



# RETRACTED: Transcriptome Analysis Reveals the Expressed Gene Complement and Acute Thermal Stress Response of *Acropora digitifera* Endosymbionts

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### Specialty section:

This article was submitted to  
Marine Biology,  
a section of the journal  
Frontiers in Marine Science

**Received:** 16 August 2021

**Accepted:** 04 January 2022

**Published:** 27 January 2022

### Citation:

Ravelo SF, Posadas N and  
Conaco C (2022) Transcriptome  
Analysis Reveals the Expressed Gene  
Complement and Acute Thermal  
Stress Response of *Acropora*  
*digitifera* Endosymbionts.  
*Front. Mar. Sci.* 9:758579.  
doi: 10.3389/fmars.2022.758579

Dinoflagellate endosymbionts from the family Symbiodiniaceae form a mutualistic relationship with corals. However, this partnership is vulnerable to temperature stress, which can result in coral bleaching. In this study, we sequenced the metatranscriptome of symbionts (dominated by ITS2 type C3u) associated with the common scleractinian coral, *Acropora digitifera*, from Bolinao, Pangasinan, northwestern Philippines, to elucidate their genetic complement and stress response mechanisms. The assembled metatranscriptome revealed a rich repertoire of genes for the utilization of nutrients and metabolic exchange with the host, as well as genes that provide protection against cellular damage. Exposure of the algal endosymbionts to 31°C for 12 h induced global changes in the transcriptome profile. While transcripts involved in cell signaling, cytoskeletal organization, and rRNA processing were upregulated, downregulation of transcripts related to translation, proteolysis, lipid storage, and immune response indicate vulnerability to acute thermal stress. Some of these stress responsive transcripts were predicted to be under microRNA (miRNA) regulation. These findings reveal conserved hallmarks of coral-algal symbiosis and provide insights into the genetic potential and acute thermal stress response of a common symbiont of corals in the region.

**Keywords:** RNA-Seq, *Cladocopium*, symbiosis, holobiont, microRNA, coral

## INTRODUCTION

Reef building corals host dinoflagellate endosymbionts of the family Symbiodiniaceae in their tissues (Kirk and Weis, 2016). These symbionts provide the majority of the energetic requirements of the coral and are therefore critical for the maintenance of coral health (Muscatine and Porter, 1977). However, increasing seawater temperatures brought about by global warming can disrupt this symbiosis, resulting in a phenomenon known as coral bleaching (Carpenter et al., 2008). In recent decades, mass bleaching events have become more frequent and more extreme, leading to a global decline in coral reef cover (Hughes et al., 2017).

Symbiodiniaceae currently consists of nine genetically diverse genera: *Symbiodinium* (clade A), *Breviolum* (Clade B), *Cladocopium* (clade C), *Durusdinium* (clade D), *Effrenium* (clade E), *Fugacium* (clade F), *Gerakladinium* (clade G, clade H, and clade I) (LaJeunesse et al., 2018). Corals can host multiple symbiont genera and their associated community of symbionts may

change over time or with varying environmental conditions (Mieog et al., 2007; Stat et al., 2013; Boulotte et al., 2016). Most corals acquire symbionts from the environment, indicating that seawater and sediments are an important source of Symbiodiniaceae in their temporary free-living or *ex hospite* phase (Stat et al., 2006; Littman et al., 2008; Quigley et al., 2017). Association of corals with symbionts possessing different thermal tolerance characteristics may influence the ability of the coral holobiont to withstand elevated seawater temperature (Berkelmans and Van Oppen, 2006; Howells et al., 2011). For example, corals in the Persian Gulf or those that have experienced bleaching events usually host symbionts from genus *Durusdinium*, which are well-adapted for growth and photosynthesis at high temperature (Stat et al., 2013; Hume et al., 2015). However, different species within a genus may also exhibit a wide range of physiological differences and thermal tolerances that may reflect local adaptation (Levin et al., 2016; Klueter et al., 2017). Elucidating the diversity of symbionts in corals from different regions and their physiological responses to stress will be essential for predicting coral bleaching susceptibility.

Complete genome sequencing of acroporid corals and various species of Symbiodiniaceae have provided many important insights into the biology of the coral host, as well as the underlying dynamics of the symbiotic relationship (Shinzato et al., 2011, 2014, 2021; Shoguchi et al., 2013, 2018; Shinzato et al., 2021; Lin et al., 2015; Aranda et al., 2016; González-Pech et al., 2017, 2021; Liu et al., 2018). The obligate partnership of corals with Symbiodiniaceae may be partly explained by metabolic complementation, whereby the absence of key metabolic genes in the host are compensated with those of the symbiont. For example, cystathionine beta-synthase (CBS), a gene necessary for cysteine biosynthesis, is absent from the genome of *Acropora digitifera* (Shinzato et al., 2011) and other acroporid corals (Shinzato et al., 2021), but is present in the genomes of symbionts. Symbionts also possess complete biosynthetic pathways for other essential amino acids that the host cannot synthesize (Lin et al., 2015). Functions that reinforce the coral-algal symbiosis, such as transmembrane transport, response to reactive oxygen species (ROS), and protection against ultraviolet radiation, are enriched in most Symbiodiniaceae representatives (González-Pech et al., 2017).

Under elevated temperature conditions, symbionts *in hospite* exhibit little detectable change in gene expression, likely due to the ability of the host to insulate its endosymbionts from external conditions (Leggat et al., 2011; Barshis et al., 2014). On the other hand, studies on symbionts *ex hospite* revealed upregulation of stress-associated genes (Baumgarten et al., 2013; Krueger et al., 2014; Xiang et al., 2015), ROS scavenging molecules (Levin et al., 2016), and photosynthesis-related proteins (Gierz et al., 2017) with increasing temperature. In fact, thermally tolerant symbiont populations exhibited greater expression of ROS scavenging and molecular chaperone genes at elevated temperature compared to thermosensitive conspecifics (Levin et al., 2016). However, while it has been demonstrated that the tolerance of symbionts *in hospite* correlates with thermal tolerance in culture (Howells et al., 2011), it has been shown that *Durusdinium trenchii* mount a greater gene expression response

to thermal stress when in symbiosis with *Exaptasia pallida* compared to their free-living state (Bellantuono et al., 2019). These differences highlight the importance of investigating the gene complement of more diverse symbionts types and assessing their responses to thermal stress. Although acute stress exposure of *ex hospite* symbionts may not reflect the natural setting, it provides a window into the molecular basis of thermal tolerance of the symbionts. This also allows investigation of potential regulatory mechanisms that may be involved in the symbiont response. It has been proposed that, because algal symbionts possess few transcription factors, they may instead rely on post-transcriptional and translational regulatory mechanisms, such as small RNAs, under stress (Barshis et al., 2013; Baumgarten et al., 2013; Lin et al., 2015). microRNAs (miRNAs) are a family of small RNA regulators that can repress the translation of mRNA or mediate mRNA degradation (Millar and Waterhouse, 2005). Twenty one miRNAs were reported in *Symbiodinium microadriaticum* (Baumgarten et al., 2013), while hundreds were identified in *Fugacium kawagutii* (Lin et al., 2015). Regulation of mRNA abundance and translation rate by miRNAs may play an important role in regulating physiological responses in Symbiodiniaceae. However, it remains to be determined how miRNAs are involved in the thermal stress response of different symbiont species.

