The Shifts of Diazotrophic Communities in Spring and Summer Associated with Coral Galaxea astreata, Pavona decussata, and Porites lutea

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The coral holobiont often resides in oligotrophic waters; both coral cells and their symbiotic dinoflagellates possess ammonium assimilation enzymes and potentially benefit from the nitrogen fixation of coral-associated diazotrophs. However, the seasonal dynamics of coral-associated diazotrophs are not well characterized. Here, the seasonal variations of diazotrophic communities associated with three corals, Galaxea astreata, Pavona decussata, and Porites lutea, were studied using nifH gene amplicon pyrosequencing techniques. Our results revealed a great diversity of coral-associated diazotrophs. nifH sequences related to Alphaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria were ubiquitous and dominant in all corals in two seasons. In contrast with the coral P. decussata, both G. astreata and P. lutea showed significant seasonal changes in the diazotrophic communities and nifH gene abundance. Variable diazotroph groups accounted for a range from 11 to 49% within individual coral samples. Most of the variable diazotrophic groups from P. decussata were species-specific, however, the majority of overlapping variable groups in G. astreata and P. lutea showed the same seasonal variation characteristics. Rhodopseudomonas palustris- and Gluconacetobacter diazotrophicus-affiliated sequences were relatively abundant in the summer, whereas a nifH sequence related to Halorhodospira halophila was relatively abundant in spring G. astreata and P. lutea. The seasonal variations of all diazotrophic communities were significantly correlated with the seasonal shifts of ammonium and nitrate, suggesting that diazotrophs play an important role in the nitrogen cycle of the coral holobiont.

Keywords: diazotrophs, diversity, nifH, pyrosequencing, coral reef
INTRODUCTION

The coral holobiont is a multi-partner symbiotic system that forms associations with both external and internal microbiota (Mouchka et al., 2010). There is increasing evidence that coral-associated microbial communities are crucial for biogeochemistry and control the health and resilience of coral reef ecosystems (Mouchka et al., 2010; Thompson et al., 2014). Due to their relative size and high per cell activity, a small change in microbial biomass may signal a large reallocation of available energy in the ecosystem (McDole et al., 2012; Haas et al., 2016). Corals often reside in oligotrophic waters. Nitrogen fixation, one of the new fixed nitrogen input into the ecosystem, has long been thought to be an important linchpin to sustain biological productivity in coral reef areas (Wiebe et al., 1975; Capone et al., 1977; Shashar et al., 1994b; Cardini et al., 2014). Nitrogen fixation activity within the coral holobiont has been detected using acetylene reduction and isotopic assays in several coral species (Williams et al., 1987; Shashar et al., 1994a; Lesser et al., 2007; Chimetto et al., 2008; Grover et al., 2014; Benavides et al., 2016). Coral-associated nitrogen-fixing bacteria, capable of fixing and converting gaseous nitrogen (N₂) to biologically available nitrogen forms, contribute an important source of nitrogen to the coral holobiont (Lesser et al., 2007; Cardini et al., 2014). Both coral cells and their symbiotic dinoflagellates (Symbiodinium) have the capacity to assimilate ammonium (Perince et al., 2012) and benefit from nitrogen-fixing bacteria (Lesser et al., 2007; Cardini et al., 2014; Santos et al., 2014). Additionally, a close relationship was found between the abundance of coral-associated diazotrophs and symbiotic dinoflagellates (Lesser et al., 2007; Olson et al., 2009; Santos et al., 2014), and between coral-associated diazotrophs and coral productivity (Cardini et al., 2014).

Accumulating evidence shows that diazotrophic organisms are ubiquitous members of coral-associated microbial communities and form species-specific associations with their hosts (Lema et al., 2012, 2014b). However, the degree to which nitrogen-fixing bacterial communities are specific to their coral hosts can vary, as both species-specific (Lema et al., 2012) and site-specific (Lema et al., 2014b) nitrogen-fixing microbial communities have been reported. Nitrogen fixation activity in corals is also highly dynamic and can be rapidly affected by changes in environmental conditions (Lesser et al., 2007; Rädecker et al., 2014). It is suggested that coral holobionts harbor both a core microbiome determined by holobiont macroorganisms, and a variable microbiome to adapt to local conditions (Kelly et al., 2014; Ainsworth et al., 2015). As the disturbance of microbial nitrogen-cycling may be tightly linked to coral bleaching and disease (Rädecker et al., 2015), knowledge of the variable diazotrophic groups would help to further evaluate the importance of these communities to the coral host; however, such knowledge is currently lacking.

