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Symbiont community diversity is more variable in corals that respond poorly to stress

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Abstract

Coral reefs are declining globally as climate change and local water quality press environmental conditions beyond the physiological tolerances of holobionts—the collective of the host and its microbial symbionts. To assess the relationship between symbiont composition and holobiont stress tolerance, community diversity metrics were quantified for dinoflagellate endosymbionts (Family: Symbiodiniaceae) from eight *Acropora millepora* genets that thrived under or responded poorly to various stressors. These eight selected genets represent the upper and lower tails of the response distribution of 40 coral genets that were exposed to four stress treatments (and control conditions) in a 10-day experiment. Specifically, four 'best performer' coral genets were analyzed at the end of the experiment because they survived high temperature, high pCO_2, bacterial exposure, or combined stressors, whereas four 'worst performer' genets were characterized because they experienced substantial mortality under these stressors. At the end of the experiment, seven of eight coral genets mainly hosted *Cladocopium* symbionts, whereas the eighth genet was dominated by both *Cladocopium* and *Durusdinium* symbionts. Symbiodiniaceae alpha and beta diversity were higher in worst performing genets than in best performing genets. Symbiont communities in worst performers also differed more after stress exposure relative to their controls (based on normalized proportional differences in beta diversity), than did best performers. A generalized joint attribute model estimated the influence of host genet and treatment on Symbiodiniaceae community composition and identified strong associations among particular symbionts and host genet performance, as well as weaker associations with treatment. Although dominant symbiont physiology and function contribute to host performance, these findings emphasize the importance of symbiont community diversity and stochasticity as components of host performance. Our findings also suggest that symbiont community diversity metrics may function as indicators of resilience and have potential applications in diverse disciplines from climate change adaptation to agriculture and medicine.

KEYWORDS

Acropora millepora, alpha diversity, beta diversity, climate change, coral, generalized joint attribute model (GJAM), Symbiodiniaceae, *Vibrio owensii*

1 | INTRODUCTION

Coral reefs are undergoing rapid declines in health on a global scale following increased exposure to climate change stressors, such as warming sea surface temperatures and increased pCO_2, and conditions that favor pathogens (Hughes et al., 2017, 2018; Maynard et al., 2015; Zaneveld et al., 2016). Microbial symbionts can influence the capacity of their reef-building coral hosts to acclimatize and adapt to environmental stressors. In particular, dinoflagellates in the Family Symbiodiniaceae (including genera *Symbiodinium*, *Breviolum*, *Cladocopium*, and *Durusdinium*; LaJeunesse et al., 2018) reside in the tissues of corals, giant clams, and other marine invertebrates, and have been empirically shown to influence the ability of corals to survive stress events (e.g., Baker, 2001, 2004; Cunning & Baker, 2013; Kenkel & Bay, 2018; LaJeunesse, Smith, Finney, & Oxenford, 2009; Manzello et al., 2019; Rouze, Lecellier, Saulnier, & Berteaux-Lecellier, 2016).

Some Symbiodiniaceae species, such as *Durusdinium trenchii*, are more likely to remain associated with hosts and/or to retain photosynthetic function during acute temperature anomalies or extremes (Manzello et al., 2019; Silverstein, Cunning, & Baker, 2015; but see Silverstein, Cunning, & Baker, 2017). For example, in the Pacific coral, *Acropora millepora*, a *Durusdinium* species minimized bleaching (a diminished host health state characterized by loss of Symbiodiniaceae *en masse*) and increased host survival of acute thermal anomalies (Bay, Doyle, Logan, & Berkelmans, 2016; Berkelmans & van Oppen, 2006; Jones, Berkelmans, van Oppen, Mieog, & Sinclair, 2008; Mieog et al., 2009). Despite the stress tolerance potentially conferred by some *Durusdinium* species, symbionts in this genus typically support lower host growth rates than symbionts in *Cladocopium*. Therefore, harboring dominant communities of *Durusdinium* symbionts might present a trade-off for corals, in which increased temperature tolerance comes at the expense of growth, especially in cooler environments (Cantin, van Oppen, Willis, Mieog, & Negri, 2009; Jones & Berkelmans, 2010; Little, van Oppen, & Willis, 2004).

Other types of environmental stress can also influence associations among corals and their dinoflagellate symbionts. Elevated pCO_2 has been shown to enhance growth and photosynthetic capacity in certain Symbiodiniaceae species (Brading et al., 2011), to have no effect on other symbiont species (Brading et al., 2011), and to result in the loss of symbionts and their photosynthetic function in some coral hosts (Kaniewska et al., 2012). Thus, the impact of ocean acidification stress on Symbiodiniaceae likely varies among taxa and environmental context and needs further investigation. Interactions among Symbiodiniaceae and bacterial pathogens (e.g., *Vibrio* spp.) have also been previously correlated with coral (Rouze et al., 2016) or symbiont health (Hauff et al., 2014). Specifically, *Acropora cytherea* colonies harboring symbionts in *Durusdinium* were more resistant to infection with *Vibrio* spp. than conspecifics harboring symbionts in *Symbiodinium* (Rouze et al., 2016). However, the relationship between symbiont identity and relative resistance to *Vibrio* infection is unexplored for most Symbiodiniaceae species.

To date, studies have mainly focused on the contribution of individual symbiont species to holobiont stress responses. This approach can be described by the 'selection effect', which assumes that the independent function of dominant species drives overall community function (Loreau, 1998; Loreau et al., 2001; Tilman et al., 1997). However, symbiont community composition may also influence holobiont stress tolerance via the 'complementarity effect' (Loreau, 1998; Tilman et al., 1997). This effect describes systems in which the function of symbiont species is dependent on facilitative or competitive interactions arising from their community context (Fox, 2005). If symbiont complementarity effects are driving corals' responses, then community composition or diversity metrics may provide important insights into variation in holobiont physiology and fitness. Community diversity metrics have already shown promise (e.g., based on bacterial communities: Zaneveld, McMinds, & Vega Thurber, 2017) for identifying systems where low abundance symbionts contribute (even if ephemerally, e.g., for Symbiodiniaceae: LaJeunesse et al., 2009; Lee et al., 2016) to overall holobiont stress responses. One of the first studies to quantify how dinoflagellate symbiont community attributes are associated with host performance (Kenkel & Bay, 2018) found a decreasing trend in symbiont cooperation, in terms of autotrophically derived carbon shared with hosts, under heat stress within coral species that harbored more diverse Symbiodiniaceae communities. Another study found that coral juveniles that harbored more diverse and variable Symbiodiniaceae communities exhibited lower survival than juveniles with less diverse communities (Quigley, Willis, & Bay, 2016). Most recently, McIlroy, Cunning, Baker, and Coffroth (2019) documented that priority effects influenced the outcome of competitive interactions among Symbiodiniaceae under some environmental conditions (i.e., low light), with implications for the performance of octocoral holobionts under stress. These studies support the hypothesis that symbiont community composition can track or influence a holobiont's stress tolerance under changing conditions.