In this study, we sought to elucidate the expressed gene complement of algal symbionts associated with *A. digitifera*, a coral commonly found in the Bolinao-Anda Reef Complex, northwestern Philippines. This part of the Coral Triangle, which faces the South China Sea, experiences rising sea surface temperatures (Fang et al., 2006; Yu et al., 2020) that can be exacerbated by the El Niño Southern Oscillation (McPhaden et al., 2006). During the warmest months of the year, recorded temperatures on the reef can reach around 33°C (Guzman et al., 2019). Recurring warming conditions have resulted in two major bleaching events since the 1990s (Arceo et al., 2001; Shaish et al., 2010). Although considered to have greater susceptibility to bleaching (Marshall and Baird, 2000), acroporid corals continue to thrive in this region. To understand how the symbionts contribute to the persistence of these corals, we generated the metatranscriptome of *A. digitifera*-associated symbionts. We then compared the expressed gene complement with the genomes of the host and other Symbiodiniaceae. We also assessed gene expression responses induced by acute thermal stress and identified miRNAs that may mediate this response. This data provides an important resource on common symbionts associated with acroporid corals in the region, which may serve as a basis for designing local reef restoration initiatives.

## MATERIALS AND METHODS

### Collection, Acclimation, and Temperature Exposure of Symbionts

The samples described in this paper were derived from a previous experiment (Ravelo and Conaco, 2018). In brief, *Acropora digitifera* corals were collected from the Bolinao-Anda Reef complex (N: 16°17.286' and E: 120°00442') in May 2015

with permission from the Department of Agriculture Bureau of Fisheries and Aquatic Resources of the Philippines (GP-0102-15). The *A. digitifera* symbionts were extracted using a WaterPik and tissue homogenates were blended to disrupt coral tissues and release endosymbionts (**Supplementary Figure 1A**). The cell suspension was passed through a 60  $\mu\text{m}$  mesh to remove coral tissues and mucus, then spun down for 1 min at 1,500 rpm to collect symbiont cells (Santos et al., 2001). Cell pellets were washed three times by resuspending in sterile seawater and spinning down at 1,500 rpm to remove remaining cell debris. Cells were transferred to f/2 media at a density of approximately  $5 \times 10^5$  cells/ml (**Supplementary Figure 1B**) and acclimatized for seven days at  $26^\circ\text{C} \pm 1$  with a 12:12 light-dark cycle and illumination of  $\sim 60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  provided by 21 W Firefly Elite T5 warm white fluorescent lamps. The cells were transferred into 20 ml vials at approximately  $4 \times 10^5$  cells per vial and placed into triplicate aquaria at  $31^\circ\text{C} \pm 1$  (treatment) or at  $26^\circ\text{C} \pm 1$  (control). Cells were harvested by centrifugation from three replicate vials for each treatment at 0, 12, 18, 24, and 48 h. Cell pellets were flash frozen in liquid nitrogen. The full experiment ran for 48 h, but only the 12 h samples were selected for sequencing, as a significant decrease in cell density and integrity was observed after this time point (Ravelo and Conaco, 2018).

## RNA Extraction and Sequencing

Total RNA was extracted using the mirVana miRNA isolation kit (Ambion) following the manufacturer's protocol. Contaminating DNA was removed using the TURBO DNA-free™ Kit (Ambion). RNA was quantified using a BioSpec NanoDrop spectrophotometer (Shimadzu). RNA quality was assessed by agarose gel electrophoresis and using the mRNA Pico Series II assay on the Agilent Bioanalyzer 2100 System (Agilent Technologies). Due to resource limitations, only two RNA samples each from the 12 h heated and control treatments were sent to the Beijing Genomic Institute (BGI), Hong Kong, for preparation of barcoded libraries using the Illumina TruSeq RNA Sample Prep Kit. The mRNA-enriched libraries were sequenced on the Illumina HiSeq 2000 platform with 100 bp paired-end reads.

## Metatranscriptome Assembly and Annotation

Raw FASTQ reads were assessed using FastQC v0.11.8 (Andrews, 2010). Reads with quality score  $\leq 15$ , sequencing primers, and adaptors were removed using Trimmomatic v0.39 (Bolger et al., 2014). Trimmed reads were normalized and assembled on Trinity v2.8.5 (Grabherr et al., 2011). Redundant transcripts with 95% sequence identity were clustered using CD-HIT v4.8.1 (Li and Godzik, 2006) and transcripts with length  $< 500$  bp were removed. Assessment of the assembly quality was carried out using Benchmarking Universal Single-Copy Ortholog v4.0.2 tool (BUSCO) (Simão et al., 2015) and TransRate (Smith-Unna et al., 2016). Coding regions within the transcripts were identified using TransDecoder v5.5.0 in the Trinity package. Transcripts

and predicted peptides from the assembly were aligned against the UniProt/SwissProt and NCBI nr databases using Blastx and Blastp with an  $e$ -value cutoff of  $1 \times 10^{-5}$ . Gene ontology (GO) annotations were obtained based on the top Blastp hit for each peptide. Protein domains were annotated using the Pfam 32.0 (Finn et al., 2013) database through HMMER v3.3 (Eddy, 1998). To identify symbiont types represented in the assembly, we aligned reference sequences of ITS2, 28S, and 18S rRNA to the metatranscriptome using Blastn at an  $e$ -value threshold of  $1 \times 10^{-70}$  and nucleotide identity greater than 99%. 28S rRNA sequences were aligned to representative sequences of other Symbiodiniaceae genera (LaJeunesse et al., 2018) using ClustalO (Sievers et al., 2011). Alignments were trimmed using Gblocks (Castresana, 2000). Phylogenetic analysis was conducted using MrBayes (Ronquist et al., 2012) with two independent runs of four chains per run set for 1 million generations. Trees were sampled every 100 generations until the average standard deviation of split frequencies was  $< 0.01$ . The first 25% of trees were discarded as burn-in.

## Ortholog Analysis

Orthologous gene families in the metatranscriptome and in the genomes of *S. microadriaticum* (Aranda et al., 2016), *B. minutum* (Shoguchi et al., 2013), *Cladocopium* sp. type C93 (Shoguchi et al., 2018), *C. goreauii* type C1 (Liu et al., 2018), *D. trenchii* (Shoguchi et al., 2021), *F. kawagutii* (Lin et al., 2015), and *A. digitifera* (Shinzato et al., 2011) were identified using OrthoFinder (Emms and Kelly, 2019). Peptide sequences of *S. microadriaticum*, *B. minutum*, *Cladocopium* sp., *C. goreauii*, and *F. kawagutii* were retrieved from SAGER database (Yu et al., 2020) while the predicted peptides of *D. trenchii* and *A. digitifera* were downloaded from the Okinawa Institute of Science and Technology Marine Genomics Unit website. Intersections of orthologous groups across different species were visualized using the UpSetR package in R (Conway et al., 2017).

