Identification of MicroRNAs in the Coral *Stylophora pistillata*

Yi Jin Liew¹,², Manuel Aranda¹, Adrian Carr², Sebastian Baumgarten¹, Didier Zoccola³, Sylvie Tambutté³, Denis Allemand³, Gos Micklem²*, Christian R. Voolstra¹*

1 Red Sea Research Center, King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia; 2 Cambridge Systems Biology Centre & Department of Genetics, University of Cambridge, Cambridge, United Kingdom; 3 Centre Scientifique de Monaco, Monaco, Monaco

**Abstract**

Coral reefs are major contributors to marine biodiversity. However, they are in rapid decline due to global environmental changes such as rising sea surface temperatures, ocean acidification, and pollution. Genomic and transcriptomic analyses have broadened our understanding of coral biology, but a study of the microRNA (miRNA) repertoire of corals is missing. miRNAs constitute a class of small non-coding RNAs of ~22 nt in size that play crucial roles in development, metabolism, and stress response in plants and animals alike. In this study, we examined the coral *Stylophora pistillata* for the presence of miRNAs and the corresponding core protein machinery required for their processing and function. Based on small RNA sequencing, we present evidence for 31 *bona fide* microRNAs, 5 of which (miR-100, miR-2022, miR-2023, miR-2030, and miR-2036) are conserved in other metazoans. Homologues of Argonaute, Piwi, Dicer, Drosha, Pasha, and HEN1 were identified in the transcriptome of *S. pistillata* based on strong sequence conservation with known RNAi proteins, with additional support derived from phylogenetic trees. Examination of putative miRNA gene targets indicates potential roles in development, metabolism, immunity, and biomineralisation for several of the microRNAs. Here, we present first evidence of a functional RNAi machinery and five conserved miRNAs in *S. pistillata*, implying that miRNAs play a role in organismal biology of scleractinian corals. Analysis of predicted miRNA target genes in *S. pistillata* suggests potential roles of miRNAs in symbiosis and coral calcification. Given the importance of miRNAs in regulating gene expression in other metazoans, further expression analyses of small non-coding RNAs in transcriptional studies of corals should be informative about miRNA-affected processes and pathways.

**Introduction**

Scientific curiosity about corals has recently intensified, following observations of the deterioration of coral reefs at an unprecedented rate worldwide — for instance, in the Caribbean, Hughes [1] reported that coral cover has declined from over 50% in the 1970s to less than 5% in the 1990s; in the Indo-Pacific region, home to 75% of the world’s coral reefs, Bruno and Selig [2] estimated that coral cover declined ~1% annually in the past 20 years, and ~2% annually between 1997–2003. This trend is worrying, as coral reefs are important ecosystems, supporting more marine biodiversity per unit area than any other marine habitat [3]. There are many reasons behind the global decline of coral reefs, which include, but are not limited to, accelerated warming and acidification of oceans [4,5], overfishing [1], pollution [6,7], and disease [8].

In recent years, the increasing use of genomics has broadened our understanding of basic coral biology. The genome sequence of the coral *Acropora digitifera* [9] revealed a potential dependency of some coral species on their symbiont population for synthesis of an essential amino acid, and highlighted an unexpectedly diverse repertoire of immune-response genes [9]. Furthermore, microarray and RNA sequencing studies on several coral species have shed light on their responses to environmental cues at the transcriptional level. Shifts in transcriptional landscapes have been noted, based on the composition of symbionts in the coral cell [10,11], or as a response to stressors such as increased temperatures [12–15]; long-term darkness [16]; elevated CO₂ levels [17,18], and ultraviolet radiation [19]. Despite the increasing accumulation of genomic data, some aspects of the molecular machinery potentially involved in these processes, such as microRNAs (miRNAs), have yet to be studied in corals.

miRNAs are a class of small non-coding RNAs of ~22 nucleotides (nt) in length, which regulate gene expression through posttranscriptional degradation or translational repression via the RNA interference pathway (RNAi) [20–22]. Recent studies in plants and metazoans have discovered pivotal roles for miRNAs in regulating developmental timing [23–25]; cell cycle progression [26,27]; immune response [28,29]; metabolism [30]; response to stress [31–33]; and potentially biomineralisation [34–36]. miRNAs have been identified in more than 200 species that span major kingdoms of life: animals, plants, and protists (based on miRBase v20, June 2013) [37–40]. miRNAs have also been identified in the...
MicroRNAs in Corals

Genome and transcriptome of the coral symbiont Symbiodinium microadriaticum [41] as well as in the genomes of two other cnidarians: Chapman et al. [42] reported 17 miRNAs for Hydra magnipapillata, while Grimson et al. [43] reported 40 miRNAs in the sea anemone Nematostella vectensis. The large evolutionary distance from Hydra and Nematostella to corals (~500 million years [9]) warranted a search for the presence of miRNAs and the corresponding RNAi machinery in scleractinian corals. Here we present a first assessment of the miRNA repertoire, the RNAi machinery, and putative gene targets in the scleractinian coral S. pistillata from the Red Sea.

Materials and Methods

Ethics statement

Corals were kept in accordance with recommendations by the Centre Scientifique de Monaco and appropriate guidelines for the Care and Use of Laboratory Animals (Permit Number DCI/89/32).

Growth conditions of S. pistillata

S. pistillata was maintained in aquaria at the Centre Scientifique de Monaco, Principality of Monaco, in controlled culture conditions: semi-open circuit, Mediterranean seawater heated to 25 ± 0.5°C, salinity of 38.2 psu, illuminated with HQI-10000K; BLV-Nepturion at a constant irradiance of 175 μmol photons m⁻² s⁻¹ on a 12 h:12 h day:night light cycle. Corals were fed three times a week with a mix of Artemia salina nauplii and A. salina frozen adults and frozen krill.

mRNA sequencing and transcriptome

Total RNA extraction was performed as described previously [44]. Briefly, rubbins of coral (sampled at noon) were snap-frozen in liquid nitrogen and ground into powder in a cryogrinder (Freezer/Mill 6770, Spex Sample Prep) and then extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Total RNA was quality-checked using a Bioanalyzer 2100 (Agilent, Santa Clara, CA) and a Nanodrop 2000c (ThermoScientific, Wilmington, DE) prior to library creation and sequencing by the KAUST Biosciences Core lab. For mRNA sequencing, paired-end reads for Illumina sequencing were generated from oligo-dT selected total RNA using the Illumina TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) according to manufacturer’s instructions. A total of 152,552,099 paired-end read pairs (read length: 101 bp, insert size: 175 bp) were sequenced on the HiSeq 2000 platform (Illumina, San Diego, CA).

