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Ocean Currents May Influence the Endolithic Bacterial Composition in Coral Skeletons

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Coral endolithic microbes can be an important nutrients support for hosts while under stresses. Previous studies have found that the endolithic microbial composition of a single coral species can be biogeographical diverse. However, the potential environmental factors, such as salinity, temperature, pH, and nutrient, that might influence the composition of the endolithic microbes remain unclear. In this study, we used both amplicon sequence variants (ASV) and a kmer-based taxonomic unit (KTU) to characterize the endolithic bacterial constitution of *Isopora* spp. located in the western Pacific Ocean—where it is subjected to the Kuroshio Current (in Okinawa, Japan and Green Island, Taiwan)—and the South China Sea (Dongsha Atoll). The endolithic bacterial community compositions showed a significant geographical difference, and we suggest that the ocean current and primary productivity are the most essential environmental factors influencing the bacterial communities in the skeleton of *Isopora* spp. In addition, our results showed that, technically, compared to ASV, bacterial composition based on KTU avoids extreme data, making it a suitable tool for explaining the associations between microbes and environmental factors.

Keywords: *Isopora*, endolithic bacteria, KTU, Kuroshio Current, marine environmental factors

INTRODUCTION

Coral endolithic microbes are considered to be highly important because they play key roles in carbon and nitrogen cycles (Fine and Loya, 2002; Ricci et al., 2019; Yang et al., 2019; Pernice et al., 2020); their coral hosts can, in turn, utilize the nutrients by those endolithic microorganisms, such as the organic carbon synthesized by the green algae *Ostreobium* (Ferrer and Szmant, 1988; Fine and Loya, 2002). The abundant endolithic community is composed by bacteria, microeukaryotes and viruses (Ricci et al., 2019; Pernice et al., 2020), among them are eukaryotic algae *Ostreobium*

(del Campo et al., 2017); the photoautotrophic cyanobacteria *Plectonema terebrans* and *Mastigocoleus testarum* (Fine and Loya, 2002; Tribollet and Golubic, 2005); and fungi (Tribollet and Golubic, 2005). Those microbes are able to form clear green layers in coral skeletons beneath coral tissues (Ricci et al., 2019; Pernice et al., 2020).

Isopora spp., common reef-building corals in the offshore of the western Pacific Ocean, have such a green layer in their skeleton (Yang et al., 2016; Yang et al., 2019; Yang et al., 2020). However, instead of finding aerobic microbes in coral skeletons, we found that the endolithic microbes in *Isopora* were dominated by anaerobes, such as *Chlorobi*, *Chloroflexi* and *Deltaproteobacteria*. These anaerobic bacteria in the green layer might involve in nitrogen fixation and sulfur cycles (Yang et al., 2019). In Yang et al. (2019)'s sample, the rarely-found photoautotrophic algae or fungi endolithic communities were speculated to be host-specific.

Ricci et al. (2021) recently found that the endolithic community of *Isopora* from the southern Great Barrier Reef, Australia, was dominated by the algae *Ostreobium*. In addition, the endolithic bacterial community of *Isopora* in Okinawa is mainly dominated by aerobic *Bacteroidetes* and *Alphaproteobacteria* (Yang et al., 2020). These observations suggest that the endolithic communities in the *Isopora* skeleton might be influenced by geographical factors. Nevertheless, which local environmental factors influence endolithic composition remains unclear.

The operational taxonomic unit (OTU) has been implemented for microbial ecology studies since molecular biology tools were applied to study microbial molecular taxonomy (Edgar, 2010; Edgar, 2013). The OTU analysis was performed by either *de novo* or reference-based clustering methods with a 97% sequence similarity threshold for defining an operational species (Stackebrandt and Goebel, 1994). However, the denoising methods unbiasedly detect amplicon sequence variants (ASVs) and have revolutionized OTU-based pipelines in recent years. The ASVs are distinguished by single nucleotide variants; thus, any unique ASV is reproducible through the denoising process (by processing the same sequence regions) (Rosen et al., 2012).

More and more studies have been implementing denoising pipelines using the DADA2 (Callahan et al., 2016), Deblur (Amir et al., 2017) or UNOISE (Edgar, 2016) algorithms to obtain unbiased taxonomic composition data. However, in contrast to the OTUs, the ultra-high resolution sequence variants lead to a sparse ASV abundance table with too-many-zeros. Some studies reclustered ASVs into OTUs with 97% sequence similarity to improve the explained variance in community diversity (Liu et al., 2020). The reclustering-OTU (e.g. the VSEARCH plug-in embedded in the QIIME2 pipeline) method still follows operational classification cutoffs (e.g. 97% similarity for species or 94% similarity for genus) (Youssef et al., 2012), but it is criticized for its universality.

We performed an alternative ASV-reclustering method "KTU," a kmer-based taxonomic unit, alignment-free clustering algorithm to aggregate sparse ASVs into convergent taxonomic units (i.e. KTUs) with the closest tetra nucleotide usage patterns, which reflect to a 'genome signature' of a species (Dick et al., 2009; Liu et al., 2022). KTU reclustering reduces the zero-inflation effect in the data structure of the microbial communities. Therefore, continuous quantitative microbial abundance could precisely fit the environmental factors. In addition, the KTU assembles the ASVs into the closest phylogenetic lineages with the same genus or species names; for example, KTU reclusters *Lactobacillus* ASVs into several close sub-lineages with over 99% sequence similarities (e.g. *Fructilactobacillus* spp., *Levilactobacillus* spp., *Latilactobacillus* spp., and *Limosilactobacillus* spp.) (Huang et al., 2018; Zheng et al., 2020; Liu et al., 2022). Thus, the reclustered features not only assembles close-phylogeny features but also improves the confidence of associations between microbiota compositions and continuous environmental/clinical factors compared to using raw ASV composition data.

