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Microbial and Small Eukaryotes Associated With Reefs in the Upper Gulf of Thailand

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Reef sites of Ko Samae San (S), Khao Ma Cho (K) and Ko Tao Mo (T) in the upper Gulf of Thailand have abundant corals and represent a hotspot of marine biodiversity. Coral reefs serve as major networks of food and energy, where bacteria, microbial eukaryotes (fungi) and small eukaryotes play significant roles as primary producers that convert inorganic compounds to organic compounds, degraders of toxic substances, and recyclers. These functions sustain food and energy supplies. Advances in metagenomics and next-generation sequencing can provide knowledge of diversity without limitations imposed by media and other conditions associated with laboratory cultures. Scientists have researched bacterial diversity of coral sites; however, a database for fungi and small eukaryotes from Thailand's sites with abundant corals is lacking. The present study combined fungal ribosomal intergenic spacer analysis (F-RISA) and 18S rRNA gene sequencing to unveil the first culture-independent microbial and small eukaryotes from these sites at two times and across four species of coral (*Porites lutea*, *Platygyra sinensis, Acropora humilis,* and *Acropora millepora*), seawater and sediment. Results showed that the small eukaryotic communities on corals were distinct from communities in the surrounding seawater and sediment. The communities were relatively similar at the three sites and during the two periods of time. Pearson's correlations indicated the community diversity were associated with water quality (e.g., dissolved oxygen concentrations and density of water).

Keywords: diversity, fungi, microbiome, 18S rRNA gene, coral

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INTRODUCTION

Coral reefs provide habitat where microbes and small eukaryotes (e.g., fungi, diatoms and copepods) live in potentially symbiotic relationships with corals, and these organisms play important roles in food and energy networks. These eukaryotic assemblages have been denoted "coral holobionts." Similar to bacteria, microbial and small eukaryotes serve as primary producers and recyclers, for examples, microalgae *Symbiodinium* photosynthesizes (Mydlarz et al., 2010), copepods and fungi degrade toxic nitrate and nitrite compounds, and fungi fix nitrogen gas in

seawater to forms that can be used by other organisms (Wegley et al., 2007). These activities support colonization, reproduction, growth, and resistance to stress (i.e., against coral bleaching, pathogen infection and pollution) for corals (Massana and Pedrós-Alió, 2008; Ainsworth et al., 2010; Mydlarz et al., 2010; Pootakham et al., 2018).

The upper Gulf of Thailand (GoT) harbored pristine reefs where corals remain abundant and healthy, including reefs at Ko Samae San (S), Khao Ma Cho (K) and Ko Tao Mo (T). These sites also harbor sone of the world's most diverse assemblages (Phongsuwan et al., 2013; Ramírez et al., 2017). These sites are part of the Sea Natural History Museum of Thailand, thus they are protected from many anthropogenic activities. These fringing reefs include numerous colonies of *Acropora, Platygyra,* and *Porites* (Phongsuwan et al., 2013; Latypov, 2015). The microbes and small eukaryotes of these relatively pristine sites have not been studied in detail.

Our study provides the first data on microbial and small eukaryotic assemblages at the S, K, and T sites, using fungal ribosomal intergenic spacer analysis (F-RISA) and sequencing of the V9 region of 18S rRNA gene. F-RISA measures a DNA length that is characteristic for fungal species, so different communities yield different combinations of lengths (Ranjard et al., 2001; Gillevet et al., 2009). The next generation sequencing (NGS) provides more power to detect and identify natural microbial diversity, without limitations due to media or other conditions associated with laboratory cultures (Riesenfeld et al., 2004; Rusch et al., 2007; Somboonna et al., 2014; Mahé et al., 2015; Khitmoh et al., 2017). Our study primarily characterized and compared the eukaryotic assemblages of the prevalent coral species at the three sites (*Porites lutea*, *Platygyra sinensis, Acropora humilis* and *Acropora millepora*), and the surrounding niches (sediment and seawater) during the wet and dry seasons in 1 year (**Figure 1A**). Independent replicates were collected during all sampling events (3–10 replicates). Our study also correlated data on the relative abundances of representative genera of eukaryotes with parameters describing water quality. These initial data serve as a reference, and they may advance our understanding of similar reefs where data on microbial and small eukaryotes are inadequate.