The nifH gene encodes a conserved subunit of the dinitrogenase iron protein responsible for nitrogen fixation, and is conserved in all known diazotrophs (Zehr et al., 2003). As the agreement with 16S rRNA gene-based phylogeny, nifH is an ideal molecular target and is widely used for gene-based phylogenetic characterization of diazotrophs (Gaby and Buckley, 2012). In this study, high-throughput Illumina sequencing was used to investigate seasonal and species-specific patterns in diazotrophic communities associated with the corals Galaxea astreata, Pavona decussata, and Porites lutea. The core and variable diazotrophic microbiome were tested through comparative analysis. The aims were to: (i) investigate the diversity and abundance of diazotrophic communities associated with three coral species; (ii) determine the seasonal shifts of diazotrophic communities and the variable diazotrophic species associated with different coral species; (iii) explore the possible relationship between the coral-associated seasonal-variable diazotroph groups and environmental variables, given the important role of diazotrophic microbes in driving biogeochemical cycles.

MATERIALS AND METHODS

Study Site and Sampling Collection

Coral samples were collected from Luhuitou fringing reef (18°12’19”N, 109°28’27”E) located in Sanya Bay of South China Sea, which is affected by cold-water upwelling during the summer. Cold-water upwelling affects the distribution of a variety of dissolved and particulate forms of nitrogen in Sanya Bay (Huang et al., 2003; Zhang et al., 2010; Wu et al., 2012). Furthermore, in summer, tropical cyclones and monsoonal rainfall may also carry high nutrient loads. Differences in nutrient loads between spring and summer may influence overall diversity of coral-associated diazotrophic communities.

G. astreata, P. decussata, and P. lutea are important scleractinian species and are all a natural occurrence in the Luhuitou fringing reef region. All coral samples were collected in April and June 2013 using a punch and hammer. Three healthy coral colonies from each coral species were collected at a depth of 5–10 m. Triplicate coral fragments (∼2 cm²) for each coral colony were placed in sealed plastic bags, rinsed thoroughly with sterile seawater at the surface, placed on ice and transported to the laboratory (Tropical Marine Biological Research station in Hainan). Samples were cryopreserved at −20°C.

Seawater samples within 20 cm of the coral colonies (n = 3) for temperature and salinity were measured using a YSI 6600V2 water quality sonde. Dissolved oxygen (DO) was determined by DO meter, pH was measured using a standard hydrogen electrode and reference electrode, and chemical oxygen demand (COD) was determined by alkaline potassium permanganate method. Inorganic nutrients including nitrate, ammonium, nitrite, and phosphate were measured using standard methods as described previously (Huang et al., 2003).

DNA Extraction, Amplification, Sequencing, and Data Processing

The coral fragments were suspended in TE buffer and homogenized in a sterilized mortar and pestle with liquid nitrogen. The homogenized solution was transferred to a clean tube and the total community DNA was extracted using an E.Z.N.A.® Soil DNA Kit (Omega Biotek). The DNA was then purified with a Promega Wizard DNA clean-up system (Madison,
Diazotrophic bacteria richness and diversities were confirmed by melting-curve analysis, and the amplified fragments were checked by electrophoresis in 2% agarose gel to confirm the expected sizes of amplicon. The specificity of the amplification products was confirmed by qPCR. Triple qPCRs were performed for all samples and standard curve efficiency (E) was 1.90. The number abundance. Response ratio analysis was conducted to identify seasonal variation of nifH gene diversities and nifH copy number abundance. Response ratio analysis was conducted to detect significantly seasonally changed OTU (Deng et al., 2012). Redundancy analysis was performed to determine the relationship between variable diazotroph groups and environmental parameters.

RESULTS

Environmental Characteristics, Diazotrophic Composition, Community Structure, and nifH Gene Abundance

The temperature of ambient seawater was 24.63 ± 0.32°C in the spring, and 25.83 ± 0.45°C in the summer. The average water salinity was 33.46% in the spring and 35.01% in the summer. The nutrient concentrations of ammonia and phosphorus were higher in the spring. However, the concentration of nitrate was higher in the summer. Higher values of COD and DO were detected in the summer. The concentration of Chlorophyll a was higher in the spring (Supplementary Table S1).

