Transcriptome analyses of dark-induced bleaching octocorals and improvement in octocoral RNA extraction

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Abstract

Octocorals, vital components of reef ecosystems, inhabit a wide range of marine environments across various climate zones, spanning from tropical shallows to frigid deep-sea regions. Certain octocoral species, notably *Lobophytum* and *Sinularia*, are particularly intriguing due to their production of diverse metabolites, warranting continuous investigation. Octocorals harboring symbiotic algae are prone to bleaching when subjected to stressors like elevated temperatures and intense sunlight. Interestingly, octocorals with greater tolerance to water turbidity may experience reduced light exposure, which can disrupt photosynthesis and result in bleaching. However, in comparison to stony coral (scleractinian), our knowledge of octocorals' physiological resilience under various stressors remains limited, and the genetic expression of octocorals has been relatively understudied. To address these gaps, our study focuses on comparing the transcriptomes of healthy octocorals and those induced to bleach by darkness, specifically focusing on *Lobophytum hsiehi*. Additionally, considering the challenges posed by the high fluid content and rich bioactive compounds within octocorals, we have devised a universal protocol for RNA extraction from six different octocoral genera. We believe that the findings of this study, along with the developed RNA extraction method, will serve as valuable references for future research, particularly in the realm of octocorals.

Introduction

Octocorals are one of the important members of reef communities that are widely distributed in different marine habitats, climate zones and are also found in different depth ranges from tropical shallow reefs to arctic deep water levels (Dinesen 1983; Edmunds & Lasker 2022; Heifetz 2002; Sánchez et al. 1998; Watling et al. 2011). As one of the reef-built scleractinian corals, despite of their lower reef-formation speed (Jeng et al. 2011), octocorals provide shelter and food for reef-associated fishes and invertebrates contribute to the spatial complexity of reef habitats (Cúrdia et al. 2015; Epstein & Kingsford 2019; Ferrari 2017). In addition, Octocorals are also rich in bioactive natural products with antibacterial, anti-inflammatory, and anticancer activity, such as diterpenes, terpenoids, cembranoids, and steroids, providing enormous potential and economic values to the medicine and biotechnology industries (Rocha et al. 2011).

Octocorals are more stress-tolerant to the bleaching event caused by the environmental threats (Schubert et al. 2017). Octocorals also showed their potential for resistance and resilience to the changing environmental conditions in observed phase shifts of scleractinian corals toward the high abundance of octocorals. The shift of species composition and dominance from scleractinian corals to octocorals was observed after the massive mortality of local scleractinian corals which was caused by environmental stresses and anthropogenic factors (van de Water et al. 2018). Nonetheless, when contrasted with scleractinian, our understanding of the physiological resilience of octocorals in response to diverse stressors remains limited. There are also relatively few studies on the genetic expression of octocorals (Ledoux et al. 2020; Vargas et al. 2022; Woo & Yum 2022).

The octocorals and their symbionts produce many endogenous compounds including second metabolites, polysaccharides, and pigments which interfere RNA extraction and reduce the quality and quantity of RNA when phenol/chloroform was used as extraction reagent. The previous study was reported that SDS based method was developed for RNA extraction of *Sclereonephthya gracillimum* and *Dendronephthya gigantean* (Jeon et al. 2019; Woo et al. 2005). However, this method was difficult to extract RNA from other succulent species such as *Sinularia* and *Lobophytum*. Thus, we need to develop a new RNA extraction method that is efficient for most of the octocoral species.

In addition, some octocorals research using transcriptomic profiling to investigate the stress-related genes and their response to the environmental stressor, including thermal and seawater acidification stress (Ledoux et al. 2020; Vargas et al. 2022; Woo & Yum 2022). Transcriptome analysis also provides new insights into the ecology of octocorals and the evolution of octocoral skeleton components (Conci et al. 2019; Guzman et al. 2018). Nevertheless, the number of octocorals publicly available transcriptomic and genomic data was rare and a large number of species still lack sequenced transcriptomic and genomic data (Conci et al. 2021). Thus, the availability of octocorals transcriptome and genome sequencing data is needed to increase unsolved the interaction of gene network and environmental factors and the evolutionary relationships among octocorals.

Successful isolation of total RNA was essential for transcriptomic analysis. The RNA quality and quantity must satisfy the standard protocol of cDNA synthesis, the library preparation, and construction for RNA-seq (Riesgo et al. 2012; Shi et al. 2021). Since octocorals contain a high amount of fluid and are rich in bioactive compounds which will exclude to mucus surface layer for chemical defenses and would affect the efficiency of RNA extraction, we modified a protocol for octocorals RNA extraction from the *Dioscorea* tubers which is high mucilage and secondary metabolite content, similar to the octocorals (Barman et al. 2017). This protocol can be used for the RNA extraction of six different octocorals species from two families which were widely distributed on the northeastern coast of Taiwan, including *Sinularia acuta*, and *Lobophytum hsiehi* in *Alcyoniidae* family; *Stereonephthya* sp., *Sclereonephthya* sp., *Dendronephthya* sp., and *Litophyton* sp. in *Nephtheidae* family (Dai 2021).

Recent studies showed that *L. hsiehi* species have been found to contain compounds with antimicrobial, anti-inflammatory, and anticancer properties and these studies bring out the potential of *L. hsiehi* species for the development of new pharmaceuticals (Chao et al. 2008; Li et al. 2020; Rodrigues et al. 2019). Furthermore, the studies conducted by Michalek-Wagner and Willis in 2001 demonstrated that bleaching events have significant impacts on reproductive capacity and biochemical changes in the tissue and eggs of *L. hsiehi*, which have severe implications for their survival and reproduction (Michalek-Wagner & Willis 2001a, 2001b). However, the gene response of *L. hsiehi* to environmental stressors such as thermal or darkness-induced bleaching remains unknown due to the lack of transcriptome data for *L. hsiehi* species. Therefore, here we provide our transcriptome data for health and darkness-induced bleaching *L. hsiehi* which the total RNA was extracted by using the on-column RNA extraction method not only to further prove the efficiency of our RNA extraction protocol but also to provide the first insight into *L. hsiehi* species transcriptome analysis and first report of dark-induced bleaching in octocorals.

Materials and methods

Coral materials collection

Six octocoral species were collected at a depth of 5–15 m offshore from northern Taiwan. S*inularia acuta*, *Lobophytum hsiehi*, *Stereonephthya* sp., *Sclereonephthya* sp., *Dendronephthya* sp. were from Guishan Island, Yilan. *Litophyton* sp. was from Bitou, Keelung (Fig. 1 and Table S1). The fresh materials were kept in the seawater and transported to the Marine Fisheries Institute, National Taiwan University. The samples were then segmented into about 1 g wet-weight fragments, frozen in liquid nitrogen, and stored at -80°C before RNA extraction.

Chemicals and reagents

Phenol/chloroform and detergents such as CTAB or SDS are commonly used in RNA and DNA extraction of animal and plant tissues (Gambino et al. 2008; Ghangal et al. 2009; Woo et al. 2005). The base four lysis buffers were prepared as follows, 1)CTAB buffer [2% (w/v) CTAB, 2M NaCl, 25mM EDTA (pH 8.0), 100mM Tris-Cl buffer (pH 8.0), 2% (w/v) PVPP, 2% (v/v) β-mercaptoethanol], 2) CTAB/SDS buffer [1% (w/v) CTAB, 5% (w/v) SDS, 2M NaCl, 25mM EDTA (pH 8.0), 100mM Tris-Cl buffer (pH 8.0), 2% (w/v) PVPP, 2% v/v β-mercaptoethanol], 3) SDS buffer [10% (w/v) SDS, 2 M NaCl, 25 mM EDTA (pH 8.0), 100 mM Tris-Cl buffer (pH 8.0), 2% w/v PVPP.], 4) phenol/chloroform extraction buffer [phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v, pH 6.7) was used to extract. chloroform, isopropanol, 70% ethanol, 8 M LiCl solution, 3 M NaOAC solution, and RNase-free water were also prepared for further treatment.

