A microRNA regulates the response of corals to thermal stress

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Abstract

Coral reefs are diverse ecosystems of great ecological and economic importance. However, corals are vulnerable to a variety of stressors, including rising seawater temperatures, and yet little is known about the genetic mechanisms underlying their survival and adaptation to stress. Like other animals, corals possess genes for key members of the microRNA (miRNA) machinery. miRNAs are short RNAs that regulate diverse cellular processes, including organismal stress response, through post-transcriptional repression of gene transcripts. Through small RNA sequencing, we identified 26 miRNAs in the coral, *Acropora digitifera*. Many of the identified miRNAs are novel, while eight are conserved with miRNAs previously identified in other cnidarians. One of the identified miRNAs is differentially expressed in coral tissues exposed to acute thermal stress. This thermally responsive miRNA putatively regulates multiple pathways of the organismal stress response, DNA/RNA expression regulation, repair mechanisms, tissue morphogenesis, and signaling. We propose a model by which miRNA regulation allows the coral to mount a robust stress response through sequestration of a pool of non-translated transcripts encoding stress response proteins. Release of miRNA-mediated repression under stress conditions may result in rapid and abundant translation of proteins that help the coral maintain cellular homeostasis. These findings highlight the potential importance of miRNAs in the thermal resilience of corals.

Introduction

Coral reefs are diverse and productive ecosystems with great ecological and economic importance (Costanza et al. 1997). Philippine coral reefs provide annual economic benefits totaling about US $1.1 billion per year through services, including coastline protection, food security, tourism, and employment (Chou et al. 2002). However, coral communities worldwide are threatened by the increasing scale of anthropogenic impacts, particularly the continuing rise in ocean temperatures that is resulting in greater incidence of coral disease and bleaching (Carpenter et al. 2008; Hoegh-Guldberg et al. 2007; Hughes et al. 2003). Different corals exhibit differential susceptibility to stress, with the *Acropora* species that are the
dominant reef-building coral in the Indo-Pacific being one of the most sensitive to elevated sea surface temperatures (Loya et al. 2001). Various factors may influence and contribute to coral resilience. Exposure of coral larvae to heat stress causes the expression of heat shock proteins and results in changes in the protein translation machinery (Meyer et al. 2011). In adult corals, thermal stress causes differential expression of genes involved in oxidative stress, cellular homeostasis, and oxidation/reduction (Bellantuono et al. 2012). Preconditioning or pre-exposure to warmer temperatures provides corals with some degree of thermal tolerance (Bellantuono et al. 2012; Carilli et al. 2012) and corals in highly variable environments exhibit constitutive expression of genes that are thought to confer a protective effect (Barshis et al. 2013). The mechanisms that mediate the changes in gene expression in response to stress remain unknown and there is a need to look at the regulatory mechanisms that control the dynamic expression of genes in the face of environmental challenges.

microRNAs (miRNAs) have been shown to be critical in the maintenance of cellular homeostasis under stressful conditions (Ebert & Sharp 2012). miRNAs are 20-26 nt small regulatory RNAs sensu stricto (Fromm 2016; Fromm et al. 2015) that guide a complex of proteins to specific sequences in the 3’ untranslated regions of mRNAs, resulting in repression of translation or induction of degradation (Bartel 2009; He & Hannon 2004). They are found in almost all eukaryotic groups (Grimson et al. 2008; Wheeler et al. 2009) and are able to fine-tune mRNA and protein levels, allowing cells to maintain robust phenotypes despite the stochastic manner of gene expression or environmental perturbation (Schmiedel et al. 2015). In fact, knockdown of miRNA function typically has no observable effect unless the cells are subjected to an environmental stressor (Ebert & Sharp 2012). Cell stress has been found to affect localization of the miRNA silencing machinery (Detzer et al. 2010), suggesting that miRNAs are involved in mediating the changes in gene expression that are necessary for survival. Furthermore, miRNAs are implicated in heat stress response in C. elegans (Nehammer et al. 2015) and other mammals (Fukuoka et al. 2014; Hao et al. 2016; Yu et al. 2011; Zheng et al. 2014). In plants, many miRNAs have been found to
be directly linked to the stress regulatory network and are responsive to environmental stressors, including temperature fluctuations, salt concentrations, oxidative stress, UV exposure, nutrient deficiency, water stress, and pathogens (Zhu et al. 2011).

Cnidarians, such as corals, sea anemones and jellyfish, have an active miRNA regulatory pathway that may be critical in controlling various aspects of their development and environmental response. Some examples of these functions include the regulation of head regeneration in *Hydra magnipapillata* (Krishna et al. 2013) and possibly larval development in *Nematostella vectensis* (Moran et al. 2014). Despite the lack of information on their functions, cnidarians are known to possess a rich repertoire of miRNAs. Forty miRNAs were identified in the anthozoan *N. vectensis* by one study (Grimson et al. 2008), but this number has gone up to 87 from a deep-sequencing study conducted on early developmental stages (Moran et al. 2014). A recent study in the coral *Stylophora pistillata* revealed 31 miRNAs, 5 of which are conserved with metazoans (Liew et al. 2014). To date, there is no information on the miRNA complement of any common Indo-Pacific corals, such as *Acropora digitifera*. More importantly, the role of these coral miRNAs with respect to a dynamic environmental response still remains to be elucidated.