In this study, we targeted *Isopora* spp. from three locations subjected to the Kuroshio Current (KC) in the western Pacific Ocean to investigate the environmental factors which might affect the endolithic bacterial diversity in the skeletons. In addition, we attempted to improve the correlation of bacterial community and key environmental factors by comparing the two amplicon feature detection methods (KTUs and ASVs).

MATERIALS AND METHODS

Coral Sampling, DNA Extraction and PCR

Isopora colonies were sampled from three locations: Green Island (GI) (November 2015 at 22°75'N, 121°50'E and April 2017 at 22°40'N, 121°27'E) and Dongsha Atoll (DS) (July 2017 at 20°75'N, 116°75'E) in Taiwan, and Okinawa (OK) in Japan (October 2017 at 26°38'N, 127°51'E). The OK and DS sites are reef flat, GI site is at reef front. The location and depth of each sample are listed in **Table 1**.

The collected samples were washed twice with filtered seawater and stored at -80°C until DNA extraction. The extraction procedure followed the method in Yang et al. (2016). Slurry samples from the green layers of the coral skeleton were transferred into clean 1.5 mL tubes. The total genomic DNA of all the samples were extracted using the DNeasy PowerSoil Kit (Qiagen, Maryland, US). All the PCR protocol were followed Yang et al. (2016). In brief, PCR was performed using two bacterial universal primers: 968F (5'-AACGCGAAGAACCTTAC-3') and 1391R (5'-ACGGGCGGTGWGTRC-3'), designed for the bacterial V6-V8 hypervariable regions in the 16S ribosomal RNA gene, with 30 cycles of PCR with initial step of 94°C for 5 min, 94°C for 30 s, 52°C for 20 s, 72°C for 45 s, and finally 72°C for 10 min. Each PCR product was tagged using DNA tagging PCR (Chen et al., 2011), then sequenced using the Illumina Miseq 300 bp paired-end configuration.

Amplicon Sequence Analysis and KTU Re-Clustering

The 16S rDNA amplicon sequences were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) pipeline (version 2019.10) (Bolyen et al., 2019). The raw reads

TABLE 1 | Information on *Isopora* spp. coral colonies used to characterize bacterial communities in different locations.

Location	Sampling time	Depth	Sample name	Reads passed byDADA2 denoising	#ASVs[a]	#KTUs[a]
Okinawa, Japan	2017.10	Intertidal 0.5m	WI1	47,087	296/270	259/234
			WI2	45,842	635/619	517/501
			WI3	43,157	127/105	114/93
		Subtidal 3m	WST1	53,472	88/69	79/64
			WST2	31,553	466/456	376/366
			WST3	64,034	810/783	590/566
Green island, Taiwan	2015.11	30m	DB1	43,307	57/28	51/25
			DB2	59,318	639/593	437/412
			DB3	45,180	114/88	73/62
	2017.04	10m	GK1	56,700	287/249	229/202
			GK2	40,953	170/138	152/124
			GK3	44,600	362/313	306/272
Dongsha Atoll, Taiwan	2017.07	5m	ES1	32,672	50/36	43/29
			ES2	46,599	371/342	312/288
			ES3	40,288	43/22	39/22
			ES4	55,250	39/26	35/23
			ES5	36,396	89/71	70/61
			ES6	55,703	38/23	38/24
			ES7	39,384	87/76	75/64
			ES8	31,024	106/94	95/85

[a]*Rarefied/unassign, chloroplast, mitochondria removed.*

were first demultiplexed by cutadapt (version 1.15) (Martin, 2011), then the demultiplexed sequences were denoised using the DADA2 plugin of QIIME2 (Callahan et al., 2016). The qualified amplicon sequence variants (ASVs) were obtained *via* the denoising process with quality filtering (by truncating both ends of the reads to 235 bp) and chimera removal. ASV taxonomy was assigned using the classifier-consensus-vsearch plugin (Bokulich et al., 2018) against SILVA NR128 99% 16S rRNA gene sequences (Quast et al., 2013; Yilmaz et al., 2014). The ASV sequences were then re-clustered and aggregated using the "K-mer-based taxonomic (KTU) clustering algorithm" (https://github.com/poyuliu/KTU/) to refine the sparseness of the ASV abundance table. The KTU algorithm iteratively clustered ASV representative sequences with their tetra-nucleotide patterns (256 tetramer features, either frequencies or present scores; using the present-score option of KTU algorithm in the study) by partitioning around medoids (PAM, also called k-Medoids). A convergent KTU number was found by iteratively searching the maximum silhouette coefficient. Then we evaluated the KTU algorithm of the dataset by calculating the 1) ASV-to-KTU aggregating rate of each KTU, 2) sequence similarity within KTU and 3) within-KTU divergence based on the cosine distance. Finally, the KTUs were obtained by reclustering and used for subsequent analyses. R software (version 3.6.3) (R Core Team, 2015) was used for all data analyses. The ASV and KTU abundance tables were rarefied to the minimal read counts of the samples using the vegan R-package (31,024 reads per sample) (Oksanen et al., 2015). The unassigned taxon, chloroplast and mitochondria reads were removed from both the ASV and KTU abundance tables. The 52 removed chloroplast KTUs were then extracted to identify eukaryotic coral symbionts using Blastn (Camacho et al., 2009) (e value < 1e-5, identity ≥ 85%) by searching against the NCBI non-Redundant (NR) database.