MATERIALS AND METHODS

Sampling

Samples were collected at S, K, and T sites during April-May (dry season) and September-October (wet season) in 2014. The coral reefs at these sites appeared healthy during both sampling events. Samples included 3–10 independent replicates for seawater, sediments, and prevalent coral species (*P. lutea*, *P. sinensis, A. Humilis,* and *A. millepora*). We collected coral colonies that were 5 cm in diameter, 5 L of seawater within 1 m above a colony, and 50 g of sediment within 1 m below a colony. All samples were placed in sterile bottles or bags, transported on ice, and analyzed within 7–14 days. Water quality was characterized by measuring temperature, pH, salinity, dissolved oxygen (DO) and density of water on-site (**Figure 1A**).

Metagenomic Extraction

Metagenomic DNA were extracted using Power Water DNA Isolation Kit for seawater and Power Soil DNA Isolation Kit for corals and sediments (MoBio, Carlsbad, CA, United States) according to the manufacturer's instructions. Microorganisms in replicate 2.5 L aliquots of seawater were captured on a sterile 0.22-micron filter using a vacuum filtration system (Merck Millipore, Massachusetts, MA, United States). For coral and sediment samples, 1 g of ground coral and 1 g of sediment were used. Extractions were performed for individual replicates and checked for DNA quality and concentration by agarose gel electrophoresis and nanodrop spectrophotometry, respectively.

Fungal Ribosomal Intergenic Spacer Analysis for Subgrouping of Independent Sampled Replicates

Fungal ribosomal intergenic spacer analysis was performed according to established protocols, using universal primers 18S-2234C (5′-GTTTCCGTAGGTGAACCTGC-3′) and 28S-3126T (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Ranjard et al., 2001; Gillevet et al., 2009). To prevent potential bias in PCR, for each replicate the minimum of two PCRs were performed and pooled. Each PCR comprised 1 × EmeraldAmp® GT PCR Master Mix (TaKaRa, Shiga, Japan), 0.3 μM forward primer, 0.3 μM reverse primer, and the metagenome. The thermocycling conditions were 95°C for 4 min, followed by 30–35 cycles of 94°C for 1 min, 55∘C for 1 min and 72∘C for 2 min, and ended by 72∘C for 10 min. Different communities or subgroups were identified by different banding patterns upon agarose gel electrophoresis.

18S rRNA Gene Library Construction and NGS

The 18S rRNA V9 gene libraries were constructed following Caporaso et al. (2012), using universal eukaryotic primers Illumina_Euk_1391F (5′-GTACACACCGCCCGTC-3′) and Illumina_EukBr (5′-TGATCCTTCTGCAGGTTCACCTAC-3′), appended with 5′ Illumina adapter and 3′ Golay barcode sequences. PCR comprised 1 × EmeraldAmp® GT PCR Master Mix (TaKaRa), 0.2 μM of each primer, and the metagenome. Triplicate PCRs were performed and pooled for each sample or subgroup to address potential bias. The thermocycling conditions were 94°C for 3 min, and 28–30 cycles of 94°C for 45 s, 50°C for 60 s and 72°C for 90 s, followed by 72°C for 10 min. The amplicons were purified using GenepHlow™ Gel Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan), and quantified with Picogreen (Invitrogen, Eugene, Oregon, OR, United States). 200 ng of each sample or subgroup was pooled for NGS, along the sequencing primers and index sequence (Caporaso et al., 2012), using Miseq300 platform (Illumina, San Diego, CA, United States) housed at the Chulalongkorn Medical Research Center, Chulalongkorn University (Bangkok, Thailand).

Bioinformatic Analyses

Raw sequences were quality screened following Mothur's standard operating procedures for MiSeq (Schloss et al., 2009)