In this study, we show that the coral *A. digitifera* expresses a thermally responsive miRNA that is predicted to regulate many genes involved in the environmental stress response. We hypothesize that the function of this miRNA is to allow the coral to maintain a pool of non-translating transcripts encoding stress response proteins that can be rapidly translated upon release of miRNA repression under stress conditions. This miRNA-mediated mechanism may be a key component of the resilience of corals to thermal stress.

**Materials and Methods**

**Identification of microRNA machinery genes**
Detection of the coral gene homologs that make up the small RNA biogenesis and processing pathways was conducted using BLAST analysis against available sequence data for *Acropora digitifera* on the Compagen site (Hemmrich & Bosch 2008; Shinzato *et al.* 2011) and *Acropora millepora* (Transcriptome Shotgun Assembly on NCBI). Protein sequences for known components of the miRNA machinery were obtained from previous studies (Grimson *et al.* 2008; Moran *et al.* 2013) and from sequences deposited in NCBI.

**Collection of coral samples and thermal stress experiments**

Coral colonies were collected from Caniogan Reef, Bolinao, Pangasinan, Northwestern Philippines with permission from the Bureau of Fisheries and Aquatic Resources of the Philippines (GP-0076-14). Adult corals were fragmented into nubbins approximately 2 inches in length and allowed to heal and acclimate in aerated tanks with flowing seawater prior to experiments. Thermal stress experiments were conducted in tanks with seawater maintained at 4-8°C above ambient using submersible heaters. Controls were determined by the ambient temperature of incoming seawater and varied between experiments conducted at different times. Experiments for small RNA sequencing were conducted in August 2014. Coral fragments from different colonies were subjected to acute thermal stress at 34±1°C for 4 hours or at 31±1°C for 24 hours with the control temperature at 26±1°C. Experiments for transcriptome sequencing and miRNA qPCR were conducted in December 2014 and December 2016, respectively. For these experiments, coral fragments from different colonies were exposed to 34±1°C for 4 hours with the control temperature at 29±1°C. Coral larvae were collected in April 2014 at 9 days post-fertilization. Larvae pooled from multiple synchronously spawning colonies were placed in 15ml tubes at a density of 2 larvae per ml and subjected to thermal stress for 4 hours at 32°C with the control temperature at 28°C. Coral tissue samples were directly frozen in liquid nitrogen and larvae were stored at -20°C in RNAlater (Ambion) prior to RNA extraction.
RNA extraction, quality check and sequencing

Total RNA extraction was conducted using either the mirVana kit (Ambion) or the Trizol reagent (Invitrogen) following the manufacturer’s protocol. Contaminating genomic DNA was removed by using the DNA-free kit (Ambion). The quality of RNA for sequencing was further assessed using the Pico Series II assay on the Agilent Bioanalyzer 2100 System (Agilent Technologies). Independent biological replicates from separate thermal stress experiments were used for small RNA and transcriptome sequencing, as well as for qPCR analysis.

Small RNA sequencing and microRNA identification

Total RNA was size fractionated to select RNA 18-30nt in length. Small RNA libraries were prepared and subjected to 50 bp single-end sequencing on the Illumina HiSeq2000 platform (Beijing Genomic Institute, Hong Kong) at an average depth of 15 million reads per library (Table S1). Two small RNA libraries were sequenced for each condition, with one sample representing RNA from a single colony and the other containing pooled RNA from 3 to 4 colonies. *A. digitifera* sequencing reads were pooled (153,865,244 reads) and used to identify an initial reference set of coral miRNAs. Raw sequencing reads were first trimmed using Trimmomatic (Bolger et al. 2014) to remove low quality ends. The resulting reads were filtered to remove nucleotides with Phred scores of less than 30. Reads of length greater than and equal to 18nt with an average Phred score of 30 were retained for further analysis. miRDeep2 (Friedlander et al. 2012) was used to map miRNAs to the genome of *A. digitifera* v1.0 (Shinzato et al. 2011). Potential pre-miRNA precursor sequences were identified and folded using RNAfold (Mathews et al. 2004) to determine presence of a hairpin secondary structure. Predicted pre-miRNAs that had a miRDeep2 score of 10 or above were retained for further analyses and inspected manually. Bona fide miRNAs were those that passed the following additional criteria: (a) precursor miRNA minimum free energy of folding (MFE) less than -25 kcal mol$^{-1}$, (b) presence of a 2nt 3’ overhang for both mature and star strands, (c) consistent 5’ end position for the guide sequence, (d) no matches to known protein coding RNAs and other non-coding
RNA families. In addition, miRNA annotation criteria from Fromm et al. (2015) were applied, specifically, (e) expression of 20-26 nucleotide long reads for both strands, (f) at least 16nt complementarity between the two arms, and (g) at least 8nt loop sequence. In addition, potential miRNA homologs were mined by conducting miRDeep2 analysis on the Aiptasia genome (Baumgarten et al. 2015) using the A. digitifera small RNA sequencing reads as input and BLASTn alignment of A. digitifera mature and pre-miRNA sequences against Aiptasia sequences on the Reef Genomics webserver (Liew et al. 2016).

