Diazotroph diversity associated with scleractinian corals and its relationships with environmental variables in the South China Sea

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1   **Diazotroph diversity associated with scleractinian corals and its relationships with environmental**
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Abstract

Background: Coral reef ecosystems cannot operate normally without an effective nitrogen cycle. For oligotrophic coral reef areas, coral-associated diazotrophs are indispensable participants in the nitrogen cycle. How coral-associated diazotrophs will change in order to adapt to environmental changes resulting from global warming and human activities is a topic of concern for researchers. To this end, 68 colonies of scleractinian coral were collected from 6 coral reefs areas with different environmental variables in the South China Sea to investigate the composition of associated diazotrophs based on *nifH* gene amplification using high-throughput sequencing. The six coral reefs can be clearly divided into two types (fringing reefs and island reefs), are affected by varying degrees of human activities and are located at different latitudes from 9°20'06"N to 22°34'55"N with different seawater temperatures.

Results: Alpha- and beta-diversity analyses showed that the distribution of diazotrophs among coral reefs exhibited greater geographical fluctuations than interspecific fluctuations. The predominant bacterial phyla included Proteobacteria, Chlorobi, Cyanobacteria, and two unclassified phyla. Chlorobi exhibited an abundance of 47–96% in coral samples from the high-latitude Daya Bay fringing reef affected by eutrophication. Unclassified bacteria II, with an abundance of 28–87%, was found in all coral samples from the midlatitude Luhuitou fringing reef affected by eutrophication. However, unclassified bacteria I and Proteobacteria dominated (> 80% abundance) in most of the coral samples from the Weizhou Island fringing reef, which is far from land, and three island reefs (Huangyan Island, Xinyi Reef, and Sanjiao Reef) at relatively low latitudes. At the genus level, some core diazotrophs were found in different coral sample groups. In addition, the correlation analysis with various environmental variables revealed that the variables correlated positively or negatively with different diazotrophic genera.

Conclusions: We found that coral-associated diazotrophs were common among coral individuals. The presence of these diazotrophs was not affected by the external environment, but their population abundances were closely related to the different environmental variables. These results provide insights into the ecological characteristics of coral-associated diazotrophs and their relationships with critical environmental variables in the South China Sea.

Keywords: Coral reef ecosystem, Associated diazotrophs, *nifH* gene, Geographical differences, Different latitudes

Background

Although coral reef ecosystems are located in oligotrophic seas, their biodiversity and primary productivity are extremely high [1]. This is mainly due to the efficient biogeochemical cycles of carbon, nitrogen, phosphorus, and other basic elements in which coral symbiotic microbes participate [2]. In an ocean environment with very low concentrations of nutrients, the primary productivity of coral reefs is often limited by available nitrogen, which is one of the primary nutrients essential for the survival of all living organisms [3, 4]. The nitrogen fixation system of coral-associated diazotrophs (reduction of N_2 to ammonia) is considered the major source of available nitrogen in coral reef waters [5, 6]. Previous studies have shown that coral reefs have their own internal nitrogen circulation system and protection mechanism [7, 8]. In addition, abundant nitrogen-fixing bacteria associated with corals have been detected in different coral compartments, including mucus [9, 10], tissue [11, 12], and skeleton [7, 13]. In addition, some studies predicted that diazotrophs associated with corals not only provided sufficient nitrogen sources for coral symbionts but also supplied approximately 6% of organic nitrogen for the whole coral reef ecosystem when the available nitrogen was low [14, 15]. Recent studies found that coral-associated diazotrophs could significantly respond to human-induced environmental changes, thermal stress, and coral bleaching. For example, the key physiological traits of coral hosts, zooxanthellae, and diazotrophs associated with *Stylophora pistillata* show less resilience to thermal stress than those associated with *Acropora hemprichii*. These results revealed a drastic increase in dinitrogen fixation in daylight, particularly in *A. hemprichii*, which was more resilient to thermal stress than *S. pistillata*. The results also suggested that coral-associated diazotrophs play an important role in coral holobiont responses to ocean warming [16]. The relationship between bleaching mortality and nitrogen fixation rates of diazotrophs in the coral *Acropora aspera* showed that N_2 fixation rates in

dead colonies (caused by thermal or cold bleaching) were up to 30 times greater than those measured in live colonies [8].

Currently, the following three main routes are involved in the provision of nitrogen to coral reef ecosystems: (1) terrestrial input, (2) input from ocean currents with rich nutrients, and (3) nitrogen fixation by diazotrophs in the coral reef ecosystems [12, 17-20]. However, isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) tracer experiments showed that the sources of nitrogen for the primary producers in coral reefs (in Palau and Ishigaki) were mainly derived from biological nitrogen fixation [20]. Furthermore, the photosynthetic fixation of CO_2 occurred simultaneously with the absorption and fixation of new nitrogen. This suggested that biological nitrogen fixation plays an important role in the assimilation of carbon and nitrogen by the whole coral reef ecosystem.

Recently, diazotrophic communities associated with different coral species, including *S. pistillata* and *A. hemprichii* [16], *Cladopsammia gracilis* and *Porites* sp. [21], and *Montipora capitata* and *Montipora flabellata* [5], among others [13, 22, 23], were investigated. These coral species were investigated in different geographical locations, including the Great Barrier Reef (Kelso Reef, Knife Reef, and Davies Reef), the Luhuitou fringing reef of Sanya Bay (South China Sea), the Marine Science Station in Aqaba (Jordan), the Gulf of Aqaba (Red Sea), Leleiwi Reef (Hawaii Island), Green Island (southeastern Taiwan), and so on. However, how the coral-associated diazotrophs will change in order to adapt to environmental changes associated with global warming and eutrophication caused by human activities remains unclear.