## Differential Gene Expression and Gene Ontology Enrichment Analysis

Transcript abundance was estimated by mapping the reads to the reference metatranscriptome using RNA-Seq by Expectation Maximization (RSEM) (Li and Dewey, 2011) with Bowtie 2 (Langmead and Salzberg, 2012) alignment method. Differentially expressed genes (DEGs) were identified using edgeR with the generalized linear model and likelihood ratio testing method, which are suitable for datasets with few or no replicates (Robinson et al., 2010). To further increase stringency of the analysis, we filtered out lowly expressed transcripts [ $< 10$  counts per million (CPM) in at least two libraries] prior to edgeR analysis (23,625 out of 157,291 transcripts retained). We considered transcripts as differentially expressed if up or downregulation was greater than four-fold relative to the controls with a Benjamini-Hochberg-adjusted  $p$ -value  $\leq 1 \times 10^{-5}$ . Functional enrichment analysis for DEGs was performed using the topGO package in R (Alexa and Rahnenfuhrer, 2010). GO terms with a  $p$ -value  $\leq 0.05$  (Fisher's exact test) were considered significantly enriched.

## Sequencing and Identification of microRNAs

Small RNA libraries were prepared from size-fractionated (18–30 nt) total RNA and subjected to 50 bp single-end sequencing on the Illumina HiSeq2000 platform (BGI, Hong Kong SAR, China) at an average depth of 15 million reads per library. Two small RNA libraries were sequenced representing two sets of pooled algal cells derived from *A. digitifera*. Raw reads were trimmed using Trimmomatic (Bolger et al., 2014). Only reads with at least 18 nt length and an average Phred score of 30 were retained for further analysis. Pooled sequence reads (31,754,571 reads) from the two libraries were mapped against the genome of *C. goreau* (Liu et al., 2018) and microRNAs (miRNAs) were identified using miRDeep2 (Friedlander et al., 2012). Hairpin secondary structures on pre-miRNA precursor sequences were assessed using RNAfold (Mathews et al., 2004). Sequences were considered putative miRNAs if they had (i) a miRDeep2 score  $\geq 10$ , (ii) a precursor miRNA minimum free energy (MFE) of folding  $< -25 \text{ kcal mol}^{-1}$ , (iii) a 2 nt 3' overhang on both mature and star strands, (iv) consistent 5' end position for the guide sequence, (v) no matches to known protein coding RNAs and other non-coding RNA families, (vi) expression of 20–26 nt long reads for both strands, (vii) at least 16 nt complementarity between the two arms, and (viii) at least 8 nt loop sequence (Fromm et al., 2015).

## Identification of microRNA Targets

Targets of putative miRNAs, with either partial or extensive mRNA complementarity, were identified using miRanda (Enright et al., 2003) or RNAhybrid (Kruger and Rehmsmeier, 2006), respectively. Animal miRNAs typically bind to the 2–8 nt seed region resulting in translational repression, whereas plant miRNAs usually have extensive complementarity to the target gene and trigger mRNA cleavage (Millar and Waterhouse, 2005). The 3' untranslated regions (UTRs) from our assembly were used to predict animal type target genes. Only miRNA-target duplexes with MFE of  $\leq -20 \text{ kcal mol}^{-1}$  were included in downstream analyses. Plant type target genes were predicted by aligning mature miRNA sequences to the coding sequences (CDS) of the assembly. Only predictions with RNAhybrid *p*-value of  $\leq 0.01$  and with the following duplex characteristics: (i)  $< 4$  mismatches between miRNA and target; (ii)  $< 2$  adjacent mismatches in the miRNA/target duplex; (iii)  $< 2$  mismatches between position 1–12 of the duplex, no adjacent mismatches in this window allowed; and (iv) no mismatch in position 10–11 of the duplex (Allen et al., 2005), were included in the analysis. Functional enrichment analysis of predicted target genes was carried out using the topGO package in R (Alexa and Rahnenfuhrer, 2010).

## RESULTS

### Metatranscriptome Characterization

RNA sequencing of four symbiont libraries yielded a total of 109,515,594 reads that were assembled into 1,120,556 transcripts (Supplementary Table 1). We note that because the cultures were

not derived by single cell isolation, they represent the community of symbionts associated with the coral and may include coral-associated bacteria and some single-celled eukaryotes (e.g., diatoms) that were not effectively removed by the filtering and centrifugation steps. To minimize inclusion of sequences from non-Symbiodiniaceae components, and to eliminate fragments and misassembled transcripts, the assembly was further filtered to remove duplicates and overlapping sequences, lowly represented transcripts, and transcripts shorter than 500 bp. The final reference metatranscriptome contained 165,018 transcripts of which 157,291 (95%) were protein coding. The reference metatranscriptome had an N50 of 992 bp and Ex90N50 of 1,227 bp (Supplementary Figure 2). The overall GC content was 53.34%. To assess assembly completeness, we compared the metatranscriptome against core gene sets in BUSCO. The assembly recovered around 93% of the 303 eukaryote and 171 Alveolata genes but only 77% of the 978 metazoan genes and 55% of 148 bacterial genes. Overall assembly statistics are similar to those reported for other Symbiodiniaceae transcriptomes (Bayer et al., 2012; Ladner et al., 2012; Baumgarten et al., 2013; Rosic et al., 2015; Levin et al., 2016; Parkinson et al., 2016).