After processing, 426,335 high quality nifH sequences (310–330 bp) were retrieved from the fragments of coral G. astrea, Pavona decussata and Porites lutea. Samples were rarefied to 10,000 sequences per sample. All sequences obtained could be assigned to 2,146 OTUs (90% similarity level). The significantly seasonally changed diazotrophic OTUs identified in this study have been deposited in the GenBank database under nucleotide accession numbers KX078090 to KX078212 for nifH gene sequences.

Quantification of nifH Gene Copy Number

To quantify the number of copies of the nifH gene, the primers PolF and PolR were used. Absolute quantification was carried out on the Lightcycler 480 System (Roche). Standard curves were developed by serially diluting plasmid containing a nifH gene to final concentrations from 10^3 to 10^8 copies/µL. The qPCR efficiency (E) was 1.90. The R^2 of standards was higher than 0.99. Triple qPCRs were performed for all samples and standard curve concentrations. The specificity of the amplification products was confirmed by melting-curve analysis, and the amplified fragments were checked by electrophoresis in 2% agarose gel to confirm the expected sizes of amplicon. The nifH copy number was ultimately expressed as per µg coral colony dry weight.

Statistical Analysis

All analyses were performed using the R vegan package (R Foundation for Statistical Computing, Vienna, Austria), our R-based pipeline1, and the software package CANOCO 4.5 for Windows. Diazotrophic bacteria richness and diversities were calculated using Chao1, Shannon–Wiener’s (H’), and evenness. Principal coordinates analysis (PCoA) was used to visualize the changes of overall diazotrophic community structure. Dissimilarity tests by permutation multivariate analysis of variance (PERMANOVA) were performed with Euclidean, Manhattan, Bray–Curtis, and Jaccard for comparing seasonal variation of diazotrophic communities. Significance tests based on unpaired Student’s t-test were applied to identify seasonal variation of nifH gene diversities and nifH copy number abundance. Response ratio analysis was conducted to identify seasonal variation of nifH gene diversities and nifH copy number abundance. Response ratio analysis was conducted to detect significantly seasonally changed OTU (Deng et al., 2012). Redundancy analysis was performed to determine the relationship between variable diazotroph groups and environmental parameters.

1http://ieg.ou.edu/microarray/
Deltaproteobacteria, and Gammaproteobacteria were ubiquitous and dominant groups that constituted 76.78% of all nifH sequences (Figure 1). A dissimilarity test based on the Euclidean, Manhattan and Bray-Curtis matrix showed that the diazotrophic communities associated with coral G. astreata and Porites lutea in spring were significantly different from those in summer (PERMANOVA, \( P = 0.001 \); Table 1), indicating that for these two coral species the diazotrophic communities significantly changed according to the season. However, no significant changes were detected in coral Pavona decussata. PCoA also showed the significant seasonal variation in the diazotrophic communities associated with coral G. astreata and Porites lutea as revealed by the plot. However, Pavona decussata -associated diazotrophic communities did not vary in a seasonal manner (Figure 2).

The nifH copy number was determined by quantitative PCR with the absolute quantification method. Significant seasonal variations were detected in both G. astreata and Porites lutea coral colonies (Table 2). The nifH gene copies in spring G. astreata colonies were significantly higher than those in summer (t-test, \( P < 0.001 \)). However, the nifH gene copies in summer Porites lutea colonies were much higher than those collected in spring (t-test, \( P < 0.001 \)). No statistical seasonal difference was detected in abundance of nifH gene copies from Pavona decussata colonies (t-test, \( P = 0.841 \); Table 2).

Seasonal Variations of Diazotrophic Communities

Seasonal variable diazotrophic communities were detected at 95% confidence interval. The numbers of variable OTUs were 45, 44, and 48 in coral G. astreata, Pavona decussata, and Porites lutea, respectively (Figure 3). These variable OTUs accounted for a range from 13.92 ± 1.56 to 38.45 ± 5.12% of the total sequences within individual coral samples (Supplementary Table S3). For all coral samples, the majority of variable nifH sequences fell within Alphaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria, and a few within Cyanobacteria, Betaproteobacteria, Chlorobi, Firmicutes, and Verrucomicrobia (Figure 3).