**Quantitation of microRNA expression**

Trimmed sequencing reads from each small RNA library were mapped to the A. digitifera genome. The number of reads mapping to predicted miRNA precursors was counted using the quantifier.pl script included in miRDeep2. Differentially expressed miRNAs in ambient versus elevated temperature setups were determined using DESeq (Anders & Huber 2010). miRNAs with an FDR-adjusted p-value ≤ 0.05 were considered to be differentially expressed.

**Determination of putative coral miRNA targets and GO enrichment of targets**

Targets of the bona fide miRNAs were predicted using miRanda (Enright et al. 2003) and the PITA algorithm (Kertesz et al. 2007). Briefly, miRanda determines putative targets using two criteria: (1) complementarity of mature miRNA to a given mRNA and (2) estimate of free energy formation ($\Delta G_{\text{duplex}}$) of the miRNA:mRNA duplex. miRNAs exert regulation by binding to the 3’ untranslated region (3’ UTR) of target mRNAs. For this reason, we used the 3’UTR sequences of A. digitifera predicted transcripts as input to miRanda. We invoked strict seed binding in the algorithm to prevent the prediction of non-canonical binding and gaps between miRNA and target mRNA. Only targets with $\Delta G_{\text{duplex}} \leq -10$ kcal/mole were reported. This set was further narrowed down by including only targets with an exact seed match and an A in position 1, as these criteria are said to be the top predictors of miRNA targets (Agarwal et al. 2017).
On the other hand, PITA incorporates both the $\Delta G_{\text{duplex}}$ (energy associated with miRNA:mRNA binding) and the $\Delta G_{\text{open}}$ (energy required to open mRNA secondary structure) to calculate $\Delta \Delta G$. Targets with $\Delta \Delta G \leq -10$ kcal/mole were retained for Gene Ontology (GO) enrichment analysis. For stringency, only targets with an 8-mer seed match containing no mismatches and no G:U wobble pairs were analyzed further. In addition, we checked the targets for more extensive complementarity to the miRNA using FASTA v36 (Pearson & Lipman 1988) and scored the alignments as previously described (Moran et al. 2014). Predicted peptides of A. digitifera were aligned to peptides in the UniProt database (UniProtKB 2013_12 release) using BLASTp with an e-value cutoff of $1 \times 10^{-5}$. GO annotation of sequence matches was retrieved using Blast2GO (Conesa et al. 2005). GO enrichment was implemented in topGO (version 2.16.0) using the weight01 algorithm (Alexa et al. 2006) with a threshold $p$-value $< 0.05$. The gene names for the top BLASTp hits of predicted target mRNAs were used as input into the STRING database v10.0 (Szklarczyk et al. 2015) to draw a protein interaction network based on the human interactome. The network of interacting proteins was visualized on Cytoscape v3.1.1 (Shannon et al. 2003).

Transcriptome sequencing and differential expression analysis

Barcoded cDNA libraries were prepared from rRNA-depleted and mRNA-enriched total RNA using the Illumina TruSeq RNA Sample Prep Kit protocol. Libraries were sequenced on the Illumina HiSeq2000 platform with 100bp paired-end reads (Beijing Genomic Institute, Hong Kong) at approximately 21 million reads per sample (Table S2). Two RNAseq libraries representing different coral colonies were sequenced for each condition. Raw sequences were filtered to remove adapter sequences and low-quality reads using Trimmomatic (Bolger et al. 2014). Trimmed forward end reads were mapped to the A. digitifera genome v0.9 (Shinzato et al. 2011) using Bowtie implemented under the RNASeq by Expectation Maximization (RSEM) protocol (Li & Dewey 2011). Differential expression analysis was performed using DESeq (Anders & Huber 2010) with read counts per transcript as input. Only transcripts...
with one or more mapped reads in at least 2 libraries were included in the analysis. Transcripts with an FDR-adjusted p-value ≤ 0.05 were considered to be differentially expressed.

miRNA reverse transcription and qPCR

Total RNA was extracted using Trizol. Contaminating DNA was removed using the DNAfree kit (Life Technologies). Purity of RNA samples was assessed spectrophotometrically by measuring $\text{OD}_{260/280}$ whereas RNA integrity was determined by visual inspection of two sharp bands on an agarose gel. Three hundred nanograms of total RNA was reversed transcribed using Taqman® MicroRNA Reverse Transcription kit (Life Technologies). Briefly, 0.075 µl of 100 mM dNTPs, 0.5 µl of 50 U/µl reverse transcriptase, 0.75 µl of 10x RT buffer, 0.095 µl of 20 U/µl RNase inhibitor, 2.08 µl nuclease-free H$_2$O, and 1.5µl stem-loop RT primer specific to the mature sequence of Adi-Mir-Novel-5_3p were mixed to synthesize the miRNA cDNA. Samples for reverse transcription were incubated at 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. qPCR was performed in a CFX96 Touch™ Real-Time PCR Detection System using Taqman® smRNA assay. Briefly, 0.5 µl of the Taqman® smRNA assay mix, 5 µl of Taqman® Universal PCR Master Mix II, No UNG (Life Technologies), 3.835 µl of nuclease-free water and 0.67 µl of RT product were mixed. qPCR cycles consisted of 95°C for 10 mins followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Each treatment was represented by four biological replicates from individual coral colonies. The assay was run with three technical replicates for each biological replicate along with triplicates of the no-reverse transcriptase (NRT) and no template controls (NTC). Primer efficiency was assessed using a dilution series. A stable reference gene, $A$. digitifera U6B, was used for the normalization of miRNA abundance. Custom qPCR primers for coral sequences were designed by Life Technologies (Table S3). Analysis of qPCR data was performed using the Bio-Rad CFX software.

