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**Title:** Genotype dominates transcriptomic response to thermal stress in the coral *Pocillopora acuta*

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**Summary:**

Understanding how stony corals respond to environmental change1 can guide conservation strategies and aid assessment of reef health2 Therefore, it is critical to clarify the link between genotype and stress markers, such as transcriptome data, which are widely used in coral research3,4. To address this issue, we generated RNA-seq data from 85 randomly sampled triploid and diploid *Pocillopora acuta* corals from Kāneʻohe Bay, Hawaiʻi that were exposed to abiotic stress. Existing single-nucleotide polymorphism data from these corals identified three clades distributed throughout the bay that comprise distinct clonal lineages that have arisen *via* asexual larval propagation or fragmentation5. We assessed fit of the coral RNA-seq response to high temperature across different genotypes (including ploidies) to two divergent outcomes: 1) gene expression is largely independent of genotype, reflecting a shared response (Treatment-Driven Expression, TDE4) or alternatively, 2) genotype dominates gene expression, regardless of treatment (Genotype-Driven Expression, GDE). Surprisingly, the *P. acuta* data fit best the GDE model, showing that host genotype dominates gene expression even when colony bleaching across different genetic backgrounds follows a predictable pattern under thermal stress. These results lead to two major conclusions; First, genotype is paramount when studying gene expression in *P. acuta*, and likely other corals, setting boundaries on the transcriptomic response to stress; and second, given the inconsistency between gene expression and phenotype, there exists intermediate regulatory steps (e.g., control of translation, protein turnover) that disconnect transcript abundance from proteomic and metabolic responses. The latter most likely explains the coral bleaching response to warming oceans.

**Keywords:** asexual reproduction, clonal lineages, coral, gene expression, principal component analysis, triploidy

**Results and Discussion**

Physiological response

Coral fragments of *P. acuta* were collected from six patch reefs that span Kāneʻohe Bay (Fig. 1A). The fragments were exposed to abiotic stress (either high temperature, high pCO2 [low pH], or a combination of both) that mirrored real-world conditions, over the course of a 16-week period (see Methods). We found that *P. acuta* paled significantly in color (Fig. 1B) in response to treatment through time (3-way interaction of Temperature, pCO2, and Time [F1,10 = 71.95, *p* < 0.00000000002]; Table SX), resulting in >80% mortality by the end of the experiment (Fig. 1C). There was significant mortality in response to high temperature (*p* = 0.029) and high pCO2 (*p* = 0.030), and their interaction (*p* = 0.013, Table SX). These results demonstrate that *P. acuta* exhibits a significant physiological response to the stress treatments used in this study, often leading to colony death. Given this clear outcome, we asked whether a shared gene expression response to stress exists within and between clonal clades (Fig. 1D) to assess the role of genotype and ploidy in controlling transcript accumulation patterns.

Effect of genotype on transcript profiles

We focused our analysis on 85 *P. acuta* colonies that represent three genotypes that had been exposed to ambient (control) and abiotic stress during a 16-week thermal challenge experiment (Table 1; Table S1). Fit of the gene expression data to the competing TDE and GDE hypotheses was tested using a principal component analysis (PCA) analysis of 21,048 genes in these 85 samples. Under the TDE model (Fig. 2A), treatment dominates gene expression, whereby theoretical PC1 accounts for the vast majority of variation that is driven by treatment. PC2 under this model, explains the more minor, genotype-based variation with some separation of different genotypes based on treatment, as would be expected in a realistic scenario. This scenario supports the idea that *P. acuta* corals share a conserved transcript-based stress response that dominates the RNA-seq data (see details below). In contrast, the GDE model (Fig. 2B) predicts that genotype dominates gene expression, whereby theoretical PC1 largely accounts for variation driven by coral animal genotype. PC2 under GDE accounts for the minor, treatment-based variation among different genotypes. Given these two scenarios, it is apparent in Figure 2C, that the experimental data overwhelmingly support the GDE model. This PCA analysis show that genotype is highly correlated with PC1 (*r*s=0.9, p-value=1.32E-15, Table 2, Table SX), which accounts for the majority (23.76%) of variation, whereas PC7, which explains 2.42% of variation in the data, is significantly, but only weakly correlated with treatment (*r*s=00.54, p-value=16.43E-07, Table 2, Table SX). The two triploid clonal groups (Groups 1 and 2) are distinct from the diploids (Group 3). When all three genotypes are used in a differential gene expression analysis, no significant DEGs by treatment are found (Fig. 3). We combined RNA-seq data (Tables SX, SX) from the colonies with high pCO2 treatment with data from the control treatments because principle components analysis showed there was only 1.04% cumulative variation in gene expression across all significantly correlated principle components [p<0.05; Table 2, Table SX]. Overall, these results show the absence of a core transcriptomic stress response in *P acuta*, which contrasts strongly with studies of acroporids that identify a highly conserved group of genes across different genotypes that are consistently associated with the stress response7,8. Therefore, our results support the alternative GDE model proposed here, wherein gene expression profiles observed in this experiment are primarily governed by genotype, while treatment and timeline may have very weak effects.

