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Protein expression and genetic structure of the coral *Porites lobata* in an environmentally extreme Samoan back reef: does host genotype limit phenotypic plasticity?

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Abstract

The degree to which coral reef ecosystems will be impacted by global climate change depends on regional and local differences in corals' susceptibility and resilience to environmental stressors. Here, we present data from a reciprocal transplant experiment using the common reef building coral *Porites lobata* between a highly fluctuating back reef environment that reaches stressful daily extremes, and a more stable, neighbouring forereef. Protein biomarker analyses assessing physiological contributions to stress resistance showed evidence for both fixed and environmental influence on biomarker response. Fixed influences were strongest for ubiquitin-conjugated proteins with consistently higher levels found in back reef source colonies both pre and post-transplant when compared with their forereef conspecifics. Additionally, genetic comparisons of back reef and forereef populations revealed significant population structure of both the nuclear ribosomal and mitochondrial genomes of the coral host (F_{ST} = 0.146 P < 0.0001, F_{ST} = 0.335 P < 0.0001 for rDNA and mtDNA, respectively), whereas algal endosymbiont populations were genetically indistinguishable between the two sites. We propose that the genotype of the coral host may drive limitations to the physiological responses of these corals when faced with new environmental conditions. This result is important in understanding genotypic and environmental interactions in the coral algal symbiosis and how corals may respond to future environmental changes.

Keywords: coral resilience, local adaptation, phenotypic plasticity, *Porites lobata*, reciprocal transplant experiment, Ubiquitin

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Introduction

Although climate warming scenarios (IPCC 2007) predict the most dramatic warming at high latitudes, even a small amount of warming at low latitudes may have large impacts on marine organisms as many tropical marine organisms presently experience temperatures at or near their heat tolerance thresholds (Stillman 2003; Carpenter *et al.* 2008; Tomanek 2008). Climate anomalies (such as the El Niño Southern Oscillation) increasing in severity and frequency because of global climate change (IPCC 2007), in combination with rising levels of ocean acidification are further compromising coral reef ecosystems (Hoegh-Guldberg *et al.* 2007). While coral reefs are distributed throughout the warm tropical and subtropical oceans and seas, most corals are found

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within 1–2 °C of their upper limits of temperature tolerance during the summer months (Jokiel 2004). Consequently, conspecific corals from different geographic regions can have very different heat tolerance limits and temperatures at which bleaching (the functional loss of their endosymbiotic algae) occurs. These regional differences in bleaching susceptibility are generally attributed to host and symbiont adaptation to local thermal environments (Hughes *et al.* 2003).

During mass coral bleaching events induced by extreme environmental temperatures and irradiances, there is generally survival of scattered colonies, specific communities, or whole reef sections (West & Salm 2003; Sotka & Thacker 2005). The mechanisms underlying continued survival of some corals and not others have been the focus of considerable research over the past few decades (Lesser 1997; Rowan *et al.* 1997; Brown *et al.* 2002a; Grottoli *et al.* 2006). In many cases, results have been conflicting and questions remain as to what degree host or symbiont-specific mechanisms contribute to increased resilience of the entire holobiont (defined here as the collective unit of the coral animal, endosymbiotic dinoflagellates of the genus *Symbiodinium*, and associated community of microorganisms).

Many of the proposed mechanisms for differences in holobiont stress tolerance and resilience can be grouped into three broad categories. First, it has been shown that different genotypes of the algal endosymbiont, *Symbiodinium,* significantly affect coral thermal susceptibility (Rowan *et al.* 1997; Baker 2001; Little *et al.* 2004); with thermally tolerant symbiont types found in corals living in higher temperature and postdisturbance environments (Baker *et al.* 2004; Rowan 2004; van Oppen *et al.* 2005). Second, host genetic background can alter such physiological responses as upper thermal tolerance limits across taxonomic and latitudinal gradients (Smith-Keune & van Oppen 2006; Ulstrup *et al.* 2006), symbiont type effect on holobiont physiology (Abrego *et al.* 2008), and capacity for heterotrophic feeding during bleaching and recovery (Grottoli *et al.* 2006). Third, it is thought that the history of the thermal conditions a given coral has experienced may drive acclimatization