For the transcriptome assembly, sequence adaptors were trimmed from the raw sequences and low quality ends were cut with trimmomatic [45]. The remaining read pairs were subjected to digital normalization with Diginorm at k = 20 and C = 20 [46], reducing the dataset to 51,023,864 read pairs. Further, in order to remove contaminating sequence information from endosymbiotic dinoflagellates, remaining read pairs were mapped to the transcriptome of Symbiodinium microadriaticum [41] using Bowtie 2 [47]. This resulted in 38% of the remaining read pairs being mapped to the S. microadriaticum transcriptome, and a significant reduction in potential chimeric locus assemblies for the remaining 16,555,086 read pairs.

The transcriptome was assembled with Oases [48] using k-mer values ranging from 29 to 69. To reduce redundancy within single k-mer assemblies, only contigs with a minimum coverage of 7 were reported. Based on contig lengths, number of distinct loci, and number of transcripts, single k-mer assemblies from k = 45, 47, 49 were reassembled at k = 27, resulting in a final transcriptome assembly of 43,493 unique loci genes ≥250 bp (Supporting Information S1). For gene annotation, the longest transcript per loci was subjected to a BLASTX search (minimum e-value threshold of 10⁻⁵) against three protein databases: UniProtKB/Swiss-Prot, UniProtKB/TreEMBL, and the non-redundant GenBank nr in a subsequent manner. Hits were selected preferentially from Swiss-Prot to Swiss-Prot is manually curated, followed by TreEMBL if no matches were found in Swiss-Prot, and lastly from nr if neither Swiss-Prot nor TreEMBL yielded hits. Out of the 20,392 transcripts with an annotation, 15,177 (77.6%) were from Swiss-Prot; 4,964 (24.4%) were from TreEMBL; and 193 (0.93%) were from nr (Supporting Information S2). For transcripts with annotations from Swiss-Prot or TreEMBL, a script was written to assign GO (Gene Ontology) terms (and their parent GO terms) from UniProt-GOA [49]. 14,558 (95.9%) of the Swiss-Prot hits and 1,955 (39.4%) of the TreEMBL hits had at least 1 GO term assigned to it (Supporting Information S2).

Identification of core RNAi proteins

In order to identify homologues of the RNAi machinery in S. pistillata, sequences from six families of proteins (Argonaute, Dicer, Piwi, Drosha, Pasha, andHEN1) were drawn from five organisms (H. sapiens, D. melanogaster, C. elegans, S. pombe, and A. thaliana). These sequences were obtained from the UniRef100 database [50], and clustered into groups with 90% sequence identity using CD-HIT [51] to remove near-identical sequences. The clustered sequences were used in a TBLASTN search against the S. pistillata transcriptome to identify candidate RNAI-related transcripts. Identified homologues (TBLASTN e-value<10⁻¹⁰) of known RNAi proteins were then searched for domains that are required for the function of the protein using InterProScan [52–54]. The domains that were determined to be essential for function were: a Paz and Piwi domain for Argonaute and Piwi; a pair of RNase III domains for Dicer and Drosha; a double-stranded RNA binding domain for Pasha; and a methyltransferase (MTase) domain for HEN1. Candidate homologues were not considered further in the absence of any of these domains.

Additional support for the inferred function of candidate homologues was obtained by carrying out a reciprocal BLASTP search of these translated candidates against all proteins in the Swiss-Prot database [55] (Supporting Information S3). The candidate homologues were aligned against known RNAI proteins on a per-family basis using Clustal Omega [56], and the alignments were visualised using Jalview [57,58]. Key residues were derived from literature [59–64]. In addition, for each of the six protein families, phylogenies were constructed by aligning our candidate homologues with selected sequences from Grimson et al. [43] and Moran et al. [63] with MUSCLE [66]. Aligned regions of low quality were removed with TrimAl, using the in-built “gappyout” parameter [67] (Supporting Information S4). ProtTest [68] was used to determine the best model for amino acid substitution, and MEGA (version 6) [69] was used to construct maximum-likelihood phylogenetic trees (support values were computed from 1,000 bootstrap replicates).

Small RNA sequencing and processing

The small RNA (smRNA) fraction from S. pistillata was selectively enriched from isolated total RNA (see above) using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to manufacturer’s instructions. The small RNA fraction was quality-checked using a Bioanalyzer 2100 (Agilent, Santa Clara, CA) and a Nanodrop 2000c (ThermoScientific, Wilmington, DE). The small RNA library was created using the Illumina Small RNA
Sample Prep Kit (Illumina, San Diego, CA) according to manufacturer’s instructions, and sequenced on 1 lane on an Illumina Genome Analyzer Ix (GA2x) machine. A total of 30.5 million small RNA reads of <40 bp in length were produced. The reads, along with associated Phred quality scores for each sequenced base, were saved in a FASTQ file.

The raw FASTQ file was processed using several scripts to remove low-quality reads resulting in a more compact FASTQ file that contained high-quality reads for downstream analyses. First, low quality 3’ ends were trimmed from the reads. The 3’ end of the resulting reads had a Phred score >20, while the average Phred score of the entire read was >20 as well. After trimming, the overall error rate of the reads was calculated from the Phred scores of individual bases. Reads were discarded if the error rate exceeded 2%. Subsequently, the Illumina 5’ and 3’ adapter sequences used in library generation were trimmed off using Cutadapt v1.0 [70]. Last, in order to remove fragments of rRNA, sequences used in library generation were trimmed off using reads that matched known mRNA sequences.