In the South China Sea, there is a large area of coral reefs at a latitudinal range of 4–21°N [24]. These coral reefs have long been affected by extreme marine events, human activities, and geographical climates [24-26]. These scleractinian corals can adapt to different environments and

establish a unique holobiont [27, 28]. Therefore, coral reefs in the South China Sea are natural laboratories with which to study the patterns of coral-associated diazotroph responses to global warming and eutrophication caused by human activities. To this end, 68 scleractinian coral colonies were collected from 6 geographical locations at different latitudes and with different eutrophication levels in the South China Sea to investigate the composition of coral-associated diazotrophs. It is beneficial to understand the environmental adaptability of coral reefs and the possible changes in coral-associated diazotrophs in the face of climate change and human activities.

Results

Diversity of coral-associated diazotrophs

A total of 1,223,398 reads recovered from 68 coral samples, with lengths ranging from 421 to 440 bp, were obtained from the sequencing database. Good's coverage of each sequencing database was more than 99% (Additional file 1: Table S1). Thus, these sequencing results accurately represented the diazotrophs in the coral samples. Other indices, including the abundance-based coverage estimator (ACE) and Shannon index, are shown more intuitively in Fig. 1. The detailed data showed that the ACE, which reflects community richness, varied greatly among coral samples (Additional file 1: Table S1). The lowest values were 21.35, 30.79, and 31.54, from Pv3_Lht, P11_SjR, and P12_SjR, respectively, while the highest values were 300.4, 293.57, and 286.85, from Gr3_XyR, Fp3_WzI, and Ar3_XyR, respectively. The Shannon index (Additional file 1: Table S1), which reflects community diversity, also differed significantly among coral samples (ranging from 0.58 to 4.48). When corals of different species (CDSs) and corals from different reef regions (CDRs) were grouped, these indices showed few significant differences between most CDSs but significant differences between most CDRs (Fig. 1). The average ACE values for Weizhou Island (WzI), Huangyan Island (HyI), Xinyi Reef (XyR), and

Sanjiao Reef (SjR) (146.99, 139.83, 145.23, and 102.12, respectively) were obviously higher than those for Luhuitou (Lht) and Daya Bay (DyB) (71.07 and 75.54, respectively). Meanwhile, the average Shannon values for WzI, HyI, and XyR (3.34, 2.76, and 2.70, respectively) were also higher than those for DyB, SjR, and Lht (1.98, 1.82, and 1.70, respectively). However, the ACE and Shannon index averages for different coral species ranged from 101.73 to 150.28 and 2.12 to 2.73, respectively, with a small range of fluctuations between them (Fig. 1B and D). These findings suggest that geographical factors have a strong effect on the community richness and diversity of coral-associated diazotrophs.

The number of operational taxonomic units (OTUs) and diversity at various taxonomic levels are listed in Table 1. The results showed significant differences in the number of communities of diazotrophs between coral individuals, even for the same coral species in the same sampled location. For example, 6 phyla, 21 genera, and 280 OTUs were detected in Gr3_XyR, but the numbers were 5, 11, and 50 in Gr1_XyR, respectively.

Clustering of coral-associated diazotrophs based on similarity

The similarity among the diazotrophic communities associated with 68 coral samples from 6 locations was evaluated using principal coordinate analysis (PCoA) at the OTU level. The diazotrophic composition of coral individuals differed between coral reefs, whether or not the coral individuals were of the same species (Fig. 2A). In comparison with the grouping of regions, coral individuals from the same species did not cluster together significantly (Fig. 2B). This indicated that the diazotrophic composition associated with corals was somewhat species specific. Overall, the most significant factor affecting diazotrophic composition was geographical position, rather than interspecific differences.

Composition of diazotrophs associated with corals

Eleven bacterial phyla capable of nitrogen fixation, including 3 unclassified bacteria (I, II, and III),

were identified from the sequencing database of 68 coral samples (Fig. 3). Among these phyla, unclassified bacteria I, with a very high abundance, was present in almost all the coral samples. In addition, the dominant bacterial phyla were Proteobacteria, Chlorobi, and Cyanobacteria. The abundance of these coral-associated bacterial phyla exhibited significant differences between coral reefs. For example, unclassified bacteria I and Proteobacteria were the dominant bacterial phyla (> 80% abundance) in most of the coral samples from WzI, HyI, XyR, and SjR. In contrast, the abundance of these two bacterial phyla was low (< 35% abundance) in most of the coral samples from Lht and DyB. In particular, the abundance of Proteobacteria was generally very low (< 10%) in most of the coral samples from the two locations; the dominant diazotrophs in these locations were unclassified bacteria II (Lht) and Chlorobi (DyB). Of course, there were special cases. For example, the abundance of Chlorobi, the dominant group, was 84% in Pl3_SjR from SjR. Unclassified group II was the dominant group, with an abundance of 89%, in Hm1_SjR from SjR. Cyanobacteria, members of which are capable of nitrogen fixation, was also a common bacterial phylum in these coral samples. Its abundance ranged from 0.21% (Pl2_SjR, Pl3_Lht, Pv3_Lht, etc.) to 51% (Pl5_HyI). The other nitrogen-fixing groups, including Euryarchaeota, Firmicutes, and Verrucomicrobia, were detected in a small number of coral samples and had a very low abundance (except marine stromatolite eubacteria in Pd2_SjR). At the class level (Additional file 2: Fig. S1), the dominant taxa were unclassified_p_unclassified bacteria I and unclassified Proteobacteria in most coral samples from WzI, HyI, XyR, and SjR. Chlorobi was the dominant class in all coral samples from DyB (ranging from 47% to 96%) and Pl3_SjR (84%) from SjR. In addition, unclassified_p_unclassified bacteria II was the dominant group in most coral samples from Lht (ranging from 28% to 87%) and Hm1_SjR from SjR (89%). The abundance of most other classes, including Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria, was very low. At