The dominant variable nifH sequences retrieved from spring G. astreata samples belonged to Gammaproteobacteria, however, those dominant in summer G. astreata samples belonged to Alphaproteobacteria and Deltaproteobacteria (Figure 3A). Within the Gammaproteobacteria, three dominant OTUs were identified (OTU669, OTU991, and OTU758) and constituted 10.37% of the total sequences recovered from spring G. astreata samples. The dominant group (OTU669) was affiliated with Thiornhodospira sibirea (96% similarity). Both OTU991 and OTU758 were affiliated with Halorhodospira halophila (93 and 86% similarity). More than 74% of total summer G. astreata variable nifH sequences (29.29% of 39.57%) belonged to seven OTUs (OTU3305, OTU3604, OTU3752, OTU706, OTU608, OTU616, and OTU619). The nifH sequences within the Alphaproteobacteria were relatively abundant in summer. The dominant groups (OTU3305, OTU3604, and OTU3752) comprising up to 16.51% of sequences recovered from summer G. astreata samples, were affiliated with Gluconacetobacter diazotrophicus (87% similarity), Rhodopseudomonas palustris (92% similarity) and Azospirillum lipoforum (91% similarity), respectively. The variable OTUs within the Deltaproteobacteria were all affiliated with anaerobic sulfate-reducing bacteria of which the dominant members OTU616, OTU608, OTU706 and OTU619 were affiliated with Desulfonatromon thiosulfatophilum (95% similarity), Desulfovibrio desulfuricans (94% similarity), Desulfatibacillus alkenivorans (93% similarity) and Desulfurodomonas acetoxidans (95% similarity) respectively.

In contrast with G. astreata samples, diazotrophic communities associated with Pavona decussata samples showed different seasonal variation patterns (Figure 3B). OTU242 and OTU3228 dominated the variable nifH sequences derived from spring Pavona decussata samples. OTU3228 was closely related to a nifH sequence from cyanobacterium Leptolyngbya boryana (95% similarity). OTU242 demonstrated 86% identity with Alphaproteobacteria Mesorhizobium sp. Seven OTUs (OTU799, OTU760, OTU663, OTU602, OTU3292, OTU3253, and OTU3829) constituting more than 65% of total summer Pavona decussata variable nifH sequences (16.11 of 24.66%) fell within Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria. The dominant groups (OTU3253 and OTU3829) represented 6.42% of the total sequences recovered from summer Pavona decussata samples affiliated with nifH sequences from Betaproteobacteria Dechlorosoma suillum. OTU799 and OTU760 were members of the Deltaproteobacteria class, affiliated with anaerobic sulfate-reducing bacterial genus Desulfovibrio. Within the Gammaproteobacteria class, the dominant groups (OTU663 and OTU602) which represented 4.69% of the total sequences recovered from summer Pavona decussata samples were both closest to Halorhodospira halophila. Furthermore, OTU3292 was affiliated with nifH sequences from Alphaproteobacteria, Hyphomicrobium sp. (95% similarity).

For coral Porites lutea, Deltaproteobacteria dominated the variable nifH sequences retrieved from spring samples, and the Alphaproteobacteria dominated those from summer ones (Figure 3C). Within the Deltaproteobacteria, the majority of these sequences were affiliated with the genera Desulfovibrio and Desulfurodomonas, with six dominant ribotypes (OTU619, OTU627, OTU2272, OTU630, OTU2284, and OTU649) comprising up to 14.09% of sequences recovered from spring Porites lutea samples. All were affiliated with anaerobic sulfate-reducing bacteria Desulfovibrio aspoeonius, Desulfurodomonas acetoxidans, and Pelobacter carbinolicus. With one exception in Deltaproteobacteria, the dominant variable (OTU667) affiliated

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**Table 1** | Dissimilarity tests of diazotrophic communities’ dissimilarity between spring and summer by ADONIS.

| Community          | Euclidean F | Euclidean P | Manhattan F | Manhattan P | Bray–Curtis F | Bray–Curtis P |
|-------------------|-------------|-------------|-------------|-------------|---------------|---------------|
| Galaxea astreata  | 0.279       | 0.001       | 0.308       | 0.001       | 1.746         | 0.001         |
| Pavona decussata  | 0.215       | 0.001       | 0.258       | 0.139       | 1.388         | 0.117         |
| Porites lutea     | 0.31        | 0.001       | 0.368       | 0.001       | 2.352         | 0.001         |