For GO enrichment of target genes, we used topGO (version 2.12.0), an R script that is available through Bioconductor 2.0. topGO is a scoring algorithm that improves GO scoring by eliminating local dependencies between related GO terms [75]. The threshold for significance was set at P<0.01, using otherwise default topGO “weight01” settings, which produced GO terms that were significantly enriched in the set of transcripts targeted by each miRNA. The resulting P values were not corrected for multiple testing, as non-independent tests are carried out on each GO term by topGO [75].

**Results**

**Identification of core RNAi proteins**

The miRNA machinery that processes and mediates the function of miRNAs encompasses several key components that appear to be conserved across the animal kingdom [76]. In order to establish the presence of a functional miRNA machinery in *S. pistillata* we conducted a BLAST-based search for key proteins known to be essential for miRNA processing and function.

We identified seven candidate genes that are homologues to known RNAi proteins: one Argonaute, two Piwi, one Dicer, one Drosha, one Pasha, and one HEN1 in *S. pistillata*. We employed several key metrics (i.e. matches to known RNAi families, presence of protein domains crucial for catalytic activity, and a reciprocal BLAST search against manually curated proteins in Swiss-Prot) to identify candidate RNAi proteins (Supporting Information S3).

The per-family alignments of candidate homologues against known sequences revealed a striking conservation of functionally important amino acid residues located within the key protein domains. Examples include the strong conservation of the DDX triad in the Piwi domain of the Argonaute and Piwi homologues; the aspartate and glutamate residues essential for Dicer activity; and the pair of alanine/alanine and alanine/serine dipeptides involved in the binding of dsRNA in Pasha (Supporting Information S5, S6, S7, S8, S9, S10). Maximum-likelihood phylogenetic trees that were constructed for all six protein families (Figures 1A–1F) placed all of the candidate *S. pistillata* homologues with those from other cnidarians. Judging from the presence of the key RNAi proteins in *S. pistillata* in comparison to other organisms, the RNAi machinery in *S. pistillata* is similar in composition to those from sea anemone, worm, fruit fly, and humans (Table 1).

Besides the core RNAi proteins, we have also discovered transcripts that are candidate homologues of HYL1 (one), GW182 (two), and RdRP (RNA-dependent RNA polymerase, eight) (data not shown). HYL1 is thought to be a plant-specific partner to Dicer [77], whereas GW182 helps Argonaute repress its targets [78]. Both proteins have recently been discovered in four cnidarians (*Acropora digitifera, A. mallepora, Hydra vulgaris, Nematostella vectensis*). However, although we could identify the PAME2 and P-GG motif in one of our GW182 homologues, there were very few GW repeats in this homologue (1 in *S. pistillata*, compared to 14 in *N. vectensis* and 40 in humans) [65]. RdRPs, using small RNAs as templates, amply the silencing effect by directing the production of secondary dsRNAs [79]. Functional RdRPs have been discovered in plants [25,60] and *C. elegans* [81], but not in mammals nor flies [79]. Four candidate homologues of RdRPs have been found in *N. vectensis* [82], indicating that RdRPs might be present in cnidarians.

**Small RNA sequencing and miRNA repertoire**

Sequencing produced 30,543,433 reads, of which 23,830,932 reads (78.0%) were kept after adapter trimming. The additional step of removing short reads that matched known tRNA, rRNA, and miRNA sequences, Velvet [71] was used to assemble miRNA prediction and filtering

We used miRDeep2 [72] to identify miRNAs. Briefly, miRDeep2 mapped smRNAs to a preliminary draft genome of *S. pistillata* using Bowtie, discarding reads that occurred more than five times in the genome to avoid mapping to repetitive elements. Potential pre-miRNA precursor sequences were identified based on the pattern of the mappings, and subsequently folded using RNAfold to ascertain whether they had the canonical hairpin secondary structure [72]. Predicted pre-miRNAs that had a miRDeep2 score of 10 or above were retained for further analyses and inspected manually. A script was written to produce additional information not found in the miRDeep2 output (i.e. length of 3’ overhang, proportion of reads with consistent 5’ end, number of mismatched bases in stem) to further select a set of *bona fide* miRNAs. Conserved miRNAs were identified using BLASTN against all previously identified pre-miRNA sequences in miRBase (ver 20) [37–40].

**Functional analysis of miRNA targets**

ORFs were identified in the transcripts using TransDecoder (part of the Trinity software pipeline [73]). Sequences downstream of the longest ORF identified in the transcripts were treated as the 3’ UTR of the transcript. 3’ UTRs under 100 bp were filtered out to remove transcripts associated with short UTR sequences. Out of the 43,493 genes, 14,125 transcripts (32.4%) had a predicted 3’ UTR longer than 100 bp.

For each miRNA, we ran PITA (Probability of Interaction by Target Accessibility) [74] on the 3’ UTRs at default settings to produce a set of putative genes targeted by the miRNA. In the absence of genomic data from other closely related organisms, PITA achieves higher sensitivity and specificity than other target prediction software (e.g. miRanda, TargetScan) as the latter algorithms rely on a filter based on evolutionary conservation to reduce the false positive rate. PITA works by calculating the difference in Gibbs’ free energy (ΔG) between the energy that is required to unfold the secondary structure of the target site (ΔG_unfold) and the energy of the mature miRNA binding its target (ΔG_signature) [74]. Only miRNA targets with a ΔG of ≤−10 kcal mol⁻¹ were retained.
and transcript sequences removed a further 7.2% of reads, resulting in a dataset that contained 21,625,195 reads of at least 10 bp in length. Most of these reads were of 20–31 nt in length. Relative frequencies of the starting 5′ nucleotide showed a clear enrichment of 5′-terminal uracil among short reads of length 26–31 nt (Figure 2), which is consistent with the likely presence of functional Piwi-interacting RNAs (piRNAs) in *S. pistillata* [43].