other taxonomic levels (order, family, genus, and species), the unclassified diazotrophs were the dominant groups. These results indicated that the coral holobionts contained many diazotrophs that have not been isolated and recognized.

At the genus level, Venn diagrams showed that most diazotrophic genera were common (14 and 20 among CDSs and CDRs, respectively) in multiple sample groups (Fig. 4). Among these genera, *Vibrio* and *Chlorobium* overlapped exactly among coral reefs and coral species (Table 2). In addition to overlapping genera, there were some unique diazotrophic genera for each sample group, whether in CDSs or CDRs (Table 3). For example, *Desulfobacter* was endemic to coral individuals from DyB. *Chroococcidiopsis* appeared only in the coral species *Goniastrea retiformis*. Overall, 15 diazotrophic genera were endemic to different groups of CDRs, and 14 genera were endemic to different groups of CDSs. These endemic diazotrophic species were directly related to the coral host species and external environment.

Environmental variables affecting the distribution of diazotrophic species

The six sampling locations were distributed in different areas of the South China Sea. These coral reefs were affected by different environmental factors due to their geographical locations (Additional file 1: Table S2). The concentrations of dissolved inorganic nitrogen (DIN) and other nutrients were lower in HyI, XyR, and SjR, which were far from land, than in the other locations. In contrast, these nutrient concentrations were 2 to 10 times higher in some coastal sampling locations (e.g., DyB, WzI, and Lht) than in those far from land. Turbidity (Tur) was also significantly higher in the coastal reefs than in the offshore reefs. The correlations between various environmental parameters and communities of coral-associated diazotrophs (the top 20 taxa based on total abundance at the genus level) were then evaluated (Fig. 5). The results showed that the effects of different environmental factors on bacterial

communities were significantly different. According to similar effects, environmental factors were clustered into two groups: group I, including DIN, Tur, latitude (Lat), PO_4^{3-} (SRP), and SiO_3^{2-} , and group II, including pH, dissolved oxygen (DO), longitude (Lng), temperature (Tem), and salinity (Sal). Group I correlated positively with unclassified_f_Chlorobiaceae ($0.26 \leq R \leq 0.49$), *Chlorobium* ($0.29 \leq R \leq 0.37$), unclassified_p_unclassified Bacteria II ($0.30 \leq R \leq 0.43$) and unclassified_o_Desulfuromonadales ($0.25 \leq R \leq 0.44$) and negatively with four other bacterial genera, namely, unclassified_c_norank_p_Cyanobacteria ($-0.42 \leq R \leq -0.24$), norank_p_unclassified Bacteria I ($-0.37 \leq R \leq -0.25$), unclassified_c_Alphaproteobacteria ($-0.44 \leq R \leq -0.30$), and unclassified_p_Cyanobacteria ($-0.44 \leq R \leq -0.30$). The correlations of other environmental factors with a series of bacterial genera in group II were generally opposite to those in group I (Fig. 5).

Discussion

Microbial nitrogen fixation requires a nitrogenase gene, *nifH*, which has been confirmed to be consistent with the phylogenesis of the 16S rRNA gene [29]. Our annotation results revealed that microorganisms capable of nitrogen fixation were ubiquitously present in coral holobionts. Their presence was not related to the external environment. The six sampling locations were distributed in different areas of the South China Sea. The concentrations of DIN and other nutrients were very high in some fringing reef sampling locations (e.g., DyB, WzI, and Lht). In contrast, the concentrations of these nutrients were low in some island reefs (HyI, XyR, and SjR), which were far from land. The nutrients in coastal coral reefs could completely meet the nitrogen demand of coral holobionts, even without biological nitrogen fixation. However, diazotrophs and coral hosts coexist well and coevolve over the long term [30]. It can be speculated that coral-associated diazotrophs have many other biological functions in addition to nitrogen fixation, such as the coordination of carbon fixation. For

example, Cyanobacteria is a phylum of prokaryotic microorganisms that can carry out photosynthesis with oxygen production [31]. They can also fix atmospheric nitrogen and convert it into ammonia (NH_3), nitrite (NO_2^-), or nitrate (NO_3^-), which can provide available nitrogen to other organisms [12, 32].

