nonetheless, based on reports from others (Raghukumar, 2004; Wang and Johnson, 2009), this is not likely to be the case for the fungal-like protists.

Second, fungal 18S rRNA gene primers used in this study may have a low specificity to the other mycoplankton groups in the presence of many ribosomal genes from other marine eukaryotes. The

primer pair (NS1/NS2-10) used in this study yields the 18S rRNA gene regions with the size of 600 bp. The fragment contains useful sequence information to allow phylogenetic inference and is of suitable size for DGGE analysis (Teske *et al.*, 1996; Kowalchuk *et al.*, 1997). However, they have also been reported to match sequences from Acantharea, Amastigomonas, Alveolata, Bangiophyceae, Centroheliozoa, Cercozoa, Eumetazoa, Lobosea, Metazoa,

Rhodophyta, Stramenopiles and Viridiplantae (Pang and Mitchell, 2005). In addition, direct application of this PCR primer pair in terrestrial samples have been suggested to yield plant material (White *et al.*, 1990). In our study, direct amplification using these two primers on seawater samples did amplify 18S rRNA genes from Acantharea, Polycystinea, Alveolata, Cnidaria, Arthropoda, Streptophyta and Chlorophyta (data not shown). A nested PCR can successfully circumvent the low-specificity problem, with the first step designed to target the fungi and the second designed to amplify products suitable for DGGE analysis (Kowalchuk *et al.*, 1997; Gao *et al.*, 2008). This approach was shown to provide a satisfactory degree of success in discriminating against other environmental DNA while maintaining a broad range of fungal compatibility in fungi-infected marram grass and marine sponges (Kowalchuk *et al.*, 1997; Gao *et al.*, 2008). The drawback of PCR-based approaches is the introduction of biases due to the preferential amplification of certain templates, the nested PCR could augment such biases further (Kowalchuk *et al.*, 1997). Hence, some mycoplankton may be neglected by this strategy. However, this nested PCR protocol enabled to amplify broad taxonomic fungal groups, including members of *Ascomycota*, *Basidiomycota*, *Chytridiomycota* and *Zygomycota* from terrestrial environmental samples (Kowalchuk *et al.*, 1997) and there is no direct evidence that this nested PCR approach failed to detect any major fungal groups in our seawater samples.

Third, properties of fungal cells may enable some fungi more resistant than others to the cell lysis method used and result in a low yield of fungal genomic DNA for certain fungal groups. Finally, some marine fungal groups such as the oomycetes are known to be difficult to be amplified due to inhibitory materials in (personal communication with L Baker). Thus, the diversity uncovered in our study represents a lower boundary and there likely exists other mycoplankton phylotypes not found here.

The majority of the basidiomycete phylotypes did not cluster with any described fungal sequence in GenBank. This strongly suggests that these phylotypes are novel mycoplankton species and more novel fungal species in the world's ocean remain to be discovered. Particularly, 27 of basidiomycete phylotypes were closely related to the basidiomycete clone 8-2-5 from the Hawaiian alien sponge *Suberites zeteki*, with the sequence identity ranging from 93 to 100%. It indicates the abundant and unique presence of this group of mycoplankton abundant in the Hawaiian waters. This also reveals the incompleteness of the marine fungal 18S rRNA database. Several phylotypes found here are similar to those fungal clones found from other ocean provinces, suggesting they are potentially cosmopolitan in the marine environment (Figure 4). The close affiliation of the ascomycetes phylotypes

with other cosmopolitan fungal isolates seemed to support this observation. However, it is still premature to conclude that these novel phylotypes of marine fungi are endemic to Hawaii. Finally, none of the phylotypes discovered in this study matched with cultured fungi from Hawaiian seawaters or marine substrates (Steele, 1967; van Uden and Fell, 1968; Fell, 1976; Wang *et al.*, 2008a; Li and Wang, 2009 and Wang *et al.* unpublished data). Likely, mycoplankton phylotypes derived from cultivation and molecular methods belong to different populations similar to what has been observed in many ecological studies of other microbial communities in other natural environments (Hentschel *et al.*, 2006).

Our results suggest that mycoplankton communities show noticeable spatial diversity on both lateral and vertical gradients. In this study, mycoplankton diversity in water samples from the surface to 100 m in the coastal waters was greater than that in the open-ocean waters (Figure 3). This is consistent with the report that fewer culturable fungal isolates were found in water samples collected from pelagic regions than those from coastal waters (Steele, 1967). The biological and chemical variables (fluorescence, Chl-a, temperature, salinity) suggest that higher primary production and higher photosynthetic biomass were present at these depths at site 1 (coastal) than those in site 4 (open ocean). The primary production of the coastal waters is known to commonly exceed the consumption of herbivores, and therefore, most of such primary organic matter becomes available to the consumers as detritus (Newell, 1982). Furthermore, mycoplankton use phytoplankton exudates and detritus as nutrient sources (Kimura and Naganuma, 2001; Raghukumar, 2004). Therefore, it is not surprising to observe more diverse mycoplankton communities in the coastal waters than open-ocean waters at these depths. However, based on the scatter analysis, detrital organic carbon remains relatively high at depths of ≥ 150 m in coastal waters (data not shown) whereas phytoplankton biomass were similar (Figure 3). Thus, our results suggest that the phytoplankton and primary production has a more dominant role in determining mycoplankton diversity in deep samples. Finally, this study shows for the first time that diversity of mycoplankton communities displays vertical variations. The vertical diversity profile in coastal waters differs dramatically from those in the open ocean. Therefore, biological, geochemical and chemical factors, which govern diversity and composition of mycoplankton in the coastal waters, may differ from those in the open ocean. Because of the dynamic nature of the marine ecosystem, the relationship between planktonic fungi and their biotic and/or abiotic environmental conditions could be very complicated and should be a promising research topic for future exploration of functional ecology of marine mycoplankton.

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