VARIANCE MATTERS: INDIVIDUAL DIFFERENCES AND THEIR CONSEQUENCES FOR NATURAL SELECTION WITHIN AND AMONG CORAL HOLOBIONTS

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Editorial: Variance matters: Individual differences and their consequences for natural selection within and among coral holobionts

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Editorial on the Research Topic
Variance Matters: Individual Differences and Their Consequences for Natural Selection Within and Among Coral Holobionts

The coral reef crisis has entered a critical stage. Ongoing political efforts to reduce greenhouse gas emissions have been ineffective at slowing the global decline of reef habitats and associated biodiversity. Restoration practitioners have been forced to focus on stop-gap measures to ensure that at least some coral species persist into the future (National Academies of Sciences Engineering and Medicine, 2018). Key examples include establishing nurseries wherein corals can be grown and later outplanted onto reefs (Young et al., 2012), identifying heat-tolerant individuals for nursery propagation (Cunning et al., 2021), and facilitating the natural ability of corals to adapt through interventions like assisted gene flow (Baums et al., 2019).

Corals may uniquely benefit from such interventions because they feature high adaptive capacity owing to their diverse symbiotic microbial associations (Voolstra et al., 2021). The coral host produces a suitable micro-habitat for intracellular populations of dinoflagellate photosymbionts (Symbiodiniaceae; LaJeunesse et al., 2018), as well as intra- and extra-cellular bacteria, archaea, fungi, algae, and viruses. Taken together, the entire biological unit is termed the coral holobiont. Each member includes its own genetic and physiological diversity, and can contribute to the plasticity of the whole. In such a system, natural selection can play out at multiple levels, such as through changes in partnerships (Torda et al., 2017). Different pairings of host and symbiont genotypes may contribute to intraspecific variation in important coral holobiont phenotypes, which may ultimately scale up to affect associated reef organisms (Parkinson and Baums, 2014). For example, coral holobiont heat tolerance can be impacted by host species identity (Loya et al., 2001), the identity of individual host colonies within a species (Parkinson et al., 2015), the Symbiodiniaceae species identity

(Sampayo et al., 2008), the bacterial consortium (Rosado et al., 2019), as well as interactions between any of these players (*e.g.*, Kavousi et al., 2020).

Projections of reef decline that fail to account for the coral holobiont's evolutionary response to climate change are unrealistic (Hughes et al., 2003). Similarly, projections based on the average ecophysiological responses of coral colonies to environmental shifts might be inappropriate because only a subset of existing partnerships survive stress events and repopulate reefs (Kubicek et al., 2019). To better predict the future trajectory of reef ecosystems and to stage effective interventions, it will be critical to (1) monitor changes in coral holobiont partnerships due to heat stress; (2) identify high-performing outlier colonies; and (3) estimate the breadth of intraspecific variation among colonies, as this variability forms the foundation for natural selection (Violle et al., 2012). Inferences must also come from the wide array of "coral" holobionts, including soft corals, black corals, leather corals, blue corals, fire corals, and other sessile cnidarians.

Observations such as the divergent bleaching outcomes of two adjacent *Porites astreoides* colonies depicted in Figure 1 provide a sense of hope. Clearly, there is a high degree of variability within populations of reef-building corals. The question now is: to what extent can coral holobiont evolution keep pace with climate change? The goal of this Research Topic was to collect studies that highlight intraspecific variation among coral colonies and their associated microbial communities, as well as to evaluate the consequences of such ecophysiological diversity for the long-term survival of coral species. Here, we summarize the key findings of nine articles that directly address these concepts.

Three studies examined intraspecific variation among reef-building corals. Rodriguez et al. investigated non-contact competitive behavior between three colonies of the hard coral *Porites cylindrica* and five colonies of a soft coral competitor. The fully crossed design pitted each colony against all others, revealing unique behavioral responses depending on the combination of genotypes. Some secretory genes were only expressed by *Porites* in certain pairings, and encoded putative allelopathic toxins. Such individual-level variation in competitive interactions could be an important determinant of reef community dynamics. Marhoefer et al. used a reciprocal transplant design to detect genotype-by-environment interactions among *Pocillopora damicornis* colonies sourced from either the reef slope or reef flat. The performance of clonal fragments varied depending on whether they remained at the source habitat or were transplanted. Experimental heat stress assays indicated that some transplanted fragments began to acclimate to their new habitat, either with or without changes in their symbiont communities, highlighting the role of interactions between hosts, symbionts, and the environment in adaptive responses. Lenz et al. monitored the physiology of *Porites astreoides* colonies at four different reef sites during a warm-water bleaching event. They identified divergent impacts of heat stress on the different coral populations, as well as unique recovery trajectories at each site. These patterns reflect how prevailing environmental conditions can drive local selection despite gene flow, altering population-level responses to stress and maintaining intraspecific phenotypic variation across a coral species' biogeographic range.

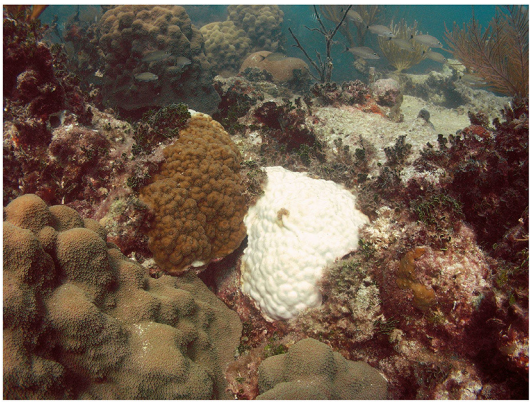

FIGURE 1
An example of intraspecific variation in a coral population: two *Porites astreoides* colonies exhibiting different bleaching responses to thermal stress despite sharing the same environment (and most likely the same Symbiodiniaceae communities). Coral bleaching results when stress breaks down the symbiosis between the host and microalgae, causing a loss of symbionts. The image was captured in the Florida Keys during a warm-water anomaly in October 2015. Photo credit: Ilsa Kuffner, PhD; USGS.

Two contributions to this Research Topic focused on other reef cnidarians. Hsu et al. characterized organismal trait composition in two octocoral species dominated by different Symbiodiniaceae but thriving within the same high-latitude environment. Divergence across multiple physiological traits suggested contrasting performances among *Gerakladium*-associated *Stereonephtya* and *Durusdinium*-associated *Litophyton*. Intraspecific variation in traits indicated a higher possibility for some *Litophyton* individuals to blend heterotropic energy with autotrophically-acquired energy in comparison with *Stereonephtya*. Fuijiwara et al. resolved three Symbiodiniaceae species associating with the zoantharian *Zoanthus sansibaricus* across a depth gradient. They identified intraspecific variation in symbiont community composition across the surfaces of shallow colonies, which suggested a capacity for acclimation via symbiont shuffling. However, after reciprocal transplantation between shallow and deep habitats, symbiont community composition remained stable in most colonies despite the stressful change in environmental conditions.

In addition to associations between hosts and their primary microalgal communities, several authors explored intraspecific variation in the bacterial component of coral

holobionts. Yang et al. examined seasonal variation in the prokaryotic communities found within the scleractinian coral *Acropora muricata* at three latitudinal settings influenced by the Kuroshio Current. They observed that local conditions had a great effect on bacterial community dynamics despite exposure to the same water current, driven primarily by the temperature gradient. In corals located at temperate latitudes, specialist bacterial phylogroups outnumbered generalist groups, whereas the opposite was true in tropical latitudes. Van de Water et al. assessed the spatial and temporal stability between the black coral *Antipathella subpinnata* and its bacterial microbiome. They further compared this stability to a sympatric octocoral, *Eunicella cavolini*, which featured a similar arborescent morphology. The black coral's prokaryotic community composition varied across space and time, whereas the octocoral community remained stable. A high degree of flexibility with respect to the microbiome may allow *Antipathella* holobionts to acclimate to a range of environmental conditions, particularly through the occurrence of bacterial taxa putatively involved in nitrogen and sulfur cycling.

One experiment tracked interactions between Symbiodiniaceae and bacteria. Diaz-Almeyda et al. compared the microbial communities associated with three *Symbiodinium* species characterized by contrasting ecophysiologies, levels of interaction with their hosts, and thermal adaptations. Microbial communities were assessed after culturing photosymbionts for 27 days at ambient and elevated temperatures. In addition to identifying a shared set of bacteria common to many Symbiodiniaceae cultures and corals, they showed that among the more variable components of the prokaryotic microbiome, community composition was better predicted by *Symbiodinium* species than by temperature. At elevated temperatures, some species displayed reduced microbial diversity along with greater individual variability in community composition. Overall, these results suggest that bacteria associated with thermotolerant Symbiodiniaceae might play a role in their thermotolerance.

Finally, one study examined how intraspecific variation may scale to impact ecosystem processes. Lin et al. investigated the response of an abundant reef fish species, *Ctenochaetus striatus*, to shifts in benthic composition in the South China Sea. Through DNA sequencing, they showed that fish stomach contents were dominated by macroalgae, filamentous algae, and microalgae. The biomass of *Ctenochaetus* exhibited a positive relationship with short algal turf cover, which tends to be more common on partially degraded reefs. Thus, an increase in fish body condition may be associated with moderate loss in coral cover. The implication is that benthic shifts triggered by the loss of the most susceptible corals could have cascading effects on closely-associated species such as reef fishes. Because *Ctenochaetus* is an important detritivore within reef communities, such shifts could impact the flow of energy in reef systems.

Although they may seem disparate at first glance, the works included in this Research Topic are all focused on intraspecific variability at some level within coral holobionts, from meta-populations to micro-communities. The diverse nature of the studies reflects the organismal diversity found within coral colonies themselves. If we are to gain a truly holistic understanding of coral ecology and evolution, particularly as reef ecosystems respond to climate change in the near future, we must continue to explore variation in coral holobionts at multiple scales.

Author contributions

JEP wrote the first draft of the article. All authors contributed to the article and approved the submitted version.

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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.

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Local Conditions Influence the Prokaryotic Communities Associated With the Mesophotic Black Coral *Antipathella subpinnata*

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Black corals are important habitat-forming species in the mesophotic and deep-sea zones of the world's oceans because of their arborescent colony structure and tendency to form animal forests. Although we have started unraveling the ecology of mesophotic black corals, the importance of the associated microbes to their health has remained unexplored. Here, we provide in-depth assessments of black coral-microbe symbioses by investigating the spatial and temporal stability of these associations, and make comparisons with a sympatric octocoral with similar colony structure. To this end, we collected samples of *Antipathella subpinnata* colonies from three mesophotic shoals situated along the Ligurian Coast of the Mediterranean Sea (Bordighera, Portofino, Savona) in the spring of 2017. At the Portofino shoal, samples of *A. subpinnata* and the gorgonian *Eunicella cavolini* were collected in November 2016 and May 2017. Bacterial communities were profiled using 16S rRNA gene amplicon sequencing. The bacterial community of *E. cavolini* was consistently dominated by *Endozoicomonas*. Contrastingly, the black coral microbiome was more diverse, and was primarily composed of numerous Bacteroidetes, Alpha- and Gammaproteobacterial taxa, putatively involved in all steps of the nitrogen and sulfur cycles. Compositional differences in the *A. subpinnata* microbiome existed between all locations and both time points, and no phylotypes were consistently associated with *A. subpinnata*. This highlights that local conditions may influence the bacterial community structure and potentially nutrient cycling within the *A. subpinnata* holobiont. But it also suggests that this coral holobiont possesses a high degree of microbiome flexibility, which may be a mechanism to acclimate to environmental change.

Keywords: 16S rRNA gene amplicon analysis, black coral, microbiome, gorgonian, mesophotic coral, *Antipathella subpinnata*, microbiome flexibility, phylosymbiosis

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INTRODUCTION

Antipatharians, commonly known as black corals, can be found throughout the world's oceans from tropical to polar latitudes (Wagner et al., 2012). This taxon of hexacorals consists of about 247 species (Brugler and France, 2007) and is particularly diverse in tropical and subtropical regions (Tazioli et al., 2007; Wagner et al., 2012). Dense assemblages of black corals have been reported in

the mesophotic (40–150 m depth) (Cairns, 2007; Bo et al., 2019) and deep-sea zones (Molodtsova and Opresko, 2017), where they provide critically important habitat. Because of their typically wide bathymetric distribution, it has been difficult to collect specimens and make direct observations of black corals. As such, many aspects of the black coral ecology and biology remain unknown. However, access to remotely operated vehicles and technical diving now allows us to explore black coral communities and investigate the physiology and microbial ecology of these organisms.

Macro-organisms live in symbioses with microbes (an assemblage termed the 'holobiont'), which are known to play important roles in host health, and contribute to their host's adaptation and acclimation to environmental change (Bosch and McFall-Ngai, 2011; McFall-Ngai et al., 2013). In corals, mutualist and commensal bacteria provide the host with nutrients (Benavides et al., 2017) and protect it against pathogens through occupation of available niches (Rohwer et al., 2002; Rypien et al., 2010) and the secretion of antimicrobial compounds (Ritchie, 2006; Nissimov et al., 2009; Kvennefors et al., 2012; Shnit-Orland et al., 2012). For the fitness of the holobiont, it is therefore important to maintain a microbial community with a stable repertoire of metabolic and protective functions. In fact, signals of phylosymbiosis in the coral holobiont have shown that there is a long-term evolutionary history between corals and various microbial taxa (Pollock et al., 2018; van de Water et al., 2018b). The coral microbiome has been extensively studied in tropical and temperate scleractinian corals (hexacorals) and Mediterranean gorgonians (octocorals) (reviewed in Bourne et al., 2007; Hernandez-Agreda et al., 2017; van de Water et al., 2018a, respectively). While the microbial community compositions of the latter have been found to be stable on both spatial and temporal scales (van de Water et al., 2016, 2017, 2018b), the microbiota of scleractinians tends to be more diverse and variable (Littman et al., 2009; Hernandez-Agreda et al., 2016; Pollock et al., 2018). It should, however, be noted that in contrast to Mediterranean octocorals, the majority of scleractinian corals studied are in an intricate symbiosis with Symbiodiniaceae and that these algal symbionts are known to affect the microbiota of marine invertebrates (Bourne et al., 2013). Generally, however, species-specific microbes and environmentally responsive microbes can be found within the holobiont of each coral species (Hernandez-Agreda et al., 2016; van de Water et al., 2017; Pollock et al., 2018), with differences observed between the microbiota in coral tissues and other compartments, such as mucus and skeleton (Sweet et al., 2011; Apprill et al., 2016; Pollock et al., 2018; Bednarz et al., 2019). Although of high ecological importance in the mesophotic zone and deep sea, the microbial ecology of black corals has so far received little attention.

Initial investigations established cultures of various microbes associated with black corals, particularly Firmicutes and Actinobacteria from *Antipathes dichotoma* (Pallas, 1766) (Zhang et al., 2012), and a range of Gammaproteobacteria and Actinobacteria from *Stichopathes luetkeni* (Santiago-Vázquez et al., 2007). However, as most microbes cannot be cultured yet, a culture-dependent approach may not provide the best overview of the black coral-associated microbiota. Early culture-independent approaches found primarily Rhodobacterales and Pseudomonadales on an unidentified deep-sea black coral (Penn et al., 2006), but a more diverse microbiota associated with *S. luetkeni*, consisting mainly of a range of Proteobacteria, Actinobacteria, Firmicutes, Cytophaga-Flavobacterium and Chloroflexi (Santiago-Vázquez et al., 2007). Two recent studies using 16S rRNA gene amplicon sequencing on the widely distributed deep-sea black coral *Leiopathes glaberrima* (Esper, 1788) and two Pacific *Antipathes* spp. found a similar microbial community composition at the phylum level (Dannenberg, 2016; Liu et al., 2018). However, significant differences in the microbiota were observed between sampling locations. Interestingly, only one phylotype, which belonged to the genus *Endozoicomonas*, was present in all colonies of *L. glaberrima* (Dannenberg, 2016). These bacteria are commonly present in the microbiota of scleractinian corals, gorgonians and various other marine invertebrates, where they are believed to be important for host health (Neave et al., 2016).

Black corals are also associated with various eukaryotic microbes. For example, several Hawaiian (Wagner et al., 2011) and Indonesian (Bo et al., 2011a) species have been found to engage in symbioses with unicellular algae of the genus Symbiodiniaceae. These algae contribute significantly to the nutrition of shallow reef-building scleractinian corals, but their relevance to black corals in the non-photic zone remains unclear. Using a transcriptomics approach, apicomplexans were found to be part of the *L. glaberrima* holobiont as well (Dannenberg, 2016). Further genomic characterization of these corallicolid apicomplexans revealed that members of this diverse clade are widespread associates of deep-sea corals. Although their ecological role remains uncertain, they do possess some characteristics of a parasitic lifestyle (Vohsen et al., 2020).

While these studies have provided initial assessments of the microbiota of black corals, a comprehensive assessment of the black coral-associated microbial communities is an important first step to understand the ecological success of these animals better. *Antipathella subpinnata* (Ellis and Solander, 1786) is the most common mesophotic black coral species in the Mediterranean Sea, but it has also been reported in the Atlantic Ocean (OCEANA, 2011; de Matos et al., 2014). Its arborescent colonies of up to 1.5 m form large (Bo et al., 2018) and dense (5.2 colonies m^{-2}) aggregations (Bo et al., 2009; de Matos et al., 2014) on hard substrates, including shipwrecks, between 55 and 500 m (Bo et al., 2008, 2012b; Mastrototaro et al., 2010). This makes *A. subpinnata* one of the most important habitat-forming species that provides structural complexity to the Mediterranean mesophotic ecosystems and serves as a refuge for a rich associated fauna, including species of economic importance, such as seabream, jack mackerel, catshark and octopus (personal observations, Bo et al., 2008, 2009; Cau et al., 2017). Unfortunately, commercial fishing activities and entanglements by lost fishing lines or nets have caused significant damage to local populations of *A. subpinnata* (Bo et al., 2014; Oliveira et al., 2015). While several aspects of *A. subpinnata*'s reproductive biology (Gaino and Scoccia, 2010; Coppari et al., 2019) and ecology, including its interactions with

various macro-epibionts (Bo et al., 2011b; Gaino et al., 2013) and feeding strategies (Coppari et al., 2020), have been studied, its microbial associates have never been investigated.

Antipathella subpinnata commonly lives in mixed assemblages with arborescent gorgonian octocorals, such as *Paramuricea clavata* (Risso, 1826) (Enrichetti, 2019; Chimienti et al., 2020) and *Eunicella cavolini* (Koch, 1887) (Bo et al., 2009; Gori et al., 2017), which are commonly found at shallow and mesophotic depths. While it is unknown how these black corals and gorgonians interact, the similar colony structures and polyps sizes (Weinberg, 1976; Bo et al., 2018) suggest that they may occupy the same trophic niche. Comparison of the natural ^{13}C and ^{15}N stable isotope signatures of the tissues of these corals indicates that this may indeed be the case (Gori et al., 2017; Coppari et al., 2020). *E. cavolini* may thus be a competitor for food resources of *A. subpinnata*, particularly, because the polyp density in *E. cavolini* is ∼3-fold higher compared with *A. subpinnata* (Weinberg, 1976; Bo et al., 2018). Besides, this gorgonian has been shown to be a strong competitor in physical interactions with other gorgonians (Turicchia et al., 2020). In contrast to *A. subpinnata*, the prokaryotic communities associated with shallow *E. cavolini* colonies have been previously described in detail (van de Water et al., 2017, 2018b), showing a relatively stable microbiota dominated by a few bacterial phylotypes, in particular *Endozoicomonas*. A comparison of the microbiota of these two distantly related but sympatric species in the mesophotic zone may provide insights into common microbial taxa, possibly linked to the trophic niche or the mesophotic environment.

Here, we describe the spatial and temporal patterns in the bacterial communities of the arborescent black coral *A. subpinnata*. In addition, we provide a comparison with the gorgonian *E. cavolini* to assess differences between the microbiota of these two sympatric species. We show that, in contrast to the gorgonian, the bacterial communities of *A. subpinnata* are highly diverse and differ significantly between locations and between autumn and spring time points. Interestingly, no bacteria were consistently associated with this species across space and time, suggesting that local environmental conditions are the main drivers of the microbiota associated with this black coral.

MATERIALS AND METHODS

Study Sites and Sampling Procedures

To assess the effect of seasonality on the microbiome of *Antipathella subpinnata*, samples (one per colony) were collected by technical diving in Portofino (coordinates 44° 17.63′ N, 9° 13.27′ E, 67 m depth) (**Figure 1**) in autumn (n = 10 colonies, 30 November 2016, seawater temperature of 17.8°C) and spring (n = 10 colonies, 10 May 2017: seawater temperature 14.2°C). Here, samples of 6 colonies of *Eunicella cavolini* were also collected at both time points. To characterize the spatial stability in the microbiome of *A. subpinnata*, samples were collected from two other locations off the Ligurian coast near Bordighera (n = 10 colonies, coordinates 43° 46.11′ N, 7° 40.82′ E, 63 m depth, 21 April 2017, seawater temperature 14.1°C) and Savona (n = 12 colonies, coordinates 44° 13.52′ N, 8° 27.61′ E, 70 m depth, 15 May 2017, seawater temperature 14.3°C) (**Figure 1**) in spring 2017. Fragments of approximately 10 cm were cut from colonies using scissors and placed in a zip-lock bag. On board the vessel, samples were rinsed twice with 0.2 μm filtered seawater and stored in RNA*later* at 4°C until further processing. From each site and time point, three replicates of 2 L seawater were collected close to the sampling site (about 2–5 m above the black coral colonies) in a Niskin bottle. The collected seawater was sequentially filtered through 3 and 0.2 μm Whatman Nucleopore Track-Etched filters (Sigma-Aldrich) and the 0.2 μm filter was kept in RNA*later* at 4°C. An overview of the sample collection can be found in **Table 1**.

The three sites are characterized by rocky shoals with a maximum height of 10 m, which emerge from a gently sloping, sandy or detritic sea-bottom. The Portofino shoal is a large, isolated rocky shoal under strong current conditions. It is dominated by a dense forest of *Eunicella cavolini* and a small population of *A. subpinnata* is located on the steep westernmost side of the shoal (**Figure 2A**). The Bordighera shoal consists of scattered large rocky boulders, which were heavily silted and host a dense population of *A. subpinnata*, occasionally co-existing with the gorgonian *Paramuricea clavata* (Risso, 1826) on the margins of this animal forest (**Figure 2B**). The Savona shoal extends parallel to the coast and is a gently sloping, coralligenous elevation mainly hosting a scattered assemblage of gorgonians. At this site, *A. subpinnata* colonies can be found with a sparse distribution (**Figure 2C**). Additional information about the benthic communities and environmental conditions at these study sites can be found in Enrichetti et al. (2019).

DNA Extraction and Sequencing Library Construction

To extract the DNA from both coral tissue and filter retentate, the DNeasy PowerBiofilm Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's protocol, with the exception that the bead beating was performed at 30 Hz for 1 min using a CryoMill (Retsch, Haan, Germany) at room temperature. Extracted DNA was shipped to Macrogen (Seoul, Republic of Korea) for sequencing library construction using the 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) primers that target the V3-V4 regions of the 16S rRNA gene (Klindworth et al., 2012), and 2x 300 bp paired-end sequencing (with a 30% PhiX control spike-in) on the Illumina MiSeq platform.

Negative DNA extraction kit controls (i.e., an extraction procedure without sample added) were not included in the sequencing run, although DNA extraction kits are known to be contaminated with microbial DNA. This may affect 16S rRNA gene amplicon sequencing results (Salter et al., 2014), especially in low bacterial biomass samples, whereas the impact will be low on high bacterial biomass samples (Glassing et al., 2016). To estimate the potential contamination in our DNA extractions, the amount of microbial DNA present in 2 μl of coral and seawater samples and negative DNA extraction kit controls was quantified using the Femto Bacterial DNA Quantification

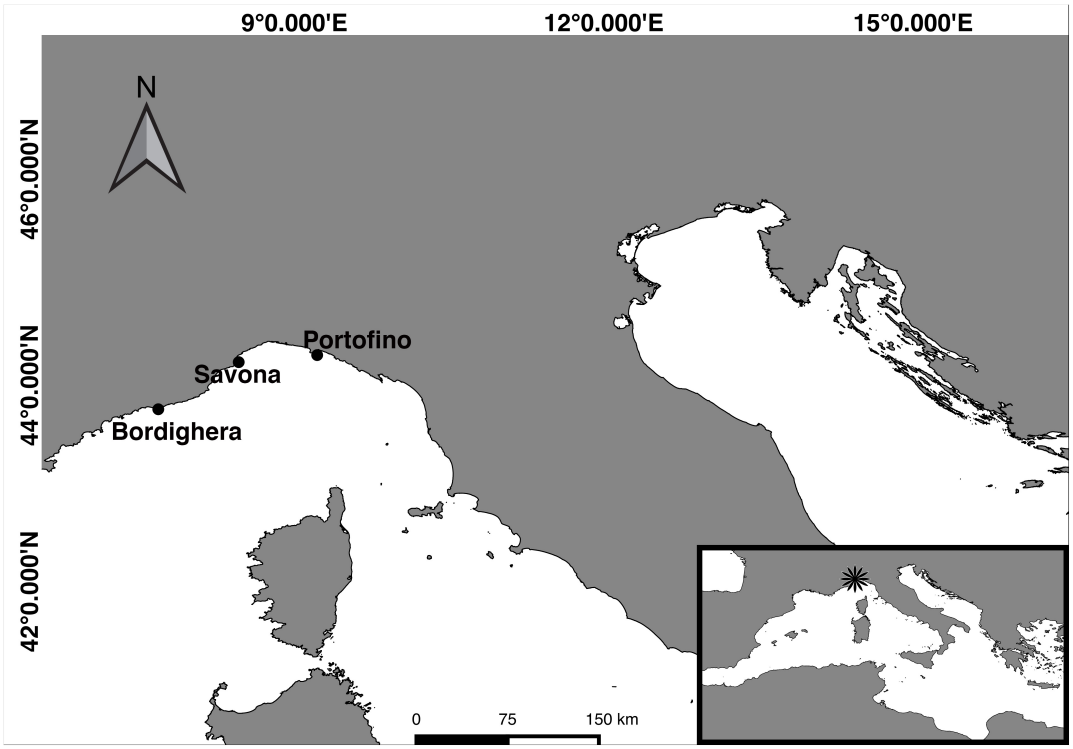

FIGURE 1 | Map of the study sites along the Ligurian coast. Samples were collected from three mesophotic shoals near Bordighera, Savona, and Portofino.

Kit (Zymo Research, Irvine, CA, United States) following the manufacturer's protocol. Concentrations were 2.88 ± 0.92 ng/μl (C_T = 15.0 ± 0.23) and 0.148 ± 0.015 ng/μl (C_T = 19.6 ± 0.08) of bacterial DNA in seawater and coral samples, respectively. The negative DNA extraction kit controls contained 0.02 ± 0.05 pg/μl (C_T = 32.4 ± 1.28) of bacterial DNA. As such, the potential contamination was estimated to be low and had likely no to little impact on our results.

16S rRNA Gene Amplicon Data Processing and Analysis

The 16S rRNA gene amplicon data was analyzed using the UNOISE2 pipeline (Edgar, 2016) as implemented in the USEARCH package (version 9.2[1]) (Edgar, 2010). The raw forward (R1) and reverse (R2) sequence fastq files of the 66 samples contained a total of 20,259,044 reads (ranging between 183,112 and 386,636 reads per sample) with an average Q20 score of 89.43% and a Q30 score of 81.64%. R1 and R2 paired reads were merged using -fastq_mergepairs. Primer sequences were trimmed using -fastx_truncate and reads were quality filtered with the -fastq_filter script, generating a filtered fasta file containing 8,163,975 reads with an average length of 390 bp. Unique sequences were identified using the –fastx_uniques script followed by denoising of the sequence dataset with the UNOISE2 algorithm, obtaining 16,450 denoised sequences or 'zero-radius OTUs' (zOTU, Operational Taxonomic Unit). The –usearch_global script was then used to generate an OTU table at the 97% similarity level, containing 13,076 OTUs and an average 116,685 reads per sample (range 73,183 and 165,196 reads). The taxonomy was assigned to each OTU based on the SILVA database (release v123) (Quast et al., 2013) using the -sintax algorithm. The OTU table was converted to the HDF5 biom format and taxonomic assignment metadata was added.

Unassigned OTUs, and OTUs classified as chloroplast or mitochondria were excluded from the dataset. The most abundant OTU, OTU1 (representing 47% of all quality filtered reads), was also removed from the dataset after it was identified as a match to the mitochondrial 12S rRNA gene of black corals. [This high background amplification reduces the sequencing

TABLE 1 | Overview of sample collection and distances between sampling locations.

	Portofino		Bordighera	Savona
	November 2016	May 2017	April 2017	May 2017
A. subpinnata	10	10	10	12
E. cavolini	6	6		
Seawater	3	3	3	3
Distance between sampling locations	Portofino – Bordighera		∼135 km	
	Portofino – Savona		∼ 60 km	
	Bordighera – Savona		∼ 90 km	

[1]https://www.drive5.com/usearch/

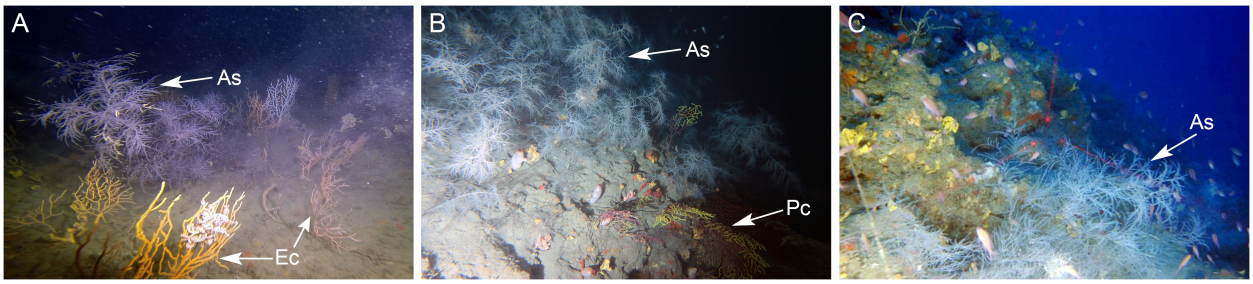

FIGURE 2 | Populations of *Antipathella subpinnata* on the shoals near **(A)** Portofino at 67 m depth, **(B)** Bordighera at 63 m depth and **(C)** Savona at 70 m depth. Colonies of *A. subpinnata* (As) and the sympatric gorgonians *Paramuricea clavata* (Pc) and *Eunicella cavolini* (Ec) are indicated with arrows.

depth of the microbial community dramatically, and thereby could underestimate the diversity. Future studies on black coral microbiomes should take into account that in order to fully capture the microbial diversity of the black coral microbiome relatively deep sequencing is required. This result also shows that the 341F/805R primer set presents a strong amplification bias for the 12S rRNA gene of black corals. We therefore also recommend the testing of a range of primer pairs to assess which pair provides the least 12S rRNA gene amplification without compromising the accuracy of the assessment of the bacterial community composition.] Three *A. subpinnata* samples collected in spring 2017 were identified as outliers (two samples from Portofino and one sample from Bordighera) during sample quality assessment of the dataset during differential abundance analysis (see below), and were also removed from the OTU table.

Because negative DNA extraction kit controls had not been included in the sequencing run, a statistical approach was employed to identify and remove potential contaminant sequences as an additional quality control step. The 'Frequency' method of the R-package *decontam* (Davis et al., 2018) was used on (1) seawater and (2) coral (*A. subpinn*ata and *E. cavolini*) samples separately because of the large difference in microbial DNA concentration in the samples. In case of contaminants, a bimodal distribution of the decontam scores is expected (Davis et al., 2018), but this was not observed in both cases (**Supplementary File S1**). In addition, all OTUs within the low decontam score ($P^* < 0.05$) range (1 and 24 OTUs in the seawater and coral datasets, respectively) had a very low prevalence (present in 2–3 samples), indicating low classification accuracy and sensitivity (Davis et al., 2018) (**Supplementary File S1**). Taken together, no potential contaminant sequences were identified in the dataset.

The final OTU table contained 5,621,675 reads belonging to 12,368 OTUs, with an average of 85,177 reads per sample (min 19,354, max 124,694). The unfiltered OTU table, sample metadata and representative sequences of each OTU are provided in the **Supplementary Data S1–S3**. Raw sequences were deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA506661.

The OTU table was rarefied to 19,354 reads per sample, containing 9,330 OTUs. Alpha diversity metrics (richness: observed OTUs, diversity: Shannon-Wiener *H*, evenness: Simpson's *E*) were calculated from the OTU table using QIIME v1.9 (Caporaso et al., 2010). The *phyloseq* package (McMurdie and Holmes, 2013) integrated in R was used to generate relative abundance plots and heatmaps. Using PRIMER 6 & PERMANOVA+ (PRIMER-E Ltd, Auckland, New Zealand) (Clarke and Gorley, 2006; Anderson and Walsh, 2013), Permutational Analysis of Variance (PERMANOVA) performed under Type III partial sums of squares and 9999 permutations under the reduced model was used to statistically assess differences in bacterial communities' alpha and beta diversity between locations, time points and species. Principal Coordinates Analysis (PCoA) on square root-transformed Bray–Curtis similarity matrices was used to visualize these differences in beta diversity. Negative binomial generalized linear modeling and Wald tests for pair-wise comparisons as implemented in the *DESeq2* package (version 1.20.0) (Love et al., 2014) in R (version 3.5.0) (R Core Team, 2018), were used for differential abundance analysis to test which bacterial OTUs were differentially abundant (adjusted p-value < 0.01) between time points and sampling sites. Overall effects of location and time point on the coral-associated bacterial communities were investigated using the adonis() function in the R-package *vegan* (Oksanen et al., 2018). Core microbiome analyses were performed on the non-rarefied OTU table to identify the core microbiome (i.e., OTUs present in 80–100% of the samples) for each coral species, as well as the microbes that are consistently present at each location (locally stable microbial associates, LSMA). Since many of the OTUs that were identified as part of the core microbiome, an LSMA and/or differentially abundant were also present in the seawater, we used *DESeq2* to assess which OTUs were more abundant in the seawater than the bacterial communities of the coral to identify potential transiently associated microbes, e.g., environmental microbes trapped in the coral mucus.

The PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) pipeline (Langille et al., 2013; Douglas et al., 2020) was used to identify OTUs putatively involved in the nitrogen and sulfur cycles. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG[2]) (Kanehisa and Goto, 2000), the relevant KEGG orthologs were selected (**Supplementary File S7-1**). OTUs that had a Nearest Sequenced Taxon Index (NSTI) value of <2 and that were

[2]https://www.genome.jp/kegg/

predicted by PICRUSt2 to contain at least one KEGG ortholog involved in the each step of a nutrient cycling process (Nitrogen Cycle: nitrogen fixation, nitrification/ammonium oxidation, nitrate reduction/ammonification and/or denitrification, Sulfur Cycle: DMSP demethylation or cleavage, sulfur oxidation and/or sulfate reduction) were selected. The nitrite-to-nitrate step in the nitrification process is, however, only performed by highly specialized microbes possessing the *nxrAB* genes, but KEGG orthologs K00370 and K00371 also contain the common *narGZHY* genes involved in denitrification and dissimilatory nitrate reduction. Therefore, we ensured that only taxa known to be capable of performing this step (e.g., *Nitrospina*, *Nitrospira*) were included. Differences in the relative abundances between locations and time points of functional groups overall were analyzed using a negative binomial generalized linear model using the R-package *MASS* (Venables and Ripley, 2002) followed by pairwise comparisons with the R-package *multcomp* (Hothorn et al., 2008).

RESULTS

Microbial Community Diversity

Beta diversity analyses (**Supplementary File S2**: **Supplementary Table S1**) showed that the prokaryotic communities of both the black coral *Antipathella subpinnata* and the gorgonian *Eunicella cavolini* were distinct from the surrounding seawater at each location and time point (all comparisons $p \leq 0.0036$, **Figure 3**).

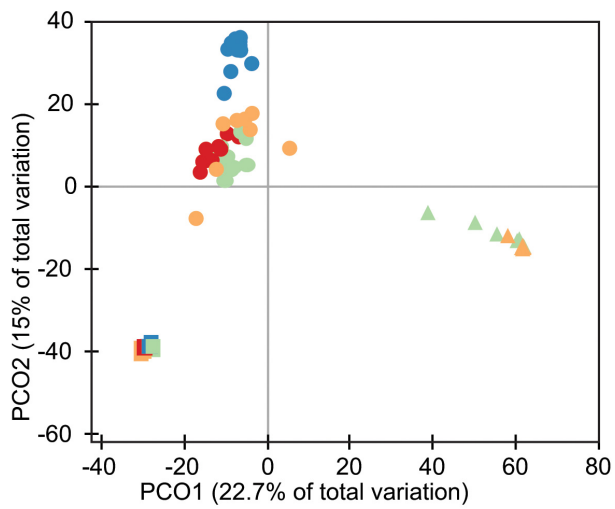

FIGURE 3 | Differences in the diversity of the *Antipathella subpinnata* and *Eunicella cavolini* microbiomes. Beta diversity of the microbiota of *A. subpinnata* and *E. cavolini* and the surrounding seawater is presented in a principal coordinates analysis (PCoA) ordination plot based on a Bray–Curtis similarity distance matrix.

However, these analyses also indicated that the prokaryotic community of *A. subpinnata* was different (I) between the three sampling locations (all comparisons $p < 0.0003$, **Supplementary Figure S1A**) as well as (II) between autumn and spring at the Portofino location ($p < 0.0005$, **Figures 1A,B**) and (III) compared with the sympatric *E. cavolini* at both time points (both comparisons $p \leq 0.0002$, **Figure 3**). In addition, the microbial communities of the seawater also showed spatial and temporal differences (spatial $p \leq 0.023$, temporal $p < 0.0068$, **Supplementary Figure S1C**). Contrastingly, no temporal differences were observed in the microbial community associated with *E. cavolini* ($p = 0.3079$, **Figure 3**).

Outcomes of the analyses of the three alpha diversity metrics richness (observed OTUs), evenness (Simpson's *E*), and diversity (Shannon-Wiener *H*) are provided in **Supplementary File S2**: **Supplementary Table S2–S3**. In the spring of 2017, no significant differences in evenness and diversity were observed between *A. subpinnata* and seawater, except at the Savona shoal ($p = 0.0001$). In addition, the prokaryotic communities of both *A. subpinnata* and the seawater showed significant spatial differences ($p < 0.02$), however, no difference was found in the microbiota of *A. subpinnata* collected near Portofino and Bordighera. At the Portofino location, significant differences in the richness of the microbiota were found between the coral species and seawater. A similar pattern was observed in microbiota diversity, with the exception that the diversity in the seawater and in the *A. subpinnata*-associated microbiota was similar. No temporal differences were observed in any of the alpha diversity metrics.

Prokaryotic Community Composition and 'Core Microbiome'

Overall, we observed 7813 different bacterial and 73 archaeal OTUs associated with *A. subpinnata*. In contrast, 343 bacterial OTUs and no archaeal OTUs were found in the microbiota of *E. cavolini*. Archaea were relatively low abundant in the microbiota of *A. subpinnata* in comparison with bacteria, representing only 0.25% of the prokaryotic community associated with this black coral near Portofino, 0.17% near Bordighera and 0.02% near Savona.

Clear differences in the prokaryotic community composition between *A. subpinnata* and *E. cavolini* were indeed observed (**Figure 4**). The bacterial community of *A. subpinnata* was composed of a range of Proteobacteria (particularly Gamma-, Alpha- and Deltaproteobacteria), Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes and Verrucomicrobia (**Figure 4**). Differences in the relative abundances of these taxa existed between locations and time points (**Figure 4**, discussed below), but also among individual colonies within the different locations (**Supplementary Figure S2**). No 'core microbiome' (i.e., those microbes that are ubiquitous and consistently present within the microbiota of a species, regardless of space and time) could be determined for *A. subpinnata*. However, we identified 94 OTUs that were present in at least 80% of the samples at one of the locations. Twenty-one of those OTUs were significantly more abundant in seawater and belonged to known

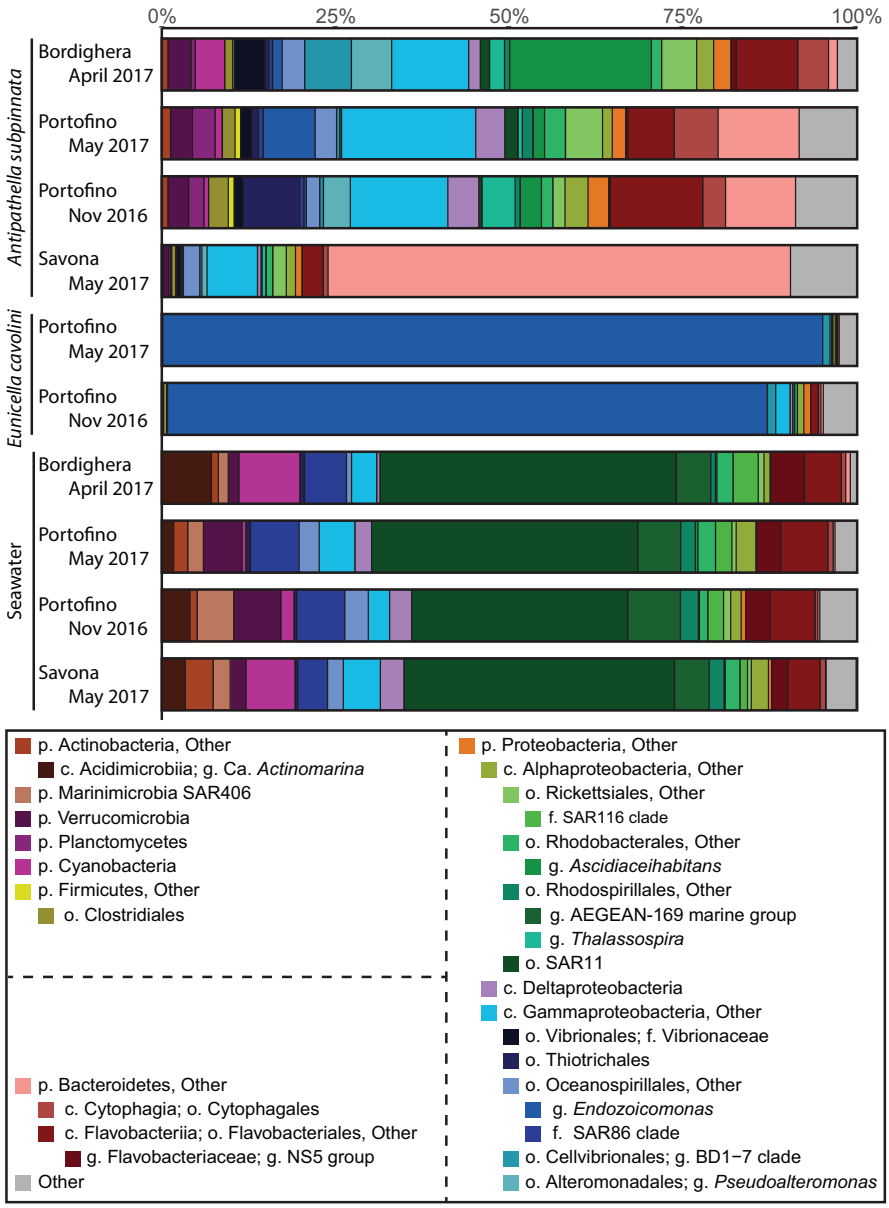

FIGURE 4 | Overview of the composition of the bacterial community associated with the black coral *Antipathella subpinnata* and in the seawater at three locations (Bordighera, Portofino and Savona), and at two time points (spring and autumn) at the Portofino location. The composition of the microbiota of the gorgonian *Eunicella cavolini*, living sympatrically with *A. subpinnata* at Portofino, is also provided. The composition of the microbial communities is presented at the various taxonomic levels (p, phylum; c, class; o, order; f, family; g, genus). The contribution of each taxon is indicated in percentages (%). Taxa with an abundance of <1% were merged into its corresponding higher taxonomic level. Higher level taxa indicated with 'Other' (e.g., p. Proteobacteria, Other) include all subtaxa not specified below. Other (gray) contains all phyla with an abundance of <1%.

bacterioplankton taxa (e.g., SAR11, SAR116, AEGEAN-169 and Flavobacteriaceae NS marine groups, **Supplementary Figure S3**) and were therefore considered transient associates. Of the remaining 73 locally stable microbial associates (LSMA) of *A. subpinnata* 54 OTUs were significantly more abundant in *A. subpinnata* than seawater (**Figure 5**), while for 19 OTUs no difference was found (**Supplementary Figure S3**). The relative abundance of these LSMAs within the bacterial communities of *A. subpinnata* differed between on average 29 and 82% depending on the location and time point (**Supplementary File S3**). LSMAs belonged primarily to the main taxa identified (relative abundance > 1%, **Figure 4** and **Supplementary File S4**), particularly Rhodobacteraceae, *Thalassospira*, Rickettsiales, *Pseudoalteromonas*, Cellvibrionales BD1-7, *Vibrio*, Thiotrichales and various taxa within the phylum Bacteroidetes. An archaeon of the order Thermoplasmatales (OTU1066) was also found commonly associated with *A. subpinnata* (relative abundance ∼0.05%) from the Bordighera (9/10 samples) and Portofino

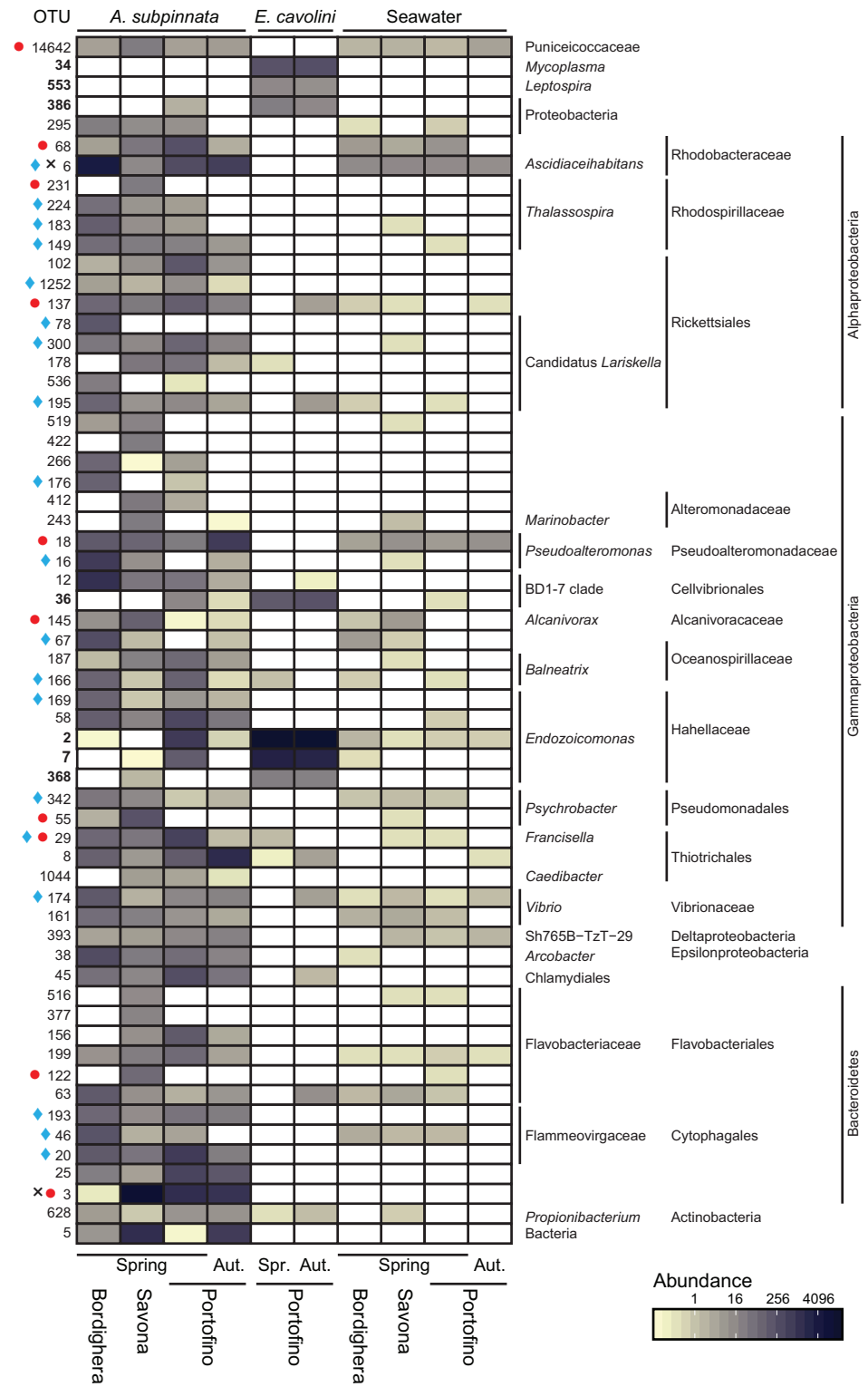

FIGURE 5 | Core and locally stable microbial associates of *Antipathella subpinnata* and *Eunicella cavolini* and their presence in the surrounding seawater. Heatmap presents the abundance of OTUs considered as part of the core microbiome (present in all samples of a species) and locally stable microbial associates (LSMA, present in at least 80% of the samples from one location). OTUs indicated in bold are core microbes of *E. cavolini*. LSMA OTUs indicated with a symbol are present in all samples of *A. subpinnata* at a given location: Bordighera (♦), Savona (●) and Portofino (**×**) in spring (Spr., April/May 2017) and autumn (Aut., November 2016). The taxonomy of each OTU is given at lowest possible taxonomic level. OTUs presented have a higher relative abundance in *A. subpinnata* than seawater. See **Supplementary Figure S3** for OTUs with equal or lower relative abundance in *A. subpinnata* than seawater.

(9/10 and 5/10 samples in November and May, respectively) populations, but it was absent in the Savona population (**Supplementary Figure S3**).

The bacterial communities associated with *E. cavolini* were dominated by bacteria from the genus *Endozoicomonas* and to a lesser extent the Cellvibrionales Clade BD1-7 (**Figure 4**). OTUs belonging to these taxa were the major constituents of the *E. cavolini* core microbiome, which consisted of 7 OTUs that represented 89–97% of the associated bacterial community. OTUs identified as *Leptospira*, *Mycoplasma* and an unidentified Proteobacterium were also part of the core microbiome of this gorgonian (**Figure 5** and **Supplementary Files S2, S3**).

Spatial Differences in the Black Coral-Associated Bacterial Communities

Using differential abundance analysis (**Supplementary Files S5A–C**), 101 OTUs were found to be primarily responsible for the spatial differences in the prokaryotic communities of *A. subpinnata*. However, 9 of these OTUs were more abundant in the surrounding seawater and were therefore likely environmental microbes trapped in the coral mucus (**Supplementary Figure S4**). Nearly half of the remaining OTUs matched the LSMA criterion (22 OTUs were present in at least 80% of samples at one location and 19 OTUs were present in at least 100% of the samples at one location) (**Figure 6** and **Supplementary Figure S3**). The spatial differences observed in the diversity of the bacterial communities of *A. subpinnata* in May 2017 (**Supplementary Figure S1A**) could be discerned in the community composition (**Figure 4**) and attributed primarily to changes in the abundances of OTUs belonging to the main phyla (**Figure 6** and **Supplementary Figure S4**).

The microbial communities associated with *A. subpinnata* at Savona were highly distinct from those at Portofino and Bordighera (**Figure 4**). This was primarily driven by the high relative abundance of Bacteroidetes LSMA OTU3 and the unknown Bacteria OTU5, which represented respectively ∼66 and 7.5% of the bacterial communities at Savona. Contrastingly, these OTUs were significantly lower or nearly absent in the Bordighera (0.0006 and 0.05%) and Portofino (7.1% and 0%) populations (**Figure 6** and **Supplementary File S3**). Consequently, the relative abundances of nearly all other taxa were lower in comparison with Portofino and Bordighera (**Figure 4**). The exceptions identified included several Flavobacteriales OTUs that were only present in Savona and higher abundances of the Gammaproteobacteria *Psychrobacter* OTU55 and Alteromonadaceae (**Figure 6** and **Supplementary Figure S4**). We also observed significantly higher numbers of the hydrocarbon-degrading gammaproteobacterial genera *Alcanivorax*, *Oleiphilus,* and *Marinobacter* in the Savona population (**Supplementary Figure S4**). These genera represented 1.90% of the bacterial community compared with 0.03 and 0.2% in Portofino and Bordighera, respectively.

The *A. subpinnata*-associated prokaryotic communities at Bordighera and Portofino in May 2017 were more similar to each other than at Savona (**Supplementary Figure S1A** and **Figure 4**), but compositional differences were still observed. Most clearly visible were the significantly higher abundances of *Ascidiaceihabitans*, *Pseudoalteromonas*, *Colwellia*, Vibrionaceae and numerous Bacteroidetes phylotypes at Bordighera (**Figures 4**, **6** and **Supplementary Figure S4**). On the other hand, the Portofino *A. subpinnata* colonies harboured significantly higher levels of Planctomycetes, Thiotrichales, Bacteroidetes (OTU25 and Cytophagales OTU20), Chlamydiales OTU45, Rickettsiaceae OTU102, *Mycoplasma* and *Endozoicomonas* (**Figure 6** and **Supplementary Figure S4**). Two of the three *Endozoicomonas* OTUs (OTU2 and 7) were the main core bacterial symbionts of the sympatric gorgonian *E. cavolini* (**Figure 5** and **Supplementary Figure S4**) and were exclusively found in *A. subpinnata* at Portofino.

In addition, we found differences in the OTUs belonging to specific genera present on *A. subpinnata* at the different sampling locations. In the case of the Rhodospirillales genus *Thalassospira*, for example, OTU13 was present in the Bordighera and Portofino populations but absent from the Savona population, whereas OTU231 was dominant in *A. subpinnata* near Savona but absent at the other locations (**Figure 6**). Similarly, OTU78 was a Rickettsiales *Ca. Lariskella* phylotype only found in Bordighera, while OTU178 was present only in Savona and Portofino (**Figure 6**). A third example concerned the genus *Rubritalea* of the Verrucomicrobia: OTU155 and OTU361 were observed in the microbiota of *A. subpinnata* near Bordighera, but OTU53 and OTU695 were detected in the Portofino and Savona populations (**Figure 6**). Although these patterns were obvious, the relevance of these spatial differences within particular genera for holobiont functioning and acclimation remains unclear.

Temporal Differences in the Black Coral-Associated Bacterial Communities

At the Portofino location, we also observed temporal changes in the diversity of the microbial community associated with *A. subpinnata* (**Supplementary Figure S1B**). Samples collected in November 2016 contained higher levels of Flavobacteriales, Rhodospirillales (genus *Thalassospira*), Planctomycetes, Alteromonadales and Thiotrichales compared with May 2017, but lower levels of *Endozoicomonas* and Rickettsiales bacteria (**Figure 4**). These results were confirmed by differential abundance analysis, identifying 129 OTUs (incl. 18 that likely belong to the bacterioplankton) that were differentially abundant between time points (**Figure 6**, **Supplementary Figure S4**, and **Supplementary File S5D**). Of these OTUs, 65 were only present in the *A. subpinnata* microbiota in November and 23 OTUs were exclusive to May, explaining the significant differences observed in the diversity of these bacterial communities. It should also be noted that we cannot exclude the possibility that the microbiota of individual colonies are stable over time but differ between neighboring colonies (**Supplementary Figure S2**), as we did not sample the same colonies at both time points. In contrast, no OTUs were found differentially abundant in *E. cavolini* between those two time points (**Supplementary File S5E**).

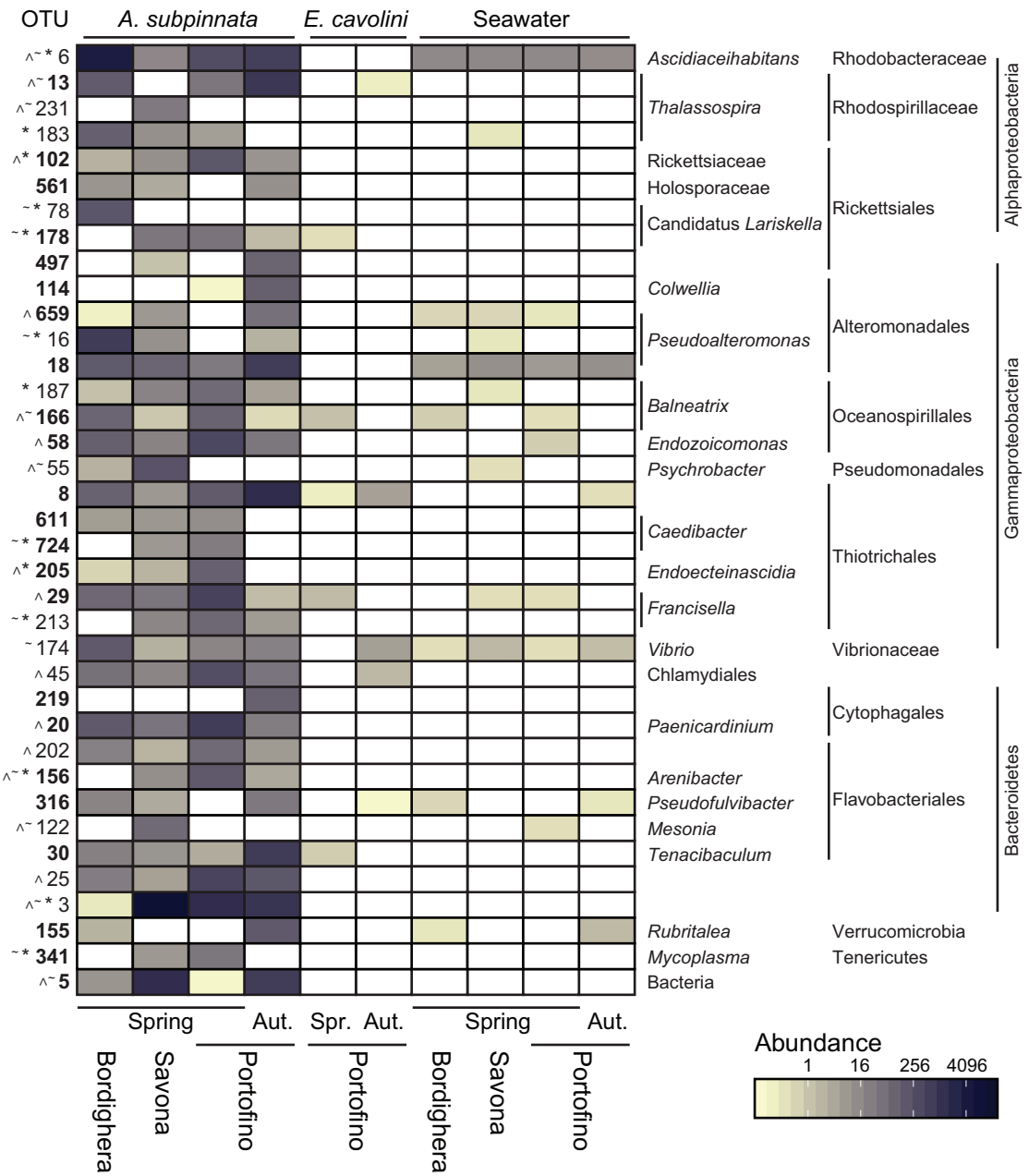

FIGURE 6 | Microbes associated with *Antipathella subpinnata* that show differential abundance between locations or time points. Heatmap presents the abundance of OTUs found differentially abundant between locations (Bordighera, Savona and Portofino) in the spring (Spr., April/May 2017) and/or between spring and autumn (Aut., November 2016) at the Portofino location. Abundance of these OTUs in the microbiota of *E. cavolini* and seawater is also provided. The taxonomy of each OTU is given at the lowest possible taxonomic level. Symbols indicate OTUs showing spatial differences (~ Bordighera versus Savona, * Bordighera versus Portofino, ^ Savona versus Portofino). OTUs in **bold** were found differentially abundant between time points. OTUs presented have a higher relative abundance in *A. subpinnata* than seawater. See **Supplementary Figure S4** for OTUs with equal or lower relative abundance in *A. subpinnata* than seawater.