In this study, the most striking finding was that the fluctuations of coral-associated diazotrophic composition, which were identified via the analysis of alpha- and beta-diversity and species differences, were highly significantly different between CDRs (Fig. 1). Notably, community richness, which was reflected by the ACE, was lower for coral-associated diazotrophs from DyB and Lht than for those from WzI, HyI, SjR, and XyR (Fig. 1A). This may be attributed to the reef types of DyB and Lht, which are a kind of typical fringing reef frequently affected by human activities and contain high concentrations of nutrients and DO. The community diversity, which was reflected by the Shannon index, exhibited the same trend as the ACE for coral-associated diazotrophs from DyB and Lht (Fig. 1C). This may be related to the fact that high concentrations of nutrients (especially DIN) can meet some of the nitrogen requirements of corals. However, this finding was contrary to the results of the analysis of coral-associated bacterial diversity obtained by high-throughput sequencing based on 16S rRNA gene amplification. Li *et al.* studied the bacterial diversity associated with *Porites lutea*, *Galaxea fascicularis*, and *Acropora millepora* sampled from Lht. The results showed that the ACE and Shannon index were 855.53–8970.90 and 4.16–7.04, respectively [27]. Additionally, our previous study found that the ACE and Shannon index of 25 scleractinian coral samples from XyR were 332.22–1500.66 and 1.91–5.88, respectively [28]. McKew *et al.* also showed that bacterial communities from Caribbean corals were significantly more diverse than those from Indonesian corals [33]. In this study, the relationships between coral-associated bacteria were structured by multiple factors at different scales.

For example, the diversity and composition of bacterial communities associated with corals are significantly affected by various factors, including coral species [34], geography [33], skeletal morphology [28], and others [35, 36]. However, the relationship between coral-associated nitrogen-fixing bacteria and other bacteria is not yet clear. In this study, the characteristics of dominant coral-associated diazotrophs, which exhibited significant geographical differences, were clearly recognized. At the same time, some core diazotrophs were found in different coral reefs or coral species. In addition, there were some unique diazotrophs in different coral reefs or coral species. The PCoA also showed that coral samples from different locations were clearly separated based on diazotrophic composition (Fig. 2). We speculated that the selection of coral-associated diazotrophs was directly related to coral species and their living environments. Because environmental variables were significantly different between these coral reefs, correlation analysis revealed that various environmental factors correlated positively or negatively with different bacterial genera. For example, the effects of group I (Lat, SRP, SiO_3^{2-} , DIN, and Tur) and group II (pH, DO, Temp, Sal, and Lng) on most of the diazotrophic communities were similar. The correlations between the distribution of nitrogen-fixing bacteria associated with corals and environmental factors were reported for the first time in this study. The findings of this study shed light on the communities and dominant groups of diazotrophs in characteristic coral reefs and their relationships with key environmental variables.

Conclusions

Our results fully reflected the diversity of diazotrophs associated with different coral species sampled from a number of coral reefs that varied in environmental variables in the South China Sea. Although many diazotrophic species were unclassified, it was shown that the predominant taxa of diazotrophs among six different coral reefs exhibited more significant geographical differences than interspecific

differences. In addition, the correlation analysis revealed that various environmental factors correlated positively or negatively with different bacterial genera. We think that corals tend to associate with unclassified_f_Chlorobiaceae, *Chlorobium*, unclassified_p_unclassified Bacteria II, and unclassified_o_Desulfuromonadales as symbiotic nitrogen-fixing bacteria under changes in nutrient enrichment. In addition, corals from high-latitude reefs may tend to associate with unclassified_c_norank_p_Cyanobacteria, norank_p_unclassified Bacteria I, unclassified_c_Alphaproteobacteria, and unclassified_p_Cyanobacteria under global warming. An understanding of diazotrophic communities associated with scleractinian corals from reefs with significantly different environmental characteristics in the South China Sea will help us anticipate possible changes in coral reefs in response to future environmental changes.

Materials and methods

Study sites, coral sample collection, and species identification

In this study, 6 locations in the South China Sea, namely, DyB, WzI, Lht, HyI, SjR, and XyR, were selected (Fig. 6). Coral samples were collected using a hammer and chisel by way of scuba diving at a depth of 5–8 m from a specific site in each selected location (Table 4). Three replicate samples (approximately 6 × 6 cm) were collected from the sides of the colonies. The distance between two colonies of the same reef was greater than 10 meters. Some widely distributed coral species, such as *P. lutea* and *G. retiformis*, needed to be collected as much as possible from different coral reefs where they grow. The collected samples were washed with sterile seawater 3 times and then placed in sterile plastic bags. All samples were briefly stored at low temperatures (0–4°C) and then immediately transported back to the laboratory for DNA extraction.

A total of 68 coral samples, which included 6 families, 9 genera, and 11 species (Table 4), were

identified and selected as the study subjects according to their ecological and morphological characteristics.

Seawater collection, nutrition, and environmental factor detection

Three to five liters of seawater was collected at a depth of 5–8 m using a water sampler around each site. The distance between two sampling sites was not less than 100 meters. The Temp, Sal, Tur, and pH values were immediately measured on site using a thermometer, salinometer, turbidimeter, and acidometer, respectively. Pore water was extracted from sediments by centrifugation (3,500 rpm, 40 min), filtered through 0.45- μ m-pore-size cellulose acetate filters, and then collected in acid-precleaned vials. Finally, all samples were stored in an icebox for transport to the laboratory and stored in deep freezers until analyses [37]. All nutrient statuses, including the concentrations of DIN ($\text{DIN} = \text{NH}_4^+ + \text{NO}_2^- + \text{NO}_3^-$), PO_4^{3-} , and SiO_3^{2-} , were measured according to “Specifications for oceanographic survey” (General Administration of Quality Supervision, Inspection and Quarantine of the People’s Republic of China, 1991) [37]. The average physical and chemical parameters from at least three samples of each coral reef were tested. Lng and Lat were detected by global positioning system (GPS).