A

Sources	Types of samples	Sample depth	Seasons	Temp. (°C)	pH	Salinity (psu)	DO (mg/L)	Density (kg/m³)	Sample names
Ko Samae San	Coral (*Porites lutea*)	2	Dry	30.2	8	31.17	5.41	1022.76	SCD1
			Wet	29.9	7.45	30.72	5.37	1018.8	SCD2
	Sediment	2	Dry	30.2	8	31.17	5.41	1022.76	SCW1
			Wet	29.9	7.45	30.72	5.37	1018.8	SSD1
	Water	2	Dry	30.2	8	31.17	5.41	1022.76	SSD2
			Wet	29.9	7.45	30.72	5.37	1018.8	SSW1
Khao Ma Cho	Coral (*Platygyra sinensis*)	3	Dry	30.2	8	31.17	5.41	1022.76	SWD1
			Wet	29.9	7.45	30.72	5.37	1018.8	SWD2
	Sediment	3	Dry	30.2	8	31.17	5.41	1022.76	SWW1
			Wet	29.9	7.45	30.72	5.37	1018.8	KCD1
	Water	3	Dry	30.2	8	31.17	5.41	1022.76	KCW1
			Wet	29.9	7.45	30.72	5.37	1018.8	KSD1
Ko Tao Mo	Coral (*Acropora humilis*)	2	Dry	30.5	8.11	32.50	5.51	1025.4	KSW1
			Wet	29.5	7.89	31.20	5.59	1021.7	KWD1
	Coral (*Acropora millepora*)	2	Dry	30.5	8.11	32.50	5.51	1025.4	KWW1
			Wet	29.5	7.89	31.20	5.59	1021.7	TCHD1
	Sediment	2	Dry	30.5	8.11	32.50	5.51	1025.4	TCHD2
			Wet	29.5	7.89	31.20	5.59	1021.7	TCHW1
	Water	2	Dry	30.5	8.11	32.50	5.51	1025.4	TCMD1
			Wet	29.5	7.89	31.20	5.59	1021.7	TCMD2
									TCMW1
									TSD1
									TSD2
									TSW1
									TWD1
									TWD2
									TWW1