Statistical Analysis

The bacterial community analyses were conducted and visualized using the MARco R-package (Liu, 2021). A Kruskal-Wallis test in R software (R Core Team, 2015), with α=0.05, was used for all statistical analyses of group comparisons and Dunn's test for *post-hoc* comparisons. Dissimilarities among microbial communities were measured by Bray-Curtis dissimilarity using a principal coordinates analysis (PCoA), and heterogeneity was tested using ADONIS (permutational multivariate analysis of variance using distance matrices). Alpha diversity indices were estimated by richness, Shannon's index, Simpson's index and Chao1 index. Pearson correlation analysis was performed to find the associations between factor analysis (FA) axes and PCoA axes. Spearman correlation analysis was performed to select FA axes-associated taxa, and taxa with $p < 0.01$ and in the top 2.5% coefficients (ρ) were selected. These taxa were visualized with a heatmap using the pheatmap R-package. An indicator species analysis was done with a chi-square test (Niemi et al., 1997); the indicator species were then identified by Pearson residuals > 5.

Analysis of the Environmental Factors

The environmental factors of the sampling sites were retrieved from the Ocean Data Bank (ODB, Ocean Data Bank of the Ministry of Science and Technology, Republic of China; http://www.odb.ntu.edu.tw/); an exploratory factor analysis was conducted by the psych package in R. An "fa" function with varimax rotation was performed and three factors were extracted. The factor scores were extracted using a regression method.

The flow rates of currents in the western Pacific Ocean and the South China Sea were also retrieved from the ODB database. The average flow rates of U (the velocity toward east) and V (the velocity toward north) vectors were averaged based on the corresponding sampling months and depths; the averaged flow

vectors were then placed onto a map of the western Pacific Ocean and the South China Sea.

Kuroshio Current Meta-Analysis

A microbiota dataset of Kuroshio Current transects from eight stations between 121.72° and 123° E at 23.75° N east of Taiwan (Cheng et al., 2020) was retrieved from the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA543843. The KC microbiota dataset was analyzed with the QIIME2 pipeline, with DADA2 denoising and reclustering ASVs with the KTU algorithm as in the pipeline mentioned above. The samples from the surface and deep chlorophyll maximum (DCM) layers of the maximum current subsets (sampling stations 2 and 3) were extracted for comparison to the current study.

RESULTS

Characterization of Endolithic Bacterial Composition in *Isopora* Coral Skeletons

The Illumina MiSeq platform generated a total of 2,655,526 high quality paired-end sequences, with an average of 912,519 non-chimeric reads, ranging from 31,024 to 64,034 reads/sample (median= 44,890 reads/sample; mean= 45,625.95 reads/sample) after DADA2 denoising. The QIIME2 pipeline coupling with KTU reclustering algorithm identified 3,927 ASVs and 1,938 KTUs (on average, 2.03 ASVs were clustered into each KTU; 98.86% mean sequence identity and 0.02 mean cosine divergence within KTUs; **Figure S1**). In general, the bacterial communities were different among the colonies from different locations (**Figure 1**). In both results of KTU and ASVs, *Proteobacteria* and *Bacteroidetes* were the dominant phyla in most of the samples (**Figures 1A, B**). In detail, *Alphaproteobacteria* occupied 31.55% of KTUs and 31.57% of ASVs; *Gammaproteobacteria* showed 10.18% of KTUs and 10.12% of ASVs; *Cytophagia* presented 22.57% KTUs and 22.47% of ASVs. Among them, *Alphaproteobacteria* was the most dominant class in GI and OK, but *Cytophagia* was the most dominant class in DS (**Figures 1C, D**).

The alpha diversity also showed a locational difference: the diversity from Dongsha (DS) was lower than those from Green Island (GI) and Okinawa (OK) (**Figure S2**). For the diversity indexes of KTU, the observed KTU (p=0.026) and Chao 1 (p=0.029) were significantly different among the three locations, while Shannon (p=0.054) and Simpson (p=0.082) did not show any significant difference. The pairwise Dunn's test showed the diversity indexes—observed KTU (p=0.004), Chao 1 (p=0.0046) and Shannon diversity (p=0.0138)—were significantly different between DS and OK. The PCoA analysis of taxa composition for both KTU and ASV showed that the difference is more prominent when examining geological locations (**Figure 2**). In the PCoA of KTU, GI is separated from OK and DS, whereas in ASV, OK is separated from GI and DS. In summary, both ASV and KTU analyses demonstrated consistent biodiversity patterns, while the KTU results reflected higher associations with the environmental facts. Therefore, the following analyses were primarily based on KTU reclustering features, not raw ASV features.

Three Main Environmental Factors Associate the Endolithic Bacterial Composition From Different Locations

We investigated which ocean-geographical factors may affect bacterial compositions. Geological location is one of the main factors that causes the diversity in the bacterial community, and our further analysis of the other 14 environmental factors showed more specific correlations in bacterial compositions (**Figure 2** and **Table 1**). Miscellaneous environmental factors were dimension reduced into three principal components (factor axes, FA). Among these, currents (current velocity, directions of the current: U. and V. component) and productivities (Chlorophyll a, phytoplankton, and primary production) explained 51.15% of FA1; inorganic factors (silicate, nitrate, salinity, temperature, and pH) explained 33.34% of FA2; and sampling depth explained 15.51% of FA3 (**Figures 2B–D**).