mRNA reverse transcription and qPCR
RNA extraction, DNase-treatment, and quality check was performed as described above. Three hundred and fifty or seven hundred nanograms of total RNA was reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Briefly 1 μl of 10mM dNTP mix, 1 μl of random hexamers, 2 μl of 10x RT buffer, 4 μl of 25 mM MgCl₂, 2 μl of 0.1M DTT, 1 μl of RNase OUT, 1 μl of SuperScript reverse transcriptase were combined with template RNA and nuclease-free water then incubated according to manufacturer’s protocol. Primers were designed for selected targets using Primer-BLAST (Table S3). PCR cycling conditions were 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 5 seconds, and a melt curve at 65°C-95°C in 0.5°C increments at 5 seconds per step. Quantitative real-time PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System with SsoFast Evagreen Supermix (BioRad). Briefly, 5 μl of SsoFast mix, 0.3 μl each of 10μM forward and reverse primers, 3.4 μl of nuclease-free water, and 1 μl of cDNA were mixed. Three biological and three technical replicates were used in the quantitation of each gene alongside NTC and NRT controls. Primer efficiency and primer specificity was assessed using the dilution curves and melt curves, respectively. The actin gene was also amplified from each sample as reference.

Results

Identification of homologs of the microRNA machinery in corals

miRNAs originate from sequential processing of transcripts produced from miRNA genes. Homologs of some components of the miRNA biogenesis pathway and the silencing machinery have previously been identified in cnidarians (Grimson et al. 2008; Liew et al. 2014; Moran et al. 2013). These include members of the processing complex involved in cropping primary miRNA (pri-miRNA) to produce the precursor miRNA hairpin (pre-miRNA), namely Ars2, Drosha, and Pasha. Dicer, which is involved in the processing of the pre-miRNA into an RNA duplex that gives rise to the mature miRNA strand that is loaded into the RNA-induced silencing complex (RISC), is also represented. Similarly, RISC components, including Argonaute and GW182, are present in corals and other cnidarians. We further identified
homologs of other RISC proteins in corals, including the decapping enzyme DCP1, the exonuclease XRN, and Piwi, a member of the Argonaute family (Table S4). The members of the RISC complex trigger transcriptional repression or mRNA degradation resulting in translational inhibition of mRNA targets (Rehwinkel et al. 2005). The presence of the core small RNA biogenesis machinery and silencing complex supports the idea that this mode of gene expression regulation is active in corals.

Identification of coral microRNAs

Deep sequencing of A. digitifera small RNA libraries produced reads with a length distribution showing a small peak at 22nt, corresponding to the expected size of miRNAs (Fig 1A). A larger peak centered at 28nt may correspond to another class of small RNAs called Piwi-interacting RNAs (piRNAs). In fact, 28% of the 28nt reads possess a 5’ uracil and an adenine at the 10th position, a signature of piRNAs produced through the “ping-pong” mechanism (Brennecke et al. 2007). The reads from 18-30nt exhibit a strong bias for 5’ uracil, which is a typical characteristic of both miRNAs and piRNAs (Brennecke et al. 2007) (Fig 1A).

miRDeep2 analysis predicted 283 putative miRNAs, 162 of which have a miRDeep2 score greater than 10. Predicted miRNAs exhibit the typical stem-loop structure of miRNAs, with a strong strand bias where the majority of reads map to the mature strand (Fig 1B, Fig S1, and Data S1). Additional stringency filters were applied against this set of predicted miRNAs to reduce false positives. In total, 26 miRNAs were identified in A. digitifera (Table S5), which is similar to the number identified in the coral S. pistillata (Liew et al. 2014). Of these, 10 miRNAs passed all the stringency criteria that were applied, although 6 exhibited low miRDeep2 scores or low MFE values. Five out of the 26 miRNAs failed some of the stringency criteria but were retained because they matched the mature or seed sequence of conserved miRNAs in miRBase. It is worth noting that, similar to miRNAs reported in N. vectensis and S. pistillata (Grimson et al. 2008; Liew et al. 2014; Moran et al. 2014), 13 out of 26 predicted A. digitifera miRNAs
possess a loop shorter than the typical 8nt found in vertebrate miRNAs. We thus retained 3 other sequences as bona fide miRNAs given that they passed all criteria except for loop length and did not match sequences of known miRNAs. Eight out of 26 miRNAs were noted as equivocal miRNAs because they passed most of the criteria proposed by miRBase (Kozomara & Griffiths-Jones 2014) but failed some of the additional annotation criteria of Fromm et al. (2015). The presence in *A. digitifera* of the miRNAs Adi-Mir-Novel-1_3p, Adi-Mir-9425_5p, and Adi-Mir-Novel-18_5p, which have seed matches to *N. vectensis* miRNAs, mir-9437, mir-9425, and mir-9463, respectively, raises the possibility that these are all bona fide miRNAs. The lack of sequence read support for the star strands of these *N. vectensis* miRNAs, as reported in a previous study (Moran et al. 2014), may have been due to low sequencing depth.