DNA extraction, PCR amplification and Illumina MiSeq sequencing

Small pieces of coral samples, including tissue, mucus and skeleton (approximately 50 mg), were cut with a pair of scissors and used for genomic DNA extraction with the TIANamp Marine Animals DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) according to the manufacturer’s instructions. The nitrogen-fixing gene (*nifH*) of diazotrophs was amplified using the specific forward primer nifH-F (5'-AAAGGYGGWATCGGYAARTCCACCAC-3') and reverse primer nifH-R (5'-TTGTTSGCSGCRTACATSGCCATCAT-3'), where the barcode was an eight-base sequence unique to each sample [38-41]. The reaction system and procedure for PCR using an ABI GeneAmp® 9700

thermal cycler and TransGen AP221-02 PCR kit (TransStart FastPfu DNA Polymerase, 20 µl reaction system) were the same as those described in a previous report [42]. The following steps were employed in the PCR: a 3-minute hot start at 95°C after the reaction system was configured according to the manufacturer's instructions; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 45 seconds; an extension at 72°C for 10 minutes; and preservation at 10°C until halted by the user. Triplicate PCR products were pooled for each sample, and fragments with size ranges of 421–440 bp were then purified and quantified using an AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, U.S.A.) and QuantiFluor™-ST fluorometer (Promega, U.S.A.). Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 250 bp) on the Illumina MiSeq platform according to standard protocols (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China).

Data analysis

Raw sequences were optimized using Trimmomatic to exclude reads with homopolymer inserts and low-quality scores (< 20) [43]. The obtained high-quality reads with more than 10 bp of overlapping sequence were merged by FLASH software [44]. The merged sequences were clustered into OTUs with a 97% similarity cutoff using UPARSE software (version 7.1 <http://drive5.com/uparse/>) and were then identified, and all chimeric sequences were removed using UCHIME software [45]. The obtained sequences were finally used for taxonomic analysis. The taxonomy of representative sequences from the most abundant sequences within each OTU was analyzed by the RDP Classifier algorithm (<http://rdp.cme.msu.edu/>) against the fgr/nifH database (release 7.3, <http://fungene.cme.msu.edu/nifH>), setting a confidence threshold of 70% [45]. The indices of community richness (ACE) [46] and community diversity (Shannon) [47, 48] were estimated for each sample by extracting the same

numbers of reads (10,000 valid sequences) using mothur (version v.1.30.1) [49]. The taxonomy was assigned and compared with that in the fgr/nifH database [50] using the QIIME platform (http://qiime.org/scripts/assign_taxonomy.html). Moreover, similarities or differences in the composition of bacterial communities were reflected by PCoA using Bray-Curtis distances at the OTU level [51]. A heatmap of the correlations between environmental variables and nitrogen-fixing bacteria associated with corals was constructed by Spearman's correlation test and GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA). Significance was assigned at $P < 0.05$ (*).

Supplementary information

Additional file 1: Additional tables.

Additional file 2: Fig. S1. Composition profiles of diazotrophs. Taxonomic classification of bacterial reads retrieved from all the coral samples at the class level using RDP Classifier; “others” represents the bacteria with an abundance less than 0.1%.

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Author contributions

KY and JL conceived the research; YW, XH, WH, and ZW contributed the materials; JL performed all experiments; ZQ and GW constructed all figures; BC and HS identified coral species; JL and KY wrote the manuscript; and all authors edited and approved the manuscript.

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Availability of data and materials

The datasets analyzed during the current study are available from the [NCBI Sequence Read Archive] repository under accession number SRP145254 [<https://www.ncbi.nlm.nih.gov/search/all/?term=SRP145254>].

Ethical approval and consent to participate

Permits for coral sampling were provided by the State Oceanic Administration, People's Republic of China, and the local Department of Ocean and Fisheries.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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500 **Table 1** Number of diazotrophs associated with different coral colonies at different taxonomic levels.

Coral code	Phylum	Class	Order	Family	Genus	Species	OTU
Gr3_HyI	5	9	11	11	12	12	68
Pv1_Lht	4	8	10	10	10	11	64
Pv3_HyI	5	10	14	16	17	17	202
Pl4_HyI	6	11	16	18	19	19	179
Pv2_WzI	5	8	10	10	10	10	34
Pc1_SjR	5	10	15	16	16	16	146
Gr2_XyR	5	10	12	13	15	15	130
Pv3_Lht	3	4	5	5	5	5	21
Gf2_DyB	5	10	16	16	17	17	143
Pv3_WzI	3	7	9	9	9	9	103
Pl1_XyR	5	6	7	9	11	11	74
Pd2_DyB	5	8	10	11	12	12	88
Pl4_WzI	7	10	14	16	17	17	215
Hm1_DyB	4	7	8	8	10	10	50
Gr4_XyR	4	9	14	14	15	15	66
Gr1_DyB	6	9	13	13	14	14	52
Pl3_XyR	4	9	12	12	12	12	153
Pc3_SjR	5	8	9	10	11	11	132
Fp1_SjR	3	7	9	9	9	9	96
Pl3_WzI	4	7	8	8	8	9	88
Me1_SjR	4	9	11	12	12	12	101
Gr1_XyR	5	9	11	11	11	11	50
Gr3_XyR	6	11	18	19	21	21	280
Pl2_WzI	4	8	16	16	17	17	117
Fp1_XyR	5	9	11	13	13	13	112
Fp1_WzI	6	9	14	15	18	18	93
Pl1_WzI	7	11	22	25	28	30	205
Pl4_XyR	4	9	14	15	16	16	52
Pv1_WzI	5	9	12	12	12	12	75
Pd1_DyB	4	5	6	6	7	7	32
Pc2_HyI	3	4	7	7	8	8	61
Pl3_Lht	4	6	6	6	6	6	24
Pd1_SjR	5	8	11	11	12	12	67
Pv2_HyI	6	9	13	13	13	13	62
Gr1_HyI	4	6	9	11	11	11	80
Hm2_SjR	5	8	9	9	10	10	33