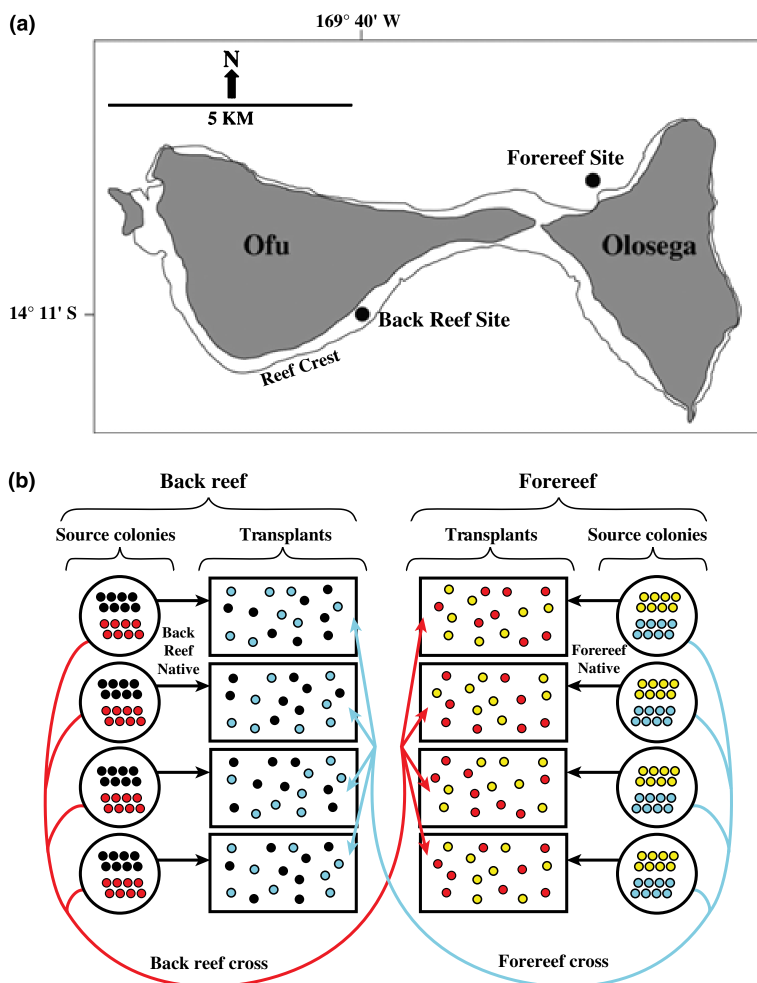

Fig. 1 (a) Map of Ofu and Olosega islands, American Samoa, showing source colony collection and transplant sites. (b) RTE transplant schematic illustrating experimental design. The RTE consisted of four replicate source colonies in each site, back reef and forereef, designated by black circles. Eight replicate cores from each source colony were designated for cross (red and blue for back reef and forereef, respectively) transplantation to the opposite environment, and native (black and yellow for back reef and forereef, respectively) transplantation to the same environment. Two cores per source colony per environment were sampled at time 0 and once daily at 13:45 for four consecutive days following a 24-h acclimation period. Figures modified based on similar in (Smith *et al.* 2007).

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responses in the form of increased capacity for cellular stress defence and thermotolerance (Brown *et al*. 2000, 2002a; b). In this study, we use an integrative approach to address the contributions of these three factors (symbiont and host genotypes, and thermal experience) to the stress resistance of corals from habitats with different thermal conditions that are extreme compared with those of most extant reefs (Coles 1997), but which could represent 'normal' thermal conditions of future reefs under projected climate warming scenarios (IPCC 2007).

Our study was conducted on Ofu and Olosega islands in American Samoa (14°11′S, 169°40′W; Fig. 1a). These islands host diverse communities of ~85 shallow reef-building coral species, many of which are consistently exposed to seawater temperatures (Craig *et al*. 2001) and irradiances (Smith & Birkeland 2007) greater than neighbouring areas. The extreme conditions on Ofu are caused by a prominent reef crest that blocks circulation between the waters of the back reef and the open ocean, limiting mixing of back reef waters during midday low tides (Smith & Birkeland 2003). This limited mixing coupled with high midday solar irradiance produces large (up to 6 °C) daily fluctuations in temperature (Figs 2 and S1, Supporting Information; Smith & Birkeland 2003, 2007). In addition, frequency of large storm events, corresponding rainfall, and potential groundwater release sporadically cause drops in back reef salinity (up to 5 ppt; Fig. S2, Supporting Information; Smith *et al*. 2007). In comparison, the shallow (<3m low tide depth) forereef of Olosega island represents a more typical, average reef environment with a more constant flow regime, significantly lower temperature, irradiance, and salinity variability, and extremes

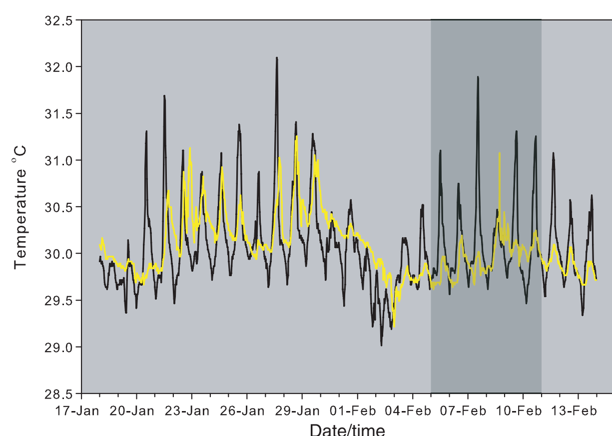

Fig. 2 Temperatures (°C) in the back reef (black line) and forereef (yellow line) recorded every 30 min continuously for 27 days surrounding the RTE using HOBO Water Temp Pro® temperature loggers (Onset Computer Corp.). The specific duration of the RTE is denoted by the shaded area.

of each (Figs S1–S5, Supporting Information; Smith *et al*. 2007; Smith & Birkeland 2003, 2007).

Using the common reef-building coral *Porites lobata*, we investigated (i) coral holobiont (cnidarian host and *Symbiodinium*) protein expression, (ii) *Symbiodinium* genetic identity (referred to herein as symbiont), and (iii) cnidarian host population structure in a reciprocal transplant experiment (RTE) between a thermally extreme back reef on Ofu island and the more stable, neighbouring forereef on Olosega. Our study revealed evidence for genotypic influence on all four of the biomarkers under investigation which correlated with differences in coral host population genetic structure. Genetic differentiation of symbionts in corals from the two study locations was not observed, thus these results suggest a host genetic influence on response differences between corals from contrasting thermal environments.

Materials and methods

Source colony selection and environmental monitoring

Individual *P. lobata* source colonies were initially identified based on surface morphology and skeletal characteristics (Veron 2000). Additionally, all sequences obtained from the host genetic study were integrated into a comprehensive *Porites* phylogeny (Forsman *et al*. 2009) and all shown to fall within Clade I, a monophyletic clade containing known *P. lobata* genotypes from other sites across the Pacific (data not shown).