No Relationship Between Differences in the Bacterioplankton and Microbiota of *A. subpinnata*

As significant spatial and temporal differences were also observed in the seawater, we hypothesized that seawater microbes trapped in the *A. subpinnata* mucus may be primarily responsible for the differences observed. However, we found that only a small portion of the OTUs differentially abundant in the coral microbiota (spatial: 7–18%, temporal 25%) were also differentially abundant in the seawater (**Supplementary File S6**). In addition, we found that some of these overlapping OTUs had contrasting abundance patterns. For example, OTU33 and OTU41 were significantly lower in the *A. subpinnata*-associated

bacterial communities in Portofino than in Savona in May 2017, however, their abundance in the seawater was higher in Portofino than in Savona (**Supplementary Figure S3**). Besides, several OTUs that were differentially abundant in both the seawater and coral microbiota had an overall higher abundance in corals than seawater (**Supplementary Figure S3**), suggesting that these may thus be coral symbionts rather than trapped environmental microbes.

Predicted Microbiome Functionality: Nutrient Cycles in the *A. subpinnata* Holobiont

PICRUSt2 analysis identified a number of taxa involved in one or multiple steps of the nitrogen (**Figure 7**, nitrogen fixation, nitrification/ammonium oxidation, nitrate reduction/ammonification and denitrification) and/or sulfur (**Figure 8**, DMSP demethylation or cleavage, sulfur oxidation, sulfate reduction) cycles (**Supplementary File S7**). Microbes likely involved in the different steps of these two nutrient cycles were present in the *A. subpinnata* holobiont at each location and at both time points at the Portofino site (**Figures 7**, **8**). However, significant temporal and spatial differences in the relative abundances of the assigned functional groups and their members were observed (statistical outcomes: **Supplementary File S8**). The relative abundances of all functional groups were significantly lower in the Savona population compared with the other locations (**Figures 7**, **8**, all comparisons $p < 0.02$, **Supplementary File S7**). This was likely related to the high relative abundance of Bacteroidetes at this location (**Figure 4**), resulting in generally low relative abundances of all other taxa, including those involved in the nitrogen and sulfur cycles. Differences in functional groups were also observed between the Bordighera and Portofino populations, with *A. subpinnata* near Bordighera harboring higher levels of potential denitrifying (**Figure 7D**, $p = 0.0001$), DMSP-demethylating ($p = 1^{*}10–9$, **Figure 8A**) and sulfide-oxidizing ($p = 1^{*}10–10$, **Figure 8D**) bacteria, especially Rhodobacterales. Temporal differences could only be discerned in the relative abundances of nitrite- and nitrate-reducing bacteria ($p = 0.0008$), which may have been particularly related to the higher relative abundances of *Endozoicomonas* in May compared with November (**Figure 7C**).

DISCUSSION

We present one of the first in-depth assessments of the bacterial communities associated with black corals, which provide crucial forest-like structural habitat in the mesophotic and deep sea, by profiling its associated microbial community on both spatial and temporal scales. Our findings show that *Antipathella subpinnata* possesses a microbiome distinct from the surrounding seawater as well as from a sympatric arborescent gorgonian octocoral. The potential lack of a true core microbiome and the significant differences in bacterial communities between locations and sampling time points suggest that the composition of this holobiont is highly influenced by local environmental conditions. Here, we discuss (1) the composition of the black coral microbiota in the context of coral microbial ecology, (2) the putative functions of the prokaryotes associated with *A. subpinnata*, (3) the potential causes of the high variability in the microbiota of this black coral, and (4) the implications that the lack of a 'core microbiome' may have for this coral species as well as for the field of coral microbial ecology in general.

Microbiome of *Antipathella subpinnata*

Our study shows that the microbiome of the black coral *A. subpinnata* was dominated by Proteobacteria (particularly Gamma-, Alpha-, and Deltaproteobacteria), Bacteroidetes and to a lesser extent by Firmicutes, Cyanobacteria, Planctomycetes, and Verrucomicrobia. When placing these results in context with the five other studies on black coral-associated microbes published to date (Penn et al., 2006; Santiago-Vázquez et al., 2007; Zhang et al., 2012; Dannenberg, 2016; Liu et al., 2018), some interesting patterns emerge: namely, the bacterial communities (classified at the genus level and higher) may be relatively conserved across black coral species. For example, *Leiopathes glaberrima*, a deep-sea species with a wide distribution (Caribbean, Pacific, and Mediterranean), possesses an associated microbial community that is composed mostly of Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (Dannenberg, 2016). In addition, initial culture-based techniques (Santiago-Vázquez et al., 2007; Zhang et al., 2012) isolated and identified various bacteria from black corals that belong to taxa which are now found widespread in the microbiota of black corals based on culture-independent techniques (here in *A. subpinnata* and Dannenberg, 2016; Liu et al., 2018). These taxa include Firmicutes (e.g., *Bacillus*), Actinobacteria (e.g., *Propionibacterium*) and Gammaproteobacteria (e.g., *Acinetobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Psychrobacter*, *Vibrio*). This also suggests that many of the bacterial symbionts of black corals may be amenable to cultivation, allowing more detailed studies of their function.

The bacterial communities of *A. subpinnata* and other black corals were also found to share significantly more characteristics of the microbial communities associated with other Hexacorallia than with Octocorallia. The holobiont of Mediterranean *A. subpinnata* showed similarities with the Mediterranean corals *Oculina patagonica* and *Cladocora ceaspitosa*. These shallow scleractinian corals also harbor relatively high levels of Bacteroidetes and Alpha-, Delta- and Gammaproteobacteria (∼60% of the overall community) and lower levels of Verrucomicrobia, Planctomycetes, Cyanobacteria and Firmicutes (Rubio-Portillo et al., 2018b; Bednarz et al., 2019). Although at relatively lower abundances, bacteria from these taxa are also commonly found in sea anemones (Brown et al., 2017; Herrera et al., 2017), tropical scleractinian corals (Sunagawa et al., 2010; Godwin et al., 2012; Morrow et al., 2012) and numerous deep-sea coral species (Kellogg et al., 2009, 2016, 2017; Gray et al., 2011; Lawler et al., 2016; Kellogg, 2019). Contrastingly, the *A. subpinnata* prokaryotic community composition did not show much similarity with the bacterial communities of the gorgonian *Eunicella cavolini*, despite living sympatrically in the same location, and having a similar arborescent colony structure and ecological function.

In conclusion, we show that, despite the lack of a 'core microbiome' at the OTU level, the *A. subpinnata*-associated prokaryotic community is primarily composed of a limited number of bacterial taxa. Most of these higher level taxa have also been found in the microbial community of other black coral species. These similarities suggest that black corals may have a relatively conserved bacterial community at the taxonomic genus level and up.

Putative Functions of *Antipathella subpinnata*-Associated Prokaryotes

Although many studies have addressed the composition of the coral-associated microbial community under natural as well as experimental conditions, still very little is known about the functions of these bacteria. As such, it is difficult to understand the role of the microbes within the *A. subpinnata* holobiont. Based on functional and genomic studies on bacteria with closely related 16S rRNA gene sequences, it might be possible to infer the role of coral-associated microbes, which may provide some insights into their niche within the holobiont. However, it is important to remain cautious when inferring microbial functions, as lateral gene transfer among bacteria and mutations may have altered a microbe's catabolic and anabolic capacities and behavior, compared with its taxonomically close relatives.

The majority of the dominant bacterial taxa found in the prokaryotic communities of *A. subpinnata* belong to taxa which are commonly found within the holobiont of benthic marine invertebrates, including corals. Some of these microbes may have a role in nutrient cycling, which would be of high importance to the health and nutritional status of the coral holobiont. For example, bacteria in the phylum Bacteroidetes play an important role in organic carbon cycling in the marine environment (Thomas et al., 2011; Fernández-Gómez et al., 2013). They are particularly recognized for their capacity to break down complex organic molecules, including chitin. As such, these bacteria may

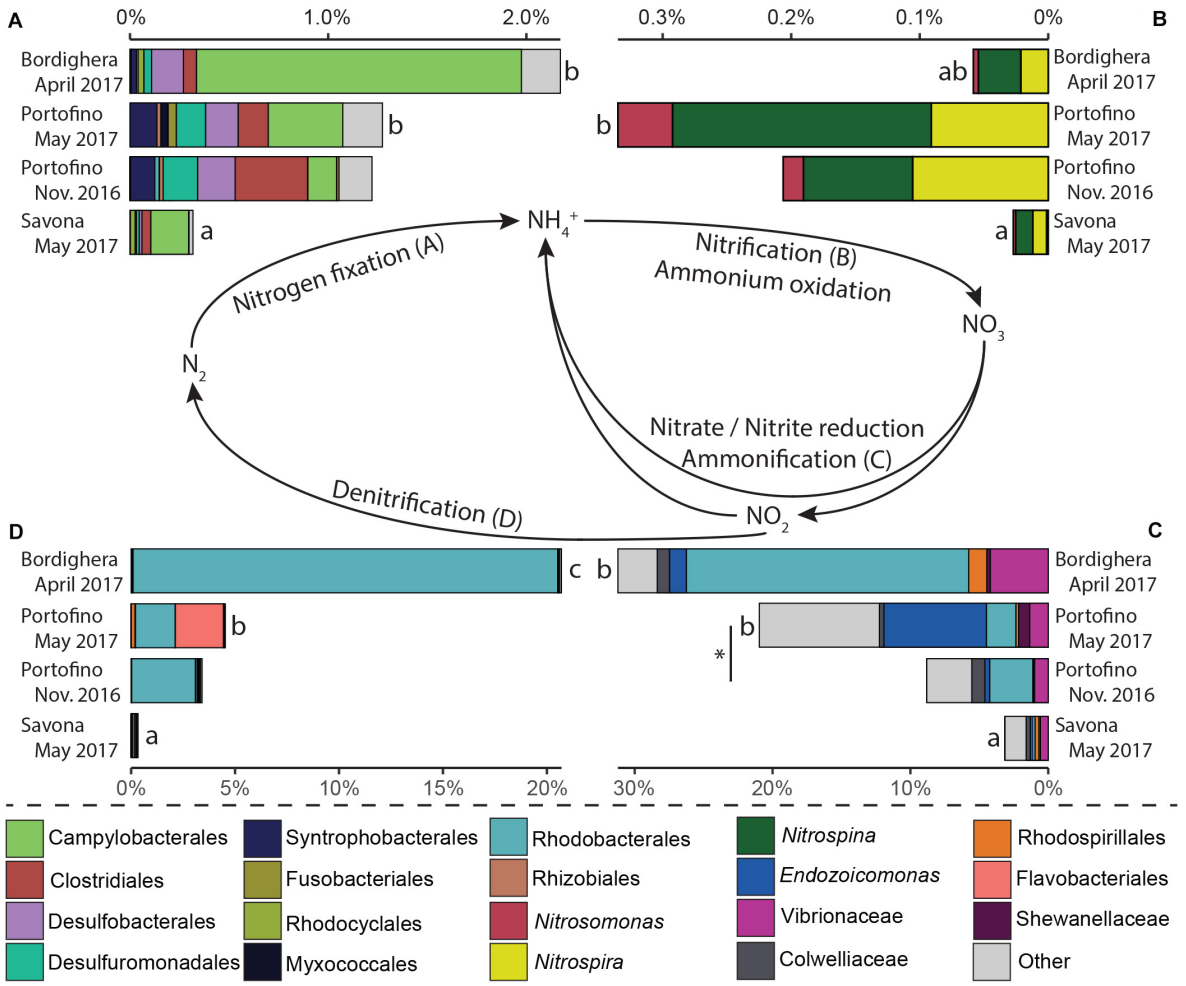

FIGURE 7 | Relative abundance of *Antipathella subpinnata*-associated bacteria with a putative role in the nitrogen cycle. The nitrogen cycle consists of four main processes and microbes putatively involved in these processes are presented: **(A)** nitrogen fixation, **(B)** nitrification/ammonium oxidation, **(C)** nitrate/nitrite reduction/ammonification and **(D)** denitrification. Differences between locations are indicated with letters, differences between autumn and spring in the Portofino population are indicated with an asterisk (*). Taxa with low abundances are combined and indicated in light gray.

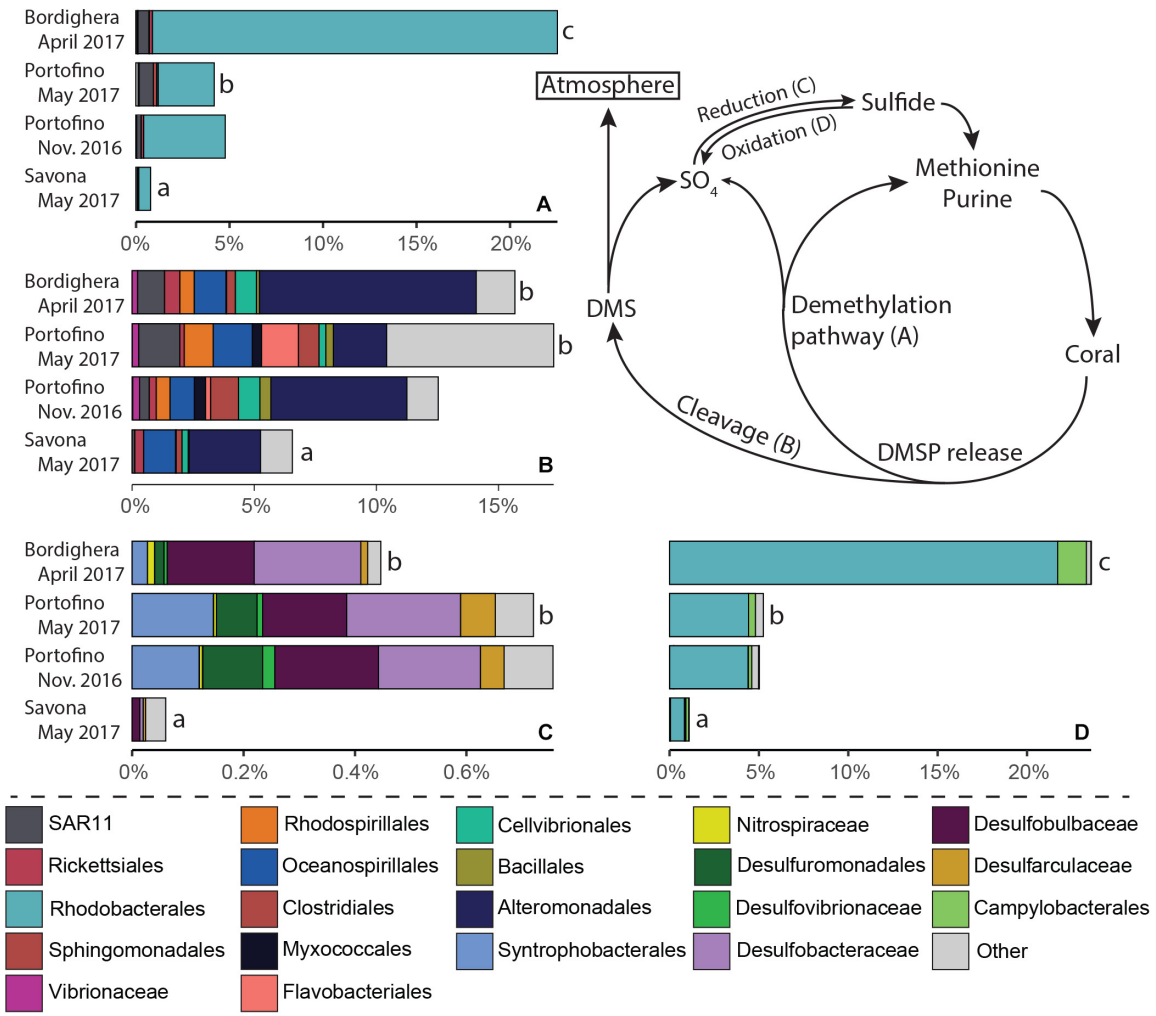

FIGURE 8 | Relative abundance of *Antipathella subpinnata*-associated bacteria with a putative role in the sulfur cycle. The sulfur cycle consists of four main processes and microbes putatively involved in these processes are presented: **(A)** DMSP demethylation, **(B)** DMSP cleavage, **(C)** sulfate reduction and **(D)** sulfur oxidation. Differences in the abundance of functional groups between locations are indicated with letters. Taxa with low abundances are combined and indicated in light gray.

aid the coral host with the digestion of captured prey. This may also be the function of the relatives of *Vibrio gigantis* that we found in *A. subpinnata*, as *V. gigantis* is a mutualist in shellfish (Le Roux et al., 2005) and sea cucumbers (Beleneva and Kukhlevskii, 2010) that aids in the host's food digestion using a broad spectrum of enzymes, including chitinases. However, as the skeletons of black corals have generally a high (∼10–15%) chitin content (Goldberg et al., 1994; Bo et al., 2012a), these bacteria may potentially also use the chitin produced by the coral as a carbon and nitrogen source.

One of the best studied coral-associated bacterial taxa, *Endozoicomonas*, was also commonly present in the *A. subpinnata* and *L. glaberrima* (Dannenberg, 2016) holobionts, although at relatively low abundances. Contrastingly, it is the main symbiont of *E. cavolini* (Bayer et al., 2013; van de Water et al., 2017, 2018b). Genome analyses have indicated that *Endozoicomonas* may provide its host with a variety of amino acids, which are synthesized using ammonium and sulfur acquired through its involvement in nutrient cycling processes of nitrogen (nitrate reduction and ammonification) and sulfur (DMSP metabolism) (Neave et al., 2016). This led us to investigate whether other bacteria involved in the (re)cycling of essential nutrients (nitrogen, sulfur, and phosphorus) were present in the microbiota of *A. subpinnata*. Overall, we found bacteria putatively involved in all steps of the nitrogen cycle in the black coral-associated microbial community (**Figure 7**). The presence of these bacteria may indicate that the *A. subpinnata*-associated bacterial communities are able to acquire (via nitrogen fixation) and retain (via nitrification and ammonification) nitrogen within the holobiont. Nitrogen fixation has been shown in the deep-sea coral *L. pertusa* (Middelburg et al., 2015) and a complete nitrogen cycle may be present in the microbiota of several deep-sea octocoral species (Kellogg et al., 2016; Lawler et al., 2016). However, the relatively high levels of potential

denitrifying bacteria in comparison with nitrogen-fixing bacteria in the microbiota of *A. subpinnata* could indicate that there might be a net loss of nitrogen, requiring the holobiont to obtain nitrogen from exogenous sources, such as predation on plankton (Coppari et al., 2020).

Phosphorus is generally a limiting nutrient for organismal growth, particularly in the marine environment and efficient (re)cycling is therefore of high importance for a holobiont. In addition to the uptake of inorganic phosphate, phosphonates are another main source of phosphorus in the marine environment. Although primarily a microbial process, some marine invertebrates (incl. corals and anemones) possess genes representing the complete pathway for phosphonate synthesis (Shoguchi et al., 2013) and contain high levels in their tissues (Henderson et al., 1972; Garrett et al., 2013; Godinot et al., 2016). In two soft corals, microbes that are able to degrade the highly stable C-P bonds of phosphonates have previously been found, including *Thalassospira*, *Vibrio*, *Pseudoalteromonas*, *Psychrobacter* and Bacteroidetes (Thomas et al., 2009). These taxa were also commonly found in the microbiota of *A. subpinnata*, indicating that phosphorus cycling likely exists within the black coral holobiont, thereby limiting the loss and facilitating the acquisition of this crucial nutrient.

We also found several microbial taxa linked to the degradation of dimethylsulfoniopropionate (DMSP), a sulfur compound shown to be produced at high levels by coral animals (Raina et al., 2013). The sulfur contained in DMSP can be recycled by a large number of bacterial taxa using the demethylation pathway (**Figure 8**), and is then used in the synthesis of purines and the essential amino acid methionine. The other DMSP degradation pathway leads to the cleavage of DMSP into DMS by bacteria (**Figure 8**). Although sulfur may be lost after oxidation by bacteria in the form of sulfate in both pathways, sulphite- and sulfate-reducing bacteria (**Figure 8**) can reduce this and environmental sulfate into bioavailable sulfide for incorporation into biomolecules. The common presence of DMSP-metabolizing and sulfate-reducing bacteria in the microbiota of *A. subpinnata* shows that sulfur cycling likely takes place within the black coral holobiont. Although the metabolic activities of these microbes are unknown, the relatively high levels of sulfide-oxidizing bacteria in comparison with sulfate-reducing bacteria may indicate a net loss of sulfur.

DMSP has also been implicated in the structuring of the microbiota of corals (Frade et al., 2016), and may be used by coral-associated microbes to produce antimicrobial compounds capable of eliminating coral pathogens (Raina et al., 2016). This shows the importance of some bacteria in microbial community regulation of the coral holobiont. This might also be important in *A. subpinnata* as *Vibrio* bacteria were often present at low abundance in its microbiota. These bacteria are commonly found in corals, particularly in the surface mucus layers (Bourne and Munn, 2005; Chimetto et al., 2009; Raina et al., 2009; Porporato et al., 2013; Ainsworth et al., 2015), where they are considered to be commensal or opportunistic pathogens because of their implication in various coral diseases (Ben-Haim et al., 2003; Ushijima et al., 2012; Munn, 2015; Rubio-Portillo et al., 2018a). Some *A. subpinnata*-associated bacteria, for example *Pseudoalteromonas*, may have a microbiome regulatory role. These bacteria are often found in association with marine animals, including cnidarians, and are known to secrete compounds with antibacterial (Holmström and Kjelleberg, 1999; Shnit-Orland et al., 2012; Richards et al., 2017), antifungal (Shiroyama et al., 2017) and alginolytic (Holmström and Kjelleberg, 1999) activities. Besides, *Endozoicomonas* (Neave et al., 2016) and Actinobacteria (phylum including *Propionibacterium*) (Ritchie, 2006; Nithyanand et al., 2011; Krediet et al., 2013; Zhang et al., 2013) have been implicated in coral microbiome regulation.

Overall, it appears that each step in the cycling of nutrients may be performed by a number of different bacterial taxa present in the *A. subpinnata* holobiont. This suggests that functional redundancy may be present within the *A. subpinnata* microbiota. However, it also implies that, rather than having a true 'core microbiome,' this black coral holobiont may ensure the presence of important core functions within its microbiome, with a potential microbiome regulatory role for some of its common microbial associates.

Potential Causes of Variability in the *Antipathella subpinnata* Microbiota

The microbial communities associated with *A. subpinnata* were found to be distinct at each of the three locations and different between the spring and autumn. As all colonies were visually healthy at the time of collection, a cause for these differences is difficult to identify. However, it was unlikely to be related to the trapping of bacterioplankton in the large amounts of mucus produced by this black coral (Bo et al., 2008), because (1) only a minor fraction of the differentially abundant OTUs in the coral-associated bacterial communities was also differentially abundant in the seawater, and (2) the relative abundance of some OTUs was higher in the coral than seawater and these may thus have been released through shedding – a known mechanism of corals to regulate their microbiome (Garren and Azam, 2012).

The genetic structure of the black coral populations, and thus host genotype, may be a driver of the spatial differences observed in the prokaryotic communities associated with *A. subpinnata*. Possible links between host genotype and microbiome were recently found in two deep-sea octocoral species from the genus *Primnoa* (Goldsmith et al., 2018) and spatial differences in the microbiota were also observed in two Caribbean populations of *L. glaberrima* (Dannenberg, 2016). However, preliminary analyses have revealed that coastal *A. subpinnata* populations on the western coast of Italy are highly connected (Costantini et al., 2019). This suggests that genotype has a limited role, and that the *A. subpinnata*-associated microbial communities were likely influenced by local environmental conditions.

Increased seawater temperature (Bourne et al., 2007; Ziegler et al., 2017; Pootakham et al., 2018) and anthropogenic stressors (van de Water et al., 2017, 2018b), including pollution (Ziegler et al., 2015; Leite et al., 2018), have previously been linked to shifts in the coral microbiome. But in our study, the seawater temperatures at the time of sampling were nearly the same at

all locations and the shoals are not exposed to heavy river run-off pollution due to their relative distance from shore and depth. We did, however, observe a significantly higher abundance of bacteria that are capable of degrading or exclusively feeding on hydrocarbons in the microbiota of *A. subpinnata* of the Savona population, such as *Marinobacter*, *Alcanivorax,* and *Oleiphilus*. This may be linked to nearby activities, as the Port of Savona (incl. Vado Ligure) handles petroleum products, harbors a large fleet of commercial fishing vessels and is frequented by cruise and cargo ships, whereas the ports of the other two localities are primarily used for yachting. It is tempting to speculate that these microbes may convert these hydrocarbons into a bioavailable carbon source for the coral host as has been previously hypothesized for deep-sea corals from the Red Sea (Röthig et al., 2017). While this may partially explain the highly different *A. subpinnata*-associated bacterial communities near Savona, this does not account for all spatial and temporal differences observed.

The local conditions at each of our sampling sites were likely somewhat different in terms of silting levels, hydrodynamism and upwellings due to nearby canyons. In addition, modeling of the coastal currents in the Ligurian coastal region has shown that coastal mesoscale eddies are common and long-lived (over a month) (Casella et al., 2011). Particularly, the anti-cyclonic eddies between the coast and the main Ligurian-Provencal-Catalan current induce strong upwelling on their coastal side and are an important source of nutrients for ecosystems in the upper sea layer (down to 200 m depth) (Casella et al., 2011). Unfortunately, we were unable to verify whether such eddies were present at the time of sampling at any of the locations or how much time had passed since the last upwelling, and no data on nutrient levels at these locations are available. Regardless, our best explanation of our results is that the observed differences in the bacterial communities of *A. subpinnata* are due to the different local environmental conditions that characterize the sites and with a potential role of anti-cyclonic vortices.

Environmental conditions may also impact the main food source of the filter-feeding *A. subpinnata*: plankton. It was demonstrated recently that *A. subpinnata* in the Portofino area feeds primarily on mesozooplankton in spring, but nano- and picoplankton (incl. the bacterioplankton) in autumn (Coppari et al., 2020), which coincides with the temporal differences observed in the microbiota. Changes in food sources are known to affect the nutritional status and the microbiomes of organisms. Although a definitive link has not been established here, it is tempting to speculate that the diet of this black coral influences its microbiome and thereby potentially its function. The observed differences in the abundance of microbes involved in various steps of the nitrogen and sulfur cycles may reflect changes in the coral's nutritional status and fitness. However, it could also be indicative of a change in the nutritional needs of the holobiont and an adjustment of the microbiome to fulfill these needs to improve host fitness. To address this, further studies linking coral physiology with the activity of host-associated bacteria will be required along with investigations into the black coral holobiont's capacity to actively regulate its microbiome.

Microbiome Flexibility in Black Corals and the Absence of a Bacterial 'Core Microbiome'

Black corals are one of the longest lived animals known, with a reported colony age of up to 4,265 years for *L. glaberrima* (Roark et al., 2009). As they are sessile animals, it is imperative for their survival to employ strategies that allow them to acclimate rapidly in response to environmental change. Actively changing the microbiota may represent one such mechanism. Given the significant differences in microbiota observed on spatial and temporal scales in healthy populations of *A. subpinnata*, our results indicate that the holobiont of this coral has indeed a high degree of microbiome flexibility. The degree of flexibility in holobiont structure and composition may, however, differ between species as demonstrated by Ziegler et al. (2019). They showed that the microbiota of the tropical reef-building hexacoral *Acropora hemprichii* responds to different levels of anthropogenic impacts and can even recover when transplanted to non-impacted sites. In contrast, they found that *Pocillopora verrucosa*'s microbiota remains stable regardless of the level of impact. Based on these differences in microbiome flexibility, the authors referred to corals that show microbial adaptation to the surrounding environment as "microbiome conformers," and to corals that maintain a constant microbiome as "microbiome regulators" (Ziegler et al., 2019). While these terms provide guidance, most holobionts can likely not be strictly assigned to one group, and a spectrum of microbiome flexibility where the microbiome "regulators" and "conformers" are on either end of the scale should be considered. For example, *E. cavolini* possesses a microbiota dominated by its core microbiome, which shows little temporal differences but some spatial differences as seen here and previously (van de Water et al., 2017, 2018b). This suggests that this gorgonian is in the 'microbiome regulator' spectrum, as its microbiome is relatively stable but exhibits some variation, mostly in the abundances of core microbes. We also observed stability in the structural complexity of the microbiome of *E. cavolini* (based on alpha diversity metrics), which has been linked to tolerance of another coral in the 'microbiome regulator' spectrum to environmental change (Röthig et al., 2020). As all colonies of *A. subpinnata* sampled were visually healthy, the differences in the microbiota among populations and time points likely did not reflect an unhealthy state of the holobiont or a pathobiome. This black coral also did not completely alter its microbiota as numerous microbes were present at the three sampling locations. Instead, it is more likely that *A. subpinnata* is on the "microbiome conformer" side of the scale, adjusting its microbiota by selecting for the most beneficial microbial community depending on the environment (Reshef et al., 2006; Rosenberg et al., 2007) allowing it to cope with change. Consequently, the structural complexity of *A. subpinnata*'s microbiota was also significantly impacted, particularly near Savona.

However, it should be noted that 90–95% of the prokaryotic community of *A. subpinnata* was still composed of the main microbial taxa (those with an abundance $> 1\%$) at all locations. This suggests that some degree of fidelity still exists within the

composition of the holobiont, but not at the 97% phylotype/OTU level. Instead, black corals may rely on a 'functional core' (i.e., a complement of metabolic and other molecular functions that are performed by the microbiome but are not necessarily provided by the same organisms, Shafquat et al., 2014). This is in line with our results on the stable presence of microbes involved in nutrient cycling. Also Jaspers et al. (2019) determined that intimate associations between a host and its microbiota may not be a *sensu stricto* criterion for functional relevance as the composition of a holobiont may depend on multiple factors, including age, sex, life history and environment. Functional and/or metagenomics studies are, however, required to investigate whether changes in the microbiota affect the functioning of the coral holobiont.

Rigorous sampling at multiple locations, depths and over time has revealed that numerous scleractinian and gorgonian species possess a bacterial core microbiome, i.e., they consistently associate with certain bacteria regardless of space and time. Black corals, however, seem to largely lack this feature, as no core microbiome has been detected in *A. subpinnata* here or the other species studied in-depth, *L. glaberrima* (Dannenberg, 2016). The high level of microbiome flexibility in *A. subpinnata* may explain this lack of a bacterial 'core microbiome.' In agreement with Ziegler et al. (2019), we believe that, due to the differences in the degree of microbiome flexibility among coral species, elucidating a universal coral core microbiome (Ainsworth et al., 2015; Hernandez-Agreda et al., 2017) is difficult. However, recent papers have described strong signals of phylosymbiosis in both tropical reef-building hexacorals (Pollock et al., 2018) and the distantly related Mediterranean gorgonian octocorals (van de Water et al., 2018b). Phylosymbiosis has been defined as "microbial community relationships that recapitulate the phylogeny of their host" (Brucker and Bordenstein, 2013; Lim and Bordenstein, 2020). It is therefore surprising to see that the Antipatharia-branch of the Hexacorallia may not fit in the pattern of phylosymbiosis in corals as it appears to lack close relationships with its microbiota. Phylosymbiosis has often been considered to arise from long-term associations between a host and its microbes (e.g., co-diversification in scleractinian and gorgonian corals, Pollock et al., 2018; van de Water et al., 2018b). However, a recent study on *Drosophila melanogaster* found that phylosymbiosis may also be driven by short-term changes in the microbiota (Rudman et al., 2019). Consequently, Lim and Bordenstein (2020) suggest that microbial communities are not passive agents of phylosymbiosis, but may have the potential to induce genomic changes in the host that could impact establishment, maintenance or breakdown of phylosymbiosis. Whether a breakdown of phylosymbiosis in the Antipatharia occurred during the evolution of the Hexacorallia or phylosymbiosis arose multiple times during coral evolution remains an open question.

CONCLUSION

The composition of the bacterial communities of black corals is more similar to the microbial community associated with the reef-building scleractinian corals than the structurally similar, but more distantly related, and sympatric gorgonian corals. The potential lack of a core microbiome and major spatial and temporal differences observed shows that environmental factors largely determine the compositional differences of the black coral-associated microbial communities. However, whether the functioning of the microbiota has changed or that there is functional redundancy remains to be assessed through functional and metagenomics studies. This may suggest that black corals, such as *A. subpinnata*, have limited microbiome regulatory capacities. Yet, microbiome flexibility may also allow these corals to tailor their microbiota to local, and potentially changing, environmental conditions by selecting the most beneficial microbes from the surrounding environment. This could thus be another strategy that may have contributed to the black corals' success in colonizing the mesophotic zone and deep sea.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive: PRJNA506661.

AUTHOR CONTRIBUTIONS

JW, MC, MB, and CF-P designed the study. MC, FE, and MB collected and processed the samples. MC performed the DNA extractions. JW analyzed the sequencing data. JW, MC, MB, and CF-P wrote the manuscript. All the authors reviewed, edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.537813/full#supplementary-material

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Locality Effect of Coral-Associated Bacterial Community in the Kuroshio Current From Taiwan to Japan

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The Kuroshio Current (KC) is one of the fastest water currents in the world, running through the western boundary of the North Pacific Ocean. The KC strongly influences the regional hydroclimate by creating temperature, salinity, and pH gradients from tropical to subtropical and temperate zones, including regions with rich coral reef habitats. Microbial community composition of corals is influenced by various environmental factors, including salinity, pH, and geographical location. However, to date, it is unclear how coral-associated microbial communities would respond to the same water current running through different locations with a time lag. Therefore, we hypothesized that the locations subjected to the KC at higher latitudes experience similar but sequential lag in environmental variability compared to those at lower latitudes, and thus the coral communities of both will respond similarly, but at different times. In this year-long study, we compared the bacterial communities of *Acropora muricata* at Taiwan, Okinawa, and Kochi subjected to the KC. We found that site-specific conditions and site latitude may have stronger effects on bacterial composition and dynamics than a water current. Consequently, we suggest that latitude largely determines the temperature tolerance range of coral microbiota. Additionally, among the dominant coral associated bacteria, *Endozoicomonas* from *A. muricata* and *Stylophora pistillata* forms distinct phylogroups, while *Acinetobacter* is more likely a host generalist.

Keywords: Kuroshio Current, coral-associated bacteria, *Endozoicomonas*, *Acinetobacter*, generalist and specialist, *Acropora muricata*

INTRODUCTION

Coral associated microbes, together with their coral host, form an integrated holobiont, and have diverse interactions with their host and maintain coral holobiont function, such as nutrient acquisition and coral's health (Rosenberg et al., 2007). With respect to the coral-associated microbial community, *Gammaproteobacteria* is dominant (Bourne et al., 2013), and the putative symbiont

Endozoicomonas-like group in particular has received notable attention for its potential role in coral fitness (Meyer et al., 2014; Morrow et al., 2015). Based on genomics evidence, *Endozoicomonas* is proposed being beneficial to coral host by modulating coral metabolisms to prevent mitochondrial dysfunction (Ding et al., 2016), and furthermore by participating in coral-associated carbohydrate cycling and protein provision (Neave et al., 2017). The relative abundance of coral microbiome varies depending on different factors, such as temperature (Shiu et al., 2017) and coral species (Neave et al., 2016). Our recent report investigating the same coral species from different locations around Taiwan further demonstrated that the coral-associated microbiome was dominated by different bacterial taxa (Yang et al., 2017), indicating local environmental factors playing an important role affecting microbial composition in corals.

Coral-associated microbial communities were reportedly influenced by environmental factors such as seawater temperature, pH, salinity, and nutrients (Guppy and Bythell, 2006; Klaus et al., 2007; Hong et al., 2009; Littman et al., 2009), all of which are largely affected by ocean currents. In fact, ocean currents transport a massive amount of waters (of distinct properties) and nutrients across a long distance and even different climate zones, resulting in latitudinal gradients of water parameters (Qu et al., 2018). Furthermore, the change in current speed and current structure also contributed to the variation of oceanic nutrient flux (Guo et al., 2012). Along the Western Australian coast, the Leeuwin Current has been proposed to explain the conserved microbial community in corals at geographically distant locations (Ceh et al., 2011). This report thus aroused the curiosity about to what extent the coral microbial communities in different climate zones are affected by the same ocean current that results in differential temperature, pH, and salinity gradients.

The Kuroshio Current (KC) is a strong northward current that runs through the western boundary of the North Pacific Ocean, transporting heat, salt, and water from low to high latitude regions. The KC is warm, low in nutrients, has a small plankton population and a high flow speed (roughly two to four knots). It passes through three climate zones, from tropical (the Philippines and southern Taiwan) to subtropical (northern Taiwan and Okinawa, Japan), and finally to the temperate zone (mainland Japan), resulting in temperatures of 15–29°C (Abbot et al., 2003) and a pH of 7.8–8.5 (Dai, 1991; Chen et al., 1995) at shallow depth above 300 m. The KC is analogous to a high-speed "freeway" that moves marine organisms from low to high latitudes in the western Pacific Ocean. Warming temperatures are associated with a poleward coral expansion (Yamano et al., 2011), and the coral reefs around Taiwan and Japan are therefore implicitly linked by the warm KC water (Fujiwara et al., 2000; Chen and Shashank, 2009).

Given its large latitudinal distance and wide temperature and pH gradients, the KC provides a unique opportunity to investigate the relationship between environmental factors driven by the current and their impacts on coral-associated microbial communities. We hypothesized that the locations subjected to the KC at higher latitudes experience similar but delayed environmental conditions compared to those at lower latitudes, and thus the coral communities of both respond similarly, but at different times.

To test this hypothesis, simultaneous observation and sampling (of the same coral species) were performed at different locations. Although the locational variations in soft coral-associated microbial composition among six sites subjected to the KC (Woo et al., 2017) were reported, there is still a knowledge gap that can be fulfilled by repetitive sampling for investigating microbial dynamics in corals under the same seasonal conditions. In addition, coral-associated bacteria can be coral species- or colony-specific (Hernandez-Agreda et al., 2018), making it difficult to compare observations obtained from different coral species. Therefore, in this study, we chose *Acropora muricata* (Linné, 1789) as our monitoring coral species to investigate the impacts of KC temperature and pH on coral-associated microbial communities across different climate zones. The sampling was performed bimonthly throughout 1 year in coastal areas of southern Taiwan and Okinawa and Kochi, Japan, which are all subjected to the KC, and their reefs are all dominated by *A. muricata*. *A. muricata*-associated microbiota is reportedly influenced by the environment (Casey et al., 2015). Therefore, we further explored the abundance variation and dynamics of *Endozoicomonas* and other genera in *A. muricata* in different climate zones but subjected to the same water current.

MATERIALS AND METHODS

Sample Collection and Measurement of Environmental Parameters

A total of 189 *A. muricata* coral and 21 seawater samples were collected in Kochi (32°46′42.95″N, 132°43′56.06″E), Okinawa (26°41′39.1″N, 127°52′31.8″E), and Taiwan (21°56′58.3″N, 120°45′11.9″E) from August 2011 to September 2012 (**Table 1**). During the sampling period, a typhoon occurred 1 month earlier than sampling O6, and shortly before O7, T1, and T7. At each site, three branches from three distinct colonies were randomly selected to represent biological replicates (n = 9) for the local ecology of *A. muricata*. The coral branches were fixed with 95% ethanol after sampling. Seawater (1 L) was collected and its parameters (including pH, salinity, and temperature) were measured at the time of sampling. Seawater was filtered with a 0.2 μm membrane (0.2 μm pore size; Adventec, Japan) to collect microbial particulate and stored at 4°C until DNA extraction.

DNA Extraction, Sequencing, and Processing

Coral and seawater samples were sent to the laboratory at Academia Sinica, Taiwan, for DNA extraction within 1 week of collection. Microbial DNA of samples was extracted via the cetyltrimethylammonium bromide (CTAB) method (Wilson, 2001). The hypervariable V1–V2 regions of bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using universal primers 27F (F: forward) and 341R (R: reverse), both

TABLE 1 | Sampling information and sample identifier of *Acropora muricata* and seawater.

Sampling location	Sample name	Sampling time	Date	Coral sample	Seawater sample
Kochi	K1	1	2011 Aug. 30	K1C1, K1C2, K1C3 (*n* = 3)	K1SW[a]
	K2	2	2011 Oct. 30	K2C1, K2C2, K2C3 (*n* = 3)	K2SW[a]
	K3	3	2011 Dec. 20	K3C1, K3C2, K3C3 (*n* = 3)	K3SW
	K4	4	2012 Feb. 23	K4C1, K4C2, K4C3 (*n* = 3)	K4SW
	K5	5	2012 April 27	K5C1, K5C2, K5C3 (*n* = 3)	K5SW
	K6	6	2012 July 10	K6C1, K6C2, K6C3 (*n* = 3)	K6SW
	K7	7	2012 Sep. 6	K7C1[a], K7C2, K7C3 (*n* = 2)	K7SW
Okinawa	O1	1	2011 Aug. 28	O1C1, O1C2, O1C3 (*n* = 3)	O1SW
	O2	2	2011 Oct. 30	O2C1, O2C2, O2C3 (*n* = 3)	O2SW
	O3	3	2011 Dec. 21	O3C1, O3C2, O3C3 (*n* = 3)	O3SW
	O4	4	2012 Feb. 24	O4C1, O4C2, O4C3 (*n* = 3)	O4SW
	O5	5	2012 April 25	O5C1, O5C2, O5C3 (*n* = 3)	O5SW[a]
	O6	6	2012 July 26	O6C1, O6C2, O6C3 (*n* = 3)	O6SW
	O7	7	2012 Aug. 30	O7C1, O7C2, O7C3 (*n* = 3)	O7SW
Taiwan	T1	1	2011 Sep. 9	T1C1, T1C2, T1C3 (*n* = 3)	T1SW
	T2	2	2011 Oct. 21	T2C1, T2C2, T2C3 (*n* = 3)	T2SW
	T3	3	2011 Dec. 29	T3C1, T3C2, T3C3 (*n* = 3)	T3SW
	T4	4	2012 Feb. 23	T4C1, T4C2, T4C3 (*n* = 3)	T4SW
	T5	5	2012 April 24	T5C1, T5C2, T5C3 (*n* = 3)	T5SW
	T6	6	2012 July 17	T6C1, T6C2, T6C3 (*n* = 3)	T6SW
	T7	7	2012 Sep. 4	T7C1[a], T7C2, T7C3 (*n* = 2)	T7SW

[a]*Not available because of less than 200 reads available for analysis.*

of which were tagged with 4-base barcodes at their 5'-end as described previously (Chen et al., 2011). Amplified DNA was quantified by Qubit Fluorometer (Thermo Fisher Scientific, United States) and pooled in equal amounts. Multiplexed sequencing was performed using the Genome Sequencer FLX Titanium System (Roche 454 Life Sciences, Branford, CT, United States) at Mission Biotech (Taipei, Taiwan).

The raw sequencing reads were sorted into different samples according to the barcodes using an in-house script, which also removed the barcode and primer sequences. Reads (1) of lengths shorter than 280 bp or longer than 350 bp, (2) with an average quality score less than 20, (3) with a homopolymer longer than 8 bp, or (4) containing ambiguous bases were quality-filtered using Mothur (Schloss et al., 2009). Chimeric reads predicted by UCHIME (Edgar et al., 2011) implemented in USEARCH (v8.0.1623; reference mode, rdp_gold database, mindiv of 3) (Edgar, 2010) were also eliminated. Non-chimeric reads (including borderline cases by UCHIME) were retained for further analysis. Reads of coral branches from the same colony were pooled as one sample, resulting in a total of 84 samples. The read processing code is available in **Supplementary Data**.

For operational taxonomic unit (OTU) analysis, quality-filtered and non-chimeric reads were analyzed on a per-sample basis with UPARSE (Edgar, 2013); the chimera removal step was excluded, as it had been performed in previous quality filtering steps. The OTU approach has been shown to have comparable validity of results to the more stringent amplicon sequence variants (i.e., ASV) methods (Glassman and Martiny, 2018). OTUs were generated at a 97% identity threshold. Each OTU was searched (with global alignment) using USEARCH against the Ribosomal Database Project's database (training set 16 and release 11.5) to find the corresponding taxonomy of the best hit. OTUs with no hit or weak hits (i.e., the average of % identity and % query coverage <93) were excluded. After filtering OTUs of the chloroplast, which occupied a mean ($\pm$ SD) of 1.35% ($\pm$ 1.65%) in coral and 27.67% ($\pm$ 25.41%) in seawater samples, samples containing $\geq$ 200 reads were used for downstream analysis.

Statistical Analysis

All the statistical analyses were performed in R software[1] unless stated otherwise. Community data were handled with the R package phyloseq (McMurdie and Holmes, 2013), by which alpha diversity indices were estimated. Non-parametric tests were performed to compare alpha diversities between groups; Kruskal-Wallis test was used for three-group and Wilcoxon test for two-group comparison. Hierarchical clustering with average linkage was performed and visualized using the R package pheatmap (Kolde, 2012). At the genus level, samples were clustered based on the Euclidean distance of a base-10 logarithmic transformation of percent relative abundance, while taxa were based on the Bray–Curtis distance of non-transformed percent relative abundance. During the distance calculation, a pseudo-count of 1×10^{-8} was added to each taxon (Costea et al., 2014), and taxa with unknown taxonomy or <0.1% average relative abundance were classified as "Others." The distance matrix was calculated using the R package vegan (Dixon, 2003). The analysis of similarities

[1]https://www.r-project.org/

(ANOSIM) was performed to statistically test whether there is a significant difference in community composition between two or more groups.

OTU Analysis of *Acinetobacter* and *Endozoicomonas*

Endozoicomonas and *Acinetobacter* OTUs were originally defined on a per-sample basis and were difficult to pool directly by taxonomy because most species of these two genera are unclassified. Therefore, the cross-sample OTUs were analyzed. After extracting amplicon reads of *Endozoicomonas* and *Acinetobacter* OTUs, reads of each genus were clustered at 97% identity by USEARCH (with –cluster_smallmem), and the abundance profiles were constructed according to the sampling source information retained on each read.

Correlation (Pearson) heatmaps were generated in R with the corrplot package (Wei and Simko, 2017) using the relative abundance and presence-absence profiles of *Endozoicomonas* and *Acinetobacter* OTUs across all samples. Correlation significance was tested at 95% confidence intervals.

Beta diversity was assessed and visualized using non-metric multidimensional scaling (nMDS) on Primer 6 (PRIMER-E, Lutton, Lvybridge, United Kingdom) and fitted with environmental factors through constrained correspondence analysis (CCA) on the R package vegan. The top-20 *Endozoicomonas* and *Acinetobacter* OTUs (in abundance) were used for phylogenetic analysis, using the maximum likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969) to obtain the tree. Phylogenetic analyses were conducted in MEGA7 (Kumar et al., 2016).

Data Availability

All sequence data generated in this study have been deposited in NCBI under the Bioproject accession PRJNA636267.

RESULTS

Comparison of Environmental Parameters

Salinity, pH, and temperature were measured from concurrently sampled seawater as environmental parameters. Kochi, Okinawa, and Taiwan were the sampling sites from north to south. The seawater salinity in Kochi exhibited large seasonal variation (showing a statistical variance of 42.2, defined as the average of the squares of difference between the data points and the mean) and remained relatively stable throughout the year in Okinawa (showing a variance of 2.2) and Taiwan (showing a variance of 6.4) (**Supplementary Figure S1A**). The pH values varied widely during summertime in all three sites, with particularly high fluctuations in Okinawa (from sampling time 6, 7, and 1; **Supplementary Figure S1B**). A distinct time-lag was observed for temperature; in Taiwan, the temperature began to rise at the third sampling, which was, however, observed in Okinawa and Kochi at the fourth sampling (**Supplementary Figure S1C**). In general, Okinawa and Taiwan shared similar salinity, pH, and temperature values. Kochi showed large fluctuations in salinity and pH, and consistently had lower temperatures than the other two sites.

Bacterial Diversity and Composition

Hierarchical clustering revealed that bacterial composition (at the genus level) was distinct in coral and seawater samples (ANOSIM $R = 0.41$, $p = 0.01$) (**Figure 1**). The bacterial Shannon diversities in coral were significantly higher in Taiwan than the other two sites ($p = 0.0012$ by Kruskal-Wallis); seawater samples were also higher in Taiwan but not significant ($p = 0.84$ by Kruskal-Wallis). Regarding each location, although with only a marginal significance, the bacterial Shannon diversities were higher in seawater than coral samples in Kochi ($p = 0.17$ by Wilcoxon) and Okinawa ($p = 0.049$ by Wilcoxon) (**Supplementary Figure S2**), while higher in coral than seawater samples in Taiwan ($p = 0.57$ by Wilcoxon) (**Supplementary Figure S2**), suggesting that bacteria at the low latitude site thrived better in corals than seawater. Results of the Inverse Simpson, richness, and Shannon analyses suggested that the coral bacterial diversities did not show any time-lag trend among the three sampling sites (**Supplementary Figure S3**). The highest diversity for each site was found during the fifth sampling in Kochi and Taiwan, and the seventh sampling in Okinawa (**Supplementary Figure S3**).

The nMDS analysis showed that the coral bacterial compositions in Kochi and Okinawa were relatively stable compared to that of Taiwan (**Figure 2**). High variation in bacterial composition was found in coral samples in Taiwan during autumn and winter (**Figure 2A**), while in Kochi similar results were identified during spring and summer (**Figure 2B**). In seawater samples, the bacterial compositions in Taiwan were similar to those in Kochi (ANOSIM $R = 0.22$, $p = 0.059$), but significantly different from those in Okinawa (ANOSIM $R = 0.26$, $p < 0.01$) (**Supplementary Figure S4**). No significant difference was found between the compositions of Kochi and Okinawa (ANOSIM $R = 0.04$, $p = 0.33$).

Among the 20 most abundant bacterial genera, corals in Taiwan harbored a greater diversity than the other two sites (**Figure 3**). *Endozoicomonas* was consistently dominant in corals from Kochi and Okinawa but not Taiwan, where it was particularly low in abundance during autumn and winter months (T1–T4 in **Figure 3**). Inspired by our recent study which reported that *Endozoicomonas acroporae* is an *Acropora*-associated dimethylsulfoniopropionate (DMSP) degrader (Tandon et al., 2020), we further investigated how many of our *Endozoiocmonas* amplicons were associated with *E. acroporae*. By analyzing the best BLASTn match, we observed 6.35 and 4.36% relative abundance of *E. acroporae* in Okinawa and Taiwan, respectively, but only 0.3% in Kochi (**Supplementary Figure S5**).

The CCA results showed that temperature and salinity had larger influences on coral bacterial composition than pH when considering all samples (**Supplementary Figure S6A**). In the site-specific analysis, salinity and temperature had the largest effect on Kochi (**Supplementary Figures S1A,C**) and were the main factors contributing to its coral bacterial community composition (**Supplementary Figure S6B**). In Kochi, the abundance of many genera was negatively correlated with temperature, indicating that they acclimated to the lower water temperature (<20°C). In Okinawa, temperature and pH were the major factors

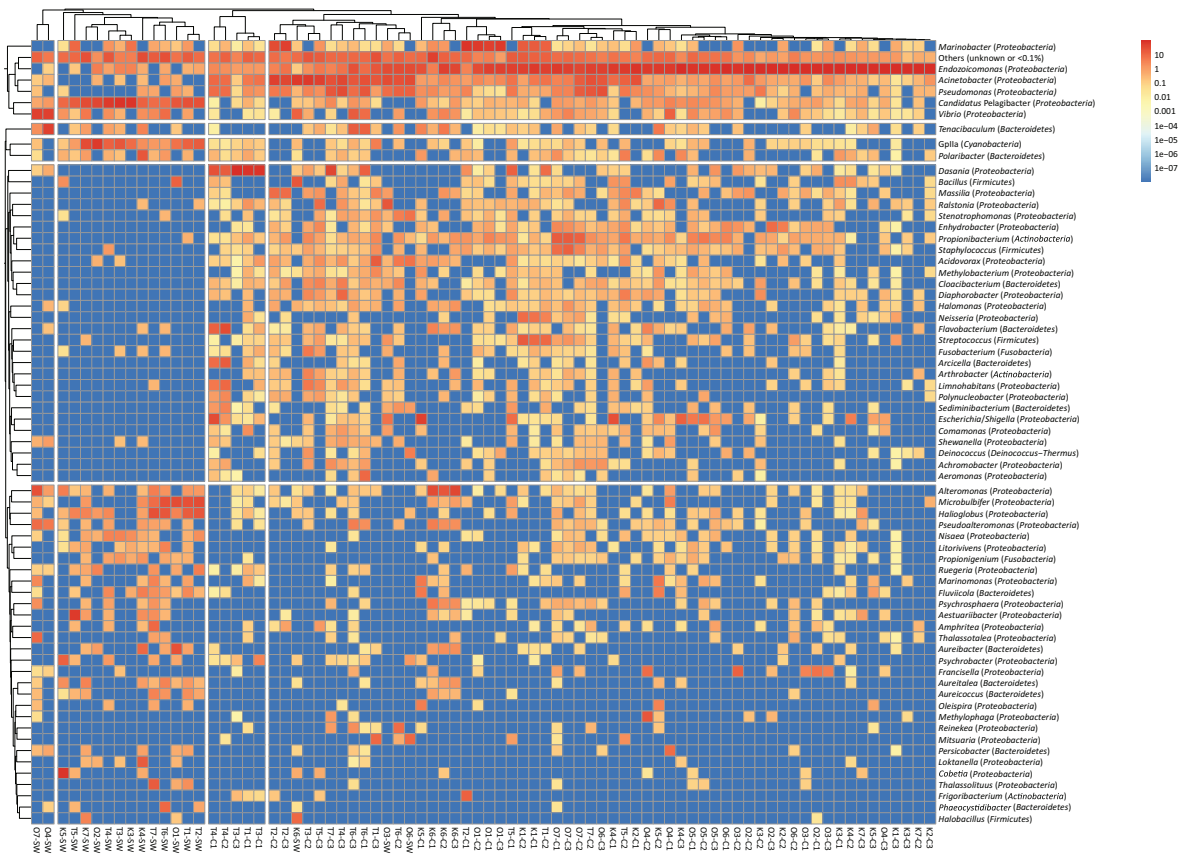

FIGURE 1 | Clustering analysis of bacterial genera in *Acropora muricata* and seawater from Kochi, Okinawa, and Taiwan. A total of 68 genera remained in the analysis after excluding genera of unknown taxonomy or < 0.1% relative abundance. Trees were cut at four and five groups in columns and rows, respectively. The color scale indicates the relative abundance (percentage). Columns are clustered by Bray–Curtis distance and rows by Euclidean distance of their abundance profile.

influencing the coral bacterial community (**Supplementary Figures S1, S6C**). In Taiwan, most genera were positively correlated with temperature, indicating an acclimation to the warmer environment (**Supplementary Figure S6D**). As a result, temperature, pH, and salinity had site-specific and differential effects, suggesting that ambient conditions have an important role in the coral-associated bacterial composition.

Endozoicomonas and *Acinetobacter*

Endozoicomonas and *Acinetobacter* were the most dominant genera in corals (**Figure 3**), so we further analyzed them to resolve their relationships to sampling times from all three sites in greater detail (see section "Materials and Methods"). Both *Endozoicomonas* and *Acinetobacter* abundance (log-transformed) demonstrated higher correlation values within sites than between sites, indicating that the compositional differences were site-specific (**Figure 4A**; ANOSIM $R = 0.695$, $p = 0.0001$ for *Endozoicomonas*; $R = 0.416$, $p = 0.0001$ for *Acinetobacter*), but the correlations diminished in Boolean transformation (i.e., presence and absence) (**Figure 4B**). Neither *Endozoicomonas* nor *Acinetobacter* demonstrated any time-lag phenomenon in OTU compositions across locations, indicating that the time-lag succession did not apply to dominant bacteria in corals along the KC. The site-specific CCA analysis revealed that temperature and salinity were the main factors affecting genera composition in both Kochi (**Supplementary Figure S7A,D**) and Okinawa (**Supplementary Figures S7B,E**), while temperature and pH were the major factors in Taiwan (**Supplementary Figures S7C,F**).

Lastly, the 20 most abundant OTUs of *Endozoicomonas* and *Acinetobacter* were phylogenetically analyzed with their counterparts from a different coral species, *Stylophora pistillata,* obtained from our previous study (Yang et al., 2017), to verify the existence of host specificity. Results showed that *Endozoicomonas* from *S. pistillata* and *A. muricata* formed distinct phylogroups (**Figure 5A**), but *Acinetobacter* OTUs did not (**Figure 5B**), suggesting that *Endozoicomonas* has a higher host specificity than *Acinetobacter*.

DISCUSSION

Influences of Current and Location

Current is driven by gradients of temperature, pH, and salinity and was expected to result in time-lagged shifts in microbial composition. However, the present study did not find enough

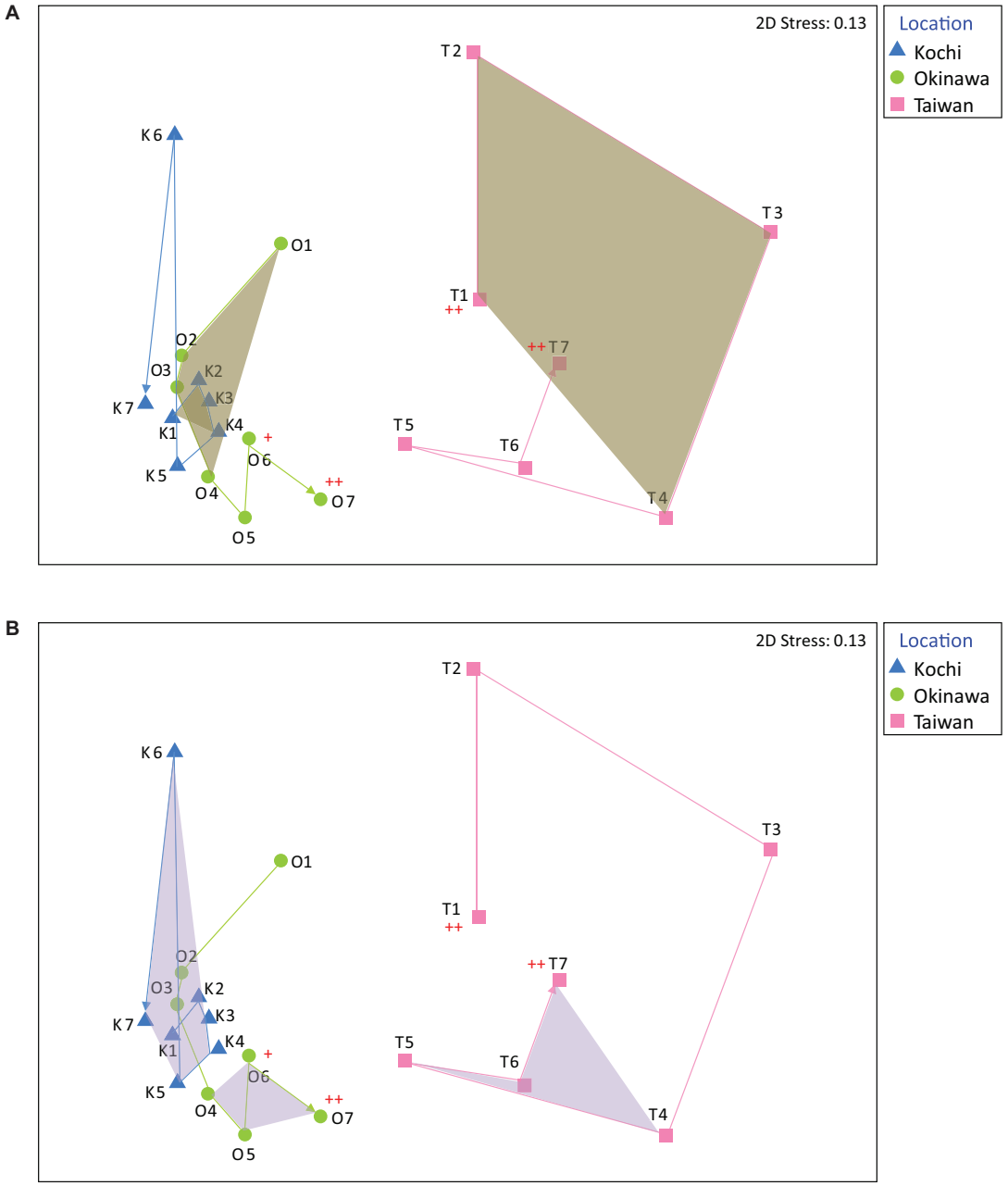

FIGURE 2 | Non-metric multidimensional scaling (nMDS) analysis of bacterial genera in *Acropora muricata* from Kochi, Okinawa, and Taiwan during **(A)** autumn and winter (samples K1-K4, O1-O4, and T1-T4) and during **(B)** spring and summer (samples K4-K7, O4-O7, and T4-T7). A cross indicates sampling after typhoon about 1 month and two crosses indicate sampling just after the typhoon.

evidence to support the hypothesis that the microbiota of coral and seawater samples can be influenced by water current from low to high latitude. Along the path of the KC, the sampling sites were more or less influenced by its water (Chu, 1974; Andres et al., 2008; Kuroda et al., 2008), but the coral-associated bacterial composition exhibited significant site-specific differences and no obvious sign of time-lag succession. In addition, further comparisons between the two most dominant genera, *Endozoicomonas* and *Acinetobacter*, repeatedly found site-specific and season-independent correlations in abundance profiles without time-lag succession. These results collectively suggest that local conditions may have stronger effects on bacterial compositions and dynamics than does the KC.