Pv1_HyI	5	9	12	13	13	13	179
Hm1_SjR	7	9	11	11	11	11	54
Gf1_DyB	5	6	7	7	8	8	55
Pl1_SjR	4	5	6	6	6	6	30
Pl5_HyI	7	12	19	20	21	21	144
Pc1_HyI	6	10	16	19	20	21	154
Gr2_DyB	5	10	11	11	12	12	86
Gr5_XyR	4	9	14	15	15	15	125
Fp1_Lht	5	8	9	9	10	10	49
Pd2_SjR	7	12	16	16	17	17	121
Pl4_SjR	6	10	14	14	15	15	69
Gr4_DyB	5	8	10	10	11	11	96
Pc2_SjR	3	7	11	12	12	12	104
Pl4_Lht	3	4	5	5	5	5	37
Fp3_WzI	5	9	16	19	21	23	291
Me2_SjR	4	8	11	12	12	12	57
Ar2_XyR	6	8	11	12	14	14	111
Ar1_XyR	6	11	17	18	18	18	136
Pl3_HyI	6	9	12	12	12	12	36
Pl2_SjR	4	6	7	7	7	7	31
Ar3_XyR	7	10	11	14	14	14	189
Pl2_HyI	4	7	10	11	11	11	62
Gr3_DyB	5	7	7	7	8	8	30
Pl3_SjR	3	6	7	7	7	7	34
Gr2_HyI	5	10	15	16	17	17	143
Pl2_Lht	5	10	15	16	17	18	199
Pl1_Lht	6	9	12	14	15	16	77
Pe1_SjR	7	11	16	16	16	16	133
Fp2_WzI	6	10	14	14	14	14	200
Pv2_Lht	6	9	10	11	12	13	52
Pl1_HyI	6	11	15	18	19	19	121
Pl2_XyR	5	8	10	10	10	10	51

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511 **Table 2** Overlapping diazotrophic genera associated with different coral sample groups.

Bacterial genera in different coral reefs ^a	Bacterial genera in different coral species ^b
[DyB (n = 9, m = 4), WzI (n = 10, m = 3), Lht (n = 8, m = 8), HyI (n = 13, m = 4), SjR (n = 15, m = 7), and XyR (n = 13, m = 4)]	[Pl (n = 21), Pv (n = 9), Gr (n = 12), Fp (n = 6), and Pc (n = 5)]
g_unclassified_f_Chlorobiaceae g_Teredinibacter g_Vibrio g_norank_c_unclassified_Cyanobacteria g_unclassified_c_Alphaproteobacteria g_unclassified_c_norank_p_Cyanobacteria g_unclassified_k_norank_d_Bacteria g_unclassified_p_Cyanobacteria g_unclassified_o_Rhizobiales g_unclassified_p_Proteobacteria g_unclassified_c_Gammaproteobacteria g_unclassified_p_unclassified Bacteria II g_Chlorobium g_unclassified_c_Deltaproteobacteria	g_unclassified_o_Rhizobiales g_Vibrio g_unclassified_o_Desulphuromonadales g_norank_c_unclassified_Cyanobacteria g_unclassified_c_Alphaproteobacteria g_unclassified_c_norank_p_Cyanobacteria g_unclassified_k_norank_d_Bacteria g_unclassified_o_Chroococcales g_Desulphuromonas g_Mastigocoleus g_unclassified_f_Chromatiaceae g_unclassified_p_Cyanobacteria g_unclassified_f_Chlorobiaceae g_unclassified_o_Chromatiales g_unclassified_p_Proteobacteria g_unclassified_c_Gammaproteobacteria g_unclassified_p_unclassified Bacteria II g_Bradyrhizobium g_Chlorobium g_unclassified_c_Deltaproteobacteria

512 n = number of coral individuals, m = number of coral species. ^a Different coral reefs are represented by

513 the abbreviations DyB (Daya Bay), HyI (Huangyan Island), Lht (Luhuitou), SjR (Sanjiao Reed), XyR

514 (Xinyi Reed), and WzI (Weizhou Island). ^b Different coral species are represented by the abbreviations

515 Pl (*Porites lutea*), Pv (*Plesiastrea versipora*), Fp (*Favia palauensis*), Gr (*Goniastrea retiformis*), and Pc

516 (*Plesiastrea. curta*). The red-labeled bacteria are the species with exact overlap between coral reefs or
517 coral species.

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558 **Table 3** Endemic diazotrophic genera from different sample groups.