A total of 2,811,736 reads (of length >17 nt) were mapped to a preliminary assembly of the *S. pistillata* genome and 46 distinct miRNAs were predicted by miRDeep2, of which a subset of 30 miRNAs passed additional filtering criteria (see Materials and Methods). An exception was made for spi-miR-temp-25 – although the precursor had a 3 bp 3′ overhang, it was included in the *bona fide* set as it was a close match to two known miRNAs. The resulting 31 miRNAs were used in all downstream analyses (Table 2, Supporting Information S11).

While the majority of these 31 predicted miRNAs were novel, 5 of them matched conserved miRNAs. spi-miR-temp-25, the predicted miRNA with the highest miRDeep2 score, was highly similar to the known miR-100 family of sequences (~2 mismatched bases, Figure 3A). This family is known to be conserved across Eumetazoa, including at least two other cnidarians (*N. vectensis* and *Metridium senile*) [43,83–87]. spi-miR-temp-25 was similar to miR-2022 from *N. vectensis* [43] and *H. magnipapillata* [88] (Figure 3B), while the other three miRNAs – spi-miR-temp-4, spi-miR-temp-40, and spi-miR-temp-30 – were similar to miR-2023, miR-2030, and miR-2036 found in *N. vectensis* [43], respectively (Figures 3C–E). For reasons of clarity, these five conserved miRNAs in *S. pistillata* will be referred to as...
Table 1. Presence of RNAi proteins in *S. pistillata* in comparison to Cnidaria and other model organisms (+: presence, −: absence, ?: not determined).

| Organism          | Ago | Piwi | Dicer | Drosha | Pasha | HEN1 |
|-------------------|-----|------|-------|--------|-------|------|
| Cnidaria          | S. pistillata | +   | +     | +      | +     | +    |
| N. vectensis      |     |      |       |        |       |      |
| A. millepora      | +   | ?     | +     | +      | +     |      |
| A. digitifera     | +   | ?     | +     | +      | +     | −    |
| H. vulgaris       | +   | ?     | +     | +      | +     |      |
| H. magnipapillata | +   | +     | +     | +      | ?     | ?    |
| Other             | H. sapiens | +   | +     | +     | +     | +    |
| C. elegans        |     |      |       |        |       |      |
| A. thaliana       | +   | −     | +     | −      | −     | +    |
| S. pombe          |     |      |       |        |       |      |

doi:10.1371/journal.pone.0091101.t001

Although nve-miR-100 has been identified in two separate studies (both of which utilised next-generation sequencing of short reads to identify miRNAs in basal metazoans), Grimson et al. [43] and Wheeler et al. [88] predicted mature miR-100 sequences which are offset by a single nucleotide. From our read data, we had >30,000 reads that exactly match the nve-miR-100 from Grimson et al. [43], but none that matched the alternative version from Wheeler et al. [88]. A negligible minority of reads (~10) did start one nucleotide upstream, but unlike the Wheeler et al. [88] version, this residue was an adenine, making this form identical to the hsa-, dme- and xtr-miR-100s.

Functional analysis of putative miRNA targets

In order to identify processes in *S. pistillata* that are potentially regulated by miRNAs, we conducted a GO term enrichment analysis. Briefly, for the set of 31 miRNAs, we searched for animal-like target sites in the 3' UTRs of those 16,513 *S. pistillata* genes that had available 3' UTR and GO annotation. This analysis was performed for all miRNAs individually, and indicated that miRNAs are likely to play roles in diverse processes (Table 3, Supporting Information S12).

We categorised the resulting enriched GO terms under several high-level groups based on known miRNA function – “immunity”, “biomineralisation”, “transcription”, “cell cycle”, “cytoskeleton”, “metabolism”, “transport/signalling”, and “differentiation/development”. Other GO terms that did not fall under one of these umbrella terms were categorised under “miscellaneous” (Supporting Information S12).

We paid particular attention to the “immunity” and “biomineralisation” high-level groups, as we considered these terms to be specifically relevant to the understanding of coral physiology (Table 3). For the former, it is likely that immunity-related transcripts are involved in the formation and retention of symbiotic relationships between the coral host and its *Symbiodinium.*
symbionts. Five miRNAs were predicted to be involved in this regulation, with a large fraction of the predictions involving spi-miR-temp-15. For the latter, 10 miRNAs were predicted to be involved in the formation of the calcium carbonate-based coral skeleton, with none of the 10 miRNAs being predominant in the predictions.

**Discussion**

**Identification of core RNAi proteins**

Advances in our understanding of miRNAs have shown that these small molecules have a big impact on the regulation of gene expression. While the biogenesis and downstream functions of miRNAs have been fairly well studied in the primary model organisms, little is known about the presence or function of these miRNAs in corals. In this study, we identified the presence of core RNAi proteins encoded by the *S. pistillata* transcriptome. The alignment of our candidate homologues against known RNAi proteins revealed the conservation of key protein domains and residues crucial for protein function. Additionally, maximum-likelihood phylogenetic trees of our candidate homologues with RNAi proteins from other cnidarian and model organisms showed broad agreement with those from other studies [43,65] – all of our seven candidate homologues clustered best with those from other cnidarians, as expected from its closer phylogenetic distance to other cnidarians than to bilaterians or poriferans. This also signifies that our homologues originate from the coral host, not from its dinoflagellate symbionts. Interestingly, the *S. pistillata* Dicer homologue clustered better with *N. vectensis* Dcr2, which is thought to be involved in processing of long dsRNA into siRNAs, and not associated with the biogenesis of miRNAs [65,89]. A reverse search of our candidate Dicer homologue against the *S. pistillata* draft genome revealed an open reading frame that encodes for another Dicer-like protein, which appears to be a good match (e-value of $1 \times 10^{-16}$) of *N. vectensis* Dcr1 (data not shown). However, the absence of transcriptomic support for that open