High-throughput amplicon, genome, and transcriptome sequencing can support comprehensive investigations of symbiont community properties and concepts such as the complementarity effect for host–microbe symbioses. A variety of markers, including the highly variable Symbiodiniaceae internal transcribed spacer-2 (ITS-2) region of rDNA, have been applied in isolation or combination to assess the diversity of Symbiodiniaceae present in hosts and make it possible to identify symbiont ITS-2 types (and some formally described Symbiodiniaceae species) occurring at abundances as low as 0.1% (Quigley et al., 2014). HTS of the Symbiodiniaceae ITS-2 region is useful for investigating symbiont contributions to host health and stress response at a fine scale (i.e., at the level of sequence variants, including those present at low abundances); this approach has revealed novel Symbiodiniaceae variants, host associations, and/or distribution patterns (Brener-Raffalli et al., 2018; Cunning, Gates, & Edmunds, 2017; Green, Davies, Matz, & Medina, 2014; Putnam, Stat, Pochon, & Gates, 2012; Quigley, Bay, & Willis, 2017; Quigley et al., 2014; Quigley, Willis, & Bay, 2016, 2017; Ziegler et al., 2017; Ziegler, Eguiluz, Duarte, & Voolstra, 2018; Ziegler, Stone, Colman, Takacs-Vesbach, & Shepherd, 2018). For example, variation in low abundance

symbionts (defined in Green et al., 2014 as <10% of total community) has been documented across reefs separated by as little as 19 km (e.g., Green et al., 2014; van Oppen et al., 2018). It has been hypothesized that these differences in Symbiodiniaceae sequence variant distribution are associated with fine scale environmental variation (Brener-Raffalli et al., 2018; Cunning et al., 2017; Quigley et al., 2014; Quigley, Warner, Bay, & Willis, 2018). However, no HTS studies have examined how symbiont diversity metrics (i.e., alpha or beta diversity) or community composition change among host genets with high versus low stress tolerance, or among host genets exposed to different stressors.

Here, we applied a HTS approach to determine whether particular Symbiodiniaceae types or community characteristics were associated with host colony stress tolerance and/or distinct environmental stressors, using samples collected from eight *A. millepora* coral colonies (hereafter called genets) at the end of a 10-day stress experiment performed by Wright et al. (2019). The eight genets investigated represent the upper and lower tails (i.e., four best performers and four worst performers) in a spectrum of responses exhibited by a total of 40 coral genets that Wright et al. (2019) exposed to a climate stressor (high temperature or pCO_2), a pathogenic bacteria (*Vibrio owensii*), all of these stressors combined, and control conditions. Symbiodiniaceae community diversity metrics and ITS-2 type (or species, where possible) identity were assessed to elucidate symbiont attributes associated with host performance. We hypothesized that (a) control fragments of best and worst performing host genets would differ significantly in symbiont alpha and beta diversity; (b) symbiont communities in fragments experiencing stress treatments would exhibit higher dissimilarity than symbiont communities in associated control fragments in both best and worst performing genets; and (c) specific symbiont ITS-2 types would be differentially associated with best and worst performing host genets.

2 | MATERIALS AND METHODS

2.1 | Study site, experimental design, and sample collection

Wright et al. (2019) conducted a tank-based experiment to determine the capacity of the stony coral, *A. millepora*, to tolerate various environmental stressors (Table 1); this experiment generated the samples analyzed in this study. Briefly, Wright et al. (2019) collected 40 colonies (genets) of *A. millepora* between October 1 and 8, 2014 from multiple Great Barrier Reef sites. The eight genets used in this study originated from the following three reefs: Pandora Island (18°48′45″S, 146°25′59.16″E, Genets Worst-27, Worst-31, Worst-34), Rib Reef (18°28′53.4″S, 146°52′24.96″E, Genets Best-12, Best-20, Best-38), and Davies Reef lagoon (18°30′3.96″S, 147°22′48″E, Genets Best-4, Worst-2; Figure S1). Genets were confirmed to be genetically unique by Wright et al. (2019) using 2b-RAD genotyping (Wang, Meyer, McKay, & Matz, 2012). After collection, genets were transferred to flow-through seawater tanks at the National Sea Simulator at the Australian Institute of Marine Science (AIMS). Each genet was fragmented into single branches, acclimatized to a common garden condition for several months (82–128 days), and then placed into 50-L tanks where stressors (elevated temperature, elevated pCO_2, and/or bacterial addition), as well as ambient conditions, were applied among five replicate tanks as described in Wright et al. (2019). Fragments were inspected and photographed daily to quantify coral bleaching and lesion progression; any fragments exhibiting significant tissue sloughing were removed from aquaria and snap frozen in liquid nitrogen with the time of death recorded. Fragments that lost all tissues (i.e., died) in between daily examinations were not snap frozen and thus could not be included in this study. Treatments were stopped after 10 days, because at this point, significant mortality (~21% of all experimental fragments) had occurred. On the 11th day, surviving coral fragments were photographed and snap frozen in liquid nitrogen. Initial samples were not collected immediately prior to the start of Wright et al.'s (2019) experiment, but unaffected fragments of each genet from control tanks were sampled at the end of their experiment. Additional experimental details can be found in Wright et al. (2019).

TABLE 1 Overview of experimental treatments

Stress type	Temperature (°C)	pCO_2 (ppm/pH)	Bacterial addition (*Vibrio owensi* exposure)
Control	27	400/8.0	Nothing added
Bacteria	27	400/8.0	6 hr bath at 10^6 cells/ml
Heat	30	400/8.0	Nothing added
pCO_2	27	700/7.8	Nothing added
Combined	30	700/7.8	6 hr bath at 10^6 cells/ml

Note: Wright et al. (2019) exposed 40 genets of *Acropora millepora* to control, bacterial addition, elevated temperature, elevated pCO_2, or combined bacteria/heat/pCO_2 stressors. On a daily basis, any fragments exhibiting significant tissue sloughing were removed from aquaria and processed for downstream analyses. After 10 days, treatments were halted and on the 11th day, all remaining fragments were sampled by Wright et al.

In this study, we analyze the Symbiodiniaceae communities from three fragments per treatment condition (n = 120) from four best performing (Best-4, Best-12, Best-20, and Best-38) and four worst performing coral genets (Worst-2, Worst-27, Worst-31, and Worst-34) sampled by Wright et al. (2019). The best performing genets (red lines in Figure 1) had the highest survival of the 40 genets tested by Wright et al. (2019). We chose four genets with low survival (blue lines in Figure 1) as representatives of the 'worst performer' group. The selected worst performing genets displayed high mortality in all treatments, as well as symbiont loss under elevated temperature and reduced calcification under increased pCO_2 (Wright et al., 2019). Six other genets (lowest grey lines in Figure 1) in Wright et al. (2019) exhibited extremely low survival during their experiment and could not be included for Symbiodiniaceae diversity analyses in this study because insufficient tissue of these genets remained for molecular analysis.

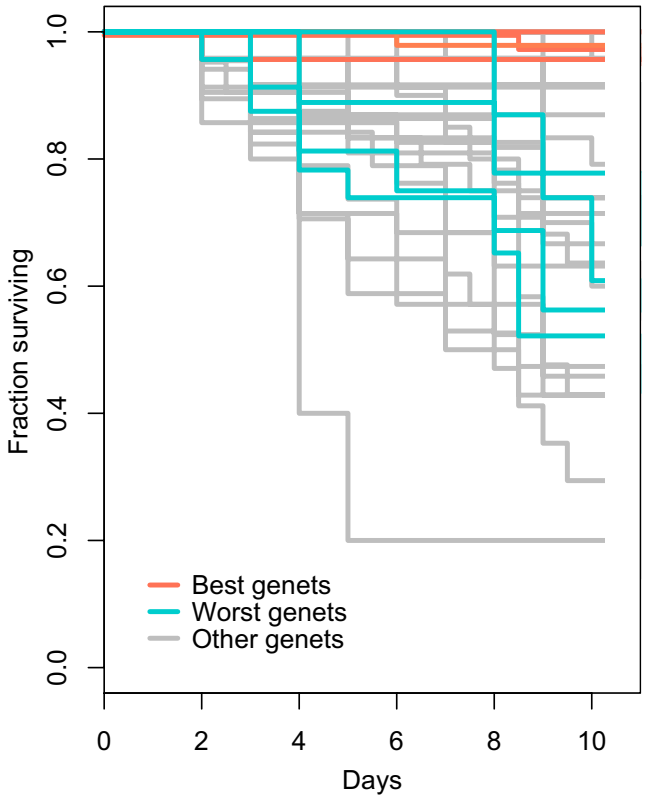

FIGURE 1 Survival of genets among treatments over a 10-day stress experiment by Wright et al. (2019). Survival was calculated as a fraction of five replicate fragments per treatment per genet in the Wright et al. (2019) experiment. The experimental treatments were conducted for 10 days, and surviving corals were preserved for downstream analyses on the 11th day. Best performing genets (Best-4, Best 38, Best-60, and Best-62) are highlighted in red, whereas representatives of the worst performing genets selected for this study (Worst-2, Worst-27, Worst-31, and Worst-34) are highlighted in blue. Three fragments per treatment per genet that survived the experimental exposure were randomly selected for analysis in this study. The 32 grey lines represent other genets from Wright et al. (2019) that were not included in this study [Colour figure can be viewed at wileyonlinelibrary.com]