To find out the optimal buffer for RNA extraction, five different lysis buffer combinations were tested in three replicates. The five methods are (1) 5 mL of trizol added with 5 mL of chloroform, (2) 10 mL of modified 2% CTAB buffer, (3) 10 mL of 1% CTAB with 5% SDS mixed before use (shorten as CTAB/SDS), (4) the base of 5 mL of 2% CTAB buffer added with 5 mL of 10% SDS solution while extracting, and no mixing beforehand (shorten as CTAB/10%SDS), and (5) 10 mL of 10% SDS buffer.

RNA extraction

For RNA extraction, 1 g of frozen samples was ground in liquid nitrogen by using mortars and pestles. 10 mL of lysis buffer was added to the samples and mixed evenly. For the CTAB/10%SDS method, the CTAB part was first added and homogenized with the sample, and then 10%SDS was added to the mixture. The mixture was then centrifuged at 4,000 xg, 10 min, and 4°C, and collect the supernatant. For the phenol-chloroform method, an equal volume of phenol-chloroform was added; for other methods, an equal volume of pre-cooled Isopropanol was added, mixed gently by inverting the tubes, and then centrifuged at 11,000 rpm, 10 min, 4°C. The supernatant was collected and repeat the previous step once. The clear supernatants were divided evenly and transferred to new 1.5 mL microcentrifuge tubes and added an equal volume of RNase-free water was for dilution, then added 1/3 volume of 8M LiCl solution, mixed gently and precipitated in -20°C overnight. The precipitated RNA was centrifuged at 13,000 rpm, 15 min, 4°C, and the supernatant was removed. The precipitate was then resuspended with 50 µl RNase-free

water and kept at room temperature for 15 min. 1 mL isopropanol and 1/10 volume of 3 M NaOAC solution were added, mixed gently, and precipitated at -20°C overnight. The supernatant was removed and the precipitate was resuspended in 70% ethanol followed by centrifugation at 13,000 rpm, 15 min, 4°C. The supernatant was removed and repeat the previous step once. After removing the ethanol supernatant, let the samples dry on the bench for 10 min. 30–50 µl RNase-free water was added and pipetted softly, kept at room temperature for 10 min to let the air-dry pellet dissolve completely. DNase treatment was done according to the manufacturer's instruction and the final total RNA samples were stored at -20°C until use.

On-column RNA extraction

For on-column RNA extraction, NAB Nanosep Device® Total RNA Extraction Kit–Tissue (Imagen, Taiwan) was used while the manufacturer's protocol was modified. 0.5 g of frozen samples were ground in liquid nitrogen by mortars and pestles. 2.5 mL of 2% CTAB buffer and 2.5 mL of 10% SDS solution were added to the samples and mixed evenly. The mixture was then centrifuged at 4,000 x g, 10 min, 4°C. The supernatant was collected and an equal volume of phenol-chloroform was added, mixed gently by inverting the tubes, and centrifuged at 11,000 rpm, 10 min, 4°C. The supernatant was collected and repeat the previous step once. The clear supernatant was transferred to a new 15 mL centrifuge tube and an equal volume of isopropanol was added, mixed vigorously, and transferred to columns (NAB Nanosep device, PALL Life Science). Each column only filters 1–2 mL of the mixture. Centrifuge at room temperature for 10,000 x g, 1min, then discard the flow-through and repeat the steps until all the mixture has been processed. 350 µL of PRW1 buffer was added into the column followed by centrifugation at 10,000 x g, 1min. DNase treatment was processed to remove DNA contamination in the samples. 5 µL of DNase I and 80 µL of RDD buffer from RNase-Free DNase I Set (Cat.RN050, ARROWTEC) were mixed and added to the center of the filter membrane. Incubate at room temperature for 30 min, then add 350 µL of PRW1 buffer and centrifuge at 10,000 x g, 1min. Discard the flow-through, 500 µL of PRW2 buffer was then added and centrifuged at 10,000 x g, 1 min. The supernatant was removed and centrifuged to remove the buffer completely. Transfer the columns to new 1.5 mL microcentrifuge tubes, add 30–50 µL RNase-free water to the center of the filter membrane, and incubate at room temperature for 5min. Centrifuge at 12,000 x g, 3min, discard the columns, and combine the samples of each tube into one microcentrifuge tube, then store the total RNA at -20°C until use.

RNA quantification and qualification

RNA quality and quantity were estimated by the spectrophotometric method (NanoDrop Lite, Thermo Scientific) by measuring absorbance at 260 nm and 280 nm. Gel electrophoresis was done to visualize the RNA samples in agarose gel. 1.5% agarose gel was prepared and 1X TAE buffer was used to prepare and run the gel. In addition, Agilent 2100 Bioanalyzer was used and RNA Integrity Number (RIN) score was obtained for RNA quality check.

cDNA synthesis and amplification of genes for a different region

The cDNA was synthesized by using ARROW-Script Reverse Transcriptase (Cat. ARP4502050, ARROWTEC), according to the manufacturer's protocol. The Folmer region of mitochondrial cytochrome oxidase I (*COI*) + adjacent intergenic region (*igr1*), octocoral-specific mitochondrial protein-coding gene (*msh1*), 28S nuclear ribosomal gene, and bacterial 16S rRNA V6–V8 hypervariable regions were amplified by using different primer set and polymerase chain reaction (PCR) program listed at the table. PCR was carried out using 2 µL of cDNA template, 0.5 µL of primers described above, 2.5 µL of 10X *Taq* buffer, 2 µL of 2.5 nM dNTP mixture, and 0.15 µL of *Taq* DNA polymerase (TaKaRa Bio Inc, Japan). (Table S2 and S3). The PCR product was then visualized by 1.5% agarose gel with 1X TAE buffer and then sequenced by TRI Biotech (Taipei, Taiwan). The sequence data was analyzed by using Basic Local Alignment Search Tools (BLAST) in the NCBI database.

Dark-induced, RNA sequencing and transcriptomic analysis of L. hsiehi

Random six colonies of *L. hsiehi* were used for the dark-induced experiment. Three were under dark treatment until visually bleached (Fig. 1g), and another three colonies were in 12/12 hr light/dark condition of irradiance of ~150 µmol photons m^{-2} s^{-1} (400 watts HQI metal halide) as control.

Total RNA from three bleaching and three non-bleaching coral fragments was extracted by on-column RNA extraction. The bleaching coral fragments had undergone dark treatment to induce a bleaching event before extraction. The RIN score ranges for six samples from 7.2 to 7.7. The RNA samples were sent to BIOTOOLS Co., Ltd. (Taipei, Taiwan) for library construction and sequencing using an Illumina NovaSeq6000 platform. Quality control of raw reads was performed using FastQC v0.11.09 (Andrews 2010). The low-quality nucleotide sequences (quality or phred score below 25; SLIDINGWINDOW 4:25) and the adapters were trimmed with Trimmomatic v0.39 (Bolger et al. 2014). Due to the absence of the soft coral reference genome, de novo assembly was performed using Trinity v0.39 with default settings (Haas et al. 2013). TransDecoder v5.5.0 was used to identify the coding region of the assembled transcripts (Haas 2016). Then, CD-HIT v4.8.1 was conducted to reduce the redundancy of nucleotide sequences with 95% similarity (Fu et al. 2012). To annotate, all retained transcripts were aligned to the NCBI-nr database and SWISS-PROT database using DIAMOND v2.0.15 with the blastx mode and the fast sensitivity mode with an e-value cutoff of 1e-3 (Buchfink et al. 2021).