The majority of the miRNAs that were identified in *A. digitifera* are of novel sequence but some show conservation in other animals ([Fig 1C](#)). Of the 26 predicted miRNAs in *A. digitifera*, 11 sequences matched *N. vectensis* miRNAs, 5 to *S. pistillata* miRNAs, and 2 to *H. magnipapillata*. *A. digitifera* possesses a miRNA similar to miR-100, which is conserved in cnidarians and bilaterians with possible loss in the hydrozoan lineage (Moran et al. 2014). Like in other cnidarians, *A. digitifera* miR-100 is offset by one nucleotide (Liew et al. 2014), however, it starts with a U instead of an A ([Fig S2](#)). Two *A. digitifera* miRNAs corresponding to miR-2022 and miR-2030 in miRBase are conserved in the cnidarians *S. pistillata, N. vectensis, and H. magnipapillata*. The hydrozoan, *H. magnipapillata*, expresses the 3’ arm of the mir-2030 hairpin whereas anthozoans express the 5’ arm. We further detected five out of the six miRNAs reported to be conserved in the anthozoans, *N. vectensis* and *A. digitifera* (Moran et al. 2014). Analysis of the recently published genome of the sea anemone, *Aiptasia* (Baumgarten et al. 2015), revealed potential matches for several *A. digitifera* miRNAs. This includes an exact match to the mature strand of Adi-Mir-2023_3p (mir-2023 family) and significant homology to its pre-miRNA sequence ([Fig S3](#)). In addition, we identified potential sequence matches to 12 of the 26 *A. digitifera* miRNAs, including Adi-Mir-Novel-5_3p, using BLASTn alignment against the *Aiptasia* genome ([Fig S3 and Table S6](#)). The
pre-miRNA sequence of six of these miRNAs exhibited partial alignment (BLASTn e-value < 0.05). Deep sequencing of the small RNAs in Aiptasia will be necessary to validate these potential miRNA homologs.

Relatively few studies have been done on cnidarian and coral miRNAs to date. Comparing A. digitifera miRNAs to those identified in S. pistillata (Liew et al. 2014) revealed no miRNAs specific to the scleractinian coral lineage that were present in both coral species. It is likely that additional sequences will be discovered through a more exhaustive interrogation of small RNAs in different species, developmental stages, and growth conditions (Krishna et al. 2013; Moran et al. 2014).

Many of the predicted bona fide miRNAs in A. digitifera are expressed in both larvae and adult stages (Fig 2A). Some miRNAs, such as Adi-Mir-100_5p, Adi-Mir-2036_3p, Adi-Mir-2050_3p, Adi-Mir-Novel-10_3p, Adi-Mir-Novel-11_3p, and Adi-Mir-Novel-18_5p are more abundant in the adult while Adi-Mir-Novel-2_5p, Adi-Mir-Novel-9_5p, and Adi-Mir-Novel-17_5p are more abundant in coral larvae (Table S7).

Identification of a thermal stress-responsive coral miRNA

Differential expression analysis revealed one miRNA, Adi-Mir-Novel-5_3p, that is differentially expressed in response to thermal stress. This miRNA is downregulated by approximately 50% in both coral adults and larvae subjected to short term exposure to elevated temperature based on small RNA sequence read counts (Fig 2B). Downregulation of Adi-Mir-Novel-5_3p was further validated by qPCR using an assay specific for the detection of the mature miRNA and that is able to discriminate among related sequences (Chen et al. 2005). A clear downregulation of Adi-Mir-Novel-5_3p was observed in corals subjected to higher temperature, although independent coral colonies showed variability in terms of the degree of miRNA downregulation (Fig 2B, Table S8).
To determine the function of the thermal stress responsive miRNA, Adi-Mir-Novel-5_3p, its mRNA targets were predicted using the miRanda (Enright et al. 2003) and PITA (Kertesz et al. 2007) algorithms. Target prediction methods work by searching for potential matches to the first 2-8nt or seed sequence of the miRNA on the 3’ untranslated regions (UTRs) of mRNAs. Aside from seed complementarity, both algorithms evaluate potential sites based on binding energy. The miRanda algorithm can also consider evolutionary conservation of target sites while PITA algorithm imposes no such restrictions (Liu et al. 2014). Furthermore, PITA examines 3’UTR target site accessibility to reduce false positives (Kertesz et al. 2007). Using these two methods, 191 mRNA targets were predicted for Adi-Mir-Novel-5_3p with 34 predicted by both methods, 54 predicted by miRanda (seed match+A1) only, and 103 predicted by PITA only (Table S9). All these targets possess canonical binding sites with an exact seed match to the miRNA. Further evaluation of the predicted targets revealed 29 transcripts with binding sites that have 16 or more nucleotides that are complementary to the miRNA and an average score of 6, based on the scoring method developed by Moran et al 2014 (Table S9). We did not identify any targets with perfect complementarity to the miRNA.