The coral-associated bacterial community in Taiwan demonstrated higher seasonal variation than those of Okinawa and Kochi, plausibly attributable to the water flow velocity. Advection in currents has been suggested to influence microbial community assemblies (Doblin and Van Sebille, 2016), and

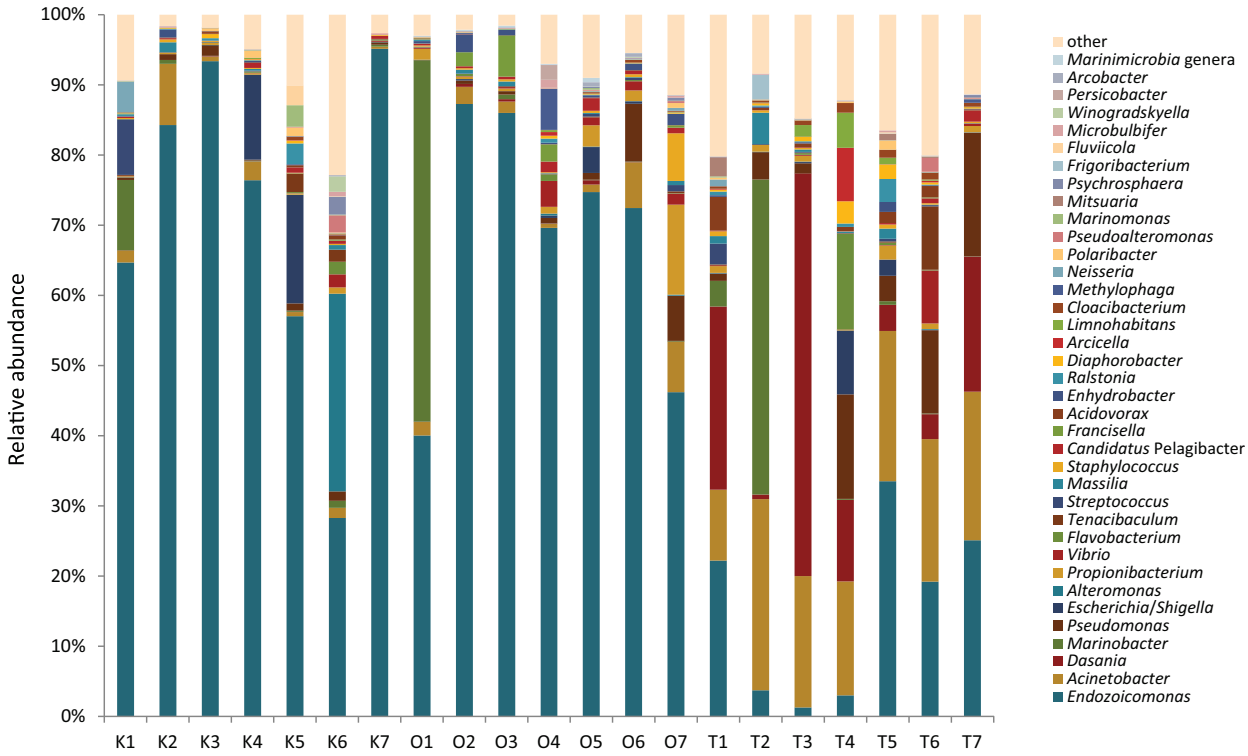

FIGURE 3 | Top 20 bacterial genera in Kochi, Okinawa, and Taiwan.

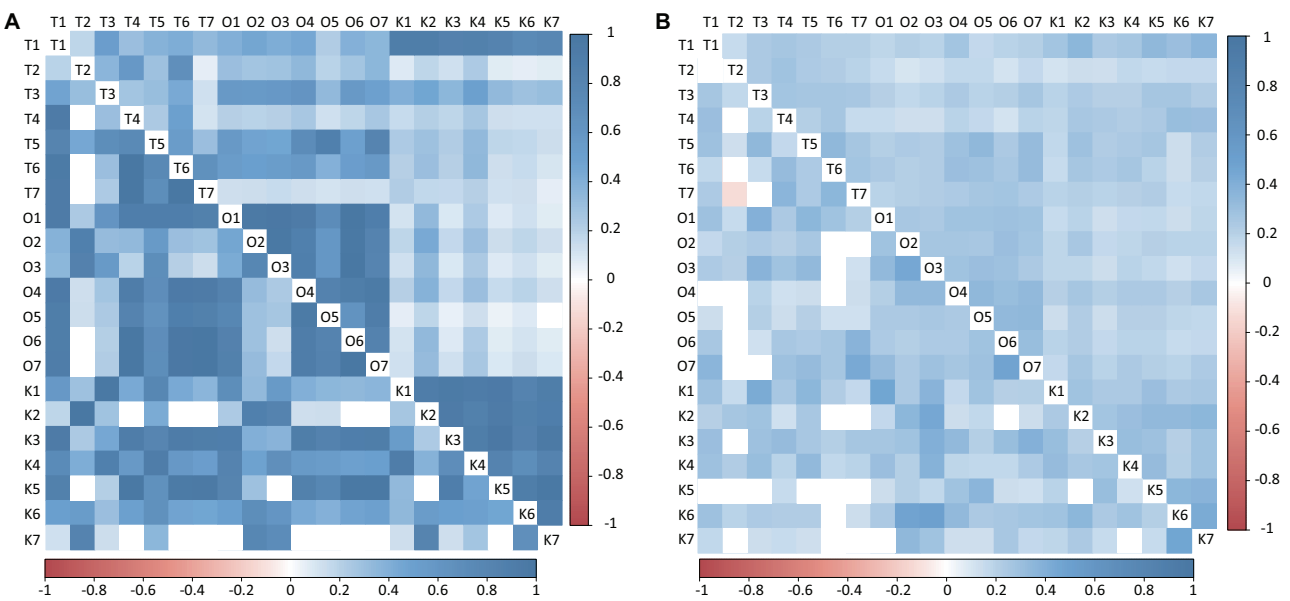

FIGURE 4 | Heatmap of Pearson's correlation between *Endozoicomonas* and *Acinetobacter* among different sampling time from Kochi, Okinawa, and Taiwan. **(A)** matrix with log transformation. **(B)** matrix with present/absent transformation. The values above the diagonal represented *Endoizoicomonas* abundance correlation values and below the diagonal represented *Acinetobacter* abundance correlation.

increased water flow velocity has also been shown to modify the bacterial composition in the coral surface mucus and tissue layers (Lee et al., 2016). Compared to Kochi, the sampling sites in Taiwan and Okinawa had faster current flow because of their physical proximity to the KC mainstream (according to the Ocean Data Bank, Ministry of Science and Technology,

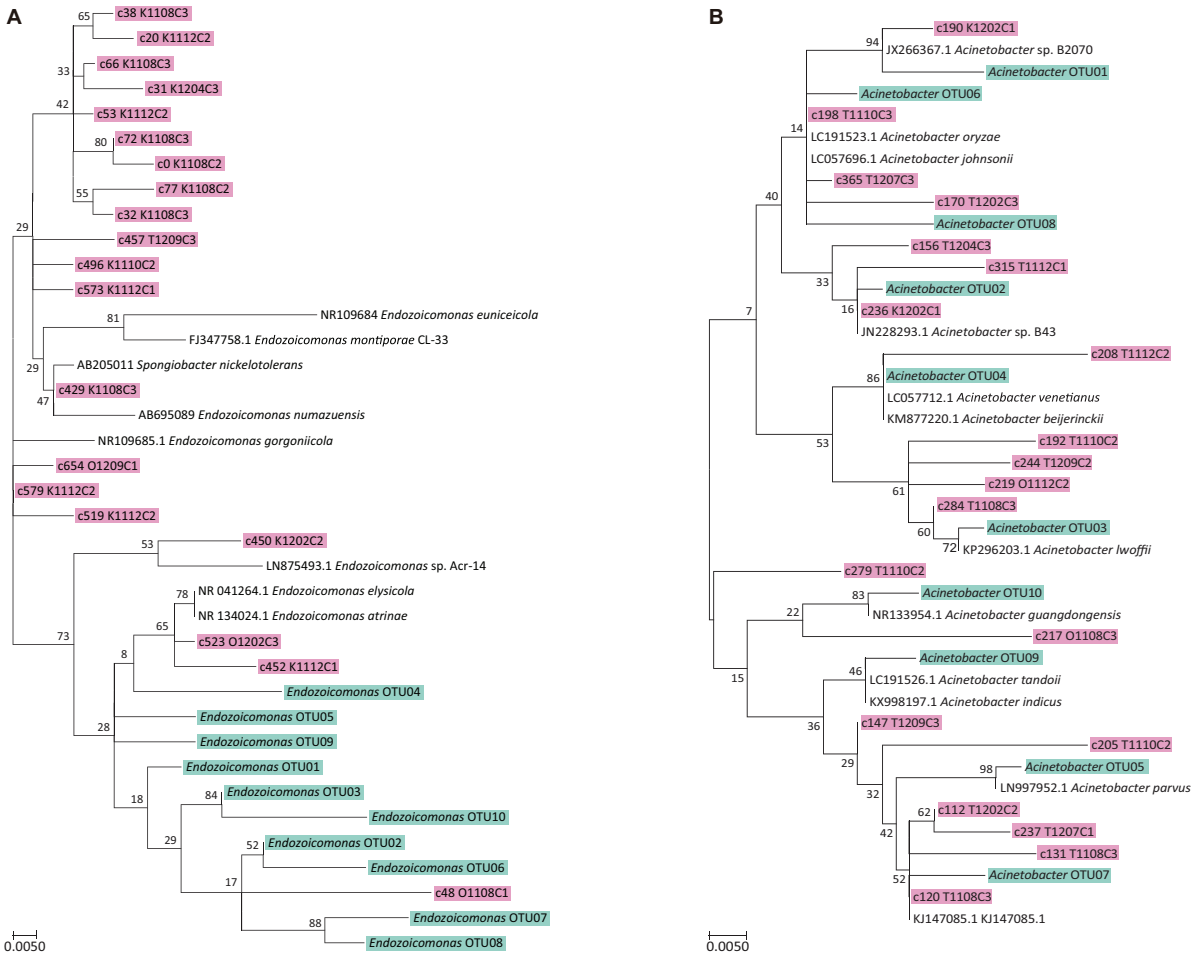

FIGURE 5 | Maximum likelihood trees of top 20 OTUs *Endozoicomonas* and *Acinetobacter*. **(A)** ML tree of top 20 *Endozoicomonas* OTUs from this study (shown in pink) and top 10 *Endozoicomonas* OTUs from coral *Stylophora pistillata* in Yang et al. (2017) (shown in blue). **(B)** ML tree of top 20 *Acinetobacter* OTUs from this study (shown in pink) and top 10 *Acinetobacter* OTUs from coral *S. pistillata* in Yang et al. (2017) (shown in blue). The trees with the highest log likelihood (−1227.6999) and 1,000 bootstraps. The analysis involved 42 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 295 positions in the final dataset.

Taiwan)[2]. Moreover, the site in Taiwan is affected not only by the KC but also waters from the South China Sea (Hu et al., 2000), which in combination may impact coral-associated bacterial compositions. Last but not least, extreme hydrodynamics (e.g., typhoons) can cause a shift in the coral ecosystem (White et al., 2017) and phytoplankton composition (Blanco et al., 2008), and lead to disease in corals (Bright et al., 2016) by disrupting local environments for coral holobionts. In Taiwan and Kochi, the first and last sampling (T1 and T7, K1 and K7) were similar in community composition (**Figure 2**), which was not observed in Okinawa (i.e., O1 and O7 were separated from each other). As O7 sampling was performed shortly after a typhoon, it was very likely that the observed microbial community has been substantially disrupted.

The coral microbiota is also associated with site latitude. Our previous study found that *Stylophora pistillata*-associated bacterial compositions differed between tropical and subtropical zones (Yang et al., 2017). A one-degree latitudinal difference was proposed to be the distribution boundary for similar bacterial compositions in the same coral (i.e., two corals of the same species separated by more than one degree would have different bacterial compositions) (Woo et al., 2017). Pollock et al. (2018) found that the coral microbiota has lower richness at higher latitudes, corresponding to the lower coral bacterial diversity in Okinawa and Kochi than Taiwan. The coral microbiota in Kochi and Okinawa shared similar compositions, but largely differed from those in Taiwan, suggesting that coral bacterial dynamics correspond with the climate zone. However, the geographic location is still attributable to this observation as they may differentially be influenced by the water currents from the South China Sea.

Several variation patterns associated with seasons in coral microbiota were discovered. Being a middle-latitude site, the coral microbiota in Okinawa highly overlapped with those

[2]http://www.odb.ntu.edu.tw/

in Kochi (the high-latitude site) during autumn and winter but became closer to those of Taiwan (the low-latitude site) during spring and summer. Also, compared to autumn and winter, the coral microbiota in Kochi largely fluctuated during spring and summer, while those in Taiwan demonstrated the opposite trend. The coral microbiota has been shown to tolerate a temperature range similar to ambient thermal history (Shiu et al., 2017). In this study, the *Acropora*-associated microbial composition in the low-latitude site (i.e., Taiwan) demonstrated a relatively small fluctuation during spring and summer; the counterparts in the high-latitude site (i.e., Kochi) demonstrated a relatively small fluctuation during autumn and winter. Therefore, the latitude largely determines the temperature tolerance range of coral microbiota. Although coral microbiota is slightly fluctuating from time to time, this phenomenon is escalated while the ambient temperature varies beyond the tolerance range.

Site and Host Specificities of *Endozoicomonas* and *Acinetobacter*

Endozoicomonas is a ubiquitous taxon in coral (Neave et al., 2016). Its high abundance has been suggested as an indicator of good coral health (Bourne et al., 2008). However, in the present study performed on healthy *A. muricata*, the abundance of *Endozoicomonas* varied across sampling sites. As our sampling was performed at inshore sites, the impact of terrestrial nutrient influx (e.g., P and N) on coral microbiota cannot be ignored (Vega Thurber et al., 2014).

It has been suggested that ubiquitously dominant taxa may be eurythermic environmental generalists or composed of multiple subtypes selected through different environmental factors (Yung et al., 2015). Although *Endozoicomonas* is common in corals, at low latitude the *A. muricata*-associated microbiota was simultaneously dominated by other genera, such as *Acinetobacter*, *Dasania,* and *Pseudomonas*. *Acinetobacter* was found particularly more in *S. pistillata* around Taiwan than was *Endozoicomonas* (Yang et al., 2017).

Among the *Endozoicomonas* species, *Endozoicomonas* amplicons associated with *E. acroporae* were detected at all three sites, and its abundance in Okinawa and Taiwan increased during the warm seasons (spring and summer). *E. acroporae* is a DMSP degrader (Tandon et al., 2018, 2020), and DMSP and its breakdown products can quickly scavenge hydroxyl radicals and mitigate oxidative stress to protect corals from bleaching (Sunda et al., 2002; Lesser, 2006). However, the coral microbiota in Taiwan was not overwhelmed by *Endozoicomonas*, and the second-most abundant genus, *Acinetobacter,* is proposed to be a DMSP degrader performing a similar protective function for *A. muricata* (Raina et al., 2010). *Acinetobacter* was abundant in different stony corals in various regions, including the tropics (Littman et al., 2009; McKew et al., 2012; Li et al., 2014; Leite et al., 2018), and its role is still elusive as being reportedly beneficial (Shnit-Orland and Kushmaro, 2009) and detrimental (Sweet et al., 2013) to corals. Given the observation that *Acinetobacter* abundance was higher and more stable in low-latitude (Taiwan) than high-latitude (Okinawa and Kochi) sites, the question of whether *Acinetobacter* could edge *Endozoicomonas* out of the microbial assemblage dominance in *Acropora* warrants further study, especially with the rising seawater temperature in the age of global warming.

CONCLUSION

This year-long study compared the bacterial communities of *A. muricata* at three sites subjected to the KC at different climate zones (Taiwan, Okinawa, and Kochi), and found an inverse correlation between coral bacterial diversity and site latitude. The bacterial composition in coral has no obvious time-lag succession along with the KC, but a strong correlation with ambient conditions, indicating that environmental parameters are more influential to the indigenous bacterial community. *Endozoicomonas* were substantially detected in temperate and subtropical sites but outnumbered by *Acinetobacter* in tropics, indicating *Acinetobacter* having a lower host specificity and better adaptability to the warmer environment than *Endozoicomonas*.

Manipulation of the coral microbiome was recently proposed as a promising means to improve coral health by strengthening coral resistance and resilience to environmental stress (Damjanovic et al., 2017; Sweet et al., 2017), and *Endozoicomonas* has been proposed as an environmental probiotic for this purpose (Peixoto et al., 2017). According to the site-specific distribution of *Endozoicomonas*, the microbial manipulation in coral would be more effective if we factor in local environmental parameters and host species. Finally, the dominance of *Acinetobacter* in tropical corals suggests its potential role of being another environmental probiotic candidate.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA636267.

AUTHOR CONTRIBUTIONS

S-LT designed the study and edited the draft manuscript. S-HY, C-HT, and KT performed data analysis and manuscript writing. H-PL, P-WC, H-JC, J-HS, H-CL, NI, TM, and HY performed sampling. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.569107/full#supplementary-material

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Stranger Things: Organismal Traits of Two Octocorals Associated With Singular Symbiodiniaceae in a High-Latitude Coral Community From Northern Taiwan

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Scrutinizing the traits of octocorals that could affect their physiological performance becomes increasingly important as several of these species are observed to become dominant on reefs pressured by the Anthropocene. In the present study, we compare the organismal traits of two branching octocorals *Litophyton* sp. and *Stereonephthya* sp. commonly populating in sympatry the high-latitude coral communities of northern Taiwan. Using 13 traits, we describe and compare performance traits in these two symbiotic species that we discuss in light of the association they maintain with their algal partners. *Litophyton* sp. and *Stereonephthya* sp. hosted *Durusdinium* and *Gerakladium,* respectively. Both genera represent singular associations, with the latter further establishing the first solid report of *Gerakladium* in octocorals. Traits distinguished two groups explained by the two partnerships considered. *Litophyton* sp. associated with *Durusdinium* had significantly higher organic matter, chlorophyll (chl) *a*, total lipid and lower chl *c*/chl *a* ratio than *Stereonephthya* sp. associated with *Gerakladium.* The $\delta^{15}N$ in the host and algae, as well as $\delta^{13}C$ in the host were also higher in *Litophyton* species. Although no significant difference was observed in the $\delta^{13}C$ of the algae, *Litophyton* sp. presented a significantly higher variance for this trait and for chl *a* content than *Stereonephthya* species. Altogether, the traits examined suggested contrasting performances among the two octocorals. Both octocoral species clearly deviate from an autotrophic diet. *Litophyton* sp. appears to complement its heterotrophic diet with photosynthetically acquired energy, while *Stereonephthya* sp. tends to be more specialized and benefits relatively little from its symbiotic relationship. Our study calls for greater consideration of the individual variation in octocoral physiology and in the definition of their ecological strategies.

Keywords: stable isotope, intraspecific variation, trade-off, plasticity, functionality, algal symbiont, trait-based approach, soft corals

INTRODUCTION

Octocorals are important contributors to the living three-dimensional structure of the marine animal forests (Sánchez, 2016). These communities, dominated by sessile suspension-feeding organisms, support a diversity of ecological functions. A number of organisms use octocorals for their food (Gerhart, 1990; O'Neal and Pawlik, 2002), their habitat (Reijnen et al., 2011) and/or as a nursery ground (Etnoyer and Warrenchuk, 2007). Octocorals further influence energy flows and nutrient cycling between the pelagic and benthic systems by capturing planktonic organisms and filtering large amounts of dissolved and particulate organic matter (OM) (Coma et al., 1998; Rossi et al., 2017). They play a key role in carbon sequestration (Coppari et al., 2019), and some species contribute substantially to the reef formation in tropical habitats (e.g., Jeng et al., 2011). Therein, octocorals appear to have a relatively high level of tolerance to environmental stressors (Schubert et al., 2017). Accordingly, they have emerged as key potential candidates to prevail in future reefs (Vercelloni et al., 2020). Yet, octocorals spread across more than 40 families and display a wide array of morphologies, sizes, and sclerite architectures (e.g., Aharonovich and Benayahu, 2012). A generalization of these resilience abilities to all reef-associated octocorals seems doubtful, especially considering the variable responses to thermal stress observed among species (Slattery et al., 2019) and the limited knowledge currently available on their physiology.

The combination of features that allows the comparison of individual performance emerged as a decisive approach in defining and comparing species fitness (Violle et al., 2007). In this context, trade-off among traits involved in the flow of energy and matter at the individual level (Kearney et al., 2010) are directly related to their "function" in the ecosystems (sensus Bellwood et al., 2018). This further supplies a basis for the estimation of intraspecific variability and the delineation of species niches (Violle et al., 2012). Nevertheless, mean field theory (the study of the behavior of the mean while ignoring variance, Violle et al., 2012) still remains widely adopted in trait-based community ecology, especially in studies focusing on hyperdiverse ecosystems such as the coral reefs (Hughes et al., 2018; McWilliam et al., 2018). It provides a wide array of opportunities to improve our understanding of organism responses, particularly in species where symbiosis is expected to mediate niche differentiation and expansion (see Gerz et al., 2018). In octocorals, it has relevance in depicting and comparing the variation in individual traits involved in the acquisition and allocation of energy in the holobiont.

A significant proportion of octocorals is mixotrophic in shallow-water tropical reefs, living in association with photosynthetic dinoflagellates of the family Symbiodiniaceae (Schubert et al., 2017). These microalgal symbionts can ensure that most of the daily metabolic needs of the animal host are met (Schlichter et al., 1983), but their contribution has been demonstrated to change in accordance with environmental conditions (Bednarz et al., 2015) or host morphology (Rossi et al., 2018). On the other hand, heterotrophic feeding (Sorokin, 1991; Ribes et al., 1998) may be preponderant in some adult and juvenile individuals of the few known aposymbiotic species (e.g., *Eunicella singularis*, Gori et al., 2012; Schubert et al., 2017). Heterotrophic diet has also been observed to vary seasonally (Coma et al., 2015) and increase with local pollution (Baker et al., 2010). It may also improve the resilience of species with generalist, facultative associations; which in return, usually appear more sensitive to bleaching than species maintaining specialized, obligate symbioses (Baker et al., 2015). The relative contribution of both heterotrophic and autotrophic feeding is probably a determinant for explaining the performance of many species in a given habitat. Furthermore, it represents a basis upon which natural selection may operate under stressful conditions.

Earlier studies emphasized the variety of responses that can be expected in octocorals and the importance of recognizing species strategies to cope with present-day environmental challenges. In the present study, we targeted octocorals populating high-latitude coral communities from the coastal waters of northern Taiwan (Lin and Denis, 2019). We used 13 organismal traits to describe and compare individual variation in two symbiotic species *Litophyton* sp. and *Stereonephthya* sp., chosen for their phylogenetic proximity (McFadden et al., 2006) and morphological similarity (van Ofwegen, 2016). We delineated species performance niches in a constant environment and discussed differences among the two targeted species in light of the association they maintained with their respective algal partners.

MATERIALS AND METHODS

Study Species and Sampling Site

Two octocoral species (F: Nephtheidae) were targeted in this study: *Litophyton* sp. and *Stereonephthya* sp. (**Figures 1A,B**). Both share similar branching morphology and their populations are observed to thrive in sympatry in the shallow water benthic communities of northern Taiwan. For each taxon, 10 large colonies were sampled in April 2019 at −10 m (water temperature: 24°C) in Bitou (25.1262°N, 121.9131°E). Colonies were tentatively diagnosed as our targeted organisms (general morphology and close-up inspection of the polyps) and were photographed *in situ* with a tag and scale. Colony fragments, representing a total surface of 10–20 cm^2 of the extended colony, were sampled with scissors and placed delicately in Ziploc bags. Samples were collected under Collection Permit No. 1083544868 issued by the Fisheries and Fishing Port Affairs Management Office, New Taipei City Government, Taiwan. Immediately after the dive, octocoral branches were subsampled and fixed in 10% formalin for histological analysis. The remaining samples were fast-frozen for 5 min at −80°C in a cryogenic dry shipper (CX100, Taylor-Wharton, United States), and transferred into an icebox for transportation to the laboratory. Samples were transferred in a −20°C freezer until further processing.

Host Distinction and Algal-Symbiont Identification

Both the confirmation of the host colonies and the identification of their dominant algal symbiont were processed independently.

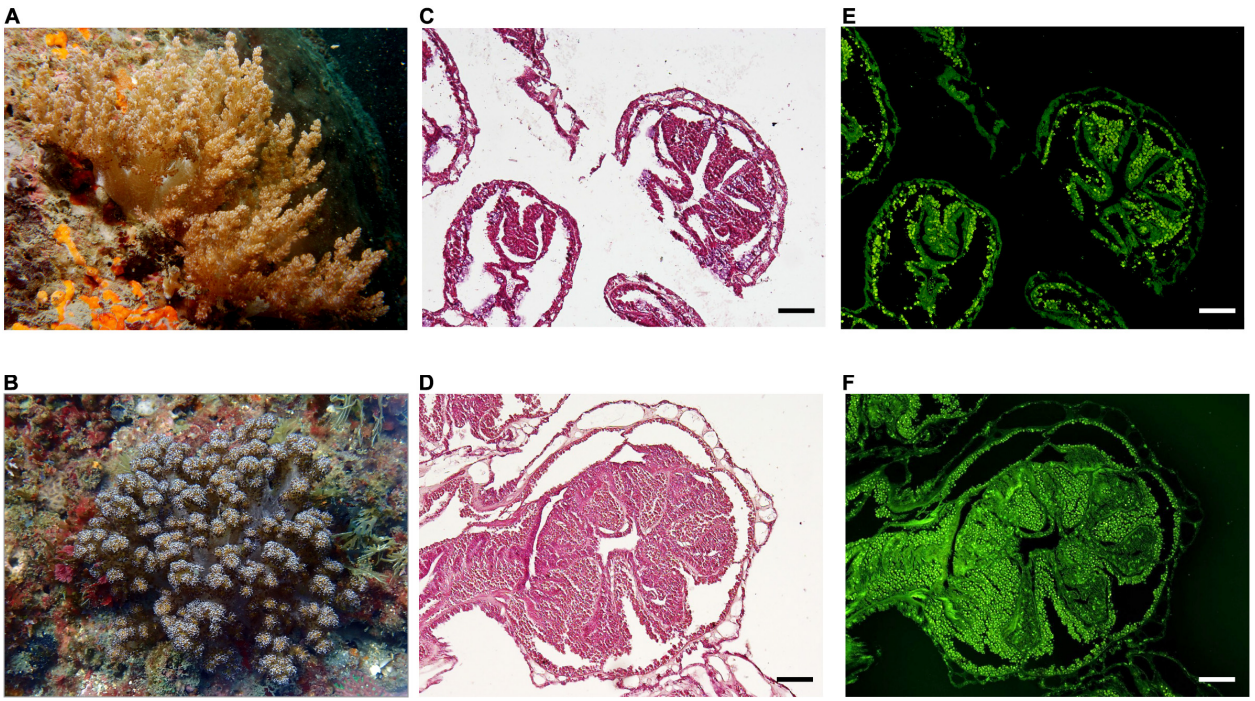

FIGURE 1 | *In situ* appearance and symbiont distribution in octocoral colonies. *Litophyton* sp. **(A)** and *Stereonephthya* sp. **(B)** at –10 m depth in Bitou (Taiwan). Histological sections of polyps from *Litophyton* sp. **(C)** and *Stereonephthya* sp. **(D)** under light microscope with an emphasis on algal endosymbionts using a U-MWB2 filter **(E,F)**. Scale bar 100 μm.

Samples were thawed on ice in the dark. For the confirmation of the host, tissue collected from the top, base, and branches of the coral fragment were bleached to isolate their skeleton elements. Dried sclerites were examined and compared to relevant taxonomic references for the identification of the host species. Other small subsamples were further collected from randomly selected coral colonies of each genus and preserved in 100% EtOH for molecular confirmation of the membership to *Litophyton* (n = 4) and *Stereonephthya* (n = 3). DNA was extracted and mitochondrial marker mtMutS was amplified using published primers and protocols (McFadden et al., 2011).

For algal-symbont identification, five colonies of each species were randomly selected to extract DNA from a small subsample preserved in 100% EtOH using a DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturer's protocol. The purity of DNA was checked with a NanoDrop 1000 (Thermo Thermo Scientific, United States). For the Symbiodiniaceae identification, the internal transcribed spacer ITS2 was amplified using zITSf (5′-CCGGTGAATTATTCGGACTGACGCAGT-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described in Noda et al. (2017). A PCR amplification of 35 cycles was performed (95°C for 30 s, 51°C for 45 s, and 72°C for 2 min). PCR products with around 724 bp were submitted to sanger sequencing, then individually checked for similarity with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database (Wheeler et al., 2008). Novel sequences have been deposited in GenBank (see accession numbers in **Supplementary Table 1**).

Algal Symbiont Distribution

Adapted from Rossi et al. (2018), Zinc Formal-Fixx (Shandon™ Thermo Fisher Scientific, CAS no.: 50-00-0) samples were treated in a series of rinsing and decalcification steps using 10% formic acid (Sigma-Aldrich, CAS no.: 64-18-6), after which 8 μM sections of tissue embedded in paraffin wax (Thermo Fisher Scientific, CAS no.: 9003-27-4) were stained with Meyer's hematoxylin (Shandon™ Thermo Fisher Scientific, CAS no.: 517-28-2) and eosin (Shandon™ Thermo Fisher Scientific, CAS no.: 17372-87-1) procedures, and coverslipped with Organol/Limonene (Sigma-Aldrich, CAS no.: 5989-27-5) mounting medium. Octocoral samples were examined with a fluorescence microscope (Olympus-BX51) under the excitation/emission wavelength of 460–490/520 nm (with U-MWB2 filter) to detect the presence of microalgal pigments in their tissue.

Organismal Trait Measurements

A small fragment (∼1 g of wet weight) was isolated from each thawed sample. The remaining tissue was ground in a mortar, transferred into a beaker, and the slurry was adjusted to an initial volume of 60 mL using artificial seawater (V_i). V_i was homogenized using a T10 basic ULTRA-TURRAX disperser (IKA Works Inc., United States) for 3 min. Dry weight (DW) was determined from a 1 mL aliquot of V_i, vacuum-filtered through a pre-weighed Whatman (United Kingdom) glass fiber filter (Grade: GF/C, Ø 100 mm, pore size 1.2 μm), and dried overnight

at 60°C. The filter was then ashed for 4 h at 450°C to calculate an ash-free dry weight (AFDW) (Pupier et al., 2018), that was used to standardize the following biochemical parameters. It was further used to estimate the % content in OM.

For photosynthetic traits below, a 1 mL aliquot of V_i was centrifuged at 3,000 *g* for 3 min to pellet algal symbionts. The pellet was re-suspended in 10 mL 90% acetone (J.T. Baker™ Avantor, CAS no.: 67-64-1) and incubated overnight at 4°C. The solution was later centrifuged at 3,000 *g* for 1 min, and the supernatant was transferred to a cuvette for measuring absorbance at 630, 647, 664, and 750 nm with a spectrophotometer (Optizen Pop, Mecasys, South Korea). Both, chlorophyll *a* (chl *a*) and *c* (chl *c*) concentrations (μg gAFDW^{-1}) were then estimated following trichromatic equations proposed by Jeffrey and Humphrey (1975). Note that the presence of chl *b* was checked, but found to be absent in the colonies examined. Algal symbiont content (cells gAFDW^{-1}) was estimated by counting cells from a 10 μL aliquot of V_i in a Neubauer chamber (Bright-Line 3100, Hausser Scientific, United States). Counts were repeated five times and averaged for each sample.

Total protein (μg gAFDW^{-1}) was determined from combining separate measurements of their concentrations in the animal host and algal fractions of a 5 mL aliquot of V_i. Both were isolated by centrifugation (3 min, 3,000 *g*), and homogenized with a dispenser after adding to the algal pellet a 2 mL 1% sodium dodecyl sulfate (SDS, J.T. Baker™ Avantor, CAS no.: 151-21-3) to ease the release of proteins from the dinoflagellate cytoplasm. Protein concentrations were calculated from triplicate spectrophotometric measurements (SpectraMax i3x, FortéBio, United States) on both fractions using a protein assay kit (Pierce™ BCA Thermo Fisher Scientific) with bovine serum albumin as a standard, following manufacturer protocol.

For the stable isotope measurements, the overall methodology was adapted from the protocol recommended for scleractinian corals in Sturaro et al. (2020) to octocorals. Notably, tissue was ground and not air-brushed. A large volume corresponding to a 50 mL of V_i was centrifuged (4°C, 10 min, 2,000 *g*) to separate both the host and the algal tissue. The purity of the host fraction was checked under an optical microscope, and the process was repeated until no algal cells were observed within the solution. It was then vacuum-filtered through a pre-cleaned (4 h, 450°C) Whatman (United Kingdom) glass fiber filter (Grade: GF/F, Ø 47 mm, pore size 0.7 μm). The algal pellet was cleaned a minimum of 10 times by adding Milli-Q water to the pellet, resuspending the cells, centrifuging, and discarding the pellet in order to remove host tissue debris from that fraction. Purity was once again checked under a microscope until no significant contamination was visible. To remove carbonates, acidification was performed by adding 1N HCl (Fluka™ Honeywell, CAS no.: 7647-01-0) droplets to the filter and the algal pellet until no bubble was observed. They were both rinsed with Milli-Q water and dried at 50°C overnight. The surface of the filters containing host tissue was then scraped and homogenized into a fine powder as the algal pellet. Both were weighed into tin capsules before stable isotope analysis.

Stable isotope ratios of carbon and nitrogen in both host tissues and algae were measured with an isotope ratio mass spectrometer (DELTA V Advantage, Thermo Fisher Scientific, United States) coupled in continuous flow with an elemental analyzer (Flash 2000, Thermo Fisher Scientific, United States) at the Institute of Oceanography, National Taiwan University. The results were reported using the common δ notation (i.e., $\delta^{15}N$ and $\delta^{13}C$) in ‰ relative to Vienna Pee Dee Belemnite and atmospheric N_2 for carbon and nitrogen isotopes, respectively. Isotope ratio equation (Coplen, 2011) is as follows:

$$\delta X(‰) = \left[\left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000\right],$$

where X stands for ^{15}N or ^{13}C, and R represents $^{15}N/^{14}N$ or $^{13}C/^{12}C$. USGS40 (L-glutamic acid: $\delta^{13}C = -26.4 \pm 0.1‰$; $\delta^{15}N = -4.5 \pm 0.1‰$) (IAEA, Vienna, Austria) and protein ($\delta^{13}C = -27.3 \pm 0.1‰$; $\delta^{15}N = 6.0 \pm 0.1‰$) are used as certified reference material and internal standard, respectively. Carbon and nitrogen contents were expressed as a percentage of the dry mass (%), and the carbon to nitrogen ratio (C:N ratio) was calculated.

Eventually, total lipid (g gAFDW^{-1}) was measured from the small fragment of the colony previously isolated. This subsample was freeze-dried for one day at −80°C and 40 mbar using a lyophilizer (FD6-4P-D-80°C, Fortelice, Taiwan). The subsample was then ground into a fine powder, from which 90% were used to extract lipids following an adapted methodology of Folch et al. (1957) replacing the chloroform by dichloromethane (Riedel-de Haën™ Honeywell, CAS no.: 75-09-2) in the extracting solution (dichloromethane-methanol, 2:1, v/v). The upper organic layer (containing the lipids) was transferred to an aluminum dish and solvents were allowed to evaporate under a chemical hood. The remaining 10% was used for a measurement of the $AFDW_L$ which was used to standardize lipid contents. Its measurement follows a similar methodology as described above, but using an aluminum dish instead of a filter.

Trait Analysis

A total of 13 quantitative traits were used to document intra- and inter-specific variations (data available the Dryad Digital Repository: https://doi.org/10.5061/dryad.qz612jmd7): (1) OM; (2) chl *a*; (3) chl *c*; (4) ratio chl *c*/chl *a*; (5) algal symbiont; (6) total protein; (7) host $\delta^{15}N$; (8) host $\delta^{13}C$; (9) algae $\delta^{15}N$; (10) algae $\delta^{13}C$; (11) host C:N; (12) algae C:N; (13) total lipid. All traits selected are hypothesized to respond to the local environment, and therefore are relevant in delineating realized species performance.

Trait variations were depicted using violin plots and data were compared between species using Welch *t*-tests, assuming normality but without assumption of the equality of variances among samples. In addition, Levene's tests was used to assess the equality of variances in traits between species. A Pearson's *r* correlation matrix was produced between each pair of trait variables while retaining only individuals with completed observations (see **Supplementary Figure 1**). Substantial correlation was detected using a cut-off at $|r| > 0.8$. A principal component analysis (PCA) was generated on scaled trait variables, and variations among individuals visualized on

a biplot. Intraspecific variations were assessed by multivariate homogeneity of species dispersions and compared between the two species using a permutation test. A permutational multivariate analysis of variance (PERMANOVA) computed on Euclidean distances was used to test for the difference between the two species. R (v 3.6.1,R Core Team, 2019) and the packages "corrplot," "factoextra," "ggplot 2," "ggpubr," "tidyr," and "vegan" were used to analyze the data and produce the figures. The source code for data analysis is available at https://github.com/vianneydenis/stranger-things.git.

RESULTS

Octocorals were confirmed to belong to two distinct genera: *Litophyton* and *Stereonephthya*. Histological sections (**Figures 1C,D**) validated the symbiotic status of both species, with algal cells clearly identified in the endoderm cells (**Figures 1E,F**). *Litophyton* sp. was populated by *Durusdinium* (former clade "D") while *Stereonephthya* sp. was populated by *Gerakladium* algal symbionts (former clade "G") (**Supplementary Table 1**).

Litophyton sp. had significantly higher OM, chl *a*, total lipid, and lower ratio chl *c*/chl *a* than *Stereonephthya* sp. (**Figure 2** and **Supplementary Table 2**). Significantly higher $\delta^{15}N$ values were observed in *Litophyton* sp. animal host and algal fractions compared to *Stereonephthya* sp. (**Figure 3**). It was also the case for $\delta^{13}C$ values of the animal host fraction, but not for the algal fraction in which there was no significant difference between the two species. In addition, $\delta^{13}C$ (Welch's *t*-test: $t = -11.2$, $p < 0.001$) and $\delta^{15}N$ (Welch's *t*-test: $t = -2.30$, $p = 0.04$) in *Stereonephthya* sp. were higher in the algal fraction than in the animal host. In contrast, no significant difference was observed in $\delta^{13}C$ (Welch's *t*-test: $t = -2.05$, $p = 0.06$) and $\delta^{15}N$ (Welch's *t*-test: $t = 0.1$, $p = 0.89$) between the algal and host fractions in *Litophyton* species. Other traits such as chl *c*, algal symbiont, total protein contents, and C:N ratios did not differ between species. The chl *a* by cell was also significantly higher in *Litophyton* sp. than in *Stereonephthya* sp. (Welch's *t*-test: $t = 5.2$, $p < 0.001$, inset **Figure 2E**). *Litophyton* sp. had a significantly higher variance in chl *a* (Levene's test: $F = 6.46$, $p < 0.05$) and algae $\delta^{13}C$ (Levene's test: $F = 13.28$, $p < 0.01$) values than *Stereonephthya* species. For host C:N, significantly higher variances were observed in *Stereonephthya* sp. than in *Litophyton* sp. (Levene's test: $F = 6.00$, $p < 0.05$). The variances in other selected traits did not significantly differ between the two species.

Substantial correlations (**Supplementary Figure 1**) identified positive relationships between the $\delta^{15}N$ in host and algae (Pearson's $r = 0.91$, $p < 0.001$), the chl *a* and *c* (Pearson's $r = 0.84$, $p < 0.001$), and the host $\delta^{15}N$ and total lipid (Pearson's $r = 0.82$, $p < 0.01$). A negative relationship was detected between the ratio chl *c*/chl *a* and host $\delta^{15}N$ (Pearson's $r = -0.82$, $p < 0.001$).

The PCA further displayed the variation in individuals for the 13 traits (**Figure 4**). The first two principal components explained up to 72.2% of the variation observed among individuals, with a clear distinction between the species in the first two dimensions. Intraspecific variations calculated as the dispersion of the species performance niches significantly differed between the two species (Permutation test, $F = 9.7$, $p < 0.05$). The multivariate pattern observed in **Figure 4** and the significance of the PERMANOVA test ($F = 8.3$, $p < 0.01$) further supported a significant difference in how the two species performed, which seemed to be mainly driven by traits differentiating species in their dependence to photosynthetic energy.

DISCUSSION

This study demonstrated that two targeted species are associated with singular Symbiodiniaceae. Traits measured in the partnerships discriminated two distinctive groups supporting contrasting performances. Individual trait variation illustrated differences in species dispersion, which further suggests contrasting niche occupation in these two octocorals living in sympatry in a high-latitude coral community of northern Taiwan.

The genus *Stereonephthya* is described as an azooxanthellate genus (Fabricius and Alderslade, 2001). However, in the present study, all *Stereonephthya* sp. colonies were associated with *Gerakladium* algal symbionts (former clade "G"). A similar partnership was reported with a single unidentified *Stereonephthya* colony from the Great Barrier Reef (Van Oppen et al., 2005). This association could be commonly occurring in colonies from northern Taiwan, yet its seasonality cannot be excluded and the possibility that this association could be facultative should be further examined. In any case, although intrageneric variation in photosymbiosis is observed in octocorals (e.g., Williams et al., 2010; Aurelle et al., 2017), this proves to be the first confirmed case of an Alcyoniina genus that either exists symbiotically or asymbiotically with Symbiodiniaceae. The association with *Gerakladium*, a rare taxon for which there is still limited ecological information (LaJeunesse et al., 2018), is also noteworthy. These Symbiodiniaceae were previously found to be associated with some foraminifera (genus *Marginopora*) (Pochon et al., 2001), excavating sponges (genus *Cliona*) (Hill et al., 2011), and a black coral (genus *Cirrhipathes*) (Bo et al., 2011). Our study confirmed its prevalence in one octocoral species, *Stereonephthya*. Also of interest is the association of *Litophyton* sp. with *Durusdinium* algal symbionts (former clade "D"), whereas previous studies documented this genus in association with *Symbiodinium* (former clade "A") (Barneah et al., 2004; Pupier et al., 2019). *Durusdinium* is not unusual in octocorals (Van Oppen et al., 2005), but its occurrence here associated with *Litophyton* sp. could be related to the environmental conditions of northern Taiwan. For instance, some microalgae symbiotic with scleractinians are known to be extremophile presenting adaptations to survive in regions with large temperature and turbidity fluctuations (LaJeunesse et al., 2018). When applied to octocorals, elucidating the ecological significance of both Symbiodiniaceae requires additional *in situ* observations and specific laboratory experiments which are beyond the aim of our study.

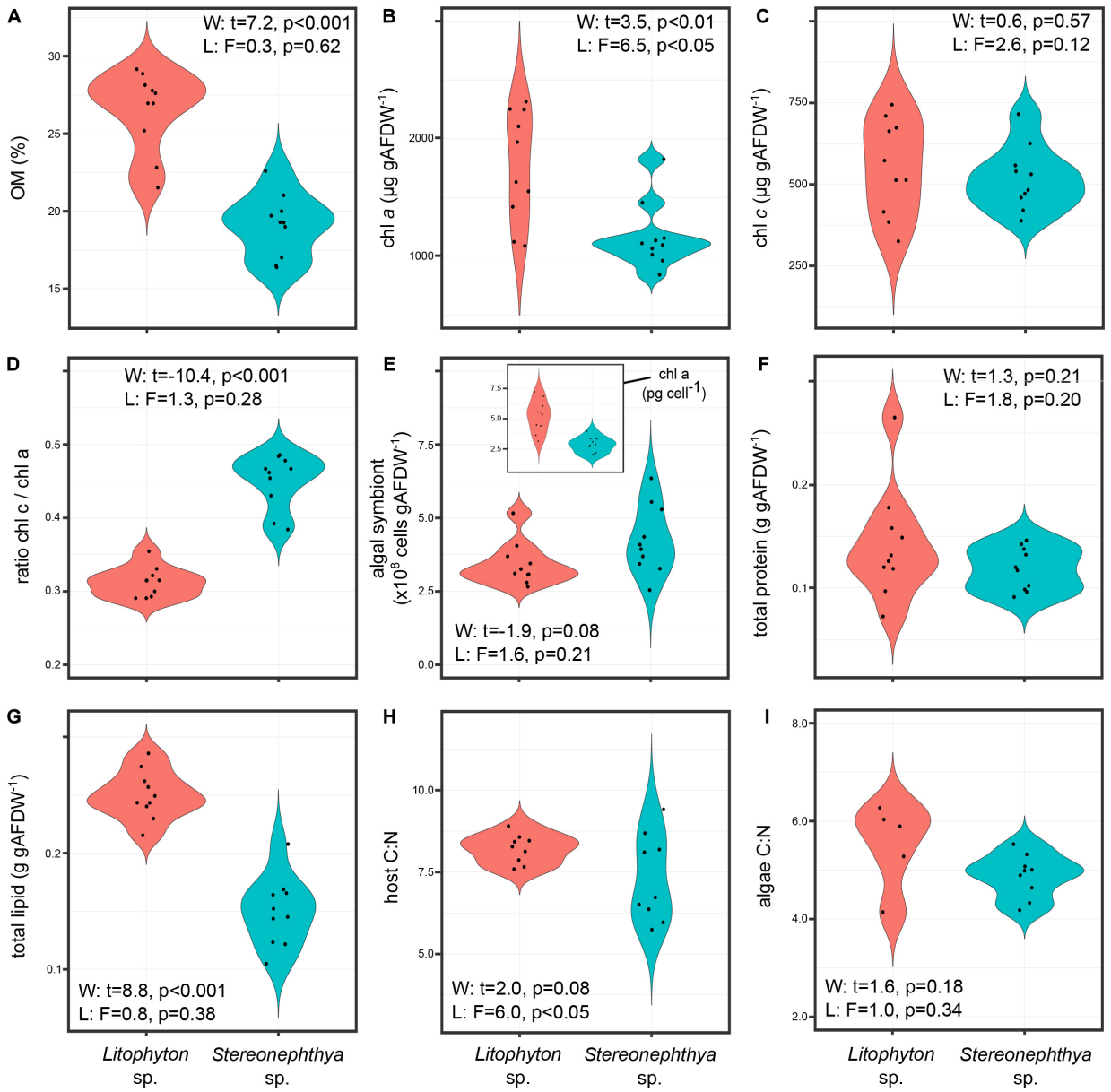

FIGURE 2 | Comparison of the distribution in individual trait values for the two study species: **(A)** OM, **(B)** chl *a*, **(C)** chl *c*, **(D)** ratio chl *c*/chl *a*, **(E)** algal symbiont content – ***inset*** content of chl *a* by algal symbiont cell, **(F)** total protein, **(G)** total lipid, **(H)** host C:N; **(I)** algae C:N.

Litophyton sp. had higher OM, total lipid, chl *a*, and lower chl *c*/chl *a* ratio than *Stereonephthya* species. Those differences were accompanied by higher host tissue $\delta^{13}C$ and $\delta^{15}N$ in *Litophyton* sp. than in *Stereonephthya* sp., while only algae $\delta^{15}N$ was significantly higher in *Litophyton* sp. ("*Durusdinium*" associated) than in *Stereonephthya* sp. ("*Gerakladium*" associated). In a shallow Caribbean reef lagoon (<3 m, Puerto Morelos, Mexico), Gorgoniidae such as the sea fan, *Gorgonia ventalina* (58 ± 4%), or the sea rods, *Antillogorgia bipinnata* (44 ± 2%) and *Antillogorgia americana* (57 ± 1%), presented higher OM contents (Rossi et al., 2018) than those reported here in the two targeted Alcyoniidae (27 ± 3% and 19 ± 2% in *Litophyton* sp. and *Stereonephthya* sp., respectively). In contrast, our values were higher than those reported on other Gorgoniidae sea whip (*Pterogorgia citrina*, 17 ± 0.2%) or Plexauridae sea rods such as *Eunicea* sp. (13 ± 1%), *Eunicea tourneforti* (12 ± 0.4%), and *Plexaurella nutans* (15 ± 0.3%). However, records of higher values in the Mediterranean Plexauridae sea whip, *Paramuricea clavata* (40%, Rossi et al., 2006), and similarities between OM content in our bushy *Litophyton* sp. with the sea rod, *Eunicea mammosa* (27 ± 1%, Rossi et al., 2018), suggest that neither the morphology nor the phylogenetic information represent a reliable proxy of the OM content in octocorals. Therefore, in contrast with previous hypotheses

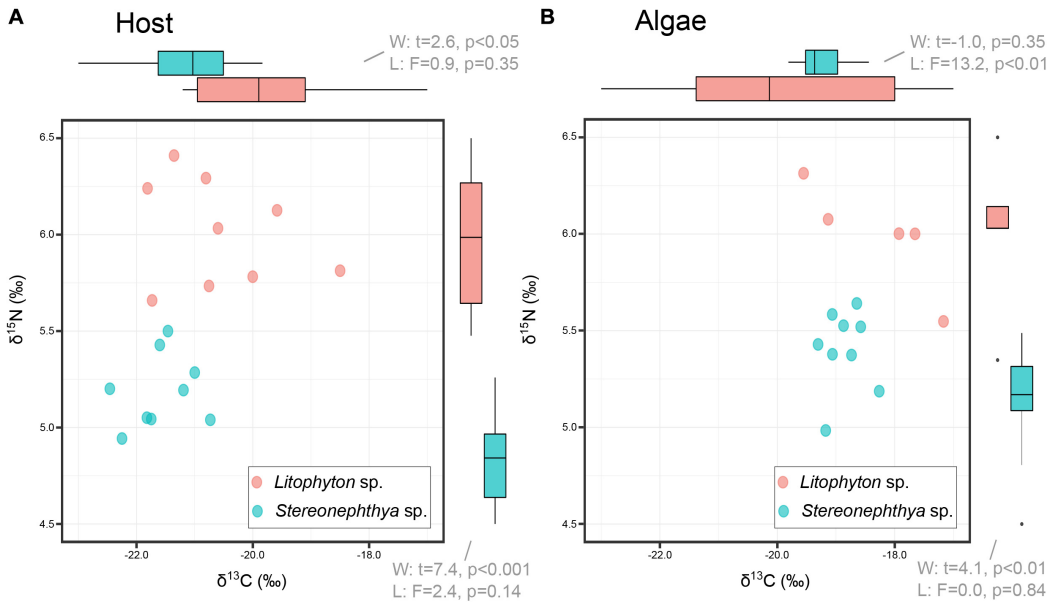

FIGURE 3 | Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) comparison between octocoral hosts **(A)** and their associated algae **(B)**. Bi-plots only display full case samples, while boxplot in margins incorporates all measurements available for the variable of interest.

(Rossi et al., 2018), we suggest that OM content is holobiont-specific while varying to some extent with the changes in environmental conditions. Lipids (0.25 ± 0.02 g $gAFDW^{-1}$ and 0.15 ± 0.03 g $gAFDW^{-1}$ in the *Litophyton* and *Stereonephthya*, respectively) were within the same range of content reported in Mediterranean *P. clavata* (0.12–0.32 g $gAFDW^{-1}$, Rossi et al., 2006). Converted to contribution to the whole-body tissue, lipid concentrations were 7 ± 1% and 3 ± 1% of the DW in our two species, respectively. These concentrations are below values reported in sexually mature colonies of *Heteroxenia fuscescens* in the northern Red Sea (11 ± 4%, Ben-David-Zaslow and Benayahu, 1999). Protein contents did not differ between species (0.14 ± 0.05 g $gAFDW^{-1}$ and 0.12 ± 0.02 g $gAFDW^{-1}$ in *Litophyton* and *Stereonephthya*, respectively), yet values reported here were lower than those in horny colonies of *P. clavata* in which stem and branches are stiffened by gorgonin. Reported to whole-body tissue contribution, proteins represent 37 ± 10% and 22 ± 3% of the DW in *Litophyton* and *Stereonephthya*, respectively. This was higher compared to the aforementioned fleshy *H. fuscescens*, for which proteins averaged 19 ± 6% (Ben-David-Zaslow and Benayahu, 1999). Both lipid and protein contents may vary seasonally with reproductive cycles and abiotic features of the water (Ben-David-Zaslow and Benayahu, 1999). However, to date, no information exists about the reproduction of the two Alcyoniidae in northern Taiwan. Algal symbiont contents (3.4 ± 0.7 × 10^8 cells $gAFDW^{-1}$ and 4.3 ± 1.2 × 10^8 cells $gAFDW^{-1}$ in *Litophyton* and *Stereonephthya*, respectively) were high compared to Gorgoniidae and Plexauridae species previously examined in the Caribbean (Rossi et al., 2018). Of nine species, only one unidentified *Eunicea* sp. presented comparable contents in algal symbionts (3.9 ± 0.6 × 10^8 cells $gAFDW^{-1}$). Values observed were in the range of the ones reported in the other Alcyoniidae (*Rhytisma fulvum fulvum*) at shallow (−8 m) and mesophotic (−40 m) depths from the northern Red Sea (Pupier et al., 2019). However, Pupier et al. (2019) also documented contents 2.7 times (9.4 × 10^8 cells $gAFDW^{-1}$) and 1.8 times (6.4 × 10^8 cells $gAFDW^{-1}$) for an unidentified *Litophyton* populating both depths, respectively. In the latter species, total chlorophyll ($a + c_2$) exhibited lower concentrations in shallow waters (1,591 μg $gAFDW^{-1}$) than in deeper waters (2,240 μg $gAFDW^{-1}$, Pupier et al., 2019). Here, total chlorophyll concentrations differed in *Litophyton* sp. (2,321 ± 613 μg $gAFDW^{-1}$) and *Stereonephthya* sp. (1,684 ± 373 μg $gAFDW^{-1}$), but both ranged within values previously reported among depths [although it is worthwhile to mention that that the concentration of the extracting solvent differed: 100% acetone in Pupier et al. (2019) vs. 90% in the present study]. These values are also within the ranges of values previously documented in species of Gorgoniidae and Plexauridae.

The chl *a* content in the Red Sea (−4 m, northern Gulf of Eilat) *Litophyton arboretum* presented intracolonial difference and increased under low light (Berner et al., 1987). Both light-acclimated (0.9 ± 0.1 pg $cell^{-1}$) and shade-acclimated (2.2 ± 0.5 pg $cell^{-1}$) areas showed lower concentration of chl *a* per cell than the two species in the present study (5.2 ± 1.3 pg $cell^{-1}$ and 2.8 ± 0.6 pg $cell^{-1}$ in *Litophyton* and *Stereonephthya*, respectively), which may infer weaker light intensity at −10 m at Bitou – while the influence of other factors such as the nutrient availability (e.g., Courtial et al., 2018) and/or differential algal symbiont content (e.g., Scheufen et al., 2017) cannot be entirely ruled out. Similarly, *Sinularia flexibilis* showed an increase of chl *a* with the attenuation of light from 1,000 to 100 μmol quanta m^{-2} s^{-1} (Khalesi et al., 2009). In

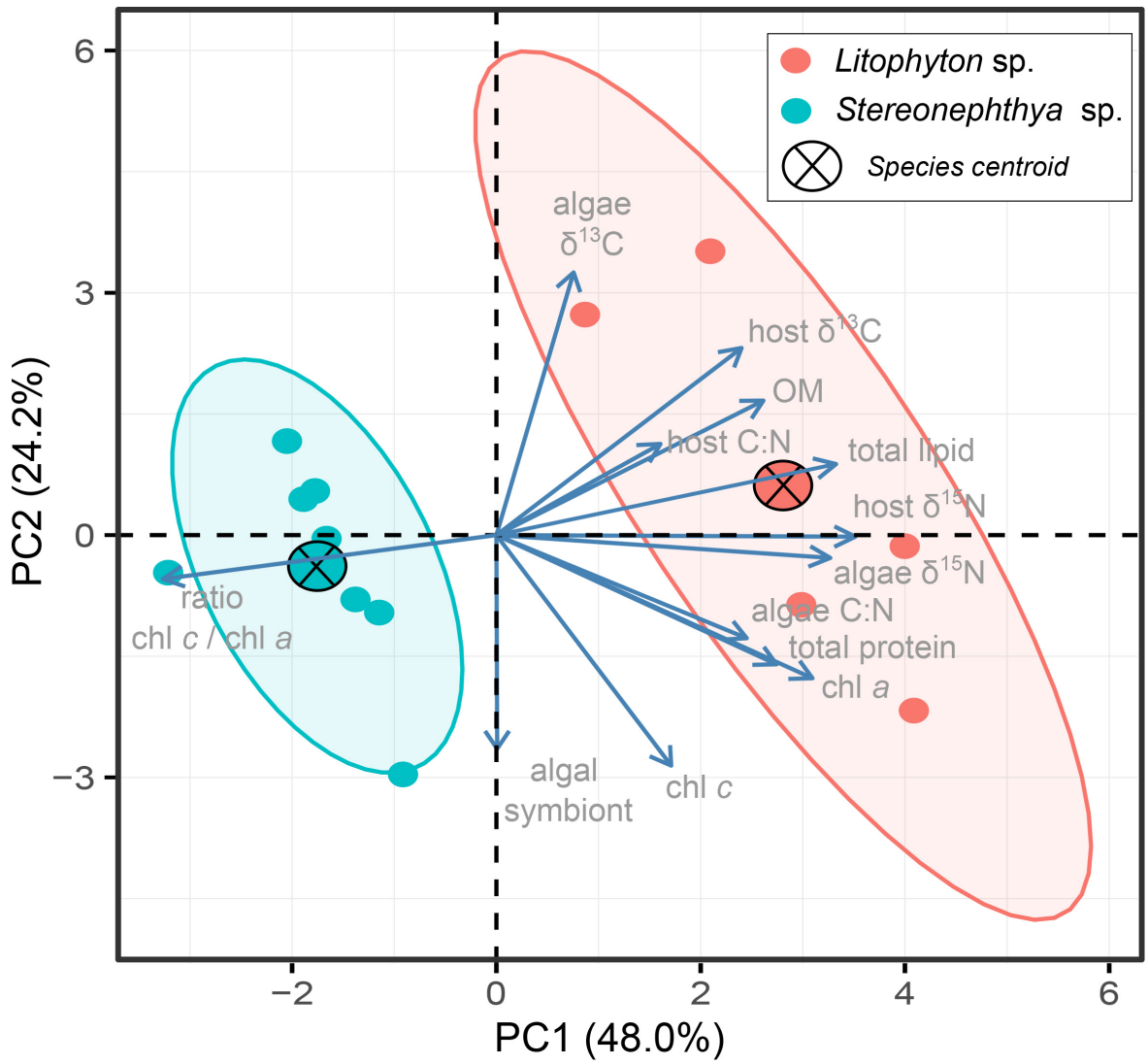

FIGURE 4 | PCA biplot displaying multivariate (trait) variation among individuals. The first two dimensions explained up to 72.2% of the variance observed. Species centroids are displayed, and ellipses are shown with an 80% confidence level for the two species.

the scleractinian coral *Stylophora pistillata*, the increase of chl *a* content in algal symbiont cells with depth represents another well documented example of photoacclimation to low light habitat (Falkowski and Dubinsky, 1981; Dubinsky et al., 1984; Mass et al., 2010), and this could also be the case here in northern Taiwan (Denis et al., 2019).