To classify coral and Symbiodiniaceae transcripts, these transcripts were aligned to a customized coral-Symbiodiniaceae database using BLASTN with an e-value cutoff of 1e-3 (Camacho et al. 2009). The database included the genome assemblies of 10 octocorals, 13 scleractinian corals, and 14 Symbiodiniaceae obtained from the NCBI Reference Sequence (RefSeq) database (Table S4). Then, the transcripts were classified into the coral transcripts or the Symbiodiniaceae transcripts based on the species identified by BLASTN. After that, BUSCO v5.4.4 was used to assess the completeness of the coral transcripts and the Symbiodiniaceae transcripts, respectively (Manni, Berkeley, Seppey, & Zdobnov 2021). The eukaryote_odb10 dataset was selected for the assessment of both coral and Symbiodiniaceae transcripts (Manni, Berkeley, Seppey, Simão, et al. 2021).

For orthologous group clustering, the coral transcripts were first translated into protein sequences by the function TransDecoder, LongOrfs5t (Haas 2016). Then, the proteomes of the other 12 cnidarians were downloaded from RefSeq, including two octocorals, six scleractinian corals, three anemones, and one Hydra as the outgroup, and used for inferring orthologous relationships using OrthoFinder (Emms & Kelly 2019) (Table S5). Finally, a total of 13 proteomes were used to infer a species tree with the STAG algorithm by the OrthoFinder (Emms & Kelly 2019).

To estimate the expression levels of the coral transcripts, the built-in function of Trinity 'align_and_estimate_abundance.pl' was implemented (Haas et al. 2013). In this step, Bowtie2 v2.5.0 was used to perform alignment and RSEM v1.3.3 was chosen to quantify transcript expression (Langmead & Salzberg 2012). Differential gene expression analysis was performed using the DeSeq2 package in R language (Love et al. 2014). DEGs were defined by the $\log_2$ fold change > 1 and the adjusted p-value < 0.05. As for over-representation analysis, the UniProt IDs of DEGs were sent to DAVID Bioinformatics Resources 2021 to carry out functional annotation (Huang et al. 2009). Gene Ontology (GO) terms with p-value < 0.05 and fold enrichment > 5 were focused.

Results

Comparison of different lysis buffer systems and RNA precipitation

The RNA results of the two soft corals extracted by five extraction methods, (1) phenol/chloroform, (2) CTAB buffer, (3) CTAB/SDS buffer, (4) CTAB/10% SDS, and (5) SDS buffer were checked by gel electrophoresis (Fig. 2) and spectrometry (Table 1). The RNA quality was low by the phenol/chloroform method, although the spectrometry showed a high yield but failed to show on the gel electrophoresis (Fig. 2, lane 1). The CTAB can extract RNA but the yield is low by both spectrometry and agarose gel (Fig. 2, lane 2). On the contrary, methods with SDS would have better yield and quality, except the mixed CTAB/SDS lysis buffer, the CTAB buffer with 10% SDS (CTAB/10%SDS), and SDS buffer methods have the highest quantity of RNA with good quality (Table 1 and Fig. 2). In a previous study, CTAB lysis buffer combined with 10% SDS was used to extract the recalcitrant species in woody plants and improve the RNA extraction efficiency (Barbier et. al, 2019). Similar to our result by using combined CTAB/10%SDS lysis buffer, the RNA yield is the highest. These results suggest both SDS-based and modified CTAB/SDS methods can efficiently extract RNA from soft coral samples and the modified CTAB/10%SDS method is even better than SDS based method (Table 1).

To remove excess genomic DNA (gDNA) and decrease DNase usage in the DNase treatment step, Lithium chloride (LiCl) is used for RNA precipitation in the extraction process. LiCl is efficient in precipitating the RNA while the contaminated matters such as DNA and proteins were unable to precipitate and were removed with the supernatant (Barman et al. 2017; Sherman et al. 2022). To examine the optimal condition for RNA precipitation, we added an equal volume of 8 M LiCl solution in diluted (water: sample

= 1:1) and undiluted RNA samples. The result showed LiCl precipitation in the diluted sample is more efficient than in the undiluted sample (Table S6 and Figure S1).

Table 1

The average total RNA concentration (ng/µL) and quality (260/280 ratio) of two octocoral species, *Sinularia acuta* and *Lobophytum hsiehi* extracted by 5 different lysis buffer combination

Species	Buffer	A260/280	Conc. (ng/µl)
Sinularia acuta	Phenol/chloroform	1.49 ± 0.28	215.8 ± 15.2
	CTAB buffer	1.67 ± 0.11	27.9 ± 16.2
	CTAB/SDS buffer	1.85 ± 0.09	65.0 ± 7.4
	CTAB buffer with 10% SDS solution	1.91 ± 0.04	466.7 ± 264.3
	SDS buffer	1.95 ± 0.08	168.1 ± 94.3
Lobophytum hsiehi	Phenol/chloroform	1.00 ± 0.17	244.8 ± 159.7
	CTAB buffer	1.72 ± 0.13	26.9 ± 8.6
	CTAB/SDS buffer	1.85 ± 0.01	48.3 ± 18.6
	CTAB buffer with 10% SDS solution	2.01 ± 0.05	428.4 ± 221.0
	SDS buffer	1.88 ± 0.04	119.5 ± 44.7

RNA extraction for different octocoral species

Based on the various metabolites and components in different species of soft corals, may affect the efficiency of lysis buffer in RNA extraction. To examine the yielding of RNA in soft corals with CTAB/10%SDS lysis buffer, samples from six octocoral species were tested, including *Sinularia acuta*, *Lobophytum hsiehi*, *Stereonephthya* sp., *Sclereonephthya* sp., *Dendronephthya* sp., and *Litophyton* sp.. Spectrometric analysis showed us the ratio of 260/280 in all samples was in the range of 1.9–2.1 indicating the quality of extracted RNA was good (Table 2 and Fig. 3a). Due to the components, texture, and endogenous metabolites, the RNA amounts are also different in these six species after extraction, but the RNA yielding of all samples is higher than 250 ng/µL. The data suggest the CTAB/10%SDS method is suitable for RNA extraction in different coral species.

In-solution RNA extraction is usually time-consuming in the whole process for two to three days. To save time for the experiment, we modified the RNA extraction process by combining it with a commercial extraction column. The extraction column and DNase treatment were also tested in the extraction process (see materials and methods). The RNA quantity of all samples was at least 100 ng/µL but the 260/280 ratio was slightly higher than 2.1 (Table 3). The 18S rRNA and 28S rRNA band were distinct and clear, without any contaminated genomic DNA on the gel electrophoresis (Fig. 3b). Based on the results, the on-column RNA extraction could be a time-saving method.