Temperatures at 1 m low tide depth in the Ofu back reef have been continuously recorded every 30 min since 1999 with Tidbit® (1999–2003) or Water Temp Pro® (2004–present) temperature loggers (Onset Computer Corp., Pocasset, MA, USA). Forereef temperatures were recorded every 30 min with Water Temp Pro loggers during the experimental period (18 January 2005 to 13 February 2005), and continuously since April, 2006 for long-term comparisons. Water quality data loggers, Sonde 6600 (YSI Inc., Yellow Springs, OH, USA), measuring salinity, conductivity, and dissolved oxygen were deployed in both forereef and back reef sites during 12 days in March 2006. A 48-h profile of nutrient dynamics in the water column was also performed during March 2006. Additional water quality measurements were taken in the back reef during a previous study (Smith & Birkeland 2003).

Sample collection and reciprocal transplant experiment

A pneumatic drill and hole saw attachment was used to remove 18 cores, 19-mm diameter, from the upward facing surfaces of each four source colonies in the back reef and forereef sites; two for immediate freezing

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(day 0) and eight each for cross (new location) or back (original location) transplantation (Fig. 1b). Holes were filled and transplants affixed to the substrate with Z-Spar, Splash Zone marine epoxy (Carboline Company, St Louis, MO, USA).

To minimize confounding factors associated with clonal structure and microhabitat differences, original source colonies were selected >5 m apart and similar in size to reduce the likelihood of selecting clones, and resulting forereef and back reef cores were grouped randomly and transplants were affixed in random orientation >5 m apart and near (<1 m) original source colony locations to include microhabitat variation. The transplant experiment was initiated on 5 February 2005 (day 0) and concluded on 10 February 2005 (day 5). After an initial 24-h recovery period (day 1), all samples were destructively sampled for four consecutive days (days 2, 3, 4 and 5) between 13:45 and 14:15. At each sampling day, two cores from each parent colony in each transplant group were placed on preincubated −80 °C ice packs and stored at −80 °C until used for protein or DNA extractions.

Coral protein extraction

Coral and algal symbiont protein was extracted using a protocol adapted from a previous study (Brown *et al.* 2002a). Each core was flash frozen in liquid nitrogen before the tissue layer (up to 1 cm below surface) was removed with bone cutting pliers and placed in a pre-frozen, 50-mL stainless steel mixing jar (Glennmills, Clifton, NJ, USA). The tissue and skeleton was crushed using a TissueLyser® (Qiagen, Valencia, CA, USA) at 25 rpm for 5 s. Resulting samples were transferred to individual 2.5-mL cryovials and stored at −80 °C until further analyses.

Between 280 and 380 mg of crushed tissue was added to a prechilled 2 mL microcentrifuge tube to which 3.5 μL/mg of protein extraction buffer was added for a total volume of 1–1.5 mL. The extraction buffer was made fresh daily and consisted of 50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 25 mM dithiothreitol (DTT), 10 mM EDTA, 4% (w/v) polyvinylpolypyrrolidone (PVPP), and 1% (v/v) DMSO. One microlitre of Halt Protease Inhibitor Cocktail (Pierce Biotechnologies, Rockford, IL, USA) was added per 100 μL of extraction buffer. Tubes were vortexed for 20 s then incubated at 90 °C for 3 min, vortexed again for 20 s, and incubated for an additional three minutes at 90 °C. The tubes were cooled at room temperature for 5 min then centrifuged at 13 000 *g* for 15 min. After the centrifugation, approximately 300–400 μL was removed from the middle phase of the tube, being careful not to collect any supernatant that had been contaminated with the whitish matrix that was the top-most phase. Total protein concentration was determined using the Bio-Rad RC/DC protein concentration assay (Bio-Rad, Hercules, CA, USA).

SDS–PAGE and Western blotting

Standard curve dilutions of 0, 2, 6, 10, 14 and 18 μg total protein for ubiquitin-conjugate assays and 0, 1, 3, 5, 7 and 9 μg total protein for all other assays of a single, standard coral sample were added to all gels for reference. Note that an increased range of standard was needed for ubiquitin-conjugate assays in order to accurately capture the larger range of the experimental samples. Two microgram total protein of each experimental sample was added to 4 μL of E-PAGE Sample Loading Buffer (Invitrogen, Carlsbad, CA, USA), and water to a final volume of 18 μL. All samples and standards were run in triplicate, boiled at 95 °C for 5 min before loading onto E-PAGE 96-well 6% gels (Invitrogen) and run for 14 min with the provided E-PAGE power supply according to manufacturers instructions.

Gels were removed, incubated for 30 min in a western transfer buffer consisting of 25mM Tris, 192 mM Glycine, 20% v/v Methanol, 0.025% w/v sodium doecyl sulphate (SDS) pH 8.3 before transfer using a Criterion blotting system (Bio-Rad) according to manufacturers instructions with PVDF (Millipore, Bedford, MA, USA) membrane. Transfers were conducted for 30–40 min at 100 V. Membranes were blocked overnight in 50 mL 1 × TBST (50 mM Tris, 150 mM NaCl pH to 7.4, 0.1% (v/v) Tween-20) and 1% (w/v) bovine serum albumin (BSA) at 4 °C. After blocking, membranes were incubated with primary antibody in TBST/BSA blocking buffer for 2 h at room temperature, washed three times for 10 min each with fresh changes of TBST, incubated with secondary antibody in blocking buffer for 1 h, washed three times for 5 min each in 1 × TBS with 0.3% Tween-20 (v/v), and three times for 5 min each in 1 × TBST before detection with SuperSignal West Dura Extended Duration Substrate (Pierce; antibody dilutions are specified below). All images were recorded using a ChemiDoc XRS molecular imager (Bio-Rad) and analysed with National Institute of Health Image J software.