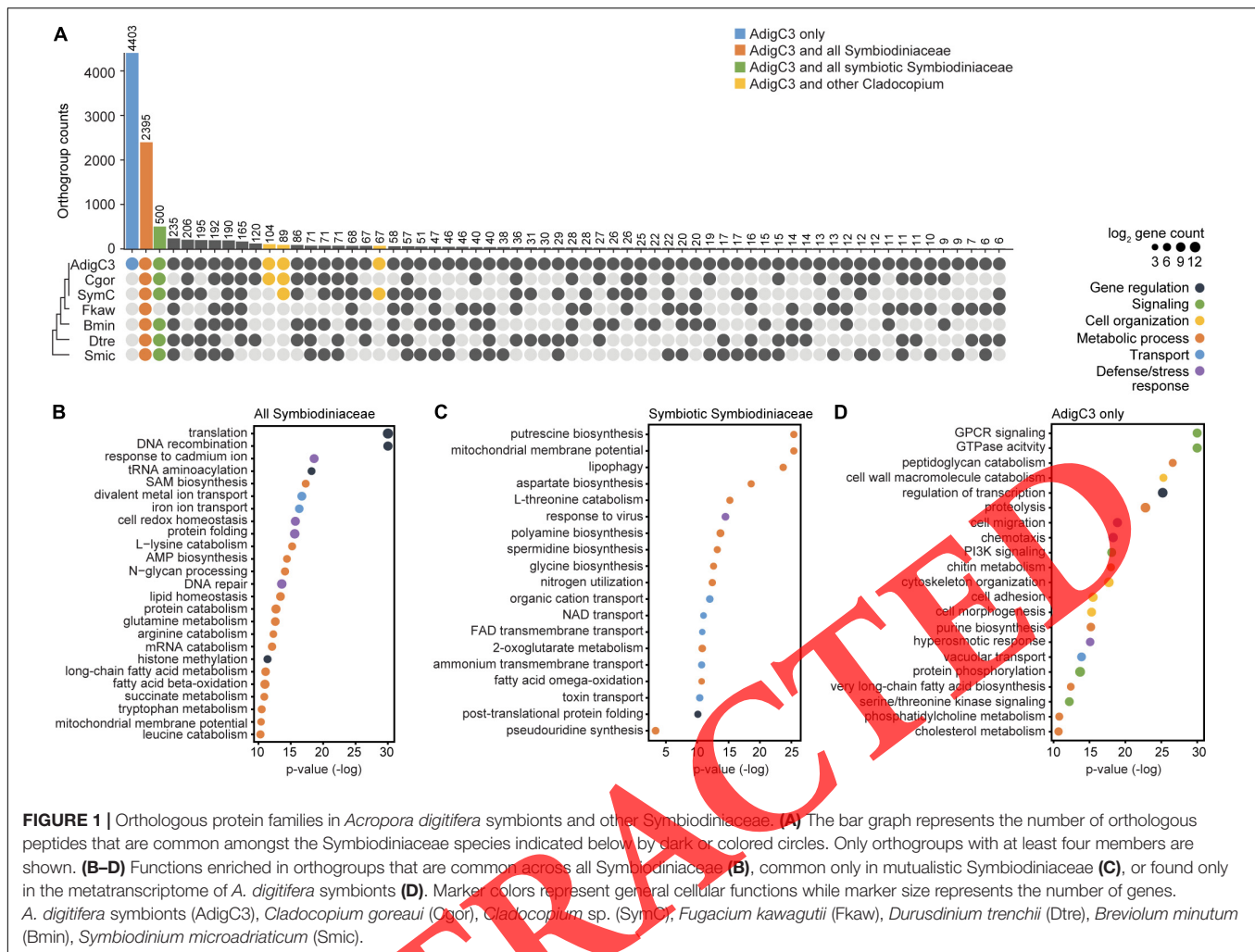
### Identification of Symbiont Types in the Assembly

Alignment of reference ITS2, 18S, and 28S rRNA sequences against the assembly captured transcripts with top hits to sequences associated with *Cladocopium* (Supplementary Table 2). The ITS2 sequences in the assembly matched ITS2 type C3u from GeoSymbio, as well as other type C3 sequences in NCBI. The 28S rRNA sequences from the assembly also matched to type C and C3, while 18S rRNA matched sequences from type C and C2 symbionts. Phylogenetic analysis of the 28S rRNA sequences derived from the assembly verified affiliation with *Cladocopium* (Supplementary Figure 3). We did not detect significant hits to marker genes affiliated with *A. digitifera* or to other organisms, suggesting that the assembly did not capture many transcripts from host cells or single-celled eukaryotes that may have been present in the culture.

### Assembly Annotation and Identification of Orthologs

Only 60% of predicted peptides could be annotated against UniProt, the NCBI non-redundant protein database, and PFAM (Supplementary Figure 4). Taxonomic distribution of UniProt matches showed that 91.8% of the best hits were affiliated to eukaryotes, 7.9% to prokaryotes, and 0.3% to viruses. Along with the BUSCO results, this finding suggests that the metatranscriptome did not capture many transcripts derived from bacteria.

Peptides predicted from the *A. digitifera* symbiont metatranscriptome clustered into 10,357 orthologous protein families (Figure 1A). Of these families, 2,395 were represented in all Symbiodiniaceae, 500 were common amongst all symbiotic Symbiodiniaceae, 260 were present only in other *Cladocopium* representatives. A total of 4,403 protein families were unique to the symbionts from *A. digitifera*.



Functions enriched in the set of genes with orthologs found in all Symbiodiniaceae include translation, protein folding, DNA repair, DNA recombination, cell redox homeostasis, and metabolic processes such as amino acid synthesis and fatty acid metabolism (Figure 1B; Supplementary Table 3). Functions common amongst all symbiotic Symbiodiniaceae species include putrescine synthesis, lipophagy, nitrogen utilization, and organic cation transport (Figure 1C; Supplementary Table 3). Functions enriched in the genes unique to the symbionts of *A. digitifera* include signaling, transcriptional regulation, chemotaxis, cell adhesion, chitin metabolism, and synthesis of very long chain fatty acids, cholesterol, and phosphatidylcholine (Figure 1D; Supplementary Table 3).

## Comparison of Symbiosis and Stress Response Genes

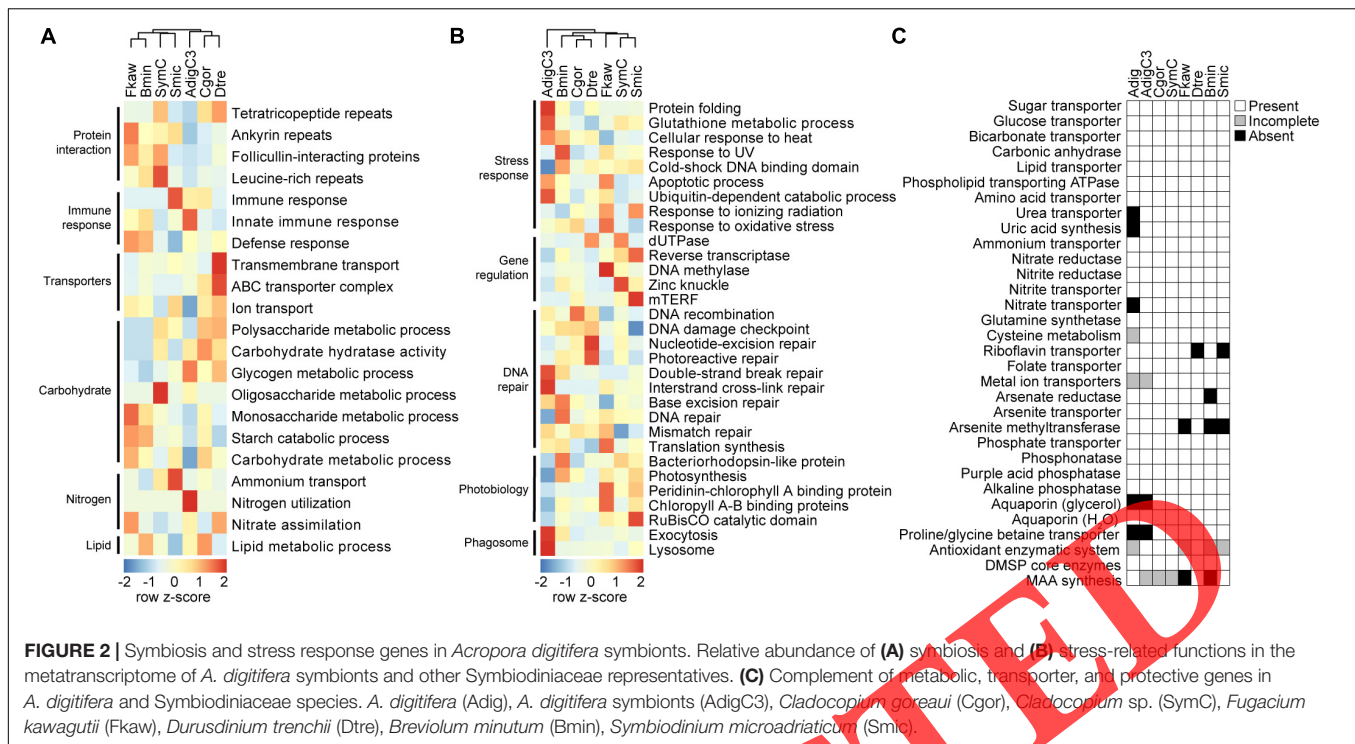
The relative abundance pattern of symbiosis-related genes in the *A. digitifera* symbiont metatranscriptome was most similar to that of *C. goreau* and *D. trenchii* (Figure 2A; Supplementary Table 4). Relative to the genomes of other Symbiodiniaceae, our assembly contained fewer peptides with tetratricopeptide,