2.2 | Sample DNA extraction and sequencing

In this study, we extracted coral holobiont DNA using Wayne's Method (Lundgren, Vera, Peplow, Manel, & van Oppen, 2013) from tissue slurry prepared by Wright et al. (2019). First, each sample was centrifuged and the ethanol was removed. The pellet was transferred to a SDS buffer and then precipitated with KOAc, followed by a series of ethanol washes. Pellets were resuspended in 30 µl of 1× tris-acetate-EDTA and stored at −20°C. DNA concentrations were determined through Quant-iT PicoGreen dsDNA assays; 96 samples out of 102 had sufficient DNA concentrations for HTS sequencing. The ITS-2 region of Symbiodiniaceae rDNA was amplified using symbiont-specific primers: SYM_VAR_5.8SII (5′-GAATTGCAGAACTCCGTGAACC-3′) and SYM_VAR_REV (5′-CGGGTTCWCTTGTYTGACTTCATGC-3′). The target amplicon was approximately 234–266 bp (Hume et al., 2018). The PCR reaction contained 5 µl of DNA (5 ng/µl), 2.5 µl of SYM_VAR_5.8SII + MiSeq Adapter (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GAATTGCAGAACTCCGTGAACC-3′; 2 µM), 2.5 µl of SYM_VAR_REV + MiSeq Adapter (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CGGGTTCWCTTGTYTGACTTCATGC-3′; 2 µM), 12.5µl 2× KAPA HiFi HotStart ReadyMix (https://rochesequencingstore.com/catalog/kapa-hifi-hotstart-readymix/), and 2.5 µl of molecular grade water for a total reaction volume of 25 µl. PCR cycles were as follows: 95°C for 3 min, 15 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and 72°C for 4 min.

PCR clean-up was completed using Agencourt AMPure XP Magnetic Beads. Illumina indexing primers were added to 50 µl of purified PCR product, and a new PCR was run to incorporate unique barcodes for each sample. The PCR reaction contained 5 µl of cleaned PCR product, 5 µl of Illumina Indexed Primer 1 (i5), 5 µl of Illumina Indexed Primer 2 (i7), 25 µl 2× KAPA HiFi HotStart, and 10 µl molecular grade water for a total reaction volume of 50 µl. PCR cycles were as follows: 95°C for 3 min, 20 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and lastly 72°C for 4 min. The resulting PCR product was purified with Agencourt AMPure XP Magnetic Beads. Samples were quantified via qPCR using the KAPA library quantification kit and normalized and pooled in equal molar amounts. Pooled samples were sequenced on the Illumina MiSeq platform using a PE300 run with 25% PhiX at the Georgia Genomics and Bioinformatics Core (University of Georgia, Athens, GA).

2.3 | Bioinformatic processing and statistical analyses

Illumina's real-time analysis was run during sequencing using the default settings to remove clusters with the least reliable data. Demultiplexed fastq files were generated with Illumina's BaseSpaceFS (version 1.5.964) and reads were processed in RStudio (version 1.1.456) through the DADA2 pipeline (version 1.11.0; Callahan et al., 2016) with modifications for the Symbiodiniaceae ITS-2 region. The DADA2 pipeline generated a table of amplicon sequence variants (ASVs). Samples with fewer than 10,000 reads (n = 2) were removed from the dataset; 94 samples remained.

The significance of individual base pair differences within Symbiodiniaceae ITS-2 sequence data can be ambiguous because of the multi-copy nature of ITS-2 (e.g., Correa & Baker, 2009; Stat et al., 2012; Stat et al., 2011), yet comparisons of species or types identified using the ITS-2 among treatments (Cunning, Silverstein, & Baker, 2015) or environmental compartments can be informative (Cunning, Yost, Guarinello, Putnam, & Gates, 2015; Ziegler, Stone, et al., 2018). To identify biologically relevant entities from Symbiodiniaceae ITS-2 sequence data, we applied a post-DADA2 clustering curation using the LULU pipeline (Frøslev et al., 2017). LULU uses co-occurrence patterns and sequence similarity to collapse ITS-2 sequences that likely represent intragenomic variants; we applied thresholds of 95% and 84%, respectively, in this

study. There is precedent for the application of this approach in the coral–Symbiodiniaceae system (Kenkel & Bay, 2018; Quigley, Willis, & Kenkel, 2019). The LULU algorithm has also been applied to the analysis of fungal ITS-2 sequence datasets (Anslan et al., 2018). Symbiodiniaceae ITS-2 types were then assigned based on BLAST results to a local Symbiodiniaceae ITS-2 database (Cunning et al., 2017). In this study, for Symbiodiniaceae, we use the term 'type' in reference to previously documented ITS-2 sequences that are not currently included in any existing Symbiodiniaceae species description, or that are not diagnostic of a single formally described Symbiodiniaceae species. Where formally described Symbiodiniaceae species names can be unambiguously related to an ITS-2 type, we apply the formal species nomenclature.

Alpha diversity was calculated using Shannon's Diversity (H') index and Simpson's Diversity (1-D) index. Shapiro–Wilk tests were used to test for normality and t tests were used to test for significant differences between best and worst performers in control (~ambient) conditions when the assumption of normality was met. Wilcoxon rank sum tests were used when samples did not have a normal distribution. A regularized log transformation was applied to raw count data to account for variation in sequencing depth using DESeq2 (version 1.22.1; Love, Huber, & Anders, 2014). After this transformation, the ordinate function in phyloseq (McMurdie & Holmes, 2013) was used to generate a non-metric multidimensional scaling plot using Bray–Curtis distances to visualize differences in Symbiodiniaceae communities. To determine whether Symbiodiniaceae community composition differed significantly between control fragments of best and worst performing genets, we first tested for homogeneity in group (genets and treatments) dispersion using 'betadisper' in the 'vegan' R package (version 2.5.6; Oksanen et al., 2019). Multivariate analyses of the variance in composition are sensitive to heterogeneity and because this was a feature of the present data, the least sensitive permutational multivariate analysis of the variance ('adonis' in 'vegan'; Anderson & Walsh, 2013) was implemented.

Next, the magnitude of differences in Symbiodiniaceae beta diversity among treatments in best versus worst performers or among treatments within genets was assessed. To accomplish this, Bray–Curtis between-group distance values for stressed fragments were normalized to the average of their respective control fragments (i.e., symbiont community composition under ambient conditions). Wilcoxon rank sum tests were used to identify significant differences in normalized community dissimilarity overall between best versus worst performing genets, and between control and stress treatments for each genet.

To investigate the strength of links among *A. millepora* genet, stress type, and Symbiodiniaceae community composition at a regional scale (among genets from all three reefs), we fitted a generalized joint attribute model (GJAM) in R to the LULU-curated dataset, using 10,000 iterations and 500 burn-in steps (Clark, Nemergut, Seyednasrollah, Turner, & Zhang, 2017). This joint probabilistic model takes into account co-dependence among ASVs and performs well in the presence of zeros. We used the regional GJAM model to inversely predict *A. millepora* genet and experimental treatment given a particular Symbiodiniaceae community. Specifically, for each Symbiodiniaceae sample (y^*), we used a Monte Carlo integration to inversely predict the covariates (x^*):

$$[x^*|y^*] = \int [x^*|\theta, y^*][\theta|x, y]\, d\theta$$

where θ is the non-informative prior distribution.