Ratio chl *c*/chl *a* was significantly higher in *Stereonephthya* sp. ("*Gerakladium*" associated) (0.45 ± 0.04) than in *Litophyton* sp. ("*Durusdinium*" associated) (0.31 ± 0.02). These values were in the higher range of values reported in Caribbean octocorals (Rossi et al., 2018), suggesting a possible chromatic adaption (quality of light) of algal symbionts to acclimatize to predominantly short-wavelength, blue-light habitat. For instance, this hypothesis was suggested for the scleractinian coral *Leptoseris fragilis* (Kaiser et al., 1993) but it, generally, remains overlooked in the interpretation of coral photo-acclimatization (Nir et al., 2011). However, this could be a dynamic pattern, as two of the species examined by Rossi et al. (2018) also presented a lower chl *c*/chl *a* ratio when sampled at the same location near Puerto Morelos reef lagoon (Ramsby et al., 2014). Algal symbiont cells in hospite being less sensitive to the quality of the light spectra than in isolation, an alternative explanation for this increase would be the total amount of light received by the algal symbionts (quantity of light) that enlarges the size of the photosynthetic antenna to enhance light harvest in low light habitats. Both hypotheses are likely to be holobiont specific [see Reynolds et al. (2008) for the differences in photoprotection among Symbiodiniaceae genera], and the photo-physiology of both partnerships should further be scrutinized in detail in future studies.

Although C:N ratio can represent a reliable proxy of lipid content in marine organisms (e.g., in crabs, Bodin et al., 2007),

this was not true of the two species examined here, which further presented similar ratios in both the host and the algal fractions. The $\delta^{13}C$ values of hosts suggested slight differences in carbon resource use. Coral host $\delta^{15}N$ values were more positive for *Litophyton* sp. than for *Stereonephthya* sp. ($\sim 1‰$), indicating a difference in their trophic positions. For both species, since the $\delta^{15}N$ values of the host and algal tissues are similar, the differential utilization of nitrogen by the symbionts that would have been translocated to the host seems limited. The most likely hypothesis is the feeding of the larger size-fractions of phytoplankton and zooplankton by *Litophyton* sp. and a probable greater contribution of detritus or small size phytoplankton for *Stereonephthya* species. Several studies have already demonstrated a depletion in $\delta^{15}N$ values with decreasing plankton size (Bănaru et al., 2013). Further, stable isotope values observed are representative of scleractinian coral hosts that have acquired carbon and nitrogen by heterotrophic feeding of suspended particulate OM (generally around −22 to −24‰ for carbon; e.g., Muscatine et al., 1989; Ferrier-Pagès et al., 2011). The same range of $\delta^{13}C$ values were reported in tissues from non-symbiotic Mediterranean octocorals (−19 to −24‰; Cocito et al., 2013). The clear differences between host and symbiont $\delta^{13}C$ values ($\Delta^{13}C = \sim -3‰$) indicate clear deviations from an autotrophic diet (Fox et al., 2018). Although heterotrophy was the main energy source of certain gorgonian species (Baker et al., 2015), findings show that autotrophic nutrition had great importance in octocorals (Rossi et al., 2020). The $\delta^{13}C$ range of *Litophyton* sp. suggests higher trophic plasticity than for *Stereonephthya* sp., indicating diversification of basal resources with varying $\delta^{13}C$ values. *Litophyton* sp. seems to vary its use of heterotrophic nutrient sources, but may also use the autotrophic pathway. In scleractinians corals, facultative associations are commonly associated with parasitic behavior (Lesser et al., 2013). Therefore, it could here explain why *Gerakladium* does not appear to contribute to the diversification of *Stereonephthya* diet. However, the paucity of information available on this Symbiodiniaceae lineage prevents further discussion. A more detailed view of the trophic ecology of these two partnerships will require additional samples from various habitats and conditions (e.g., reproduction period; Rossi et al., 2020), as well as from all their possible food sources. Those data, together with insight from ^{13}C-labeling experiment (e.g., Ezzat et al., 2017), might further support evidence of niche partitioning that may explain the coexistence of these two phylogenetically and morphologically similar species.

Altogether, the examined traits elucidated two distinct groups which could be further explained by both partnerships considered. They illustrated contrasting behaviors where *Litophyton* sp., in association with *Durusdinium* algal symbionts, appears to complement its heterotrophic diet with slight autotrophically acquired energy. This diversification was reflected among individuals of *Litophyton* with chl *a* and $\delta^{13}C$ measured in the algal fraction presenting higher variance than *Stereonephthya* colonies. This variation may have further relevance at a biogeographical level and/or within the *Litophyton* genus. Pupier et al. (2019) typified a Red Sea *Litophyton* characterized by its high ratio between photosynthetically acquired carbon and respiration. In contrast, *Stereonephthya* sp. seems to be much more specialized and may only receive limited benefit from its symbiotic relationship with *Gerakladium*. Partnership performance niches are clearly distinctive in terms of their positions and sizes, which could be of major importance in individuals naturally selected through exposure to stressors. Interestingly, recent bleaching survey (August 2020) revealed that *Litophyton* sp. was virtually the only species affected −10 m at the study site. All colonies were severely bleached while no sign of mortality was recorded (V. Denis, personal observation). Quantification of performance traits of both species in variable (extreme) conditions would lead to a better understanding of their individual variation and physiological flexibility. Although species niche delineation will eventually require a detailed examination of the temporal and spatial variation in traits and partnerships, our study already paves the way for the integration of the intraspecific variance in the definition of octocoral strategies.

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 in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication. VD conceived and designed the experiments. T-HH, LC, YH, T-YL, C-WW, C-YH, S-HY, and P-LW performed the experiments. T-HH and LC analyzed the data. T-HH, LC, NS, and VD wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.606601/full#supplementary-material

Supplementary Figure 1 | Pearson correlation matrix of trait values. Correlation coefficients (*r*) are provided with a color scale indicating either a highly positive (r = 1) or negative (r = −1) correlation. Only significant and substantial correlations with a $|r| > 0.8$ were mentioned in the text. Non-significant coefficient ($p < 0.05$) is shown crossed.

Supplementary Table 1 | Samples examined for the identity of dominant algal symbiont and accession numbers for the corresponding sequences deposited in GenBank.

Supplementary Table 2 | Mean, standard deviation (SD), coefficient of variation (CV), maximum (Max), and minimum (Min) values of the 13 traits and chl *a* content by symbiont algal cell in the two octocoral species. Significant differences detected by Welch *t*-tests are indicated by level of significance $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$; details on statistics in **Figures 2**, **3** (English editing).

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BRIEF RESEARCH REPORT
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The Significance of Genotypic Diversity in Coral Competitive Interaction: A Transcriptomic Perspective

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Competitive interactions shape coral assemblages and govern the dynamics of coral ecosystems. Although competition is an ecological concept, the outcomes of competitive interactions are ultimately determined by patterns of gene expression. These patterns are subject to genotypic variation on both sides of any interaction. Such variation is typically treated as "noise", but it is sometimes possible to identify patterns within it that reveal important hidden factors in an experiment. To incorporate genotypic variation into the investigation of coral competitive interactions, we used RNA-sequencing to study changes in gene expression in a hard coral (*Porites cylindrica*) resulting from non-contact competition experiment with a soft coral (*Lobophytum pauciflorum*). Hard coral genotype explained the largest proportion of variation between samples; however, it was also possible to detect gene expression changes in 76 transcripts resulting from interaction with the soft coral. In addition, we found a group of 20 short secreted proteins that were expressed as a coordinated unit in three interacting *Porites-Lobophytum* pairs. The presence of this secretion response was idiosyncratic in that it could not be predicted based on polyp behaviour, or the genotype of hard or soft coral alone. This study illustrates the significance of individual variation as a determinant of competitive behaviour, and also provides some intriguing glimpses into the molecular mechanisms employed by hard corals competing at a distance.

Keywords: coral competition, coral behaviour, gene expression, allelopathy, hard coral

INTRODUCTION

Competition is an important ecological interaction, especially in highly productive tropical systems such as rainforests and coral reefs where it is a driver of ecosystem dynamics (Connell et al., 2004; Álvarez-Noriega et al., 2018). Competition also plays an important role in determining the impacts of climate change and other anthropogenic impacts on these systems. On coral-reefs, where hard corals (Scleractinia) have historically been dominant, many locations have seen shifts in species composition in favour of other reef taxa such as macroalgae (Roff and Mumby, 2012), octocorals

(Lenz et al., 2015; Lasker et al., 2020), zoanthids (Cruz et al., 2016), and sponges (Bell et al., 2013). These shifts may themselves result from altered competition between reef taxa, and on non-Scleractinia dominated reefs the frequency of interactions between scleractinians and other major reef taxa is increased (Ladd et al., 2019). Even on reefs where Scleractinia still dominate, the role of competition, and the nature of competitive hierarchies is changing (Horwitz et al., 2017; Johnston et al., 2020), leading to shifts in the structure and function of reef communities.

The effects of competitive interactions between reef taxa are challenging to measure because the outcomes of competition play out slowly, are not strictly hierarchical (Precoda et al., 2017) and can reverse over time (Bak et al., 1982). In addition, most field surveys and experiments to date have relied on visible signs to detect competitive interactions and determine their order of dominance. Visible competitive strategies of corals include: overtopping to starve competitors of light; deployment of mesenteric filaments to externally digest a competitor; and elongation of polyps or development of sweeper tentacles to enable contact followed nematocyst discharge [reviewed by Lang and Chornesky, 1990; Chadwick and Morrow, 2011; Yosef et al., 2020). Although these physical signs are reliable indicators of competition when competitors are in contact, it is now clear that a wide range of reef taxa including scleractinian corals, octocorals, sponges, and algae (Coll and Sammarco, 1983; Sammarco et al., 1983; Fearon and Cameron, 1996; Koh and Sweatman, 2000; Chadwick and Morrow, 2011) all produce toxins that could mediate competitive interactions without close contact. Despite abundant evidence of non-contact competitive capability in a variety of reef taxa, research on competitive strategies in corals has overwhelmingly focussed on interactions that involve contact (Chornesky, 1983; Sebens and Miles, 1988; Tanner, 1995; Fleury et al., 2004; Shearer et al., 2012). This bias could lead to underestimates of key competitive interactions, especially those for which non-contact competition is the primary mode.

Molecular techniques such as transcriptomics and metabolomics have the potential to resolve key gaps in our understanding of competition between reef taxa but have so far seen little use in coral competition research (except: Shearer et al., 2012, 2014; Quinn et al., 2016). Importantly, these techniques can directly measure molecules involved in both defensive and aggressive responses to competition and can therefore be employed to study non-contact interactions or interactions that do not generate clear physical effects. This is underscored by the results of Shearer et al. (2012) who studied molecular responses of *Acropora millepora* to four species of macroalgae and found the greatest change in gene expression in a competitive regime that showed the least physiological evidence of competitive impact. In addition, molecular analyses may reveal the mechanisms that underpin individual variation in competitive outcome that have been shown to exist between and within species. Interspecific competitive outcomes between reef taxa can be difficult to predict, with highly idiosyncratic dominance relationships between individual species pairs (Precoda et al., 2017). Dominance relationships may also depend on variations in genotype or physiological state of individual competitors. Although this has not been explicitly explored in the context of competition, evidence from molecular studies across a range of other extrinsic factors suggests that such intraspecific variation in response to stressors is likely to be high (Marshall and Baird, 2000; Loya et al., 2001; Obura, 2001; Fitt et al., 2009; Hughes et al., 2017; Sekizawa et al., 2017; Wright et al., 2017).

In this study we explore the transcriptomic response of *Porites cylindrica*, a hard coral, to competition with *Lobophytum pauciflorum*, a soft coral. The experiment was designed to investigate non-contact competition, although, as we show below, some limited contact via elongated mesenterial filaments was also observed. Since both coral types can be fragmented, we were able to pair each genotype of *Porites* with five genotypes of the competing soft coral. This design allows us to describe a core molecular response, which appears to be consistent across competing pairs, as well as a more specialised response involving up-regulation of secreted proteins that is restricted to a subset of competing pairs.

MATERIALS AND METHODS

Competition Experiment

Molecular and behavioural responses to non-contact competition were investigated using an experiment conducted at Orpheus (Goolboddi) Island Research Station, in the central Great Barrier Reef, Australia (18'34 'S; 146'29'E). Five colonies of the soft coral *Lobophytum* and 18 nubbins (∼3 cm) from each of three colonies of the hard coral *Porites* were collected with a bonecutter from reefs around Orpheus Island (GBRMPA Permit No. G16/38499.1). The *Porites* nubbins were fixed onto ceramic tiles. Each soft coral colony was cut into 12 pieces containing one or two lobes/fingers (∼5 cm) and these were placed on top of the tiles but not attached. The hard and soft coral fragments were then allowed to recover for three weeks prior to the start of the experiment.

After the acclimatization period, corals were placed in tanks (1,300 ml, open system, 400 ml/min of 10 μm filtered sea water) for 60-days. In each tank, a soft and a hard coral piece were placed purposely <3 cm apart to prevent corals touching each other, and simulate a non-contact interaction from the start of the experiment, while isolated hard and soft corals were used as controls (**Figure 1A**). This pair-wise design was built with five biological replicates of the soft coral (colonies: La, Lb, Lc, Ld, and Le) and three biological replicates of *Porites* (colonies: Pd, Pe, and Pf). Combinations will be referred to by listing the hard coral followed by the soft coral (e.g., Pd–La) while controls are denoted with a C (e.g., Pd–C). In total, the experiment was composed of 15 biological combinations of interacting corals (e.g., Pd-La and Pe-La), and 3 hard coral controls (Pd-C, Pe-C, and Pf-C). For each combination and control there were two technical replicates/clones, resulting in a total of 36 experimental tanks.

Collection and Analysis of *Porites* Behavioural Data

Behavioural observations were recorded throughout the competition experiment to determine if *Porites* interacting with

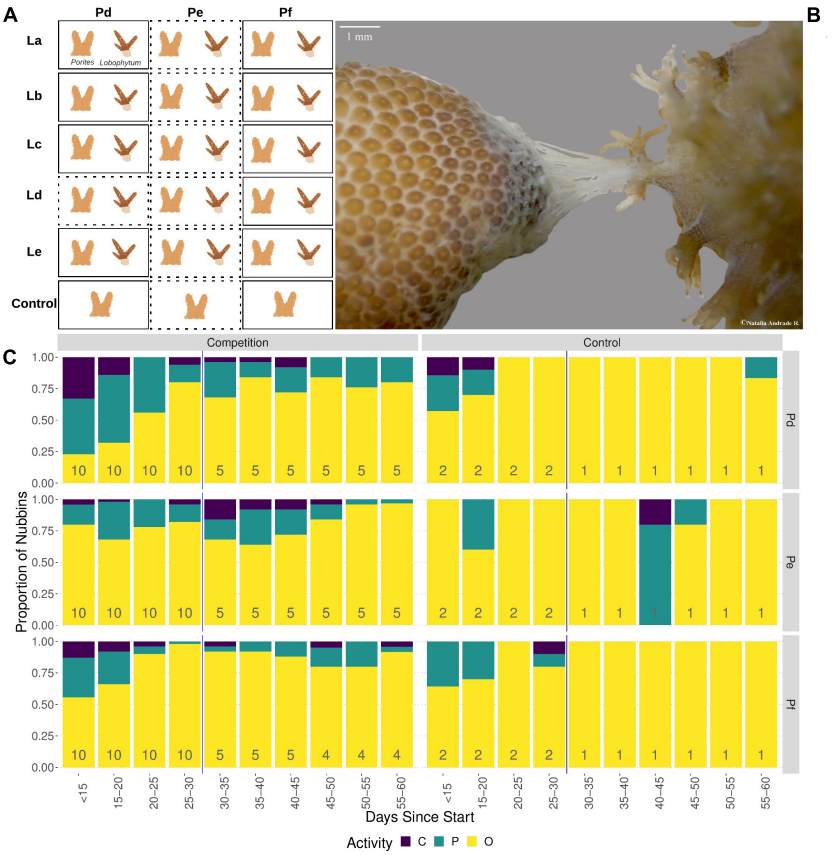

FIGURE 1 | Behaviour of *Porites* in the presence of *Lobophytum*. **(A)** Experimental design showing pairing of three competing *Porites* colonies (columns) with the five *Lobophytum* (rows). Dotted rectangles indicate samples not included in differential expression analysis; **(B)** Photograph (OLYMPUS TG-3, focal length 18 mm) showing *Porites* Pf (right) attacking *Lobophytum* (left) with mesenteric filaments at interaction day 50. **(C)** Barplot showing variation in polyp activity over time. Each bar shows relative counts of nubbins in each of the three activity states: black representing closed polyps, green partially open and yellow open polyps. Total numbers of nubbins contributing to each bar are shown at its base. The activity state represented per nubbin corresponds to the average of the three polyp activities observed per day. Reduction of number of the nubbins after day 30 corresponds to sampling time point of a technical replicate of each genotypic pair.

Lobophytum were showing signs of competitive behaviour or if their polyp activity was affected by the interaction. *Porites* polyp activity and competitive behaviour were observed three times per day to avoid bias due to highly variable diel polyp activity patterns (Levy et al., 2006). Observation times were between: 8 am–11 am, 12 pm–4 pm, and 6 pm–9 pm. Polyp activity was categorized as open, partially open, or closed. **Supplementary Figure 1** illustrates the different states of polyp activity. Then, the observations were summarized to a majority consensus value (open, partially open, or closed) using the key shown in **Supplementary Table 1**. Polyp activity measurements were taken starting from day eight of the experiment and continuing until day 60.

In addition to basic polyp activity, competitive behaviour of *Porites* towards *Lobophytum*, such as elongated polyps (Sammarco et al., 1985; Rinkevich and Sakamaki, 2001) and/or mesenteric filaments, was also recorded.

These data were analysed using a cumulative link mixed effect model (clmm) with the package "ordinal" (Christensen, 2015) in the statistics program R (R Core Team, 2016), to determine if competition affected *Porites* polyp activity. A range of models were explored within this framework and the most parsimonious was selected on the basis of AIC[1]. The final model (Eq. 1) included polyp activity (Activity) as an ordered factor (Closed < Partially-open < Open) dependent on the following fixed effects; time categorized in eight groups of ∼5 days each (Time), the *Porites* colony the nubbin came from (Colony) and the nubbin's treatment (competition or control) (Treatment). In addition, tank was modelled as a random effect (Tank).

$$Equation\ 1 : Activity \sim Time + Colony + Treatment \mid Tank$$

RNA Sequencing, Assembly and Transcript Quantification

Porites nubbins from one of the two replicates in each experimental condition were randomly sampled for RNA sequencing after 30 days of interaction with *Lobophytum* to determine the effects of competition on *Porites* gene expression.

[1]https://github.com/China2302/Porites_competition/blob/master/03_polyp_activity_exploration.md

Samples were taken by quickly crushing the nubbin with a hammer and immediately, snap-freezing it in liquid nitrogen. Samples were stored at −80°C until required. RNA was extracted with TRIzol Reagent (Ambion, catalogue#15596-026). RNA quality checks and library preparation were performed as described in supplementary methods. High-quality RNA extractions were obtained for nubbins from colonies of *Porites* Pd and Pf. It was not possible to extract RNA from nubbins of colony Pe, therefore 12 samples (10 nubbins in competition and two nubbins in control) from colonies Pd and Pf were sequenced. Samples were sequenced by the Australian Genome Research Facility (AGRF: Melbourne, Australia) using an Illumina HiSeq2500, to obtain approximately 14.5 million reads (100 bp paired-end) per sample. Reads were checked for quality, adapter content and other sequencing artefacts using FastQC (version 0.11.9). All sequencing data have been deposited with Genbank under bioproject (PRJNA706467).

A *de novo* transcriptome assembly for *Porites* was constructed by adapting evidence-based best practices (MacManes, 2016) to deal with data from two distinct genotypes (Pd and Pf). Data for each genotype were processed separately for read error correction with Rcorrector (Song and Florea, 2015), followed by initial assembly with Trinity (version2.4.0; Grabherr et al., 2011) using options to enable read trimming and normalization. Independent assemblies produced by this process were then merged together using the software TransFuse (version0.5.0[2]) with a 95% identity threshold for merging clusters. The merged transcriptome was analysed with the software TransRate (version1.0.3; Smith-Unna et al., 2016), which scores contigs based on agreement with mapped raw reads. All high quality (called "good" by TransRate) contigs retained from this process were subjected to analysis with software Psytrans[3] to remove those likely to originate from algal symbionts (family Symbiodiniaceae). The completeness of the clean assembly was assessed with the software BUSCO version4 (Simão et al., 2015).

To assess the effectiveness of Psytrans, and to check for additional contaminating organisms, we performed two additional analyses on the transcriptome data. Firstly, diamond blastx (version 2.0.7.145; Buchfink et al., 2015) was used to identify the best match and its corresponding phylum of origin in the NCBI nr database (*E*-value < 0.01 in very-sensitive mode) for all transcripts remaining after processing with Psytrans. Secondly, the lowest common ancestor was inferred for all reads using kraken (version 1; Wood and Salzberg, 2014) for each sample. See supplementary methods for detailed information on construction of the kraken database.

Corrected reads (from Rcorrector) were first trimmed using Trimmomatic (version 0.36) and then mapped to the final transcriptome assembly using Bowtie2 with recommended settings (end to end alignments, report all alignments, min alignment score 0.3) to suit downstream quantification. Corset (version1.05; Davidson and Oshlack, 2014) was then used to cluster transcripts likely to have originated from the same gene and count reads assigned to clusters.

Results from Corset were used to identify a set of 17,093 transcripts suitable for genetic analysis. These transcripts were expressed with at least three reads in all samples and belonged to a singleton cluster (likely a single gene copy with no alternative splicing variants). Analysis of mapped reads for these contigs with ANGSD (Korneliussen et al., 2014) revealed 30,289 SNPs which were then used to calculate the relatedness statistic, theta between each pair of samples with ngsRelate (Hanghøj et al., 2019). This analysis confirmed that the two colonies used for sequencing (Pd and Pf) were unrelated to each other (distinct genotypes; theta = 0) while individual fragments from a single colony were clones (theta = 0.5).

Preliminary differential expression analysis with DESeq2 (Love et al., 2014) revealed a small set of clusters that were exclusively expressed in three samples and that appeared to be of barnacle origin (>90% sequence similarity to barnacle transcripts, via blastx to the NCBI non redundant protein database). In order to identify and remove all barnacle clusters, minimap2 (Li, 2018) was used to map all transcripts to a genomic database consisting of the *Porites lutea* (Robbins et al., 2019), *Cladocopium goreaui* (Liu et al., 2018), and *Amphibalanus amphitrite* genomes (Kim et al., 2019). Mapping was performed using the xsplice option to allow gapped alignment and up to 10% sequence divergence. Any transcript that produced a valid alignment to the barnacle (*Amphibalanus amphitrite*) genome was deemed to be of barnacle origin. This resulted in identification of 2,297 transcripts and 492 clusters likely to be of barnacle origin, the expression of which was almost entirely restricted to three samples (Pf-Lc, Pd-Le, and Pd-La) (**Supplementary Figure 2**). Although all *Porites* nubbins were subject to frequent visual inspection throughout the experiment, it is likely that these barnacle reads originated from commensal, coral associated barnacles (Tsang et al., 2014), which tolerate overgrowth by the coral skeleton and coexist with the coral (Liu et al., 2016). Our analysis assumes that this relationship (likely commensal) had minimal effect on gene expression in the affected nubbins.

After removal of all barnacle clusters the gene expression profiles of barnacle-affected samples clustered together with other samples suggesting that the effect of barnacles on host gene expression was minimal. We therefore retained barnacle-affected samples for further analysis.

Statistical analysis of cleaned gene expression data was performed using DESeq2 with the goal of detecting transcript clusters consistently differentially expressed between control and treatment nubbins. To do this, a single factor capturing all experimental sample groupings (*Porites* genotype, *Lobophytum* genotype, and Control) was fitted and hypothesis testing was performed based on a contrast between all conditions involving competitive interactions (non-control samples) and controls. Clusters were deemed to be differentially expressed under competition if they had an adjusted *p*-value (padj) < 0.1 under this contrast. Complete details and code used to perform this analysis are provided as an online repository[4].

[2]https://github.com/cboursnell/transfuse

[3]https://github.com/jueshengong/psytrans

[4]https://github.com/China2302/Porites_competition

Functional Annotation of the *Porites* Transcriptome

The Trinotate protocol (version3[5]) was used to infer functional information for each of the transcripts in the *de novo* assembled *Porites* transcriptome. This annotation process included: protein prediction with TransDecoder (version4.1.0; Haas et al., 2013), identification of homologous proteins in SwissProt (2017) using blastp on predicted proteins and blastx on raw transcripts ($E < 10^{-5}$), signal peptide prediction with SignalP (version4.1; Nielsen, 2017), identification of conserved Pfam domains with hmmer (version3.1b2; Finn et al., 2011), ribosomal RNA prediction with rnammer (version1.2; Lagesen et al., 2007) and identification of transmembrane regions with tmhmm (version2.0c; Krogh et al., 2001).

Additional manual annotation was performed to supplement results for 76 transcript clusters found to be differentially expressed under competition. For each of these transcripts InterProScan (version5.48–83.0; Jones et al., 2014) was first used to identify conserved domains. If the transcript had a blast hit to a protein from SwissProt, the domain structure of this hit was compared with the domain structure of the transcript to determine whether genuine functional homology could be inferred. If domain structure was not conserved, an attempt was made to infer function based on conserved domains alone. Cnidarian-specific functional information was then identified by using google scholar to search for papers containing the combination of conserved domain names, gene names and the words coral or cnidarian. Manual curation of these search results yielded a small number of gene expression studies with relevant functional information. Finally, an attempt was made to assign the transcript to one or more of the following functional categories: immune response, stress response, secreted proteins and toxin. **Supplementary Table 2** lists evidence of homology and inferred functions for these 76 differentially expressed transcripts.

RESULTS

Polyp Activity and Behaviour

Analysis of the polyp activity data (**Figure 1C** and **Supplementary Table 3**) showed that polyps were more likely to be closed or partially closed in colonies under competition compared with controls ($p < 0.01$; based on a clmm) and differed significantly between *Porites* genotypes ($p < 0.001$). Polyp activity also changed over time but this seemed to vary between *Porites* genotypes, with Pd and Pf showing a gradual increase in open polyps during the first 30 days of the experiment, whereas genotype Pe showed little change.

In addition to basic polyp activity, competitive behaviour in the form of mesenteric filament formation was observed for six of the ten competing *Porites* nubbins, including representatives of genotypes Pd (four nubbins) and Pf (two nubbins) but not for Pe (**Supplementary Table 4**). Although filaments were clearly visible when present (**Figure 1B**) they were short-lived and it is likely that some instances of filament formation were not observed.

Transcriptome Assembly

Assembly of the *Porites cylindrica* transcriptome with Trinity resulted in 532,484 and 502,263 raw transcripts for Pf and Pd genotypes, respectively. Merging these assemblies with transfuse resulted in a total of 709,417 contigs and of these 422,222 were found to be good contigs by transrate (see section "Methods"). Splitting this assembly using Psytrans resulted in a coral fraction (340,399 contigs) and a Symbiodiniaceae fraction (81,823 contigs). The average mapping rate of the raw corrected reads to the combined coral-Symbiodiniaceae transcriptome was 83.6% while the mapping rate to the coral-only fraction was 53.4% due to a high proportion of reads being of Symbiodiniaceae origin (**Supplementary Table 5**). The coral-only assembly had a longest contig of 43 kb and average contig length of 992 bp. It contained complete copies of 95.7% of metazoan BUSCO genes (35.2% single copy; 60.5% duplicated) as well as 2.2% fragmented BUSCOs. This percentage of completeness is similar to that observed in other *de novo* coral transcriptome assemblies from adult tissue, such as *Acropora gemmifera* (94.4%; Oldach and Vize, 2018).

Assessment of the taxonomic composition of assembled transcripts with blastx was challenging because only 155,814 of the 340,399 transcripts (46%) in our transcriptome could be classified via homology, probably due to poor representation of cnidarian sequences in the nr database. Of the transcripts classified to phylum level by blastx, 86% were of cnidarian origin and tended to have better matches (higher bitscores) than the remaining transcripts matching other phyla (**Supplementary Figure 4**). The two most abundant phyla in this long-tail of non-cnidarian sequences were Arthropoda (6%) and Chlorophyta (4%) possibly reflecting the presence of barnacle and Symbiodiniaceae transcripts. Analysis with kraken recovered the known pattern of barnacle contamination in three samples, but otherwise suggested that taxonomic composition was consistent (**Supplementary Figure 3**) and not confounded with key groupings identified in our differential expression analysis (see below).

Effect of Genotype and Competition on Gene Expression

Gene expression data obtained at 30 days after the onset of competition were dominated by differences between the two sequenced *Porites* genotypes (Pd and Pf). Nubbins from these two colonies separated clearly into two groups along the first principal component of a PCA, and accounted for 81% of total variance in expression for the top 500 most variable genes (**Figure 2A**). Variation in gene expression due to competition with *Lobophytum* appeared to be associated with the second principal component of this PCA but could not simply be attributed to *Porites* or *Lobophytum* genotype alone. Sample clustering based on 76 genes differentially expressed between control nubbins and those in competition (**Figure 2B**) revealed four groups of samples, the composition of which was linked to

[5]https://trinotate.github.io/

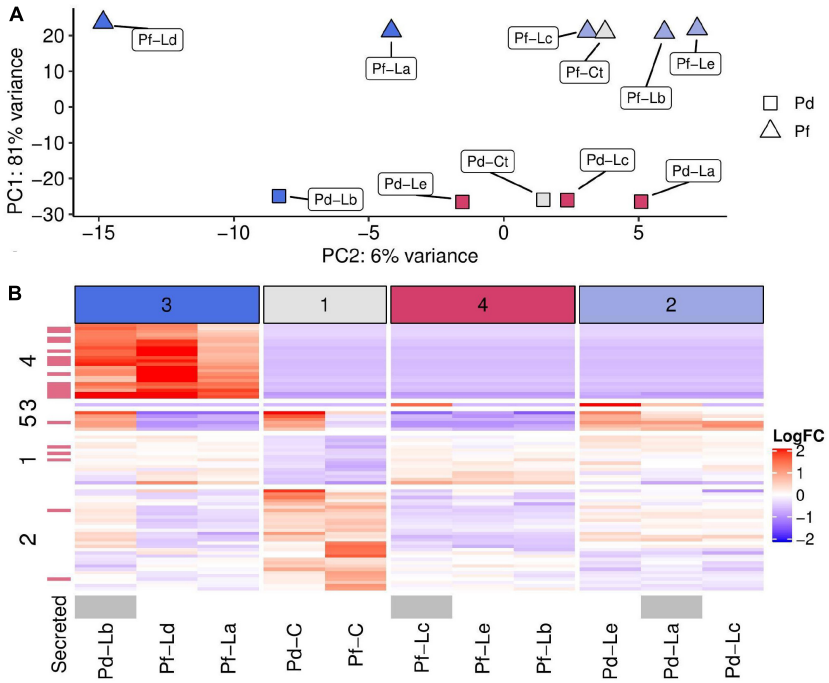

FIGURE 2 | Dominant sources of variation in gene expression between samples. Sample labels in both plots show the *Porites* genotype (Pd, Pf) followed by *Lobophytum* genotype (La–Le) or Control (C). **(A)** Principal components plot showing relative position of samples based on PC1 and PC2. Point shapes indicate the *Porites* genotype (Pd represented by triangles and Pf by squares) and point colours indicate sample groupings shown as coloured and numbered columns in part B (group 1– grey; group 2–light blue, group 3–dark blue, group 4–red). **(B)** Heatmap showing $\log_2$ fold change (Log_2FC) in expression compared to the mean (across all samples) for 76 genes found to be differentially expressed between treatment and control samples. Column clusters are indicated with numerical values and colours (top) and row clusters are labelled numerically (left). Transcripts highlighted in red (left strip) have predicted secretion signals via SignalP in their corresponding protein translations. Samples where mesenteric filaments were observed are shown in grey (bottom strip).

position along PC2. In particular, sample group three (dark-blue) (Pf–Ld, Pf–La, and Pd–Lb) included all those at one extreme of PC2, while control samples (sample group one; grey) occupied a central position and the remaining samples (groups 4 and 2; red and light-blue, respectively) occupied the other extreme of PC2 (**Figure 2B**).

Row clustering also revealed key groups of genes with expression profiles that differ between these sample groups (**Figure 2B**). The most striking example of this is demonstrated by row cluster four, which included genes that were exclusively expressed under competition in all samples from column group three (dark-blue) and not at all in other samples. Genes in row clusters one and two had less consistent expression within sample groups but generally partitioned genes into those that were overexpressed (row cluster one) or under expressed (row cluster two) in competition versus controls. Finally, row cluster five captured six genes which differed strongly between samples from genotype Pd and Pf, and where the response to competition was in the opposite direction depending on *Porites* genotype (**Figure 2B**).

Genes Differentially Expressed in Response to Non-contact Competition

A total of 76 transcripts were found to be differentially expressed in response to non-contact competition (DESeq2 adj p-value < 0.1). Of these, it was possible to obtain functional annotations for 30 by combining information from homology to proteins with known function, the presence of conserved domains, and gene expression or population genetic studies in cnidarians (see **Supplementary Table 2** for a full list including a summary of evidence). Very few could be assigned meaningful gene ontology terms because although some (24) had highly significant blast matches to SwissProt proteins, analysis with InterProScan revealed differences in domain structure that cast doubt on conservation of function. Nevertheless, it was possible to manually assign 35 functionally annotated genes as having putative roles in immunity (10) or cellular stress (9) including response to ROS and unfolded proteins. In addition, 20 genes were classified as secreted proteins and four of these had hallmarks of toxins (short, secreted and with ShKT, CRISP or protease domains).

Examination of correspondence between row groups in **Figure 2B** and functional categories revealed a striking enrichment [14/23 (60%) transcripts] in numbers of secreted proteins in row group four compared with differentially expressed genes as a whole [20/76 (20%) transcripts]. None of the proteins in this group were close homologs to each other (no clusters at 70% similarity with cd-hit) but many had the hallmarks of toxins, including two with ShKT domains, one secreted peptidase and five others that were secreted and short (<200 AA).

Transcripts related to stress response and immunity had more complex and variable expression profiles than the secreted group (row cluster 4) and were therefore not associated with specific row clusters in **Figure 2B**. When considering the distribution of transcripts according to their effect size rank (**Figure 3**), transcripts putatively assigned to immunity were more often up-regulated (8/10 genes) in response to competition while those associated with the core cellular stress response included a more equal mix of genes up and down regulated (three up-regulated; five down-regulated). The strong tendency for secreted proteins to be up-regulated under competition reflects enrichment for those in row group 4 (**Figure 2B**).

DISCUSSION

The dominant effect of colony as a driver of gene expression profiles in this study agrees with, and adds to, a growing body of evidence that this can be a major source of transcriptional variability in corals (Seneca et al., 2010; Granados-Cifuentes et al., 2013; Parkinson et al., 2018). While this overall inter-colony variability can be broadly viewed as a source of noise, it also has important ecological implications, especially in the context of competition where it could underpin the apparently idiosyncratic responses seen in past experiments (Precoda et al., 2017). Here we show that, at the transcriptional level, this variability is not random, but governed by latent factors that divide samples into groups with distinct expression profiles. Even with the relatively small number of samples analysed here it was possible to identify a distinct subset defined by exclusive expression of a suite of secreted proteins (**Figure 2**).

This association between a distinct pattern of expression and functional category (secretion) suggests that the genes encoding these proteins are part of a coordinated response, perhaps via a common transcriptional regulatory mechanism. Although this response was not correlated with any behavioural factors such as mesenterial filaments or polyp activity, the sequences of genes involved provide some indication of its functional significance. One possibility, supported by the presence of putative toxins among these secreted proteins, is that they are involved in aggression towards the competitor. If this is the case, the mechanism of target delivery for putative toxins remains unclear as the only means of contact between competitors was via mesenterial filaments. These filaments were observed sporadically, and their presence was not correlated with samples showing a secretion response, which suggests that this mode of delivery is unlikely, although it cannot be ruled out. Another working hypothesis is that these candidate toxins could reach the competitor through tentacle contact. Tentacle attack is usually seen through the development of sweeper tentacles where the tip is enriched in nematocyst and other toxins, as documented for *Galaxea* (Yosef et al., 2020) but this strategy has neither been registered for *Porites* nor was it observed in this experiment. Another possibility is that these proteins could be secreted into the surrounded water and reach a competitor via diffusion through the water column. Although there is evidence to suggest that a wide range of cnidarians, including *Porites cylindrica*, may use allelopathy a form of non-contact competition- to inhibit growth (Sammarco et al., 1983) and alter settlement of other corals (Maida et al., 1995; Da-Anoy et al., 2017), the toxic molecules involved have either not been identified, or are small organic compounds (Coll and Sammarco, 1983; Aceret et al., 1995) rather than proteins or peptides. Although peptides are metabolically expensive to produce (and therefore less likely to be released into the surrounding water) they can be highly

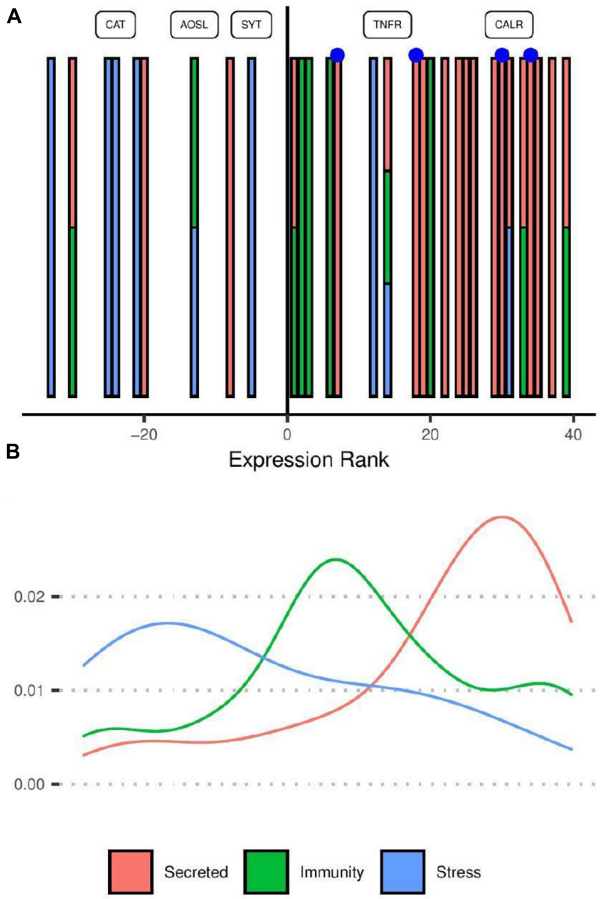

FIGURE 3 | Ranking distribution of differentially expressed transcripts under non-contact competition coloured by functional category. **(A,B)** Share the same colour scheme and horizontal axis. **(A)** Individual transcripts are shown as vertical bars positioned along the horizontal axis according to the rank of their effect size (log_2 fold change under competition compared with controls). Positive ranks represent transcripts up-regulated in competition compared to controls. Transcripts belonging to multiple categories are shown as multi-coloured bars. Blue points represent putative toxins. Category "Stress" includes transcripts associated with response to reactive oxygen species as well as those involved in the unfolded protein response. Labelled points correspond to transcripts with strong evidence of homology to named proteins. CAT, catalase; AOSL, allene oxide synthase-lipoxygenase; SYT, synaptotagmin-like; TNFR, tumor necrosis factor receptor; CALR, calreticulin. **(B)** Smoothed density plot showing the density of genes in each category according to expression rank.

specific and potent, and are known to be used by cyanobacteria as allelochemicals (Gross, 2003). Our results indicate these short-secreted proteins could be part of a peptide-mediated allelopathic response in corals and warrant further investigation to clarify their roles in competition and determine their bioactivity and mode of delivery.

In addition to the secretion response observed for a subset of *Porites/Lobophytum* pairs, it was also possible to identify genes with consistent responses to non-contact competition across all *Porites* samples. This "core" response included genes with putative roles in immunity and with roles in responding to cellular stress. The closest comparable experiments are those of Shearer et al. (2012, 2014) who explored gene expression responses in acroporid corals (*Acropora millepora* and *Montipora digitata*) to acute (6 and 48 h) and longer-term (20 days) competition with a range of macroalgal species. Short-term exposure experiments highlighted differential expression of genes across a wide range of putative roles, including immunity and cellular stress, but found that responses were highly species specific. Long-term exposure resulted in differential expression of a suite of genes involved in cellular stress, however, the specific genes involved were almost entirely different from those found differentially expressed by *Porites* nubbins in this experiment. Specifically, they included up-regulation of six heat shock proteins which we did not observe, and a change in expression of calreticulin in the opposite direction to that seen here.

Traditionally *Porites* spp. have been regarded as weak or non-aggressive competitors (Sheppard, 1979; Dizon and Yap, 2005), but both the morphological and molecular data presented here challenge this idea. Mesenteric attack (**Figure 1B**) is a form of aggression employed by some corals in competitive interactions, but not previously documented for any *Porites* spp. The molecular data implying secretion of candidate toxins by *Porites* under challenge are also inconsistent with the response being passive, and investigation of the properties of these novel proteins should be a priority. Although genotype is typically the strongest influence in transcriptomic experiments on corals, the work presented here illustrates the complexity and heterogeneity of responses in competitive interactions, and the extent to which these cannot be accounted for by genotype alone.

DATA AVAILABILITY STATEMENT

The transcriptomic sequencing data presented in this study are deposited in Genbank under bioproject PRJNA706467.

AUTHOR CONTRIBUTIONS

NA, AM, and DM designed the study. NA and AM performed sampling and process samples. IC and NA performed transcriptomic data analysis and the manuscript writing. RJ, IC, and NA performed the statistical analysis. DM, IC, NA, and RJ edited the draft manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.659360/full#supplementary-material

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Zoantharian Endosymbiont Community Dynamics During a Stress Event

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Coral reefs are complex ecosystems composed of many interacting species. One ecologically important group consists of zoantharians, which are closely related to reef-building corals. Like corals, zoantharians form mutualistic symbioses with dinoflagellate micro-algae (family Symbiodiniaceae), but their associations remain underexplored. To examine the degree to which zoantharians exhibit altered symbiont dynamics under changing environmental conditions, we reciprocally transplanted colonies of *Zoanthus sansibaricus* between intertidal (2 m) and subtidal (26 m) depths within a reef in Okinawa, Japan. At this location, *Z. sansibaricus* can associate with three Symbiodiniaceae species from two genera distributed along a light and depth gradient. We developed species-specific molecular assays and sampled colonies pre- and post-transplantation to analyze symbiont community diversity. Despite large environmental differences across depths, we detected few symbiont compositional changes resulting from transplantation stress. Colonies sourced from the intertidal zone associated with mixtures of a "shallow" *Symbiodinium* sp. and a "shallow" *Cladocopium* sp. independent of whether they were transplanted to shallow or deep waters. Colonies sourced from the subtidal zone were dominated by a "deep" *Cladocopium* sp. regardless of transplant depth. Subtidal colonies brought to shallow depths did not transition to the presumably high-light adapted shallow symbionts present in the new environment, but rather bleached and died. These patterns mirror observations of highly stable coral-algal associations subjected to depth transplantation. Our results indicate that *Zoanthus*-Symbiodiniaceae symbioses remain stable despite stress, suggesting these important reef community members have relatively low capacity to shuffle to more stress-tolerant micro-algae in response to ongoing climate change.

Keywords: light intensity, quantitative PCR, reciprocal transplant, Symbiodiniaceae, *Zoanthus sansibaricus*

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INTRODUCTION

Climate change continues to threaten environmentally-sensitive marine mutualisms, the most ecologically important of which are associations between reef-building corals (order: Scleractinia) and dinoflagellate micro-algae (family: Symbiodiniaceae). These nutritional symbioses form the foundation of tropical reef ecosystems, creating reef habitat in nutrient-poor waters *via*

light-enhanced calcification (Roth, 2014). Such partnerships are sensitive to biotic and abiotic stressors, which can drive symbiont loss – a process termed coral bleaching (Fitt et al., 2001). Although bleached corals may not lose all of their symbionts, they are nevertheless compromised, and often expire or suffer reproductive consequences if conditions do not improve and the symbiont community does not recover (Baird and Marshall, 2002). During stress events, partner fidelity can vary greatly across different coral holobionts (the host and its microbes). Some corals exhibit a "shuffling" response, whereby the numerically dominant micro-algal symbiont changes to favor a more resilient species (Bay et al., 2016). Other corals are more stable, such that the dominant symbiont never alters even during bleaching events (Thornhill et al., 2006). This range of dynamics has been recorded across many different scleractinian-Symbiodiniaceae partnerships under both natural and experimental stress conditions.

However, scleractinian corals are not the only organisms on reefs that associate with Symbiodiniaceae, nor are they the only organisms that can bleach. Other sessile cnidarian hosts include octocorals (soft corals), as well as the hexacorallian sea anemones (order Actiniaria), and zoantharians (Trench, 1974). Zooxanthellate zoantharians in the genera *Zoanthus* and *Palythoa* are often common components of coral reef ecosystems in both the Atlantic and Indo-Pacific oceans (Karlson, 1980; Sebens, 1982; Burnett et al., 1997; Irei et al., 2011), but have received comparatively little attention with respect to their symbiont dynamics (but see Reimer et al., 2007). One such symbiotic zoantharian is *Zoanthus sansibaricus*, a species that is widely distributed across coral reefs in the Indo-Pacific (Reimer et al., 2017b). It can be found in a variety of environments – from the shallow intertidal zone to mesophotic depths (>50 m) – and is considered to be a generalist species (Kamezaki et al., 2013; Albinsky et al., 2018).

At one reef site (Manza) on the west coast of the main island of Okinawa, Japan, *Z. sansibaricus* colonies are present on the reef flat in intertidal and shallow subtidal waters as well as on the outer reef wall up to depths > 35 m (Kamezaki et al., 2013). Morphological, phylogenetic, and population genetic data indicate that *Z. sansibaricus* at Manza exhibit no obvious differences across depths and therefore together represent a homogenous population of a single species (Reimer et al., 2004; Kamezaki et al., 2013; Wham et al., 2013; Wham, 2015; Albinsky et al., 2018). However, the host can associate with at least three Symbiodiniaceae lineages that likely represent distinct species (Wham et al., 2014; Wham, 2015). Two of the lineages are primarily found in the shallows (*Symbiodinium* A1z in direct sunlight and *Cladocopium* C1z-intertidal in shadow; Reimer, 2008), while the other is primarily found at greater depth (*Cladocopium* C1z-subtidal), suggesting that they are adapted to different light levels and possibly different temperatures (Kamezaki et al., 2013; Leal et al., 2016).

We have previously examined Symbiodiniaceae biogeography and bleaching patterns among zoantharians (Parkinson et al., 2016; Noda et al., 2017; Reimer et al., 2017a), but to our knowledge, no studies have attempted to track zoantharian symbiont community dynamics throughout a stress event. As a result, it remains unclear to what extent zoantharian-Symbiodiniaceae associations may shuffle or remain stable in response to stressors associated with climate change. To fill this gap in our understanding, we reciprocally transplanted colonies of *Z. sansibaricus* from shallow and deep locations at Manza, exposing them to novel light and temperature conditions. We tracked symbiont community composition – the proportion of each Symbiodiniaceae species in each colony – using species-specific molecular markers. We detected almost no shuffling 1 month after transplantation, with excessive mortality in the deep-to-shallow (DS) transplants, indicating that the association is relatively stable despite stress. Therefore, *Z. sansibaricus* holobionts are unlikely to acclimate to climate change *via* alterations in symbiont community composition.

MATERIALS AND METHODS

All raw data and the R code used to generate the results can be found in the **Supplementary Figure S1** and online at https://github.com/parkinson-lab.

Study System

The study site was located at Manza, Okinawa Island, Japan (26° 50'N, 127° 85'E; for map, see Figure 1 of Kamezaki et al., 2013). Manza features a reef lagoon with a gradual shallow slope leading to a precipitous drop-off starting at approximately 10 m depth. During a typical summer, the light and temperature levels in the shallow intertidal zone are greater and more variable than in the deep dropoff zone. To quantify these differences, *in situ* data loggers (Onset Computer Co., Massachusetts) were deployed from 19 August to 2 September, 2015, and environmental readings were recorded hourly. At Manza, *Z. sansibaricus* exhibits a discontinuous distribution, with an "intertidal" (<2 m) group and a "subtidal" (7–35+ m) group (for images, see Figure 2 of Kamezaki et al., 2013). Based on previous ITS2 genotyping, the numerically dominant symbiont within intertidal *Z. sansibaricus* colonies is typically *Symbiodinium* sp. (ITS2 type A1z; *sensu* Reimer, 2008) or a shallow *Cladocopium* sp. (ITS2 type C1z-intertidal; *sensu* Reimer, 2008; Kamezaki et al., 2013). The subtidal *Z. sansibaricus* colonies are usually dominated by a distinct deep *Cladocopium* sp. (ITS2 type C1z-subtidal; *sensu* Reimer, 2008; Kamezaki et al., 2013). As yet, none of these symbiont lineages have formal names. For clarity and pending taxonomic description, hereafter, we refer to the three primary Symbiodiniaceae species at this location as A1z-shallow, C1z-shallow, and C1z-deep.

Reciprocal Transplants

Reciprocal transplant experiments were carried out from 24 July to 10 October, 2013. Substrates with whole *Z. sansibaricus* colonies (n = 40 per depth) were collected from the intertidal (2 m) or subtidal (26 m) using a hammer and chisel and brought to the surface. Pieces of substrate with colonies attached

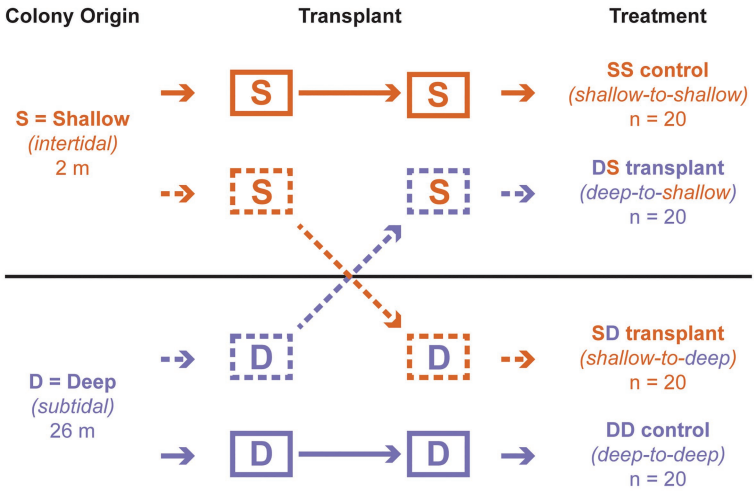

FIGURE 1 | Reciprocal transplant design. The first letter of each treatment indicates depth of origin, while the second indicates depth of transplantation. "S" corresponds to "shallow" (intertidal; 2 m); "D" corresponds to "deep" (subtidal; 26 m).

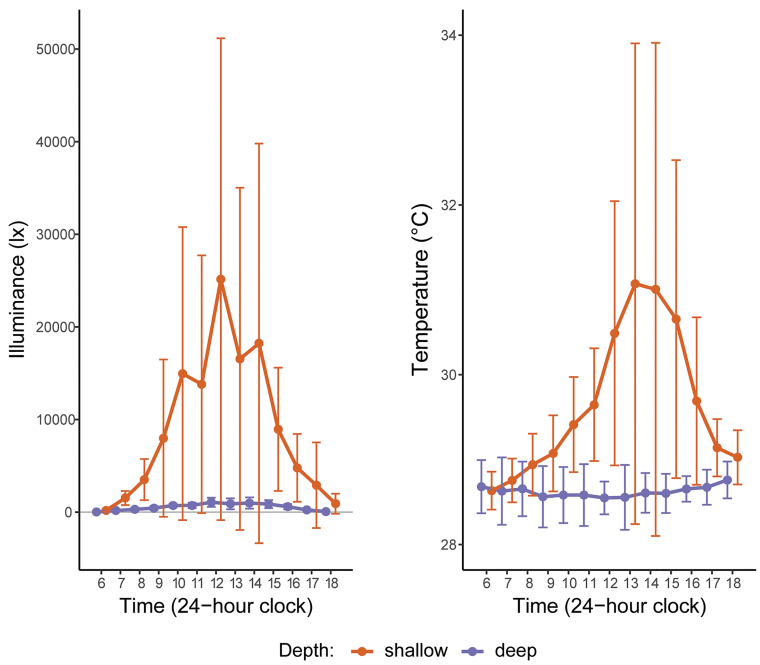

FIGURE 2 | Hourly light (illuminance) and temperature readings at two depths in Manza, Okinawa Island, Japan, averaged over 2 weeks in Summer 2015. "Shallow" corresponds to the intertidal (2 m); "deep" corresponds to the subtidal (26 m). The gray horizontal line indicates zero illuminance. The large error bars for shallow depth reflect high hourly variation in these metrics, as expected in the intertidal zone.

(~5 cm in diameter) were adhered to four concrete blocks (two per depth of origin) by applying Krazy Glue (Toagosei America, Inc., North Carolina, United States). The block dimensions were 39 cm × 19 cm × 15 cm, with a mass of 14 kg each. A subset of polyps from each colony were preserved immediately in 99.5% ethanol for subsequent molecular analyses of symbiont communities. The blocks were then returned to the depth of origin to acclimate. Colonies were not subfragmented, so the colonies on each block from each depth of origin were distinct (there was no pseudoreplication of clonal fragments) and likely represented unique genotypes, though this was not confirmed.

After 3 days of acclimation, the blocks were transplanted among depths, following a standard reciprocal design with four treatments (**Figure 1**). One intertidal origin block remained at 2 m as a control ("shallow-to-shallow" or SS; n = 20 colonies),

while another was moved to 26 m ("shallow-to-deep" or SD; n = 20); one subtidal origin block remained at 26 m as a control ("deep to deep" or DD; n = 20), while another was moved to 2 m ("deep-to-shallow" or DS; n = 20). Blocks remained at each depth for approximately 2 months. Individual polyps from each living colony were sampled on SCUBA at the start of the experiment (all transplants), at 1 week (DS transplant only), and at 4 weeks (all transplants) and fixed in 99.5% ethanol. All colonies were observed at these time points as well as at 8 weeks to record their health status as alive (apparently healthy), dead (>95% tissue loss), or missing (cases where the entire substrate was absent).

DNA Extraction and Phylogenetic Analyses

All samples were initially screened to identify the numerically dominant symbiont species *via* direct Sanger sequencing. Total genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN Inc., Tokyo, Japan). Symbiodiniaceae DNA was amplified with a HotStarTaq Plus Master Mix Kit (QIAGEN, Inc., Tokyo, Japan) targeting the nuclear internal transcribed spacer 2 (ITS2) region with the primers ITSintfor2 (5'-GAATT GCAGAACTCCGTG-3') and ITS-reverse (5'-GGGATCCATA TGCTTAAGTTCAGCGGGT-3'; LaJeunesse and Trench, 2000), as this marker can clearly resolve *Symbiodinium* spp. from *Cladocopium* spp., and weakly resolve C1z-shallow and C1z-deep (Kamezaki et al., 2013). For additional resolution, the noncoding region of the chloroplast psbA minicircle (psbA^{ncr}) was amplified using the primers 7.4-Forw (5'-GCA TGAAAGAAA TGCA CACAACTTCCC-3') and 7.8-Rev (5'-GGTTCTCTTATTCCA TCAATATCTACTG-3'; Moore et al., 2003).

Amplifications were performed in 20 µl reaction volumes containing 10 µl of Master Mix, 7 µl of ultrapure water, 1 µl of each forward and reverse primer (final concentration: 1 µM), and 1 µl of template DNA. Thermocycler conditions for ITS2 were as follows: 5 min at 95°C; 35 cycles of 45 s at 94°C, 45 s at 54°C, and 30 s at 72°C; and a final extension of 10 min at 72°C. Thermocycler conditions for psbA^{ncr} were as follows: 5 min at 95°C; 40 cycles of 10 s at 94°C, 30 s at 55°C, and 2 min at 72°C; and a final extension of 10 min at 72°C. Products were cleaned by the addition of 2.55 µl of ultra-pure water, 0.15 µl of Exonuclease I, and 0.3 µl of Shrimp Alkaline Phosphatase (TAKARA Co., Ltd., Shiga). Thermocycler conditions were as follows: 20 min at 37°C, followed by 30 min at 83°C. Cleaned products were shipped frozen to FASMAC (Kanagawa, Japan) for sequencing.

The ITS2 and psbA^{ncr} nucleotide sequences were aligned within Geneious v9.1.3 (Biomatters, New Zealand) and inspected manually. Sequences were aligned to references in GenBank (National Center for Biotechnology Information) to confirm identity. All ITS2 sequences matched previous references. Due to the short length of some of the psbA^{ncr} sequences, they could not be deposited in GenBank; instead, alignments are provided in the **Supplementary Figure S2**. Distance, parsimony, and maximum likelihood bootstrap consensus cladograms were generated in PAUP* v4a169 (Swofford, 2001), using the optimum inferred nucleotide evolution model (Jukes-Cantor for both *Symbiodinium* and *Cladocopium* alignments) and 1,000 bootstraps to calculate node support. Genetic distances among representative psbA^{ncr} sequences for C1z-shallow and C1z-deep were calculated with MEGA v6.0 (Tamura et al., 2013) and compared to divergence among other closely-related *Cladocopium* species (Thornhill et al., 2014).

Relative qPCR

Three species-specific primer sets targeting the Symbiodiniaceae psbA^{ncr} region were designed for A1z-shallow, C1z-shallow, and C1z-deep (**Table 1**). Appropriate, unique primers for each sequence were identified using the online tool Primique (Fredslund and Lange, 2007). Reactions were performed using SYBR Green chemistry on an ABI StepOne Plus Real-Time PCR System (Thermo Fisher Scientific, MA, United States). Reaction volumes consisted of 10 µl Fast SYBR Green Master Mix (Thermo Fisher Scientific), 7 µl purified water, 1 µl of each forward and reverse primer (final concentration: 0.5 µM), and 1 µl template DNA. Thermocycler conditions were as follows: 10 min at 95°C; 40 cycles of 3 s at 95°C followed by 30 s at 60°C; 15 s at 95°C; 1 min at 60°C; concluding with a melting curve (continuous ramping of +0.3°C per cycle up to 95°C for 15 s each). Each reaction was performed in duplicate, and for any given sample all three species assays were included on the same plate. Each plate also included no-template controls and four 10-fold serial dilutions of reference samples (10–0.01 ng/µl) for all three species. The dilutions were used to generate plate-specific calibration curves accounting for differences in reaction efficiencies among assays and runs. Reference samples were chosen based on initial symbiont identification using direct PCR and subsequent quantitative PCR (qPCR) confirmation that the target species represented >99% of the symbiont community in each reference. Because the goal was relative quantification rather than absolute quantification, it was not necessary to normalize to a reference gene.