---

**Table 2. Set of 31 bona fide miRDeep2-predicted miRNAs in *S. pistillata***

| miRNA name | Predicted mature miRNA (5’ – 3’) | Matches to known miRNAs |
|------------|----------------------------------|-------------------------|
| spi-miR-temp-1 | accgccugauccgacuccugugg | miR-100 family |
| spi-miR-temp-2 | uacgcaucucgcuaaagaga | |
| spi-miR-temp-3 | uacgccgagugagagaaugugu | |
| spi-miR-temp-4 | aacaggauagggagagaaugaa | nve-miR-2023 |
| spi-miR-temp-5 | gaggucggagggagaga | |
| spi-miR-temp-6 | uacgcaccaucucgcuuuuagagg | |
| spi-miR-temp-7 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-8 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-9 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-10 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-11 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-12 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-13 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-14 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-15 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-16 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-17 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-18 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-19 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-20 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-21 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-22 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-23 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-24 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-25 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-26 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-27 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-28 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-29 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-30 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-31 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-32 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-33 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-34 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-35 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-36 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-37 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-38 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-39 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-40 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-41 | uacgcacacucucgcuuuuagagg | |
| spi-miR-temp-42 | uacgcacacucucgcuuuuagagg | |

1 Note: the names of the miRNAs are temporary. miRBase (the miRNA registry) only accepts submissions of new miRNAs after the manuscript has been accepted for publication.

doi:10.1371/journal.pone.0091101.t002
reading frame excluded it from being a candidate Dicer in *S. pistillata* in this study. Nonetheless, both observations serve to indicate the presence of a functional miRNA-processing machinery in *S. pistillata*. This, to our knowledge, has not been demonstrated previously for any other coral.

**Small RNA sequencing and miRNA repertoire**

Besides a functional RNAi machinery, and based on our analysis of short reads, we also predicted the presence of 31 *bona fide* miRNAs (out of a total of 46), of which 5 were conserved: the miR-100 family found in many other metazoans; miR-2022, which is conserved in *N. vectensis* and *H. magnipapillata*; miR-2023, miR-2030, and miR-2036, which are conserved in *N. vectensis* only.

The dearth of conserved Hydra miRNAs in *S. pistillata* echoes the findings of Chapman et al. [42,43], who found only one conserved *N. vectensis* miRNA among the *H. magnipapillata* miRNAs. This might be due to the evolutionary distance separating the anthozoans and hydrozoans, or, more likely, due to the incomplete coverage of short reads used in the identification of miRNAs in *H. magnipapillata* – only 9,654 reads were used to identify potential miRNA genes in *H. magnipapillata* [42]. In contrast, we [and Grimson et al. [43]] identified miRNAs from a much larger pool of short reads. We believe that the repertoire of miRNAs that are conserved across both cnidarian classes (i.e. Anthozoa and Hydrozoa) could be expanded if miRNA predictions were ran on a larger pool of small RNA reads.

The conservation of miRNA families across and within different bilaterian phyla have been fairly well-covered, with the general consensus that the continuous acquisition of miRNA families with minimal secondary losses rapidly expanded the bilaterian miRNA repertoire relative to cnidarians, which contributes to the increased morphological complexity of bilaterians [83,88,90–92]. As one of the few cnidarians with its small RNA fraction extensively sequenced, *S. pistillata* has demonstrated that conservation of miRNA families does occur within cnidarians too, as five of its miRNAs are conserved in *N. vectensis* despite the ~500 mya evolutionary distance that separate both species. However, due to the dearth of sequenced small RNA reads from other cnidarians, we are unable to make further conclusions regarding the rate at which cnidarians acquire their own phylum-specific miRNA families. Also, recent evidence has surfaced that demonstrated the gradual loss of conserved (up to 50% in more derived species) and gain of novel miRNA families in Platyhelminthes, the first that was reported for a major lineage within Bilateria, and might be related to morphological simplifications in some of the studied flatworms [93]. Similar observations could apply to specific classes of cnidarians, but this type of study would need to include more than just a few species of cnidarians in order to elucidate the true rate underlying the gains and losses of miRNA families.

**Functional analysis of putative miRNA targets**

Functional analysis of all 31 miRNAs, using target predictions for each miRNA followed by a GO enrichment analysis on the
predicted target genes, revealed several putative processes and pathways that are regulated by miRNAs in corals. For the mir-100 homologue in *S. pistillata*, the GO terms “embryonic forelimb morphogenesis” and “bone development” were enriched (*P* < 0.01, Supporting Information S12) in the predicted targets, which is reminiscent of its reported function: in humans, miR-100 has been shown to target genes involved in growth and development. Examples include Plk1, a key mitotic checkpoint regulatory protein [26]; RBSP3, involved in cell proliferation and myeloid cell differentiation [27]; BMPR2, involved in osteogenesis [94]; and FRAP1/mTOR, which regulates cell growth [95]. It is possible that miR-100 plays an analogous role in coral calcification, making this miRNA a potentially important piece of the puzzle in coral physiology, as well as a gene of interest when investigating coral responses to ocean acidification. However, as miRNA-mRNA target recognition depends critically on the miRNA seed sequence (bases 2–7 of the mature RNA), it is possible that the targets of bilaterian and cnidarian miR-100 will differ due to the one nucleotide offset between the two miRNA sequences. This 5’ offset has also been observed for miR-2, miR-10, miR-133, and miR-210 that are otherwise well-conserved across two phylogenetically-related taxa, and presumably able to regulate non-overlapping sets of target mRNAs [91]. Thus, further experimentation is required to confirm the bona fide function of cnidarian miR-100 in corals. Nonetheless, our spi-miR-100 adds to the existing literature documenting the strong conservation of miR-100 within metazoans.

Besides the only miRNA with documented function, we identified miRNAs whose targets are involved in high-level functions such as immunity, biomineralisation, regulation of cell cycle, cellular motility, metabolism, signalling, and development, analogous to functions that were previously ascribed to miRNAs in other organisms [23–36]. We were interested in the first two high-level groups, as immunity genes might regulate the relationship with the symbiotic dinoflagellate *Symbiodinium*, and biomineralisation genes may control the rate of coral skeleton growth, two processes that are arguably of importance to corals under conditions of environmental change.