We also estimated the Symbiodiniaceae community sensitivity to host genets and treatment. After fitting the regional GJAM model, we obtained a predictors (genet and treatment)-by-ASV matrix of coefficients $\boldsymbol{B}$ that holds all predictor-by-ASV responses. Elements of $\boldsymbol{B}$ are the individual sensitivities of each symbiont ASV to each predictor. As part of the fitting process, we also estimated an ASV-by-ASV covariance matrix Σ that holds residual indirect relationships between symbiont ASVs. Sensitivity across the entire Symbiodiniaceae community is then:

$$f = \mathrm{diag}(B\Sigma^{-1}B)$$

Finally, since Rib Reef genets were all best performers and Pandora Island genets were all worst performers, additional GJAM models were fitted to genets originating from these locations, respectively, to test for treatment effects on more local scales. The model parameters and run conditions for Pandora Island and Rib Reef subsets were identical to the regional GJAM model.

3 | RESULTS

For the eight *A. millepora* genets analyzed in this study, the highest coral mortality among replicate fragments was observed in the pCO_2 and combined stressor treatments (black and white hatched bars in Figure 2), whereas none of the control fragments experienced mortality. The Worst-2 genet experienced the most mortality (n = 8 of 15 fragments) out of the genets in this study, followed by Worst-34 (n = 4), and then Worst-27 and Worst-31 (n = 3 each; Figure 2).

3.1 | Symbiodiniaceae types identified from *A. millepora* genets

Of the 102 *A. millepora* fragments that survived the experimental conditions, 96 of these were able to be amplicon sequenced. A total of 29,466,474 raw sequence reads were recovered from these samples; the raw amplicon sequencing dataset is available at NCBI's SRA (sequence read archive; accession #PRJNA596498). After processing through the DADA2 pipeline, paired reads per sample ranged from 591,680 to 1,069. Two samples that contained <10,000 reads (one Best-4 Control and one Best-4 Heat sample) were removed, leaving a total of 9,979,596 paired reads (Table S1) from which 262 ASVs were

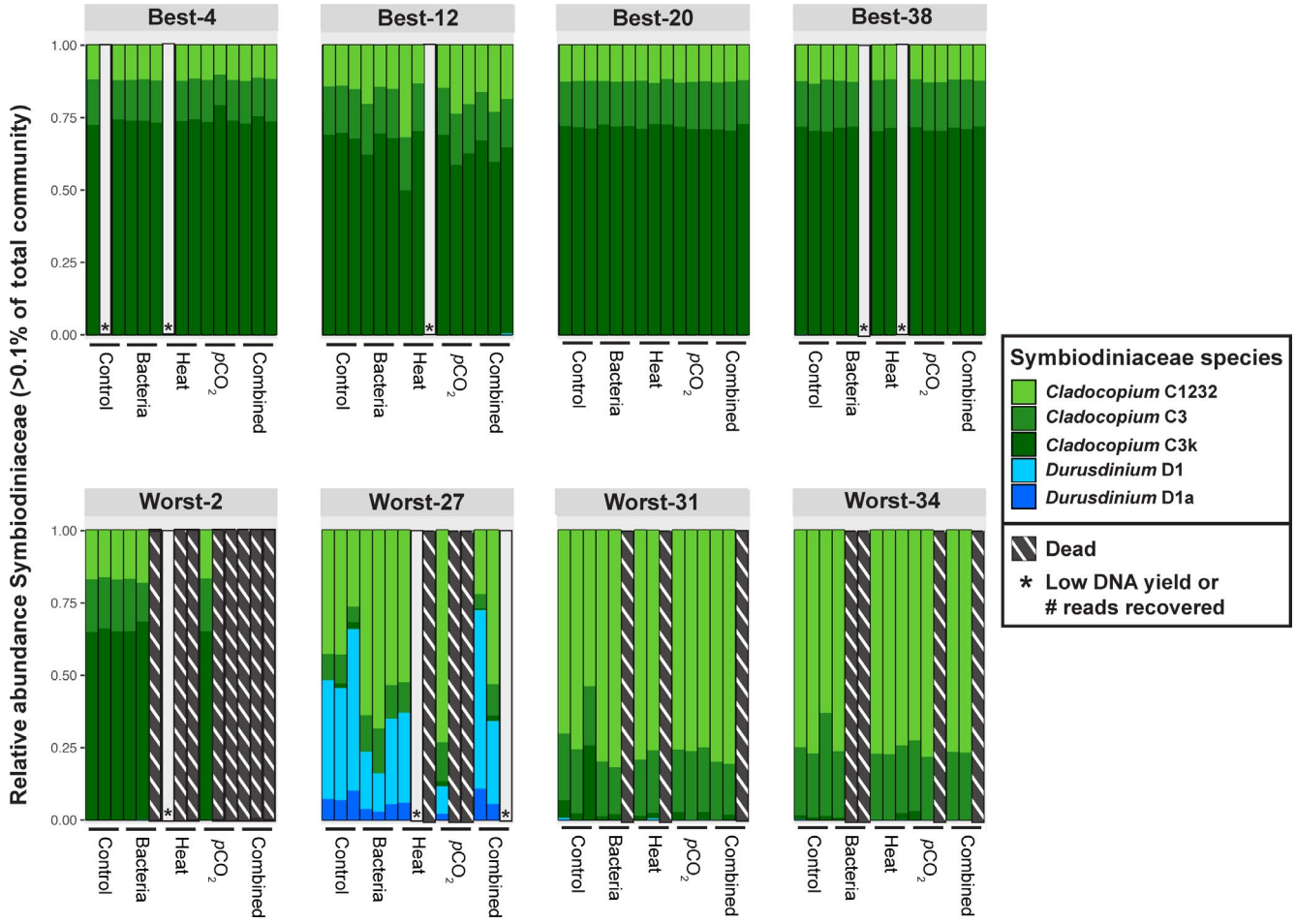

FIGURE 2 Relative abundance of the dominant (>0.1% of total community) Symbiodiniaceae ITS-2 types in *Acropora millepora* genets exposed to various stress treatments. Each vertical bar represents an individual *A. millepora* fragment of a given genet [Colour figure can be viewed at wileyonlinelibrary.com]

identified. The LULU pipeline resolved 12 ASVs from this dataset. One of these ASVs (represented by 12 reads total among two samples) was discarded because it did not produce any sequence similarities during the taxonomic assignment process (see Table S3 for the curated ASV table). Some of the remaining 11 ASVs were subsequently assigned to the same Symbiodiniaceae types (Table S4). Type assignment post-LULU ultimately identified four Symbiodiniaceae genera from the dataset, which contained a total of eight ITS-2 types: *Symbiodinium* A101, *Breviolum minutum*, *Cladocopium* C1232, *Cladocopium* C3, *Cladocopium* C3k, *Durusdinium* D1, *Durusdinium* D1a, and *Durusdinium* D2 (Table 2). To confirm that our LULU ASV curation was sufficiently conservative, the algorithm was also run with 70% co-occurrence and 84% sequence similarity thresholds, which yielded comparable results (data not shown). Fragments of Best-4 and Worst-31 each harbored the highest number of Symbiodiniaceae types (seven) observed in a genet, whereas genets Best-38 and Worst-2 each harbored the fewest (four) Symbiodiniaceae types (Table 2). All individual coral fragments (Table S2) analyzed in this study harbored more than one Symbiodiniaceae type (see Table 2 for all detected symbionts; Figure 2 for species comprising >0.1% of total Symbiodiniaceae community).