Antibodies against heat shock protein 70 (hsp70; Cat. #SPA-822), Ubiquitin (Cat, #SPA-200), Manganese superoxide dismutase (MnSOD; Cat. #SOD-110), anti-rabbit- and anti-mouse-conjugated (Cat. #SAB-300, SAB-100) horseradish peroxidase were obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Anti-4-Hydroxynonenal (4HNE; Cat. #AB5605) and anti-goat-conjugated horseradish peroxidase (Cat. #AP180P) antibodies were obtained from Chemicon International (Temecula, CA, USA). Primary antibody dilutions were 1:10 000 for hsp70, 1:5000 for MnSOD,

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and 1:2000 for Ubiquitin and 4HNE, secondary antibody dilutions were all 1:10 000.

Antibody specificity was verified by molecular weight comparisons with known standards and protein extractions from *Symbiodinium* cultures and holobiont (host and symbiont) tissue homegenates. Gels run for hsp70, ubiquitin-conjugates, MnSOD, and 4HNE-conjugates were 8%, 10%, 15%, and 10% SDS–PAGE, respectively. Positive control lanes (+) consisted of 10 μg of Heat Shocked HeLa Cell Lysate for hsp70 and 4HNE-conjugates, and Rat Brain Tissue Extract for ubiquitin-conjugates (Stressgen LYC-HL101F and LYT-RB100F, respectively). Gels were run at 200 V for 45 min with a 100 V, 50 min wet transfer. No positive control was available when running the MnSOD validation gel, however, the prominent band observed was ~25 kDa, the expected size of MnSOD; and a similar size was observed using rat brain tissue extract previously (data not shown).

Antibody reactivity against *Symbiodinium* protein was investigated using protein extracts from three different *Symbiodinium* cultures (KB8, AipB and MCap) graciously provided by the laboratory of Dr Robert Kinzie III at the Hawai'i Institute of Marine Biology (Fig. 3). These cultures are believed to represent *Symbiodinium* types from taxonomic clades A, B and D, respectively. Antibody recognition of host/symbiont homogenates was investigated using extractions prepared from a heat shocked *P. lobata* colony collected at the Hawai'i Institute of Marine Biology (Hom1, Hom2 and Hom3; Fig. 3). Reactivity against Cnidarian host protein is assumed based on manufacturers recommendations and previous study (Downs *et al.* 2000).

Protein biomarkers were chosen based on their functional roles in stress defence and stress-caused damage: heat shock protein 70 (hsp70) is a molecular chaperone that helps maintain protein structure and function