Within the StepOne Plus software, cycle threshold (C_T) values were converted to initial concentrations based on the species-specific calibration curves for the plate on which the sample was run. These values were directly compared to compute the relative proportions of different Symbiodiniaceae species

TABLE 1 | Species-specific qPCR primer sets developed to amplify the chloroplast psbA^{ncr} region of Symbiodiniaceae associated with *Z. sansibaricus* in Okinawa, Japan.

Species	Abbreviation	Forward primer	Reverse primer	Mean efficiency
Symbiodinium sp. A1z	A1z-shallow	5'-CCACGAGGGTGGAAATGAGCTG-3'	5'-AATGCGAAGTATTGCGCTGGAC-3'	109.4%
Cladocopium sp. C1z-intertidal	C1z-shallow	5'-ACCCATAATCTTGGCCTGCTTG-3'	5'-CTTTCTCCTGCGGGCTCCTG-3'	87.1%
Cladocopium sp. C1z-subtidal	C1z-deep	5'-GACCACAATTTAGGCCACATC-3'	5'-GCCCTCTAATGCACTTCGTG-3'	82.5%

in each sample and plotted within the R statistical environment. Although amplification efficiencies were comparable between the *Cladocopium* assays, the *Symbiodinium* assay could not be optimized to fall within a similar range (**Table 1**). The over-efficiency of this assay likely shifted C_T values such that it appeared there were more *Symbiodinium* cells and fewer *Cladocopium* cells when combinations of both genera were present in a sample. However, high consistency between the output of direct sequencing and qPCR methods indicate that despite the qPCR bias toward *Symbiodinium*, the assays captured which species were dominant, and any inaccuracies in the observed relative abundances did not influence the key findings (see Results and Discussion).

Intracolony Sampling

Results from the reciprocal transplant time series seemed to suggest that in the "shallow-to-shallow" treatment, intertidal *Z. sansibaricus* shuffled their symbiont communities back and forth among A1z-shallow and C1z-shallow in less than a month. Such rapid shuffling under control conditions was unexpected. An alternative explanation was that *Z. sansibaricus* in the shallows featured spatial variation in the distribution of symbiont species across the colony surface. Under such a scenario, the strategy of collecting a few polyps at each time point for molecular analyses may have created an artificial pattern of symbiont shuffling, as regions of the colony with distinct symbiont communities may have been sampled at different times. To test for this possibility, four additional intertidal *Z. sansibaricus* colonies were sampled from Manza in late Spring 2017. Five sub-samples were taken per colony: one central polyp, and four polyps located 20 cm away and oriented in the four cardinal directions (north, south, east, and west). All sub-samples were analyzed *via* qPCR as described above to assess symbiont community composition.

RESULTS

Environmental Parameters and Mortality

Based on 2 weeks of data from *in situ* loggers deployed in Summer 2015, Manza's average daily maximum light intensity at 2 m was ~25-fold greater than at 26 m (25,142 lx ± 26,013 vs. 1,065 lx ± 493, respectively), and average daily maximum temperature was ~2.5°C warmer (31.1°C ± 2.8 vs. 28.5°C ± 0.2, respectively), with greater variability in the shallows (**Figure 2**). *Zoanthus sansibaricus* mortality varied by transplant, as did the incidence of missing colonies due to detachment of the substrate from the concrete block (**Figure 3**). All SS transplants that did not go missing survived, though this treatment also exhibited the greatest number of missing colonies (only three of 20 substrates remained attached after 8 weeks (8 W), but at 4 W there were still 11 living colonies). The SD transplants also showed low mortality (one of 11 colonies that did not go missing after 8 W expired). Missing colonies in the shallows likely resulted from large waves generated by summer typhoons (White et al., 2013). In the deep-to-deep (DD) transplant,

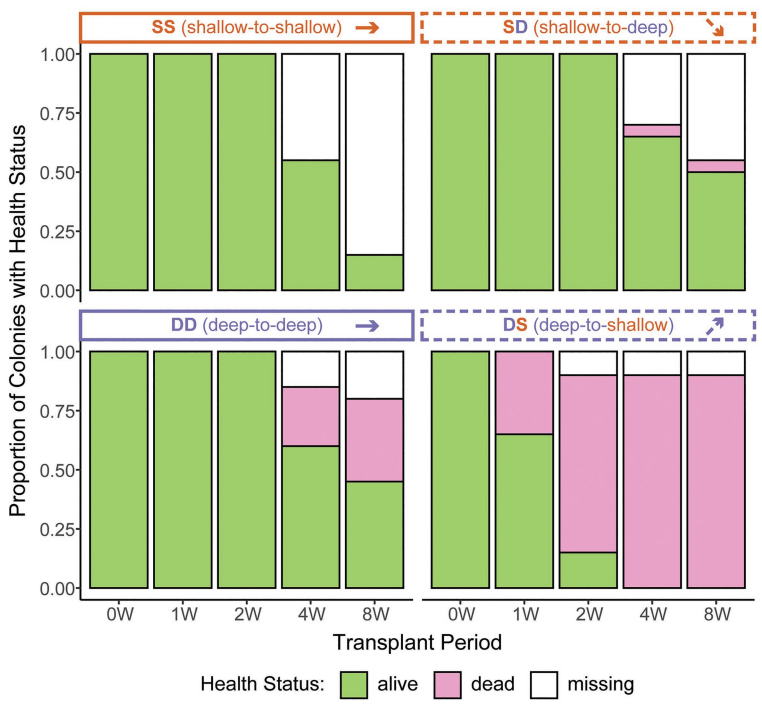

FIGURE 3 | *Zoanthus sansibaricus* colony mortality over the course of the 2-month reciprocal transplant experiment at Manza in Summer 2013. "W" corresponds to the week of observation. Note the rapid mortality in the deep-to-shallow (DS) transplant, necessitating early sampling to characterize symbiont community composition. Anecdotally, all the living DS colonies were visually bleached at 1 and 2 W.

mortality was greater (seven of 15 colonies that did not go missing after 8 W expired). Finally, the DS transplant was the most sensitive. After just 1 W in the shallows, seven of 20 colonies expired, and several of the 13 living colonies were completely bleached, while others were starting to pale (Fujiwara et al., personal observation). By 2 W, an additional eight colonies had expired, and two had gone missing, leaving only three living colonies. By 4 W, all remaining colonies had died. Given the rapid mortality observed in the DS transplants after 1 W, emergency sampling was carried out. Thus, symbiont communities in the DS transplants could only be compared at 0 W (pre-transplantation) and 1 W (non-bleached or pale colonies only). In the other treatments, it was possible to compare 0–4 W. Logistical issues prevented tissue sampling at 8 W. Due to mortality, missing colonies, and occasional DNA preservation failures, only the subset of colonies with complete data pairs for both pre- and post-transplantation were analyzed and presented (n = 9 per treatment).

Confirming Symbiodiniaceae Species Identities

Direct sequencing of ITS2 revealed the numerically dominant Symbiodiniaceae species in each sample (69 of 72 cases, with three amplification failures). Across the entire experiment, only three unique ITS2 sequences were recovered. We aligned these sequences against previously published entries in the NCBI Genbank database to confirm they corresponded to the expected *Symbiodinium* and *Cladocopium* lineages. Consistent with previous characterizations of *Z. sansibaricus* symbiont communities at Manza (Kamezaki et al., 2013), the single *Symbiodinium* sequence was identical to that of ITS2 type A1z (accession JQ762357), which differs by one base pair from that of *Symbiodinium microadriaticum* (ITS2 type A1; accession AF333505). The two *Cladocopium* sequences were identical to that of ITS2 type C1z-shallow (accession JQ762324) and ITS2 type C1z-deep (accession JQ762330), which differ by 1–2 base pairs from that of *Cladocopium goreaui* (ITS2 type C1; accession AF333515). All A1z-shallow and C1z-shallow sequences were recovered from intertidal origin colonies, while all C1z-deep sequences were recovered from subtidal origin colonies.

Direct sequencing of the psbA^{ncr} also revealed the numerically dominant Symbiodiniaceae species in each sample. Where these sequences were successfully recovered, the results were in complete agreement with the ITS2 data (60 of 60 cases). Though more variable than ITS2, the *Symbiodinium* psbA^{ncr} sequences formed a single monophyly corresponding to A1z-shallow (no node support > 75%) and the *Cladocopium* psbA^{ncr} sequences split into two major monophylies corresponding to C1z-shallow and C1z-deep (node support = 100%; **Figure 4**). In a previous study, microsatellite data confirmed that sympatric populations of *Cladocopium* lineages corresponding to Caribbean ITS2 types C3, C7, and C7a were reproductively isolated and thus likely represent distinct species (Thornhill et al., 2014). The psbA^{ncr} sequences from these Caribbean *Cladocopium* species provided a frame of reference for inferring whether C1z-shallow and C1z-deep are divergent enough to constitute separate species. The genetic distance between C1z-shallow and C1z-deep (0.37) exceeded the average genetic distance among ITS2 types C3,

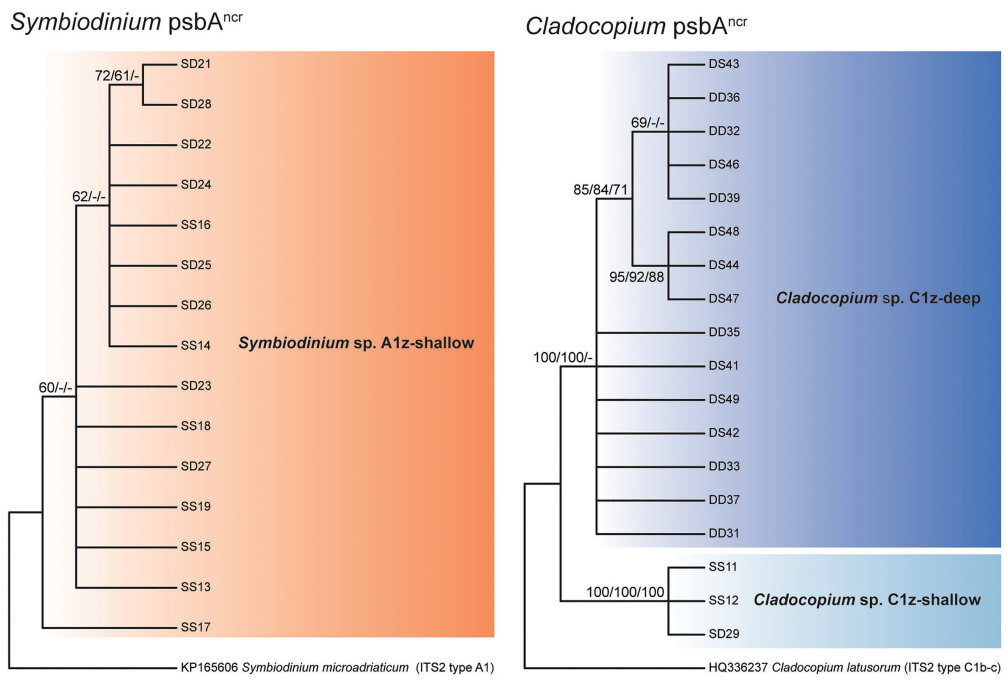

FIGURE 4 | Consensus cladograms of the noncoding region of the chloroplast psbA minicircle (psbA^{ncr}) sequences for *Symbiodinium* spp. (left) and *Cladocopium* spp. (right) based on distance methodology. Node values indicate bootstrap support (values below 60% excluded). Values correspond to different analyses in the following order: distance/parsimony/maximum likelihood. Dashes indicate where nodes were not supported by the given method.

C7, and C7a (0.17; maximum 0.23), so they are likely to represent distinct species, and we treat them as such in this study. The C1z-deep monophyly segregated into two subgroups (node support > 75%), but the genetic distance between these subgroups (0.01) fell below the average within-species distance among ITS2 types C3, C7, and C7a (0.05; minimum 0.02), so the subgroups are unlikely to represent distinct species, and we treat them here as one lineage.

Changes in Symbiont Community Composition After Transplantation

Quantitative PCR of the psbA^{ncr} primers revealed not only the numerically dominant Symbiodiniaceae species, but also the abundance of background levels of the other focal species. These qPCR results were consistent with the psbA^{ncr} direct sequencing results in all but two instances (63 of 65 cases). For colony SD21 at 0 W, direct sequencing indicated that A1z-shallow dominated, while qPCR indicated that C1z-shallow dominated; for colony SD26 at 4 W, direct sequencing indicated that C1z-shallow dominated, while qPCR indicated that A1z-shallow dominated. Thus, even though the qPCR primer efficiencies were unequal across assays (**Table 1**), the bias toward *Symbiodinium* does not appear to have had a great effect in most samples, at least in terms of detecting the dominant species. The bias also reinforces that we did not detect A1z-shallow in nearly all deep-sourced colonies (see below), as any A1z-shallow signal would have been exaggerated.

After transplantation, the first consideration was whether any colonies shuffled between intertidal Symbiodiniaceae species (A1z-shallow and/or C1z-shallow) and subtidal Symbiodiniaceae species (C1z-deep). Based on species-specific qPCR assays, there was no successful transplantation-induced shuffling of symbionts to favor species originating from different depths. All colonies of shallow origin (SS, n = 9 of 9; or SD, n = 9 of 9) began with a community composition consisting of >90% shallow symbionts prior to transplantation and ended with >90% shallow symbionts 4 W after transplantation (**Figure 5**). All but two colonies of subtidal origin (DD, n = 9 of 9; or

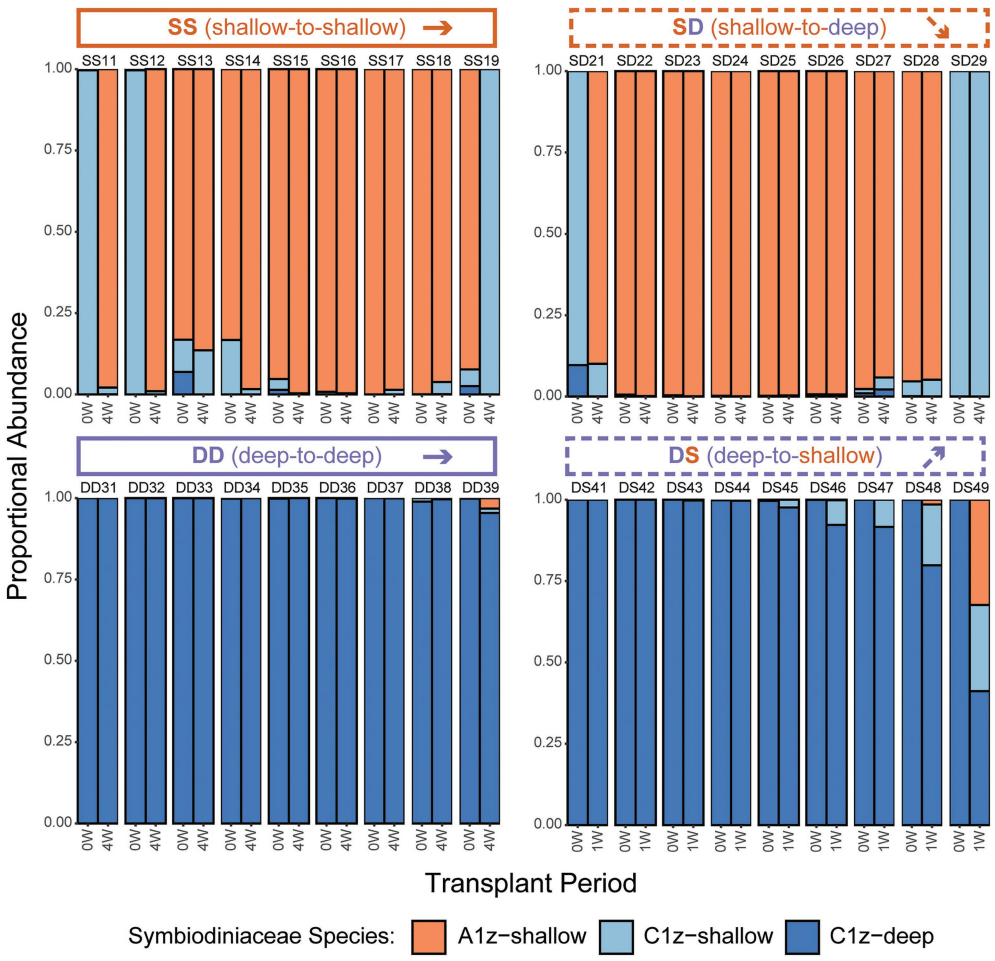

FIGURE 5 | Changes in Symbiodiniaceae community composition for individual *Z. sansibaricus* colonies over the course of the reciprocal transplant experiment based on species-specific quantitative PCR (qPCR) during Summer 2013. "W" corresponds to the week of observation. Note that in the DS transplant, the final symbiont community characterization was made at 1 W instead of 4 W due to the onset of rapid mortality and visual bleaching among these colonies. Designations above barplots indicate transplant treatment (two letters) and colony ID (two numbers).

DS, n = 7 of 9) began with >90% deep symbionts prior to transplantation and ended with >90% deep symbionts either 4 W (DD) or 1 W (DS) after transplantation (**Figure 5**). The two colonies with larger shifts were both in the DS treatment, which was experiencing rapid mortality after just 1 W. One colony (DS48) shuffled from ~100% C1z-deep to ~80:20% C1z-deep:C1z-shallow, while the other (DS49) shuffled from ~100% C1z-deep to 40:25:30% C1z-deep:C1z-shallow:A1z-shallow. Recall that the A1z-shallow signal in this colony may have been exaggerated by primer inefficiencies.

Because there were two intertidal Symbiodiniaceae species, the second consideration was whether there was any shuffling between these shallow symbiont species (A1z-shallow and C1z-shallow) within intertidal-origin colonies (SS or SD). Such transitions were present in both SS and SD transplants, but there was no predominant pattern. Some colonies began ~100% A1z-shallow and transitioned to ~100% C1z-shallow after 4 W (e.g., SS11), some showed the opposite (e.g., SS19), and others were intermediate between these extremes (**Figure 5**).

Intracolony Spatial Variation in Symbiont Community Composition

Because rapid shuffling among intertidal Symbiodiniaceae species within SS and SD transplants was unexpected, an alternative hypothesis of spatial variation in symbiont community composition was tested by subsampling four separate intertidal colonies five times each across the colony surface. Based on the same species-specific qPCR assays, it was confirmed that different regions of shallow colonies had different symbiont compositions (**Figure 6**). One colony (Colony 2) was uniformly dominated by C1z-shallow, while the remainder featured different mixtures of A1z-shallow and C1z-shallow at different points along the surface. The different species distributions appeared to be random with respect to cardinal direction.

DISCUSSION

Consistent with previous studies (Kamezaki et al., 2013; Noda et al., 2017), we confirmed that zoantharians in Okinawa can host several distinct symbiont species over small spatial scales (**Figure 4**). In the case of *Zoanthus sansibaricus*, not only were symbionts structured along a depth gradient representing distinct light and temperature regimes (**Figure 2**), we also detected spatial variation in symbiont distribution within individual intertidal colonies (**Figure 6**). In several cases, the surfaces of colonies were dominated by A1z-shallow in some regions and C1z-shallow in others. These patterns

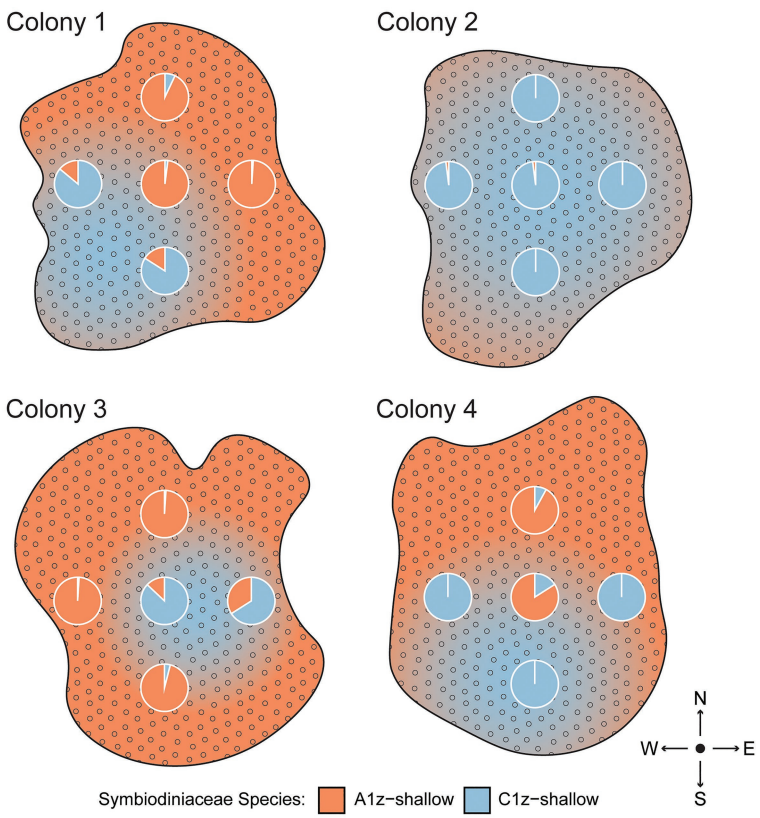

FIGURE 6 | Spatial differences in Symbiodiniaceae community composition across the surfaces of four intertidal *Z. sansibaricus* colonies collected from Manza's intertidal zone (2 m) in Spring 2017. Each colony was sampled at five points, represented by the pie charts of proportional abundance for each symbiont species. The gradients are subjective interpretations of symbiont distributions inferred from these points.

did not align with cardinal direction, indicating the distribution was unlikely to have been determined simply by orientation relative to the sun. During field collection, we noted that the shallow *Z. sansibaricus* colonies were often situated in small cracks in the intertidal zone reef substrate, with some portions protected in the shade and others more exposed to direct light (Fujiwara et al., personal observation), similar to other reefs in Japan (Reimer, 2008). This variation in irradiance at the scale of centimeters may explain the intra-colony symbiont community distribution, though this hypothesis will require further testing. Similar symbiont diversity within colonies has been documented in scleractinian corals, and has led to unique bleaching outcomes (Rowan et al., 1997; Brown et al., 2002; Kemp et al., 2014). The presence of spatial variation in shallow colonies is important to consider when interpreting apparent changes in symbiont community composition during the reciprocal transplant experiment.

Prior to transplantation, all shallow *Z. sansibaricus* colonies at Manza hosted A1z-shallow, C1z-shallow, or a combination of these two species of intertidal origin, while all deep colonies started with C1z-deep of subtidal origin (**Figure 5**). After transplantation, we found that nearly all colonies maintained stable associations: they were essentially "locked in" to the symbionts typical of their depth of origin, at least over the short time period examined in this study (**Figure 5**). The apparent shuffling between A1z-shallow and C1z-shallow in transplants of shallow colonies (the SS control and SD treatment) is most likely an artefact of intra-colony spatial variation in symbiont distribution (**Figure 6**), rather than rapid community changes. Although we cannot rule out rapid shuffling, it is a less parsimonious explanation than inadvertently sampling different regions of the colony at different time points, given our experimental design and the otherwise stable patterns of symbiont diversity. In addition to SD and DS transplants retaining their original symbionts, deep subtidal colonies visibly bleached and expired within 2 weeks of being brought to the shallow intertidal zone (**Figure 3**). These results match well with previous studies of reciprocally transplanted scleractinian corals (Baker et al., 2004; Frade et al., 2008; Sampayo et al., 2016) and experimentally manipulated sea anemones (Gabay et al., 2019; but see Herrera et al., 2020), and can be explained at least in part by specificity owing to symbiont niche-specialization.

As is true in other cnidarian-algal systems (Iglesias-Prieto et al., 2004; Frade et al., 2008), we can assume that the shallow Symbiodiniaceae at Manza are photo-adapted to the high-light, variable environment characteristic of the intertidal zone, while deep Symbiodiniaceae are photo-adapted to the low-light, constant environment of the subtidal zone. While shallow Symbiodiniaceae may still thrive at depth, especially over the short term, deep Symbiodiniaceae accustomed to lower light levels may face acute photoinhibition and possibly temperature stress in shallow environments, which can lead to dysbiosis, bleaching, and mortality (Baker et al., 2004; Frade et al., 2008; Sampayo et al., 2016; this study). One exceptional DS colony did appear to shuffle to a slight majority of shallow symbionts 1 week after being transplanted upward. However, there are reasons to think this may have also been an artefact. It is difficult to classify the extent to which zoantharians have visibly bleached (Parkinson et al., 2016), and although it was obvious that some DS transplants had started bleaching after just 1 week, the majority still looked healthy and were therefore sampled. The colony that shuffled between deep and shallow symbionts had relatively low DNA yield in the post-transplantation sample, indicating it may have already been bleaching despite appearing healthy. Therefore, it would be incorrect to interpret the outcome as successful shuffling – the symbiont community may have simply been in a state of flux due to stress-induced non-visible bleaching. Regardless of its bleaching status at 1 week, this colony ultimately died after 2 weeks, so while there may have been some variation among colonies in the capacity to change symbiont communities, such change was insufficient for survival. This suggests *Zoanthus*-Symbiodiniaceae associations are relatively stable, despite their ability to associate with more than one symbiont within a colony, and thus they appear unlikely to shuffle their symbiont communities to acclimate or adapt to rising sea surface temperatures caused by climate change. Further experimentation with more gradual manipulations over longer time scales will be required to confirm this prediction.

More generally, our results demonstrate that Symbiodiniaceae ecology is more important than phylogenetic relatedness in establishing a successful mutualism. This conclusion follows from the fact that intertidal *Z. sansibaricus* colonies that typically associate with the high-light adapted C1z-shallow will more readily accommodate the high-light adapted but evolutionarily divergent A1z-shallow than the low-light adapted but extremely closely related C1z-deep. The primacy of ecological compatibility is consistent with the distribution of other phylogenetically divergent Symbiodiniaceae within scleractinian coral colonies that can associate with multiple species simultaneously (e.g., Kemp et al., 2014). It is unclear to what extent the symbiosis discerns between C1z-shallow and C1z-deep *via* mechanisms like microbe-associated molecular patterns and pattern-recognition receptors (Davy et al., 2012; Parkinson et al., 2018). The exclusion of one *Cladocopium* species when both could be present might also stem from competitive interactions inherent to the symbionts (Gabay et al., 2019; McIlroy et al., 2019, 2020), which theory predicts to be of greater intensity among sister lineages than among more divergent lineages. Both host habitat and depth are major drivers of functional specialization in Symbiodiniaceae (Finney et al., 2010; Thornhill et al., 2014). This symbiont family has undergone multiple adaptive radiations over its evolutionary history, co-speciating with scleractinian corals and branching out to fill varied environmental niches and functional roles (LaJeunesse, 2005; Prada et al., 2014; Parkinson et al., 2015; LaJeunesse et al., 2018). The *Zoanthus*-Symbiodiniaceae association at Manza is unique in that the C1z lineage appears to have diverged along a depth gradient in the absence of genetic barriers in *Z. sansibaricus*. This highlights that Symbiodiniaceae typically exhibit more genetic structure than their hosts (Thornhill et al., 2017) and supports the contention that dinoflagellate endosymbionts may evolve more rapidly than cnidarians.

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 in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

YF, JR, and JP: conceptualization and formal analysis. YF, IK, JR, and JP: methodology, investigation, and writing–review and editing. JP: software. JR and JP: resources, supervision, project administration, and funding application. YF and JP: writing–original draft preparation and visualization. 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/fmicb.2021.674026/full#supplementary-material

Supplementary Figure S1 | Raw data and R code.

Supplementary Figure S2 | *Symbiodinium* and *Cladocopium* psbA^{ncr} sequence alignments.

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Physiological Differences in Bleaching Response of the Coral *Porites astreoides* Along the Florida Keys Reef Tract During High-Temperature Stress

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The Florida Keys reef tract (FKRT) has a unique geological history wherein Holocene sea-level rise and bathymetry interacted, resulting in a reef-building system with notable spatial differences in reef development. Overprinted on this geologic history, recent global and local stressors have led to degraded reefs dominated by fleshy algae, soft corals, and sponges. Here, we assessed how coral physiology (calcification rate, tissue thickness, reproduction, symbiosis, and bleaching) varies seasonally (winter vs. summer) and geographically using 40 colonies of the mustard hill coral *Porites astreoides* from four sites across 350 km along the FKRT from 2015 to 2017. The study coincided with a high-temperature event in late summer 2015 that caused heterogeneous levels of coral bleaching across sites. Bleaching severity differed by site, with bleaching response more aligned with heat stress retroactively calculated from local degree heating weeks than those predicted by satellites. Despite differences in temperature profiles and bleaching severity, all colonies hosted Symbiodiniaceae of the same genus (formerly Clade A and subtypes). Overall, *P. astreoides* at Dry Tortugas National Park, the consistently coolest site, had the highest calcification rates, symbiont cell densities, and reproductive potential (all colonies were reproductive, with most planula larvae per polyp). Corals at Dry Tortugas and Fowey Rocks Light demonstrated strong seasonality in net calcification (higher in summer) and did not express visual or partial-mortality responses from the bleaching event; in contrast, colonies in the middle and southern part of the upper keys, Sombrero Key and Crocker Reef, demonstrated similar reduced fitness from bleaching, but differential recovery trajectories following the heat stress. Identifying reefs, such as Dry Tortugas and possibly Fowey Rocks Light that may serve as heat-stress refugia, is important in selecting candidate sites for adaptive reef-management strategies, such as selective propagation and assisted gene flow, to increase coral-species adaptation to ocean warming.

Keywords: scleractinia, *in situ* calcification rates, reproduction, coral-algal symbiosis, coral reef degradation, buoyant weight

INTRODUCTION

Coral reefs have declined in live-coral cover by 50–80% over the last four decades from the culminating impacts of local and global stressors (Gardner et al., 2005; De'ath et al., 2012; Hughes et al., 2018), putting at risk the vast ecological goods and services that coral reefs provide such as coastal protection, fisheries, and tourism (Moberg and Folke, 1999; Pratchett et al., 2014; Storlazzi et al., 2019). Ocean warming is identified as the greatest threat to coral reefs (Intergovernmental Panel on Climate Change (IPCC), 2018), particularly because of recent severe and repeated marine heatwaves resulting in widespread coral bleaching (Eakin et al., 2019), exacerbated disease outbreaks (Randall and van Woesik, 2015; Precht et al., 2016), and increasingly severe storm events (Gardner et al., 2005; Webster et al., 2005), with little reprieve between disturbances (Hughes et al., 2003, 2018). Coral reef communities in the Caribbean and Florida Keys have already shifted from three-dimensional, reef-building structures to flattened reefs dominated by soft-bodied benthic dwellers (Alvarez-Filip et al., 2009; Norström et al., 2009; Ruzicka et al., 2013; Lenz et al., 2015). These structurally degraded ecosystems leave coastlines, islands, and their communities vulnerable to erosion and inundation, especially as hurricanes (Webster et al., 2005), sea-level rise, and wave stress intensify (Storlazzi et al., 2019). Identifying sites where corals maintain biological performance that supports reef building, coral-population recovery, and ecological resilience is critical as local and global stressors continue to impact coral reef ecosystems (Beyer et al., 2018; Guest et al., 2018; Darling et al., 2019).

Exceptional reefs that stand out against the current backdrop of degradation can greatly influence how sites are prioritized in conservation and management strategies (Beyer et al., 2018; Guest et al., 2018; Hoegh-Guldberg et al., 2018). Some sites and regions have been described as "reefs of hope" (McClanahan et al., 2009), "bright spots" (Cinner et al., 2016), and "reef oases" (Guest et al., 2018). These reefs were identified based on percent coral cover, reef biodiversity, or fish assemblages as being influenced by factors including location, socioeconomic status, and environmental and biological traits (Cinner et al., 2016; Beyer et al., 2018; Guest et al., 2018; Darling et al., 2019). Although metrics on percent coral cover and reef assemblages are readily available to compare sites, there is a general lack of information on the critical biological processes that promote coral recovery and resilience. Identifying locations where *in situ* coral physiology outperforms that at other sites could further help prioritize reef management, protection, and restoration strategies (Beyer et al., 2018; Guest et al., 2018; Darling et al., 2019).

The Florida Keys reef tract (FKRT) consists of a unique, subtropical and largely submerged spur-and-groove and patch-reef system that stretches 350 km approximately 6–10 km from shores of the Florida Keys islands (Stephenson and Stephenson, 1950). Based on geological characteristics, the reef system is traditionally divided into subregions including the upper, middle, and lower Keys, and the Marquesas/Dry Tortugas (Murdoch and Aronson, 1999; Lidz et al., 2006; Toth et al., 2017, 2018) featuring strong spatial and temporal variability in reef development (Lidz and Shinn, 1991; Precht and Aronson, 2004; Lidz et al., 2006; Toth et al., 2018). Mean percent coral cover throughout the FKRT is now less than 5% (Ruzicka et al., 2013) and current spatial patterns in coral net calcification rates along the FKRT correlate with average Holocene reef thickness with the greatest suppression in reef development between the middle and lower Keys (Kuffner et al., 2013; Manzello et al., 2015; Toth et al., 2018). The suppression of reef development in the middle Keys may be driven by unfavorable environmental conditions of water traveling out of tidal passes, connecting the offshore reefs to Florida Bay and bringing large fluctuations in temperature, salinity, turbidity, and nutrients; this theory is known as the "inimical waters hypothesis" (Schlager, 1981; Neumann and Macintyre, 1985; Hallock and Schlager, 1986; Ginsburg and Shinn, 1994; Smith, 2002; Precht and Miller, 2007).

In recent decades, *in situ* sea temperatures throughout the FKRT have increased, with mean maximum daily temperatures exceeding 30.5°C at least once every year since 1994 (Kuffner et al., 2015; Manzello, 2015). As thermal stress along the FKRT persists on a near-annual basis, it is important to understand the *in situ* response of coral physiology (i.e., calcification, coral-algal symbiosis, reproduction, and survivorship); in particular, how traits vary in response to differences in water-residence time, bathymetry, proximity to tidal passes, and other physical or environmental gradients along the FKRT. Given the environmental history of the FKRT and recent thermal-stress events, the goal of our 2-year study was to quantify differences in coral response to temperature stress that may be driven by reef location. Using four, previously established sites where coral-calcification assessment has occurred since 2009 (Kuffner et al., 2013, 2019), we assessed coral colony-level traits of 40 *Porites astreoides* colonies followed from May 2015 to May 2017 across the length of the FKRT from Miami to the Dry Tortugas. We compared calcification rates (e.g., Kuffner et al., 2013; Manzello et al., 2015), tissue thickness, reproductive potential (Chornesky and Peters, 1987; Serrano et al., 2016), and Symbiodiniaceae concentration, identity, and diversity (Kenkel et al., 2013; Hauff et al., 2016; Cunning et al., 2017) as proxies for coral fitness that reveal information about resistance and resilience against heat stress. Few studies have simultaneously measured these essential physiological traits in the field to determine possible trade-offs as corals experience the natural spatial and temporal variability of environmental conditions along the FKRT (Manzello et al., 2018). We provide evidence based on our colony-level traits that corals at specific sites (Biscayne and Dry Tortugas National Parks) maintained physiological function despite the severe thermal anomaly while corals in the middle Keys and southern part of the upper Keys were more vulnerable, further supporting the inimical waters hypothesis.

MATERIALS AND METHODS

Study Sites and Coral Collection

Four sites spanning the ∼350-km Florida Keys reef tract (FKRT) (**Figure 1A**) were established for the calcification-assessment network by the United States Geological Survey (USGS) Coral Reef Ecosystems Studies (CREST) project in 2009

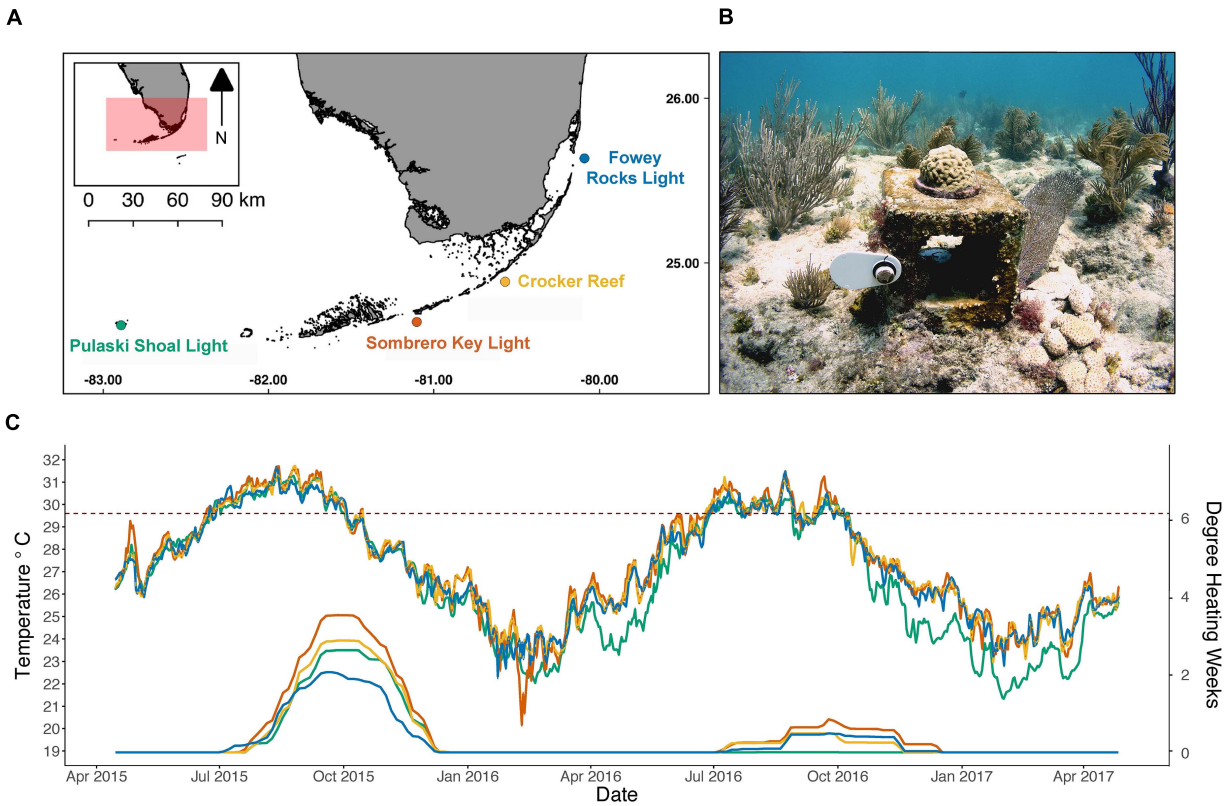

FIGURE 1 | (A) Map of sites established by the U.S. Geological Survey for the calcification assessment network along the Florida Key Reef Tract, with Pulaski Shoal Light (green) in Dry Tortugas National Park, Sombrero Key Light (orange) in the middle Keys, Crocker Reef (yellow) in the southern upper Keys, and Fowey Rocks Light (blue) in the northern upper Keys in Biscayne National Park. **(B)** Underwater photograph (image by I.B. Kuffner) of one of the 40 calcification stations with a *Porites astreoides* colony fastened to the top. The cement block is 19 × 19 × 19 cm for scale. **(C)** Mean daily temperature (top, left axis) at each site with a red dashed line indicating the 29.6_C maximum monthly mean in the Florida Keys (Manzello et al., 2015) and degree heating weeks calculated using climatology specific to each respective site (bottom, right axis).

(Kuffner et al., 2013). Sites were chosen because of historical oceanographic data collected by the Sustained Ecological Research Related to Management of the Florida Keys Seascape (SEAKEYS) program (Ogden et al., 1994), and meteorological data from the National Data Buoy Center (NDBC) C-MAN stations (Kuffner et al., 2013). The study sites are located at Pulaski Shoal Light (Pulaski, 24.694°N 82.773°W) in Dry Tortugas National Park, Sombrero Key Light (Sombrero, 24.627°N 81.109°W) and Crocker Reef (Crocker, 24.563°N 80.527°W) in the Florida Keys National Marine Sanctuary (FKNMS), and Fowey Rocks Light (Fowey, 25.591°N 80.096°W) in Biscayne National Park (**Figure 1A** and **Supplementary Table 1**). All sites are in 3–5 m water depth on the outer reef in low to moderate relief reef-crest habitat. Fowey, Crocker, and Sombrero are 5–10 km off the coast of the Florida Keys island chain, and Pulaski is about 100 km west of the city of Key West, FL.

The mustard hill coral, *Porites astreoides* (de Lamarck, 1816), was chosen for this study because of its abundance throughout the FKRT, year-round reproductive strategy with peak larval release in late April and May (Chornesky and Peters, 1987), and its potential as a bioindicator of thermal stress (Kenkel et al., 2013). *P. astreoides* is a fast-growing "weedy" species common throughout the Caribbean in shallow and deep reefs with higher relative abundance compared to other coral species (Green et al., 2008; Darling et al., 2012). Colonies in shallow reefs are commonly dominated by *Symbiodinium* spp. (formerly Clade A; LaJeunesse et al., 2018), particularly "A4/A4a" (Thornhill et al., 2006; Kenkel et al., 2013; Serrano et al., 2016; Cunning et al., 2018).

At the previously established sites (Kuffner et al., 2019), concrete cinder-block stations (19 × 19 × 19 cm, n = 10 per site), placed 2–4 m apart from one another and fastened to the benthos with two 15 cm stainless-steel threaded rods drilled and epoxied into the substratum (Morrison et al., 2013), were used to monitor coral survival and growth for 2 years. Each block was designed to hold a single coral colony that was epoxied to a polyvinyl chloride (PVC) disc that can be reversibly bolted to the top of the cinder block using a wingnut (**Figure 1B**). In April 2015, 10 *P. astreoides* colonies of the yellow/green color morph measuring 4–6 cm in diameter were collected from each site at 3 to 5 m water depth within ∼200 m using a hammer and chisel to remove colonies from the substratum. A total of 40 colonies were used in the study. Care was taken to collect colonies

no closer than 20 m apart to minimize the chance of collecting genetic clones (Baums et al., 2019). Corals were epoxied onto the PVC discs, weighed, measured, photographed and deployed to the cinder-block stations.

Seawater temperature was recorded every 15 min (n = 96 per day) with HOBO Water Temp Prov2 temperature loggers (Onset, Pocasset, MA, United States ± 0.2°C) attached to two of the cinder-block stations at each site. All temperature data are publicly available (Kuffner, 2016). Mean daily temperature, mean monthly maximum, and mean monthly minimum were used to characterize sites. Degree heating weeks (DHW) for each site during this study were calculated using *in situ* temperature data and were also compared to the National Oceanic and Atmospheric Administration (NOAA) Coral Reef Watch Satellite Regional Stations in the Florida Keys based on local climatology (Wyatt et al., 2020). To characterize the light environments at each site, monthly mean (± SD) photosynthetically active radiation (PAR) data were collected from satellite-derived water-quality data products for the Florida Keys made publicly available through the Optical Oceanography Laboratory[1] using the virtual buoys closest to the sites (Pulaski = FK01, 24.693N 82.773°W; Sombrero = FK07, 24.628°N 81.112°W; Crocker = FK08, 24.816°N 80.676°W; Fowey = FK11, 25.591°N 80.096°W). Daily precipitation was used as a proxy for salinity and was acquired from the NOAA Tropical Rainfall Measuring Mission from the Environmental Research Division's Data Access Program (ERDDAP) that corresponded closest to the sites and summarized by month[2].

Seasonal Measurements: Coral-Calcification Rates, Tissue Loss, and Condition Scores

Every 6 months during the 2-year study (**Supplementary Table 1**), the 40 colonies of *P. astreoides* were retrieved from the field to measure seasonal changes in coral condition and calcification rates. Colonies were brought to shore in coolers or 5-gallon buckets filled with ambient seawater, buoyant weighed (Jokiel et al., 1978), measured with calipers, photographed, and returned to the study site within 6 hrs. The difference in buoyant weight for each colony at each time point was converted to dry mass using 1.02 g cm^{-3} as seawater density and 2.93 g cm^{-3} as aragonite density of the coral skeleton (Jokiel et al., 1978). The rate of coral calcification was calculated using the number of days between each measured interval and normalized to the initial planar-area footprint of the coral colony (using formula for the area of an ellipse; Kuffner et al., 2013). Photographs of each colony were captured on land and were white-balance corrected with the same white, gridded-photography background in each image to assess colony pigmentation over time based on a condition score of (1): no bleaching, (2): paling to normal, (3): 1–50% bleached, (4): 51–99% bleached, (5): 100% bleached, and (6): dead (Baird et al., 2018).

Final Timepoint: Tissue Thickness, Reproductive Potential, and Symbiodiniaceae Communities

At the end of the study in April 2017, coral colonies were sampled to compare coral-tissue thickness, reproduction (i.e., gametogenesis and planulation), symbiont cell densities, and the composition of Symbiodiniaceae taxa across sites. Using a masonry-tile saw, two 4-mm-wide sagittal sections were taken from the center axis of the colony with the saw wetted with ambient seawater. Slices from each colony were photographed and archived. An additional 4 cm × 2 cm section was taken from the center of the colony and processed as described in Chornesky and Peters (1987) for histology to measure tissue thickness (Loya et al., 2001) and reproductive potential. If the center of the colony had died, a section was taken from the colony side. The tissue sample was placed in buffered zinc formalin fixative (Z-fix, Anatech Ltd.) diluted in filtered seawater (1:4) (Padilla-Gamiño et al., 2014) for at least 48 h and then transferred into 70% ethanol (diluted in distilled water) until processed by Histotech Ltd. (Cleveland, OH, United States) where samples were decalcified in diluted HCl and EDTA. The 4–6 μm thick sagittal sections along the tissue and cross sections of the top, middle, and bottom thirds were mounted on slides and stained with hematoxylin and eosin (Chornesky and Peters, 1987). One of the slices was used to examine tissue thickness using microscopic images and the FIJI package of the opensource image software ImageJ (Schneider et al., 2012). Each slice mounted on a microscopic slide consisted of approximately 5–20 polyps. The number of oocytes, spermatocytes, and planula larvae per polyp were counted in ten haphazardly chosen polyps of each colony.

For Symbiodiniaceae, symbiont cell densities were first measured by taking a 4 cm × 2 cm section from the third sagittal slice and removing the tissue from the skeleton using an airbrush filled with filtered seawater (0.1 μm). The tissue slurry was homogenized, and 1 mL was aliquoted into a buffered zinc formalin fixative to later count symbiont cell densities, with n = 10 samples per colony, using a hemocytometer (n = 10 per site, n = 8 at Sombrero). Cell densities were normalized to skeletal surface area (cm^2) determined by the aluminum foil wrapping technique (Marsh, 1970). Second, a sterilized razor was used to remove approximately 5 mm^2 of coral tissue and placed in 1% sodium dodecyl sulfate DNA buffer and shipped to the Hawai'i Institute of Marine Biology (HIMB) where genomic DNA was extracted and isolated using a cetyl trimethylammonium bromide protocol[3]. The amplification of the ribosomal Internal Transcribed Spacer 2 (ITS2) region, a multicopy genetic marker, was used to identify Symbiodiniaceae diversity with next-generation amplicon sequencing (NGS) (LaJeunesse, 2001; Thornhill et al., 2007, 2014). DNA samples were sent to the HIMB Genetics Core Facility for library preparation using "itsD" and "its2rev2" primers (Stat et al., 2009) and sequenced on an Illumina MiSeq platform with 2 × 300 paired-end read chemistry. The paired forward and reverse reads

[1]https://optics.marine.usf.edu/

[2]https://oceanwatch.pifsc.noaa.gov/erddap/griddap/hawaii_soest_5687_3d16_a6d4.html

[3]https://www.protocols.io/view/Bulk-gDNA-extraction-from-coral-samples-dyq7vv

from each sample (provided in fastq.gz files outputted from the Illumina sequencing) were submitted directly to SymPortal[4]. The SymPortal analytical framework is designed to resolve genetically differentiated taxa of Symbiodiniaceae (formerly *Symbiodinium* spp. LaJeunesse et al., 2018) using NGS data of the ITS2 amplicon and its high intragenomic diversity by identifying defining intragenomic ITS2 sequence variants (DIVs) as specific profile types (Hume et al., 2019).

Statistical Analysis

Environmental parameters including temperature, light, and precipitation were compared across the four FKRT calcification network sites using a linear mixed-effects model with *lmer* in package *lme4* (Bates et al., 2015), site as a fixed effect, and date as a random effect. For the biological response variables measured over time (calcification rates, tissue loss and colony-condition scores), univariate analyses were conducted to assess interactive effects of time interval (Summer 2015, Winter 2016, Summer 2016, and Winter 2017) and site (Pulaski, Sombrero, Crocker, and Fowey). Specifically, calcification rates and colony condition scores were analyzed with linear mixed effect models in *lme4* with site and time interval as fixed effects and coral colony as a random effect. ANOVA tables for all environmental and biological metrics were generated using Type II sum of squares Satterthwaite approximation of degrees of freedom using *lmerTest* (Kuznetsova et al., 2017). Percent tissue loss was analyzed using *glmmTMB* for a zero-inflated mixed model with the *glmmTMB* package (Brooks et al., 2017) with the same fixed and random effects. *Anova* in the package *car* (Fox et al., 2013) was used to generate a Type II Wald Chi-square test of the zero-inflated mixed model. *Post hoc* Tukey tests were conducted using the package *emmeans* (Lenth, 2018). Assumptions of analysis of variance were confirmed of all models using graphical inspection of the residuals with the *DHARMa* package (Hartig, 2017) and the *simulateResiduals* command.

The coral-level biological responses (tissue loss, tissue thickness, fecundity, spermatocytes, symbiont cell density, calcification rate, and condition score) for *P. astreoides* were analyzed using a permutational multivariate analysis of variance (PERMANOVA) with 999 model permutations using *adonis2* in the *vegan* package (Oksanen et al., 2007). First, the three response variables were assessed by site and time interval as main effects and second, all response variables measured in the final time interval. Principal components analysis (PCA) was used to visualize the multivariate relationship of the response variables by sites for each time interval separately with the *factoextra* package (Kassambara and Mundt, 2017).

RESULTS

Environmental Conditions

Of the environmental data available for each site, only *in situ* temperature revealed site-level differences (**Figure 1C**). Mean daily temperature measured throughout the study significantly differed across sites (**Figure 1C**; $P < 0.001$) except for mean daily temperatures between the Crocker and Sombrero reef sites in the middle and lower Keys, respectively ($P = 0.247$). There were season-specific differences in mean temperature across site ($P < 0.001$), except in Summer 2015 between Pulaski and Crocker (Tukey *Post hoc*, $P = 0.998$) and between Sombrero and both Crocker and Fowey (Tukey *Post hoc*, $P \geq 0.468$). During the height of the coral bleaching event in August 2015 (Manzello et al., 2018), the maximum mean daily temperatures reached 32.3°C at Fowey, 32.1°C at Crocker and Sombrero, and 31.7°C at Pulaski (**Figure 1C**), exceeding the 30.5°C bleaching threshold in the Florida Keys (Manzello, 2015). These values are above the maximum monthly mean from NOAA Coral Reef Watch (2020) Version 3.0 Daily Global 5-km Satellite Virtual Station for each site which is 29.53°C at Fowey (2.7-km from site), 29.63°C at Crocker (1.7-km from site), 29.71°C at Sombrero (1.7 km from site), and 29.59°C at Pulaski (2-km from site). *In situ* temperature measured at Pulaski in Dry Tortugas National Park was generally cooler than the other sites, and DHW reached 2.6 in September 2015 and 0 in 2016 while Sombrero reached 3.6 and 0.9 DWH in 2015 and 2016, respectively (**Figure 1C**). Precipitation (mm), as a proxy for salinity, was similar across the four sites ($P \geq 0.099$) with mean ± SE seasonal precipitation for all sites ranging from 258.7 ± 30.9 mm at minimum in Winter 2017 to 801.2 ± 103.8 mm at maximum in Summer 2016. Mean ± SE of PAR estimated at the seafloor did not differ by site ($P = 0.999$) but differed by season, with winter having lower values of 40.6 ± 1.6 mol m^{-2} d^{-1} than the summer values of 55.5 ± 1.3 mol m^{-2} d^{-1} ($P < 0.001$).

Calcification, Tissue Loss and Coral Condition Through Time

From 2015 to 2017, the mean ± SE net calcification rate of *P. astreoides* across all sites was 3.1 ± 0.2 mg cm^{-2} d^{-1} ($n = 40$, all data are available in Kuffner et al., 2021). Net calcification rates varied significantly by site and time period, and sites responded differently across time periods (**Supplementary Table 2**). If only corals considered to be "live" (retaining > 50% live tissue) are included, mean net calcification rates across the 2-year study were 4.8 ± 1.2 mg cm^{-2} d^{-1} ($n = 10$) at Pulaski Shoal and 2.9 ± 0.8 mg cm^{-2} d^{-1} ($n = 21$) at the other Florida Keys sites. The highest rate of net calcification measured during any one time period at any site was 9.4 mg cm^{-2} d^{-1} ($n = 10$) at Pulaski Shoal. Mean net calcification rates of colonies at Pulaski and Fowey were 56 and 47% higher in the summer than winter, respectively (**Figure 2A**, $P < 0.001$). In the middle Keys, mean net calcification rates were lowest at Sombrero, 1.7 ± 0.4 mg cm^{-2} d^{-1}, and continued to decline over time. In the southern end of the upper Keys at Crocker, mean net calcification rate was 2.4 mg cm^{-2} d^{-1}, but in summer 2016 was 49% higher than the other time periods (**Figure 2A**; $P = 0.006$).

Mean percent live-tissue loss by the end of the 2-year study was low for corals at Pulaski and Fowey Rocks with 0.04 and 13%, respectively, and higher at Sombrero and Crocker reefs, with 47 and 13%, respectively ($n = 40$; **Figure 2B**). There was a significant interaction of site and time interval ($P < 0.001$). Sombrero had colonies that underwent a threefold increase in

[4]http://symportal.org, http://github.com/SymPortal

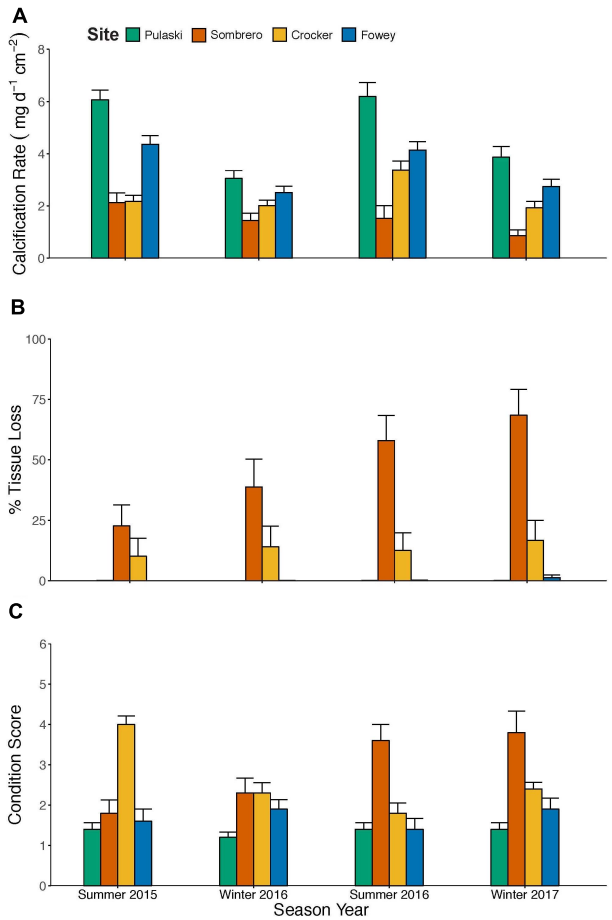

FIGURE 2 | Coral traits measured after the winter and summer seasons between April 2015 and 2017 for 40 colonies of the coral *Porites astreoides* from four sites on the Florida Keys Reef Tract. **(A)** Mean net calcification rates normalized to planar-footprint area (± SE), **(B)** accumulative percent-planar tissue loss (± SE), and **(C)** condition scores at the end of each 6-month time period based on a scale from 1 to 6, with six indicating the poorest condition based upon paling, bleaching, and mortality (Baird et al., 2018).

tissue loss from the initial to final time point of the study (Tukey *Post hoc* $P < 0.001$), including one dead colony and four other colonies with over 90% tissue loss. Percent tissue loss remained steadier at Crocker (Tukey *Post hoc* $P \geq 0.868$) and higher than at Pulaski and Fowey.

The coral condition scores based on visual paling, bleaching and mortality had a significant interactive effect with site and time interval ($n = 40$; $P < 0.001$), demonstrating that Pulaski and Fowey had consistently healthy colonies throughout the study. Corals at Crocker were highly stressed in Summer 2015 and recovered, while corals at Sombrero accumulated stress over time in response to the high temperature anomalies (**Figure 2C**).

Two-dimensional PCA plots explained 83.9–92.1% of the variation and highlight differences in overall coral condition among sites at each time interval (**Figures 3A–D**). The high (poor) condition score at Crocker in Summer 2015 isolated this site from the rest. The consistently higher calcification rates at Pulaski across seasons distanced corals at that site from those at Sombrero.

Final Holobiont Responses

Tissue thickness varied by site (n = 3–10 per site; $P < 0.001$; **Supplementary Figure 1A**) and was >50% lower at Sombrero than colonies from all other sites (Tukey *Post hoc*, $P \geq 0.075$), while tissue thickness of colonies from Pulaski, Crocker, and Fowey did not differ (Tukey *Post hoc*, $P \geq 0.075$). Symbiont cell densities in colonies from Pulaski had a 2 to 3-fold higher concentration, 6.08×10^6 cells cm^{-2}, than the other three sites ($n = 10$ per site except Sombrero which had $n = 3$; **Supplementary Figure 1B**; Tukey *Post hoc*, $P \leq 0.015$).

For reproduction, oocytes, spermatocytes, and planula larvae were present in all colonies from Pulaski; Sombrero only had three reproductive colonies (**Supplementary Figure 2**). Mean oocytes, spermatocytes, and larvae differed by sites (**Supplementary Table 3**; $P \leq 0.039$). Colonies at Sombrero had 85% less oocytes per polyp than at both Pulaski and Fowey (**Supplementary Figure 1C**; $P \leq 0.025$). The number of spermatocytes per polyp marginally differed between Pulaski and Crocker (Tukey *Post hoc* $P = 0.065$). Pulaski had 35% more larvae per polyp than Sombrero, Crocker, and Fowey (Tukey *Post hoc* $P < 0.001$).

Using the SymPortal pipeline, we were able to determine different relative abundance of defining-intragenomic variants (DIVs) by site with colonies from Pulaski consisting of only three DIVs, whereas the other sites had five (n = 7 per site). *Symbiodinium* spp. (formerly Clade A, LaJeunesse et al., 2018) was ubiquitous in all colonies sampled at all four sites. Seven Symbiodiniaceae ITS2 type profiles were generated from SymPortal, with three being unique to the colonies sampled at Pulaski, while five occurred throughout corals at Sombrero, Crocker, and Fowey (**Supplementary Figure 3**).

Taken together, the suite of holobiont response variables (calcification rate, condition score, tissue loss, tissue thickness, oocyte and larval density, spermatocyte density, and symbiont cell density) demonstrated clear overall differences by site (PERMANOVA $P = 0.001$). There was no significant effect of symbiont ITS2-profile type DIV ($P = 0.720$), nor of the interaction between site and symbiont type ($P = 0.081$; **Supplementary Table 3**). The site-level differences were evident with calcification, symbiont cell densities, and reproductive potential highest for Pulaski in Dry Tortugas National Park and in the poor status of colonies at Sombrero in the middle Keys showing high (poor) coral condition scores and high tissue loss. This multivariate representation of the overall coral response can be visualized in the PCA plot, showing that the analyses accounted for 53% of the variation associated with the PC1 axis and 20% on PC2 (**Figure 4** and **Supplementary Table 2**). Separation by site was most apparent between Sombrero and Pulaski. **Figure 4** illustrates that symbiont cell density, fecundity, and calcification rates respond together and high levels of those variables were most associated with Pulaski. Similarly, high levels of tissue loss and poor coral-condition score responded together with the inverse of tissue thickness (**Figure 4**).