KTU Improves the Correlations Between Bacterial Quantification and Environmental Factors

In the PCoA ordination of endolithic microbial beta diversity measured by Bray-Curtis dissimilarity using KTU (**Figure 2E**) and ASV (**Figure 2I**), PCoA1 and PCoA2 using KTU explained a higher ratio than they did using ASV. Even so, both KTU and ASV methods showed that bacterial communities were significantly separated by location. From both PCoA results, the PCoA1 separated OK from the other two locations, while PCoA2 separated GI from the other two locations.

According to the correlation analyses of KTU with three FAs (**Figures 2F–H**) and the correlation analyses of ASV with three FAs (**Figures 2J–L**), FA1 and FA3 showed a significantly positive correlation with the PCoA2, whereas FA2 showed a positive correlation with the PCoA1. Both KTU and ASV analyses showed that the current and productivity had high correlation with the *Isopora* endolithic bacterial community (in **Figure 2F**, KTU: FA1 R=0.5, p=0.026; in **Figure 2J**, ASV: FA1 R=0.63, p=0.003). Variables associated with FA2 and FA3 had higher correlation in KTU than ASV—in FA2, based on KTU, R=0.4, p=0.081 (**Figure 2G**), based on ASV, R=0.34, p=0.142 (**Figure 2K**); in FA3, based on KTU, R=0.48, p=0.031 (**Figure 2H**), and based on ASV, R=0.35, p=0.128 (**Figure 2L**). In addition, the correlation analyses of four alpha diversities with three FAs showed that alpha diversities were positively correlated with FA2, negatively correlated with FA3, and not correlated with FA1 (**Table 2**).

We compared the top 2.5% of bacterial taxa that were correlated with three factors in the three locations (**Figure 3**) and found that the same bacterial species in all three locations showed different relationships to the factors. For example, *Endozoicomonas* had a stronger positive association with factors of current and productivities only in GI. Additionally, OK yielded the most

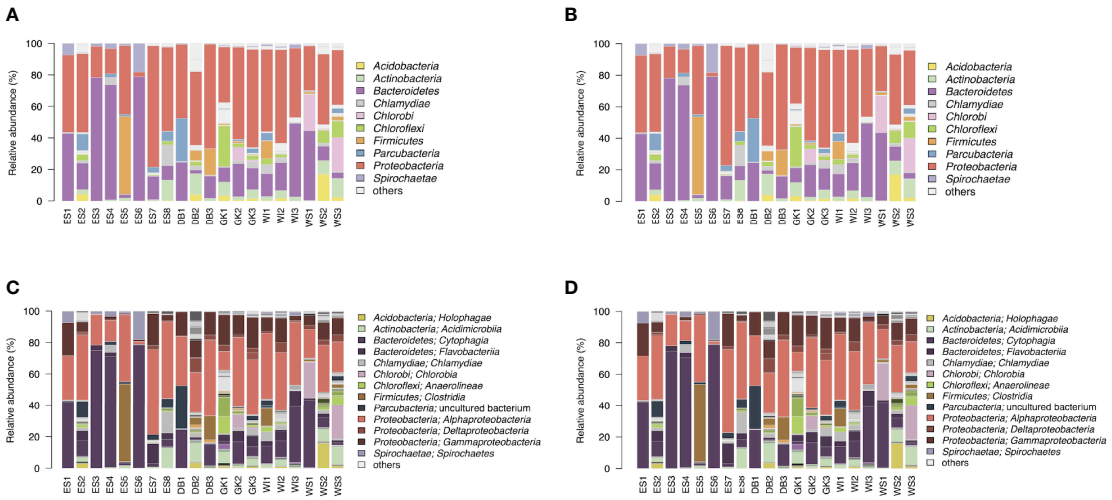

FIGURE 1 | Endolithic bacterial composition in *Isopora* spp. corals from three locations. **(A)** and **(C)** are bacterial phyla and classes, respectively, retrieved by KTU. **(B**, **D)** are bacterial phyla and class, respectively, retrieved by ASV. Location acronymous of samples: ES (Dongsha Atoll, Taiwan), DB (Green Island, Taiwan), GK (Green Island, Taiwan), WI (Okinawa, Japan), WS (Okinawa, Japan); please refer the **Table 1** to see the detail sampling information.