Table 2
The average total RNA concentration (ng/µL) and
quality (260/280 ratio) of six octocoral species,
Sinularia acuta, *Lobophytum hsiehi*, *Stereonephthya* sp.,
Sclereonephthya sp., *Dendronephthya* sp., and
Litophyton sp. in three replicates by using modified RNA
extraction

Soft coral sample	A260/280	Conc. (ng/µl)
Sinularia acuta	2.02 ± 0.03	253.9 ± 125.3
Lobophytum hsiehi	2.02 ± 0.03	285.5 ± 115.5
Stereonephthya sp.	2.08 ± 0.03	603.0 ± 145.3
Sclereonephthya sp.	2.06 ± 0.03	803.1 ± 452.9
Dendronephthya sp.	2.03 ± 0.03	545.5 ± 449.9
Litophyton sp.	2.08 ± 0.01	1213.1 ± 111.0

Table 3
The average total RNA concentration (ng/µL) and
quality (260/280 ratio) of six octocoral species,
Sinularia acuta, *Lobophytum hsiehi*, *Stereonephthya*
sp., *Sclereonephthya* sp., *Dendronephthya* sp., and
Litophyton sp. in three replicates by using on column
RNA extraction

Soft coral sample	A260/280	Conc. (ng/µl)
Sinularia acuta	2.11 ± 0.01	292.8 ± 21.0
Lobophytum hsiehi	2.15 ± 0.02	152.7 ± 50.8
Stereonephthya sp.	2.14 ± 0.01	300.1 ± 12.1
Sclereonephthya sp.	2.15	562.0 ± 87.5
Dendronephthya sp.	2.14 ± 0.01	362.4 ± 66.5
Litophyton sp.	2.11 ± 0.02	230.7 ± 54.8

Assessment of extracted RNA

After RNA was extracted, we first verified the integrity of RNA with Agilent 2100 Bioanalyzer. As shown in Fig. 4, the major peaks represented 18S rRNA and 28S rRNA.There were no significant peaks near the marker (peak in 25 nt). The RIN score of six octocoral samples was around 6.4 to 8.0. These data suggested integrity of RNA from six extracted coral species is fit for further studies. To examine the quality of extracted RNA for gene expression or transcriptomic studies, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for RNA quality verification. We used the primers from *COI+ igr*1 and *msh*1 to test RNA integrity and species identification. Folmer region of mitochondrial cytochrome oxidase I and adjacent intergenic region (*COI+ igr*1), and octocoral-specific mitochondrial protein-coding

gene region (*msh*1) are usually used to identify the coral species (McFADDEN et al. 2011). As shown in Fig. 5a, the length of the PCR fragment of about 1000 bp was amplified in six coral samples by using primers (*COI*+ *igr*1) indicating the integrity of extracted RNA. The amplification of *msh*1 gene region (about 870 bp) also showed a successful result (Fig. 5b). Additionally, PCR amplification with RNA instead of cDNA was used to rule out the possibility of genomic DNA contamination (data not shown). The PCR amplified fragments were proceeded for sequencing to identify coral species. The sequencing results matched with the sampled species which was identified by morphology (Table S7 and S8). Furthermore, the coral acts as a host harboring many symbiotic organisms, such as bacteria, archaea, and algae. The extracted RNA contained not only from soft coral but also from their symbiotic organisms. We also performed PCR amplification by specific primers designated from 16S rRNA V6-V8 hypervariable regions to examine the existence of bacterial RNA. The results showed bacterial RNA is also extracted by the modified CTAB/SDS method (Fig. 5c). This showed the CTAB/10%SDS method can extract high-quality whole RNA from both soft corals and their symbionts.

RNA sequencing and transcriptome assembly of L. hsiehi

A total of 145 million raw reads were obtained by Illumina paired-end sequencing from six samples. About 98 million reads were retained after quality control and trimming. De-novo transcriptomic assembly produced 466,918 transcripts with an average size of 600 bp and an N50 of 868 bp. 143,464 (30.7%) transcripts were predicted as coding regions and 98,083 (68.4%) of them were further clustered with 95% nucleotide sequence identity. Raw RNA-seq data has been deposited in NCBI in bioproject, accession number: PRJNA1037697.

Identification of the coral transcripts and the phylogenetic analysis

Since *L. hsiehi* holobiont consists of a mix of coral host, Symbiodiniaceae, and potentially other microorganisms, we need to select the transcriptome sequences originating from the coral host and Symbiodiniaceae. All transcripts were aligned to a customized coral-Symbiodiniaceae database, which was made up of available genomes from octocorals, scleractinian corals, and Symbiodiniaceae. There were 21,941 (22.4%) transcripts and 51,251 (52.3%) transcripts were classified into coral and Symbiodiniaceae, respectively (Figure S2a). The average GC contents are 41% in *L. hsiehi* and 55% in Symbiodiniaceae (Fig. 6a). The assembly completeness in both of them was assessed by software, Benchmarking Universal Single-Copy Orthologues (BUSCO). By analyzing coral transcripts with BUSCO score, 83.2% of highly conserved orthologs in eukaryotes (Figure S2b). The phylogenetic analysis was conducted based on the proteome predicted from our coral transcripts and the proteomes from other cnidarian genomes (Table S5). The species tree successfully distinguished octocorals and hexacorals (including scleractinian corals and sea anemones). Among octocorals, *L. hsiehi* seemed to reveal a closer phylogenetic relationship with *Dendronephthya gigantea* than *Xenia* sp. (Fig. 6b).

Differential gene expression analysis between bleaching and non-bleaching coral

After getting the real coral transcripts, we identified 2,215 DEGs (913 were upregulated; 1,302 were downregulated) in the bleaching coral (Fig. 7a, Figure S3 a,b,c). 2,054 (93%) and 861 (39%) of them had homologous sequences with NCBI-nr database and SWISS-PROT database, respectively. Stress response protein, heat shock protein 90 (Lo_93629) was highly upregulated in bleaching coral ($\log_2$ fold change = 10.9, p-value = 1.31e-22), and microbial recognition associated genes, scavenger receptor proteins (Lo_18454, Lo_66404) was highly downregulated (Lo_18454: $\log_2$ fold change = -9.9, p-value = 4.6e-14; Lo_66404: $\log_2$ fold change = -7.8, p-value = 3.3e-98) (Figure S2 b,c; Table S9). Among the upregulated genes, over-representation analysis revealed that 7 GO terms were enriched in the bleaching coral (p-value < 0.05 and fold enrichment > 5), including 2 biological processes (BP): ubiquitin-dependent protein catabolic process (GO:0006511), and locomotory exploration behavior (GO:0035641); 1 cellular component (CC): tenascin complex (GO:0090733); 4 molecular functions (MF): ubiquitin-protein transferase activity (GO:0004842), catalytic activity (GO:0003824), extracellular matrix structural constituent (GO:0005201), and transferase activity (GO:0016740). Among them, the tenascin complex showed about 234-fold enrichment in bleaching coral (Fig. 7b). On the other hand, 25 GO terms were enriched in non-bleaching coral. The highly enriched BP terms included Tie signal pathway (GO:0048014), meiotic attachment of telomere to the nuclear envelope (GO:0070197), protein poly-ADP-ribosylation (GO:0070212), and induction of bacterial agglutination (GO:0043152). Heparan sulfate binding (GO:1904399) and protein ADP-ribosylase activity (GO:0003950) were the most enriched MF terms. Furthermore, the extracellular matrix-related genes seemed to change their expression in both bleaching and non-bleaching conditions (Fig. 7c). On the other hand, the difference in the Symbiodiniaceae transcript amounts between bleaching and non-bleaching coral might be mostly influenced by algae cell number. Therefore, differential expression analysis mainly focused on the coral transcripts.