Significant differences (\( P < 0.05 \)) are indicated in italics.
with Desulfonatronospira thiodismutans (90% similarity) was relatively abundant in spring *Porites lutea* samples. Three dominant ribotypes (OTU3604, OTU3305, and OTU3276) falling within the Alphaproteobacteria, constituted 18.39% of total summer *Porites lutea* nifH sequences. The most dominant group (OTU3604), which represented 14.51% of the total sequences recovered from summer *Porites lutea* samples, affiliated with purple non-sulfur bacterium *Rhodopseudomonas palustris* (92% similarity). Two other Alphaproteobacteria groups (OTU3305 and OTU3276) affiliated with *Glucanacetobacter diazotrophicus* (87% similarity) and *Azospirillum lipoferum* (92% similarity), respectively. There were four dominant variable ribotypes recovered from *Porites lutea* samples falling within the Gammaproteobacteria. Two ribotypes (OTU991 and OTU672) were relatively abundant in spring samples affiliated with *Halorhodospira halophila* (93% similarity) and *Pseudomonas stutzeri* (96–97% similarity). Within the Cyanobacteria, the dominant group (OTU1414), affiliated with *Phormidium* sp. (95% similarity), was relatively abundant in summer *Porites lutea* samples. In addition, the dominant ribotype (OTU3989) affiliated with *Firmicutes, Clostridium arbusti* (93% similarity), was relatively abundant in spring *Porites lutea* samples.

The majority of variable nifH groups from coral *Pavona decussata* were species-specific. Only one overlap variable OTU was found between the coral *G. astreata* and *Pavona decussata* samples and two overlap OTUs were detected between the coral *Pavona decussata* and *Porites lutea* colonies (Supplementary Table S4). These overlap OTUs were generally present in relatively low abundance in all *Pavona decussata* colonies. Ten overlap OTUs were recovered from both *G. astreata* and *Pavona decussata*, of which the majority of dominant groups showed the same seasonal variation (Supplementary Table S4). Two Alphaproteobacteria OTUs (OTU3604 and OTU3305) were relatively abundant, representing 14.74 and 16.57% of the total sequences recovered from summer *G. astreata* and *Porites lutea*, respectively. The Gammaproteobacteria variable group (OTU991) was dominant in both *G. astreata* and *Porites lutea* spring colonies. One exception to this pattern is the Deltaproteobacteria variable (OTU619), which dominated summer *G. astreata* samples and spring *Porites lutea* samples.

**The Relationship between Seasonal Variable Diazotrophic Community and the Surrounding Seawater Environmental Factors**

To explore the possible relationship between the seasonal variable diazotrophic microbial community and environmental variables,

![FIGURE 2 | Principal coordinates analysis (PCoA) of diazotrophic community based on high-throughput nifH sequencing data. The percentage of variation explained by each axis is shown. DSP, spring *P. decussata*. DSU, summer *P. decussata*. GSP, spring *G. astreata*. GSU, summer *G. astreata*. LSP, spring *P. lutea*. LSU, summer *P. lutea.*](image-url)
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**FIGURE 3** | Significantly seasonal variable OTUs between spring and summer in *G. astreata* (A), *P. decussata* (B), and *P. lutea* (C) samples determined using the response ratio method at a 95% confidence interval based on the relative abundances. The variable OTUs are represented with their closest sequence match determined from GenBank BLAST, with corresponding accession number and taxonomic affiliation.

**TABLE 3** | Monte Carlo permutation test of environmental attributes with *nifH* high-throughput sequencing data.

|                | *G. astreata* | *P. decussata* | *P. lutea* |
|----------------|--------------|---------------|------------|
| Ammonium       | 0.009        | 0.045         | 0.044      |
| Nitrate        | 0.005        | 0.022         | 0.048      |
| Nitrite        | 0.061        | 0.083         | 0.092      |
| Phosphate      | 0.013        | 0.022         | 0.661      |
| Chlorophyll a  | 0.296        | 0.223         | 0.026      |
| pH             | 0.076        | 0.077         | 0.951      |
| COD            | 0.052        | 0.072         | 0.099      |
| DO             | 0.083        | 0.07          | 0.224      |
| Salinity       | 0.045        | 0.020         | 0.396      |
| Temperature    | 0.018        | 0.047         | 0.995      |

Significant differences (*P < 0.05*) are indicated in italics.