ankyrin, and leucine-rich repeat domains. We also found fewer monosaccharide, starch, and carbohydrate metabolism genes. On the other hand, we identified more genes that function in the immune response, lipid metabolism, glycogen metabolism, and nitrogen utilization.

Stress-related genes were over-represented in our metatranscriptome compared to the Symbiodiniaceae genomes (Figure 2B; Supplementary Table 4). We observed a higher relative abundance of stress response and DNA repair-related functions, such as protein folding, glutathione metabolic process, cellular response to heat, DNA damage repair, apoptotic process, double strand break repair, and inter-strand cross-link repair. However, there were fewer functions related to response to UV, cold shock, photoreactive repair. Our assembly also contained relatively fewer functions related to gene regulation and photobiology compared to other Symbiodiniaceae.

## Complementation of Genes Between Host and Symbionts

We compared the complement of specific genes in the metatranscriptome of *A. digitifera* symbionts and other



Symbiodiniaceae against the genome of the coral, *A. digitifera*, to reveal pathways that may indicate functional complementation between host and symbionts (Figure 2C; Supplementary Table 5). Both coral and symbionts possessed all the genes for carbon, vitamin, and phosphorus and arsenic exchange. However, while the symbionts expressed nitrate and urea transporters and uric acid synthesis enzymes, these genes were missing from the coral host. The symbionts also expressed a complete set of genes for cysteine synthesis to complement the lack of CBS in the host. The *A. digitifera* symbionts expressed metal transporters that were not present in the coral genome, including CBS-pair domain divalent metal cation transport mediator (CNNM), iron-sulfur cluster transporter (ATM), and high-affinity potassium transport protein. The high-affinity nickel transport protein, as well as the osmoregulators, aquaporin (glycerol transport) and proline/glycine betaine transporter, were not detected in *A. digitifera* nor in the transcriptome of its symbionts.

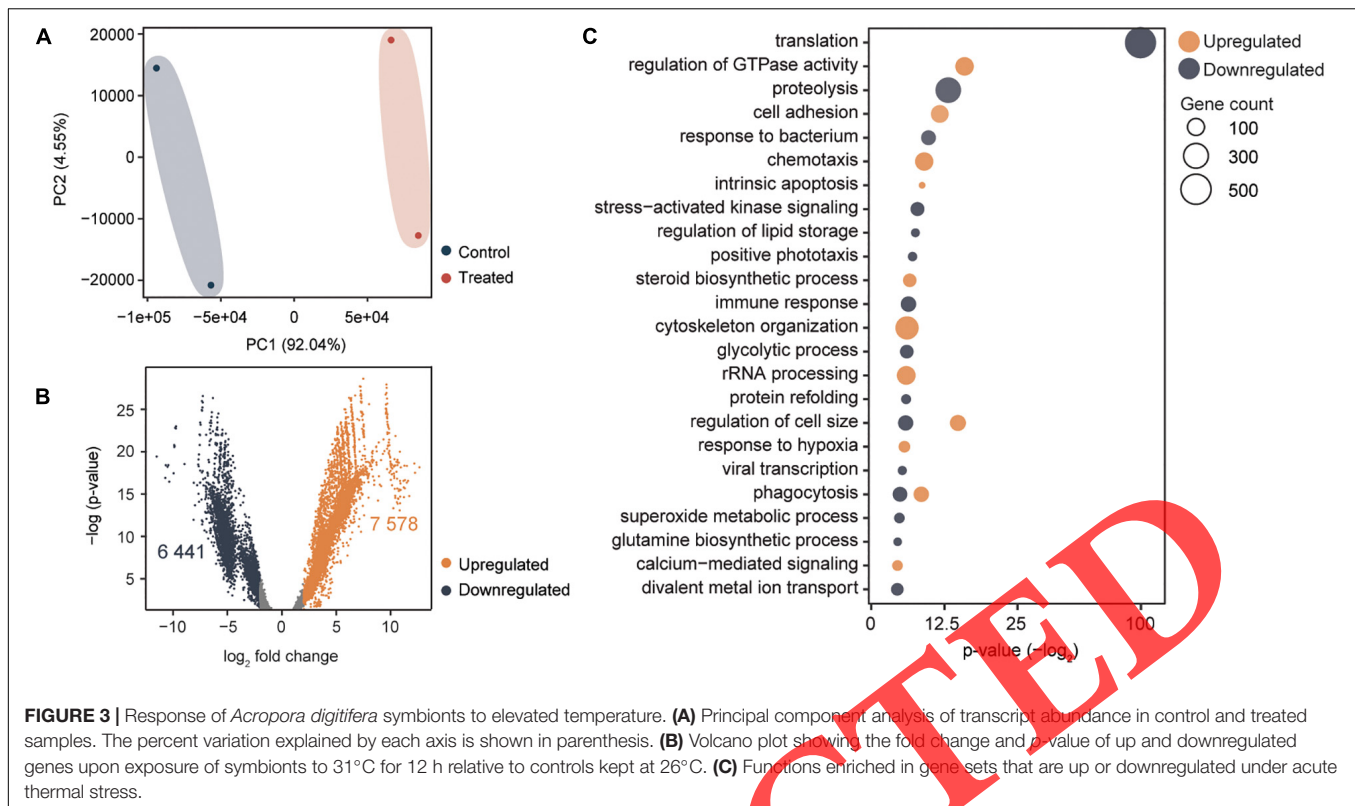
All *Cladocopium* representatives and *D. trenchii* possessed a full complement of antioxidant genes, including superoxide dismutase, ferritin, thioredoxin, and glutaredoxin. On the other hand, *Breviolum* and *Symbiodinium* lacked orthologs to glutathione peroxidase, while *Fugacium* lacked 1-cys peroxiredoxin and peroxiredoxin3 alkylhydroperoxide reductase. Nickel-type superoxide dismutase (SOD Ni) and ascorbate peroxidase (APx) were absent from *A. digitifera*. The DMSP core enzymes were present in *A. digitifera* and all Symbiodiniaceae. In contrast, the complement of mycosporine-like amino acid (MAA)-synthesis enzymes was mostly absent in all Symbiodiniaceae and was complete only in *S. microadriaticum*, *D. trenchii*, and in *A. digitifera*.