Discussion

An efficient CTAB/10%SDS extraction method

RNA extraction is not easy in octocoral due to interference by polysaccharides, polyphenolics, and secondary metabolites (Sammarco & Coll 1992). The result of different five extraction methods showed the methods mixed with SDS have better performance than others, indicating that an anion detergent such as SDS is more suitable for soft coral extraction. The optimal method from this study is the CTAB/10%SDS method, this method not only adjusts the proportion of CTAB and SDS, but also the buffer mixture in the extraction process. Interestingly, the critical part of the successful CTAB/10%SDS method is to add SDS post-homogenized to the sample, which can increase RNA yield at least fivefold. Complete cell lysis is important in nucleic acid extraction. Low RNA yielding in the pre-mixed buffer may cause the micelles aggregation to form beforehand and lower the efficiency of the tissue lysis. In the

CTAB/10%SDS method, coral tissue was lysed in CTAB before adding SDS, the CTAB can first react and then form the micelles with SDS to remove interference components, improve cell lysis and increase RNA yielding as the previous reporter mentioned (Tah et al. 2011). In the previous study, the SDS-based method was reported to extract RNA from *Sclereonephthya* and *Dendronephthya* (Woo & Yum 2022; Woo et al. 2005). But until now, there is no common RNA extraction method reported for many octocorals. In our data, CTAB/10%SDS lysis buffer is not only suitable for above two species but also other species. In addition, we are successful in extracting total RNA from *Sarcophyton* sp., one of the members of *Alcyoniidae* family (data not shown).

The traditional "non-column" method is a simple and cost-down treatment, however, the downside is it requires longer experimental time. The RNA precipitation step takes overnight, and the extra DNase digestion and complex process may increase the risk of RNA degradation. On the contrary, the column method provided a rapid and easy method but with higher expense. For different purposes, we suggest that the column method is suitable for preparing RNA for transcriptomic analysis, and the traditional method can be applied for regular or quantitative RT-PCR analysis.

Transcriptomic analysis of dark-induced bleaching L. hsiehi

Lobophytum is one of the common soft coral genera in the Indian Pacific region. Its bleaching is due to temperature and the intensity of light, which can either bleach under intense or weak light exposure. However, there is limited research on bleaching caused by the lack of light, especially in the case of soft corals. In this study, we successfully identified the transcripts originating from the host, *L. hsiehi*. The GC content was similar to other corals, for instance, *Dendronephthya gigantea* (37%) (Jeon et al. 2019), *Heliopora coerulea* (43%, 38%) (Guzman et al. 2018; Ip et al. 2023), and *Acropora millepora* (39%) (Ying et al. 2019). The BUSCO results indicated that the assembly of transcriptome had high completeness. Octocorals and hexacorals were separated in the phylogenetic analysis. These results demonstrated that the column RNA extraction method was suitable for downstream analysis.

With the coral transcripts, the Differential Expression Gene (DEG) analysis was conducted to further discuss the expression patterns of dark-induced bleaching and non-bleaching *L. hsiehi*. The genes related to tenascins, a family of extracellular matrix proteins, were highly enriched in the dark-induced bleaching coral. Tenascin proteins can promote the adhesion of cells to various substrates (Chiquet-Ehrismann & Tucker 2011) while the extracellular matrix provides mechanical support to tissues and is involved in cell adhesion and organization of cytoskeleton (Kuschel et al. 2006).

In previous octocorals bleaching studies, genes related to extracellular matrix (GO: 0005201, GO: 0031012, GO: 0030198) were highly enriched in both thermal and low pH-induced bleaching of *Scleronephthya gracillimum* (Woo & Yum 2022). The change in extracellular matrix gene regulation is also common for thermal-induced bleaching events in scleractinian corals (DeSalvo et al. 2010; Rose et al. 2015). From these results, only tenascins complex (GO: 0090733) and extracellular matrix structural constituent (GO:0005201) were upregulated, while other DEGs related to extracellular matrix and cytoskeleton organization, such as, beta-tubulin binding (GO: 0048487), microtubule cytoskeleton

organization (GO: 0000226), extracellular matrix (GO: 0031012), extracellular matrix structural constituent (GO: 0005201), and extracellular matrix organization (GO: 0030198), were downregulated in dark-induced bleached *L. hsiehi*. These results showed that the change in extracellular and cytoskeleton organization gene expression is also associated with dark-induced bleaching, which might have different expression levels between different species and bleaching-induced factors.

Nuclear factor-kappa B (NF-kappa B), a major regulator of immunity found in cnidarians, and its pathway was involved in not only immunity but also symbiosis between cnidarians and algal symbionts. In this study, the NF- kappa B signaling (GO: 1901224) was downregulated in dark-induced *L. hsiehi*, whereas in other coral bleaching studies, NF-kappaB showed the greatest increase in expression in response to thermal stress both in a scleractinian coral *Acropora palmata* and an octocoral *Sclereonephthya gracillimum* (DeSalvo et al. 2010; Woo & Yum 2022). Furthermore, NF-kappa B which activates innate immunity and the apoptotic pathway was triggered during heat and light stress in cnidarian and in sea anemone *Aiptasia*, where NF kappa B was found to have high gene expression during the early stage of heat stress (Cleves et al. 2020; Weis 2008). The opposite response of the expression of NF-kappa B between dark and light bleaching suggests the bleaching mechanisms might triggered differently.

Genes related to an immune response like bacterial agglutination downregulated in *L. hsiehi*. The induction of bacterial agglutination (GO: 0043152) was under the immune system processes GO category (GO: 0002376). This GO category was previously observed downregulated in bleached colonies of *Orbicella faveolata* and showed that coral bleaching changes the expression of innate immune genes of corals (Pinzón et al. 2015). More in the downregulated DEGs also includes scavenger receptor (GO: 0005044; Lo_18454 and Lo_66404), which is a diverse superfamily of innate immunity genes that recognize microbial ligands and are involved in phagocytosis of invading microbes (Areschoug & Gordon 2009). Scavenger receptors are involved in cnidarian immunity and regulation of cnidarian-dinoflagellate symbioses (Davy et al. 2012; Neubauer et al. 2016). Therefore, we suggest that downregulated of the scavenger receptor in *L. hsiehi* might potentially cause symbiosis dysfunction and the loss of symbiotic dinoflagellate algae during dark-induced bleaching.

Conclusion

The environment in which corals grow can be influenced by human activities, such as water quality and turbidity. High water turbidity can result in insufficient light exposure. While octocorals are considered to be more tolerant of environments with higher water turbidity compared to scleractinian corals, our current understanding of the effects of low light or darkness on soft corals is limited. Hence, this study provides the first results of gene expression related to bleaching in octocorals induced by dark conditions. Generally, bleaching is observed when symbiotic algae depart from their coral host under stress. However, this study suggests that octocorals may exhibit different physiological responses to bleaching under various circumstances, suggesting that the mechanisms of bleaching induced by darkness may differ from other common bleaching triggers.

The modified RNA extraction protocol reported in this study extracts the total RNA of octocorals that tissue contains highly fluid and secondary metabolite successfully. This universal protocol is suitable for six different octocoral species and might be useful for other octocoral species. The total RNA product can be used in the downstream analyses of octocoral transcriptomes. Understanding the genetic basis of octocoral biology and stress responses can be informative to conservation and management strategies for these which are ecologically important organisms, highlighting the significance of continued research in octocoral RNA extraction and sequencing. We believe that the outcomes of this study and the RNA extraction method employed can serve as valuable references for future octocorals research.

Declarations

Data availability

The sequence has been deposited in NCBI in bioproject, accession number: PRJNA1037697.

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Author Contribution

Jing-Wen Michelle Wong performed the experiment and wrote the manuscript; An-Chi Liu designed the research and wrote the manuscript; Hsuan-Tung Lin and Chuya Shinzato analyze the data and editing manuscript; Sung-Yin Yang provide resources and editing manuscript; Shan-Hua Yang as supervisor and funding aquisition. All authors revised and approved the final manuscript.