Out of the 5 miRNAs that were predicted to regulate coral immunity genes, we speculate that spi-miR-temp-15 should warrant further investigation due to the significant enrichment of multiple immunity-related GO terms in the transcripts targeted by this miRNA. Indeed, several of the predicted target genes of spi-miR-temp-15 have homologues that are known to be regulated by other miRNAs: Nod2 is repressed by miR-122 [96]; TLR2 is regulated by miR-19 and miR-105 [97,98]; while caspase-8 is targeted by miR-874 [99]. Interestingly, this miRNA is not

### Table 3. Enriched immunity- and biomineralisation-related GO terms (default topGO settings, *P* < 0.01) associated with predicted miRNA target genes from *S. pistillata*.

| miRNA          | GO ID          | Description                                         | P value  |
|----------------|----------------|-----------------------------------------------------|----------|
| **Immunity**   |                |                                                     |          |
| spi-miR-2022   | GO:0044003     | modification by symbiont of host morphology or physiology | 0.0015   |
| spi-miR-2022   | GO:0019048     | virus-host interaction                              | 0.0086   |
| spi-miR-temp-15| GO:0044130     | negative regulation of growth of symbiont in host  | 0.00634  |
| spi-miR-temp-15| GO:0032735     | positive regulation of interleukin2 production      | 0.00119  |
| spi-miR-temp-15| GO:0034134     | toll-like receptor 2 signaling pathway              | 0.00188  |
| spi-miR-temp-15| GO:0034142     | toll-like receptor 4 signaling pathway              | 0.00626  |
| spi-miR-temp-15| GO:0038123     | toll-like receptor TLR1:TLR2 signaling pathway      | 0.00314  |
| spi-miR-temp-15| GO:0038124     | toll-like receptor TLR6:TLR2 signaling pathway      | 0.00314  |
| spi-miR-temp-16| GO:0070498     | interleukin-mediated signaling pathway              | 0.00019  |
| spi-miR-temp-16| GO:0042102     | positive regulation of T cell proliferation         | 0.0366   |
| spi-miR-temp-20| GO:0032088     | negative regulation of NF-kappaB transcription factor activity | 0.0038 |
| spi-miR-temp-26| GO:0051703     | intraspecies interaction between organisms          | 0.0086   |
| **Biomineralisation** |                |                                                     |          |
| spi-miR-100    | GO:0060348     | bone development                                    | 0.00475  |
| spi-miR-temp-7 | GO:0030574     | collagen catabolic process                          | 0.00733  |
| spi-miR-temp-8 | GO:0001958     | endochondral ossification                           | 0.0069   |
| spi-miR-temp-12| GO:0003417     | growth plate cartilage development                  | 0.00891  |
| spi-miR-temp-13| GO:0070588     | calcium ion transmembrane transport                 | 0.0046   |
| spi-miR-temp-15| GO:0060346     | bone trabecula formation                            | 0.00629  |
| spi-miR-temp-15| GO:003417     | growth plate cartilage development                  | 0.00119  |
| spi-miR-temp-15| GO:0043931     | ossification involved in bone maturation            | 6.50E-05 |
| spi-miR-temp-16| GO:0045672     | positive regulation of osteoclast differentiation   | 0.00776  |
| spi-miR-temp-18| GO:0030509     | BMP signaling pathway                               | 0.0084   |
| spi-miR-temp-18| GO:0030501     | positive regulation of bone mineralization          | 0.0031   |
| spi-miR-temp-19| GO:0015701     | bicarbonate transport                               | 0.00172  |
| spi-miR-temp-23| GO:0061036     | positive regulation of cartilage development        | 0.00066  |

doi:10.1371/journal.pone.0091101.t003
MicroRNAs in Corals

conserved in *N. ocellata*, which does not form long-term symbiotic relationships with *Symbiodiniun*.

In contrast to the previous category, 10 miRNAs were predicted to have roles in biomineralisation – one of which being miR-100, which regulates growth and development in humans [26,27,94,95]. Further, among the targeted transcripts, we found several transcripts which are predicted homologues of genes involved in calcium and bicarbonate ion transport that are directly regulated by miRNAs (miR-506 targets human anion exchange protein 2 [100], while miR-17 targets polycystin-2 [101]). A potential involvement of miRNAs in regulating ion transport is intriguing, given the significance of these processes in relation to ocean acidification and associated consequences to coral calcification [102]. However, future experiments (e.g. in-situ hybridisations, gene expression assays, or immunoprecipitation studies) are essential in unequivocally verifying these predicted interactions.

In conclusion, our study provides strong support for the presence of a functional RNAi machinery in *S. pistillata* as highlighted by our phylogenetic analyses, the strong conservation of key RNAi protein domains, and the presence of conserved miRNAs. miRNAs seem to affect a variety of biological processes in corals, but further studies that focus on the coordinated expression of miRNAs and associated target miRNAs under different conditions, as well as their interaction with RNAi proteins, are needed in order to identify, characterise, and understand the operational miRNAome in scleractinian corals.

Data access
All small RNA and RNASeq data are available in the NCBI SRA (Sequence Read Archive) under accessions SRR1130519 and SRR1125978 respectively. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GARY00000000. The version described in this paper is the first version, GARY01000000. Names of the miRNAs are temporary, as miRBase (the microRNA registry) requires acceptance of manuscripts prior to assigning names to newly identified miRNAs. Other data are available as Supporting Information.