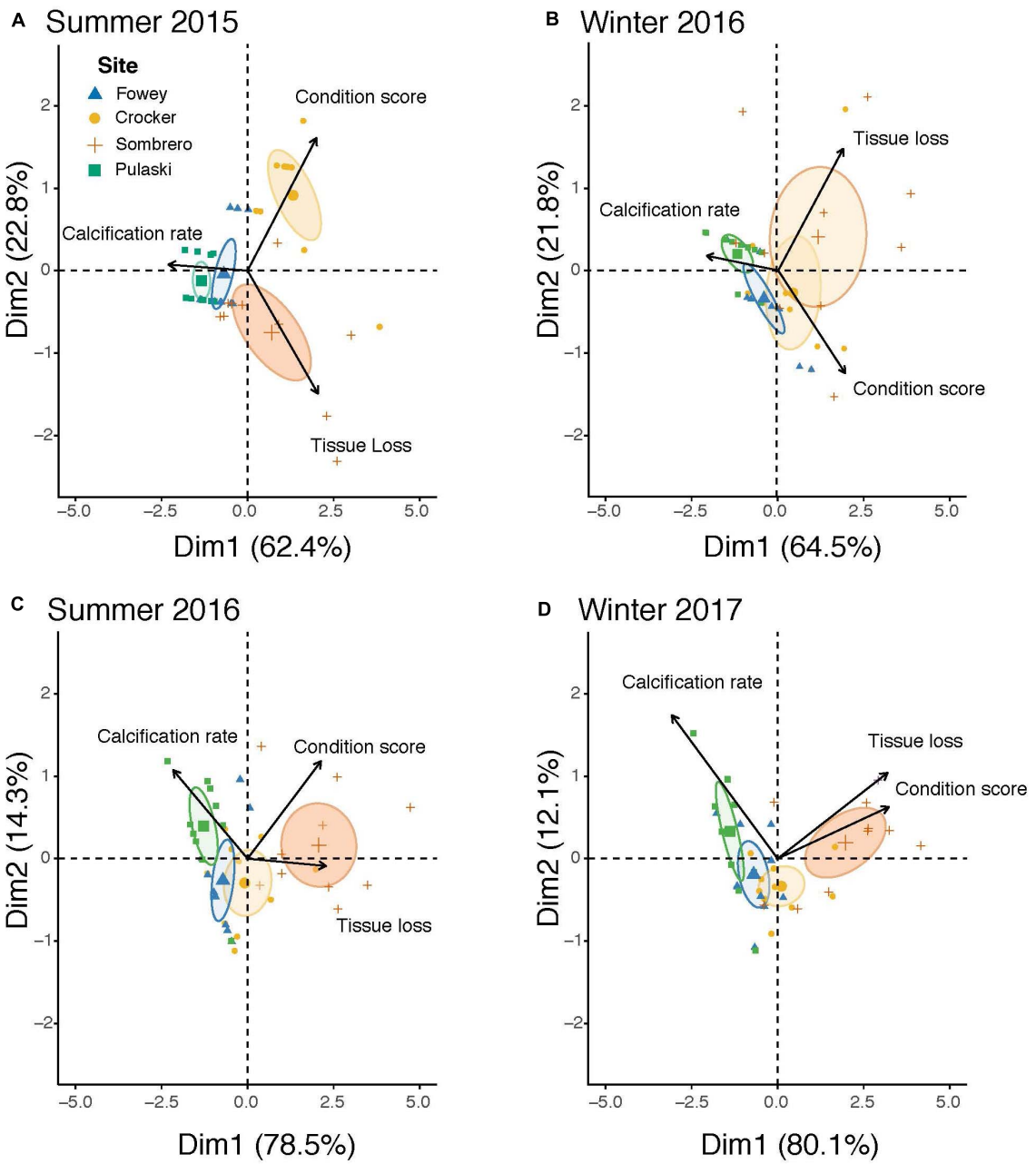

FIGURE 3 | Principal components analysis (PCA) of dimension 1 and 2 with percent variation explained in parentheses consisting of the three traits measured for corals on the Florida Key Reef Tract at each site at each time interval: **(A)** Summer 2015, **(B)** Winter 2016, **(C)** Summer 2016, and **(D)** Winter 2017. 95% confidence ellipses are placed around the mean of each site.

DISCUSSION

Against a backdrop of historical and modern changes in reef condition along the Florida Keys reef tract, Dry Tortugas National Park represents an exceptional location, demonstrated by thicker and longer duration of Holocene reef development (Toth et al., 2018) and higher rates of net coral calcification (Kuffner et al., 2013, 2020). Our study, based on quantitative comparisons of coral performance and proxies for fitness including colony-level calcification, coral-algal symbiosis, and reproduction, provided even more evidence supporting the exceptionality of the Dry Tortugas area. Understanding the capacity for corals to continue high physiological performance, even in extreme, fluctuating environments with prolonged temperature anomalies, can determine resilient from susceptible coral reefs and specific coral species under anthropogenic climate change (Loya et al., 2001; Fabricius et al., 2011; Grottoli et al., 2014). As thermal stress intensifies, it is important to identify

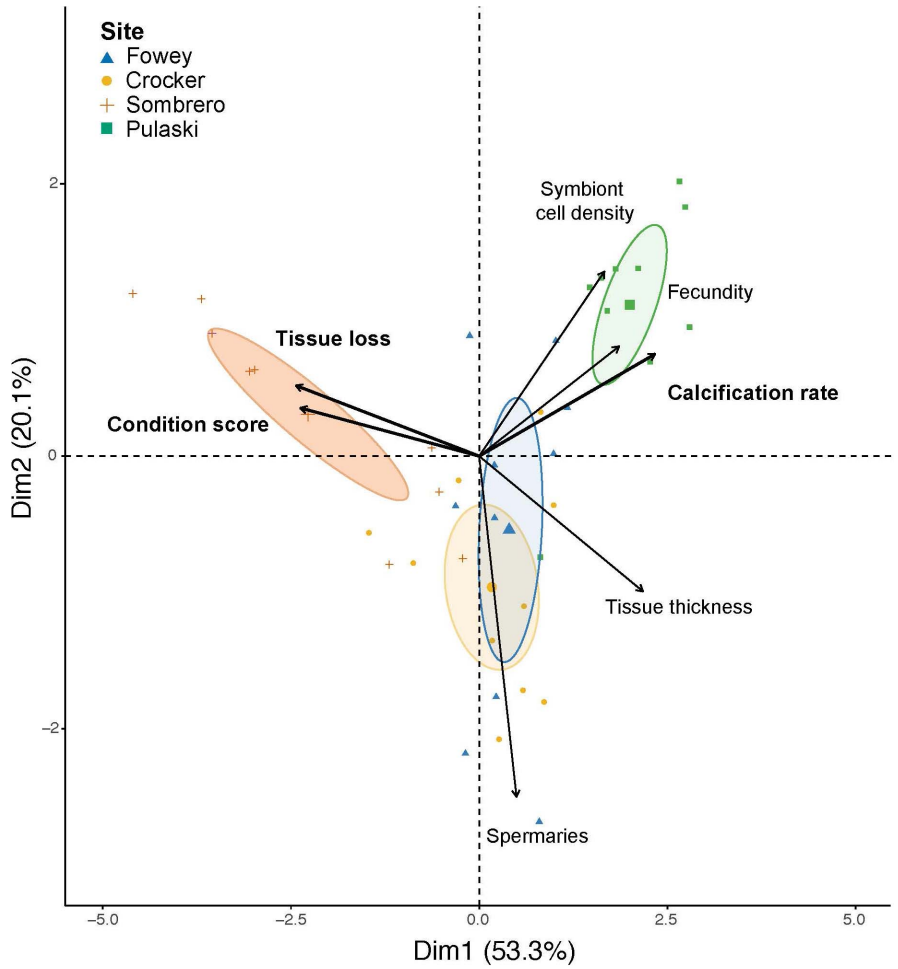

FIGURE 4 | Principal components analysis (PCA) of dimensions 1 and 2 explaining 83.4% of the variation when incorporating traits in corals destructively sampled at the end of the study: fecundity (oocytes and planula larvae per polyp), spermaries per polyp, Symbiodiniaceae cell densities, and tissue thickness with the three non-destructive traits measured and bolded (calcification rates, tissue loss, and condition score). Corals are from 4 sites on the Florida Key Reef Tract; 95% confidence ellipses are placed around the mean for each site.

coral traits that can serve as proxies for fitness and determine potential trade-offs among these traits, specifically regarding *in situ* coral-algal symbiosis and reproduction concurrent with calcification along the FKRT (Kuffner et al., 2013; Manzello et al., 2015; Toth et al., 2018).

Our study demonstrated site-specific differences in *P. astreoides* bleaching and recovery response during and after a high-temperature stress event, suggesting that either environmental setting, coral genetics, or both elicited a gradient in "sensitive" to "resistant" responses across the Florida Keys reef tract. During and after the 2015 bleaching event in the Florida Keys (Gintert et al., 2018; Manzello et al., 2018), *P. astreoides* at Pulaski in Dry Tortugas N. P. consistently outperformed colonies at the other three sites, based on high net-calcification rates and symbiont cell densities, low levels of tissue loss, and high reproductive potential. Our results lend additional evidence supporting the hope that "oases" reef sites may still exist (Guest et al., 2018), allowing some corals to persist and continue thriving under the increased thermal stress impacting the FKRT. We documented that critical biological processes, including calcification, symbiosis, and reproduction, were maintained at certain sites while failing at others, suggesting that some populations can persist even when major regional disturbances like the 2015 bleaching event continue to occur.

While *P. astreoides* has been referred to as a "winner" species in the Caribbean because of its weedy life history (Darling et al., 2012), potential resistance to stony coral tissue loss disease (Aeby et al., 2019), and increasing relative abundance (Green et al., 2008), our results indicate caution in that even such weedy winners are vulnerable and that site-specific responses can inform ecological limits of a resistant species (Grottoli et al., 2014; Manzello et al., 2015). For instance, even though *Symbiodinium* spp. hosted by shallow-dwelling *P. astreoides* colonies are mildly tolerant of ultraviolet and high-temperature stress (Loram et al., 2007; Reynolds et al., 2008), *P. astreoides* is still sensitive to rapid temperature fluctuations and susceptible

to bleaching (Edmunds et al., 2005; Gleason et al., 2006; Grottoli et al., 2014; Manzello et al., 2015). The prevalence of *P. astreoides* throughout the Caribbean and FKRT is likely due to its high-output reproductive strategy and mode as an asexual, hermaphroditic and gonochoric brooder, releasing larvae for multiple months (Chornesky and Peters, 1987; McGuire, 1998; Green et al., 2008; Schlöder and Guzman, 2008; Edmunds, 2010; Yakob and Mumby, 2013; Serrano et al., 2016). This reproductive ability supports high success in recruitment and population recovery following disturbance (Edmunds, 2010), and a high level of larval dispersal is suggested by the lack of population structure in shallow coral reefs of FKRT (Serrano et al., 2016; Gallery et al., in press). However, it is important not to take for granted the reproductive savviness of this species and consider its potential limitations, as many previously thriving species are declining in the FKRT and Caribbean, such as *Acropora palmata,* because of disease susceptibility and reproductive failure (Baums et al., 2005; Williams et al., 2008; Randall and Szmant, 2009).

Patterns in Calcification Rates Across the FKRT

Coral calcification, or the net accumulation of calcium-carbonate skeleton, is an integrated measure of coral performance responsive to environmental changes over geological and ecological time scales (Kuffner et al., 2013; Toth et al., 2018). Here, we demonstrated that the corals at our middle Keys and southern upper Keys sites had consistently lower net calcification rates that reflected no seasonal influence, whereas corals in the Dry Tortugas and at the northern upper Keys had overall higher calcification that was seasonal (greater in summer). A study by Manzello et al. (2015) showed similar geographic results, with lower rates of calcification observed in the middle Keys for *P. astreoides* and *Montastraea cavernosa*. On a geologic timescale, there is a similar geographic pattern observed in Holocene reef development, with the thickest reefs at either end of the FKRT and the middle Keys suppressed (Toth et al., 2018). Thus, evidence continues to mount in support of Ginsburg and Shinn's (1964) inimical-waters hypothesis explaining these spatial patterns in coral calcification and reef development. Pinpointing what environmental variables are most important in driving rates of calcification and reef development remains elusive. Temperature (Shinn, 1966; Jokiel and Coles, 1977, 1990), salinity (Coles and Jokiel, 1978; Lirman and Manzello, 2009), carbonate mineral saturation states (Langdon et al., 2000; Albright et al., 2016), light attenuation (Falkowski et al., 1984), and flow (Dennison and Barnes, 1988) can greatly influence coral growth. Our *in situ* temperature data do suggest that temperature variability may be an important difference among the sites in determining bleaching vulnerability, as Sombrero Reef experienced the highest and the lowest mean daily temperatures. As reef managers update marine-spatial planning and plan extensive reef-restoration activities, it is important to develop finer scale capabilities in the collection of remotely sensed and *in situ* environmental data (Ogden et al., 1994) so that we can better understand and characterize the conditions that are conducive to high coral-calcification rates and positive reef accretion.

Key Biological Traits to Assess Coral Condition

Most reef-monitoring programs that track the long-term health and status of reefs focus primarily on the percent cover of live corals, algae, and other benthic organisms (Edmunds, 2002; Brown et al., 2004; Ruzicka et al., 2013). In our study, we measured several physiological variables critical to biological processes (i.e., calcification, symbiosis, and reproduction), and these measurements allowed us to make clear distinctions in coral condition among sites. Using a dual approach, we measured traits both non-destructively over time (using buoyant weight and photographic image analysis) and destructively at the end of the study to assess tissue thickness, reproductive potential, and maintenance of the coral-algal symbiosis. We discovered that responses were generally correlated, indicating a lack of trade-offs among processes such as calcification and larval production. Through the 2-year study we identified Sombrero and Pulaski as the worst and best locations for *P. astreoides*, respectively, with Crocker and Fowey as moderate sites. It should be noted that even though there was a loss and thinning of tissue at Sombrero with few Symbiodiniaceae cells and nominal to no oocytes or larvae present, spermaries were still functional and producing spermatocytes in the remaining tissue. Work on other spawning marine invertebrates (e.g., oysters, urchins, and ascidians) has demonstrated reproductive plasticity in response to environmental changes, particularly continued functioning of male-gamete production after egg production and traits were altered; the production of male gametes is considered less energetically demanding and may serve as a last-ditch effort for genetic persistence (Levitan and Petersen, 1995; Crean and Marshall, 2008). Ward (1995) observed substantial energetic costs in reproduction, lipid storage, and calcification in the brooding coral *Pocillopora damicornis*; colonies that released larvae required more lipids for developing larvae and calcified more slowly than colonies that only released sperm. For *P. astreoides* colonies from Dry Tortugas, we found no evidence of trade-offs, as the colonies maintained high calcification rates and produced the most larvae and gametes of both sexes, as well as having higher symbiont cell densities compared with corals at the other sites. Determining why coral condition was so universally better in Dry Tortugas compared to the other Keys reef tract sites will require further testing. Given the importance of heterotrophic feeding in determining lipids stores and the resilience they impart during bleaching events (Grottoli et al., 2006; Solomon et al., 2020), testing the hypothesis that corals in Dry Tortugas are receiving nutritional subsidies (more or higher quality plankton) from upwelling events could reveal important insights regarding the mechanism behind the enhanced growth rates we observed.

Symbiodiniaceae species and community composition greatly influence the strength of coral-algal symbiosis under elevated temperatures (Baskett et al., 2009; Putnam et al., 2012; Cunning et al., 2015; Parkinson et al., 2015), including survivorship

and maintenance of calcification (Stat et al., 2008). For instance, symbiosis with *Cladocopium* spp. and *Durusdinium* spp. (formerly Clade C and D, respectively) is commonly identified as being thermally tolerant but it is also associated with reduced coral-calcification rates (Stat and Gates, 2011; Pettay et al., 2015). Further, symbiont communities or shuffling of symbionts housed within the coral may be shaped by environmental conditions (Jones et al., 2008; LaJeunesse et al., 2010; Cunning et al., 2015). Our study demonstrated that the association of *Symbiodinium* spp. (A4 and other subtypes) with *Porites astreoides* was ubiquitous (A4 and other subtypes), which is consistent with previous studies (Thornhill et al., 2006; Kenkel et al., 2013; Serrano et al., 2016). *Symbiodinium* spp. are considered tolerant of high ultraviolet and thermal conditions; however, knowledge of the physiological functioning of subtypes (e.g., A4a, A4e) or the combination of subtypes (i.e., A4-A4e-A4a) remains limited. Since symbiont communities were similar across our sites, we do not have any evidence to support that site differences in bleaching response resulted from differences in zooxanthellae populations. According to Grottoli et al. (2014), the inflexibility of shallow-dwelling *P. astreoides* under repeated warming events can result in accumulated stress, turning former Caribbean coral "winner" species into a "loser." Accumulated stress from back-to-back marine heatwaves (2014 was also a bleaching year in the Florida Keys Precht et al., 2016) paired with the inflexibility of *P. astreoides* with respect to symbiont shuffling and lack of upregulated feeding under temperature stress could explain the observed poor performance of colonies located at Sombrero Reef. *Porites astreoides* has demonstrated high adaptive plasticity between inshore and offshore colonies (Kenkel et al., 2013; Kenkel and Matz, 2016); therefore, a common garden or reciprocal transplant experiment would be required to address if local adaptation or acclimatization of corals along the FKRT contributes to differential susceptibility to heat stress (Sanford and Kelly, 2011; Torda et al., 2017).

Identifying Environmental and Biological Drivers

The SEAKEYS program served as an invaluable monitoring resource that characterized water-column parameters at several offshore navigational structures across the FKRT subregions (Ogden et al., 1994); however, with the discontinuation of this program in 2009 we relied on our own *in situ* temperature loggers, virtual buoys, and remote satellites to contextualize the environment at each site. Understanding the species-specific responses of corals and reef-building processes to environmental and biological drivers is important and would benefit from more attentive monitoring for effective management and restoration. With the intensity and frequency of marine heatwaves increasing, repeated bleaching events are becoming a new normal. This study is a snapshot within a 2-year window with limited ability to fully contextualize environmental conditioning, selection, and position along a performance curve at each study site. With recent events, there have been conflicting outcomes demonstrating how colonies are able to retain (Ritson-Williams and Gates, 2020); strengthen (Brown et al., 2002; Putnam, 2021), or weaken (Grottoli et al., 2014; Gintert et al., 2018) resistance to bleaching. Through increased monitoring and identification of key environmental parameters beyond temperature, higher-resolution maps of potential reef refugia could be developed (Guest et al., 2018; Lyons et al., 2020). For example, modeling the extent and dynamics of inimical waters impacting coral performance within reefs of the middle and upper Keys as warming continues could better explain the differential responses to coral bleaching within and among species as observed by Gintert et al. (2018). Further understanding of the biological drivers such as local adaptation, gene expression, nutritional provisioning, and symbiosis can be further examined to explain differences in coral performance and reef-building potential in the FKRT subregions. While outlier sites exist along and within subregions of the FKRT (Kuffner et al., 2013, 2020; Ruzicka et al., 2013; Gintert et al., 2018; Toth et al., 2018), it remains unknown why these sites are relatively more "exceptional," and a better understanding of biophysical settings could help differentiate the mechanisms by which they can escape, resist, or recover from disturbance events (Guest et al., 2018).

Reef Protection and Restoration

The Florida Keys Reef Tract is in a precarious state given that live-coral cover is less than 5% (Ruzicka et al., 2013; Toth et al., 2014) and reef development is net negative in many locations (Perry et al., 2013; Yates et al., 2017; Kuffner et al., 2019). The reignition of successful sexual reproduction and coral recruitment is of utmost importance to the re-establishment of coral populations and reef accretion, for currently, the reefs of the Florida Keys are not in a positive-growth phase and have not been since the mid to late Holocene (Toth et al., 2018). Locating reefs where coral populations historically maintained ecological function, such as maintaining three-dimension structure, biodiversity, and productivity, especially in regions that have undergone warming in the past (Richey et al., 2007; Stathakopoulos and Riegl, 2015), could advance research in determining site selection for reef restoration efforts along the continuum of environmental and physical conditions throughout the Florida Keys. Our results demonstrated that measuring calcification rates *in situ* is a readily accessible approach that can serve as a proxy for other coral-performance traits to identify successful populations.

Here, we provide evidence that higher calcification rates do not translate to resource partitioning or trade-offs as seen in our *P. astreoides* colonies living in Dry Tortugas National Park (DTNP). The Pulaski study site within DNTP is remote at the western terminus of the reef system, furthest away from anthropogenic sources of degradation (e.g., coastal development and run-off). DTNP potentially serves as a source for both larval delivery to FKRT sites as the Florida Current could disperse larvae northward (Lee et al., 1992, 1994; Serrano et al., 2016) and larval retention due to the presence of mesoscale eddies that extend down to 100 m depths (Hitchcock et al., 2005; Kourafalou and Kang, 2012). The transport of larvae from healthy populations could be critical in supplying reefs in FKRT impacted by multiple stressors. Recent work by Schoepf et al. (2019) showed that exposure to elevated temperatures does

not guarantee stress-resistance, and naïve corals from suitable and cooler conditions were more able to withstand heat stress than counterparts periodically conditioned. Reefs in DTNP are repeatedly bathed in cooler waters and thus may benefit from episodic upwelling (**Figure 1C**; Ogden et al., 1994; Kuffner, 2016), and depending on pelagic larval duration of coral species, the populations naturally occurring or those potentially restored there in the future could assist in repopulating reefs in FKRT, if those habitats are suitable for recruitment and coral growth.

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: https://www.ncbi.nlm.nih.gov/, PRJNA727078. NCBI BioProject: https://www.ncbi.nlm.nih.gov/bioproject/727078 and the two USGS data release [Kuffner et al. (2021), https://doi.org/10.5066/P955KBD3 and Kuffner (2016), https://doi.org/10.5066/F71C1TZK].

AUTHOR CONTRIBUTIONS

IK and EL designed the study and wrote the manuscript. EL analyzed the data. All authors edited and approved the final version of the manuscript, and collected the data.

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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.2021.615795/full#supplementary-material

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Disturbance-Mediated Changes in Coral Reef Habitat Provoke a Positive Feeding Response in a Major Coral Reef Detritivore, *Ctenochaetus striatus*

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Coral reefs are undergoing global phase shifts from coral-dominated to algae-dominated stages. The negative effects of this substratum shift on the diversity and abundance of fish have been well documented, but the influence on fish feeding is less studied, which may limit a deeper understanding of trophic pathways in such a disturbed system. In this study, we investigated the feeding response of a numerically dominant fish species *Ctenochaetus striatus* to different substrate types, including hard coral, short algal turfs (SATs, <5 mm), and long algal turfs (LATs, >5 mm), on reefs in the South China Sea. The biomass of *C. striatus* showed an inverted U-shaped relationship with coral coverage and a significant positive correlation with SAT coverage ($p < 0.05$), indicating that rising SAT coverage associated with moderate coral loss provoked a feeding response in *C. striatus*. Stomach contents of *C. striatus*, analyzed using high-throughput sequencing (HTS), were dominated by algal sequences (relative read abundance, RRA > 80.0%), including macroalgae, filamentous algae, and microalgae (e.g., *Symbiodinium* and *Prorocentrum*). The sequence number and diversity of microalgae (mainly dinoflagellates) tended to be abundant (RRA 13.5–36.5%) with increased SAT cover, but brown algae sequences (RRA 17.2–57.8%) or green algae sequences (RRA > 50.7% except one site) dominated the stomach content DNA in reefs with high coral cover and high LAT or macroalgal cover, respectively. Considering the limited ability of *C. striatus* to remove mature algae, macroalgal DNA might be from algal debris. Our results indicate that *C. striatus* populations respond positively to conditions of moderate coral loss through increases in body condition identified as increased biomass. These responses are correlated to the expansion of SAT's as coral cover declined, however, this relationship reverses if coral loss is high due to the

succession of LAT's over SAT's and a corresponding decrease in the quality of food available. Our use of HTS has nevertheless identified the importance of detritivory in the flow of energy through reefs in the Anthropocene which are increasingly becoming depauperate in hard coral.

Keywords: epilithic algal matrixes, phase shift, habitat degradation, *Ctenochaetus striatus*, microalgae, high-throughput sequencing

INTRODUCTION

Coral reefs are degenerating worldwide under anthropogenic and natural stresses, causing phase shifts from coral-dominated to algae-dominated reefs (Bellwood et al., 2006; Cheal et al., 2010). In several cases, this has led to a rise in coverage of highly productive and stress-tolerant algal turfs (Connell et al., 2014). Algal turfs contain filamentous algae, sediments, microalgae, and detrital material and can occupy 30–80% of the reef substratum after coral degradation (Wilson and Bellwood, 1997; Goatley and Bellwood, 2011; Kramer et al., 2014; Tebbett et al., 2020). These substratum changes will not only influence coral recruitment (Bellwood and Fulton, 2008; Spear et al., 2019; Tebbett and Bellwood, 2020), but also influence consumers relying on benthic primary productivity, such as detritivorous/herbivorous fishes (Bellwood and Fulton, 2008; Marshell and Mumby, 2015; Bellwood et al., 2019). Given shifting benthic configurations on coral reefs toward algal turfs (Goatley et al., 2016), detritivorous/herbivorous fishes feeding on algal turf are becoming critical in Anthropocene coral reefs (Max et al., 2013; Bellwood et al., 2019).

Although extensive coral degradation will exert negative effects on the diversity and abundance of many reef fishes, species with resource utilization patterns less dependent on live coral may differ in their responses to disturbances (Pratchett et al., 2011). Compared with coral-dependent species, detritivorous/herbivorous fishes may benefit from moderate coral reduction (Wilson et al., 2006). Russ et al. (2018) found that detritivorous surgeonfishes typically feed on dead substrata and thus increase in density with the reduction in live hard coral cover after large environmental disturbances. Several recent studies have also shown that the distribution of detritivorous/herbivorous fishes is often correlated with their food resources, such as algal turfs and fleshy macroalgae (Russ et al., 2015; Tootell and Steele, 2016). Indeed, coral loss will usually be followed by the expansion of benthic algae and other non-coral organisms, and food resources for detritivorous/herbivorous fishes may become more available than on coral-dominated reefs. However, previous studies have paid more attention to the distribution or variation in abundance of fish, with the effect of these substratum changes on the feeding of fish less studied, limiting insights into the adaptability, trophic interactions, food webs and functional delivery of reef fish (Wilson et al., 2006; Brandl and Bellwood, 2014; Leal and Ferrier-Pages, 2016; Tebbett et al., 2020).

Extensive coral reef degeneration will also exert negative effects on detritivorous/herbivorous fishes, not only due to the decline in habitat and topographical complexity (Pratchett et al., 2011), but also due to the change in food quality and palatability (Tebbett et al., 2020). Algal turfs have a spectrum of forms from short productive algal turfs (SATs) to long sediment-laden algal turfs (LATs), with the latter more abundant on disturbed reefs (Goatley et al., 2016; Tebbett and Bellwood, 2020). Compared with LATs, SATs have a higher detrital proportion and fewer sediments, which make them more palatable for detritivorous fish (Tebbett and Bellwood, 2020). A recent study found that high sediment loads on algal turfs would limit the abundance and feeding activity of *C. striatus* (Tebbett et al., 2020), a major detritivore on tropical reefs (Trip et al., 2008; Cheal et al., 2012). *Ctenochaetus striatus* is involved in a series of ecosystem functions, such as detritivory and sediment removal (Goatley and Bellwood, 2010; Marshell and Mumby, 2015), but it is sensitive to sediments remaining in algal turfs (Tebbett et al., 2017a,b), and LATs are also not suitable for its distinctive "brushing" feeding habit (Tebbett et al., 2017c). It is interesting that while *C. striatus* is sensitive to substratum traits it is widely distributed on various disturbed reefs, and more details about the dietary changes behind substratum shifting may provide important information for revealing the responses and adaptability of this species to disturbances (Tebbett et al., 2017b, 2020; Bellwood et al., 2019). However, few studies have investigated the correlation between the specific species within the algal turfs that *C. striatus* feeds on, which may limit an extensive analysis of the function and trophic roles of this important detritivore on Anthropocene reefs (Bellwood et al., 2019; Tebbett et al., 2020).

Obstacles to documenting the diversity of the *C. striatus* diet include the complex dietary sources and the high level of mechanical processing during the digestive process of this fish (Choat et al., 2004). It is difficult and time-consuming to identify the stomach contents of *C. striatus*. Most previous studies have classified food items into different morphological types, such as turf algae, macroalgae, sediments, and other unidentified materials (mostly categorized as detritus; Kelly et al., 2016; Tebbett et al., 2017c), without identifying the specific species or components in the diets of *C. striatus*. For example, microbes and microalgae that are often classified as detritus are also a potential nutrition source for *C. striatus* and other detritivores (Wilson et al., 2003; Tebbett and Bellwood, 2020). However, the species information of these cryptic organisms has often been deficient, which may ignore some subtle but important variations in the resource utilization of detritivores.

In recent years, high-throughput sequencing (HTS) has been widely used for trophic analyses of animals, including fish and other marine animals, because of its accuracy, comprehensiveness, and ability to process large databases (O'Rorke et al., 2012). HTS is highly suitable for screening

certain cryptic species, which are difficult to precisely identify using traditional gut morphological identification methods (Leal and Ferrier-Pages, 2016), such as microalgae in the diets of gelatinous zooplankter, ciliates ingested by juvenile fish and dietary partitioning among cryptobenthic species (Lin et al., 2018a; Walters et al., 2019; Brandl et al., 2020). Meanwhile, the sources of detritus in the algal turfs are from a variety of organisms (Wilson et al., 2003), which implies that the DNA of these organisms remaining in the detritus could provide some useful information about the possible sources of detritus. Hence, we applied HTS to investigate the specific components of the diets of *C. striatus* and how this varies across coral reef habitats in the South China Sea with differing levels of degradation. The study objectives were to (1) identify the exact species removed or ingested by *C. striatus* and (2) determine whether and how the abundance, biomass, and specific food species of *C. striatus* are influenced by the substratum. These results will offer an improved and more detailed perspective on the diets of detritivorous fish and take a key step forward in understanding the influence of coral degeneration on detritivory and trophic interactions on coral reefs.

MATERIALS AND METHODS

Study Design and Location

Seven reefs with different levels of hard coral coverage located at the same latitude and relatively far away from the mainland (N: 8°51′–10°57′ and E: 112°50′–115°35′, **Figures 1A,B**), were surveyed in the southern part of the South China Sea in May 2016 and 2017. Considering that detritivorous/herbivorous fishes may benefit from moderate coral loss, three reef categories characterized by different coral dominance levels were classified: HC reefs (the highest coverage of hard living coral with more than 35%), IC reefs (intermediate coral coverage of 15–30%), and LC reefs (the lowest coral coverage $\leq$ 15%). The classification standards were mainly based on the historical data of surveyed reefs and relatively healthy reefs in other sea areas (Kingman Reef and Palmyra Atoll reef; Huang et al., 2012; Williams et al., 2013; Zhao et al., 2013). For site selection, at least three sites from each reef category were included. Surveys were conducted on the seaward reef crest (between 2 and 4 m depth; **Figures 1C,D**) to ensure that all sites had similar geographical structures and environmental conditions. Finally, a total of ten sites met all of the criteria and were selected for field surveying and fish sampling: three sites for HC reefs, four sites for IC reefs, and three sites for LC reefs.

Fish and Benthic Surveys

At each site, a 60 m long transect line was laid parallel to the crest between depths of 2–4 m. The abundance and biomass of *C. striatus* was quantified first at each site by an experienced surveyor using underwater visual census along the 2.5 × 60 m belt transects. All individuals of *C. striatus* were counted and placed into 5 cm size categories. Biomass of *C. striatus* was estimated from the individual fish-length data through length-weight relationships extracted from FISHBASE (Froese and Pauly, 2020). The calculated results were compared with those calculated from fish sampled at the same reefs to ensure the accuracy of fish weight data.

Substrate surveys were carried out by another diver after the visual fish counts at the same transects. Thirty quadrats (0.5 m × 0.5 m) were placed randomly along each transect to maximize spatial coverage and minimize frame overlaps. The substrate and associated benthic community were recorded in each quadrat using an underwater video camera (Olympus TG-4). The quadrats were divided into a 4 × 4 grid using strings placed at 12.5 cm intervals along the vertical and horizontal axes. Substrate identifications were summarized into the following general categories, hard living coral, fleshy macroalgae, crustose coralline algae (CCA), sands, short algal turf (SATs, algal turf length $\leq$ 5 mm), and long algal turf (LATs, algal turf length $>$ 5 mm). To distinguish LATs from SATs, algal turf length was measured using a depth probe of vernier calipers *in situ* (Tebbett and Bellwood, 2019) and recorded using a digital camera. The substrate identifications were compared with the algal turf length records to classify the forms of algal turf. Partial substrate composition was done *in situ* and was used to regulate the results identified from the photos. The percentage cover per category and transect variance were automatically generated using Excel Extensions v.3.0 (Kohler and Gill, 2006).

Fish Sampling

Fish sampling was conducted at the same area where transect surveys were conducted. Adult individuals of *C. striatus* were caught using a trammel net (10 m length × 1.5 m height, the maximum and minimum mesh sizes were 9.0 and 3.0 cm, respectively). Fish were captured between 0900 and 1100 h, which is when feeding intensities of *C. striatus* are higher (Zemke-White et al., 2002), and stored at −20°C within 1 h. After completing the weight and body length measurements, stomach contents were carefully collected and preserved in 95% ethanol at 4°C before dietary analysis. The stomach contents of fish samples were identified at two magnifications: 4 × 10 magnification using a dissecting microscope and 10 × 40 magnification under higher power before DNA extraction.

Identification of Stomach Contents Using High-Throughput Sequences

At least three fish were randomly selected from the samples at each site. Their stomach contents were ground and homogenized with silicate sand in a FastPrep® instrument (MP Biomedicals, Santa Ana, United States) for 40 s at a speed setting of 6.0 m/s to ensure that all material was completely broken and homogenized. The DNA of the stomach contents was extracted using the FastDNA® Spin Kit for Feces (MP Biomedicals, Santa Ana, United States) following the manufacturer's instructions and eluted in 30 μL of 10 mM *Tris*–HCl (pH 8.0). DNA quality was assessed by spectrophotometry at a 260/280 nm ratio, and DNA concentrations were quantified using a Nanodrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). DNA samples were stored at −20°C until further use.

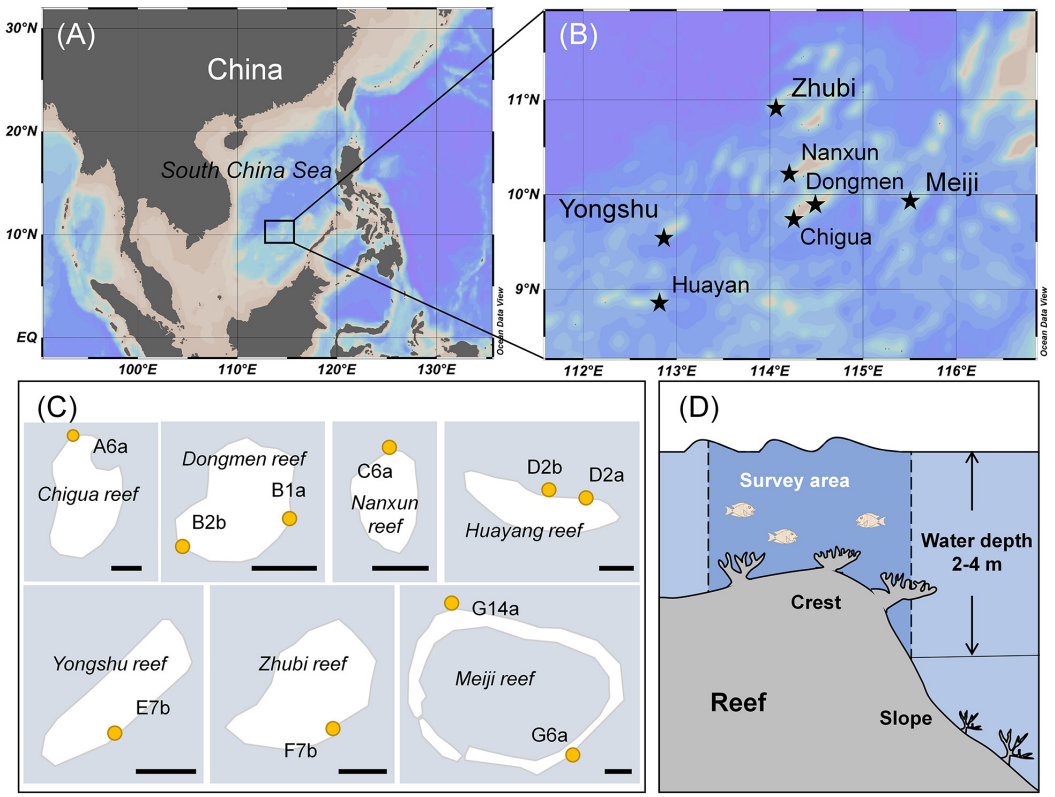

FIGURE 1 | Location of surve yed sites and area on the reefs in the southern part of the South China Sea. Seven reefs were surveyed **(A,B)** and all the sites were located on the windward side of reefs. The solid line on the scale at the bottom right of the figure represents 1 km **(C)**, and surveyed areas were the seaward reef crests at a depth of 2–4 m **(D)**.

Because the exact dietary components of *C. striatus* were not clear, whole DNA extracts were amplified using the universal TAReuk454FWD1-TAReukREV3 primer pair (TAReuk454FWD1: 5-CCAGCASCYGCGGTAATTCC-3; TAReukREV3: 5-ACTTTCGTTCTTGATYRA-3), which targets the V4 region (~380 bp) of eukaryotic 18S rDNA (Stoeck et al., 2010). 18S rDNA was selected because it has been widely used for the identification of eukaryotic organisms and has a comprehensive reference library (Corse et al., 2010). In addition, the accuracy of its semiquantitative data on the dietary analysis of fish feeding on benthic organisms was shown in our previous study (Lin et al., 2018a,b). However, considering the low species resolution of 18S rDNA V4 metabarcoding (Lin et al., 2018b), the highest identification resolution and associated analyses were limited to the genus level in this study. PCR was conducted in a 20 μL reaction volume composed of 4 μL 5 × FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL each of 5 μM universal forward and reverse primers, 0.4 μL FastPfu Polymerase, and 10 ng genomic DNA. The PCR conditions were as follows: an initial denaturation step at 95°C for 5 min; 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; and a final elongation step at 72°C for 10 min. The PCR products were prepared for sequencing at 10°C, examined by electrophoresis with a 2% agarose gel, and then sequenced using the Illumina HiSeq platform.

The raw data obtained from the Illumina HiSeq platform were subjected to quality control in accordance with the Illumina HiSeq platform workflow (Pompanon et al., 2012). After removing the primer sequences, data splitting, and splicing of paired-end reads, the sequences were filtered and intercepted. Only high-quality, long (>300 bp) sequences were retained for further analysis. To evaluate the composition and diversity of feeding items, the effective sequences were clustered into operational taxonomic units (OTUs) using Uparse v7.0.1001 software with a 97% threshold.

Representative OTU sequences were aligned with the GenBank database using the basic local alignment search tool (BLAST). A species name was assigned only if a single best hit achieved 100% similarity. A genus name was accepted only if the similarities of the five best hits were ≥98%. A family name was retained only if the similarities of all the best hits were ≥95%. A phylum or subphylum name was retained only if the similarities of all the best hits were ≥90%. Sequences with maximum similarities <90% were labeled "No Account (NA)." These were most likely due to the GenBank deficiency. The number of effective sequences was then calculated for each phylum. Sequences that were identified as of fish and human origin, and low-frequency OTU sequences (sequence abundance in each sample was less than 10) were removed from each data set. To reduce the risk of misidentification at the

lower taxonomic levels, a proportion of the different OTUs was returned for each level and those labeled "NA" were removed prior to dietary composition analysis. To calculate the alpha diversity, two metrics were calculated (Chao1 and OTU numbers) and rarefaction curves were generated based on the filtered OTU table using the QIIME software[1].

Data Analysis

The relationship between substrate categories and *C. striatus* distribution was analyzed by using Pearson's product–moment and investigating the correlation of the percentage of substratum coverage with the density and biomass of *C. striatus*. The correlation matrix and corresponding significance level were calculated using the "Hmisc" package in R statistical software (version 4.0.3; Harrell, 2019; R Core Team, 2019).

To measure the dissimilarity of the diets of *C. striatus* among reefs and the relationships between relative read abundance (RRA) and substrate categories, redundancy analysis (RDA) was performed using the "vegan" R package software (Oksanen et al., 2009). However, in some cases, the RRA-based data from fecal or stomach content samples were affected by recovery biases, and occurrence data were included to present results that were more reliable (Deagle et al., 2019). A minimum sequence percentage threshold (1%) was defined to determine occurrences, which balanced the data, thereby maximizing the inclusion of real diet sequences and excluded low-level background noise (secondary predation, contamination, and sequencing errors). Following this, a relative abundance read table was converted to a presence/absence matrix. Non-metric multidimensional scaling (NMDS) ordination was calculated using the Jaccard metric within the vegan package to provide a graphical representation of different species relations (and thus differences in diet) based on the presence/absence matrix.

To compare the feeding diversity and specific food taxa of *C. striatus* among reefs, the food items in the presence/absence matrix were selected, heatmaps were created based on the RRA of these food items, and the maximum-likelihood tree of 18S rDNA sequences was established using MEGA 4.0 software. All graphic visualizations were prepared using the "ggplot2" package in R (Wickham, 2016). Differences were considered significant at $p < 0.05$, and highly significant at $p < 0.001$.

This study was reviewed and approved by the Laboratory Animal Management and Ethics Committee of the South China Sea Institute of Oceanology, Chinese Academy of Sciences.

RESULTS

Substrate Composition and Correlation With the Abundance/Biomass of *C. striatus*

Apart from being higher in coral cover, the reefs categorized as HC had low algal turf coverage and a considerable amount of CCA compared with other reefs (**Figure 2A** and **Table 1**). Substrates in the IC reefs were mostly covered by SATs (**Figure 2D**) and had a low coverage of macroalgae (**Figure 2B**). In the LC reefs, LATs were the dominant group with a coverage of up to 55% (**Figure 2C** and **Table 1**) and containing a large amount of sediment (**Figure 2E**).

The highest individual density of *C. striatus (*44.7 individuals 100 m^{-2}) was found at site D2a, while the highest biomass of 365.1 kg ha^{-1} was found at site E7b (**Table 2**). Both individual density and biomass of *C. striatus* tended to increase with moderate coral reduction (**Figures 3A,B**). There was no significant correlation ($p = 0.56$) between individual density of *C. striatus* and the percentage ratio of SAT cover (**Figure 3C**), however, there was a significant positive correlation between the biomass of *C. striatus* and SAT coverage ($p < 0.05$, **Figure 3D**). There was no significant correlation between the abundance of *C. striatus* and LAT cover (**Figures 3E,F**), as with the other substrate categories (**Supplementary Figure 1**).

Species Detected in the Stomach Contents of *C. striatus*

Most visual matter consisted of sand, pieces of algae, and unidentified organic matter, which provided limited information about the feeding preferences of *C. striatus* (**Supplementary Figure 2**). In total, 511,684 raw sequences were generated from the stomach contents of *C. striatus* (**Supplementary Table 1**). After removing sequences identified as fish and low-frequency OTU sequences, a total of 316,687 effective sequences remained. These sequences belonged to 764 OTUs and 29 biological taxa. Most of the sequences and OTUs were identified at the genus or family level, and 14,708 remained unassigned ("NA"). To reduce the risk of misidentification at the lower taxonomic levels, a proportion of different OTUs was returned for each level, and those labeled "NA" were removed prior to dietary composition analysis. The alpha diversity (Chao1 and OTU numbers) and rarefaction curves generated based on the OTU table showed that the sequencing number covered the range of species diversity (**Supplementary Figure 3**).

Most of the identified taxa were algae (RRA > 80%, proportion of total number of OTUs > 45.0%), with microalgae (Dinoflagellata) being the most abundant taxa, followed by Rhodophyta, Chlorophyta, and Ochrophyta (**Supplementary Figure 4**). There were also a few taxa of micro-invertebrates, such as Arthropoda, Platyhelminthes, and Nematoda. Rhodophyta showed the highest diversity, including genus *Hypnea* and *Champia*, followed by Dinoflagellata, mainly from orders Suessiales and Prorocentrales, with genera such as *Symbiodinium*. There was also a high diversity of arthropod species, with no obvious dominant species. Chlorophyta and Ochrophyta had relatively low diversities, but were often abundant. For example, the RRA of brown algae *Sphacelaria* and *Lobophora* reached 55.5 and 51.2% at some sites, respectively. Similarly, green algae *Neomeris* and *Parvocaulis* also reached 36.3 and 73.4% at some sites, respectively (**Figure 4**).

[1]http://qiime.org/scripts/alpha_diversity.html

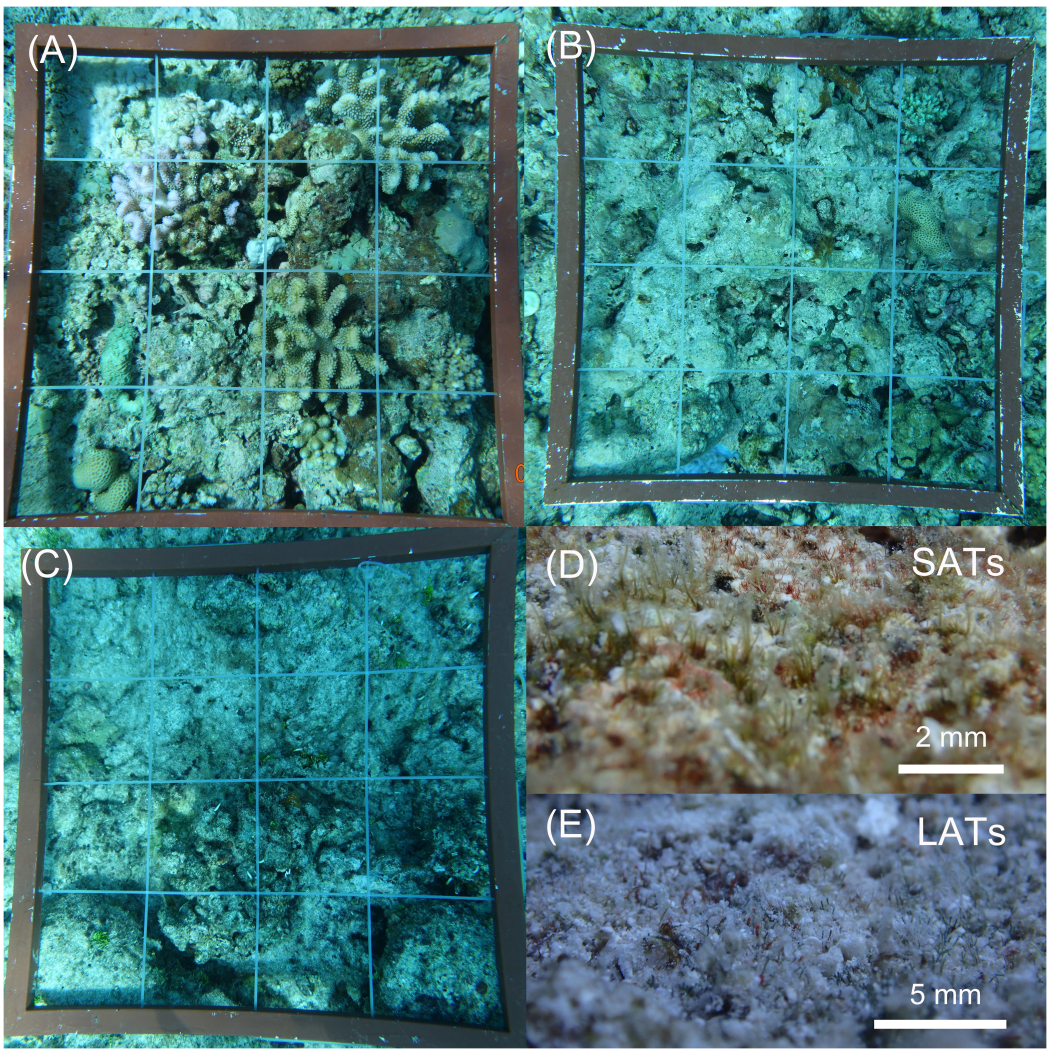

FIGURE 2 | Benthic coral reef conditions of the different study reefs. The 0.25 m^2 quadrats were divided into a 4 × 4 grid using strings at 12.5 cm intervals along the vertical and horizontal axes. **(A)** Coral-dominated reefs with a complex benthic structure and high coral coverage; **(B)** Reefs with low macroalgae cover had a large area and flat, hard substratum. **(C)** Reefs with the lowest coral cover and highest coverage of dense algal turfs and macroalgae; **(D)** The "dead hard substratum" was primarily covered by short (length was approximately 2 mm) and sparse algal turfs (SATs). **(E)** Long (length was more than 5 mm) and dense algal turfs (LATs) laden with large amounts of sediments dominated in low coral cover reefs.

Correlation Between Stomach Contents and Different Substrate Categories

Based on the RRA data of stomach content DNA, the RDA showed that the composition of stomach contents differed significantly according to the different benthic conditions (**Figure 5A**). The divergence among sites was reflected in a few groups, including dinoflagellates and red, brown, and green algae. Sites with the highest coral cover showed high similarity, with stomach content DNA in these sites dominated by brown and red algae. Stomach content DNA sampled from G14a and F7b sites, which had high macroalgal cover and low coral cover, was dominated by green algae. Dinoflagellates tended to be diverse and dominated the stomach content DNA from sites with higher SAT coverage and intermediate levels of coral (**Table 3** and **Figure 5A**). The Jaccard metric, which is based on data presence/absence, also showed that samples of different reefs separated well in NMDS plots, with IC reefs showing less variance between sites than the other reef groups (**Figure 5B**). The main species in the stomach content DNA showed distinct divergence among the reefs (**Figure 4**). Ochrophyta species belonging to genus *Sphacelaria*and *Lobophora*, were abundant in the HC reef sites, with RRAs of up to 55.0 and 51.2%, respectively. The dominant species on the reefs with high SATs mostly belonged to Dinoflagellata. The order Suessiales belonging to Dinoflagellata was abundant at all sites; however, some Suessiales were only identified to the family level (RRA ranged from 9.4 to 14.6%), followed by the genus *Symbiodinium* (RRA 5.1–6.6%). Genus *Prorocentrum*, belonging to Dinoflagellata, was also abundant (RRA up to 17.6%). Food items belonged to Arthropoda also had high diversity, especially genus *Anomalocera*. There were

TABLE 1 | Benthic community composition of the study sites revealed different habitat characteristics among the reefs.

Sites	Coral (%)	SATs (%)	LATs (%)	Fleshy macroalgae (%)	CCA (%)	Soft coral (%)	Sands (%)	Others (%)
C6a	45.8	20.1	4.6	1.3	1.4	0.0	0.0	3.0
B1a	39.2	22.3	5.9	0.1	1.9	0.0	0.0	0.0
D2b	35.5	43.7	1.6	0.0	0.9	0.0	0.0	5.0
D2a	30.0	37.9	2.7	0.0	1.0	1.7	0.2	6.0
B2b	21.2	42.6	2.2	0.0	0.2	0.3	1.3	5.3
G6a	15.7	64.4	4.2	0.3	0.8	0.0	0.0	0.0
A6a	15.7	42.2	3.5	0.2	1.0	0.7	0.0	0.7
F7b	14.5	42.8	12.6	1.8	0.6	0.0	0.0	0.0
E7b	9.3	33.2	43.3	0.0	0.2	1.2	8.2	4.7
G14a	0.8	7.3	55.0	7.2	0.6	0.0	0.0	0.0

High coral cover reefs (HC, green area) were often characterized with moderate short algal turfs (SATs) and crustose coralline algae cover, reefs (IC, yellow area) with intermediate coverage of coral were characterized with high SATs cover, and low coral cover reefs (LC, red area) was often characterized with either high long algal turfs (LATs) cover or fleshy macroalgae cover.

TABLE 2 | The distribution and sampling information of *Ctenochaetus striatus* at each site.

Sites	Density (indivudals·100 m^{-2})	Biomass (kg ha^{-1})	Sampling individuals' number (n)	Weight		Body length	
				Mean (g)	STDEV.P (±)	Mean (cm)	STDEV.P (±)
C6a	1.67	3.16	4	28.49	13.45	8.57	1.32
B1a	34.00	124.95	11	68.50	13.14	11.75	0.83
D2b	14.00	165.14	10	51.72	17.59	9.74	1.10
D2a	44.67	258.75	11	57.31	11.20	11.00	0.67
B2b	9.00	157.16	6	61.12	10.31	10.50	0.89
G6a	19.67	330.44	4	61.70	7.69	11.13	0.41
A6a	24.33	292.37	12	55.47	14.63	10.93	1.08
F7b	12.33	271.41	10	52.84	8.06	9.90	0.26
E7b	18.33	365.06	3	115.00	40.82	11.53	2.13
G14a	6.00	51.96	5	57.86	25.52	10.18	1.73

different species along different sites on the reefs with high macroalgae or LAT coverage. Chlorophyta species, such as genus *Neomeris* and *Parvocaulis*, were dominant in the G14a and F7b sites. However, the dominant taxa at the E7b site were mainly dinoflagellates of the genus *Symbiodinium* (38.0%).

DISCUSSION

The High Biomass of *C. striatus* Related to High SAT Cover

In our study, both the abundance and biomass of *C. striatus* showed an inverted U-shaped relationship with coral coverage and were highest with a moderate reduction in live hard coral cover (**Figures 3A,B**). Similarly, Russ et al. (2018) found that the density of *C. striatus* had a negative relationship with hard coral cover. Where coral cover was very low, LATs tended to be abundant on reefs (**Table 1**). Although both the abundance and biomass of *C. striatus* were not directly correlated with LATs, sediments trapped by LATs constrain the area suitable for *C. striatus* grazing (Tebbett and Bellwood, 2020), with LATs also less nutritious and palatable to *C. striatus* than SATs (Tebbett et al., 2017c). Indeed, site G14a which had the lowest hard coral coverage, highest LATs coverage and lowest coverage of SATS, had a low density and biomass of *C. striatus* (**Table 1**). Russ et al. (2018) also found that the density of *C. striatus* had a strong positive relationship with the amount of dead substratum. The "dead substratum" on coral reefs was often covered by SATs (**Figures 2D,E**), and the biomass of *C. striatus* had a significant correlation with the coverage of SATs (**Figure 3D**). However, the correlation between the density of *C. striatus* and SATs coverage was slightly but not significantly positive (**Figure 3C**), which may be due to the limited number of survey sites. Hence, the increase in SAT cover following coral cover reduction may be the actual factor for attracting *C. striatus*. Although the intensive presence of *C. striatus* with sparse algal turfs may have been found on other trophic reefs of the world (Russ et al., 2018), these results are mainly based on the abundance of *C. striatus*, and biomass data are often deficient. Our results indicated that the biomass of *C. striatus* may be a more sensitive indicator of changes than abundance, when changes in resources are involved.