Coral reefs ^c	Bacterial genera	Coral species ^d	Bacterial genera
DyB (n = 9, m = 4)	<i>g_Desulfobacter</i>		<i>g_Stenotrophomonas</i>
	<i>g_Calothrix</i>		<i>g_Zoogloea</i>
	<i>g_Stenotrophomonas</i>		<i>g_norank_p_unclassified bacteria</i>
	<i>g_unclassified_f_Desulfobacteraceae</i>	Pl (n = 21)	<i>g_norank_f_Deltaproteobacteria</i>
WzI (n = 10, m = 3)	<i>g_norank_p_unclassified bacteria</i>		<i>g_unclassified_c_Clostridia</i>
	<i>g_norank_f_Deltaproteobacteria</i>		<i>g_Rhodospirillum</i>
	<i>g_Klebsiella</i>		<i>g_Tolumonas</i>
	<i>g_unclassified_f_Rhodocyclaceae</i>		<i>g_unclassified_p_Euryarchaeota</i>
	<i>g_Rhodospirillum</i>		<i>g_unclassified_f_Desulfobacteraceae</i>
Lht (n = 8, m = 3)	<i>g_Azotobacter</i>	Pv (n = 9)	<i>g_Klebsiella</i>
			<i>g_unclassified_f_Rhodocyclaceae</i>
HyI (n = 13, m = 4)	<i>g_Desulfarculus</i>	Gr (n = 12)	<i>g_Chroococcidiopsis</i>
	<i>g_unclassified_o_Clostridiales</i>	Fp (n = 6)	<i>g_Skermanella</i>
	<i>g_Rhizobium</i>	Pc (n = 5)	<i>g_unclassified_o_Clostridiales</i>
SjR (n = 15, m = 7)	<i>g_unclassified_c_Clostridia</i>		
	<i>g_unclassified_p_Euryarchaeota</i>		
XyR (n = 13, m = 4)	none		

559 The indications for n, m, ^c, and ^d are consistent with those in Table 2.

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577 **Table 4** The locations, coral species, and number of sampled corals.

Site location (Longitude, Latitude)	Sampling date	Coral species	Coral code [#]	Colony number
Daya Bay (114°38'40"E, 22°34'57"N)	01 September 2015	<i>Galaxea fascicularis</i>	Gf1_DyB, Gf2_DyB	2
		<i>Goniastrea retiformis</i>	Gr1_DyB, Gr2_DyB, Gr3_DyB, Gr4_DyB	4
		<i>Pavona decussata</i>	Pd1_DyB, Pd2_DyB	2
		<i>Hydnophora microconos</i>	Hm1_DyB	1
Weizhou Island (109°06'40"E, 21°04'30"N)	23 October 2015	<i>Porites lutea</i>	Pl1_WzI, Pl2_WzI, Pl3_WzI, Pl4_WzI	4
		<i>Favia palauensis</i>	Fp1_WzI, Fp2_WzI, Fp3_WzI	3
		<i>Plesiastrea versipora</i>	Pv1_WzI, Pv2_WzI, Pv3_WzI	3
Luhuitou (109°29'16"E, 18°13'18"N)	15 October 2016	<i>Porites lutea</i>	Pl1_Lht, Pl2_Lht, Pl3_Lht, Pl4_Lht	4
		<i>Plesiastrea versipora</i>	Pv1_Lht, Pv2_Lht, Pv3_Lht	3
		<i>Favia palauensis</i>	Fp1_Lht	1
Huangyan Island (117°44'49"E, 15°13'08"N)	15 July 2015	<i>Porites lutea</i>	Pl1_HyI, Pl2_HyI, Pl3_HyI, Pl4_HyI, Pl5_HyI	5
		<i>Goniastrea retiformis</i>	Gr1_HyI, Gr2_HyI, Gr3_HyI	3
		<i>Plesiastrea versipora</i>	Pv1_HyI, Pv2_HyI, Pv3_HyI	3
		<i>Plesiastrea curta</i>	Pc1_HyI, Pc2_HyI	2
Sanjiao Reef (115°12'41"E, 10°13'24"N)	19 May 2015	<i>Porites lutea</i>	Pl1_SjR, Pl2_SjR, Pl3_SjR, Pl4_SjR	4
		<i>Montipora efflorescens</i>	Me1_SjR, Me2_SjR	2
		<i>Pavona decussata</i>	Pd1_SjR, Pd2_SjR	2
		<i>Hydnophora microconos</i>	Hm1_SjR, Hm2_SjR	2
		<i>Pocillopora eydouxi</i>	Pe1_SjR	1
		<i>Favia palauensis</i>	Fp1_SjR	1
		<i>Plesiastrea curta</i>	Pc1_SjR, Pc2_SjR, Pc3_SjR	3
Xinyi Reef (115°55'49"E, 9°20'06"N)	21 May 2016	<i>Porites lutea</i>	Pl1_XyR, Pl2_XyR, Pl3_XyR, Pl4_XyR	4
		<i>Goniastrea retiformis</i>	Gr1_XyR, Gr2_XyR, Gr3_XyR, Gr4_XyR, Gr5_XyR	5
		<i>Acropora rosaria</i>	Ar1_XyR, Ar2_XyR, Ar3_XyR	3
		<i>Favia palauensis</i>	Fp1_XyR	1

578 [#] The letters before the underscore are the initials of the coral genus and species, which represent the

579 species of corals. The numbers indicate the order of coral individuals. The letters after the underscore

580 represent the abbreviations of coral reef locations.