3.2 | Symbiodiniaceae community characteristics in best and worst performing coral genets under control conditions

To explore how Symbiodiniaceae community characteristics differed in best versus worst performing genets selected from Wright et al. (2019), we first examined metrics of symbiont diversity in fragments that experienced ambient (control) conditions. Symbiodiniaceae alpha diversity in control fragments varied categorically by genet performance (Figure 3a); symbiont communities of worst performer control fragments were significantly more diverse by Shannon's Diversity (H') Index estimates (worst performer genets: 1.21, best performer genets: 0.88, Wilcoxon rank sum test; $p < .001$) and Simpson's (1-D) Diversity estimates (worst performer genets: 0.65, best performer genets: 0.47, Wilcoxon rank sum test, $p < .001$). *A. millepora* Worst-27 was the most diverse genet ($H' = 1.43$; 1-$D = 0.70$), whereas Best-4 was the least diverse genet ($H' = 0.83$; 1-$D = 0.45$; Figure 3a).

A permutation test for homogeneity of multivariate dispersions also indicated that variances, a proxy for beta diversity, were significantly lower among Symbiodiniaceae communities associated

TABLE 2 Symbiodiniaceae amplicon sequence reads analyzed and Symbiodiniaceae ITS-2 types resolved from *Acropora millepora* fragments among genets and stress treatments

Host genet or treatment	#Reads	Symbiodiniaceae ITS-2 types
Best-4	1,615,553	*Breviolum minutum*, C3, C3k, C1232, D1, D1a, D2
Best-12	1,203,848	*B. minutum*, C3, C3k, C1232, D1
Best-20	2,292,477	C3, C3k, C1232, D1, D1a, D2
Best-38	1,506,374	C3, C3k, C1232, D1
Worst-2	660,409	C3, C3k, C1232, D1
Worst-27	667,066	C3, C3k, C1232, D1, D1a
Worst-31	749,608	*B. minutum*, C3, C3k, C1232, D1, D1a, D2
Worst-34	1,284,261	A101, *B. minutum*, C3, C3k, C1232, D1
Control	2,296,857	A101, *B. minutum*, C3, C3k, C1232, D1, D1a
Bacteria	1,754,156	*B. minutum*, C3, C3k, C1232, D1, D1a
Heat	1,898,163	*B. minutum*, C3, C3k, C1232, D1, D1a, D2
pCO_2	2,064,763	*B. minutum*, C3, C3k, C1232, D1, D1a, D2
Combined	1,965,657	C3, C3k, C1232, D1, D1a

Note: The 'Symbiodiniaceae ITS-2 types' column lists the ITS-2 types (or species) cumulatively detected across replicate fragments of a host genet or stress treatment. A101 = *Symbiodinium* A101 (NCBI accession #AF427468), C3 = *Cladocopium* C3 (accession #AB778606), C3k = *Cladocopium* C3k (NCBI accession #AY589737), C1232 = *Cladocopium* C1232 (accession #EU118163.1), D1 = *Durusdinium* D1 (accession #AF334660), D1a = *Durusdinium* D1a (accession #JN558078), D2 = *Durusdinium* D2 (accession #AY686649).

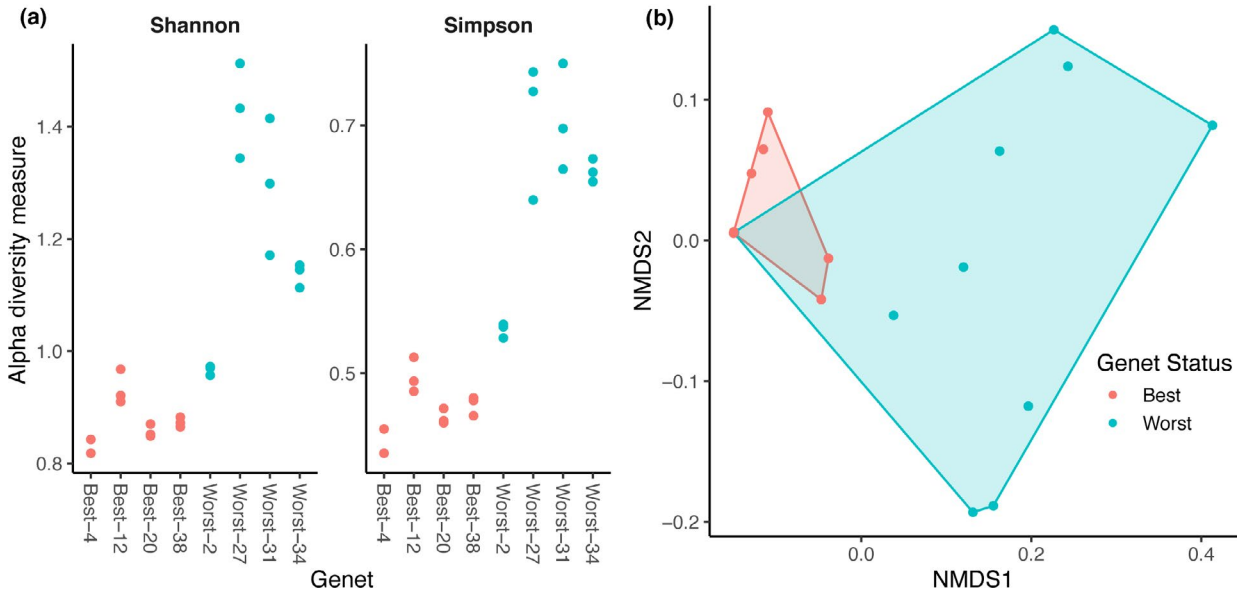

FIGURE 3 Symbiodiniaceae community diversity characteristics in control fragments of best and worst performing *Acropora millepora* genets. (a) Symbiodiniaceae alpha diversity; Shannon's Diversity Index results are reported as *H′*, Simpson's Diversity Index results are reported as 1-*D*. Symbiont communities of worst performer control fragments were significantly more diverse than best performer control fragments by Shannon's Diversity (*H′*) Index estimates (Wilcoxon rank sum test $p < .001$) and Simpson's (1-*D*) Diversity estimates (Wilcoxon rank sum test, $p < .001$). (b) Non-metric multidimensional scaling plot of control Symbiodiniaceae communities (depicted as individual dots) using Bray–Curtis distances. Spread of dots visualizes the difference in variance (~beta diversity) among best and worst performing *A. millepora* genets (betadisperser, $p < .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

with control fragments of best performing *A. millepora* genets (smaller red polygon; Figure 3b), than among communities associated with control fragments of worst performing genets (betadisperser, $p < .001$, larger blue polygon, Figure 3b). Permutational multivariate analysis of variance supported that Symbiodiniaceae communities differed significantly between best and worst performing *A. millepora* genets (Adonis with Bray–Curtis distance; $p = .003$; Figure 3b).

3.3 | Impact of stressors on Symbiodiniaceae beta diversity

Since Symbiodiniaceae beta diversity was significantly different in best versus worst performing genets under ambient (control) conditions, differences in symbiont beta diversity associated with stress treatments could only be compared through normalization to controls within each genet. This produced a metric of the relative difference in beta diversity distance values between the stress-treated fragments and the control fragments for each genet, which could be directly compared among best versus worst performers. This metric showed that fragments experiencing stress treatments exhibited greater relative differences in beta diversity in worst performing genets, compared to best performing genets (Wilcoxon test; $p < .001$; Figure 4a). Specifically, the dissimilarity of symbiont communities in bacterial treatments and heat treatments was significantly higher than that of control communities in worst performing genets (Pairwise Wilcoxon test; $p = .032$ and $p = .048$, respectively; Figure 4a). There were no significant differences in community dissimilarity in best performing genets (Figure 4a). Within the Worst-34 genet, the symbiont community exposed to combined stressors was significantly more dissimilar than control Worst-34 fragments (*t* test, $p < .001$; Figure 4b). Within the Worst-31 genet, the symbiont community exposed to the bacterial treatment bordered on being significantly more dissimilar than its control (*t* test, $p = .055$; Figure 4b). In worst performing genets, there was a general trend of increased dissimilarity in bacteria and/or heat treatments relative to controls (Figure 4b). In best performing genets, there were no general trends in dissimilarity apparent in the stress treatments relative to controls (Figure 4b).