**Expression profiles of putative miRNA targets**

miRNAs regulate target mRNA expression through mRNA degradation and translational repression, thus mRNA targets behave in a manner reciprocal to their cognate miRNA (Bazzini et al. 2012; Djuranovic et al. 2012; Huntzinger & Izaurralde 2011). To validate predicted targets of Adi-Mir-Novel-5_3p, we performed differential expression analysis on RNAseq data from corals exposed to thermal stress. We observed that most of the predicted targets of Adi-Mir-Novel-5_3p show an increasing trend in expression that correlates with the decrease in miRNA abundance under thermal stress conditions (Fig 3A) but only 9.4% of predicted targets exhibit a statistically significant change in mRNA abundance at an FDR-adjusted p-value ≤ 0.05. Independent quantification of selected target transcripts by qPCR verify that these are indeed upregulated, although there is some variability with expression estimates based on
transcriptome read counts (Fig S4 and Table S8). The reciprocal pattern of expression for the miRNA and many of its predicted targets supports the idea that the miRNA exerts a repressive effect on these mRNAs and that repression is released upon exposure to stress. On the other hand, downregulation of some predicted targets may signify transcripts that may be modulated by other downstream regulatory controls or that are erroneous target predictions.

Functional analysis of the targets of the thermal stress-responsive coral miRNA

Individual miRNAs typically exert regulatory effects on numerous genes and thus can control multiple cellular processes. To determine the pathways that may be regulated by Adi-Mir-Novel-5_3p, we performed functional characterization by Gene Ontology (GO) enrichment analysis to identify gene categories that are overrepresented within the set of predicted targets of the miRNA (Fig 3B). Putative targets of Adi-Mir-Novel-5_3p are involved in multiple pathways that are activated under acute stress.

Stress activates signal transduction pathways that rapidly communicate external stimuli into the cell and induce intracellular responses. Receptor catabolism and activation of downstream kinase cascades initiate rapid intracellular responses, which ultimately result in global changes in gene expression through chromatin modification and transcription factor activity. The miRNA also regulates functions related to repair of DNA and RNA (Fig 3B). Intuitively, if a cell cannot cope with stress and repair DNA and cellular damage, the pathways associated with cell cycle arrest may be triggered.

Functions related to the immune response, including cytokine production and toll-like receptor signaling, are enriched in the targets of Adi-Mir-Novel-5_3p (Fig 3B). This is of interest particularly because the success of reef-building corals is largely due to their symbiotic relationship with the dinoflagellate, Symbiodinium, as well as with a diverse microbial and viral community (Rosenberg et al. 2007; van Oppen et al. 2009). Thermal stress events result in coral bleaching, which marks the breakdown of
symbiosis (Weis 2008). Upregulation of the immune response during thermal stress events may help to prevent the collapse of symbiosis, protect against pathogen invasion, and maintain tissue integrity (Palmer & Traylor-Knowles 2012). Enrichment of GO terms associated with tissue morphogenesis and the skeletal system further suggests that the miRNA controls functions that may be linked to biomineralization and deposition of the coral calcium carbonate skeleton.

The miRNA putatively influences genes in the circadian regulatory network (Fig 3B), which is important for synchronizing biological process with environmental stimuli. As shallow water species, corals are likely to have mechanisms in place that allow it to regulate metabolic activity to compensate for natural temperature fluctuations in the environment (Davy et al. 2012; Sorek & Levy 2012a, b).

Some enriched GO terms further hint at the complexity of gene regulatory mechanisms in corals. For instance, functions related to histone dephosphorylation, DNA demethylation, transcription, and RNA transcription corepressor activity (Fig 3B) are enriched in the targets of Adi-Mir-Novel-5_3p, pointing to a potential crosstalk between the miRNA and transcriptional machinery to regulate and sustain responses through fine-tuning of mRNA levels.

Furthermore, miRNAs can simultaneously regulate the expression of multiple transcripts at different levels of a regulatory network to achieve a robust response that may not be possible through direct regulation of single genes (Bracken et al. 2016). Thus, a network analysis was conducted on the predicted targets of Adi-Mir-Novel-5_3p based on existing annotations for their human homologs. Only interconnected proteins were retained in the final interaction network (Fig S5). Similar to results from GO analysis of the entire set of predicted miRNA targets, the dominant functions represented by the subset of genes within the network include stress response, RNA processing, protein processing, tissue morphogenesis, signaling,
and metabolism. This lends further support to the idea that the miRNA may regulate multiple genes that contribute to the organismal stress response.

Discussion

In this study, we identified members of the miRNA machinery in coral by interrogating publicly available transcriptomic and genomic data of acroporid corals. We also revealed the repertoire of miRNAs in *A. digitifera* through small RNA sequencing. Most of the miRNAs discovered in *A. digitifera* appear to be novel. Very few miRNAs are common with *S. pistillata*, another scleractinian coral. This comparison, however, is not enough to draw conclusions about the lineage-specificity of coral miRNAs. Further small RNA sequencing endeavors would be needed to explore the full diversity of the miRNA gene cohort in other coral species.