Acknowledgments

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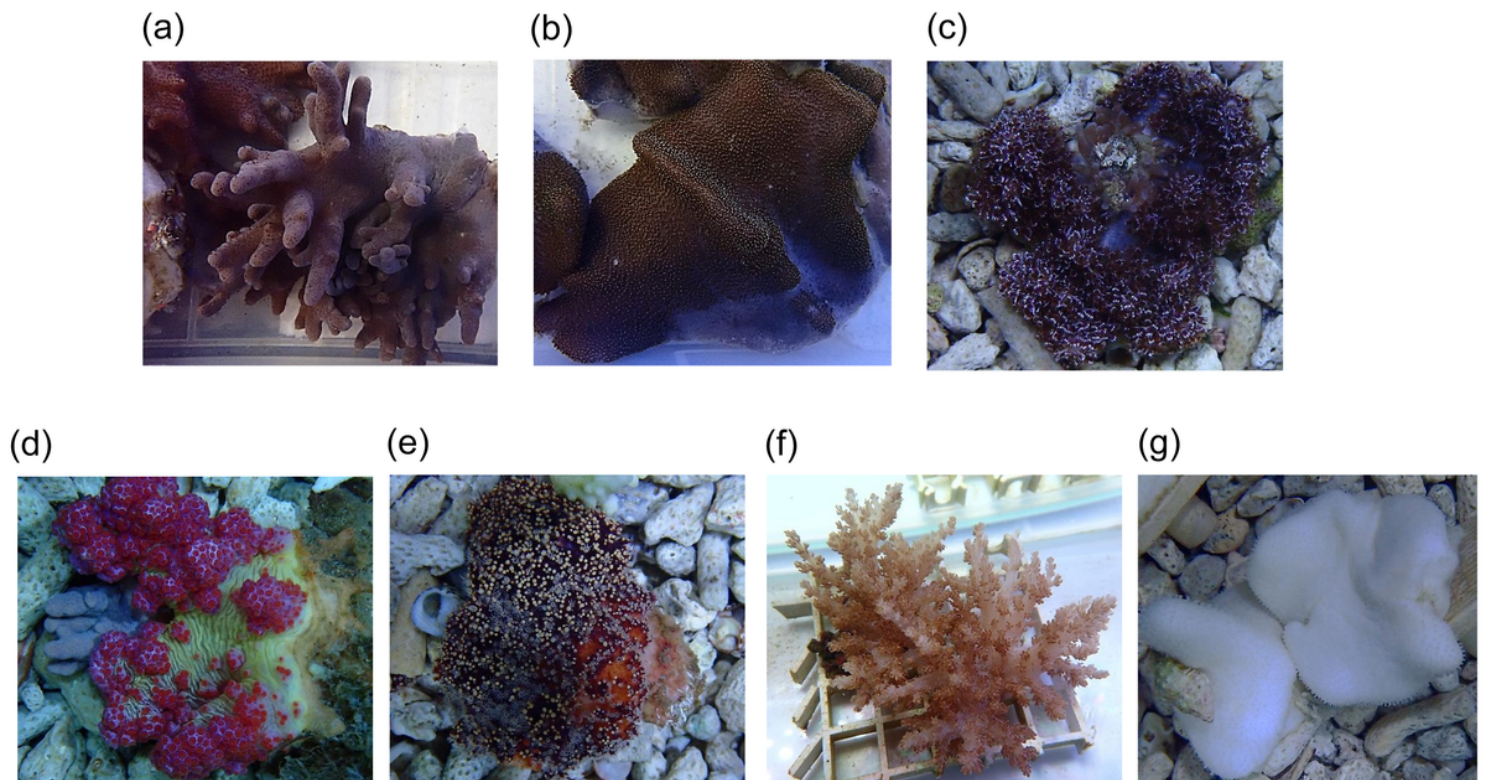
Figures

Figure 1

Octocoral species used in this study: (a) *Sinularia acuta*, (b) *Lobophytum hsiehi*, (c) *Stereonephthya* sp., (d) *Sclereonephthya* sp., (e) *Dendronephthya* sp., and (f) *Litophyton* sp. (g) Bleached *Lobophytum hsiehi* after dark treatment
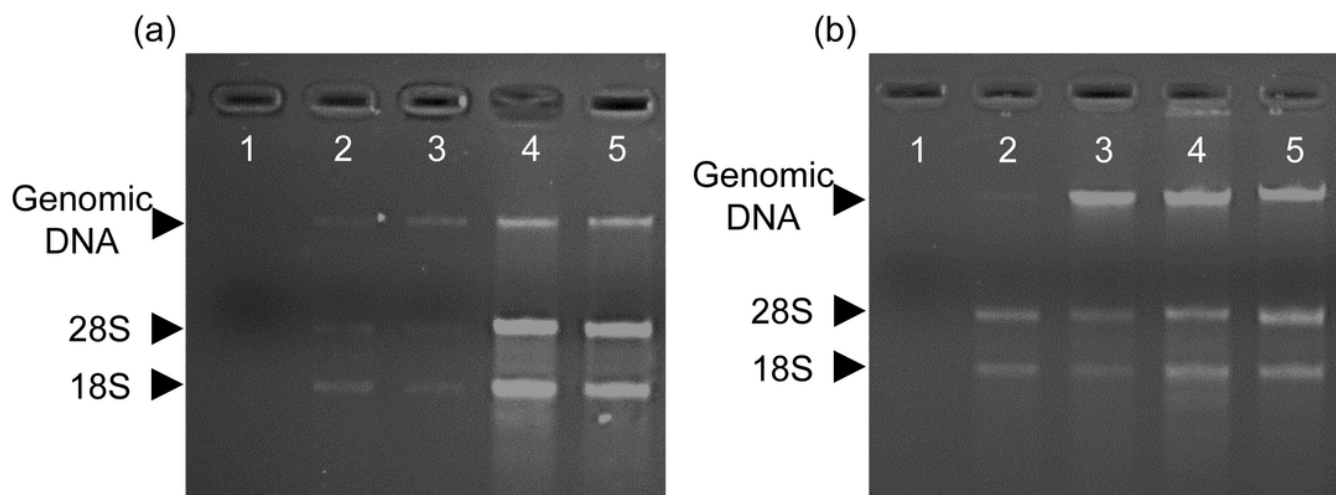
Figure 2

Gel electrophoresis results of (a) *Sinularia acuta and* (b) *Lobophytum hsiehi* total RNA extracted by 5 different lysis buffer combinations: Phenol/chloroform (1), CTAB buffer (2), CTAB/SDS buffer (3), CTAB buffer and 10 % SDS solution (4), and SDS buffer (5)
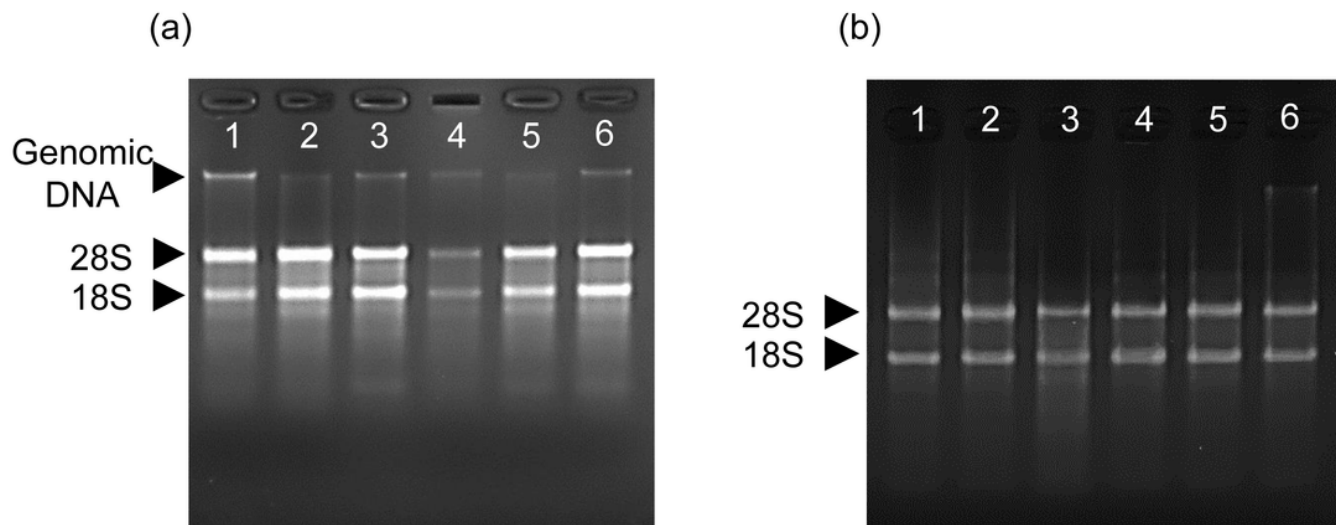
Figure 3