## Symbiont Response to Acute Thermal Stress

Principal component analysis showed that the global expression pattern of transcripts in the treated samples was distinct from the control samples (Figure 3A). Differential expression analysis revealed that the exposure of *ex hospite* symbionts to 31°C for up to 12 h resulted in a significant change in expression (> four-fold) for 8.5% (14,019) of the transcripts, with 7,578 upregulated and 6,441 downregulated (Figure 3B; Supplementary Table 6). Functions enriched in the upregulated set included regulation of GTPase activity, cell adhesion, chemotaxis, apoptosis, steroid biosynthesis, cytoskeletal organization, and rRNA processing (Figure 3C; Supplementary Table 7). Conversely, downregulated functions included translation, proteolysis, response to bacterium, stress-activated kinase signaling, regulation of lipid storage, immune response, protein folding, and superoxide metabolic process.

## Symbiont microRNAs and Potential Targets

Core components of the plant miRNA machinery (Moran et al., 2017) were detected in the metatranscriptome of the *A. digitifera* symbionts (Supplementary Table 8). miRDeep2 analysis predicted eight putative miRNAs (Supplementary Table 9). The highest scoring and most abundant miRNA, SymbC1.scaffold301\_3664 (Figure 4A), had the same mature sequence as the highest scoring miRNA predicted from *F. kawagutii* (Lin et al., 2015). Another less abundant miRNA, SymbC1.scaffold532\_5607, had a similar seed sequence.



SymbC1.scaffold301\_3664 had a total of 2,198 potential mRNA targets in the symbiont metatranscriptome (with 2,169 animal-like and 53 plant-like binding sites), of which 563 (26%) were differentially regulated under stress (Supplementary Tables 10, 11).

Prediction of the functions represented within the miRNA target set revealed enrichment for processes related to signaling (regulation of GTPase activity, response to stimulus, GPCR signaling, calcium ion homeostasis), stress response (cell death, osmotic stress, phototaxis, chemotaxis, response to heat), reproduction (cytokinesis, regulation of cell size), and interactions with the host or other symbionts (transmembrane transport, aggregation) (Figure 4B).

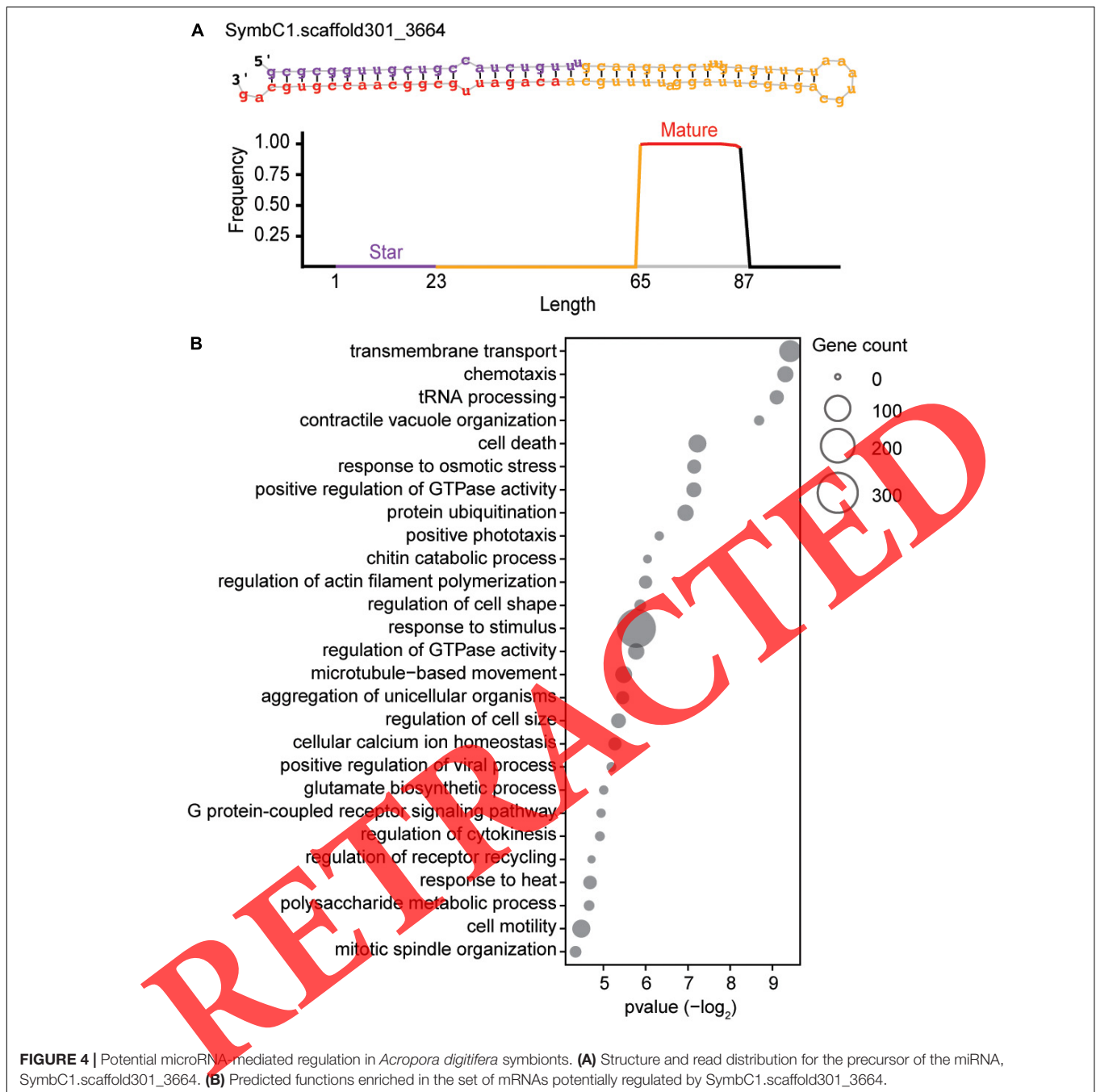
## DISCUSSION

Here, we described the metatranscriptome of symbiont cells derived from *Acropora digitifera* from Bolinao, Pangasinan, in northwestern Philippines. The population of symbionts residing in this coral was dominated by *Cladocopium* (ITS2 type C3u), as determined by metatranscriptome mining and direct ITS2 sequencing (Ravelo and Conaco, 2018). Alignment of reference marker genes to the assembly, however, suggests that the sequenced population may include other Symbiodiniaceae types. *Cladocopium* type C3u has also been identified as the dominant symbiont of *A. millepora*, *A. tenuis*, and *Favites colemani* in the Bolinao-Anda Reef Complex (Ravelo and Conaco, 2018; Da-Anoy et al., 2019). ITS2 type C3 is a generalist species and may be

found in many other corals with horizontal symbiont acquisition strategies (Stat et al., 2008).