Questions raised by our study

Here, we show that in *P. acuta*, a coral that primarily reproduces asexually, gene expression during a heat stress challenge is primarily driven by genotype, despite a clear phenotypic response to treatment that spans all genotypes. Based on these data, we propose an alternative model for gene expression in corals in which genotype, not environment, drives gene expression variation, and we discuss the role that genotype has in terms of the adaptive capacity of corals to withstanding disturbance. Our findings lead to two critical questions that should be addressed in future coral studies. The first and most important is, given that gene expression is under strong selection, why does it not shift under prolonged thermal stress, that in many cases leads to colony death in *P. acuta* (see Fig. 3)? One possible answer is that gene expression reflects past adaptive responses to local conditions that are not rapidly changed in different host animal genotypes. For this reason, downstream post-transcriptional mechanisms such as differential access to the translation machinery, regulation of protein degradation, and protein level buffering9,10 may be more informative when considering coral stress responses. These feedback systems will ultimately control enzyme abundance and activity, thereby, metabolite production, which underlie the physiological (bleaching) response that we report. Our system is peculiar in the respect we have controlled for genetic background by exploiting the natural reproductive system of *P. acuta* in Kāneʻohe Bay that relies on clonal propagation. We speculate that past stressful episodes such as freshwater incursion and heating events11 elected for fitter genotypes, both diploid and triploid, that have spread throughout the bay *via* clonal propagation to ensure survival in different micro-environments. Under this “everything is everywhere” model, which fits the data for Kāneʻohe Bay5 (Fig. 1C), gene expression, although limited by genotype, provides the foundation for post-transcriptional mechanisms that elicit the overall physiological response, which may be largely independent of genotype. Elucidating these mechanisms will be key to understanding coral biology to underpin conservation efforts.

***Montipora capitata analysis***

A second important question is, how widespread is the GDE model in different coral species, or is it specific to *P. acuta*? To test this idea in more detail, we analyzed RNA-seq data from a sympatric coral species in Kāneʻohe Bay, *Montipora capitata*, that relies on a radically different strategy to ensure survival. This is a hermaphroditic, mass spawner that is a strict outbreeder and widespread in the bay5. Under the same experimental conditions used for *P. acuta*, RNA-seq data was generated from 132 colonies (nTemperature=66 ) of *M. capitata* and analyzed as described above. While support for the GDE model could not be explicitly tested for because, with the exception of two samples, each sample constituted a distinct genotype5, we were able to test for fit of the TDE model. These RNA-seq results (Tables SX, SX) clearly do not support the TDE model; Spearman correlations between the principal coordinates and sample attributes shows very weak correlation of temperature with PC2 (*r*s=0.28, p-value=0.0014) and PC8 (*r*s=-0.28, p-value=0.0012), which explain 4.55% and 1.56% of variation in the gene expression data, respectively (FigX). Additionally, like the *P. acuta* differential expression analysis, edgeR identified 62 potentially differentially expressed genes out of 22,587 in the dataset that upon further inspection show no consistent response across the two treatments (FigX) and were thereby classified as not significantly differentially expressed. Therefore, these results, combined with differential expression analysis demonstrate that stress treatment explains very little of the variation in these RNA-seq data, and does not fit the TDE model. Previous work in *Acropora* cf. *pulchra*12shows that this species follows the TDE model…

Conclusions

In summary, our findings are worthy of serious consideration by the coral community because many studies of coral stress resilience hinge on the assumption that corals share a core transcriptomic response to stress that will drive the variation in gene expression according to the TDE model13, (Fig. 1A) whereby treatment drives gene expression, with a weak influence of colony genotype. In contrast, our results support the GDE model and demonstrate a clear need to thoroughly characterize the genetic structure and reproductive behavior of corals for informed interpretation of ‘omics’ data. The underpinnings of coral resistance and resilience involve not only algal symbiont and prokaryotic microbiome contributions, but also fundamentally reflect host genotype(s) and their provenance. This is particularly pertinent with the acceleration of the search for transferrable mechanisms for use as human interventions to assist coral resistance and resilience and maintain these invaluable coral reef ecosystems under increasing global change.