Gel electrophoresis results of six octocoral species: *Sinularia acuta* (1), *Lobophytum hsiehi* (2), *Stereonephthya* sp. (3), *Sclereonephthya* sp. (4), *Dendronephthya*sp. (5), and *Litophyton* sp. (6) in three replicates by using (a) CTAB/10%SDS RNA extraction and (b) on column RNA extraction
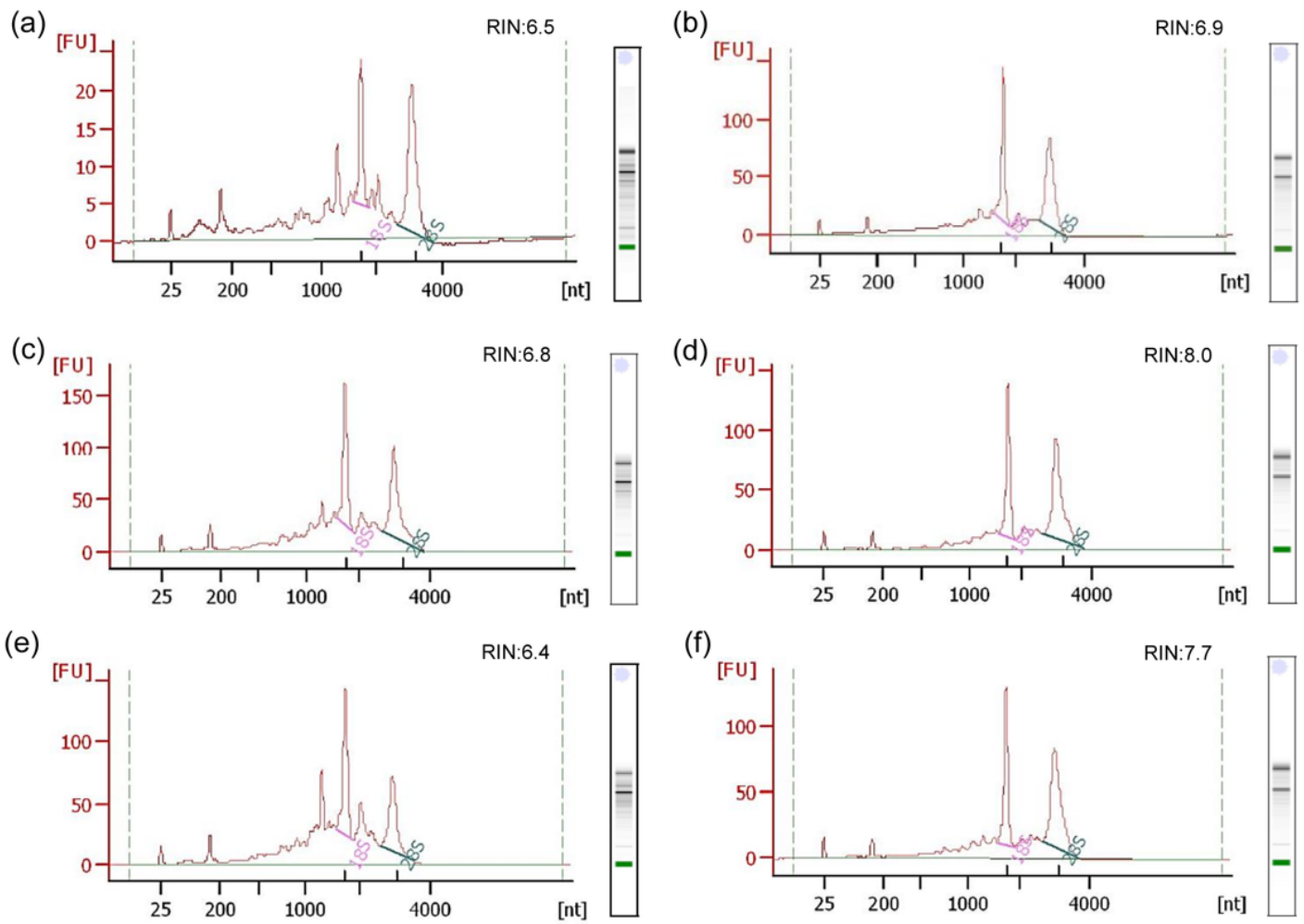
Figure 4

Bioanalyzer results of six octocoral species: (a) *Sinularia acuta* , (b) *Lobophytum hsiehi*, (c) *Stereonephthya* sp., (d) *Sclereonephthya* sp., (e) *Dendronephthya* sp., and (f) *Litophyton* sp. extracted by on column RNA extraction
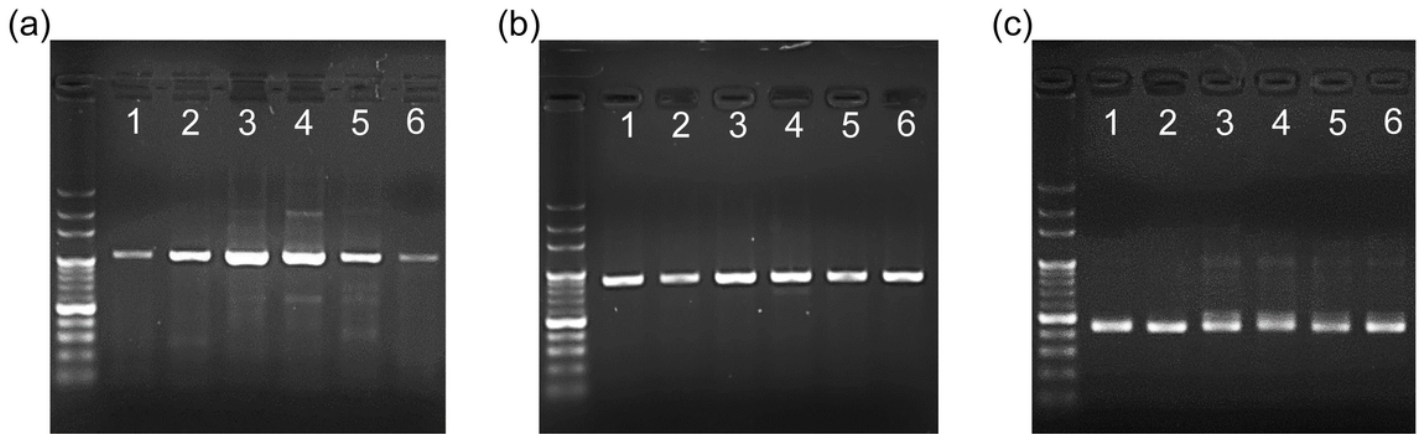
Figure 5