The heterogeneous nature of the symbionts derived from *A. digitifera* is reflected in our metatranscriptome assembly, which has relatively small N50 and a large number of transcripts. The metatranscriptome likely overestimates the number of genes expressed by a single symbiont type, as has been observed in transcriptomes from non-monoclonal cultures (Rosic et al., 2015; Levin et al., 2016). The prevalence of alternative splicing and *trans*-splicing in dinoflagellates may also add to the high diversity of transcripts in the assembly (Erdner and Anderson, 2006; Zhang et al., 2007). It is important to note, however, that while we did not detect marker genes affiliated with other organisms in the metatranscriptome, we cannot rule out the possibility that inclusion of some non-symbiont sequences may have also contributed to the greater number of transcripts and unique gene orthologs detected in the assembly. Further refinement of the assembly will be possible once more genomes for comparison become available. Nevertheless, the assembly has a high rate of completeness based on comparison to alveolate and eukaryote benchmarking genes in BUSCO. The overall GC% of the assembly is also within the range reported for other symbionts (González-Pech et al., 2017).

Comparison of the *A. digitifera* symbiont metatranscriptome to genomes of other Symbiodiniaceae revealed differences in gene complement that may reflect the extensive divergence within this genus (González-Pech et al., 2017, 2021). Variation in gene repertoire could also be attributed to changes in the genetic and epigenetic program of symbiont cells that have been kept in



long-term culture, which could lead to DNA sequence variations, methylation changes, and transposon activation (Neelakandan and Wang, 2011). As symbionts in the natural environment encounter more variable conditions, they may accumulate genomic changes that could result in greater sequence divergence compared to symbionts kept in a stable culture environment.

Our metatranscriptome reflects the expressed gene complement of a heterogeneous symbiont population grown with and without thermal stress and collected at a single time point. As such, the observed gene complement may overestimate the content from one particular species and over-represent

genes that are responsive to elevated temperature. On the other hand, genes that were not expressed at the time that the samples were collected (e.g., photosynthesis genes), will not be detected. Thus to capture a more complete transcript set, it would be necessary to sequence additional growth conditions and time points, particularly before dawn and after sunset when the highest expression of transcripts for a photosynthetic organism occurs (McClung, 2001; Van Dolah et al., 2007). Despite these limitations, the metatranscriptome provides a trove of information that allows initial exploration of the expressed gene complement of symbionts associated with *A. digitifera*.



## Adaptations for Symbiosis

Common functions represented in genes that are conserved across all Symbiodiniaceae, particularly in symbiotic species, indicate processes that are key for establishing and maintaining symbiosis, as well as for responding and adapting to the environment (González-Pech et al., 2017, 2021). These include general cellular processes, such as translation, recombination, metabolism, and molecular transport and exchange, which allow symbionts to acquire nutrients from its environment and to export metabolic products. For example, both the coral host and associated symbionts have the enzymatic machinery to incorporate ammonium. However, as only the symbionts express transporters for nitrate and urea, they are more efficient at utilizing dissolved inorganic nitrogen from seawater (Pernice et al., 2012). Nitrogen may be stored in the symbionts in various forms or transformed into other compounds, such as amino acids, that are transported into the host (Wang and Douglas, 1999). Symbionts possess xanthine dehydrogenase and can store nitrogen in the form of uric acid crystals, which are readily mobilized under nitrogen poor conditions (Clode et al., 2009). Nitrogen can also be converted into amino acids, such as cysteine, through the activity of diverse amino acid synthesis pathways in the symbionts.

Lipophagy is another function enriched in symbiotic dinoflagellates. Dinoflagellates store triacylglycerides (long-chain saturated or monounsaturated fatty acids and very long-chain polyunsaturated fatty acids), sterol esters, and free fatty acids in cytosolic lipid bodies (Leonard et al., 1994; Guéguen et al., 2021). Degradation and mobilization of these lipid bodies through lipophagy plays a central role in the exchange of lipid metabolites between host and endosymbionts (Chen et al., 2017).

Biological processes enriched in orthologous gene families that were unique to our metatranscriptome include GTPase and GPCR signaling processes, cell migration, and cytoskeleton organization. Expression of these genes may reflect processes that are activated in the symbionts under *ex hospite* culture conditions. Enrichment of genes involved in the synthesis of very long chain fatty acids, cholesterol, and phosphatidylcholine suggest active lipid metabolic processes that are important for energy storage and for building or modifying cell membranes.

## Adaptations for Defense and Environmental Response

Symbiodiniaceae are usually found in shallow reef environments where they may be exposed to heat, intense sunlight, UV rays, and ROS generated during photosynthesis. DNA repair enzymes, chaperones, and cell redox control mechanisms, which are enriched in the genes common amongst symbionts, form an important line of defense against the damaging effects of ROS and UV radiation (Roberty et al., 2016; Jones and Baxter, 2017). The extensive repertoire of defense and stress response-related genes in the metatranscriptome of *A. digitifera* symbionts suggest that this *Cladocopium* lineage is well-adapted to dealing with local environmental stressors.

Both *A. digitifera* and its symbionts possess core biosynthetic genes for DMSP, suggesting that both parties contribute to the

production of this known osmolyte and anti-stress compound (Raina et al., 2013; Broy et al., 2015). On the other hand, most genes for MAA synthesis were absent from *Cladocopium* representatives, except for a dimethyl 4-deoxygadusol (DDG) synthase ortholog, which is consistent with the report that the MAA gene cluster was lost in the common ancestor of *Breviolum* and *Cladocopium* (Shoguchi et al., 2018, 2021). Hence, these symbiont species may rely on their coral host for UV protection. Indeed, *A. digitifera* possesses most of the genes for MAA synthesis (Shinzato et al., 2011), indicating that it is able to produce these compounds that serve as UV-absorbing sunscreens, antioxidants, and osmotic regulators (Rosic and Dove, 2011). Orthologs of MAA synthesis enzymes were present in the *Symbiodinium* and *Durusdinium* genomes and, although we did not detect the complete MAA pathway in *Fugacium*, improvement of gene model prediction from its genome revealed the presence of DDG synthase and O-methyltransferase genes in this species (Li et al., 2020).

## Symbiont Thermal Stress Response

Thermal stress exposure of the symbiont cultures induced global changes in transcriptome profile. The relatively large response observed in our experiment may be due to the *ex hospite* nature of the symbionts, as also observed in similar studies (Levin et al., 2016; Gierz et al., 2017). It is important to note that *ex hospite* exposure may not capture the same transcriptome dynamics as when symbionts are inside coral tissues (Barshis et al., 2014; Bellantuono et al., 2019). In addition, because we only observed responses from a single time-point and had limited replicates for the thermal stress experiment, independent validation of the differentially expressed genes will be needed. Nonetheless, our findings provide initial insights into the molecular basis of thermal tolerance of the endosymbionts of Philippine acroporids and point to response pathways that can be investigated further.