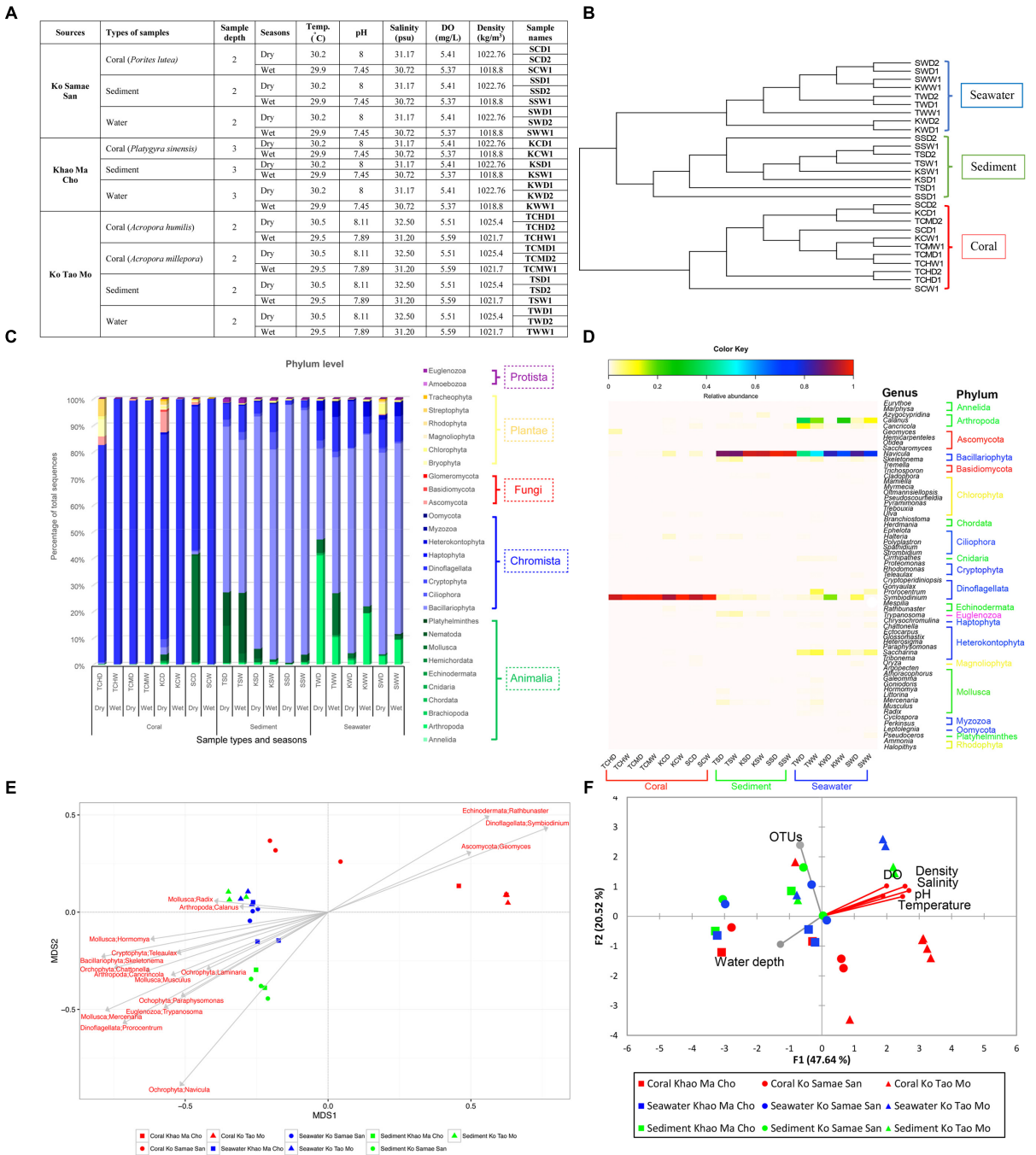

FIGURE 1 | Samples collected **(A)**, unweighted pair group with arithmetic mean (UPGMA) clustering to differentiate communities **(B)**, relative abundances at the phylum **(C)** and genus **(D)** levels, and Pearson's correlations for genera **(E)** and variables characterizing water quality **(F)**. Unclassified phyla were not displayed in **(C,D)**. In **(D)**, organisms with relative abundances <1% were omitted. In **(F)**, the vector direction and length represent the direction and strength of the correlation of that variable with the relative abundances of organisms. Red vectors represent significant correlations ($p < 0.01$), and gray vectors represent non-significant correlations ($p \geq 0.01$).

that included removal of sequences that have (i) ambiguous bases, (ii) >1 mismatch base in the primer region, (iii) ≥1 mismatch base in the barcode, (iv) >10 homopolymers, (v) a minimum sequencing quality score of <35 over a 50-bp window, (vi) a read length of <350 bases, and (vii) chimera sequence (Huse et al., 2010; Edgar et al., 2011). Sequences were aligned against Silva database (Schloss et al., 2009). Operational taxonomic units (OTUs) were assigned using Naïve Bayesian method (Wang et al., 2007). Data for all samples (and subgroups) were normalized to an equal sequencing

depth (78,068 sequences). Mothur was used to compute Good's coverage to estimate a sequencing coverage, rarefaction curve, alpha diversity (species richness and evenness in a sample: Chao1 richness, Shannon diversity and inverse Simpson index), beta diversity (thetayc dissimilarity coefficients, unweighted pair group with arithmetic mean (UPGMA) clustering, and principal coordinates analysis (PCoA)), and AMOVA (Schloss et al., 2009). For sample with 2 subgroups, a student's *t*-test was computed, and the subgroups were merged if $p > 0.01$. Correlations between the relative abundances of common genera and water quality variables were determined using Pearson's correlation (Schloss et al., 2009) and *XLSTAT*-Ecology software[1].

Availability of Supporting Data

Nucleic acid sequences were deposited in an open access Sequence Read Archive database of NCBI, accession number SRP095762.

RESULTS

Fungal Ribosomal Intergenic Spacer Analysis for Subgrouping of Independent Sampling Replicates

The 3–10 replicate samples exhibited similar F- RISA banding patterns, suggesting limited diversity among replicates (no subgroup classification). Nonetheless, we analyzed bacterial ribosomal intergenic spacer analysis (B-RISA), and some samples showed two B-RISA patterns (e.g., SCD1 and SCD2 represent 2 B-RISA subgroups in **Figure 1A** and SCW1 represents one B-RISA subgroup). To ensure we did not miss any eukaryotic subgroup, we constructed and sequenced the 18S rRNA gene libraries based on the B-RISA subgroups.

18S rRNA Gene Sequencing

Libraries of 18S rRNA genes were constructed successfully for all subgroups, because the numbers of quality sequences per subgroup were high (avg. 218,885 sequences/subgroup). This sequencing depth was statistically sufficient, as indicated by Good's coverage of 99.99–100% at genus-level OTUs (**Supplementary Table S1A**). These results are consistent with estimates of richness at the genus level derived from rarefaction curves that plateaued at this sequencing depth. The 18S rDNA data for fungi were analyzed separately, and they yielded Good's coverage values of 87.33–97.91% (**Supplementary Table S1B**).