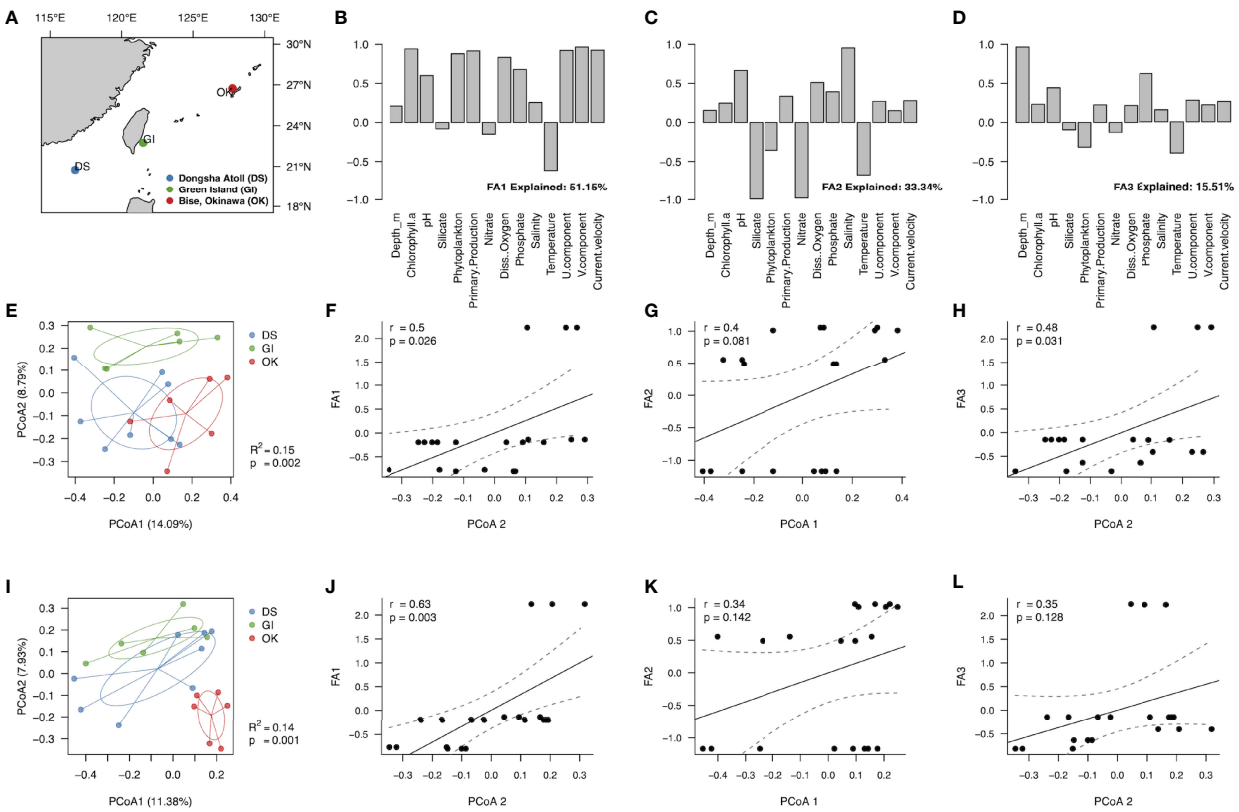

FIGURE 2 | Associations between the *Isopora* spp. endolithic microbiome and environment factors. **(A)** Geographical locations of three sampling sites (blue: Dongsha Atoll, DS; green: Green Island, GI; red: Bise-Okinawa, OK) in the western Pacific Ocean and South China Sea. **(B–D)** three components (factor axis, FA) for factor analysis of 14 environmental variables: **(B)** FA1, the Kuroshio Current and productivity factors; **(C)** FA2, the inorganic salts factors; **(D)** FA3, the minor local environmental factors. **(E, I)** the PCoA ordination of endolithic microbial beta diversity measured by Bray-Curtis dissimilarity using KTU and ASV, respectively. **(F–H)** and **(J–L)** the correlation analyses of KTU and ASV, respectively, with three FAs. Dash cubic lines represent the 95% confidence intervals of the correlations.

TABLE 2 | The correlation analyses of alpha diversities basing on KTU and ASV with three FAs.

	KTU			ASV		
	FA1	**FA2**	**FA3**	**FA1**	**FA2**	**FA3**
Richness (p values)	-0.20	0.58	-0.51	-0.15	0.56	-0.46
	(0.39)	(0.01)	(0.02)	(0.52)	(0.01)	(0.04)
Shannon	-0.05	0.48	-0.40	-0.08	0.48	-0.42
(p values)	(0.83)	(0.03)	(0.08)	(0.73)	(0.03)	(0.06)
Simpson	0.05	0.40	-0.37	0.08	0.34	-0.32
(p values)	(0.83)	(0.08)	(0.11)	(0.75)	(0.14)	(0.16)
Chao1	-0.20	0.57	-0.50	-0.16	0.54	-0.47
(p values)	(0.41)	(0.01)	(0.03)	(0.49)	(0.01)	(0.04)

bacteria with positive associations to factors (**Figures 3A–C**). Although the results based on ASV (**Figures 3D–F**) showed a similar pattern to the results based on KTU, the ASV results showed fewer factors correlated with taxa than did the KTU results. For instance, there were 27 KTUs correlated with FA2, but only 13 ASVs correlated with FA2. Even most correlated ASVs were assigned to the same taxa, which are just the intra-species variants. Instead, the correlated KTUs reflected more independent taxa associated with environmental factors.

Coral Skeletons and Seawater Share Dominant Bacteria in the Mainstream of the Kuroshio Current

According to our above-mentioned analysis of factors, FA1 factors were more related to bacterial composition in the *Isopora* skeletons than FA2 and FA3. In the map showing the average flow rate of currents in the western Pacific Ocean and South China Sea (**Figure S3**), both GI and OK were located in the mainstream of the KC, but DS was located in a branch