3.4 | Symbiodiniaceae community sensitivity to treatment condition and genet at regional and reef scales

Since exposure to stress treatments was associated with increased Symbiodiniaceae beta diversity in the eight genets tested, we next investigated whether Symbiodiniaceae communities responded differently (i.e., were sensitive) to treatment condition (and host genet) using the GJAM model. When all three reef locations were

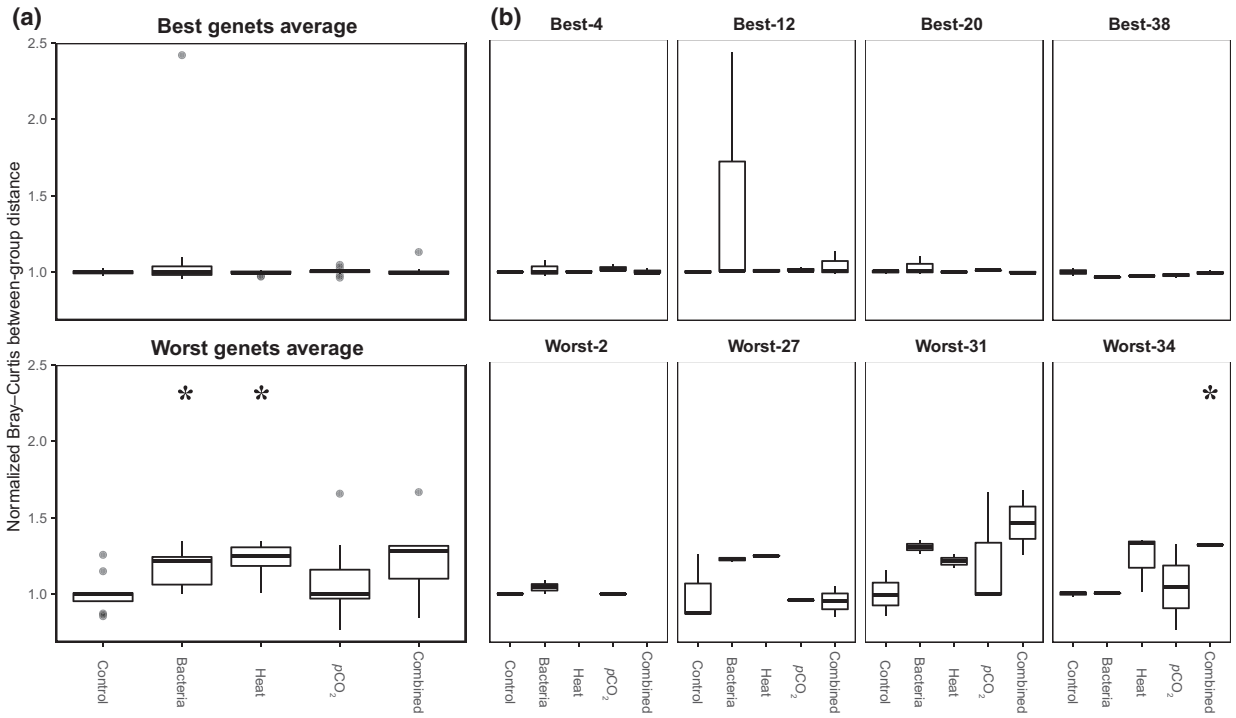

FIGURE 4 Differences in Symbiodiniaceae beta diversity among best and worst performing *Acropora millepora* genets exposed to various stress treatments. (a) Average normalized Bray–Curtis between-group distance in control versus stressed fragments for the four best performing genets combined and the four worst performing genets combined (categorically). (b) Average normalized differences in Bray–Curtis distance among communities in control versus stressed fragments within each host genet. Normalized difference is the proportion generated by dividing a fragment's between-group distance value by the average distance value of the control fragments from the same genet. Thus, control values fall around 1. In bacteria, heat, pCO_2, and combined treatments, values greater than 1 indicate that stress-treated fragments had higher between-group distance values compared to the average of their respective control fragments; values less than 1 indicate that stress-treated fragments had lower between-group distance values than the average of their control fragments. Thick lines indicate median values, with hinges extending to 25% and 75% quartiles and whiskers extending to most extreme points no further than 1.5 times the interquartile range. Single lines indicate a sample size of $n = 1$; treatments in individual genets have a maximum of $n = 3$ samples. Significant differences between a stress treatment and control are denoted by a *(Wilcoxon test, $p < .05$)

incorporated into the regional GJAM model, it fit the data well (Figure S2) and indicated that Symbiodiniaceae communities were more sensitive to genet than to treatment (Figure 5 and sensitivity analysis shown in Figure S3). Using this regional model, we inversely predicted the *A. millepora* genet and experimental treatment given a particular Symbiodiniaceae community. Based on this process, some *A. millepora* genets were easily predicted; they had distinctive Symbiodiniaceae communities associated with them. This includes Best-4, Best-12, and Best-20, and to a lesser extent, Worst-27 (Figure 5). Prediction of experimental treatments from Symbiodiniaceae communities was not robust using the regional model (Figure 5). In contrast, Symbiodiniaceae community was more sensitive to treatment in local GJAM models, which were run for Symbiodiniaceae communities at Pandora Reef and Rib Reef (Figure S4). In particular, stress treatment sensitivity values for bacteria and heat in Rib Reef were higher than all of the genet sensitivities (Figure S4).

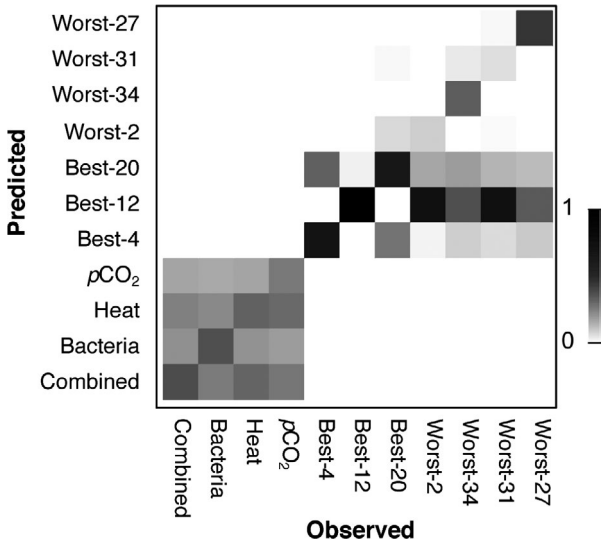

FIGURE 5 Generalized joint attribute model (GJAM) inverse predictions of *Acropora millepora* genet and stress treatment based on Symbiodiniaceae community composition. Darker shading indicates that the *A. millepora* genet or treatment condition was more readily predicted via the regional GJAM. A value of 1 indicates that a genet or treatment was always predicted

3.5 | Symbiodiniaceae taxa in best and worst performing coral holobionts

The dominant Symbiodiniaceae types among best performer *A. millepora* genets were more similar to each other than the dominant types among worst performer genets (Figures 2 and 3). We therefore used the regional GJAM model to investigate whether specific Symbiodiniaceae ASVs were differentially associated with best or worst performing *A. millepora* genets or stress treatments (Figure 6). A sequence variant assigned to *Cladocopium* C3k was strongly associated with all best performing genets and disassociated with most worst performing genets (Worst-27, Worst-31, and Worst-34). In contrast, a variant assigned to *Cladocopium* C1232 was disassociated with Best-38 (labeled as 'intercept' in Figure 6), Best-4, and Best-20 and strongly associated with all worst performing genets and Best-12. *Durusdinium* D1 and *Durusdinium* D1a were strongly associated with Worst-27 (Figure 6). The regional GJAM model did not identify any strong relationships between particular Symbiodiniaceae sequence variants and stress treatments.