Shannon diversity indices indicated greater taxonomic richness in sediment and seawater (**Supplementary Table S1A**). These results highlighted the specificity of the community on corals. Eukaryotes in the Kingdoms Chromista (88%: Dinoflagellata 66% and Bacillariophyta 22%) and Animalia (mostly worms and molluscs) were more common than fungi (**Supplementary Figures S1A,B**). Among the four fungal phyla, Ascomycota was the most dominant (>90%), followed by Basidiomycota (5.609%), an unclassified phylum (3.741%) and Glomeromycota (0.003%; **Supplementary Figure S1C**).

[1]www.xlstat.com/en/solutions/ecology

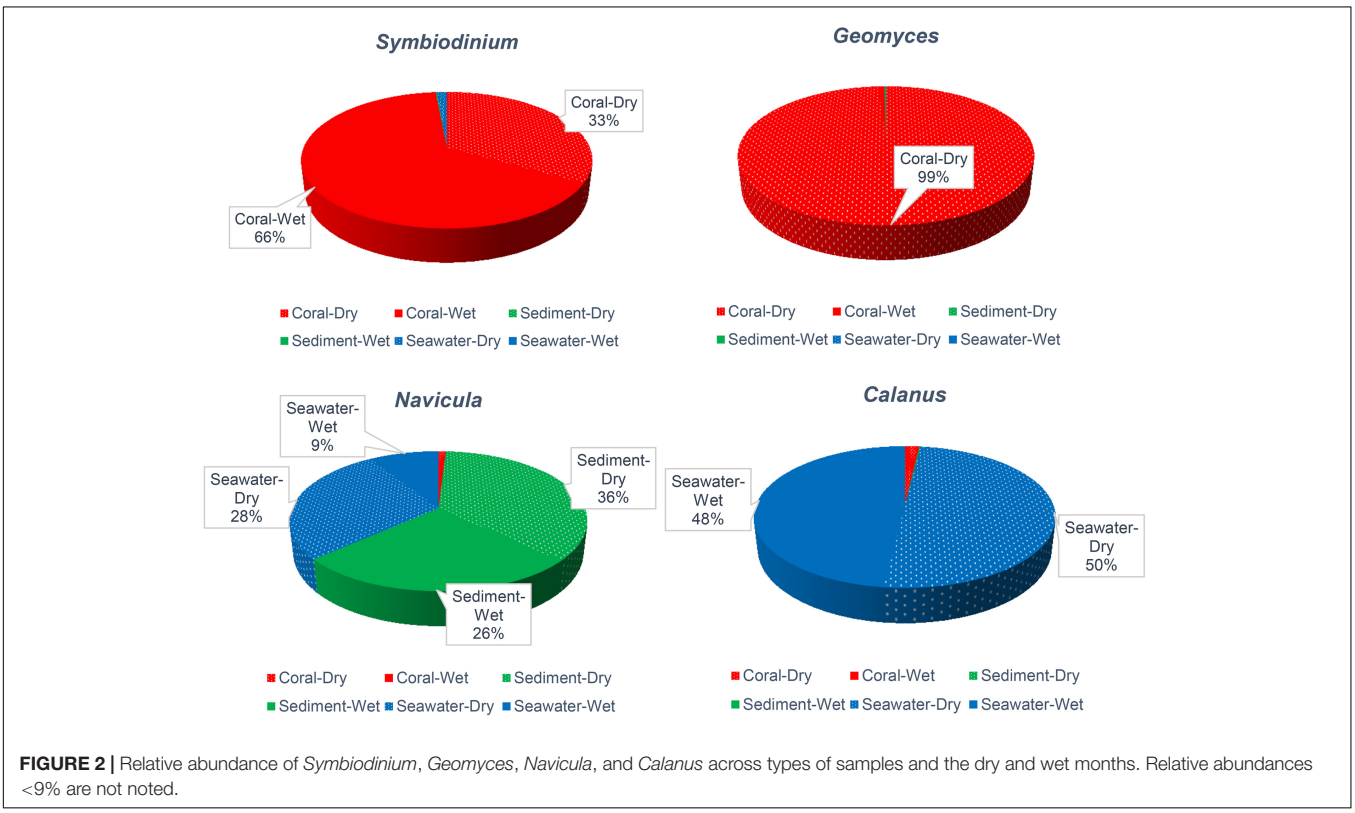

FIGURE 2 | Relative abundance of *Symbiodinium*, *Geomyces*, *Navicula*, and *Calanus* across types of samples and the dry and wet months. Relative abundances <9% are not noted.