Supporting Information

Supporting Information S1 Stylophora pistillata transcriptome (43,493 genes/loci ≥250 bp, DDBJ/EMBL/GenBank accession GARY00000000). (ZIP)

Supporting Information S2 Stylophora pistillata transcriptome BLASTX and GO annotation (43,493 genes/loci ≥250 bp). (ZIP)

Supporting Information S3 Candidate RNAi proteins in Stylophora pistillata. (DOCX)

Supporting Information S4 Alignment of sequences used to construct maximum-likelihood phylogenetic trees (FASTA format). (FA)

Supporting Information S5 Graphical alignment of the PAZ domains in Argonaute and Piwi proteins. Of note are the strong conservation of glutamate (E) at position 137 (mutants produce insoluble protein) and phenylalanine (F) at position 72 (required for RNA binding). However, the phenylalanine at position 48 in *D. melanogaster* AGO2 (also required for RNA binding) was not conserved at all. Key residue positions are marked with red asterisks. (EPS)

Supporting Information S6 Graphical alignment of the Piwi domains in Argonaute and Piwi proteins. The catalytic DDX triad, which contributes to the slicing activity of the ribonuclease (marked in red asterisks), is located at positions 46, 140 and 284 or positions 46, 140 and 155. This triad is present in one *S. pistillata* candidate, but not in two others, most likely due to the transcript sequences being incomplete. (EPS)

Supporting Information S7 Graphical alignment of the first RNase III domain in Dicer and Drosha proteins. Remarkably, all of the key acidic aspartate (D) and glutamate (E) residues, which are involved in the coordination of a divalent metal cation, are conserved across the candidate homologues and known sequences. (EPS)

Supporting Information S8 Graphical alignment of the second RNase III domain in Dicer and Drosha proteins. Similarly, most of the aspartate (D) and glutamate (E) residues involved in the coordination of a divalent metal cation are conserved - perfectly conserved for the Drosha candidate (“Locus_18820”), while the Dicer candidate (“Locus_10081”) only has the first two key residues. Both sequences however align well to known Dicer and Drosha proteins. (EPS)

Supporting Information S9 Graphical alignment of the dsRNA-binding domain in Pasha. The key alanine/alanine pair (AA, positions 21 and 22) and alanine/serine pair (AS, positions 139 and 140) involved in the binding of dsRNA are also present in the *S. pistillata* candidate Pasha. As Pasha is an essential cofactor of Drosha, it lends support to the positive discovery of Drosha in *S. pistillata*. (EPS)

Supporting Information S10 Graphical alignment of the methyltransferase domain in HEN1. The residues involved in Mg2+ coordination (positions 118, 121, 122 and 123) are well-conserved across the aligned sequences; residues associated with the cofactor AdoHcy and 3’ terminus (other positions marked by a red asterisk) are also well conserved. (EPS)

Supporting Information S11 List of additional criteria used to select bona fide miRNAs in *S. pistillata* from miRDeep2 results. (XLSX)

Supporting Information S12 Enriched GO terms (P<0.01) associated with the set of 31 bona fide miRNAs identified in *Stylophora pistillata*. (XLSX)

Acknowledgments
We thank the KAUST Bioscience Core Lab and S. Ali for Illumina library generation and sequencing, and Till Bayer and Mike Lyne for helpful discussions in regard to data analyses.

Author Contributions
Conceived and designed the experiments: CRV GM MA. Performed the experiments: YJL MA AC SB DZ. Analyzed the data: YJL MA CRV.
References

1. Hughes TP (1994) Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science 264: 1547–1549.
2. Bruno JF, Selig ER (2007) Regional decline of coral cover in the Indo-Pacific: timing, extent, and subregional comparisons. PLoS One 2: e711.
3. Knowlton N, Brainard RE, Fisher R, Moews M, Paine L, et al. (2010) Coral reef biodiversity. Life in the World’s Oceans: Diversity and Abundance: 63–93.
4. Hughes TP, Baird AH, Bellwood DR, Card M, Connolly SR, et al. (2003) Climate change, human impacts, and the resilience of coral reefs. Science 301: 929–933.
5. Hess S, Goldberg O, Munzigh PJ, Hooten AJ, Steneck RS, Greenfield P, et al. (2007) Coral reefs under rapid climate change and ocean acidification. Science 318: 1737–1742.
6. Bak R (1987) Effects of chronic oil pollution on a Caribbean coral reef. Marine Pollution Bulletin 18: 534–539.
7. Pasterik RA, Bikard GR (1985) Effects of sewage pollution on coral-reef communities. Marine ecology progress series Oldendorf: 21: 173–189.
8. Green EP, Bruckner AW (2000) The significance of coral disease epizootiology for coral reef conservation. Biological Conservation 96: 347–361.
9. Shinzato C, Shoguchi E, Kawashima T, Hamada M, Hisata K, et al. (2011) Using the Acropora digitifera genome to understand coral responses to environmental change. Nature 476: 320–323.
10. DeSalvo MK, Sunagawa S, Fisher PL, Voolstra CR, Iglesias-Prieto R, et al. (2010) Coral host transcriptomic states are correlated with symbiont genotypes. Mol Ecol 19: 1174–1186.
11. Voolstra CR, Schwarz JA, Schnetzer J, Sunagawa S, Desalvo MK, et al. (2009) The host transcriptome remains unaltered during the establishment of coral-algal symbioses. Mol Ecol 18: 1823–1833.
12. DeSalvo MK, Voolstra CR, Sunagawa S, Schwarz JA, Stillman JH, et al. (2008) Differential gene expression during thermal stress and bleaching in the Caribbean coral Montastrea faveolata. Mol Ecol 17: 3952–3971.
13. Polato NR, Voolstra CR, Schnetzer J, DeSalvo MK, Randall CJ, et al. (2010) Location-specific responses to thermal stress in larvae of the reef-building coral Montastrea faveolata. PLoS One 5: e11221.
14. Voolstra CR, Schnetzer J, Peshkin L, Randall CJ, Szmant AM, et al. (2009) Effects of temperature on gene expression in embryos of the coral Montastrea faveolata. BMC Genomics 10: 6.
15. Meyer E, Agyalamova GV, Matz MV (2011) Profiling gene expression patterns of coral larvae (Acropora millepora) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. Mol Ecol 20: 3599–3616.
16. Trends E, Estrada A, Sunagawa S, Medina M (2012) Transcriptomic responses to darkness stress point to common coral bleaching mechanisms. Coral Reefs 31: 213–228.
17. Moya A, Husman L, Ball EE, Hayward DC, Grasso LC, et al. (2012) Whole transcriptome analysis of the coral Acropora millepora reveals complex responses to CO2-driven acidification during the initiation of calcification. Mol Ecol 21: 2440–2454.
18. Vitali-Dupiol J, Zoccola D, Tambutte E, Grunau C, Cosseau C, et al. (2013) Transcriptomic analysis of the coral Acropora millepora reveals complex responses to darkness stress point to common coral bleaching mechanisms. Coral Reefs 31: 215–228.
19. Kim NI, Kim JL, Yang SK, Ko KH, Park H, et al. (2011) Cloning and use of a coral 36B4 gene to study the differential expression of animals. Nature 455: 1193–1197.
20. Moya A, Tambutte S, Beranger G, Guarna B, Scimeca JC, et al. (2008) Cloning and use of a coral 36B4 gene to study the differential expression of coral genes between light and dark conditions. Mar Biotechnol (NY) 10: 653–663.
21. Lohe M, Bolger AM, Nagel A, Fernie AR, Lunn JE, et al. (2012) RoboRNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. Nucleic Acids Res 40: W622–627.
22. Brown CT, Kaiser A, Zhao Q, Pykou AB, Brom TH (2012) A reference-free approach for computational normalization of shotgun sequencing data. arXiv preprint arXiv:12034802.
23. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9: 357–359.
24. Scholz ML, Zerbino DR, Vinorgin M, Birney E (2012) Oasis: robust de novo RNA-seq assembly across the dynamic range of expression levels. Bioinformatics 28: 1086–1092.
25. Derrien T, HayASHI UC, Goff LG, Haury AM, Veyrieres JL, et al. (2012) The host transcriptome remains unaltered during the establishment of coral-algal symbioses. Database (Oxford) 2011: bar009.
82. Zong J, Yao X, Yin J, Zhang D, Ma H (2009) Evolution of the RNA-dependent RNA polymerase (RdRP) genes: duplications and possible losses before and after the divergence of major eukaryotic groups. Gene 447: 29–39.