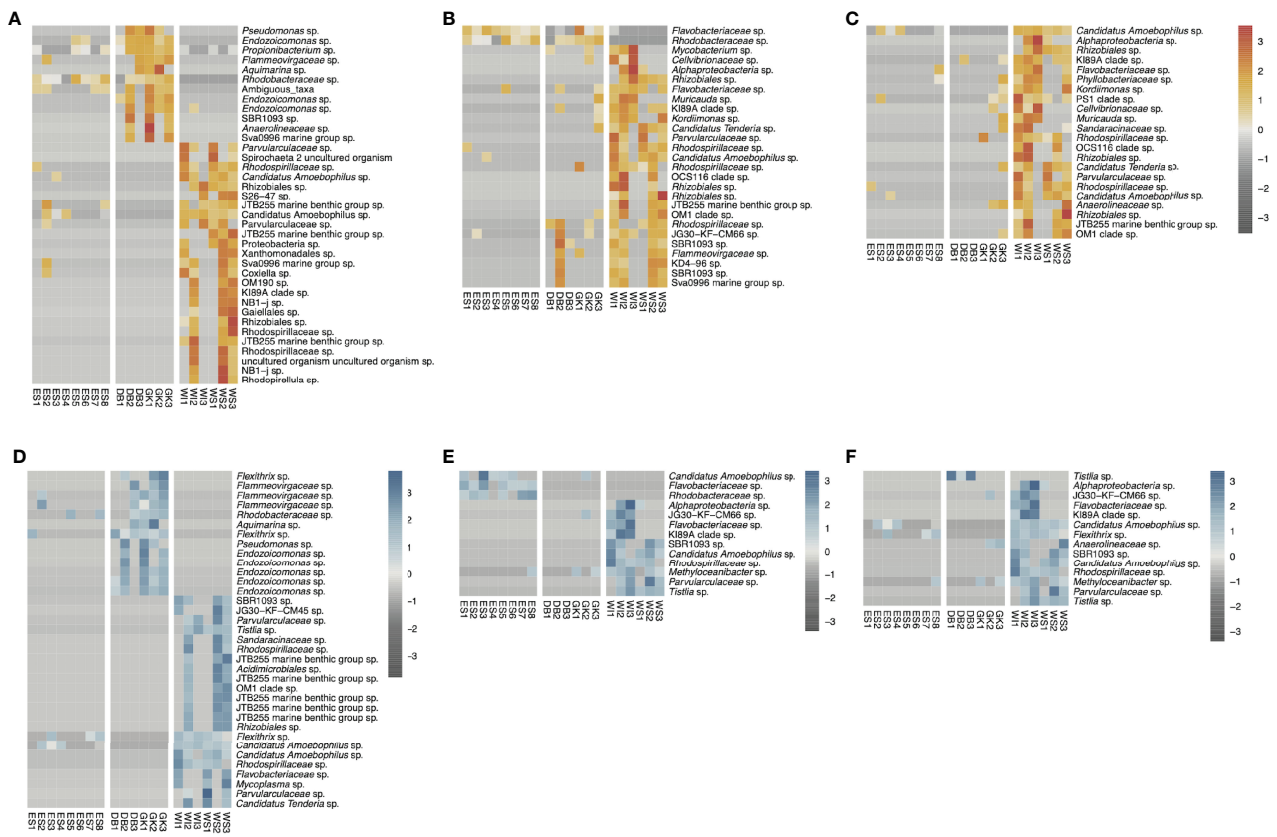

FIGURE 3 | Heatmaps of three FAs correlated with the top taxa at the three locations. **(A)** FA1 (Kuroshio Current and productivity factors)-correlated KTUs. **(B)** FA2 (inorganic salts factors)-correlated KTUs. **(C)** FA3 (minor local environmental factors)-correlated KTUs. The top 2.5% KTU high Spearman correlation coefficients, $P < 0.01$ were selected for visualizing. Heatmap of three FAs correlated with the top taxa in the three locations. **(D)** FA1 (Kuroshio Current and productivity factors)-correlated ASVs. **(E)** FA2 (inorganic salts factors)-correlated ASVs. **(F)** FA3 (minor local environmental factors)-correlated ASVs.

current; in addition, GI was subjected to the KC more than OK (**Figure S3**).

To investigate the commonality of KC and *Isopora* endolithic bacteria, we retrieved a seawater microbiota dataset (Cheng et al., 2020) of the KC mainstream in the open sea of eastern Taiwan. Comparing the bacteria in seawater of the KC and dominant endolithic bacteria in the coral skeleton at all three locations, we found that overlapped indicator species in DS and the KC showed lower abundances in other two locations. Overlapped indicator species of GI and the KC showed a lower abundance in DS, but higher abundance in OK. Similarly, overlapped indicator species of OK and the KC showed lower abundance in DS, but higher abundance in GI (**Figure S4**).

Local Environmental Factors Constrain the Colonization of Anaerobes in Coral Skeletons

To identify the anaerobes in coral skeletons, we consulted the taxonomy information of anaerobes and their physiological-metabolic potentials from Bergey's Manual of Systematic Bacteriology. OK had the highest average relative abundance of anaerobes (20.43%), followed by GI (13.36%) and DS (9.43%) (**Table 3**). Furthermore, environmental factors showed different correlations to anaerobic and aerobic endoliths. In **Figure 2**, the FAs represent three features of environments (FA1 represents current velocity and productivities, FA2 represents inorganic nutrients or salts, and FA3 represents minor local environmental factors). Among these features, FA1 describes factors at a large spatial scale, whereas FA2 and FA3 are local features. According to the relationship between FAs and endolithic anaerobes, FA1 (r=-0.17), FA2 (r=0.29) and FA3 (r=0.0013) were positively correlated to the endolithic anaerobes. This result indicated that the endolithic composition in the *Isopora* skeleton may have stronger relationships to local environments when the large spatial scales of geographic difference are partialized from compounded environmental factors.