**Acknowledgments:**

**Author contributions:**

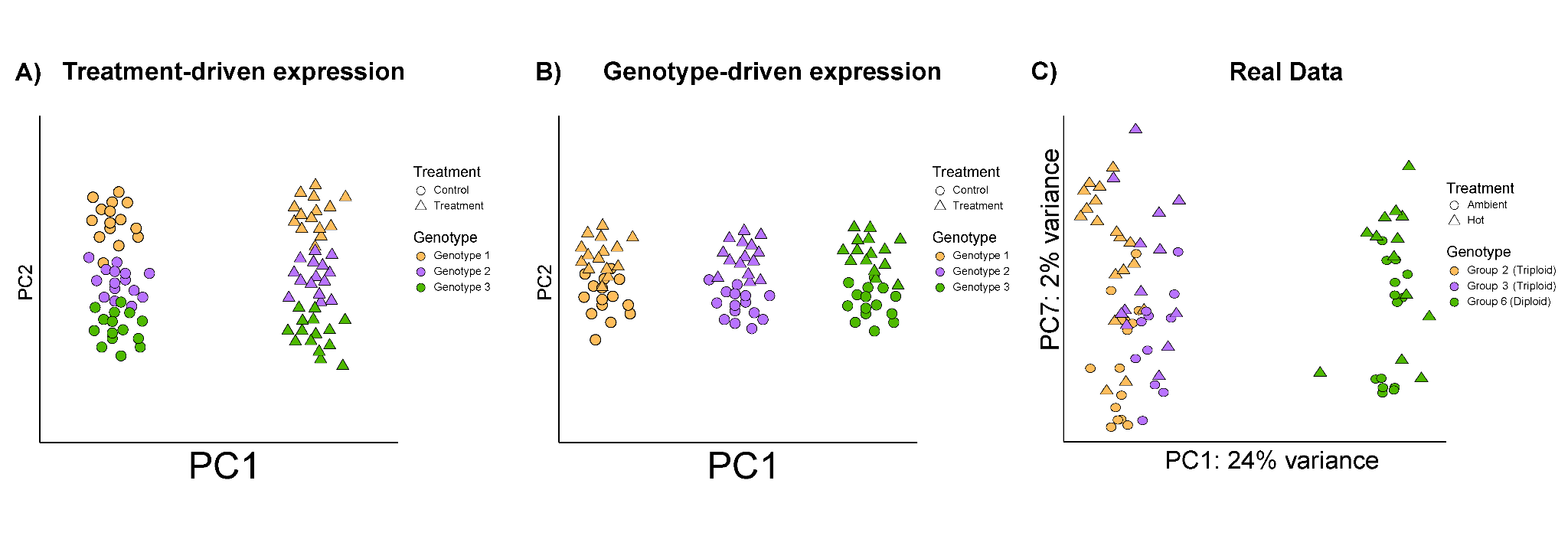
**Declaration of interests:**

**Figure legends:**

Map

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Figure 1 Map of Kbay and PCA of genotypic structure



**Figure 2.** PCA analysis of *P. acuta* RNA-seq data…

**Tables:**

Table 1. Number of samples of each group per treatment

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Treatment | Group 1 (Triploid) | Group 2 (Triploid) | Group 3 (Diploid) | Grand Total |
| Amb | 16 | 11 | 14 | 41 |
| Hot | 18 | 13 | 13 | 44 |
| Grand Total | 34 | 24 | 27 | 85 |



|0| |1|

Table 2. Summary of spearman correlation coefficients and significance of sample attributes and principal components for P. acuta. See Table SX for full table.