83. Sempere LF, Cole CN, McPeek MA, Peterson KJ (2006) The phylogenetic distribution of metazoan microRNAs: insights into evolutionary complexity and constraint. J Exp Zool B Mol Dev Evol 306: 575–588.

84. Mourélatos Z, Dostie J, Paukštienė S, Sharma A, Charroux B, et al. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev 16: 720–728.

85. Sempere LF, Sokol NS, Dubovsky EB, Berger EM, Ambros V (2003) Temporal regulation of microRNA expression in Drosophila melanogaster mediated by hormonal signals and broad-Complex gene activity. Dev Biol 259: 9–16.

86. Chen PY, Manningh K, Chaney M, Russo JJ, et al. (2005) The developmental miRNA profiles of zebrafish as determined by small RNA cloning. Genes Dev 19: 1201–1209.

87. Michelak P, Malone JH (2008) Testis-derived microRNA profiles of African clawed frogs (Xenopus) and their sterile hybrids. Genomics 91: 158–164.

88. Wheeler EM, Heinberg AM, Moy VN, Sperling EA, Holstein TW, et al. (2009) The deep evolution of metazoan microRNAs. Evol Dev 11: 50–68.

89. Mukherjee K, Campos H, Kolarczkowski B (2015) Evolution of animal and plant de-ces: early parallel duplications and recurrent adaptation of antiviral RNA binding in plants. Mol Biol Evol 30: 627–641.

90. Campo-Paysaa F, Semon M, Cameron RA, Peterson KJ, Schubert M (2011) microRNAs complements in deuterostomes: origin and evolution of microRNAs. Evol Dev 13: 15–27.

91. Peterson KJ, Dietrich MR, McPeek MA (2009) MicroRNAs and metazoan macroevolution: insights into canalization, complexity, and the Cambrian explosion. Bioessays 31: 736–747.

92. Prochnik SE, Raksha DS, Absolom HA (2007) Evidence for a microRNA expansion in the bilaterian ancestor. Dev Genes Evol 217: 73–77.

93. Fromm B, Worren MM, Hahn C, Hoving E, Bachmann L (2013) Substantial loss of conserved and gain of novel microRNA families in flatworms. Mol Biol Evol 30: 2619–2628.

94. Zeng Y, Qu X, Li H, Huang S, Wang S, et al. (2012) MicroRNA-100 regulates osteogenic differentiation of human adipose-derived mesenchymal stem cells by targeting BMPR2. FEBS Lett 586: 2373–2381.

95. Nagaraja AK, Creighton CJ, Yu Z, Zhu H, Gumaratne PH, et al. (2010) A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer. Mol Endocrinol 24: 447–463.

96. Chen Y, Wang C, Liu Y, Tang L, Zheng M, et al. (2013) miR-122 targets NOD2 to decrease intestinal epithelial cell injury in Crohn’s disease. Biochem Biophys Res Commun 430: 133–139.

97. Philippe L, Asalch G, Suffert G, Meyer A, Georgell P, et al. (2012) TLR2 expression is regulated by microRNA miR-19 in rheumatoid fibroblast-like synoviocytes. J Immunol 188: 454–461.

98. BenakaneKere MR, Li Q, Eskin MA, Singh AV, Zhao J, et al. (2009) Modulation of TLR2 protein expression by miR-105 in human oral keratinocytes. J Biol Chem 284: 21307–21315.

99. Wang K, Liu F, Zhou LY, Ding SL, Long B, et al. (2013) miR-674 regulates myocardial necrosis by targeting caspase-8. Cell Death Dis 4: e769.

100. Banaszek JM, Saez E, Urte M, Sarvise S, Urrabari AD, et al. (2012) Up-regulation of microRNA 506 leads to decreased CD1+/CD11c+ anion exchanger 2 expression in biliary epithelium of patients with primary biliary cirrhosis. Hepatology 56: 687–697.

101. Banaszek JM, Saez E, Urte M, Sarvise S, Urrabari AD, et al. (2012) Up-regulation of microRNA 506 leads to decreased CD1+/CD11c+ anion exchanger 2 expression in biliary epithelium of patients with primary biliary cirrhosis. Hepatology 56: 687–697.

102. Erez J, Reynaud S, Silverman J, Schneider K, Allemand D (2011) Coral Calcification Under Ocean Acidification and Global Change. In: Dubinsky Z, Stambler N, editors. Coral Reefs: An Ecosystem in Transition. Springer Netherlands. pp. 151–176.