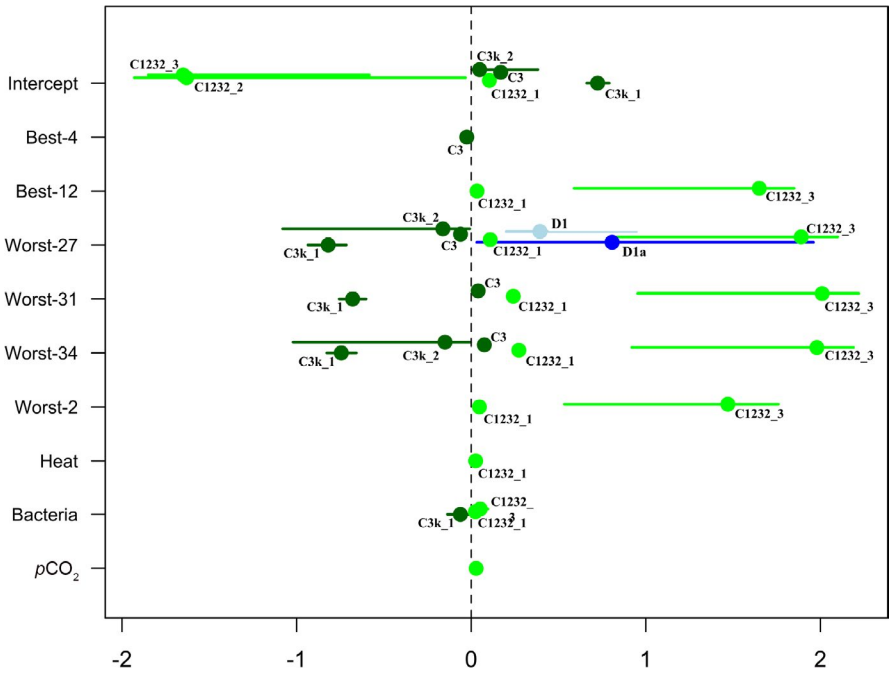

FIGURE 6 Posterior distributions of the significant effects of coral genet and treatment on Symbiodiniaceae internal transcribed spacer-2 amplicon sequence variants. Dots indicate the median and segments extend to the 95% credible intervals. C1232 = *Cladocopium* C1232; C3 = *Cladocopium* C3; C3k = *Cladocopium* C3k; D1 = *Durusdinium* D1; D1a = *Durusdinium* D1a. Numbers following the underscore in point labels distinguish different amplicon sequence variants that were assigned to the same Symbiodiniaceae type. Best-20 and the combined stress treatment are not visualized on the figure because they do not differ from the intercept, which is Best-38 [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

This study examined symbiont communities associated with strong or poor host performance under various stress treatments as well as ambient (control) conditions; an ultimate goal of this work was to identify symbiont community attributes (or specific symbionts) that can be leveraged to support coral reef resilience. We determined that best performing host genets contained remarkably consistent symbiont communities with relatively low alpha and beta diversity, even when exposed to stress. In contrast, under ambient conditions, worst performing host genets contained relatively high alpha and beta symbiont diversity and were differentially associated with some specific symbionts. Furthermore, symbiont community dissimilarity in worst performing genets was exacerbated by exposure to environmental stress treatments, underscoring symbiont community stochasticity as a potential indicator of declining reef health and emphasizing the need to understand how symbionts function in a community context (within hosts) if we are to promote naturally beneficial symbiont types and combinations.

4.1 | Metrics of symbiont diversity are higher in hosts with low stress tolerance

This work constitutes the first study from a marine system to demonstrate the potentially important role of non-bacterial symbiont alpha and beta diversity in hosts experiencing anthropogenic stressors. Across genets, symbiont community dissimilarity was higher in fragments of worst performers exposed to potentially pathogenic bacteria and temperature stressors (relative to controls for these genets; Figure 4a). This trend was generally consistent across most individual worst performing genets, but only one significant increase was detected (Figure 4b; combined stressors in Worst-34). We interpret that increased dissimilarity in stress-treated worst performing genets is a biologically meaningful pattern across genets, but that this study lacked statistical power to detect the pattern at the genet level (Figure 4b). Destabilization of bacterial symbiont communities and increased beta diversity was similarly observed in coral hosts exposed to high temperature, overfishing and eutrophication stressors by Zaneveld et al. (2016), but the same host genets were not compared across treatments in that study.

These findings are relevant to a recent general observation that dysbiotic host individuals vary more in microbial community composition than healthy host individuals—the 'Anna Karenina Principle' or AKP (Zaneveld et al., 2017). The AKP specifically predicts how microbial communities within an individual host should change over time based on declines in host health (e.g., based on exposure to stress). In this study, data on symbiont community composition at the start of the 10-day experiment are not available and therefore we cannot directly test the AKP. However, our detection of increased symbiont community dissimilarity in fragments of worst performers exposed to stressors (Figure 4) suggests that the AKP could be relevant for Symbiodiniaceae communities; direct tests of this principle (ideally over longer experimental time periods) in future studies of the coral–Symbiodiniaceae mutualism are warranted. Nonetheless, this is the first study to discuss the applicability of the AKP to endosymbiotic dinoflagellates, and these symbionts can now be added to the growing list of microbial communities, particularly those associated with various organ systems in primates (Chen et al., 2015; Halfvarson et al., 2017; Moeller et al., 2013; Wu et al., 2016) that exhibit increased stochasticity under environmental stressors.

In this study, the higher alpha diversity of the worst performing genets (relative to the best performing genets) and the increase in dissimilarity in symbiont communities of worst genet fragments exposed to stress (relative to control fragments of worst genets) suggest that interactions among diverse symbionts may destabilize coral–Symbiodiniaceae symbioses. Harboring diverse symbionts is often assumed to be advantageous for holobionts in that it allows hosts to 'hedge their bets' in dynamic environments. If a holobiont maintains a pool of symbiont diversity, at least one symbiont type is likely to retain function if environmental conditions change (Ludka, Levan, & Holway, 2015; Palmer et al., 2010). However, this symbiont diversity may come at a cost if competitive or antagonistic interactions occur among symbiont types. Symbiont competition, which was recently documented by McIlroy et al. (2019), is one potential mechanism through which destabilizing complementarity effects could have reduced symbiont benefit to worst performing hosts in this study (Miller, 2007; Moeller & Peay, 2016; Poland & Coffroth, 2019; Stanton, 2003). In contrast, members of the lower diversity (more uniform) symbiont communities associated with best performing genets could require fewer host resources to persist in polyculture, or even interact synergistically, contributing to a more stable holobiont.

Manipulative experiments using Symbiodiniaceae cultures and bleached or aposymbiotic hosts are a next step in testing whether the selection or complementarity effects are important for symbiont communities in coral hosts. Unfortunately, various symbionts identified in this study need to be established as isoclonal cultures before such manipulative experiments can be performed. Establishment of *Cladocopium* C3k and *Cladocopium* C1232 cultures would be particularly advantageous, since these symbionts were differentially associated with best and worst performing host genets, respectively (Figure 6). Investigating whether selection or complementarity effects contribute to the emergent physiological properties of colonies supports a thematic intervention strategy (i.e., physiological interventions) recently reviewed in a consensus study report on interventions to increase the persistence and resilience of coral reefs (National Academies of Sciences, Engineering, and Medicine, 2019). An alternative interpretation of the more uniform Symbiodiniaceae communities in best performing genets is that these hosts were more successful at maintaining normal physiological function under changing conditions, which could minimize stress responses by symbionts and subsequent host immune feedbacks against symbionts (e.g., Palmer, 2018). Various components of the host immune system could also contribute to the maintenance of specific symbionts in hospite (e.g., Ratzka, Gross, & Feldhaar, 2012). Large-scale meta-analyses of the relationship between symbiont beta diversity and host immunity can further support or dispute this interpretation.