Possible Dietary Resources for *C. striatus*

A previous study revealed that the diets of *C. striatus* consisted of plant cellular fragments, meiofaunal elements, very fine sediment grains, and relatively more unidentified material (Choat et al., 2002). Instead of merely classifying these unidentified

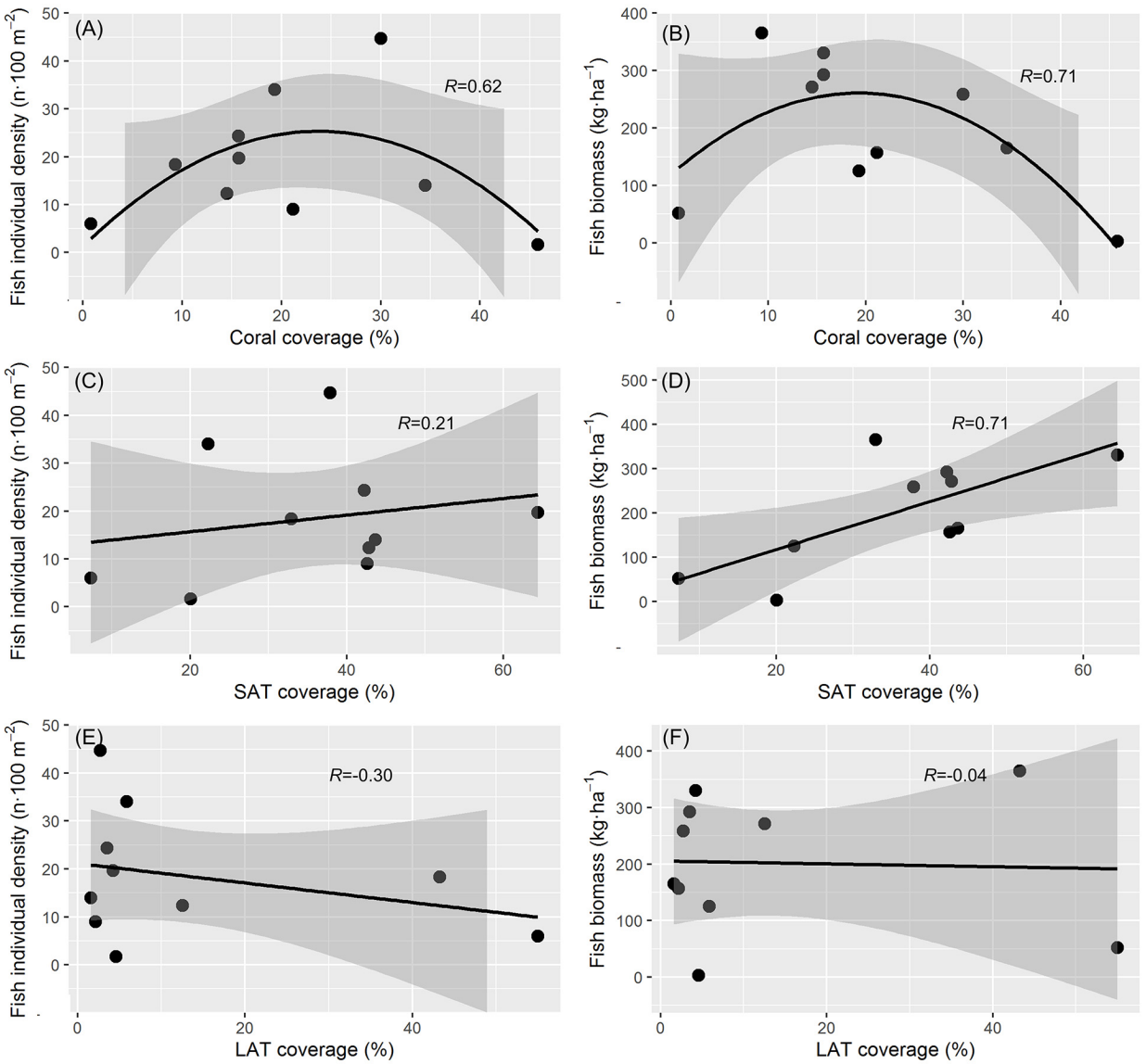

FIGURE 3 | Relationship of *Ctenochaetus striatus* with benthic conditions. Hard living coral coverage and **(A)** individual abundance, (p = 0.18) **(B)** biomass (p = 0.08) of *C. striatus*. The linear relationship between short algal turfs (SATs) coverage and **(C)** individual abundance (p = 0.56) and **(D)** biomass of *C. striatus* (p = 0.02). The linear relationship between SATs coverage and **(E)** the individual abundance (p = 0.40) and **(F)** biomass of *C. striatus* (p = 0.91).

contents as organic matter or algal debris, by using HTS multiple eukaryotic organisms were identified at the family or genus level in the stomach contents of *C. striatus*. *Ctenochaetus striatus* was confirmed to have a complex dietary intake comprised of arthropods, dinoflagellates, and red, brown, and green algae. Although DNA from these organisms was detected in stomach contents, this does not imply that they were consumed directly, at least not in a living state. Marshell and Mumby (2012) found that *C. striatus* consistently fed more intensively on SATs and that their removal from artificial substrates was more effective than removal by another surgeonfish. However, this removal effect was limited to natural reefs, and *C. striatus* was observed to consume only detritus and sediments, leaving mature algal turfs relatively intact (Purcell and Bellwood, 1993; Tebbett et al., 2017c). Although the RRA of macroalgae/filamentous algae in the diets of *C. striatus* was high, with an average ratio of 46.71% per site (**Supplementary Figure 4** and **Supplementary Table 2**; Guiry and Guiry, 2020), the special bristle-like teeth of *C. striatus* would be more suitable for "brushing" organic matter and debris from algal turfs instead of directly feeding on mature algae (Purcell and Bellwood, 1993; Tebbett et al., 2017c). This "brushing" feeding process over algal turfs has the possibility for *C. striatus* to ingest debris or early settlers of algae nevertheless. McMahon et al. (2016) found that macroalgae provide an average of 14 ± 7% (up to 30%) of the C consumed and assimilated by *C. striatus*. Therefore, the DNA of macroalgae/filamentous algae

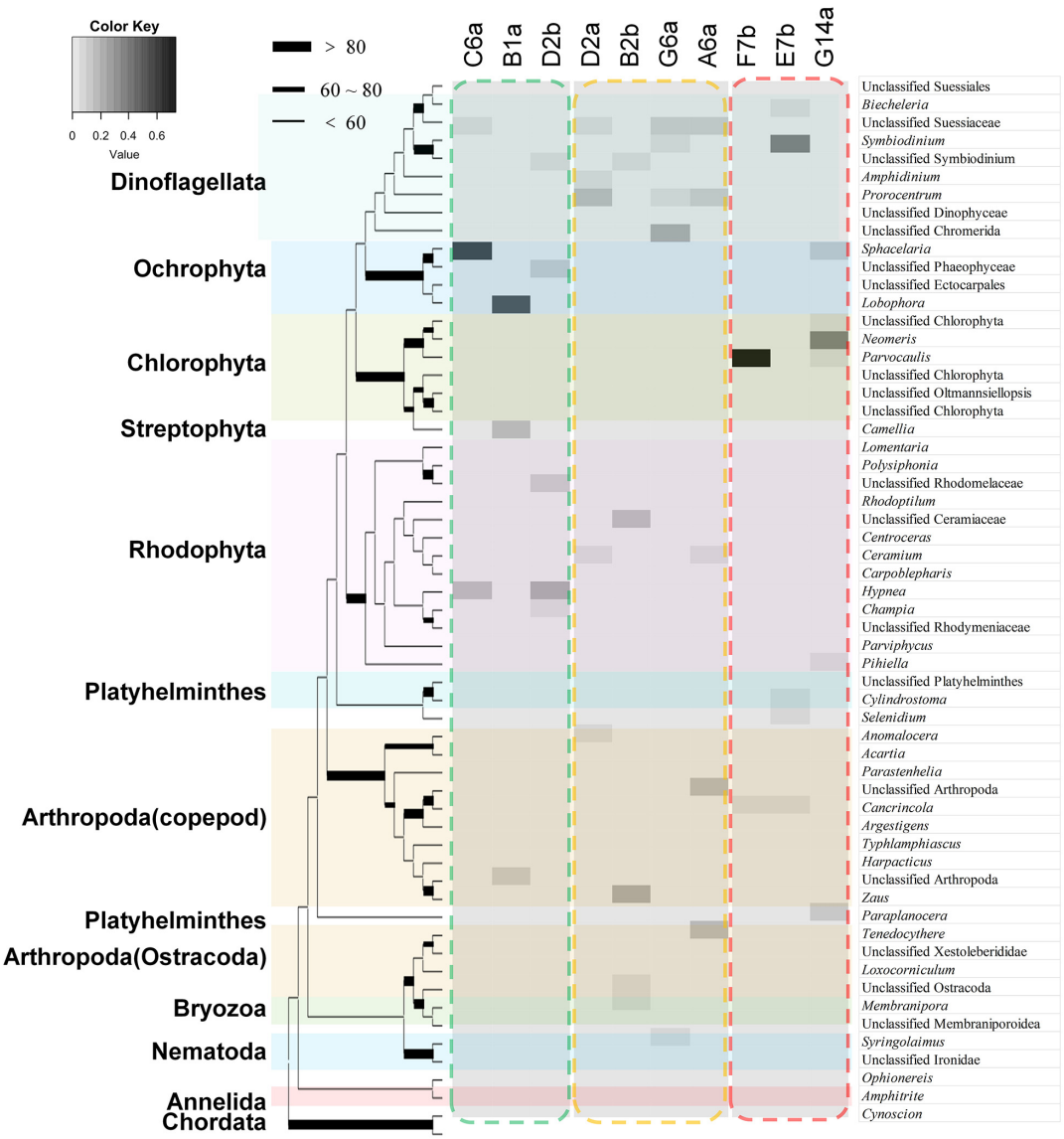

FIGURE 4 | The main species in the stomach contents of *Ctenochaetus striatus* (as revealed by DNA analysis) at the genus level showed distinct divergences among reefs. The characters above the picture represent the IDs of the sampled sites. The taxonomic species in the green dashed frame was the main species detected in the stomach contents of *C. striatus* sampled from high coral cover (HC) reefs, the orange dashed frame surrounds the main feeding items on intermediate coral cover (IC) reefs, and the red dashed frame represents the detected species on the samples of low coral cover reefs. Brown and red algal species were more frequently detected in the stomach contents within HC reefs. On IC reefs, the stomach content DNA tended to be more diverse and was mainly comprised of dinoflagellates. Green algae were the most abundant taxa on the reefs with high macroalgae or long algal turfs coverage, except site E7b.

detected in the present study is also likely to be from debris or early successional communities of these algae and consumed by *C. striatus*.

A considerable number of microalgae sequences were found in the stomach contents of *C. striatus*, and some were identified for the first time in this study. These consisted of mainly dinoflagellates (RRA 2.3–53.8%), for example, *Symbiodinium* and *Prorocentrum*. Most published studies generally agree that *C. striatus* targets detritus in algal turfs and possibly includes microalgae (Polunin and Klumpp, 1989; Choat et al., 2002; Wilson et al., 2003; Clements et al., 2017; Tebbett et al., 2017c). However, there is less information about microalgae species that can be drawn from previous studies. Microscopic observations have not revealed significant amounts of microalgae previously (Choat et al., 2002), even though diatoms and dinoflagellates are generally common in the marine environment and are easily recognizable (Hatcher, 1988; Luo et al., 2017). It is possible that the digestion process may have destroyed the cell structures of microalgae (Choat et al., 2004). Additionally, *C. striatus* is considered to be a high risk species for contracting ciguatera poisoning (Mak et al., 2013; Rongo and van Woesik, 2013) and a primary vector of water-soluble toxins

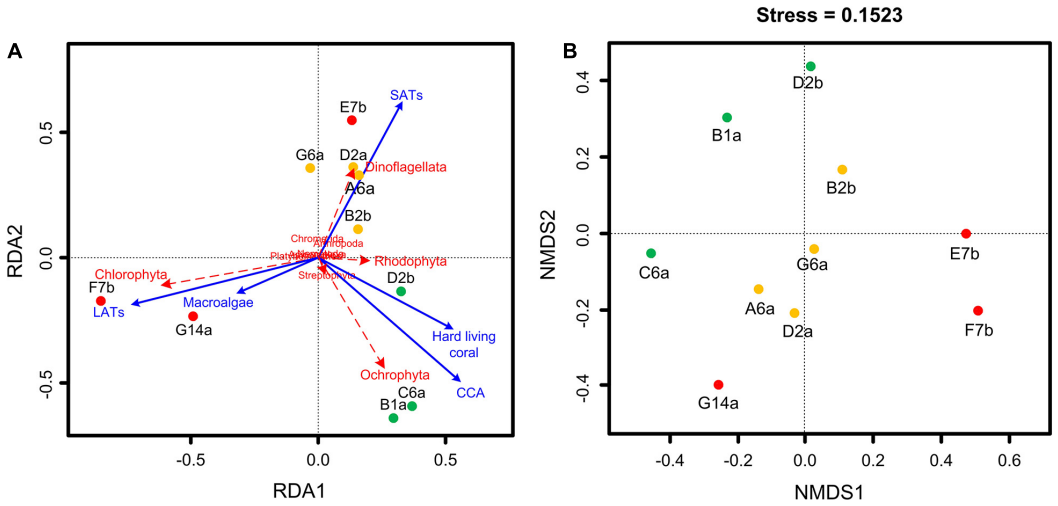

FIGURE 5 | Ordination results of **(A)** redundancy analysis (RDA) and **(B)** non-metric multidimensional scaling (NMDS). RDA was based on the relative read abundance of food taxa DNA and different substrate-type coverage. NMDS was based on a presence/absence matrix of stomach content genera. Sites with the same colors represent similar benthic conditions. The green points indicate high coral cover reefs, the red points indicate low coral cover reefs and the orange points represent sites from reefs with intermediate coral cover.

TABLE 3 | Number of operational taxonomic units (OTUs) detected in the stomach contents of *Ctenochaetus striatus* from different reefs.

Taxa	HC reefs		IC reefs		LC reefs	
	Mean (±SD)	Range	Mean (±SD)	Range	Mean (±SD)	Range
Dinoflagellata	12.0 ± 4.4	9:17	31.8 ± 9.0	25:45	19.3 ± 7.5	12:27
Ochrophyta	5.0 ± 3.0	2:8	3.5 ± 1.0	2:4	1.3 ± 0.6	1:2
Chlorophyta	3.3 ± 2.5	1:6	15.0 ± 2.4	12:17	13.0 ± 7.0	5:18
Rhodophyta	19.0 ± 16.5	3:36	28.3 ± 17.1	10:51	13.0 ± 4.4	10:18
Arthropoda	7.0 ± 3.0	4:10	11.8 ± 4.5	5:14	9.3 ± 3.1	6:12
Bacillariophyta	1.3 ± 1.5	0:3	1.5 ± 1.7	0:3	1.7 ± 0.6	1:2
Others	12.0 ± 8.7	6:22	25.0 ± 4.1	21:29	33.3 ± 13.1	21:47
UN	10.3 ± 13.7	1:26	24.3 ± 17.1	12:49	19.3 ± 11.5	8:31
Total	70.0 ± 38.6	30:107	141.0 ± 15.4	123:157	110.3 ± 18.9	89:125

produced by benthic dinoflagellates (Yasumoto et al., 1976; Lewis, 2006), which indicates that *C. striatus* can intake and ingest microalgae.

The dominant microalgae genera detected in this study were mainly from the order Suessiales and the genus *Prorocentrum* (**Figure 4**). The representative genera were *Symbiodinium*, a common symbiotic dinoflagellate in coral reef ecosystems (Hatcher, 1988), and *Prorocentrum*, which are also common dinoflagellates in the sand, corals, and macroalgae in the South China Sea (Luo et al., 2017). *Prorocentrum concavum* and *P. lima* often coexists with filamentous algae and may occasionally be consumed by surgeonfish (Kohler and Kohler, 1992), and coral mucus is an important source of detritus on coral reefs (Wilson et al., 2003), which might explain the prevalence of *Symbiodinium* in the diets of *C. striatus*. However, whether *Symbiodinium* is derived from coral mucus or is naturally associated with algal turfs needs to be confirmed. Diatoms account for 1–14% of the organic matter in detrital aggregates in algal turfs (Wilson et al., 2003), but diatom sequences detected in this study were low (even with RRA < 1% in some sites). Considerable diatom species and sequences were detected in our previous study, in which DNA extraction methods targeting phytoplankton and samples were preserved in alcohol without freezing (Lin et al., 2018a). Therefore, the underrepresentation of RRA counts on diatoms was likely a result of DNA extraction bias or the differences in the recovery of DNA from different organisms from frozen specimens (Maki et al., 2017).

Micro-invertebrates, such as crustaceans and nematodes, found in the stomach contents of *C. striatus* (**Figure 4**), may be incidentally consumed during feeding. Previous studies have found that when fish remove benthic algae from reef bases, they may also consume a significant number of benthic micro-invertebrates (Polunin et al., 1995; Trip et al., 2008; Kramer et al., 2013). Many broadly categorized herbivorous fishes (including detritivorous fish) on coral reefs are functionally distinct from traditional herbivores in nutritional terms, and it is possible for these fishes to

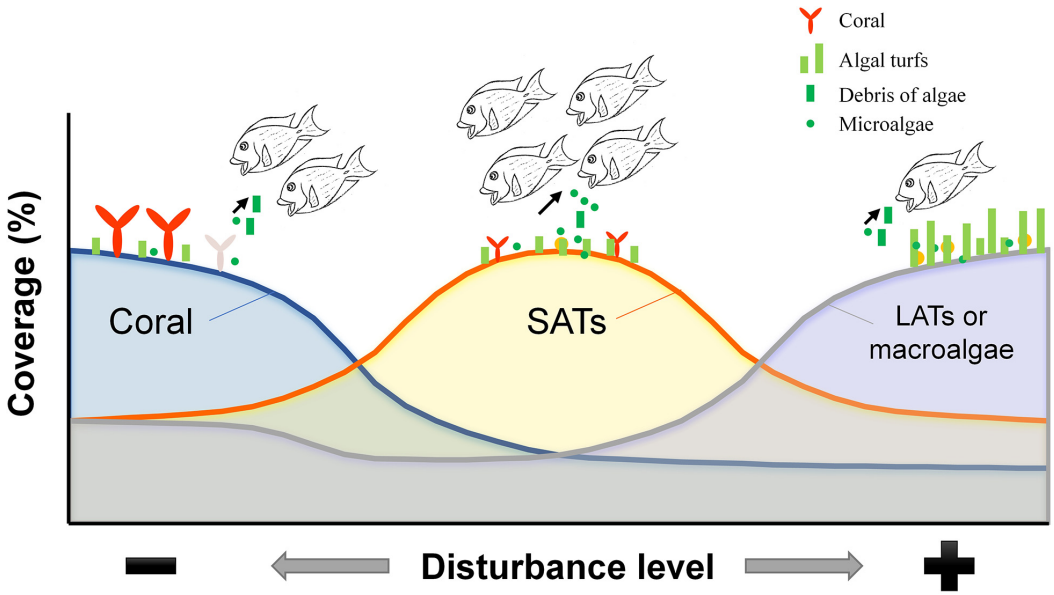

FIGURE 6 | Schematic diagram showing the influence of the substratum on the feeding of *C. striatus* during algal succession. The DNA sequences of macroalgae and filamentous algae were more frequently detected in the diets of *C. striatus* sampled from either coral-dominated reefs or macroalgae-dominated reefs, where the biomass of *C. striatus* was relatively lower. Reefs with intermediate levels of coral cover have more areas of substrate exposed which are covered by short algal turfs, which *C. striatus* preferentially feeds on. This can be seen from the high biomass of *C. striatus* and the high diversity and relative read abundance of dinoflagellates detected in the stomach contents of *C. striatus* on these reefs.

ingest mixtures of different food items to achieve adequate nutrient intake (Clements et al., 2009). Considering the wide distribution of micro-invertebrates and nutritional support for reef fishes, the contribution of micro-invertebrates to higher trophic levels may be an important topic for future research (Kramer et al., 2012).

The Positive Feeding Response of *C. striatus* to Disturbed Habitat

In our results, the stomach content of *C. striatus* was often associated with benthic conditions (**Figures 4**, **5**). Previous studies have shown that feeding of *C. striatus* was less influenced by substratum organic loads, and more dependent on the availability of organic sources on the substratum (Tebbett et al., 2017a). This implied that the DNA of detected food species can possibly reflect the availability of algal resources on coral reefs. Indeed, the high RRA of macroalgae and filamentous algae often correlated with the high coverage of macroalgae or LATs on LC reefs (**Figure 5**). Meanwhile, with the protection of scleractinian corals, the biomass of brown algae *Lobophora* was often 20 times greater in branching than in planar habitats (Brandl and Bellwood, 2016), and, thus, they increase the chances that *C. striatus* may consume algal debris derived from "shelters" in branching habitat (HC reefs). This could explain the high RRA of *Lobophora* and other brown algae in the diets of *C. striatus* on HC reefs. Microalgae and other microbes add essential nutrients and improve the nutritional value of the detritus, and increase the palatability and digestibility of detritus for fish (Wilson et al., 2003; Tebbett et al., 2017c). Dinoflagellates in the stomach contents DNA were abundant and diverse with high SATs cover, which had a higher detrital proportion. However, RRA cannot yield results in terms of biomass without adequate preliminary work (Deagle et al., 2019). For this reason, semi-quantitative data based on 18S rDNA metabarcoding were demonstrated in our previous study (Lin et al., 2018a,b). Meanwhile, the occurrence data of food items also proved that the ingested dinoflagellate species correlated with high SAT cover reefs (**Figures 4**, **5B**). Even with some problems in converting biomass from RRA data directly, the increasing or decreasing tendency in the RRA of specific taxa could still provide useful information on the influence of the substratum on the feeding of *C. striatus*, especially for the positive feeding response on microalgae on the high SAT cover reefs.

Benthic algae did not appear on the reef as mature plants, and the forces acting on the early life stages of algae may determine the ultimate development and dominance patterns of algal blooms (Lotze et al., 2000). Thus, the removal of early algal settlers and microalgae from substrata by *C. striatus* would also exert a top-down effect on benthic algae and potentially influence the primary succession of algal communities (**Figure 6**), especially considering the high biomass and intensive feeding on microalgae by *C. striatus* on the high SAT cover reefs after moderate coral loss. Indeed, previous studies have found that dinoflagellates, for example, *Prorocentrum*, often coexist with filamentous algae growing on dead coral at the early stage of coral degradation and are ingested by surgeonfish (Kohler and Kohler, 1992). In addition, this positive feeding response on microalgae may result in secondary disasters after coral

degeneration, such as ciguatera poisoning (Kohler and Kohler, 1992). Rongo and van Woesik (2013) confirmed that the density increase of *C. striatus* was a consequence of increased algal cover after coral loss and directly coincided with increased hospital cases of ciguatera poisoning. These studies were not only consistent with the correlation between the presentation of *C. striatus* and SAT cover (**Figure 3D**), but the increasing ciguatera poisoning could also be explained by the increasing chances of dinoflagellates ingested by *C. striatus* on reefs with moderate coral cover (**Figures 4**, **5A**). Even though microalgae are important food resources for consumers in other marine ecosystems (Ding et al., 2018), they are of less concern in coral reef ecosystems (Clements et al., 2017). Technically, the exact biomass or abundance of dinoflagellates ingested by *C. striatus* could not be converted from RRA data in this study, for example, due to the differences between dinoflagellates and other algae in copy number. The abundant sequences of dinoflagellates derived from HTS could draw more attention to this cryptic species and shed some light on the early algal succession and algal toxin transfer on coral reefs. The expansion of algal turf after coral degradation would actually promote the ecological status of microalgae in detritivory by affecting the feeding habit of *C. striatus* (**Figure 6**). This information would be helpful for risk assessment and prediction in the coral-algae phase shift.

Instead of simply classifying the contents into organic matter or algal debris, multiple eukaryotic organisms were firstly identified to the family or genus level in the diets of *C. striatus* using HTS. Regrettably, a weak quantitative relationship may exist between the biomass and the sequences produced (Lamb et al., 2019), and the exact proportion of different dietary taxa could not be precisely determined based on current data. A series of factors, such as DNA extraction, primer bias, and differences in dietary taxa in copy number, could influence the accuracy of RRA data. Despite some flaws and methodological problems, current data could still resolve the previous assumptions: (a) *C. striatus* could ingest early successional algae, such as microalgae, which may be an important process on coral reefs, and (b) *C. striatus* could also be influenced by benthic conditions, as its biomass was significantly positively correlated with SAT cover and its stomach contents were often associated with benthic cover (**Figures 5**, **6**). Although massive coral loss may lead to a decline in both the diversity and biomass of reef fishes, moderate coral loss often has limited or even positive effects on generalist-feeding species (Pratchett et al., 2011), such as detritivorous reef fish. Considering the expansion of algal turfs coverage and the wide distribution and high abundance of detritivorous fish on trophic reefs, the increase in both algal turfs and detritivores will potentially change the basic energy flow after coral degradation, and HTS may provide new insight into the trophic roles of this important functional group. Ultimately, no method can possibly resolve all the problems in dietary analysis. Nevertheless, HTS can provide a comprehensive eukaryotic species list of potential dietary resources, which provides useful quantified dietary analysis of this major detritivorous reef fish for future research, especially in the collection of stable isotope characteristics of fish diets.

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: https://www.ncbi.nlm.nih.gov/genbank/, MN188827–MN189943.

ETHICS STATEMENT

The animal study was reviewed and approved by Laboratory Animal Management and Ethics Committee, South China Sea Institute of Oceanology, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

SL conceived the study. XL and SH designed the study and experimental protocols. XL performed the experiments, data analyses, and wrote the manuscript. HH contributed the habitat characterization and fish abundance data. YL, SL, SH, and LZ contributed significantly to the improvement of the manuscript and reviewed the final draft. 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.2021.682697/full#supplementary-material

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Signatures of Adaptation and Acclimatization to Reef Flat and Slope Habitats in the Coral *Pocillopora damicornis*

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Strong population-by-habitat interactions across environmental gradients arise from genetic adaptation or acclimatization and represents phenotypic variation required for populations to respond to changing environmental conditions. As such, patterns of adaptation and acclimatization of reef-building corals are integral to predictions of the future of coral reefs under climate warming. The common brooding coral, *Pocillopora damicornis*, exhibits extensive differences in host genetic and microbial symbiont community composition between depth habitats at Heron Island in the southern Great Barrier Reef, Australia. An 18-month reciprocal field transplant experiment was undertaken to examine the environmental and genetic drivers behind variation in survival, weight gain, heat tolerance and algal symbiont community between the reef flat and slope habitats. We observed population-by-habitat interactions for *in situ* partial mortality and weight gain, where trait-related fitness of natives was greater than transplants in most cases, consistent with local adaptation. On average, flat colonies transplanted to the slope had a relatively low partial mortality but minimal weight gain, whereas slope colonies transplanted to the flat had relatively high partial mortality and average weight gain. Experimental heat tolerance was always higher in colonies sourced from the flat, but increased when slope colonies were transplanted to the flat, providing evidence of acclimatization in these colonies. The performance of certain slope to flat transplants may have been driven by each colony's algal symbiont (Symbiodiniaceae) community, and flat variants were observed in a small number of slope colonies that either had a fixed flat composition before transplantation or shuffled after transplantation. Host genotypes of previously identified genetic outlier loci could not predict survival following transplantation, possibly because of low sample size and/or polygenic basis to the traits examined. Local environmental conditions and Symbiodiniaceae composition may provide insight into the adaptive potential to changing environmental conditions.

Keywords: population-by-habitat interaction, local adaptation, acclimatization, reciprocal transplant, *Pocillopora damicornis*, trait-related fitness

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INTRODUCTION

Global surface temperatures are increasing, and climatic extremes are becoming more widespread and pronounced (Hansen et al., 2010; Cheng et al., 2019). This exposes whole ecosystems to conditions outside long-term baselines that result in severe stress and mortality across key species such as coral and kelp (Hughes et al., 2003; Wernberg et al., 2016; Smale, 2020). For these ecosystems to persist into the future, key habitat-forming species will either need to adapt, acclimatize or shift geographic range (Parmesan and Yohe, 2003; Burrows et al., 2011; Blois et al., 2013; Price et al., 2019). Adaptation arises when variation in fitness has a genetic basis and adaptive alleles can be selected for by local conditions over generations (Ellegren and Sheldon, 2008). Acclimatization is dependent upon an individual's potential for phenotypic plasticity, and can also be heritable and thus facilitates adaptation (Galloway and Etterson, 2007; Marshall, 2008; Putnam and Gates, 2015; Torda et al., 2017). For species with complex microbial communities, such as corals, the ability to respond to environmental change not only depends on the host, but also the adaptive and plastic potential of its symbiotic microorganisms (Chakravarti et al., 2017; Ziegler et al., 2017; Quigley et al., 2018). The ability of coral populations to adjust to multiple environmental conditions is important for long-term resilience, especially under rapidly changing climatic conditions. Therefore, comprehensive knowledge of the adaptive potential of species is required to understand and predict the future state of climate impacted ecosystems and best practice management (Van Hooidonk et al., 2016; Anthony et al., 2017; Hoegh-Guldberg et al., 2017).

Transplantation of organisms into environmental conditions that mimic climate scenarios predicted for their native habitat may uncover the likelihood of a species' persistence into the future. Reciprocal transplant experiments (RTEs) are used in evolutionary ecology to estimate the degree of genetic adaptation and plasticity that underpin organismal performance between local populations, where fitness-related traits are measured in individuals from two or more populations exposed to their distinct native and novel environments (Kawecki and Ebert, 2004; Hoeksema and Forde, 2008; Blanquart et al., 2013). Local adaptation is inferred when natives outperform foreign transplants (Blanquart et al., 2013; Savolainen et al., 2013), and acclimatization of a population manifests shifts in performance toward the locals in the novel environment and consequently outperforms its conspecifics that remained at the native environment (Kawecki and Ebert, 2004; Henn et al., 2018). The physiological responses of multiple populations to reciprocal transplantation may be coupled with genetic data to explore genotype-by-environment (G × E) interactions (Blanquart et al., 2013; Drury and Lirman, 2021).

Diverse responses have been reported for corals reciprocally transplanted among various habitats. Local adaptation was evident in traits like survival, gene expression and Symbiodiniaceae community composition along environmental gradients such as depth, water quality and temperature. For example, coral species *Acropora hyacinthus*, *Porites lobata*, and *Porites astreoides* had divergent population phenotypes between environmentally variable and moderate habitats (Barshis et al., 2010; Palumbi et al., 2014; Bay and Palumbi, 2017; Kenkel and Matz, 2017). Reciprocal transplantation also revealed adaptive divergence among depth habitats in the coral species *Seriatopora hystrix* (Bongaerts et al., 2011), as well as the octocoral species *Eunicea flexuosa* (Prada and Hellberg, 2013) and *Briareum asbestinum* (West et al., 1993). Most recently, *Acropora cervicornis* genotypes reciprocally transplanted among numerous replicate reef sites with different thermal regimes in the Florida Reef Tract indicated G × E interactions for bleaching tolerance, where the greatest variability in bleaching tolerance among genotypes was within each site, and no genotype had superior bleaching tolerance in all transplant locations (Drury and Lirman, 2021). Studies of reciprocal transplantation of corals continue to reveal that performance is affected by environmental conditions, but varies significantly by colony, highlighting the need to expand replicate RTEs among small spatial scales to elucidate colony differences.

The reefs in the Capricorn Bunker Group in the southern Great Barrier Reef, Australia, provide an ideal study system to test for local adaptation. Many reefs in this region have well-developed reef crests and ponding lagoons that lead to distinct habitats across small spatial scales (Takabayashi and Hoegh-Guldberg, 1995; van Oppen et al., 2018). For example, lagoon reef flat habitats have more variable and extreme water temperature and light irradiance compared to the slope at Heron Island (van Oppen et al., 2018). Despite being within the dispersal distance for most coral larvae including brooding species, *Pocillopora damicornis* were found to be genetically, physiologically, and morphologically partitioned among depth habitats at Heron Island (Takabayashi and Hoegh-Guldberg, 1995; van Oppen et al., 2018) [similar patterns exist for *P. damicornis* populations at Kāneʻohe Bay, Hawaiʻi (Gorospe and Karl, 2015)]. Sixteen host associated genetic outlier loci distinguished slope and flat populations at Heron Island and communities of dinoflagellate photosymbionts and prokaryotes also diverged between habitats (van Oppen et al., 2018). The present evidence of divergence between colonies that originated from the flat or slope supports the potential for local adaptation to these habitats, although it is unknown if and how these differences affect physiological performance under novel and stressful conditions.

To further understand the relative importance of acclimatization and adaptation of reef building corals to increasing and more extreme environments, we used *P. damicornis* colonies from Heron Island in a two-part experiment consisting of a replicated RTE across depth habitats and a common garden stress test. In particular, we were interested in testing whether the genetic differences unveiled in van Oppen et al. (2018) were reflected in phenotypes and to tease apart the relative contribution of genetic adaptation versus acclimatization of phenotypic traits. We measured three vital traits over 18 months in the field, and then measured relative heat tolerance by exposing the surviving colonies to 14 days of *ex*

situ experimental heat stress. The resulting information provides clarity to the influence of environmental selection on trait-related fitness of populations within a close spatial proximity and better serves as a reference for the management of coral species.

MATERIALS AND METHODS

Experimental Design

Replicate RTEs were carried out between two depth habitats (reef flat and slope) and at two adjacent reef sites at Heron Island between March 2012 – August 2013. Experiments were carried out using the source colonies of *P. damicornis* in van Oppen et al. (2018) and collected under Great Barrier Reef Marine Park Authority permit G12/34752.1. The flat and the slope habitats sampled here are separated by less than 100 m in horizontal distance and approximately 5 m in depth at each of the two replicate sites, Canyons and Gardens. These reef sites are separated by ∼1 km (Canyons flat: 23°27′14.50″S, 151°55′34.55″E; Canyons slope: 23°27′18.80″S, 151°55′28.29″E; Gardens flat: 23°26′48.50″S, 151°54′46.34″E; Gardens slope: 23°26′50.54″S, 151°54′54.46″). Over the 18-month experiment, mean annual temperature did not vary between habitats, but the range did, with colonies on the flat experiencing temperatures between 15.8 and 33.2°C, while colonies on the slope experienced temperatures between 18.7 and 28.0°C (van Oppen et al., 2018). Ambient light (measured as photosynthetic active radiation) also varied annually and was three times higher and four times more variable in the flat versus the slope (flat: 150 – 1,400 μmol photons m^{-2} s^{-1}; slope: 100 – 400 μmol photons m^{-2} s^{-1}).

At each site and habitat, 23 – 26 colonies were collected (total n = 97) and fragmented into two halves. Within a day, one half of each colony was placed onto experimental racks in its habitat of origin, and its genetic clone was transplanted to the reciprocal habitat within each site, resulting in four treatment groups replicated at two sites: flat to flat (FF), flat to slope (FS), slope to flat (SF), and slope to slope (SS). At each site, two 2 m by 2 m wire mesh racks were erected approximately 0.5 m above the substrate, and fragmented coral colonies were attached in a haphazard manner across these racks with cable ties. At each sampling time, colonies were removed from the racks, sampled and measured, then reattached to the rack.

Physiological and Genetic Measurements

Partial Colony Mortality and Cumulative Weight Gain

Partial colony mortality and weight gain of coral colonies were measured in the field over four time points in March and August of 2012 and 2013. Colonies were visually scored for partial mortality to the nearest 5% using the Coral Watch health chart as a reference (Siebeck et al., 2006). Wet buoyant weight was also obtained underwater using calibrated OHAUS Triple Beam 700 series balancing scales. Any single colony with less than 100% mortality was considered to be alive. Percent cumulative buoyant weight gain was calculated using the first and final time points [(current weight – initial weight)/initial weight]. Small samples (∼5 g and < 7 cm) taken for DNA analysis underwater were later weighed and added to the weight gain at that time point.

Heat Tolerance

At the end of the field transplant experiment on 17 August 2013, the remaining living colonies were transported under flow-through conditions aboard the RV Cape Ferguson to the Australian Institute of Marine Science headquarters in Townsville where they were kept first in outdoor 1000 L tanks for 10 days. On 27 and 28 August 2013, they were further fragmented into single branches then acclimatized in indoor 30 L flow-through tanks at 24°C conditions. Depending on the amount of live tissue per colony remaining, there were between 3 and 20 replicate fragments per colony for each level of heat stress: 24°C, 29°C, 30°C, 31°C (**Supplementary Table 1**). Water temperatures were ramped up from ambient (24°C) to its designated temperature within 1 day on 1 October 2013, and thereafter maintained at these levels for 13 days, for a total of 14 days of heat stress. Lights were sustained under a slightly shifted 12:12 light-dark cycle at a constant level of 250 μmol photons m^{-2} s^{-1}. Heat tolerance was quantified by proxy of the maximum quantum yield (Fv/Fm), measured with Pulse-Amplitude Modulation (PAM) fluorometry using a diving PAM (Walz) with default settings. Fragmented colonies were measured at the end of their dark cycle before experimental dawn at 10:00AM.

Coral Host Outlier Loci Analysis

We analyzed the genotype frequencies of 16 highly significant single nucleotide polymorphism (SNP) outliers from 13 loci identified by BayeScan (Foll, 2012) and Fdist between habitat in van Oppen et al. (2018). If genotypes of outlier loci were closely linked to survival, we anticipated the genotype frequencies of the survivors to resemble the local habitat it was transplanted into. To identify differences in allelic frequencies among site and treatment locations, pairwise F_{ST} and their corresponding p-values on the 16 outlier loci between the survivors were calculated in ARLEQUIN v3.5.2.2 (Excoffier and Lischer, 2010). F_{ST} tests were done with and without sites combined for the four treatment groups. As considerable mortality occurred between March and August 2013, the March 2013 time point (12 months post-transplantation) was used to maximize the number of survivors per treatment and increase the power of the analysis (**Table 1**).

DNA Extraction, ITS2 Sequencing and Assignment

We extracted DNA from samples at two time points, FF and SS treatments in April 2012 and all treatment groups in March 2013 (**Supplementary Table 2**). The native treatment group samples were taken 1 month after initial transplantation due to a mild bleaching response in the SF colonies immediately following the transplantation in March 2012. Following Wayne's method (Wilson et al., 2002), pieces of coral tissue and skeleton previously stored at −80°C were vortexed in a fresh

TABLE 1 | The number of survivors (n), average partial mortality (PM) ± SE, and average cumulative weight gain (WG) ± SE per site and treatment group at each sampling time point.

Treatment group	Date	Canyons survivors (n)	Gardens survivors (n)	Canyons PM	Gardens PM	Canyons WG	Gardens WG
FF	March-12	24	26	0.00 ± 0.00	0.00 ± 0.00	–	–
	August-12	24	26	0.00 ± 0.00	9.31 ± 4.16	38.97 ± 3.64	44.40 ± 4.28
	March-13	23	22	3.70 ± 1.34	45.42 ± 9.65	111.80 ± 10.93	82.40 ± 11.93
	August-13	23	18	4.35 ± 1.52	56.77 ± 11.37	145.39 ± 11.93	116.00 ± 19.01
FS	March-12	24	26	0.00 ± 0.00	0.00 ± 0.00	–	–
	August-12	22	26	7.18 ± 4.24	25.15 ± 6.06	31.57 ± 2.55	2.18 ± 3.17
	March-13	21	10	19.96 ± 7.85	84.52 ± 8.78	48.80 ± 6.79	−6.36 ± 7.89
	August-13	20	7	24.91 ± 8.80	88.00 ± 9.62	69.93 ± 8.79	19.04 ± 7.27
SF	March-12	24	24	0.00 ± 0.00	0.00 ± 0.00	–	–
	August-12	23	24	26.88 ± 6.83	13.96 ± 6.07	18.02 ± 2.65	19.70 ± 2.10
	March-13	13	5	77.38 ± 9.53	88.29 ± 12.41	29.21 ± 7.84	41.69 ± 22.07
	August-13	3	3	91.67 ± 15.87	95.38 ± 11.79	76.84 ± 12.12	91.91 ± 47.00
SS	March-12	24	23	0.00 ± 0.00	0.00 ± 0.00	–	–
	August-12	24	23	0.08 ± 0.08	1.30 ± 0.65	43.04 ± 3.86	25.89 ± 1.66
	March-13	24	22	15.96 ± 6.70	21.74 ± 5.82	97.14 ± 11.06	56.47 ± 5.24
	August-13	20	19	24.38 ± 8.90	31.74 ± 9.09	128.67 ± 15.19	82.46 ± 8.26

FF, flat to flat; FS, flat to slope; SF, slope to flat; SS, slope to slope.

grinding buffer and processed using a salting-out protocol. The resulting pellet was washed with 70% ethanol, then resuspended in 70 μL milli-Q water and stored at −20°C. The quality of DNA was checked using a NanoDrop NDTND-1000 and electrophoresis, then diluted to 1:10 in Milli-Q water. The ITS2 locus was amplified with PCR with negative controls, using AmpliTaq Gold 360 Master Mix and the following primers: ITS2-F (5′-GTGAATTGCAGAACTCCGTG-3′) and ITS2-R (5′-CCTCCGCTTACTTATATGCTT-3′). PCR was performed in a total volume of 30 μL per sample, with 2× AmpliTaq Gold Master Mix, 1.2 μL of each 10 μM F and R primer, 9.6 μL PCR-grade water, and 3 μL of DNA dilution, nominating the final primer concentrations as 0.4 μM. The PCR consisted of 10 min at 95°C of initial denaturation, then 30 cycles of 30 s 95°C denaturation, 60 s 57°C annealing, and 30 s 72°C extension. Final elongation was for 7 min at 72°C and held at 4°C. The PCR product was checked for correct amplicon size and yield on 2% agarose gel at 90 V for 60 min and shipped cold to Ramaciotti Centre for Genomics (University of New South Wales, Sydney, NSW, Australia) for sequencing on the Illumina MiSeq platform. Raw sequences are available on NCBI Sequence Read Archive under Accession no. PRJNA741589. Demultiplexed paired fastq.gz ITS2 files on Illumina were processed through the SymPortal.org database (Hume et al., 2019). SymPortal utilizes Mothur and minimum entropy decomposition to assign sequences and taxon type, with intragenomic groupings based on 97% similarity. Sequences from the samples that matched the SymPortal online database are identified as defining intragenomic variants (DIVs), which were analyzed here, and hereon referred to as "variants".

Statistical Analysis

All statistical analyses were carried out in R v4.0.2 (R Core Team, 2020). The three physiological traits (partial colony mortality, cumulative weight gain, and heat tolerance) were evaluated using generalized linear mixed effects models with the glmmTMB package (Brooks et al., 2017). Partial mortality and heat tolerance were modeled against the beta family with the logit link function because the data values were between 0 and 1. Weight gain was modeled against the Gaussian family because the data were a continuous measurement with some negative values. The model for partial mortality and weight gain included site (Canyons, Garden), source (flat, slope) and transplant (flat, slope) locations as fixed effects, and colony genetic identity as a random effect. Partial mortality and weight gain were both analyzed for the final time point (August 2013). Because of reduced numbers of survivors at the end of the field transplant experiment, heat treatment effects were combined by site and examined between flat and slope habitats only. Thus, the model for heat tolerance included temperature (24°, 29°, 30°, 31°C), source and transplant locations as fixed effects, and colony genetic identity as a random effect. Wald's Chi-square Tests were implemented on the glmmTMB models and were set to type III based on significant interaction terms. Contrasts were investigated with pairwise estimated marginal mean trait-related fitness with the emmeans package (Lenth et al., 2019) to make population-level predictions based on the small sample size for each treatment. The *p*-values for contrasts were adjusted using Holm's method for all models, but with 16 comparisons for partial mortality and cumulative weight gain models, and 24 comparisons for the heat stress model.

Symbiodiniaceae community data were trimmed to include sequence variants that were represented with >0.005% total abundance and samples with >20,000 reads. The trimmed community data plus the sample data frame were combined in Phyloseq (McMurdie and Holmes, 2013) and were tested for permutational multivariate analysis of variance using the Vegan package (Oksanen et al., 2019) on Bray-Curtis distances with 999 permutations, and subsequent pairwise comparisons

with Holm's method of adjustments for 16 ecologically relevant comparisons. Analysis of dispersion with 999 permutations were run for comparisons that had significantly different variance. The community composition of the habitats (flat vs. slope) was compared between the sampling years (April 2012 and March 2013), and all other tests were performed on treatment groups (FF, FS, SF, SS) with samples and data collected in March 2013.

Multivariate analysis of variance with 999 permutations were also used to test for associations between Symbiodiniaceae community composition with partial mortality and cumulative weight gain among source and transplant habitats with data from March 2013. These tests were run separately per site to prevent false interpretation of results. Heat tolerance was not assessed for an association with community composition given the lack of replicate samples per treatment group that had both genetic data and yield data (**Supplementary Tables 1**, **2**). Specifically, there were six total SF colonies (258, 262, 267, 453, 455, 471) that had Symbiodiniaceae composition data and three SF colonies (267, 458, 464) that were further fragmented and exposed to the heat stress experiment at 31°C. Permutational analysis of dispersion tests were not able to be conducted for associations of Symbiodiniaceae composition and trait physiologies due to lack of complexity in the R function.

RESULTS

Partial Colony Mortality

Whole colony mortality was observed in all treatment groups, especially in transplant treatments, resulting in uneven sample sizes (**Table 1**). Partial mortality of colonies in August 2013 was affected by the interaction between source and transplant habitats, i.e., treatment groups pooled by replicate reef sites (Wald's Chisq = 18.31, $p < 0.0001$; **Supplementary Table 3**), and between source and site averaged over the transplant habitats (Wald's Chisq = 6.15, $p = 0.013$). Slope-sourced colonies in both habitats showed similar levels of partial mortality between sites, but the flat-sourced colonies at the Gardens site had higher partial mortality in both habitats than at the Canyons site (**Figure 1A**). The partial mortality of colonies in the SF treatment group was two times greater than their SS counterparts and followed initial bleaching of this treatment group. At the Canyons, the partial mortality of the FS treatment group was not significantly different from the SS treatment group at the habitat transplanted into, or the FF treatment group in the habitat of origin. At the Gardens, the partial mortality of FS treatment group was 20% greater than their FF counterparts, though not significant, and 35% greater than the SS treatment group (estimated marginal means Adj. $p = 0.0048$; **Supplementary Table 4**). Between the two sites, the FS treatment group had 43% greater partial mortality at the Gardens than at the Canyons (estimated marginal means Adj. $p < 0.0001$).

Cumulative Weight Gain

The difference in cumulative weight gain in colonies after 18 months of transplantation (March 2013) was significant for the interaction between source and transplant habitats

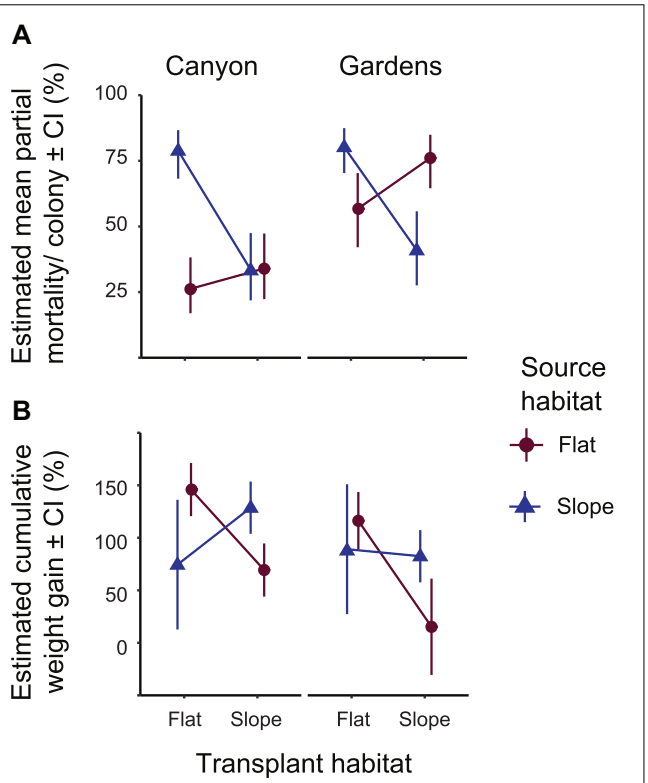

FIGURE 1 | Estimated means of **(A)** percent partial colony mortality and **(B)** percent cumulative weight gain per colony after 18 months in the field (August 2013). Positive numbers designate weight gain, negative numbers designate weight loss. Error bars on both **(A,B)** indicate range of possible means with 95% confidence. Significance tables, **Supplementary Table 4** accompanies **(A)** and **Supplementary Table 6** accompanies **(B)**.

(Wald's Chisq = 13.00, $p = 0.0003$; **Supplementary Table 5**), and insignificant between sites. The mean percent cumulative weight gain per colony in the FS treatment group was only 69% greater than its initial weight at the Canyons and 15% greater than its initial weight at the Gardens (**Figure 1B**). Some FS colonies at the Gardens had up to 30% cumulative weight loss, demonstrated by the confidence interval reaching negative values. Transplants from the SF treatment group had highly variable weight gain as seen in the large range in confidence from the estimated means at both sites. At the Canyons, the SF treatment group had significantly lower estimated weight gained compared to their SS counterparts (estimated marginal means Adj. $p > 0.0001$; **Supplementary Table 6**) and the FF treatment group at the habitat transplanted into (estimated marginal means Adj. $p > 0.0001$). Conversely, at the Gardens, the cumulative weight gained in the SF treatment group was 6.6% greater, but not significantly different, than their SS counterparts, and was also not significantly lower than the FF treatment group (**Figure 1B**).

Heat Tolerance

Heat tolerance was measured using the maximum quantum yield (Fv/Fm) as a proxy. Fv/Fm declined with increasing

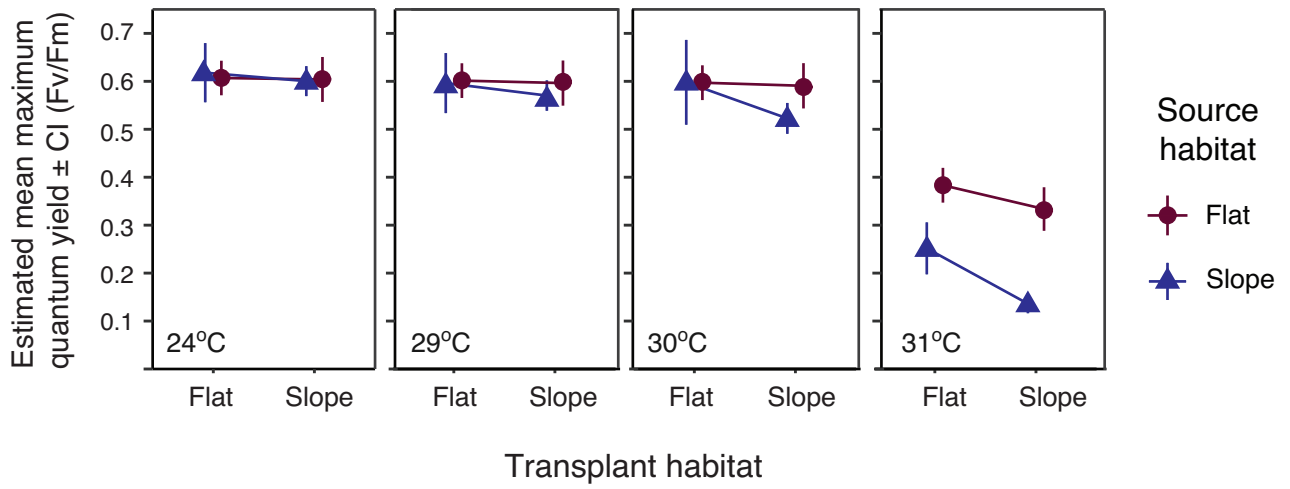

FIGURE 2 | Estimated mean maximum quantum yield ± SE after 14 days of *ex situ* heat stress at ambient and elevated temperatures, post 18-month reciprocal transplant. Error bars indicate range of possible means with 95% confidence. Significance table, **Supplementary Table 8**, accompanies this figure.

temperature (**Figure 2**) and was significantly affected by the interaction between temperature condition and source habitat (Wald's Chisq = 11.03, p = 0.012; **Supplementary Table 7**). At 30°C, the maximum quantum yield of the FF treatment group was significantly higher than that of the SS treatment group (estimated marginal means Adj. p = 0.010). At 31°C, a large drop in maximum quantum yield was observed for all treatment groups. Flat-sourced treatment groups (FF and FS) were not significant from one another, and had between 13 and 20% greater yield values than the slope-sourced treatment groups (SS and SF), regardless of transplant habitat. The yield for colonies in the SF treatment group was 11.5% greater than the SS treatment group (estimated marginal means Adj. p = 0.0003; **Supplementary Table 8**). All pairwise comparisons for temperature conditions at 24 and 29°C were not significantly different.

Coral Host Genetics

Outlier SNP pairwise F_{ST} values between surviving colonies of the Gardens and Canyons sites indicated that there was no genetic differentiation between site replication treatments (i.e., FF, SS, FS, and SF at each location), whereby all F_{ST} values where not significantly different from zero and the genetic composition of the replicated treatment groups between the sites could be considered the same (**Supplementary Table 9**). When combining treatment group data, pairwise F_{ST} averaged over all 16 outliers support the resemblance of transplant treatment groups to colonies sourced from their native rather than novel habitat (**Supplementary Figure 1**) as averaged F_{ST} values between native and transplant treatment groups were not significantly different from zero (flat-sourced: −0.012 and slope-sourced: −0.042; **Table 2**). Other pairwise comparisons between transplant group and the natives at the novel habitat had high F_{ST} values (range 0.491 – 0.531) and statistical significance (p = 0.000) indicating robust allelic differentiation. All combined site differentiation patterns were also directly replicated in the individual site comparison data (**Supplementary Table 9**).

Symbiodiniaceae Dynamics

A quality-controlled dataset yielded a total of 123 Symbiodiniaceae ITS2 sequence variants in 159 samples, 100% of which were from the genus *Cladocopium*. The majority of the *Cladocopium* variants were C42 in flat-sourced colonies, C33 in slope-sourced colonies, and C1 throughout (**Figure 3A**). Permutational multivariate analysis of variance results confirmed that samples from the native FF and SS treatment groups were distinct ($F_{1,127}$ = 345.86, p = 0.001; **Supplementary Table 10**) and did not statistically change over time ($F_{1,127}$ = 2.47, p = 0.110). The Symbiodiniaceae community composition was significantly affected by the interaction of site, source and transplant location, as in site by treatment group (permutational MANOVA: $F_{1,70}$ = 3.470, p = 0.039; **Supplementary Table 11**), and was not significantly dispersed for the same interaction (permutational analysis of dispersion; **Supplementary Table 12**).

Pairwise comparisons of permutational multivariate analysis of variance revealed that FS colonies did not change after

TABLE 2 | Pairwise F_{ST} values (bottom diagonal) and F_{ST} p-values (top diagonal) between surviving corals in all treatment groups among both sites 12 months after reciprocal transplantation (March 2013) for 16 single nucleotide polymorphism (SNP) outliers.

Treatment group	FF	SS	FS	SF
FF	–	**0.000**	0.892	**0.000**
SS	0.501	–	**0.000**	0.942
FS	−0.012	0.531	–	**0.000**
SF	0.491	−0.042	0.516	–

Bold p-values indicate statistical significance. FF, flat to flat; FS, flat to slope; SF, slope to flat; SS, slope to slope.

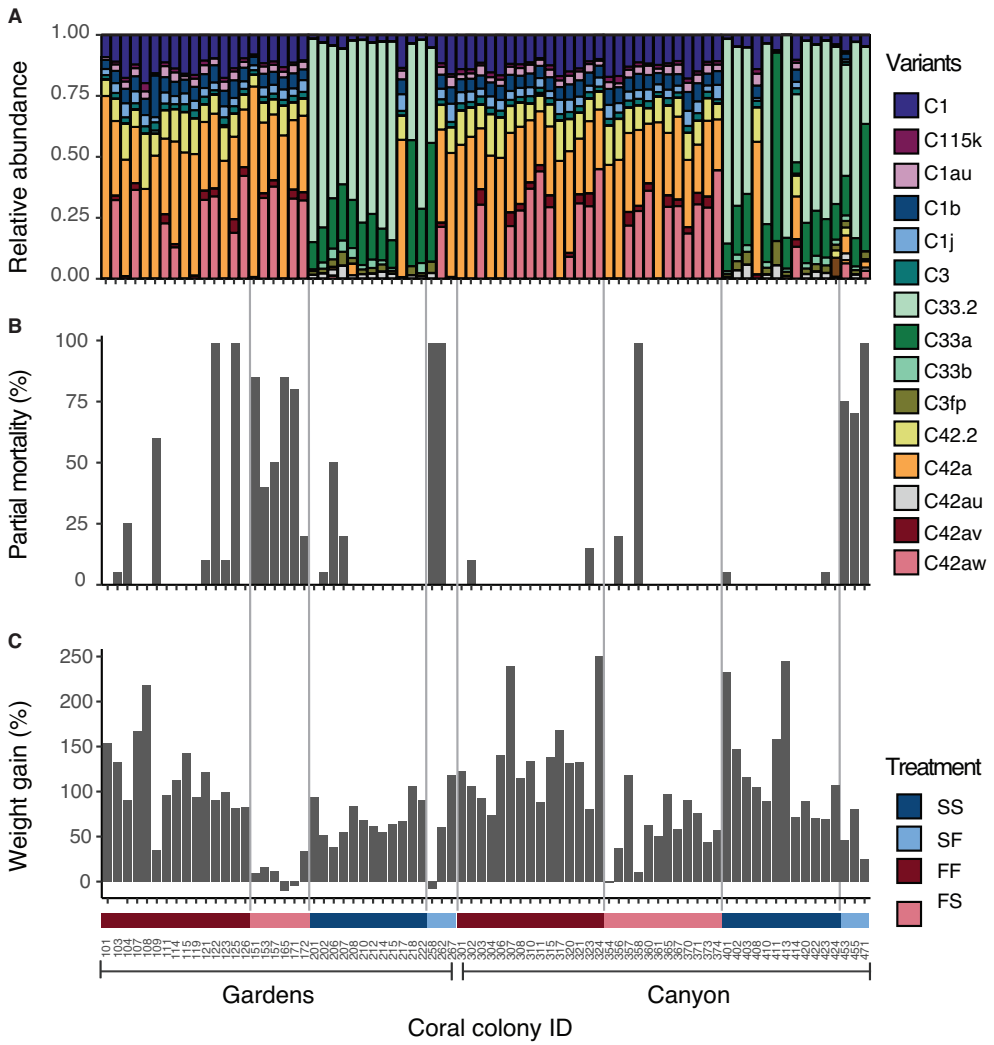

FIGURE 3 | Responses of individual coral colonies organized by site and treatment group after 12 months of reciprocal transplantation (March 2013). Numbers on the *X*-axis are colony ID and each ID number may correspond with a matching genet in the opposite habitat (i.e., FF ID 101 matches FS ID 151). All matching genetic pairs are listed in **Supplementary Table 16**. **(A)** Symbiodiniaceae community in coral tissue. Each color denotes relative abundance of different ITS2 sequence variants present. **(B)** Percent partial colony mortality. **(C)** Percent cumulative weight gain. Positive numbers designate weight gain, negative numbers designate weight loss.

transplantation, exhibited by statistical significance from the SS colonies (Canyons: Adj. p = 0.014; Gardens: Adj. p = 0.026; **Supplementary Table 13**), and none of them harbored sequence variants from the slope (**Figure 3A**). At the Canyons site, the colonies in the SF treatment group primarily maintained slope variants, and had significantly different community composition from the native FF colonies (Adj. p = 0.024; **Supplementary Table 13**), but two of these three colonies harbored a low abundance of flat variants. One Canyons SF colony increased abundance of flat variants after transplantation (SF ID 453 and SS ID 403), and one could not be compared to its SS counterpart due to loss of samples after the experiment (ID 471). In contrast, at the Gardens, two of the three SF colonies were completely dominated by flat, C42 variants (**Figure 3A**), and were not statistically different from the FF treatment group (**Supplementary Table 13**). Of these two Gardens SF colonies, one already maintained the flat variants in the SS treatment group (ID 267/217), while the other changed after transplantation into the flat habitat (ID 262/212).

Overall, the Symbiodiniaceae community composition 12 months after transplantation was not associated with the percent of partial mortality per colony at either site (permutational MANOVA Canyons: **Supplementary Table 14**, Gardens: **Supplementary Table 15**; **Figure 3B**), but was associated with the interaction of percent cumulative weight gain and transplant habitat at the Gardens site (permutational MANOVA Gardens: $F_{1,35}$ = 4.817, p = 0.015; **Supplementary Table 15**). Typically, there was greater weight gain for colonies that harbored flat variants at the flat habitat and lesser weight gain with flat variants at the slope habitat (**Figure 3C**). The

SF colonies at the Gardens site that were comprised of flat Symbiodiniaceae variants had weight gain comparable to colonies in their native habitat. Further, the Gardens SF colony (ID 267) that maintained flat variants prior to transplantation had greater weight gain than most of the slope colonies at the slope, and also exhibited 0% mortality. Although heat tolerance was not tested statistically against Symbiodiniaceae composition, SF ID 267 had higher heat tolerance than its matching SS ID 217, with an average yield value (Fv/Fm) $\pm$ standard error of 0.37 ± 0.02 and 0.34 ± 0.07, respectively.

DISCUSSION

Lower partial mortality and greater weight gain in individuals living in native versus non-native habitats confirmed strong local adaptation to depth habitat across small spatial scales in *P. damicornis* colonies from Heron Island as proposed by Takabayashi and Hoegh-Guldberg (1995) and van Oppen et al. (2018). We also detected signatures of adaptation and acclimatization to heat stress. Native reef flat colonies were more tolerant of elevated temperatures, regardless of transplantation location, demonstrating adaptation, and slope colonies transplanted to the flat increased heat tolerance compared to slope colonies that stayed at the slope, showing acclimatization. The importance of the Symbiodiniaceae symbionts for coral holobiont physiological trait-related fitness was evident from the significant association between the presence of a "flat" Symbiodiniaceae community and an increase in weight gain in a small number of slope colonies following transplantation to the flat at the Gardens site. Variation in physiological performances, host genotypes and symbiont communities between colonies native to the flat or slope habitat are characteristic of local adaptation and indicates that these colonies should be managed as separate populations despite their close physical proximity.

Physiological performance of *P. damicornis* colonies in the Capricorn Bunker Island group may be reliably predicted based on the environmental regimes in the habitat of origin, similarly to *Porites lobata* in American Samoa (Barshis et al., 2018). The flat habitat at Heron Island had greater variability in both water temperature and light irradiance (van Oppen et al., 2018), and we found that flat genotypes had less partial mortality and higher experimental heat tolerance [measured as algal photosynthetic yield (Fv/Fm)] after transplantation to the slope compared to slope genotypes transplanted to the flat. This supports the general observation that individuals exposed to more variable environments are more tolerant and/or plastic, and thereby experience less stress when transplanted into more moderate environments (Smith et al., 2007; Barshis et al., 2010, 2013; Oliver and Palumbi, 2011; Kenkel and Matz, 2017). Another reciprocal transplant study reported a loss of heat tolerance in colonies transplanted to a more moderate environment (Palumbi et al., 2014), but here, though there was a slight reduction in heat tolerance for FS colonies, the FS and FF treatment groups' heat tolerance 18 months after transplantation was similar, revealing heat tolerance as an adaptation in the native flat population. Slope colonies transplanted to the flat had acclimatized in that they exhibited significantly greater heat tolerance compared to those that stayed at the slope. Thus, acclimatization or adaptation to a highly variable environment may promote survival and increased heat tolerance in coral populations.