**Methods:**

*Coral Collections*

This study focused on two dominant coral species that vary in their environmental tolerance in Kāneʻohe Bay: Montipora capitata and Pocillopora acuta (Bahr et al. 2016; Gibbin et al. 2015). About 900 coral samples (one ~5x5cm fragment per colony from 75 colonies per species per site) were collected from six reef areas ranging across the north to south span and fringing to patch reefs of Kāneʻohe Bay (Lilipuna Fringe: 21°25'45.9"N 157°47'28.0"W; HIMB: 21°26'09.8"N 157°47'12.7"W; Reef 18: 21°27'02.9"N 157°48'40.1"W; Reef 11, 13: 21°27'02.9"N 157°47'41.8"W; Reef 35, 36: 21°28'26.0"N 157°50'01.2"W; Reef 42, 43: 21°28'37.9"N 157°49'36.8"W; supplementary fig. S11A and B) under Hawaiʻi Department of Aquatic Resources Special Activity Permit 2019-60, between 4-10 September 2018. Corals were affixed in an upright position to individually numbered plastic coral fragment mounting plugs by hot gluing the base of the skeleton to the plug. Coral fragments were allowed to acclimate for ~14 days and then were randomly allocated to mesocosm tanks. The 450 samples collected from each species were randomly allocated to different projects, the results of which are not presented here; after treatment 132 M. capitata and 119 P. acuta samples had RNA-Seq libraries generated (the increased mortality of the P. acuta samples, and problems with the extraction of some samples, resulted in the difference in the number of samples between the two species; a more detailed description is presented in a following section).

*Experimental Setup*

Treatment conditions (n=3 tanks treatment-1) were randomly assigned to twelve outdoor mesocosm tanks (122 cm x 122 cm x 30 cm; 510 L). Flow rates, measured daily with a graduated cylinder and timer, averaged 84.36 ± 1.20 mL second-1 (mean ± sem; n=826), resulting in the mesocosm tank turnover every ~1.6 hours. Temperature treatment conditions were programmed to mimic the natural daily fluctuations (0.75 °C ± 0.06) of the environment at the collection sites in Kāneʻohe Bay, Hawaiʻi (NOAA Moku o Loe Buoy data from September 2018). A Neptune Apex control system was set-up to generate a pH-stat feedback system. Seawater temperature and pH were constantly monitored using an Apex Aquacontroller (Neptune System). A microprocessor-controlled power strip (Apex Neptune Energy Bar 832) and wifi base unit (Apex Neptune Controller Base Unit) controlled 12 individual control units (Apex Neptune PM1 pH/ORP Probe Module). Each tank was measured using Neptune Systems Apex Extended Life Temperature Probe (accuracy = ± 0.05 °C) and a Neptune Systems Apex pH Probe (accuracy = ± 0.01 pH) that measured temperature and pH every second. Temperature was monitored with Apex, but controlled separately by underwater heaters (ProHeat D-1500 Heater Controllers, precision ± 1 °C), whereas pH was both monitored and controlled with the Apex system.

Daily fluctuating pH levels were generated with an independent pH-stat feedback system in each tank in order to simulate predicted future higher CO2 concentration conditions (Pachauri et al. 2014). Two 99.99% food-grade CO2 cylinders were connected to an automatic gas cylinder changeover system (Assurance Valve Systems, Automatic Gas Changeover Eliminator Valves #6091) to prevent an abrupt shortage of CO2 supply. CO2 was brought into the system on-demand, based on the pH reading in the tank (Neptune Apex control system) using gas flow solenoids (Milwaukee MA955) with airlines plumbed into a venturi injector (Forfuture-go G1/2 Garden Irrigation Device Venturi Fertilizer Injector), which was connected to a water circulating pump (Pondmaster Pond-mag Magnetic Drive Water Pump Model 5). Gas injected into the system was either CO2 or ambient air and bubbling was constant due to the pressure driven pump moving water and gas through the venturi injector.

OnSet HOBO loggers (HOBO Water Temp Pro v2, accuracy = ± 0.21 °C, resolution = 0.02 °C) were placed in each tank at the same height as the coral fragments for the duration of the experiment in order to log temperature in 10 minute intervals. Mesocosm tanks were 60% shaded from full irradiance, and photosynthetically active radiation was measured continuously with the Apex cosine corrected PAR Sensor (accuracy = ± 5%) that was cross calibrated to the Li-Cor cosine corrected PAR sensor (LI-192). Measurements were also made in 6 positions in each tank using the Li-Cor 193 spherical underwater quantum sensor (LI-193), which revealed there was no significant difference in light between the tank positions (n=4, p=0.948). Light was then assessed in the center of the tank ~daily throughout the experiment using the Li-Cor 193 spherical underwater quantum sensor. To further reduce any potential position effects with respect to incoming water, heater position, or bubble stream, the positions of the coral fragments in the tank were changed weekly during buoyant weighing.