4.2 | Differential associations of specific symbionts in best versus worst performing genets

We predicted that best performing host genets would contain higher relative abundances of stress-tolerant Symbiodiniaceae (e.g., *D. trenchii*) than worst performing genets. Contrary to this, *Durusdinium* D1 and *Durusdinium* D1a were strongly associated with only one genet, a worst performer (Worst-27; Figure 6). In Worst-27, these *Durusdinium* types represented from 11% to 72% of the total Symbiodiniaceae community (Figure 2; Table S2). In all other coral fragments analyzed in this study, *Durusdinium* represented <1% of the total Symbiodiniaceae community (Table S2). Although some *Durusdinium* species have previously been associated with holobiont thermotolerance (e.g., the ITS-1 D in Berkelmans & van Oppen, 2006; Jones, 2008), species such as *D. trenchii* are not necessarily tolerant of changes in thermal conditions (Howells, Berkelmans, Oppen, Willis, & Bay, 2013). Given the mortality suffered by Worst-27, there is no indication that *Durusdinium* types provided that genet with tolerance to elevated temperature, pCO_2, additions of *V. owensii*, or combinations thereof in this experiment. Although significant hopes for the survival of reefs into the future often rest upon corals acclimatizing to stress by harboring *Durusdinium* symbionts, this finding and some other studies indicate that hosting *Durusdinium* is not a panacea; only some *Durusdinium* types/species may impart stress tolerance to some coral hosts under certain circumstances and/or abundances (e.g., Hoadley et al., 2019; LaJeunesse et al., 2009; Morikawa & Palumbi, 2019). If complementarity effects are important in coral–Symbiodiniaceae mutualisms, then symbiont community diversity metrics may help reveal particular contexts in which *Durusdinium* (or other Symbiodiniaceae) types/species contribute to holobiont stress tolerance.

Certain *Cladocopium* types were also found to be differentially associated with best and worst performing genets. GJAM identified strong associations among a variant of *Cladocopium* C3k and best performing genets (Figure 6). *Cladocopium* C3k has previously been reported from a diversity of *Acropora* species (Barshis, Ladner, Oliver, & Palumbi, 2014; Smith, Pinzon, & LaJeunesse, 2009), including *A. millepora* (LaJeunesse et al., 2004). Most recently, *Cladocopium* C3k was reported from diverse host genera at mesophotic depths (*Echinophyllia*, *Fungia*, *Pachyseris*, and *Pavona* from 45 to 70 m, Bongaerts et al., 2011) suggesting that this symbiont can cope with (or has locally adapted to) diverse light and temperature profiles. However, *Cladocopium* C3k has not previously been linked to stress tolerance; it is more often predicted to be thermally susceptible, for example, relative to *Durusdinium* 2 in American Samoan acroporids (e.g., Barshis et al., 2014). Based on recent coral transcriptomic studies, we hypothesize that *Cladocopium* C3k may have contributed to the success of best performer host genets through differences in its baseline expression of various genes (relative to other Symbiodiniaceae types examined in this study), rather than transcriptional responses to stressors (Barshis et al., 2014; Leggat et al., 2011; Putnam, Mayfield, Fan, Chen, & Gates, 2013; but see Baumgarten et al., 2013). Finally, GJAM identified strong associations among a variant of *Cladocopium* C1232 with worst performing genets, as well as strong dissociations among that variant and three of four best performing genets. *Cladocopium* C1232 has previously been documented in *Acropora pagoensis* in American Samoa (Cunning, Yost, et al., 2015), but characterizations of its physiology and stress tolerance are not available in the published literature. The physiologies of *Cladocopium* C3k and *Cladocopium* C1232 should be further characterized in monoculture and polyculture, as well as in the diverse Pacific *Acropora* coral species that host them. Improved understanding of the physiologies and functions of specific symbiont types/species will ultimately contribute to delineations between host versus symbiont contributions to stress tolerance. Such research efforts will also help identify key symbiont types/species that can be developed as biomarkers and/or added to corals or reef locations to enhance their resilience to stress.

4.3 | Symbiont diversity is not strongly associated with stressor type

In this study, Symbiodiniaceae community composition in *A. millepora* was more strongly structured by the host (or biotic interactions) than imposed by the environment (Figures 5 and 6). Similar to this finding, Manzello et al. (2019) reported that 73% of the distribution of *D. trenchii* in a dominant reef-building Caribbean coral (*Orbicella faveolata*) was attributable to host identity. Yet, within a reef, where there is less variation in host genet, some influence of stress treatment on symbiont community composition became apparent (Figure S4). For example, we documented that symbiont communities associated with best performing genets on Rib Reef responded strongly (i.e., were sensitive) to the addition of a potential bacterial pathogen (Figure S4b). This local scale GJAM result hints that subtle shifts in symbiont communities might have occurred within best performing genets had the experimental treatments been run over a longer time period. These overall patterns fit the general expectation that history and evolutionary processes, in this case contributing to a heterogeneous distribution of host genets, might have a greater impact at regional (among reefs) as opposed to local (within reef) scales (e.g., Witman, Etter, & Smith, 2004).

It is possible that the influence of stressor (treatment) type on symbiont community composition was masked to some extent by survival bias and/or the relatively short duration (10 days) of this study. Survival bias may have occurred in that we cannot examine post-hoc whether individual worst performer fragments that experienced mortality had unique symbiont community characteristics (relative to surviving worst performer fragments of the same genet) that ultimately contributed to fragment death. It is important to remain cognizant of the fact that this bias potentially affects the findings of any experiment in which unintentional mortality occurs. Despite this, the significant differences and patterns detected from surviving fragments continue to advance the field.

We did not expect to observe severe bleaching or large shifts in Symbiodiniaceae community composition over the relatively short experimental duration and the level of stress applied; weeks to months are generally required for this (Bay et al., 2016; LaJeunesse et al., 2009). Instead, higher dissimilarity in stress-treated worst

performing fragments (relative to their controls) likely arose through incipient bleaching or other subtle processes. These processes could be detected because this study employed high-resolution amplicon sequencing and tested for potential differences in both symbiont types and metrics of symbiont community diversity. During their 10-day experiment, Wright et al. (2019) reported a decrease in symbiont-related metrics (color scores and chlorophyll measurements) in the heat treatment and an increase symbiont-related metrics in the bacterial treatment. We interpret that by the time fragments in this study were sampled, mild bleaching had begun in heat-stressed fragments, and in hospite symbiont growth and division was likely occurring in the bacterial treatment (perhaps through a surplus energy budget from heterotrophic feeding on the bacteria added to tanks). Thus, a longer experimental duration (with more mild stressors to avoid full mortality early on) would have also provided more time for selective expulsion and/or differential growth of symbiont types in hospite; this may have led to stronger patterns among treatments.

4.4 | Implications for future reefs

This study is unique in that metrics of symbiont community diversity were examined from host genets that were known best or worst performers, based on their survival under various stressful conditions. Importantly, we found that symbiont diversity and composition were distinct among the control fragments of host genets with different stress tolerances; these metrics can potentially be developed into useful predictors of coral resilience and can be included in genet selection for restoration and/or captive breeding efforts. Although symbiont physiology and function likely contribute to holobiont tolerance, the observed dissimilarity of symbiont communities in stressed fragments of worst performing genets argues for the elevated importance of focusing on symbiont community stochasticity as an explanation of host performance. The presence of a similar symbiont community across diverse stressors in best performer host genets provides some hope for coral reefs: up to a point, best performer host–symbiont combinations can potentially resist diverse or multiple stressors. The extent to which we can identify and promote specific symbionts (e.g., *Cladocopium* 3k, if stress tolerance is confirmed by additional physiological studies), communities or their characteristics (e.g., target levels of alpha, beta diversity) across diverse host genets and species is a logical next goal in managing for coral reef resistance and resilience.

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DATA AVAILABILITY STATEMENT

Raw Illumina sequence data are available at NCBI's SRA (accession #PRJNA596498). R scripts for analyses are available at https://github.com/LaurenHK/GeneticContstraints-Symbionts.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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