TABLE 3 | Percentage of anaerobes in each sample.

Location	Samples	Anaerobe %
Okinawa, Japan	WST1	26.67
	WST2	26.89
	WST3	40.91
	WI1	14.88
	WI2	11.59
	WI3	1.62
Green island, Taiwan	DB1	0.76
	DB2	28.99
	DB3	18.08
	GK1	11.94
	GK2	10.9
	GK3	9.51
Dongsha Atoll, Taiwan	ES1	0
	ES2	6.72
	ES3	0.11
	ES4	0.14
	ES5	51.52
	ES6	0.16
	ES7	3.79
	ES8	13.02

Unpurposely Sequenced Chloroplast DNA Reveals Symbiotic Algae Composition in Coral Skeletons and Its Environmental Association

However, even though amplicon sequencing targets the 16S rRNA gene of prokaryotes, the organelle rDNA, including mitochondrial and chloroplast, from mixed coral symbionts, can be sequenced. Therefore, we did not directly analyze the endolithic eukaryotic composition (e.g. 18S rDNA); we still captured the 16S rDNA of endolithic algae in the coral skeleton. Then we found that the endolithic algae in GI are different from those in DS and OK (**Figures S5A, C**). Moreover, the endolithic algae composition was correlated with the environmental factors of the Kuroshio Current and productivity (FA1; r=-0.72, $P < 0.001$; **Figure S5B**).

DISCUSSION

Past studies have confirmed that microbial composition is influenced by light intensity, oxygen concentration, inorganic nutrient and pH in the microenvironment of the coral skeletons (Pernice et al., 2020; Chen et al., 2021). The internal structure of coral skeletons depends on the coral genus or species, suggesting that endolithic microbes have host specificity. In addition, the environment that corals are located in can change the morphology and texture of the skeleton (Tambutté et al., 2015), driving the microenvironment where microbes live (Yang et al., 2020). In this study, we processed the ASV features with KTU re-clustering and then identified the latent coral-microbe-environment interactive factors, such as concentrations of inorganic chemicals at the sampling sites.

Comparison of KTU and ASV Based Microbiome Analysis

Isopora corals were widely distributed in the warm, open ocean ecosystem, and lacked apparent physical barriers. However, their endosymbiotic bacterial compositions varied among populations of different locations. To identify variations in the endolithic microbiome based on ocean-geographical factors, we conducted amplicon sequence analyses with both high-resolution methods—ASV and KTU.

Regarding the comparison between KTU- and ASV-based microbiome analyses, the amplicon denoising methods correct sequencing errors, improve taxonomic resolution, and replace the traditional OTU clustering methods for microbiome analysis (Rosen et al., 2012). However, the high resolution of sequence features is a double-edged sword for ASV-based microbiome analysis. The high resolution ASVs aggravate the zero-inflation effect of sparse microbiome data. Here, we used the KTU re-clustering algorithm to aggregate trivial ASV features in the dataset. The KTU clustering method successfully calibrated ASV features in closed systems (gastrointestinal tracts, bioreactor, and

sourdough microbiome) in previous studies (Liu et al., 2022). Since the KTU features are clustered by their tetra-nucleotide patterns, which represents the resolution of variation in sequences at global-wise scale rather than the local variation in nucleotides, we suggest that this method is applicable for open systems, such as soil, lake, and ocean microbiomes.

There were 3,927 ASVs identified and clustered into 1,938 KTUs in this study. Data explanation efficacy, such as multiple environmental factors correlation to microbial composition, was improved after re-clustering (**Figure 2**). The explanation percentage of the first two coordinates of beta diversity increased from 19.31% (ASV) to 22.88% (KTU). Both the ASV and KTU were significantly correlated to FA1, with medium-high correlation coefficients. This indicates that the ocean currents (Kuroshio) and productivity (represented as FA1) would be the major physical factors analyzed in this study that contribute to the *Isopora*-microbes interactions in geographical differentiation. The re-clustering method would reduce zero-inflation in sequence-based microbial community surveys, thus truly reflecting environmental associations (Lin et al., 2020). Even if the ASV improves the resolution of amplicon variants, it also eliminates the gradient variations of biological dynamics by over zero-inflation. It is prone to false correlations or significant differences caused by extreme values (Liu et al., 2022). Moreover, in any microbiome-associated study, we suggested that both data types of the microbiome and environmental factors would be better as a continuous distribution rather than discrete.

Kuroshio Current and Local Water Chemistry Act as Constitutive Factors of Differentiating Coral Skeleton Microbiome

According to previous studies (Yang et al., 2016; Yang et al., 2019; Yang et al., 2020), *Isopora* spp. have a dense and non-porous skeletal structure. Therefore, it forms an anaerobic microenvironment to the symbiotic bacteria. The abundance of anaerobic endolithic bacteria in coral skeletons and the average relative abundance of anaerobes were different in the three locations.

In this study, we conducted KTU re-clustering to aggregate the sparse data on the coral skeleton microbiome. The results showed significant correlations between microbial composition and broad- and local-scale environmental factors, including the ocean currents, productivity, composition of inorganic salts, and local environment factors. We provide comprehensive results that illustrate the local topographical differences and environmental factors related to endolithic microbial diversity and constitution.