RT-PCR results of (a) Folmer region of mitochondrial cytochrome oxidase I (*COI*)+ adjacent intergenic region (*igr1*), (b) octocoral-specific mitochondrial protein-coding gene (*msh1*), and (c) bacterial 16S rRNA V6–V8 hypervariable regions in six octocoral species: *Sinularia acuta* (1), *Lobophytum hsiehi* (2), *Stereonephthya* sp. (3), *Sclereonephthya* sp. (4), *Dendronephthya* sp. (5), and *Litophyton* sp. (6) in three replicates
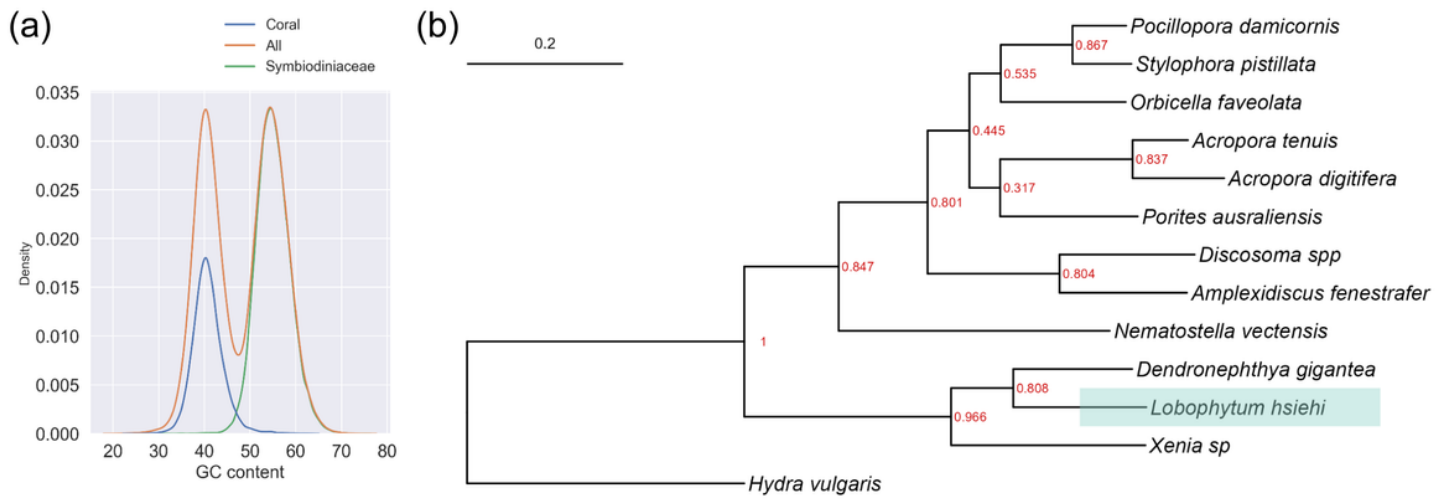
Figure 6

De novo assembly of *Lobophytum hsiehi* (a) Distribution of GC contents of assembled transcripts. Orange: all transcripts; blue: the coral transcripts; green: the Symbiodiniaceae transcripts. (b) The species tree of 13 cnidarians inferred by OrthoFinder. *Hydra vulgaris* was an outgroup. The distance of the tree is displayed on the upper left side. Red character: support value of the node inferred by STAG algorithm; green mark: our sample
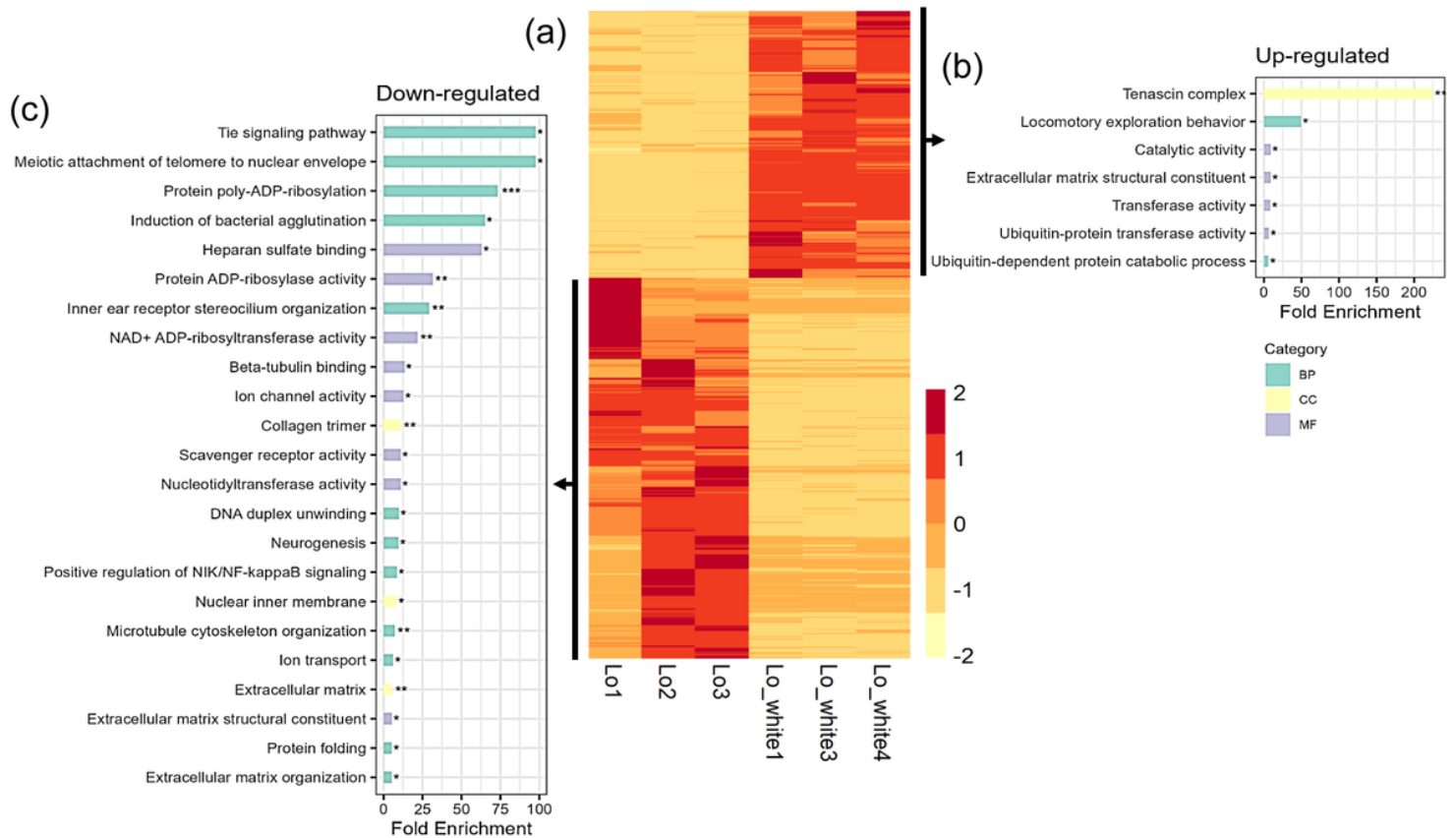
Figure 7

DEG and functional analysis between the bleaching and the non-bleaching coral. (a) 913 upregulated genes and 1302 downregulated genes were found in the bleaching coral. DEGs were defined by |the log2 fold change|> 1 and the adjusted p-value < 0.05. (b) The enriched GO terms of the upregulated genes in the bleaching coral. (c) The enriched GO terms of the downregulated genes in the bleaching coral. GO terms with p-value < 0.05 and fold enrichment > 5 were focused. *: p-value < 0.05; **: p-value < 0.01; p-value < 0.001. BP: biological Process, CC: cellular Component, MF: molecular function

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.pdf
- Supplementarymaterials2.pdf
- TableS9.csv