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Figure captions

Fig. 1 Test for differences in alpha-diversity indices of coral-associated diazotrophs between sample groups at the OTU level. (A) ACE between coral reefs, (B) ACE between coral species, (C) Shannon index between coral reefs, and (D) Shannon index between coral species. The test used was Student's t-test. The black square (■) shows the average. n = number of coral individuals, m = number of coral species. Different coral reefs are represented in (A) and (C) by the abbreviations DyB (Daya Bay), HyI (Huangyan Island), Lht (Luhuitou), SjR (Sanjiao Reef), XyR (Xinyi Reef), and WzI (Weizhou Island); different coral species in (B) and (D) are represented by the abbreviations Pl (*Porites lutea*), Pv (*Plesiastrea versipora*), Fp (*Favia palauensis*), Gr (*Goniastrea retiformis*), and Pc (*Plesiastrea curta*).

Fig. 2 PCoA plot at the OTU level of all the coral samples collected from six different coral reefs. Coral samples were grouped according to (A) collection site and or (B) species with ≥ 5 colonies from the same coral reef. Scatter plot showing principal coordinate 1 (PC1) vs principal coordinate 2 (PC2). PC1 and PC2 represent the principal factors affecting the composition of coral-associated diazotrophs. Abbreviations are the same as in Fig. 1.

Fig. 3 Composition profiles of diazotrophs. Taxonomic classification of bacterial reads retrieved from all the coral samples at the phylum level using RDP Classifier.

Fig. 4 Venn diagrams showing the number of diazotrophs at the genus level from different coral reefs (A) and coral species (B). Abbreviations are the same as in Fig. 1.

Fig. 5 Correlation analyses between environmental parameters and populations of diazotrophs at the genus level. The diazotrophs analyzed were the top 20 genera in terms of total abundance. Hierarchical clustering of environmental variables was performed based on the raw data, while diazotrophic species were clustered based on averages. Significant differences are indicated by different numbers of asterisks ($0.01 < p \leq 0.05$ *, $0.001 < p \leq 0.01$ **, $p \leq 0.001$ ***). Nonsignificant correlations do not have an asterisk. The R value represents the correlation coefficient, and the closer it is to 1, the more significant the correlation is. Some of the environmental variables are represented by abbreviations, including Lat (latitude), SRP (PO_4^{3-}), SiO3 (SiO_3^{2-}), DIN (dissolved inorganic nitrogen, $\text{NH}_4^+ + \text{NO}_3^- + \text{NO}_2^-$), Tur (turbidity), DO (dissolved oxygen), Sal (salinity), Tem (temperature), and Lng (longitude).

Fig. 6 Location map with labeled coral reef areas including 6 sampling sites in the South China Sea. The map was constructed using ArcGIS software (ver. 10.1). The offshore reef area was drawn using remote sensing images (fusion of Landsat 8 multispectral bands and a panchromatic band) with a resolution of 15 meters.

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Figures

Figure 1

Test for differences in alpha-diversity indices of coral-associated diazotrophs between sample groups at the OTU level. (A) ACE between coral reefs, (B) ACE between coral species, (C) Shannon index between coral reefs, and (D) Shannon index between coral species. The test used was Student's t-test. The black square (■) shows the average. n = number of coral individuals, m = number of coral species. Different coral reefs are represented in (A) and (C) by the abbreviations DyB (Daya Bay), Hyl (Huangyan Island), Lht (Luhuitou), Sjr (Sanjiao Reef), Xyr (Xinyi Reef), and Wzl (Weizhou Island); different coral species in (B) and (D) are represented by the abbreviations Pl (*Porites lutea*), Pv (*Plesiastrea versipora*), Fp (*Favia palauensis*), Gr (*Goniastrea retiformis*), and Pc (*Plesiastrea curta*).

Figure 2

PCoA plot at the OTU level of all the coral samples collected from six different coral reefs. Coral samples were grouped according to (A) collection site and or (B) species with ≥ 5 colonies from the same coral reef. Scatter plot showing principal coordinate 1 (PC1) vs principal coordinate 2 (PC2). PC1 and PC2 represent the principal factors affecting the composition of coral-associated diazotrophs. Abbreviations are the same as in Fig. 1.

Figure 3

Composition profiles of diazotrophs. Taxonomic classification of bacterial reads retrieved from all the coral samples at the phylum level using RDP Classifier.

Figure 4

Venn diagrams showing the number of diazotrophs at the genus level from different coral reefs (A) and coral species (B). Abbreviations are the same as in Fig. 1.

Figure 5

Correlation analyses between environmental parameters and populations of diazotrophs at the genus level. The diazotrophs analyzed were the top 20 genera in terms of total abundance. Hierarchical clustering of environmental variables was performed based on the raw data, while diazotrophic species were clustered based on averages. Significant differences are indicated by different numbers of asterisks ($0.01 < p \leq 0.05$ *, $0.001 < p \leq 0.01$ **, $p \leq 0.001$ ***). Nonsignificant correlations do not have an asterisk.

The R value represents the correlation coefficient, and the closer it is to 1, the more significant the correlation is. Some of the environmental variables are represented by abbreviations, including Lat (latitude), SRP (PO_4^{3-}), SiO3 (SiO_3^{2-}), DIN (dissolved inorganic nitrogen, $\text{NH}_4^+ + \text{NO}_3^- + \text{NO}_2^-$), Tur (turbidity), DO (dissolved oxygen), Sal (salinity), Tem (temperature), and Lng (longitude).