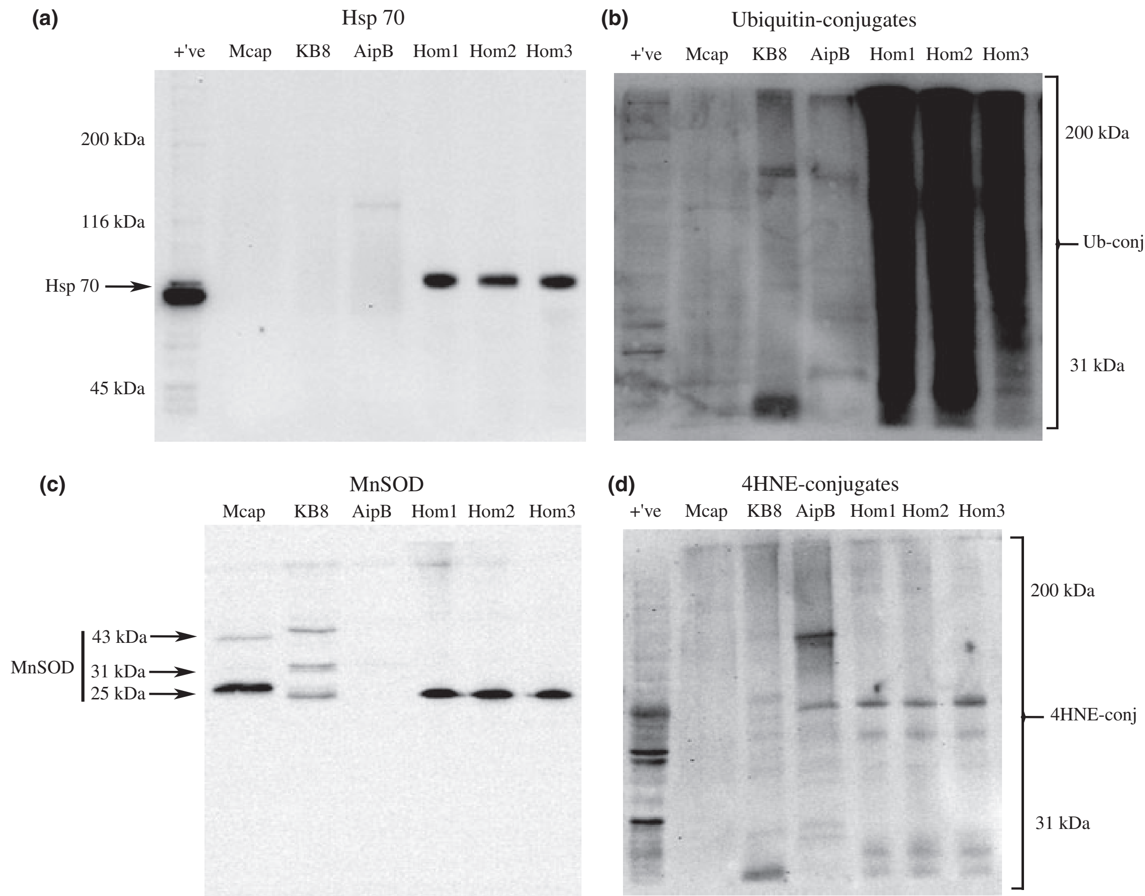

Fig. 3 Western blots used for validation of (a) Hsp70, (b) Ubiquitin-conjugate, (c) MnSOD and (d) 4HNE-conjugate antibody specificities against protein extractions from *Symbiodinium* cultures and holobiont (host and symbiont) tissue homegenates. Approximate molecular weights (kDa) are based on standard migration charts for gels of identical SDS–PAGE composition. Lanes MCap, KB8 and AipB are from three separate extracts of cultured *Symbiodinium* while lanes Hom1, Hom2 and Hom3 are from host and symbiont homegenate extractions. Arrows and brackets identify targets of interest at the predicted molecular weight (~70 kDa for hsp70, ~25–43 kDa for MnSOD isoforms, and full conjugate smear < 31 to > 200 kDa for Ubiquitin and 4HNE-conjugates).

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under heat related denaturation, functionally serving as a cellular defence against temperature related stress (Feder & Hofmann 1999). Manganese superoxide dismutase (MnSOD) deactivates the reactive superoxide anion that is formed under oxidative stress and symbiont photosynthesis before it can damage other proteins involved in cellular processes (Halliwell & Gutteridge 1999). 4-Hydroxynonenal (4HNE) is a reactive lipid peroxide that causes oxidative protein damage (Halliwell & Gutteridge 1999). Finally, ubiquitin is a cellular protein tag that is bound to damaged proteins, marking them for degradation and reuse (Hawkins 1991). Increases in ubiquitinated proteins are generally thought to be indicative of elevated levels of stress induced protein damage, although nondamage related functions of ubiquitination related to protein turnover, trafficking, and maintenance of cellular protein homeostasis could also produce similar increases (Hawkins 1991; Welchman *et al.* 2005).

Protein statistical analysis

Experimental protein concentration values were calculated relative to a standard coral sample dilution curve for each blot image based on integrated optical density using National Institute of Health software Image J. Statistical analyses were performed using Minitab® 15 (Mintab Inc., State College, PA, USA) and JMP Ver. 8 (SAS Institute Inc., Cary, NC, USA). A repeated measures ANOVA was performed with marker concentration as the response variable for each transplant day compared with source colony origin, transplant destination, and source colony number nested within origin. To investigate detailed responses within each day, five separate ANOVAs were conducted as above for ubiquitin-conjugate data using marker concentrations split by individual days. All data were natural log transformed prior to the repeated measures analysis and a weighted least squares correction was used on untransformed data for the within day tests to correct for unequal variances.

DNA extraction, amplification and sequencing

DNA was extracted following a previous study (Concepcion *et al.* 2006) for subsequent use as template in all PCRs. Optimal annealing temperatures for each primer set were determined empirically by temperature gradient PCR on a MyCycler Thermal Cycler (Bio-Rad). An approximately 650 bp sequence of coral host nuclear ribosomal DNA was amplified using primers 'ITSZ1' (5′-TAAAAGTCGTAACAAGGTTTCCGTA-3′; Forsman *et al.* 2009) and 'ITSZ2' (5′-CCTCCGCTTATTGATATGCTTAAAT-3′; Forsman *et al.* 2009). This fragment consisted of partial sequences of the 18S and 28S ribosomal RNA genes, and complete sequences of the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2), and the 5.8S ribosomal RNA gene. ITS hot-start PCR amplification was performed as follows: 95 °C for 7 min (one cycle), followed by 35 cycles consisting of 94 °C for 30 s, 50 °C for 30 s and 70 °C for 2 min, and a final extension at 72 °C for 1 min.

A 498-bp sequence of host coral mitochondrial DNA from the *NAD5* intron region was amplified using the primers 'NAD_700F' (5′-TGCCGGATGCYATGGAG-3′; Concepcion *et al.* 2006) and 'NAD_157R' (5′-VCCATCYGCAAAAGGCTG-3′; Concepcion *et al.* 2006) under the following conditions: 95 °C for 10 min (one cycle), followed by 35 cycles consisting of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, and a final extension at 72 °C for 1 min. A second mitochondrial host coral fragment from the putative control region was amplified with primers 'CRf' (5′-GCTTAGACAGGTTGGTTGATTGCCC-3′; Vollmer & Palumbi 2002) and 'CO3r' (5′-CTCCCAAATACATAATTTGAACTAA-3′; Vollmer & Palumbi 2002) under the following conditions: 95 °C for 7 min (one cycle), followed by 35 cycles consisting of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 5 min.

For symbiont genetic analysis a ~280 bp fragment of the ITS2 region of the symbiont ribosomal DNA array was either amplified with primers 'Sdino' (5′-CGCTCCTACCGATTGAGTGA-3′; Pochon *et al.* 2001) and 'L0' (5′-GCTATCCTGAGRGAAACTTCG-3′; Pochon *et al.* 2001), or primers 'ITSintfor2' (5′-GAATTGCAGAACTCCGTG-3′; LaJeunesse & Trench 2000) and 'ITS-Reverse' (5′-GGGATCCATATGCTTAAGTTCAGCGGGT-3′; Coleman *et al.* 1994) under the following conditions: 95 °C for 7 min (one cycle), followed by 35 cycles consisting of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 5 min.