RDA was performed. The results showed that all seasonal variable diazotrophic communities of three coral species were significantly correlated with ammonium and nitrate (*P < 0.05*, Table 3). Axis 1 and 2 of the RDA biplot together were shown to contribute 84.8, 87.6, and 93.5% to the overall pattern of three coral species, respectively (Figure 4). Most of the dominant variable diazotrophic OTUs of spring samples were highly positively correlated with ammonium and phosphate (Figure 4). In contrast, those from summer samples were negatively correlated with ammonium and phosphate, but positively correlated with nitrate, salinity and temperature (Figure 4).

**DISCUSSION**

Although high-throughput sequencing approaches are now commonly applied to investigate coral-associated microbial communities (Ceh et al., 2011; Chen et al., 2011; Lee et al., 2012; Mckew et al., 2012; Sunagawa et al., 2009), it has not been widely used to target nitrogen-fixing functional genes (*nifH*) to explore the coral-associated diazotrophic communities (Lema et al., 2014b). Compared with previous high-throughput sequencing studies on diazotrophic communities associated with the common Great Barrier Reef (GBR) coral *Acropora millepora* (Lema et al., 2014b), a much greater diversity of bacteria having the potential to fix nitrogen (possess the *nifH* gene) associated with *G. astreata*, *Pavona decussata*, and *Porites lutea* from Luhuitou fringing reef, South China Sea (Table 1), and might be attributed to the differences in coral species. *Alphaproteobacteria* was reported as the dominant diazotrophic group associated with the common GBR coral *Acropora* (i.e., *A. millepora* and *A. muricata*) (Lema et al., 2012), while *Gammaproteobacteria* was the most commonly found class in the Hawaiian *Montipora* corals (*M. capitata* and *M. flabellata*) (Olson et al., 2009). In the present study, *nifH* sequences related to *Alphaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* were the ubiquitous and dominant groups of three coral species in two seasons. These three groups constitute from 46.65 to 91.1% of the sequences within individual coral samples (Figure 1), suggesting that those groups play an important functional role in the coral holobiont.
FIGURE 4 | The redundancy analysis (RDA) ordination biplot showing the relationship between environmental variables and significantly seasonal variable OTUs in G. astreata (A), P. decussata (B), and P. lutea (C) samples. Only abundant variable OTUs (1%) are shown in the biplot.

coral holobiont is a complex habitat, and microenvironmental variability can strongly influence the abundance of associated microbial communities (Rohwer et al., 2002; Ainsworth et al., 2010). Previous results reported that the coral-associated bacterial community showed rapid seasonal changes for several coral species distributed throughout different regions (Ceh et al., 2011; Chen et al., 2011). However, season did not influence the diazotrophic communities associated with common GBR coral A. millepora (Lema et al., 2014b). Significant differences were detected in the diazotrophic abundance of G. astreata and Porites lutea, indicating that for these two coral species the diazotrophic communities significantly changed according to the season.

Understanding the seasonal dynamics of this association is important, as they have ecological implications. In this study, seasonal variable diazotrophic sequences were detected based on the relative abundances at 95% confidence interval. The analyses showed that three corals have a low diversity group of seasonal variable species (range from 44 to 48 OTUs) whose abundance varies widely across individuals (Supplementary Table S3). These results suggested that there are core diazotrophic microbiomes associated with corals and these cores are complemented with seasonal variable diazotrophic microbiomes. Most of the variable nifH groups from Pavona decussata were species-specific. Only a few variable OTUs overlap with two other coral species. This may be an indicator of a specific association between coral and diazotrophic microorganisms. The majority of overlapping OTUs in G. astreata and Porites lutea showed the same seasonal variation suggesting that except host species, the variation of diazotrophic communities might be related to the biogeochemical cycling processes within the holobiont, given the important role of diazotrophs in driving biogeochemical cycles (Rädecker et al., 2014).