*Experimental Timeline*

Coral fragments were exposed to four experimental conditions: Ambient Temperature Ambient pCO2 (ATAC: 27.47 °C ± 0.13; 8.03 pH ± 0.01, 396 µatm ± 12), Ambient Temperature High pCO2 (ATHC: 27.46 °C ± 0.12; 7.68 pH ± 0.014, 1045 µatm ± 35), High Temperature Ambient pCO2 (HTAC: 29.37 °C ± 0.06; 8.01 ± 0.01, 418 µatm ± 13), and High Temperature High pCO2 (HTHC: 29.51 °C ± 0.06; 7.64 ± 0.02, 1174 µatm ± 55) for two months (22 September - 17 November 2018), and then ramped down to ambient conditions over two months (18 November 2018 - 12 January 2019) (supplementary fig. S11C and D). During the two-month stress exposure period, one fragment per species per tank (n=3 per treatment) was sampled at random for each molecular time point (0h, 6h, 12h, 24h, and 1, 2, 4, 6, 8, 12, and 16 weeks; supplementary table S11). Sampled fragments were placed in sterile whirlpak bags and snap frozen in liquid nitrogen at 13:00 each day (with the exception of 6 h, 12 and 24 hr time points) and stored at -80 °C.

*Total Alkalinity and Carbonate Chemistry*

To assess the efficacy of the treatments, tank parameters (temperature in °C, pH on the total scale, and salinity in psu) were tracked up to several times daily using a handheld digital thermometer (Fisherbrand Traceable Platinum Ultra-Accurate Digital Thermometer, accuracy = ± 0.05 °C, resolution = 0.001 °C) and a portable multiparameter meter (Thermo Scientific Orion Star A series A325). A pH probe (Mettler Toledo InLab Expert Pro pH probe #51343101; accuracy = ± 0.2 mV, resolution = 0.1 mV) and conductivity probe (Orion DuraProbe 4-Electrode Conductivity Cell Model 013010MD; accuracy = 0.5% of psu reading, resolution = 0.01 psu) were used with the Orion A Star meter. To ensure pH (total scale) measurement quality, a tris standard (Dickson Laboratory Tris Batch T27 Bottle 269, 236 and Batch T26 Bottle 198) was measured daily to assess mV as a function of temperature.

To determine carbonate chemistry of each tank, pH, temperature, and salinity measurements were taken daily (as described above; supplementary table S12). Twice a week, water samples were taken simultaneously to measure total alkalinity (TA). An automated titrator (Mettler Toledo T50) was used to titrate water samples with 0.1M hydrochloric acid (Dickson Laboratory Titrant A3, A14) and accuracy was determined using certified reference material (Dickson Laboratory CO2 CRM Batches 132, 137, 176). A non-linear, least-squares procedure of the Gran approach (SOP 3b (Dickson et al. 2007)) was used to calculate TA (µmol kg−1 seawater). Carbonate values were calculated with SEACARB(v3.0.11) (Gattuso et al. 2015) in R Studio.

*~~Mortality and Color Score~~*

~~Survivorship was recorded daily and analyzed using a Cox proportional hazards regression model including Temperature and pCO2, with individual fragment ID assayed through time with the calls coxph and Surv in the package survival (Therneau 2020) (supplementary table S13). To assess tissue color change over time, each fragment was photographed once a week for all 16 weeks with a red, blue, green color standard ruler. ImageJ (Schneider et al. 2012) was used to extract the mean blue, green, and red color score for each coral fragment and these values were normalized to the mean of the red, blue, and green color standards. The color score was quantified as PC 1 from principal component analysis (Edmunds et al. 2003) (PCA) and analyzed statistically using a repeated measures ANOVA with Type III Wald Chi Square test (lmer function in the lme4 package (Bates et al. 2015) in R), for the fixed factors of Temperature, pCO2, and Time, with individual fragment ID as the repeated measure (supplementary table S6).~~