Animal evolution is marked by periods of miRNA gains and losses (Berezikov 2011; Tarver *et al.* 2013). New miRNAs can evolve from various sources of unstructured transcripts (Berezikov 2011) or can be created from existing miRNAs through point mutations, alternative processing, or post-transcriptional editing (Liu *et al.* 2008). Unlike highly conserved miRNAs, new miRNAs are often weakly expressed, imprecisely processed, and possess few targets (Cuperus *et al.* 2011). However, the functionality of a miRNA gene relies only on a short sequence complementarity with their target mRNAs. Thus, new miRNAs may be readily incorporated into existing gene regulatory networks. Functional miRNAs eventually evolve to regulate more mRNAs and become more abundantly expressed (Meunier *et al.* 2013). The flexibility of miRNAs have resulted in their incorporation into a wide range of biological processes (Berezikov 2011). The emergence of novel miRNA sequences may provide opportunities for combinatorial miRNA regulation that contribute to a greater potential for genetic robustness and morphological or functional complexity (Fromm *et al.* 2015; Peterson *et al.* 2009).
Thermal stress responsive miRNA

We identified a miRNA that is significantly downregulated in coral adults and larvae exposed to acute thermal stress. This miRNA is highly expressed and potentially regulates many target transcripts, all of which have perfect seed matches to the miRNA with some exhibiting more extensive complementarity. Cnidarian miRNAs have been shown to regulate their targets through cleavage of highly complementary mRNA target sites, similar to the typical mode of action of plant miRNAs (Moran et al. 2017; Moran et al. 2014). However, it is likely that cnidarian miRNAs may also function through translational inhibition of targets with imperfectly complementary binding sites, as has been reported in plants (Brodersen et al. 2008; Brodersen & Voinnet 2009; Dugas & Bartel 2008; Iwakawa & Tomari 2013). It should be noted that while target prediction methods are effective at identifying canonical binding sites for miRNAs, they tend to include false positive targets and overlook some bona fide targets, including those with effective non-canonical binding sites (Agarwal et al. 2015; Pendergrast & Volpe 2013; Peterson et al. 2014). There is also a possibility that many of the predicted transcripts are interactors rather than direct targets of the miRNA (Salmena et al. 2011; Wilczynska & Bushell 2014). This emphasizes the need for further experiments to test the ability of the coral miRNA to bind to its predicted targets and to cause either mRNA cleavage or translational inhibition. The predicted targets and their functional roles reported here indicate areas of interest that should be further validated using independent methods.

The predicted targets of the thermal stress-responsive coral miRNA coincide with genes that have been found to be differentially expressed under thermal stress conditions in other corals, including Orbicella faveolata (DeSalvo et al. 2008), Porites astreoides (Kenkel et al. 2013), Acropora tenuis (Yuyama et al. 2012), Acropora millepora (Bellantuono et al. 2012; Meyer et al. 2011), and Acropora hyacinthus (Barshis et al. 2013). Most of the predicted targets of the miRNA show only a small upregulation in gene expression under thermal stress, which may be attributed to the variability between independent coral colonies. Alternatively, this could suggest that the miRNA regulates many of its targets through
translational repression with little effect on mRNA quantity (Wilczynska & Bushell 2014). On the other hand, the downregulation of other predicted target transcripts suggests that some mRNAs may be indirectly regulated as a downstream effect of miRNA-mediated regulation of transcripts with functions related to transcriptional and translational regulation and signaling. Target transcript downregulation could also be an effect of the switching of miRNA function that is triggered by cell cycle arrest (Vasudevan et al. 2007). Further studies to determine association of Adi-Mir-Novel-5_3p-loaded RISC complexes with mRNA transcripts will better elucidate direct targets of the miRNA.

Functions that are putatively regulated by the thermal stress-responsive miRNA include heat shock proteins, genes involved in the oxidative stress response, maintenance of tissue integrity, and transcriptional and translational processes. By targeting multiple players in a signaling cascade, Adi-Mir-Novel-5_3p can coordinate the cellular program in a manner that is responsive to external stimuli. These functional analyses provide a starting point for future interaction studies to verify miRNA-target regulation. Although Adi-Mir-Novel-5_3p was not detected in the coral, S. pistillata, it is likely that other corals may also possess miRNAs that play a similar role in the regulation of the stress response. A miRNA-mediated regulatory mechanism may also be at play in the dinoflagellate symbionts of corals, as suggested by the presence of miRNAs in S. microadriaticum and S. kawagutii (Baumgarten et al. 2013; Lin et al. 2015).

**microRNAs and coral resilience**

Cells need to be able to balance temporal adaptive responses and the rapid kinetics of the stress response (de Nadal et al. 2011). Signaling mechanisms and adaptive responses need to be reversible and fast acting, a feature that requires a highly coordinated regulatory system. Because of the relatively slower process of protein production from mRNAs that are transcribed *de novo* in response to external stimuli, fast-acting responses, such as the translation of previously transcribed mRNAs, ensures that the cells are able to
quickly produce the proteins needed to deal with stress. The mode of action of miRNAs makes them perfectly suited for these rapid gene expression requirements.