All mitochondrial PCR amplifications (25 μL) consisted of 2 μL of DNA template, 2.5 μL of 10 × ImmoBuffer, 0.1 μL IMMOLASE DNA polymerase (Bioline Inc., London, UK), 1.5 μL of 50 mM $MgCl_2$, 0.5 μL of 10 mM total dNTPmix (Bioline), 0.325 μL of each 10 μM primer, and deionized H_2O to volume. All ITS host and symbiont PCR amplifications (50 μL) consisted of 2 μL of DNA template, 5 μL of 10 × ImmoBuffer, 0.2 μL IMMOLASE DNA polymerase (Bioline), 2 μL of 50 mM $MgCl_2$, 1 μL of 10 mM total dNTPmix (Bioline), 1 μL of each 10-μM primer, and deionized H_2O to volume. All PCR products were visualized using 1.0% agarose gels (1 × TAE) stained with 0.5% v/v Gelstar®. PCR products were treated with 2 U of exonuclease I and 2 U of shrimp alkaline phosphatase (Exo:SAP) at 37 °C for 60 min, followed by inactivation at 80 °C for 10 min. Cleaned mitochondrial PCR products were then cycle-

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sequenced using the ABI Prism Big Dye Terminator Reaction Kit (PerkinElmer–Applied Biosystems, Foster City, CA, USA) and run on an ABI-3100 automated sequencer. Clone libraries were constructed for each host and symbiont ITS PCR product using the pGEM®-T easy vector system (Promega Corp., Madison, WA, USA). Positive inserts were verified by PCR using M13 primers and plasmids (8–10 per library) were sequenced as above.

Sequence analysis

Resulting sequences were inspected using Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned using Bio-edit (Hall 2001) and by eye. Population genetic structure was estimated using an AMOVA in Arlequin 3.1 (Excoffier & Schneider 2005). Molecular phylogenetic networks were constructed using the median-joining algorithm and maximum parsimony postprocessing calculation in NETWORK ver 4.5.0.0 (Fluxus Technology Ltd; Polzin & Daneschmand 2003). Host mtDNA fragments from the *NAD5* and putative mitochondrial control regions were concatenated into a single sequence for alignment and analysis.

It is known that a range of copy number and intragenomic variability exists within the Scleractinian ITS region (Vollmer & Palumbi 2004; Wei *et al.* 2006; Coleman & van Oppen 2008). Thus, multiple approaches were used in analyses of the Host ITS sequences in this study. Results presented herein are from calculation of population differentiation performed on the entire data set including all sequence variants from each individual because this approach treats each cloned sequence as an individual. However, host ITS intragenomic variants were also combined into single consensus sequences for each individual using Sequencher for additional alignment and analysis (Supporting Information). No difference in statistical outcome for host ITS analysis was observed between sequences built by consensus by inclusivity vs. plurality, hence data presented are from analysis of consensus sequences generated by inclusivity. By incorporating all nucleotide polymorphisms detected within the clone libraries from individuals using International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes, the entire diversity of an individual's ITS region is represented by a single inclusivity consensus sequence. This approach tests the level of genetic differentiation between the entire suite of ITS diversity contained within individuals among the sampling populations. Network constructions were made using both the complete and consensus-based data sets (Fig. 6 and S6); however, for simplicity, only the consensus-based network is presented in the main text.

There is a similar occurrence of intragenomic variation in Symbiodinium rDNA (van Oppen & Gates 2006; Thornhill *et al.* 2007), which dictates a level of caution when handling cloned symbiont sequences. Hence, all symbiont ITS2 sequences were screened for potential errors following a previous study (Stat *et al.* 2009). Briefly, unique sequence types were only included in the analysis if they were found from multiple (three or more) clone libraries or if they matched previously published sequence types. The remaining clone singletons were assumed to be errors or rare intragenomic repeats and the nucleotide at the site of polymorphism was converted to the consensus. Additionally, IT2S folding was conducted using previously published Symbiodinium ITS2 structures as templates (Hunter *et al.* 2007; Thornhill *et al.* 2007) in the software package 4SALE (Seibel *et al.* 2006, 2008). Potential psuedogenes were characterized by significant changes to the secondary structure of the ITS2 RNA molecule that probably disrupt correct folding (Thornhill *et al.* 2007). These sequences were treated similar to singletons and reverted to the consensus sequence as determined by statistical parsimony. This approach is thought to greatly reduce the potential artefact on diversity estimates caused by the presence of singletons and pseudogenes generated by cloning based methodologies (Thornhill *et al.* 2007; Sampayo *et al.* 2009; Stat *et al.* 2009).

Results

Protein biomarker investigation

Antibody recognition of proteins extracted from cultured *Symbiodinium* was observed for MnSOD, Ubiquitin-conjugated proteins, and 4HNE-conjugated proteins (Fig. 3). Antibody recognition of *Symbiodinium* hsp70 protein was not observed (Fig. 3), possibly because of the specificity of the antibody for animal hsp70 or to limited expression or nonrecognition of hsp70 in these specific cultured *Symbiodinium* strains. Additionally, multiple SOD bands were observed in extracts from *Symbiodinium* cultures Mcap and KB8 (Fig. 3c), a phenomenon which has been found previously in coral symbionts (Richier *et al.* 2003) because of the increased number of SOD isoforms found within plant tissues. For the host/symbiont homegenates, a single band of the predicted molecular weight was visualized for the MnSOD (~25 kDa) and hsp70 (~70 kDa) antibodies, while for the ubiquitin and 4HNE antibodies, a variety of bands were observed due to the conjugation of these markers to a wide range of proteins (Fig. 3). There were striking differences between the profiles for cultured *Symbiodinium* extracts and those of the host/