*Coral samples used for RNA-Seq*

During the stress experiment 450 samples per species, were subjected to one of four treatment conditions. Half of the samples collected (225 per species) were used for a separate physiology experiment, the other half underwent DNA and RNA extraction (described below). During the course of the treatment the P. acuta corals severely bleached, resulting in the mortality of nubbins from the final timepoint (TP12; 16 weeks). No samples were therefore available for processing and this timepoint is absent from all P. acuta treatments. In addition, one P. acuta sample, from the HTHC treatment at timepoint 11 (12 weeks) failed during RNA extraction and processing; leaving two samples from this timepoint. For M. capitata, due to sample degradation, one sample for HTAC timepoint 1 (0hr) could not be sequenced and a sample from timepoint 4 (12hr) was sequenced in its place; there are now n=2 for M. capitata HTAC timepoint 1 and n=4 for timepoint 4. In total, 119 RNA-Seq samples from P. acuta and 132 from M. capitata were sequenced and analyzed in this study (supplementary tables S4 and S11).

*DNA and RNA Extractions*

DNA and RNA were extracted simultaneously from the coral fragments that had been snap frozen and stored at -80 °C. A small piece was clipped off using clippers sterilized in 10% bleach, deionized water, isopropanol, and RNAse free water, and then placed in 2 mL microcentrifuge tube containing 0.5mm glass beads (Fisher Scientific Catalog. No 15-340-152) with 1000 μL of DNA/RNA shield. A two-step extraction protocol was used to extract RNA and DNA, with the first step as a “soft” homogenization to reduce shearing of RNA or DNA. Tubes were vortexed at high speed for 1 and 2 minutes for Pocillopora acuta and Montipora capitata fragments, respectively. The supernatant was removed and designated as the “soft extraction”. Second, 500 μL of DNA/RNA shield was added to the bead tubes and placed in a Qiagen TissueLyser for 1 minute at 20 Hz. The supernatant was removed and designated as the “hard extraction”. Subsequently, 300 μL of sample from both soft and hard homogenate was extracted with the Zymo Quick-DNA/RNA Miniprep Plus Kit Protocol with the following modifications. A first elution of 10 μL of warmed 10 mM Tris HCl was added to DNA columns for a 15-minute room temperature incubation and centrifuged at 16,000 rcf(g) for 30 seconds. A second elution of 100 μL of warmed 10 mM Tris HCl was added for a 5-minute incubation and centrifuged at 16,000 rcf(g) for 30 seconds. Only the second DNA elution, a higher quality portion of the sample, was kept for downstream analysis. Broad-range DNA and high-sensitivity RNA quantity (ng\_μL) were measured with a ThermoFisher Qubit Fluorometer, DNA quality was assessed using gel electrophoresis, and RNA quality was measured with an Agilent TapeStation System. RNA-Seq samples were sequenced by GENEWIZ (https://www.genewiz.com) using the Illumina NovaSeq 6000 platform. DNA from the “soft” extract of a single P. acuta sample was send to DNA Link Sequencing Lab (https://www.dnalinkseqlab.com) for sequencing on their PacBio Sequal 2 and Illumina NovaSeq 6000 platforms. A whole M. capitata nubbin was frozen and shipped to Dovetail Genomics (https://dovetailgenomics.com) for processing using their Omni-C assay and workflow.

*RNA-Seq read processing*

Adapters and low-quality regions were trimmed from the RNA-Seq data generated in this study using Cutadapt14 v2.9 (--nextseq-trim 10 --minimum-length 25 -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA; Table SX). A second round of trimming with Cutadapt, using the output from the first round, was used to remove poly-G regions from the 5′-ends of the second read in each pair (-G G(20) -e 0.0 -n 10 --minimum-length 25). Read quality was assessed at each stage using FastQC v0.11.7 (default parameters; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC13 v1.9.

*Quantification of gene expression*

Expression of predicted protein-coding genes from the *M. capitata* (Version 3; <http://cyanophora.rutgers.edu/montipora/>) and *P. acuta* (Version 2; <http://cyanophora.rutgers.edu/Pocillopora_acuta/>) reference genomes15 were quantified for each sample using Salmon14 v1.6.0 (--validateMappings --seqBias --gcBias --libType ISR) with the associated reference genomes used as decoys during index building. The Salmon read mapping rate of each sample is presented in supplementary table SX.

*Assessment of global gene expression*

*Differential gene expression analysis*

*Functional enrichment analysis*

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