The physiological performance of corals can be significantly affected by their Symbiodiniaceae community composition (Oliver and Palumbi, 2011; Palumbi et al., 2014; Howe-Kerr et al., 2019; Eckert et al., 2020). Here, we found that Symbiodiniaceae community composition was associated with the interaction of the amount of weight gained and transplant location at the Gardens site, and may also have contributed to heat tolerance. After 12 months of transplantation, two of three Gardens SF colonies were entirely comprised of C42 Symbiodiniaceae variants representative of the flat, one shuffled symbiont types to increase relative abundance of flat variants, and one had fixed flat C42 dominant community. Shuffling symbiont types in low abundance was also observed for one of three Canyons SF colonies. Minor bleaching in corals may allow hosts to obtain or increase optimal symbiont types to cope with environmental conditions (Baker, 2001; Berkelmans and van Oppen, 2006; Silverstein et al., 2015). The bleaching observed in most of the SF colonies after transplantation lead to high mortality overall, but also may have enabled the surviving colonies to successfully persist in a novel environment by shuffling algal symbionts. Sequence variant, C42.2, which was present in all flat-native and some slope-native colonies, was consistently found free-living in water samples taken at Heron Island reef flat habitats (Fujise et al., 2021), and points to the accessibility for uptake of C42 variants post-bleaching. Our results also point to the possible advantage of having a fixed symbiont community representative of a more variable environment as conditions continue to change. Some species within the genus *Cladocopium* are thought to be specialized to more variable or moderate habitats (Barshis et al., 2018; Eckert et al., 2020), and it is plausible that C42 variants may be attributed as specialized for species in the genus *Pocillopora*. C42 variants are repeatedly found in *P. damicornis* (van Oppen et al., 2018; Fujise et al., 2021) and were found to be dominant in 97% of *Pocillopora grandis* samples at Kiribati Island (Claar et al., 2020). Symbiodiniaceae variants that can withstand greater ranges in environmental conditions may subsequently increase resilience of the coral holobiont to environmental extremes.

Predictions of coral trait-related fitness in time and space require carefully designed field and lab experiments. Here, we show that replicate RTEs might yield differing population-by-habitat interactions, and other replicated experiments have also uncovered variation in physiological performance among sites (e.g., Drury and Lirman, 2021). The physiological differences between the Canyons and Gardens reef sites at Heron Island may be due to local environmental conditions such as hydrodynamic conditions and/or the coral tissue-associated prokaryote community. Both of the reef sites are located on the leeward side of the lagoon and are within a channel (Wistari Channel), but the Canyons site is located further southeast where the channel is wider and is relatively more exposed to southeastern waves. Due to the sharp reef crest found around Heron Island, waves coming from the southeast

create wave-generated water flow over the reef flat in the same direction (Gourlay and Colleter, 2005). Further, the Canyons site has a more complex topography, the self-described underwater canyons create fingers of reef that stretch toward the reef edge, whereas the Gardens site has a straight shelf. The amount of water motion can affect the rate of gas and nutrient exchange between the coral tissue and the surrounding water (Dennison and Barnes, 1988), and the southeasterly water flow may flush through the underwater canyons, potentially causing the lower partial mortality seen in the Canyons FS treatment group. Minor differences in prokaryotic community composition between flat habitats at the Canyons and Gardens reef sites were previously reported (van Oppen et al., 2018), which may also have impacted physiological outcomes. Community composition of associated prokaryotes plays a role in the survival of *Porites astroides* colonies post-antibiotic treatment (Glasl et al., 2016) and *Acropora digitifera* colonies during heat stress (Gajigan et al., 2017), but were not measured here.

Genetically based variation in fitness is fuel for natural or artificial selection to promote performance under local prevailing conditions. Bleaching tolerance to thermal stress has a strong genetic basis in acroporid corals (Oliver and Palumbi, 2011; Howells et al., 2013; Bay and Palumbi, 2014; Dixon et al., 2015; Drury and Lirman, 2021) and *Platygyra* sp. (Elder et al., 2020), and here we extend knowledge to a representative of the genus *Pocillopora*. While bleaching tolerance is recognized to be polygenic (Fuller et al., 2020), host outlier loci may be used as biomarkers if they vary in individuals with differing performance to environmental conditions (Parkinson et al., 2020). Here, we could not detect variation in allelic or genotypic frequencies between surviving colonies in novel transplant treatment groups and their counterparts in their native habitat, likely because our sample sizes and experimental design lacked the power to examine this question with confidence, particularly if the trait is polygenetic and controlled by several genes of small effect. Single locus or polygenic DNA markers linked to performance (i.e., QTLs) are immensely useful to further study natural rates of adaptation and to identify stock for active seeding onto coral reefs. Our results highlight the additional importance of local environmental regimes and Symbiodiniaceae community to best describe variation in traits that affect fitness.

The active seeding of corals onto reefs is increasingly considered and applied in the management and conservation of these ecosystems (National Academies of Sciences, Engineering, and Medicine. (NAS), 2019; Bay et al., 2019). Seeded corals arise from a variety of methods (Randall et al., 2020) which may, or may not, consider the genetics of source stock (Baums et al., 2019). Selective breeding of standing genetic variation for heat tolerance has been proposed over latitudinal scales of the Great Barrier Reef (Dixon et al., 2015; Quigley et al., 2019). Here, we found extensive variation in traits that affect fitness, including heat tolerance of colonies among habitats separated by very short geographical distances. Brooding species make up 14% of 444 Scleractinian species with known modes of sexual production (Harrison, 2011). Our results will likely be applicable for other brooding species, which are generally known to settle close to their parents, compared to spawning species (Ayre and Hughes, 2000). It is possible that seeding heat tolerant flat colonies to the slope habitat may assist in increasing tolerance in the slope population through gene flow of host putative adaptive loci and through maternal transmission of flat (C42) Symbiodiniaceae variants, as maternal transmission has been documented in *P. damicornis* (Epstein et al., 2019). The benefits of selecting coral stock for propagation and breeding must be balanced against potential risks (Aitken and Whitlock, 2013) including trade-offs among traits, as observed here. It should be recognized that applied reef restoration and adaptation studies use classic evolutionary experimental designs such as those presented here and thus will also further our fundamental understanding of microevolutionary processes of reef building corals under climate change.

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 PRJNA741589.

AUTHOR CONTRIBUTIONS

SM lead the project and compiled data, extracted DNA, ran statistics, interpreted data and wrote, and edited the manuscript. KZ was second leading advisor, helped analyze coral host outliers, and edited manuscript. JS was third leading advisor and edited manuscript. ML helped write code for statistics, interpret statistical results, and edited manuscript. MO designed experiment, field data collection, and edited manuscript. CK helped with data exploration and algal symbiont interpretation, and edited manuscript. LB was first leading advisor and PI of program that data was collected under, designed experiment, field data collection, and edited the manuscript. 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.2021.704709/full#supplementary-material

Supplementary Figure 1 | Absolute abundance of genotype frequencies of 16 single nucleotide polymorphism (SNP) outliers by habitat of surviving colonies combined by site after 12 months of reciprocal transplantation (March 2013). Native and foreign treatments are compared between source habitat by row, and between transplant habitat by column. Ref/ref = homozygote dominant, alt/alt = homozygote alternate and ref/alt = heterozygote.

Supplementary Table 1 | The number of colonies sampled in each treatment group and site used in the laboratory heat stress experiment 18 months after reciprocal transplantation in the field. Water was conditioned to temperature treatments at 24 (ambient), 29, 30 and 31°C.

Supplementary Table 2 | The number of colonies sampled for Symbiodiniaceae community in coral tissue per site and treatment group at two time points. Native treatment groups were sampled in April 2012 and March 2013, and transplant treatment groups were sampled in March 2013.

Supplementary Table 3 | Partial mortality analysis of deviance table from Wald's Chi-square type III test on its generalized linear mixed model with data taken 18 months after reciprocal transplantation (August 2013). Bold values indicate statistical significance.

Supplementary Table 4 | Partial mortality pairwise contrasts based on estimated marginal means after 18 months of reciprocal transplantation (August 2013). Bold values indicate statistical significance.

Supplementary Table 5 | Weight gain analysis of deviance table from Wald's Chi-square type III test on its generalized linear mixed model with data taken 18 months after reciprocal transplantation (August 2013). Bold values indicate statistical significance.

Supplementary Table 6 | Weight gain pairwise contrasts based on estimated marginal means after 18 months of reciprocal transplantation (August 2013). Bold values indicate statistical significance.

Supplementary Table 7 | Heat stress analysis of deviance table from Wald's Chi-square type III test on the generalized linear mixed model of yield with data taken 18 months after reciprocal transplantation (August 2013). Bold values indicate statistical significance.

Supplementary Table 8 | Heat stress pairwise contrasts based on estimated marginal means among different temperatures and contrasts of treatment groups. Bold values indicate statistical significance.

Supplementary Table 9 | Pairwise F_{ST} values (bottom diagonal) and F_{ST} *p*-values (top diagonal) between surviving colonies after 12 months of reciprocal transplantation (March 2013) for 16 single nucleotide polymorphism (SNP) outliers. Bold *p*-values indicate statistical significance. FF = flat to flat, FS = flat to slope, SF = slope to flat, SS = slope to slope, G = Gardens site, C = Canyons site.

Supplementary Table 10 | Permutational multivariate analysis of variance of the Symbiodiniaceae composition in native treatment groups (FF, SS) between sampling time points in April 2012 and March 2013. Bold values indicate statistical significance.

Supplementary Table 11 | Permutational multivariate analysis of variance of the Symbiodiniaceae composition across source and transplant habitats (flat, slope) and sites (Canyons, Gardens) in March 2013 only. The combination of Source:Transplant refers to treatment group. Bold values indicate statistical significance.

Supplementary Table 12 | Permutational analysis of homogeneity of multivariate dispersions of the Symbiodiniaceae composition between site and treatment group combinations in March 2013 only.

Supplementary Table 13 | Pairwise permutational multivariate analysis of variance of Symbiodiniaceae community composition among site and treatment group comparisons in March 2013 only. Bold values indicate statistical significance.

Supplementary Table 14 | Permutational multivariate analysis of variance of the Canyons reef site Symbiodiniaceae community composition by source and transplant habitats (flat and slope) and physiological traits, percent partial mortality (PMORT) and percent cumulative weight gain (PCWEIGHT). Bold values indicate statistical significance.

Supplementary Table 15 | Permutational multivariate analysis of variance of the Gardens reef site Symbiodiniaceae community composition by source and transplant habitats (flat and slope) and physiological traits, percent partial mortality (PMORT) and percent cumulative weight gain (PCWEIGHT). Bold values indicate statistical significance.

Supplementary Table 16 | Matching genetic pair ID numbers used in the analysis of Symbiodiniaceae community composition and physiological traits shown in **Figure 3**.

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Thermal Stress Has Minimal Effects on Bacterial Communities of Thermotolerant *Symbiodinium* Cultures

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Algae in the dinoflagellate family Symbiodiniaceae are endocellular photosymbionts of corals and other cnidarians. This close relationship is disrupted when seawater temperature increases, causing coral bleaching eventually affecting entire coral reefs. Although the relationship between animal host and photosymbiont has been well-studied, little is known about the bacterial community associated with Symbiodiniaceae in culture. We compared the microbial communities of three isolates from different species of the genus *Symbiodinium* (formerly known as *Symbiodinium* clade A) with different ecophysiology, levels of interaction with the animal host, and thermal adaptations. Two species, *Symbiodinium microadriaticum* and *Symbiodinium necroappettens*, exhibit intermediate thermotolerance, with a decrease of both growth rate and photochemical efficiency with increased temperature. The third species, *Symbiodinium pilosum*, has high thermotolerance with no difference in growth rate or photochemical efficiency at 32°C. Microbial communities were characterized after 27 days of growth under control (26°C) and high temperature (32°C). Data shows stronger grouping of bacterial assemblages based on *Symbiodinium* species than temperature. Microbial communities did not group phylogenetically. We found a shared set of fifteen ASVs belonging to four genera and three families that remained in all three Symbiodiniaceae species. These included *Labrenzia*, Phycisphaeraceae (SM1A02), *Roseovarius*, and *Muricauda*, which are all commonly associated with corals and Symbiodiniaceae cultures. Few ASVs differed significantly by temperature within species. *S. pilosum* displayed significantly lower levels of microbial diversity and greater individual variability in community composition at 32°C compared to 26°C. These results suggest that bacteria associated or co-cultured with thermotolerant *Symbiodinium* might play an important role in thermotolerance. Further research on the functional metabolic pathways of these bacteria might hold the key to understanding *Symbiodinium*'s ability to tolerate thermal stress.

Keywords: microbiome, holobiont, thermotolerance, microbial communities, photosymbionts

INTRODUCTION

The holobiont, a host and its associated microbial community, is a concept that we are beginning to understand is of crucial importance for host health, and in some cases for ecosystem health as well. Not only does the holobiont serve as a unit for natural selection, but each constitutive member can increase the plasticity of the whole by their physiological responses to the environment (Ye et al., 2019). For example, lichen microbiota may shift their metabolism to a fasting state under dry conditions, thereby increasing holobiont tolerance to drought stress (Cernava et al., 2019). Furthermore, the resilience of symbiotic microbial communities can directly affect host fitness. Certain bacteria found growing in the roots of desert plants were found to promote drought resistance in wheat (Zhang et al., 2020). While animal-microbe and plant-microbe holobionts have been intensely studied (Bronstein, 2015; Sitaraman, 2015; Hacquard, 2016; Hassani et al., 2018; Mayoral-Peña et al., 2020), microbe-microbe interactions such as phytoplankton-bacteria, mycorrhiza-helper bacteria, and symbiotic bacteriophages (Rodriguez et al., 2008; Seymour et al., 2017) are only recently being recognized as important components of the holobiont. The coral holobiont encompasses the animal (cnidarian host), its endosymbiotic photosymbionts, bacteria, archaea, fungi, and viruses (Thurber et al., 2009). Coral-associated microorganisms are critical to host fitness and survival (Glasl et al., 2016; Sweet and Bulling, 2017). Coral holobionts are highly sensitive to increases in temperature, with only a +1°C increase above long-term summer maximum temperature resulting in bleaching (i.e., disruption of symbiosis between the coral and endosymbiotic dinoflagellate), and increasingly, in coral mortality (Hoegh-Guldberg, 1999; Webster et al., 2016; McDevitt-Irwin et al., 2017; Peixoto et al., 2017).

A growing body of research has started to reveal the role of bacteria in the coral holobiont (Bourne et al., 2016; van Oppen and Blackall, 2019). They can protect the host against pathogenic microbes by preventing colonization through the physical occupation of their potential niches (Ritchie and Smith, 2004). Coral-associated bacteria are known to contribute to carbon cycling (Kimes et al., 2010), sulfur cycling (Wegley et al., 2007; Raina et al., 2009; Tandon et al., 2020), phosphorus fixation, metal homeostasis, organic remediation, secondary metabolism (Zhang et al., 2015), and production of antibiotics (Ritchie, 2006). Changes in environmental stress can alter the fitness advantages of having such functional traits represented in the microbiome. The Coral Probiotic Hypothesis proposed by Reshef et al. (2006) posits that when environmental conditions are altered, a coral's populations of associated bacteria will undergo rapid change that effectively establishes a new, context-dependent coral holobiont. The modified bacterial community is thought to provide an advantage to cope with the new environment (i.e., warmer water). Recently, experiments involving transplantation of bacterial consortia showed improvements to coral host physiology and temperature tolerance (Doering et al., 2021). Elucidating microbial community dynamics during thermal stress and holobiont acclimatization is increasingly important in understanding the long-term persistence of coral reefs in the face of global climate change (Bellantuono et al., 2012).

For decades, dinoflagellate algae of the family Symbiodiniaceae have been recognized as the driving force behind the high productivity of coral reefs (Freudenthal, 1962; Hoegh-Guldberg et al., 2007; Roth, 2014). Symbiodiniaceae can supply up to 96% of its host's energy budget (Muscatine, 1990) and may help with other important functions such as irradiation and thermal tolerance, with different assemblages being associated with different light and temperature conditions (Rowan et al., 1997; Rowan, 2004; Goulet et al., 2005; Oliver and Palumbi, 2011). Symbiodiniaceae therefore play a key role in coral microbiomes, and likely in their resilience to environmental disturbance as well (Trench and Blank, 1987; Warner and Suggett, 2016). Although the roles of Symbiodiniaceae in coral reefs have been fairly well-documented, these dinoflagellates harbor microbial communities of their own that remain poorly understood (Matthews et al., 2020). Taking advantage of the ability to grow these algae in culture, only three studies have aimed to identify the core microbiome of Symbiodiniaceae (Lawson et al., 2018; Camp et al., 2020; Maire et al., 2021). These studies used highly evolutionarily divergent lineages with diverse physiology, finding little overlap across isolates as well as closely related, conspecific strains. While such studies offer great insight into the similarities and differences between microbial communities of Symbiodiniaceae species with differing physiologies, comparing divergent taxa (what now includes multiple genera) of Symbiodiniaceae overgeneralized the ecology and evolution of diverse lineages (LaJeunesse et al., 2018). Comparing closely-related species with similar physiology can allow for a more detailed understanding of the ecological and evolutionary mechanisms of host-symbiont interactions.

Understanding symbiotic microbial communities holistically is critical to conservation and restoration efforts, as microbial communities may influence holobiont fitness in the face of highly stressful disturbances such as climate change (Putnam et al., 2017). In order to predict future shifts and mitigate coral reef decline, discerning shifts in host- and photosymbiont-associated microbial species during and after thermal stress is crucial. This study aims to further characterize the microbes associated with three thermotolerant photosymbionts (two intermediately thermotolerant and one highly thermotolerant) within the genus *Symbiodinium* (formerly known as Clade A) (Díaz-Almeyda et al., 2017). Herein, we compare three closely related species of *Symbiodinium* with known thermotolerance, grown under normal and high temperature conditions. We hypothesize that phylogenetically related species of *Symbiodinium* (e.g., *Symbiodinium pilosum, Symbiodinium microadriaticum,* and *Symbiodinium necroappettens*) with relatively similar thermotolerance will have overlapping microbial communities. We observe bacterial assemblages strongly grouping according to *Symbiodinium* species, with these communities remaining stable under high temperature conditions. We are yet to determine if the bacteria present in each group have redundant functions.

MATERIALS AND METHODS

Sample Culture, Collection, and DNA Extraction

Algal isolates of three species of *Symbiodinium*, formerly known as clade A, were used for this study (**Table 1**). Cultures were grown in ASP-8A media [pH 8.5, 35ppt, Blank (1987)] at control (26°C) and high (32°C) temperature conditions. Cultures were grown on Percival incubators and placed on a glass shelf on top of full-spectrum fluorescent lights at 100 μmol quanta m^{-2} s^{-1} facing up, with a 12:12 light/dark cycle photoperiod. Light intensities were measured inside a 300 ml culture flask with media using a 4π sensor (Biospherical, United States). Cultures were maintained by adjusting cell density to 1×10^5 cells ml^{-1} every 10 days by adding fresh media, acclimated to the control temperature (26°C) for at least 2 months (**Supplementary Figure 1A**). To measure cell density, a sterile rubber policeman was used to scrape all cells from the flask walls to ensure collection of all cells. The culture was homogenized manually and 1 ml aliquot was collected into a 2 ml screwcap tube. To detach cell aggregations, lugol was added (200 μl) and tubes were vortexed for 5 min. Cells were then counted manually with a hemocytometer. To begin the experiment, replicate 15 ml clear glass tubes containing 8 ml of culture with 1×10^5 cells ml^{-1} were placed vertically on top of a glass shelf with full spectrum lights facing up, in an incubator set at 26 or 32°C (n = 5 per isolate per temperature) (**Supplementary Figure 1B**). Tubes were undisturbed until after 27 days of incubation, when F_v/F_m was measured for all replicates (n = 5 per isolate per temperature) and cells were collected for DNA extraction (n = 3 per isolate per temperature). Cells were collected by scraping all cells attached to the glass tube with a disposable inoculating loop and centrifuged at approximately 2,000 g for 1 min, removing the supernatant and flash freezing with liquid nitrogen immediately. DNA was isolated using the MO BIO PowerSoil DNA isolation Kit following manufacturer's instructions. A previous study established that *S. microadiaticum* (strain CassKB8) and *S. necroappetens* (strain RT80) have intermediate thermotolerance, since they were able to grow at high temperature but not as well as at normal temperature. *S. pilosum* (strain RT130) grew at the same rate in normal and high temperature conditions, suggesting that it has higher thermotolerance (Díaz-Almeyda et al., 2017). *S. pilosum* and *S. necroappetens* were collected in the Caribbean and are part of the same isolate collection (formerly known as Trench collection) while *S. microadriaticum* was isolated in the North Pacific (formerly known as BURR collection) (**Table 1**).

TABLE 1 | Cultures used in this study, invertebrate species from which the culture was isolated, and geographic location where sample was collected.

	Symbiodinium microadriaticum	*Symbiodinium necroappetens*	*Symbiodinium pilosum*
Culture name	CassKB8	RT80	RT130
Collected from	*Cassiopea xamachana*	*Condylactis gigantea*	*Meandrina* sp.
Origin	North Pacific	Caribbean	Caribbean
Temperature tolerance	intermediate	intermediate	tolerant
Growth rate (*K*) at 26°C	0.446 ± 0.163	0.423 ± 0.005	0.42 ± 0.008
Growth rate (*K*) at 32°C	0.354 ± 0.014	0.331 ± 0.112	0.407 ± 0.125
F_v/F_m at 26°C	0.594 ± 0.047	0.538 ± 0.045	0.631 ± 0.014
F_v/F_m at 32°C	0.373 ± 0.069	0.354 ± 0.035	0.545 ± 0.027

For detailed physiological data summarized here go to Díaz-Almeyda et al. (2017). Cultures are kept at The Pennsylvania State University.

16S rRNA Gene Amplification and Sequencing

Samples were amplified using specific primers pairs (Caporaso et al., 2011) for the V4 region of the 16S rRNA gene. A 25 μL PCR reaction was prepared with 10 μL 5Prime HotMaster Mix (QuantaBio), 0.5 μL forward primer 515F (GTGCCAGCMGCCGCGGTAA) (10 μM), 0.5 μL reverse primer 806R (GGACTACHVGGGTWTCTAAT) (10 μM), 1 μL of BSA (10 mg/ml), 1 μL template DNA (10 ng), and 12 μL PCR-grade water. PCR amplifications consisted of a 3 min denaturation at 94°C; 30 cycles of 45 s at 94°C, 60 s at 50°C and 90 s at 72°C; and 10 min at 72°C. Samples were submitted for sequencing to the Department of Energy's Joint Genome Institute (JGI) and were processed according to their iTag sequencing protocol. Individual libraries were prepared and barcoded before being multiplexed and sequenced on an Illumina MiSeq sequencer machine in a 2 × 300 run mode.

Sequence Generation and Processing

Sequences were demultiplexed and processed according to the JGI's iTag amplicon sequencing protocol for taxonomic identification. Initial processing of 16S rRNA gene sequence libraries was performed using the Quantitative Insights Into Microbial Ecology (QIIME2; 2019.7.0) package (Bolyen et al., 2019). Read trimming, filtering, denoising, and amplicon sequence variant (ASV) inference was performed with the DADA2 implementation of QIIME2 (Callahan et al., 2016). Taxonomy was assigned to ASVs using the Naive Bayes classifier pre-trained on the Silva database version 138 clustered at 99% sequence similarity and optimized for the 515F/806R primer pairs. Extraction blanks and template PCR controls were not included as part of our sequencing effort. We therefore applied additional filters to the reads to remove possible contaminants. Initially, ASVs were removed if they were not present in a minimum of three of the 18 samples sequenced. Additionally, low abundance ASVs totaling less than 63 reads (0.2 percent of the total reads from the sample with the fewest reads) were removed from each sample. The additional filtering removed rare ASVs, many of which were present in a single sample and highly likely to be contamination of the culture. Filtering reduced the total ASV count from 255 to 124. Phylogenetic diversity metrics were generated with alignments performed with MAFFT and trees generated with FastTree (**Supplementary File 1**), which was followed by alpha and beta diversity calculations.

Differential Abundance and Distance Contribution

Differential abundance testing was performed with DESeq2 [v1.32.0, Love et al. (2014)], against sample abundances normalized by the geometric mean of positive counts. Fold change values were adjusted for dispersion using the adaptive shrinkage estimator from the ashr package [v2.2-47, Stephens et al. (2022)], and ASVs differentially abundant beyond a false discovery rate of 0.01 were collected (**Supplementary Table 1**). Contributions of each ASV to Bray-Curtis dissimilarity were calculated for using the SIMPER function from the vegan package (v2.5-7) (**Supplementary Table 2**). Permutational multivariate analysis of variance (PERMANOVA, 999 permutations) was performed as well (**Supplementary Table 3**). Initially, samples were rarefied to 30,000 reads and pairwise comparisons were performed between 26 and 32°C (**Supplementary Table 4**).

Data Visualization

Data visualization, including PCoA plots of unweighted and weighted UniFrac distance results calculated in QIIME2 were generated with qiime2R (v0.99.6)[1] and phyloseq [v1.36.0, McMurdie and Holmes (2013)] R packages (RStudio Team, 2020). Overlapping taxa (grouped at the genus level) between species and temperature were identified using the online tool from VIB/Ugent Bioinformatics and Evolutionary Genomics Website.[2]

RESULTS

Bacterial Communities Are Specific to *Symbiodinium* Species

The microbial communities of each *Symbiodinium* sp. were characterized via sequencing of the V4 region of the 16S rRNA gene. We recovered an average of 50,623 raw reads per sample. After filtering and processing the sequences, the average number of sequences per sample was 48,507, with a minimum of 31,090 and maximum of 68,716. After removal of low abundance taxa, we identified 124 bacterial ASVs across all samples and we summarized them at the genus level by replicate (**Supplementary Figure 2**) and by treatment and species (**Supplementary Figure 3**). Samples grouped largely by *Symbiodinium* species when clustered by Bray-Curtis dissimilarity (**Figure 1**). Permutational multivariate analysis of variance (PERMANOVA) confirmed diversity composition significantly varied by species (999 permutations, q-value < 0.05), indicating *Symbiodinium* species harbor specific microbial communities (**Supplementary Table 3**).

Comparison of community composition with SIMPER identified less than 20 ASVs driving 85% of the observed dissimilarity between species (**Supplementary Table 2**). Additionally, OM182_clade, which accounted for the greatest percentage of dissimilarity between *S. microadriaticum* and *S. necroappetens,* was absent from *S. microadriaticum*, while present in both *S. necroappetens and S. pilosum* (**Figure 2**). *Labrenzia* accounted for 18.9% of dissimilarity between *S. pilosum* and both other *Symbiodinium* species. These findings were further corroborated by differential abundance analysis with DESeq2. We found the aforementioned taxa to be differentially abundant between *Symbiodinium* species (**Supplementary Table 1**).

A closer inspection of these ASVs revealed that they belong to a small subset of the genera found across the three species, including *Muricauda*, *Balneola*, *Labrenzia*, *Phaeodactylibacter, Sphingorhabdus,* and OM182_clade, each with >8% influence in respective species comparisons (**Figure 3** and **Supplementary Table 2**). Notably, four genera (*Muricauda*, *Labrenzia*, *Roseovarius*, and the uncultured SM1A02 group of the family Phycisphaeraceae) were commonly found across the three *Symbiodinium* species at both 26 and 32°C, with each dinoflagellate taxa associating with presumably a unique strain represented by a single ASV.

Symbiodinium microadriaticum harbored the greatest number of unique microbial genera (37 at 26°C and 36 at 32°C), whereas *S. pilosum* harbored the least, with seven species specific genera at both 26 and 32°C (**Figure 4**). No additional genera were shared between *S. pilosum* and *S. necroappetens* beyond the aforementioned four taxa, whereas *S. microadriaticum* and *S. necroappetens* shared 13 to 14 genera at 26 and 32°C, respectively.

While the four shared genera were often found to exhibit high relative abundance within the samples, other taxa were represented in greater abundance within each *Symbiodinium* species. In *S. microadriaticum*, *Phaeodactylibacter* (15.21–16.75%) and usually a member of the OM60(NOR5) clade of Halieaceae (6.08–15.80%) were the dominant genera. In *S. necroappetens,* OM182_clade of Gammaproteobacteria (17.51–21.96%), *Balneola* (13.89–24.78%), *Marinobacter* (5.89–17.18%), and *Labrenzia* (11.14–18.01%) were the dominant genera. In *S. pilosum, Muricauda* (15.09–21.61%), *Sphingorhabdus* (8.60–18.06%), and *Labrenzia* (38.32–49.74%) were the dominant genera (**Figure 3**).

Diversity of Intermediately-Thermotolerant *Symbiodinium* Remains Relatively Stable Under Elevated Temperatures

Shannon Diversity Index (H') values were calculated for each sample (**Figure 5**). *S. microadriaticum* had the highest average H' with no significant differences between temperatures (4.67 at 26°C and 4.78 at 32°C), followed by *S. necroappetens* (H' = 3.60 at 26°C and 3.61 at 32°C). *S. pilosum* had the lowest average H' of 3.04 at 26°C and 2.67 at 32°C. One-way ANOVA analysis showed significant differences between species [$F_{(5,12)} = 218.3432$, $p < 0.0001$] but only *S. pilosum* had a significant change in H' between the two temperatures (Tukey HSD, $p = 0.0053$).

Our samples did not cluster well by temperature (q-value > 0.05). We did, however, find ASVs that were significantly

[1] https://github.com/jbisanz/qiime2R

[2] http://bioinformatics.psb.ugent.be/webtools/Venn/

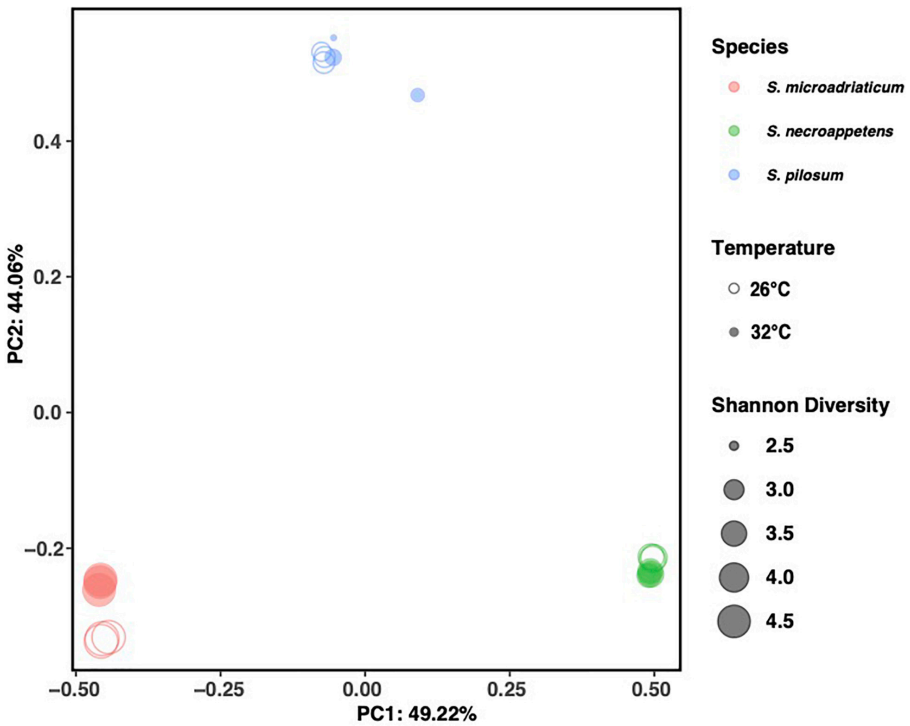

FIGURE 1 | Principal coordinate analysis (PCoA) plot of bacterial communities, with three replicates for *Symbiodinium pilosum*, *Symbiodinium microadriaticum*, and *Symbiodinium necroappettens* under 26 and 32°C conditions. All data shows strong bacterial assemblages grouped on *Symbiodinium* species rather than temperature.

differentially abundant between 26 and 32°C. Seventeen ASVs were differentially abundant in cultures of *S. microadriaticum*. Two of the differentially abundant taxa belonged to the shared genera *Labrenzia* and *Muricauda*, and increased by 8- and 2-fold (log_2), respectively. In *S. necroappetens,* both bacterial genera exhibited differential abundance, with *Labrenzia* increasing 5-fold (log_2) and *Muricauda* decreasing in relative abundance by 5-fold (log_2). Nine ASVs were differentially abundant in *S. pilosum*, although none belonged to the four genera commonly found between the three dinoflagellate species.

DISCUSSION

We compared the bacterial communities of closely related *Symbiodinium* isolates that have been previously characterized with similar thermotolerance in culture (Díaz-Almeyda et al., 2017). Similar to previous studies, we found microbial communities of *Symbiodinium* clustered primarily by species (Lawson et al., 2018; Camp et al., 2020; Maire et al., 2021). Of the three *Symbiodinium* species included in our study, two intermediately thermotolerant species (*S. necroappetens* and *S. microadriaticum*) had greater microbial diversity than the highly thermotolerant species (*S. pilosum*). The community composition of *S. necroappetens* remained relatively stable at 32°C, while that of *S. microadriaticum* clustered separately by temperature, and the community composition of *S. pilosum* became more dispersed at 32°C (**Figure 1**). Relatively few ASVs differed by temperature, and there were no ASVs that consistently increased or decreased in abundance across all species, although we detail why some of them may be notable. More evidence is needed to determine whether differences in the relative abundance of any thermally differing taxa influence *Symbiodinium* thermotolerance (or vice-versa). Our results demonstrate that even closely related *Symbiodinium* species with similar physiology are host to unique assemblages of microbes which may differ in their responses to increased temperature.

Symbiodinium Species Share Few Associated Bacterial Genera

All three *Symbiodinium* species shared members of *Muricauda* (Flavobacteriaceae), *Labrenzia* (Rhodobacteraceae), *Roseovarius* (Rhodobacteraceae), and a member of Phycisphaeraceae (SM1A02). These findings are consistent with previous studies of Symbiodiniaceae-associated microbial communities (Lawson et al., 2018; Camp et al., 2020). Importantly, although microbial communities grouped by *Symbiodinium* species, they did not group phylogenetically by *Symbiodinium* species. Rather, they grouped by location and collection of origin (**Supplementary Figure 4**). *S. necroappetens* and *S. microadriaticum* are more closely related phylogenetically than *S. pilosum* (LaJeunesse et al., 2015). However, *S. necroappetens* had a microbial community more similar to *S. pilosum* than to *S. microadriaticum*. *S. necroappetens* was isolated by Robert

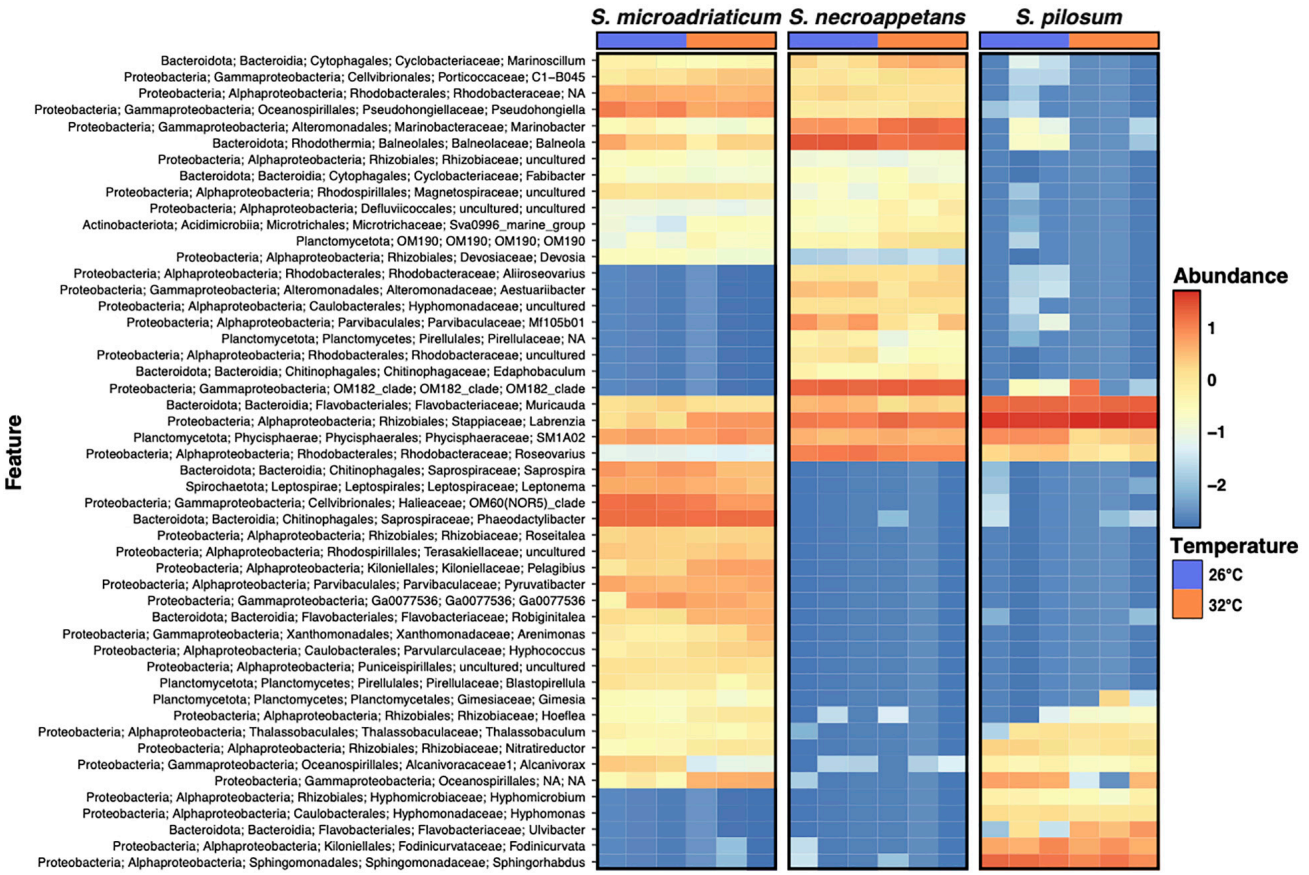

FIGURE 2 | Heatmap of relative abundances calculated in $\log_{10}$ for the top 50 most abundant ASVs.

K. Trench at least 20 years ago (LaJeunesse, 2001), *S. pilosum* was isolated at least 27 years ago (Iglesias-Prieto and Trench, 1994), and both were isolated from the Caribbean by the same laboratory with protocols which included adding antibiotics and using a low nutrient culture media to remove bacteria (Schoenberg and Trench, 1980). This may explain the low bacterial diversity we observed in *S. pilosum*. In contrast, *S. microadriaticum* was isolated at least 35 years ago by a different laboratory using f/2 media (Taguchi and Kinzie, 2001). It is therefore unknown whether grouping of bacterial communities is due to long-term culturing artifacts or true symbiotic associations, as these differing protocols may have resulted in very different founder effects, leading to the differences we observed. Maire et al. (2021) found *in situ* samples of Symbiodiniaceae differed greatly from corresponding cultures, suggesting that co-culturing may have influenced our results rather than true symbiosis. The same study also found the presence of intracellular bacteria in Symbiodiniaceae to be common, diverse, and highly distinct from extracellular bacteria. We are unable to separately characterize the responses of intracellular and extracellular communities to thermal stress because we did not distinguish these communities. With these considerations, our results provide insight into the potential connection between *Symbiodinium* thermotolerance and associated bacterial communities, and point toward future studies on *in situ Symbiodinium* that distinguish between closely- and loosely-associated bacteria.

Microbial Community Response to Temperature Stress May Correlate With Host Thermotolerance

Bacterial diversity of *S. microadriaticum* and *S. necroappetens* remained stable at high temperatures while that of *S. pilosum* was significantly lower at 32°C. Stable *H'* values across different environmental conditions can indicate a lack of substantive change in community composition, or that the combined richness and evenness of the community is relatively stable (Kimbro and Grosholz, 2006; Willis, 2019). The flexibility that we observed in *S. pilosum* may be related to its higher thermotolerance, as acclimating to rapid changes in environmental conditions has been associated with dramatic shifts in bacterial community composition (Vargas et al., 2020; Voolstra and Ziegler, 2020). In contrast to our results, Camp et al. (2020) found that the microbiome of *Durusdinium trenchii,* a thermotolerant Symbiodiniaceae, remained stable at high temperatures.

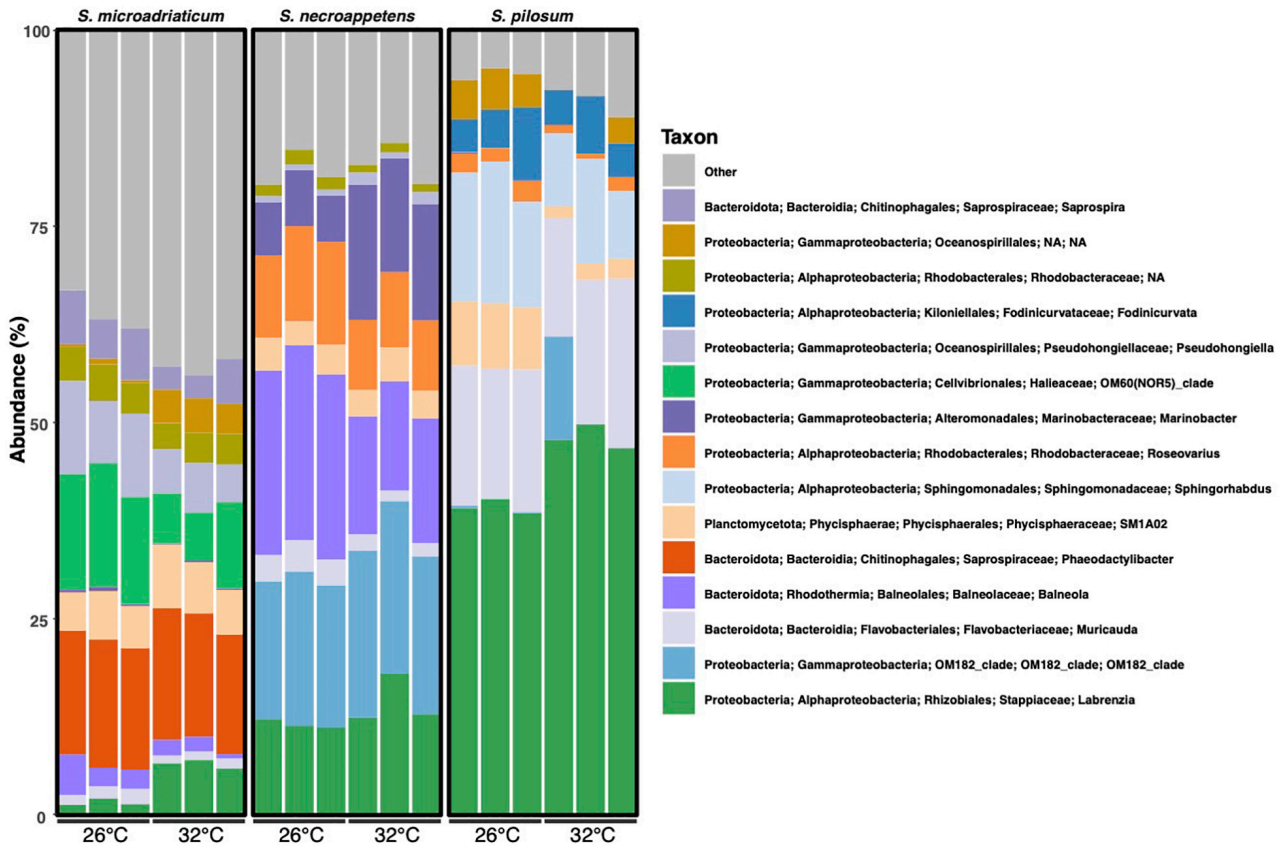

FIGURE 3 | Bacterial community composition (relative abundance in percentage) of *Symbiodinium* species in culture under 26 and 32°C at the genus level, based on the 16S rRNA gene v4 region amplification. The relative bacterial abundance is similar within *Symbiodinium* species, independent of temperature (n = 18).

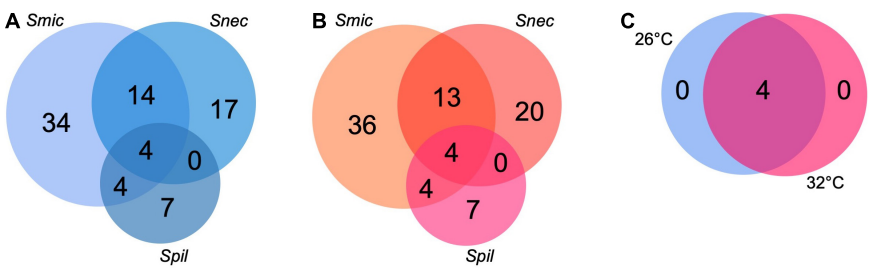

FIGURE 4 | Shared number of genera present in each core microbiome for two temperatures, independent of Symbiodinium species **(A,B)**. Combined core microbiome identified at 26°C and 32°C, across the three species of Symbiodinium **(C)**. To be considered part of the core microbiome at each temperature, the OTU must be present in all replicates (n = 18). See **Supplementary Figures 2, 3** for details.

The bacterial communities of *S. microadriaticum* and *S. pilosum* also clustered by temperature. *S. necroappetens* samples clustered together but not separately by temperature, most likely due to experiencing a much smaller change with temperature. These results support the idea that microbiome responses to heat stress are unique to *Symbiodinium* species, perhaps regardless of dinoflagellate thermotolerance. Our results also suggest that increased thermotolerance may correlate with lower levels of microbial diversity and greater individual variability in community composition. According to the "Anna Karenina principle," the increased prevalence of environmental stressors may reduce a host's ability to regulate its microbiome composition (Zaneveld et al., 2017). While our results did not offer evidence for this principle, if well-supported with future research, the Anna Karenina principle has strong implications for coral reefs in the context of climate change, as a strong link between rising ocean temperatures and abundance of coral pathogens within communities of coral-associated microbes has been described (Tout et al., 2015), and microbial communities without functional redundancy can be subject to environmental

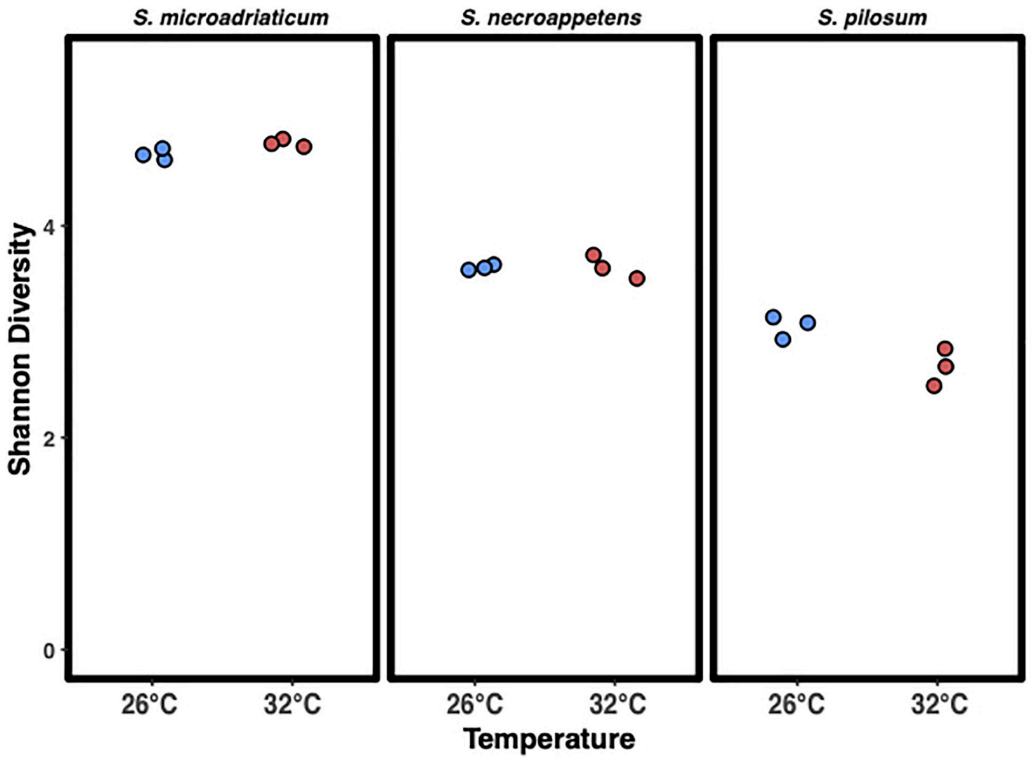

FIGURE 5 | Shannon diversity index of microbial communities associated with *Symbiodinium* in culture. Blue 26°C and red 32°C. There was a significant effect of species and temperature on the Shannon diversity index at the $p < 0.0001$ level for the three conditions with *Symbiodinium pilosum* being the only species different by temperature [$F_{(5,12)} = 154.07$, $p < 0.0001$].

stressors such as ocean warming (Voolstra and Ziegler, 2020). Further research using controlled studies to investigate this relationship may find a link between the thermotolerance of a microbial community and instability of the holobiont under high temperatures. The potential benefits of greater flexibility and diversity in microbiome community composition for thermotolerance should be further explored in future studies using larger sample sizes and other *Symbiodinium* species. Additionally, while thermotolerance is known to be affected by other factors such as genetics, environmental factors, and host-symbiont type mismatches (Brown et al., 2002; Mieog et al., 2009), the degree of contribution (positive or negative) by certain microbial taxa, their potential symbiotic interactions with *Symbiodinium,* and their relationships to *Symbiodinium* thermotolerance remain unexplored and should be considered for future studies.

Notable ASVs and Their Potential Roles in *Symbiodinium* Thermotolerance

No ASVs consistently increased with temperature across all *Symbiodinium* species. Rather, different ASVs were differentially abundant by temperature for each *Symbiodinium* species. While some or all of these differences may be a result of long-term culturing, we offer insights on the potential importance of key ASVs for *Symbiodinium* thermotolerance.

We found *Labrenzia* to be particularly abundant across all *Symbiodinium* species, but especially in *S. pilosum*, the most thermotolerant species. Additionally, *Labrenzia* were not differentially abundant between temperature treatments in *S. pilosum* but were significantly less abundant at 32°C in the two intermediately thermotolerant *Symbiodinium*. *Labrenzia* are known for their ability to produce dimethylsulfoniopropionate, which scavenges reactive oxygen species and thus may play a role in increasing *Symbiodinium* stress tolerance (Diaz et al., 2016; Curson et al., 2017). It is also plausible that these bacteria could be directly reducing pathogenic microbes, which might opportunistically take advantage of heat stressed *Symbiodinium*. An oyster-colonizing *Labrenzia* sp. exhibited broad antimicrobial activity and reduced the growth of a pathogenic *Roseovarius* (Amiri Moghaddam et al., 2018), and labrenzbactin, a siderophore produced by a species of *Labrenzia*, has been shown to exhibit antimicrobial activity *in vitro* (Sharma et al., 2019). However, its effect on *Symbiodinium*-specific pathogens has not yet been explored. The increased prevalence of *Labrenzia* in *S. pilosum* and its relatively stable abundance with temperature stress may be related to thermotolerance, or it may be a result of having lower levels of diversity in general. Research on the ecological and physiological roles of *Labrenzia* for *Symbiodinium* fitness is necessary to discern whether these bacteria are generally mutualistic, parasitic, or commensal partners in heat-stressful conditions.

Roseovarius were also present in all three host species, in much lower relative abundances than *Labrenzia,* and was highest in *S. necroappetens.* Other studies have similarly found *Roseovarius* in relatively low abundance with Symbioiniaceae (Camp et al., 2020; Maire et al., 2021). Notably, Pootakham et al. (2019) found that *Roseovarius* were one of the two most prevalent genera associated with heat-stressed corals. Members of *Roseovarius* have also been implicated in coral diseases such as black band disease (Ng et al., 2015). However, we did not find that *Roseovarius* significantly increased in relative abundance with increased temperature in *Symbiodinium* (**Figure 2**). Our results therefore suggest that *Roseovarius* may not play a similar role in heat stressed *Symbiodinium,* or perhaps only in more thermosensitive species. Further research on the functional ecology of *Roseovarius* with *Symbiodinium* under heat stress is necessary to investigate these hypotheses.

Muricauda was also shared by all three *Symbiodinium* species we cultured and were differentially abundant by temperature in the two intermediately thermotolerant *Symbiodinium,* but not in *S. pilosum.* Several studies have demonstrated that *Muricauda* are intimately associated with Symbiodiniaceae (Han et al., 2016; Lawson et al., 2018). In the first comparison of intracellular, closely-associated, and loosely-associated bacterial communities, Maire et al. (2021) found *Muricauda* present in most *in situ* samples of different Symbiodiniaceae species, but consistently in less than 1% abundance. We found *Muricauda* in greater than 1% abundance in all *Symbiodinium,* but especially in *S. pilosum,* in which *Muricauda* consistently accounted for over 15% of each sample. This discrepancy may be the result of culturing, as cultured samples have previously been found to differ substantially from *in situ* Symbiodiniaceae samples (Maire et al., 2021). Regardless of its potentially low abundance in wild Symbiodiniaceae, *Muricauda* is one of 12 bacterial genera ubiquitously associated with various reef-building corals across the globe, suggesting its functional importance not only to Symbiodiniaceae but also to the coral holobiont (Bernasconi et al., 2019). Furthermore, *Muricauda* may serve a functional role in *Symbiodinium* heat resistance. A close relative to *Muricauda lutaonensis* enhanced thermal and light stress resistance of cultured Symbiodiniaceae (Motone et al., 2020). We did not find significant differences in *Muricauda* by temperature for any *Symbiodinium* species, but *in situ* sequencing may indicate whether they play a key role in *Symbiodinium* thermotolerance.

Sphinghorabdus were one of the dominant taxa in *S. pilosum* but were not present in greater than 1% abundance in the intermediately thermotolerant host species (**Figure 3**). *Sphinghorabdus* have been isolated from Gorgonian coral and have been successfully cultivated (Keller-Costa et al., 2017; Silva et al., 2018), but otherwise are not well-known, so further research may reveal their importance for *Symbiodinium* thermotolerance.

In *S. pilosum,* a member of Phycisphaeraceae (SM1A02) accounted for the greatest amount of community dissimilarity between temperature treatments, displaying greater abundance at 26°C. This may be due to strong competitive pressure from the highly dominant *Labrenzia* in thermally-stressed *S. pilosum*, as ASVs from *Phycisphaeraceae* did not significantly decrease in abundance in the other two *Symbiodinium* species. Kellogg (2019) found three OTUs belonging to Phycisphaerales to be bacterial associates across seven deep-sea corals (Eloe et al., 2011; Allers et al., 2013). Sequences similar to those three OTUs were not associated with tropical corals, possibly suggesting a role specific to cold-water corals (Kellogg, 2019). Contrarily, members of Phycisphaerales were associated with tropical stony corals, and in greater abundance under highly variable environmental conditions by Ziegler et al. (2017). Together with our results, these findings suggest that the observed decrease in Phycisphaeraceae with temperature may not be indicative of thermotolerance, but rather a consequence of the increase in relative abundance of *Labrenzia* in our most thermotolerant host.

In total, no ASVs were indicative of thermotolerance across all *Symbiodinium* species. Rather, we found ASVs that were differentially abundant by temperature differed by *Symbiodinium* species. These differences may be explained by functional redundancy of thermotolerance within and between bacterial communities (Stilianos et al., 2018). While *Symbiodinium*-associated bacterial communities may differ substantially, even among similar *Symbiodinium* species, these communities are likely to converge on key functional traits. For example, members of Rhodobacteraceae have generally been found to associate with other algae and provide vitamins and other amino acids to their hosts (Rosales et al., 2020), and play important roles in marine sulfur and nitrogen cycles (Chen et al., 2011). In microbial communities, many species fulfilling similar functions often coexist (Louca et al., 2018). Since all our cultures contained diverse members of Rhodobacteraceae, functional stability with stress could theoretically be maintained despite each culture containing different representatives of this family. For example, soil microbial communities showed high functional stability in long-term drought (Yuste et al., 2014). Future research on the relationship between phylogenetic diversity and functional stability may evidence similar consistencies in functional stability of dinoflagellate microbial communities under long-term increases in heat stress driven by climate change.

Additionally, high rates of horizontal gene transfer in long-term co-cultures could result in changes in the functional roles of different taxa, including those that may affect *Symbiodinium* thermotolerance (Cairns et al., 2018). This could have led to the development of similar thermotolerance-related functions in different taxa, leading to dissimilar patterns in bacterial community change with heat stress across cultures. While shifts in the functional roles of different community members have not been well-documented in long-term co-cultures, research on the human gut microbiome suggests that high rates of horizontal gene transfer and selective pressure have promoted functional redundancy in gut microbial communities despite wide diversity in microbiome community composition (Tian et al., 2020). More similar to our study system, biofilm bacterial communities of the kelp *Ecklonia radiata* commonly had horizontal gene transfers between bacterial members of the same class or order, and these transfers often included genes involved in stress response (Song et al., 2021). More research on Symbiodiniaceae is necessary to determine whether functional redundancy may be important for thermotolerance rather than any one bacterial taxa in particular.

CONCLUSION

In conclusion, we show that closely related species of *Symbiodinium* cultures with similar thermotolerance have specific bacterial assemblages that remain relatively stable under high temperature conditions. While differences in relative abundance of key bacterial genera were not consistent across *Symbiodinium* species, significant differences in certain genera by temperature treatment within *Symbiodinium* species suggest functional redundancy within bacterial communities. Future studies should focus on isolating symbiotic bacteria and assessing their interactions with *Symbiodinium* and with each other. Experimenting with axenic *Symbiodinium* isolates in culture with specific bacterial isolates can allow us to determine the effects of the specific microbes, which species might be essential, and whether those species can influence the physiology of *Symbiodinium*. Since multiple strains of *Symbiodinium* can be cultured and their physiology is well-known, this phylogenetic group can aid in characterizing the role of microbes in functional and physiological diversity of their symbionts. Additionally, metagenomes of bacteria associated with *Symbiodinium* can inform about the metabolic capabilities to hypothesize functional roles and if the interactions between these bacteria and *Symbiodinium* are mutualistic, neutral or pathogenic.

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: https://www.ncbi.nlm.nih.gov/, PRJNA758393.

AUTHOR CONTRIBUTIONS

ED-A and MMe contributed to conception and design of the study. All authors contributed to the data analysis. AHO, TR, and FP performed the statistical analysis. ED-A wrote the first draft of the manuscript. All authors wrote sections of the manuscript. All authors contributed to manuscript revision, read, 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/fevo.2022.764086/full#supplementary-material

Supplementary Figure 1 | Cultures were grown on top of a glass shelf under lights facing up to prevent shading. **(A)** Cultures in flasks were acclimated for 2 months at 100 μM quanta m^2 s^{-1} in a 12:12 hr/light:dark cycle. Cell densities were adjusted to 1×10^5 cells/ml before starting experiments. **(B)** Cultures were moved to their experimental conditions with the previous light and circadian rhythm into their respective temperatures in glass tubes. Cultures started at 1×10^5 cells/ml, F_v/F_m was measured for $n = 5$ on day 27, cells for DNA extraction were collected for $n = 3$ on day 27.

Supplementary Figure 2 | Shared number of genera present in each species core microbiome of *Symbiodinium* under two temperatures (26 and 32°C). Each treatment had three replicates per *Symbiodinium* species per temperature.

Supplementary Figure 3 | Shared number of genera present in each species core microbiome of *Symbiodinium* **(A–C)** over two temperatures (26 and 32°C). The shared core microbiome at 26 and 32°C, independent of *Symbiodinium* species is also represented **(D)**. To be considered part of the core microbiome at each temperature, the genera must be present in all samples ($n = 18$).

Supplementary Figure 4 | Heatmap of relative abundances of bacteria. At the top, a Euclidean distance cladogram shows samples grouped by *Symbiodinium* species.

Supplementary Table 1 | Differential abundances calculated with DESeq2.

Supplementary Table 2 | Differential abundances calculated with SIMPER.

Supplementary Table 3 | Permanova comparisons per species **(A)** and per treatments **(B)**.

Supplementary Table 4 | Number of observed features per sample after alpha rarefaction. Reads were rarefied using the Qiime2 alpha-diversity function. Rarefaction was performed to a maximum depth of 30,000 reads over 20 steps. Feature tables were generated over 10 iterations. The variability in observed features for each iteration are presented as box plots for each sampling depth.

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