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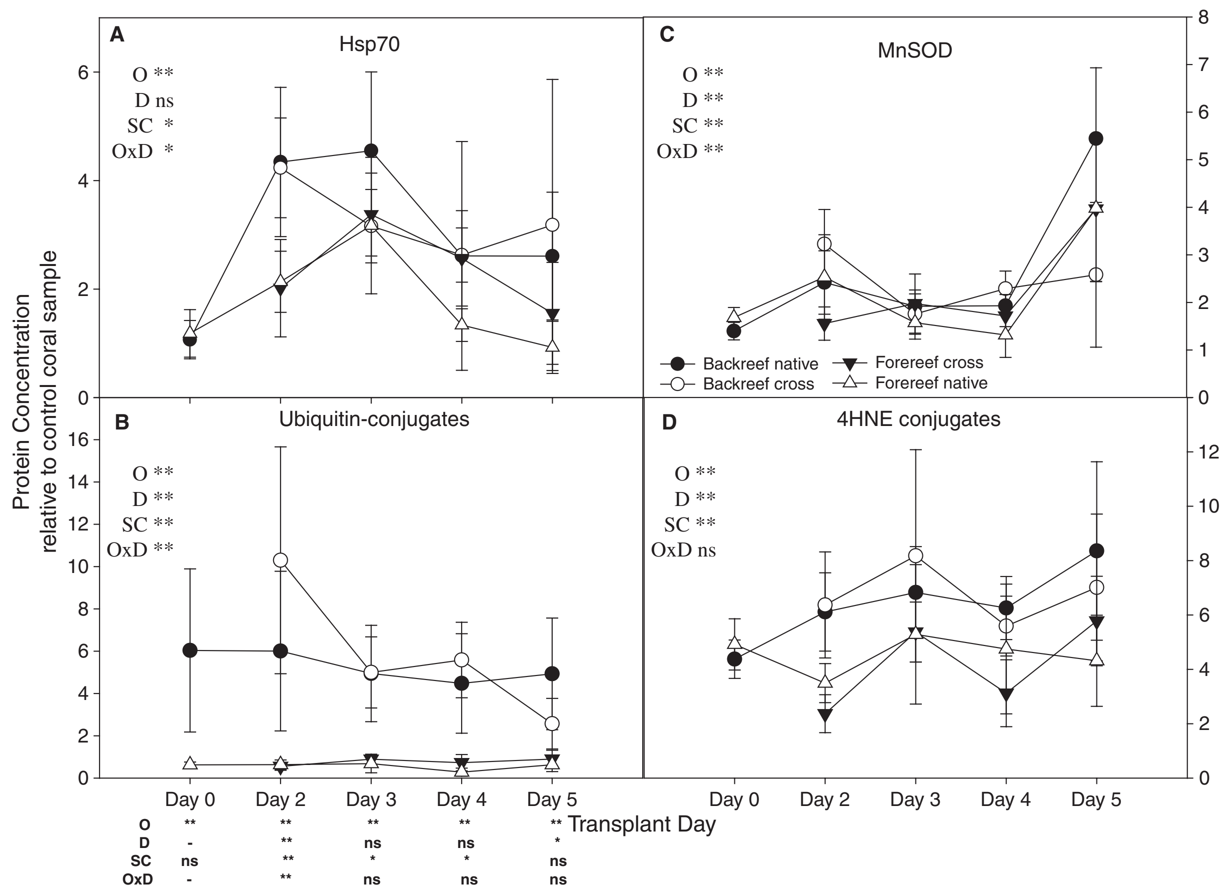

Fig. 4 Molecular marker concentrations (a) Hsp70, (b) Ubiquitin-conjugates, (c) MnSOD and (d) 4HNE-conjugates calculated from Western blots of host and symbiont homegenates relative to a standard coral sample (optical density of marker concentration per 2 μg total soluble protein) for pre (day 0) and subsequent days of the RTE. Means ± 1 SD. Black and white circles and black and white triangles represent back reef native and cross transplants and forereef native and cross transplants respectively. Statistical significance at $^*P < 0.05$ and $^{**}P < 0.01$ is presented in the legend contained within each graph for comparisons of source colony origin (O), transplant destination (D) and source colony individual (SC), and below the ubiquitin-conjugate panel for within day tests. For detailed results of statistical tests see Table S1, Supporting Information.

symbiont homegenates. The multiple MnSOD bands, lack of hsp70 reactivity, and overall weaker signal in *Symbiodinium* extracts despite greater amounts of total protein, all suggest that the majority of the signal in the homogenate extracts is likely of host origin. While we cannot rule out the influence of *Symbiodinium* protein on the signal, the suggestion that the protein complement of host tissues is driving the antibody signal has important implications given the results from the experimental samples presented below.

All four protein biomarker concentrations were evaluated from the RTE consisting of four source colonies of *P. lobata* in each location selected for cross (new location) or native (original location) reciprocal transplantation (Fig. 1b). Comparison between native and cross transplant groups examines variability by transplant destination (back reef native vs. back reef cross, forereef native vs. forereef cross) and by source colony origin (back reef native vs. forereef cross, forereef native vs. back reef cross; Smith *et al.* 2007). Within the RTE framework, variability by transplant destination is indicative of environmental influence on response (i.e. phenotypic plasticity), while variability by source colony origin indicates potential genetic influence on the response variable (Schluter 2000; DeWitt & Scheiner 2004; Smith *et al.* 2007).

Figure 4 shows the relative concentrations of each biomarker protein observed throughout the time-course of the experiment. The results for hsp70, MnSOD, and 4HNE indicated similar trends as the ubiquitin-conjugates although with much greater response variability on both temporal and treatment scales. Significant source colony origin and individual effects were observed for all three markers in addition to an effect

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of transplant destination (MnSOD and 4HNE) and a source colony origin by transplant destination interaction (MnSOD and hsp70; Fig. 4, Table S1a, Supporting Information). Additionally, all four markers showed a significant effect of transplant day (Table S1, Supporting Information), probably indicating a response to handling stress and/or some component of daily variability between measurements.