Upon exposure of symbionts to elevated temperature, processes involved in the maintenance of cellular homeostasis were activated. This included upregulation of transcripts involved in regulation of GTPase activity, cell adhesion, chemotaxis, apoptosis, and cytoskeletal organization. GTPases are molecular switches that regulate cytoskeleton dynamics, vesicular transport, and intracellular stress signaling pathways (Hodge and Ridley, 2016; Nielsen, 2020). Expression of cell adhesion, cytoskeletal organization, and steroid synthesis genes may indicate cellular reorganization or membrane maintenance activities (Ladner et al., 2012). We also observed upregulated expression of some transcripts encoding heat shock proteins, heat shock transcription factors, and antioxidant enzymes, such as glutathione peroxidase, glutathione reductase, superoxide dismutase, and cytochrome p450, which is likely a response to the overproduction of ROS and protein denaturation that occurs at high temperature (Rosic et al., 2010, 2011).

On the other hand, we observed downregulation of genes involved in translation and proteolysis, which suggests that the cells may be reallocating available energy for processes such as protein refolding and DNA repair owing to the elevated cost of basal metabolism and inhibition of pathways for energy generation at elevated temperature (Sokolova, 2013;

Kaniewska et al., 2015). However, upregulation of ribosomal RNA processing indicates that the cells may still be able to translate necessary proteins. Genes implicated in glycolytic process and regulation of lipid storage were also downregulated under thermal stress. The shift in expression of these pathways may explain the heat stress-induced changes in glycolipid composition that were observed in *Cladocopium* C3 cells but not in thermotolerant *Durusdinium* (Rosset et al., 2019). Changes in the efficiency of lipid storage mechanisms could affect the ratio of polyunsaturated fatty acids in thylakoid membranes, which has been linked to thermal tolerance in different Symbiodiniaceae species (Tchernov et al., 2004).

Other stress-related changes in gene expression, such as downregulation of functions related to stress-activated kinase signaling, response to bacterium, and immune response further suggest that the symbionts may become susceptible to invasion by opportunistic pathogens upon prolonged exposure to elevated temperature. Increasing damage due to oxidative stress, accompanied by depression of symbiont defenses and disruption of symbiont-host interactions, may eventually result in deterioration of symbiont cellular integrity and breakdown of the coral-algal symbiosis. The observed decrease in intact symbiont cells after 24 h of acute thermal stress exposure (Ravelo and Conaco, 2018) indeed shows that the symbionts are no longer able to effectively maintain cellular homeostasis or repair cellular damage under these conditions. Altogether, these findings mirror the transcriptomic response of *Cladocopium* type C1 after prolonged exposure to 32°C, which was accompanied by upregulation of genes linked to ROS scavenging and protein folding and downregulation of some genes for translation and metabolism (Levin et al., 2016). Further examination of transcriptome dynamics over time will be needed to resolve the sequence of processes leading up to the point at which symbionts reach their tolerance limit and are no longer able to maintain cellular homeostasis. In addition, comparison of our results to the stress response of *Cladocopium* symbionts *in hospite* would be important to better understand how response dynamics of members of this genus may be influenced by symbiotic state or by association with different host corals.

## Potential Role of microRNAs in the Symbiont Stress Response

Previous studies suggest that various functions in the algal symbionts of corals could be under miRNA control (Baumgarten et al., 2013; Lin et al., 2015). We identified the miRNA processing machinery and eight putative miRNAs in the symbionts of *A. digitifera*. It is likely that more miRNAs will be detected once the ITS2 type C3 genome is sequenced. The most abundant miRNA, SymbC1.scaffold301\_3664, was predicted to regulate a wide array of biological processes, including functions that were also affected by thermal stress. The functions of the putative targets of SymbC1.scaffold301\_3664 were consistent with miRNA-regulated functions in *S. microadriaticum*, which included protein modification, immunity, signaling, DNA damage, gene expression, translation, and metabolism (Baumgarten et al., 2013). These findings support the idea

that post-transcriptional mechanisms are key in the symbiont thermal stress response. This mode of regulation may provide a rapid response mechanism that allows immediate translation of needed proteins from a pool of post-transcriptionally repressed mRNAs (Gajigan and Conaco, 2017). Further investigation of the co-expression patterns of symbiont miRNAs and their target genes, coupled with biochemical verification, are needed to validate the role of miRNAs in the symbiont stress response.

## CONCLUSION

Sequencing of the metatranscriptome of the ITS2 type C3 symbionts associated with *A. digitifera* revealed a rich complement of genes for the maintenance of symbiosis and environmental defense. We detected genes that were conserved in other *Cladocopium* species, as well as in other members of Symbiodiniaceae, but we also identified many genes that were unique to the *A. digitifera* symbionts. The symbionts were responsive to acute thermal stress, exhibiting shifts in the expression of protective genes and damage repair mechanisms, although depression of key cellular functions related to translation and immune response suggest that *ex hospite* symbionts are vulnerable to acute thermal stress. Exploration of the miRNA complement revealed potential involvement of miRNA-mediated regulation in the symbiont thermal stress response. These findings provide insights into the genetic potential and temperature response of a common symbiont of corals in northwestern Philippines. Further studies to explore the gene complement and stress responses of other symbiont types, as well as their host corals, will be essential for predicting the fate of coral reefs in the region under future climate scenarios.

## DATA AVAILABILITY STATEMENT

Sequence data for transcriptome and small RNA analysis are available in the NCBI Sequence Read Archive under BioProject PRJNA386227 and PRJNA753218, respectively. Additional datasets supporting the conclusions of this article are included as **Supplementary Material** or available on Figshare ([https://figshare.com/projects/Transcriptome\\_analysis\\_of\\_Acropora\\_digitifera\\_endosymbionts/120309](https://figshare.com/projects/Transcriptome_analysis_of_Acropora_digitifera_endosymbionts/120309)).

## ETHICS STATEMENT

This study follows all prevailing local, national and international regulations and conventions, and normal scientific ethical practices. Collections were done with permission from the Department of Agriculture Bureau of Fisheries and Aquatic Resources of the Philippines (GP-0102-15).

## AUTHOR CONTRIBUTIONS

SFR and CC designed the study. SFR conducted the experiments. SFR, NP, and CC analyzed the data and wrote the manuscript.

All authors contributed to the article and approved the submitted version.

## FUNDING

This work was funded by the Department of Science and Technology Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (DOST-PCAARRD) Coral Genomics Program and a grant from the International Foundation for Science (A/5380-1) to CC. SFR was supported by the Department of Science and Technology ASTHRDP scholarship grant and thesis grants from the University of the Philippines Marine Science Institute and the Bolinao Marine Laboratory.

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