In responding to stress, cells can restore or reprogram their gene expression patterns through the regulation of miRNA and mRNA target abundance or by modulating the activity or mode of action of miRNA-protein complexes (Leung & Sharp 2010). miRNAs can influence the global gene expression pattern during stress by subjecting target transcripts to degradation or by sequestering them to inhibit translation. miRNAs can also determine the endpoint of the cellular response, whether homeostasis will be restored or whether a new gene expression program will be implemented to adapt to the new environmental conditions (Leung & Sharp 2010).

miRNAs enhance evolvability by canalization, a process of evolved robustness through the stabilization and precise control of regulatory networks to reduce the effect of the stochastic gene expression (Ebert & Sharp 2012; Hornstein & Shomron 2006; Peterson et al. 2009). They may thus be an important factor in the evolution of resilience in corals. It has been shown that corals exposed to highly variable temperatures “frontload” or constitutively express certain genes at higher levels (Barshis et al. 2013; Palumbi et al. 2014). It is tempting to speculate that these frontloaded genes represent a pool of transcripts under miRNA-mediated translational repression. Release of miRNA-mediated repression in the presence of stress may result in rapid translation of the encoded proteins, which protect the cells during stress, restore cellular homeostasis, maintain tissue integrity, and provide adaptive capacity to survive the change in environment (Fig 4). However, further experiments are needed to test the hypothesis of miRNA-mediated translational repression of constitutively expressed genes in corals.

In conclusion, this study elucidates the potential role of miRNA regulatory mechanisms in the coral thermal stress response. The presence of members of the miRNA machinery and a unique set of miRNAs
in the coral, *A. digitifera*, support the importance of miRNA-mediated regulatory networks in corals. The involvement of miRNA regulatory mechanisms potentially provides the coral with the ability to mount a rapid protective response under adverse conditions. Corals that are adapted to higher temperatures or large temperature variations constitutively and abundantly express many genes in readiness for stress events (Barshis et al. 2013). The translational repression or expression fine-tuning of these mRNA transcripts under normal conditions may be one important function of coral miRNAs. This study provides a glimpse into the diversity of genes that may be regulated by a thermal stress responsive miRNA and points to the potential role of miRNAs in the adaptive response to thermal stress and the evolution of coral resilience. In light of climate change and its continuing effects on coral reef ecosystems, it is important to arrive at a more comprehensive understanding of the mechanisms that underlie coral responses to the environment. This information and the tools that evolve from it will be a vital contribution to ongoing efforts to enhance reef resilience (van Oppen et al. 2015).

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**References**


**Data Accessibility**

The data used in this paper have been deposited in the NCBI Short Read Archive as BioProject PRJNA298499 for small RNA sequencing and PRJNA298496 for transcriptome sequencing.

**Author Contributions**

C.C. designed the research. A.P.G. performed experiments. A.P.G. and C.C. analyzed data and wrote the paper.

**Conflict of Interest**

The authors declare no conflict of interest.
Fig 1. miRNA identification in *A. digitifera*. (A) Sequence length distribution of pooled sequencing reads from *A. digitifera* small RNA libraries showing the proportion of reads starting with A, C, G or U. The small peak at 22nt corresponds to the expected length of miRNAs. The larger peak at 28nt may include reads from Piwi-interacting RNAs. (B) Predicted precursor stem-loop structure of Adi-Mir-Novel-5 showing the mature strand (red), hairpin loop (yellow), and star strand (blue). The 2nt overhang at the 3’ ends of the mature and star strand are marked by black lines. The frequency of read mapping to the different parts of the precursor sequence is represented by the line graph. Numbers above the line represent the number of mapped reads. (C) Origins of conserved miRNAs in cnidarians. The number of conserved miRNAs at each ancestral node is indicated in parentheses. Numbers in boxes are the total number of miRNAs reported in each cnidarian species.
Fig 2. miRNA expression under thermal stress. (A) Expression of *A. digitifera* miRNAs based on small RNA read counts in larval and adult tissues subjected to different temperatures. Row-scaled expression values of the miRNAs are shown to better visualize the differences between samples subjected to control or elevated temperatures (red, high; blue, low). Heatmap columns represent small RNA sequence libraries and rows are individual miRNAs. The figure was generated using pheatmap in R. (B) Differential expression of Adi-Mir-Novel-5_3p in adult tissue and larvae subjected to acute thermal stress. Box plots show the degree of change in miRNA expression in different tissues subjected to different temperature regimes. Open circles represent the values for each replicate library (small RNA sequencing) or replicate colony (qPCR) and thick lines represent the average log₂ fold change. Asterisks indicate significant differential expression based on DESeq (FDR-adjusted p-value <0.05). The number of biological replicates for each independent experiment is shown below the bars.
Fig 3. Expression and gene ontology enrichment of predicted targets of Adi-Mir-Novel-5_3p. (A) Volcano plot showing the change in expression versus FDR-adjusted p-value for predicted miRNA targets in tissues subjected to elevated temperature. Predicted miRNA targets are shown in blue, targets with more extensive complementarity are in red, and other coral transcripts are in gray. (B) Gene ontology (GO) analysis for the predicted targets of Adi-Mir-Novel-5_3p. Enrichment p-values for selected functions are shown.
Fig 4. Model of miRNA-mediated regulation of stress response genes. The miRNA potentially represses transcripts that protect cells against damage, repair tissues, and modify the global gene expression profile to effect a sustained response. Downregulation of the miRNA during stress exposure may release transcripts from repression and promote their rapid translation. Negative regulation is indicated by bars; positive regulation is represented by arrows.