Alphaproteobacteria affiliated with the order Rhizobiales were reported as the continuous and dominant diazotrophic assemblages associated with the common GBR Acropora corals (Lema et al., 2012, 2014a,b). Additionally, the rhizobia found within pure mucus samples of New Caledonia reefs (predominantly composed of Acropora sp.) was much higher than in the surrounding seawater in the summer period and 400-fold higher in the winter (Camps et al., 2016). In the present study, the variable Alphaproteobacteria affiliated with Rhizobiales and Rhodospirillales. One rhizobial group (OTU3604) was most closely affiliated with purple non-sulfur phototrophic bacterium Rhodopseudomonas palustris. This group dominated all spring G. astreata and Porites lutea colonies, representing up to 6.57 and 14.51% of the total nifH sequences in spring G. astreata and Porites lutea samples, respectively (Figure 3). The Rhodospirillales groups closely related to Gluconacetobacter diazotrophicus and Azospirillum lipoferum were also the dominant variable groups found in all G. astreata and Porites lutea colonies. It is notable that the most dominant variable group of G. astreata was closely related to Gluconacetobacter diazotrophicus. Here Rhodopseudomonas palustris- and Gluconacetobacter diazotrophicus-affiliated OTUs were not correlated with investigated environmental factors, which suggested the seasonal variations of these diazotrophs are not affected by environmental factors. Rhodopseudomonas palustris is notable for its ability to flexibly switch between four different modes of metabolism: photoautotrophic, photoheterotrophic, chemoautotrophic, and chemoheterotrophic (Larimer et al., 2010).
variations of Deltaproteobacteria et al., 2014). The present study results showed that the seasonal and fermentation to occur within the holobiont (Thompson and skeletons enable anaerobic forms of bacterial respiration in stagnant water or sediment and coral surfaces (mucus layer) (Guldberg and Williamson, 1999) and microaerophilic regions oxygen-depleted conditions within the gastrodermis (Hoegh-Guldberg et al., 2003; Richardson and Kuta, 2003; Myers et al., 2007). The nifH phylotypes affiliated with Firmicutes Clostridium ljungdahlii were only detected showing significant seasonal variation in Porites lutea samples, comprising 2.77% of nifH phylotypes derived from spring Porites lutea and positively correlated with ammonium and phosphate.

The seasonal variations in coral-associated bacterial and diazotrophic communities may be influenced by the nutrient loads between spring and summer. The sample collection location Sanya Bay is affected by cold-water upwelling during the summer (Huang et al., 2003; Zhang et al., 2010; Wu et al., 2012). Cold-water upwelling affects the distribution of a variety of dissolved and particulate forms of nitrogen in Sanya Bay (Huang et al., 2003). Ammonium was the predominant dissolved nitrogen in spring, while nitrate was the predominant dissolved nitrogen in summer (Supplementary Table S1). In our previous study, the functional gene composition of the microbial community was significantly correlated with the concentrations of inorganic nitrogen and phosphate (Zhang et al., 2015). Here, all diazotrophic communities of the three coral species were significantly correlated with ammonium and nitrate (Table 3). In addition, the diazotrophic communities of coral G. astrea and Pavona decussata were significantly correlated with phosphorus (Table 3). This suggested that the apparent seasonal change in diazotrophic communities of corals could be linked to the seasonal shifts of nutrients. A priority for future studies should be to identify environmental variables contributing to these shifts in coral bacterial communities and to determine how they influence the health of the coral host.

CONCLUSION

This study revealed a much greater diversity of diazotrophs associated with G. astrea, Pavona decussata, and Porites lutea. Alphaproteobacteria, Deltaproteobacteria, and
**Gammaproteobacteria** were the ubiquitous and dominant groups in all corals in two seasons. Seasonal factors did not cause shifts in diazotrophic richness and diversities of the three coral species; even no shifts in diazotrophic communities and abundance were observed in coral *Pavona decussata*. In contrast, the diazotrophic communities and *nifH* gene abundance of both *G. astreata* and *Porites lutea* showed significant seasonal changes. Most of the variable *nifH* groups from *Pavona decussata* were species-specific. The dominant overlap OTUs in *G. astreata* and *Porites lutea* showed the same seasonal variation. The seasonal variations of diazotrophic communities were significantly correlated with the seasonal shifts of nutrients. Variable diazotroph groups are widely distributed in the environment and may be of relevance to diverse metabolic potential, such as carbon fixation and sulfate reduction. This suggests that their potential to provide additional sources of fixed nitrogen to the coral holobiont may be functionally important. However, almost all metabolic potential of these diazotrophs was referred from crops and land plants. The physiological roles of these nitrogen-fixing symbionts in the nitrogen budget and cycling within corals need to be investigated in detail.

### AUTHOR CONTRIBUTIONS

YZ and JD conceived the research. YZ and QY performed the experiments. YZ wrote the manuscript. JVN and JZ edited the manuscript. QY and ZS contributed sampling or data analysis pipeline. All authors reviewed and accepted the manuscript.

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### SUPPLEMENTARY MATERIAL

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