Comparison of Communities From Species of Corals, Sites, and Times

In the UPGMA, the communities from corals clearly clustered separately from the communities from seawater and sediment across all sites and the two periods of time (**Figure 1B**). These results further highlight the unique community associated with corals, and AMOVA also identified a statistically significant difference between these communities ($p < 0.001$). However, the communities at the three sites and during the wet and dry sampled months were not statistically different (S vs. K vs. T, $p = 0.082$; months, $p = 0.707$). Moreover, pairwise comparisons between F-RISA subgroups supported the F-RISA results, because all corresponding subgroups were statistically similar (avg. $p = 0.33$; **Supplementary Table S2**). The corresponding subgroups were thereby merged.

Figures 1C,D exhibited phyla and genera that dominated and differentiated the communities. The genus *Symbiodinium* in phylum Dinoflagellata dominated samples of corals, whereas the genus *Navicula* in Bacillariophyta dominated in sediments and seawater. Fungi, although relatively rare, were 25–96 times more abundance in samples of corals (avg. 1.67%) than in sediments (0.017%) and seawater (0.066%; **Figure 1C**), and helped differentiate the communities on corals (phylum Ascomycota: genus *Geomyces*; **Figure 1E**). Additional genera specific to corals were *Symbiodinium* and *Rathbunaster* (**Figure 1E**).

To better understand the community associated with corals, genera that were reported to affect coral health were assessed. *Symbiodinium*, a well-known coral holobiont, was most abundant on all species of coral and more abundant in the dry (66%) months. In contrast, the relative abundance of *Symbiodinium* in sediment and seawater was only 1% (**Figure 2**). The presence and abundance of *Geomyces* on corals in the dry months raises some concern, because *Geomyces* can cause a skin disease called "white-nose syndrome" (Work and Meteyer, 2014). However, the corals we collected appeared healthy, so *Symbiodinium* and echinoderm *Rathbunaster* might limit the effects of *Geomyces*.

The diatom *Navicula* was relatively abundant in sediment, and the free-living copepod *Calanus* was relatively abundant in seawater (**Figure 2**). These organisms may play important roles in the system. *Navicula* has been reported to play major role in cycling of nitrogen and phosphate (Kwon et al., 2013; Stock et al., 2014), and it is a food source for *Calanus*, which itself is a food for corals and other animals.

Correlation With Water Quality

Dissolved oxygen, density of water, salinity, pH, and temperature were correlated in the similar vector direction to the coral communities ($p < 0.01$; **Figure 1F**). The community diversity were not significantly correlated with numbers of OTUs and water depth. Given a sufficient sequencing depth, additional OTUs should not alter relative abundances substantially, and all samples were collected the similar depth (2–3 m) so the communities would not be expected to exhibit substantial variation.

CONCLUSION

This study provided initial data on the diversity of microbes and small eukaryotes on coral reefs in the upper GoT. The community on corals was consistently different from the communities in seawater and sediments, and all communities were similar at the three sites (S, K, and T) and in the two periods of sampling. Pearson's correlations indicated that the relative abundances of various genera were correlated in different vector directions with several metrics characterizing water quality.

AUTHOR CONTRIBUTIONS

DB did molecular biology experiments and performed the data analysis. AW helped the data analysis. ST and VV conceived the study. SC provided the samples and conceived the study. NS conceived the study, coordinated the experiments and data analysis, and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00436/full#supplementary-material

FIGURE S1 | Overall eukaryotic distribution by kingdoms **(A)** and phylum **(B,C)**. In **(C)**, only fungal phyla were displayed.

TABLE S1 | Estimates of sample coverage and diversity indices at the phylum and genus levels for all 18S rDNA profiles **(A)** and for fungal profiles **(B)**.

TABLE S2 | Student's *t*-test to evaluate statistically significant differences in the community structures of genus-level operational taxonomic unit (OTU) compositions between corresponding subgroups.

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