According to our results based on both KTU and ASV, water current velocity and primary productivity are positively correlated to PCoA2, which effectively distinguished bacterial compositions between samples from the reef flat (DS and OK) and from other places. Cyronak et al. (2020) found that shallow reef systems (e.g. reef flat) exhibit wider changes in environmental conditions, including pH and temperature. In addition, the topography of lagoon-like areas decides the current velocity, which influence the dissolved organic matter in the seawater (Vanwonterghem and Webster, 2020). These factors may result in changes to the seawater carbonation chemistry, which likely influences the coral skeleton properties and microbe niches inside. Therefore, we suggest that the local topology of reefs influences the endolithic microbial composition in coral skeletons.

In addition to the seawater chemistry of reef flat environments, sampling depths may influence the endolithic microbial composition in the coral skeleton. Regarding our results, the minor local environmental factors (FA3)—including depth—showed a positive correlation with PCoA2, which may echo the influence from the local topology of reefs. Furthermore, bacterial communities and chloroplast compositions in DS and OK are closer to each other than they are to GI. Samples from DS and OK are from a shallower environment (1-5 meters) than those in GI. Corals at shallower depths are subjected to wider tidal changes, light intensity and dissolved oxygen in seawater (Cyronak et al., 2020). Hence *Isopora* may experience a stronger light intensity and oxygen concentration in their skeletons, promoting the growth of endolithic green algae and photoautotrophic bacteria.

The KC strongly influences the regional hydroclimate by creating temperature, salinity, and pH gradients from tropical to subtropical and temperate zones (Dai, 1991; Abbot et al., 2003). In our previous studies, we found that latitude may be one of the factors influencing microbial composition in tissues of a coral species that was sampled along the KC (Woo et al., 2017; Yang et al., 2017; Yang et al., 2020). In this study, PCoA1, which was positively correlated with FA2, distinguished between the tropical (DS and GI) and the subtropical sampling sites (OK). Additionally, FA2, correlated with the top bacterial taxa, showed that the taxa from DS and GI are more similar. Hence, we suggest that the latitude of the sampling site along the KC (**Figure S4**) may also be a factor deciding the endolithic microbial composition. However, unlike coral tissues associated with bacterial richness—which are lower at higher latitudes (Pollock et al., 2018; Yang et al., 2020)—in the present study, coral skeleton-associated bacterial richness was higher at higher latitudes. Further investigations combining spatial and temporal conditions are needed to elucidate the role of latitude in the bacterial composition of coral skeletons.

To determine whether endolithic microbial compositions are influenced by the KC, we compared the bacterial constitutions in the coral skeleton and seawater from the KC mainstream in our study to those of Cheng et al. (2020). In this study, bacteria in the GI that were highly correlated with FA1 were *Pseudomonas*, *Rhodobacteraceae*, Sva0996, and bacteria in the OK that were highly correlated to FA1 were *Rhodospirillaceae*, *Coxiella*, *Rhodopirellula*, and Sva0996. These bacteria overlapped the indicator species among the KC. In addition, both the alpha diversities and overlapped bacterial taxa were higher in the locations in the KC mainstream (GI and OK) than the KC branch (Dongsha). Hence, we suggested that the dominant bacterial taxa in the KC may be involved in the microbial composition in the coral skeleton.

Interestingly, compared to the bacterial taxa in DS and OK, we found a higher relative abundance of bacteria related to the sulfur cycle in the GI, such as *Deltaproteobacteria*, *Chlorobi* and *Firmicutes*. This finding echoes our previous results, that *Chlorobi*, *Deltaproteobacteria* and *Firmicutes* were dominant in coral skeletons (Yang et al., 2016; Yang et al., 2019; Chen et al., 2021). Unlike the other two locations—which are reef islands—the GI is a volcanic island. There are hot springs close to the island's shore, and the hot spring water and seawater mix (Zheng et al., 2017). Although the exact sulfide concentration of seawater samples is unknown, a previous study found that the seawater surrounding volcanic islands and the host spring environment have higher concentrations of sulfide (Chen et al., 2005). This may explain why sulfur reducers and sulfur oxidizers are consistently dominant taxa in the skeletons of GI corals. Although further geochemistry data is needed, we suggest that the geological substance of the locations may also play a role in deciding the microbial composition in coral skeletons.

CONCLUSION

The influence of the environment on the microbial composition in coral is complex, especially for coral endolithic communities. Here, by investigating the relationship between microbial composition and locational environmental factors, we expand on the knowledge of potential biotic and abiotic factors that correlate with the microbial constitution in skeletons of *Isopora* from different locations. Although endolithic microbes may show host specificity because of the different skeleton structures of different hosts, endolithic microbes in *Isopora* spp. were mainly related to water currents, Chlorophyll a, phytoplankton and primary production in the surrounding water. In addition, using the KTU algorithm to re-cluster over-sparse sequence variants, we retrieved a continuous, not discrete, distribution of microbiome and environmental factors, showing gradient variations in the biological dynamics that were eliminated by ASV algorithm. Therefore, we suggest that KTU algorithm reclustering is an effective method for analyzing the relationships between microbial diversity and environment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA686201].

AUTHOR CONTRIBUTIONS

P-YL: data analysis and manuscript writing (result and discussion). S-YY: sampling (Dongsha), data analysis, and manuscript writing. C-YL: performed molecular experiment. NW: sampling (Okinawa). SP: sampling (Green Island). S-SY: data analysis. HY: sampling (Okinawa). S-LT: conceived of the idea and research design. S-HY: conceived of the idea, manuscript writing, and research design. All authors contributed to the article and approved the submitted version.

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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.2022.850984/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.