The consistent differences observed in the ubiquitin-conjugate response allowed for a more detailed analytical approach for this marker only. Ubiquitin-conjugate levels were consistently higher in back reef when compared with forereef source colonies for both transplant treatments and across all days. There were significant effects of both source colony origin and individual on Ubiquitin response (Fig. 4), suggesting a genotypic influence at both the population and individual level. Tests within each day revealed that source colony origin was significant pretransplant and for all four days post transplant while source colony individual was significant only on transplant days two, three and four (Fig. 4 and Table S1b, Supporting Information). Significant effects of transplant destination, and a transplant destination by source colony origin interaction were also observed (Fig. 4). However these effects were only significant for within-day tests on days two (destination and interaction) and five (destination only), were opposite in nature, and appear to be driven by the changing response of back reef cross transplants for those given days (Fig. 4b and Table S1b, Supporting Information).

Symbiodinium *community analysis*

An additional 11 and 12 source colonies of *P. lobata* were sampled from the forereef and back reef, respectively, for the *Symbiodinium* genetic analysis and were included with the original four source colonies from each location used in the RTE to ensure robust comparisons. A total of 154 back reef and 143 forereef *Symbiodinium* cloned sequences from the ITS2 of the nuclear ribosomal tandem array were collected, comprising two previously published sequence types and four novel sequence identities (Fig. 5 and Table S2). Two sequences from separate clone libraries matched the unpublished GenBank sequence C60 (AY589764.1); however, this resulted in a disrupted ITS2 fold and was excluded from the analysis. Likewise, a singleton sequence from one individual clone library was more closely related to another unpublished sequence C31c (FJ646570.1) than C15, yet this sequence also resulted in a nonfunctional ITS2 fold, has never been found in any *Porites* species previously, and was also considered potential artefact and excluded from analysis. Molecular phylogenetic network examinations of the back reef and

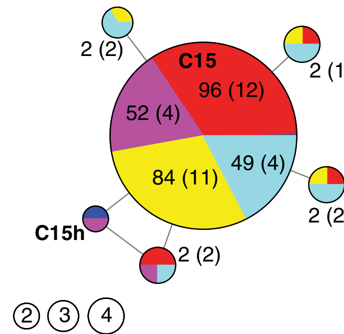

Fig. 5 Maximum parsimony phylogenetic network reconstruction of *Symbiodinium* sp. ITS2 isolates from symbiont genetic survey compared with previously published sequences. Lines between nodes represent single substitutions, gaps and/or indels. Gaps and/or indels were weighted as single changes regardless of length. Diameter of circle at each node is proportional to the number of clones with identical sequences. Nodes with >1 sequence per sample group have the corresponding number of sequences marked within the circle followed by the number of individuals from which those sequences were obtained in parentheses. Sequences from back reef individuals are shown in red, with those from back reef colonies used in the reciprocal transplant experiment shown in light purple, forereef individuals shown in yellow and forereef RTE colonies shown in light blue, and previously published sequence types shown in dark blue. Sequence accession numbers can be found in Table S2, Supporting Information.

forereef *Symbiodinium* populations showed no systematic differences in symbiont identity between back reef and forereef corals with 94.6% of sequence types identical to ITS2 type C15 (Fig. 5), a genotype known to associate with Pacific *Porites* sp. (LaJeunesse *et al.* 2003). All colonies sampled contained sequences identical to type C15, with the four additional sequences exhibiting no discernable pattern based on source location.

Porites lobata *genetic population structure*

Multilocus sequence data from the ITS of the nuclear ribosomal tandem array (rDNA) and two mitochondrial (mtDNA) regions (the putative control region, and the NAD5 intron) were obtained from 27 and 28 back reef and 22 and 26 forereef *P. lobata* source colonies (mtDNA and rDNA, respectively). The two mtDNA fragments were concatenated to produce six unique haplotypes, two shared between forereef and back reef and four unique to the forereef. Cloning of ITS intragenomic variants resulted in 96 and 126 unique sequence types for the back reef and forereef populations, respectively, with three sequence types shared between them. All coral samples were found to contain at least one unique sequence type not found in any other individual, hence no individual samples are thought to be clonal pseudoreplicates.

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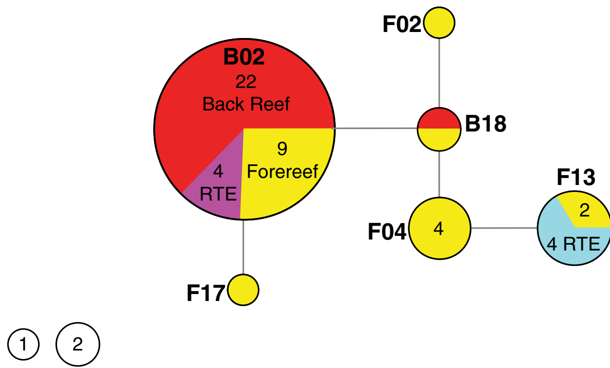

Fig. 6 Maximum parsimony network of mtDNA haplotypes. Colour scheme is the same as in Fig. 5 excluding dark blue. Individual sequence names are shown in black with a B or F for back reef and forereef individuals respectively followed by colony number.

Host genetic comparisons using both ITS and mtDNA revealed significant genetic differentiation between these back reef and forereef corals ($F_{ST} = 0.146$ $P < 0.0001$, $F_{ST} = 0.335$ $P < 0.0001$ for ITS and mtDNA, respectively). Four of the six mtDNA haplotypes were unique to the forereef, while 10 forereef individuals shared haplotypes with back reef samples (Fig. 6). Overall, the back reef population had lower haplotype diversity with only two of the six total mtDNA haplotypes occurring in the back reef samples while all six occurred in the forereef samples. The ITS network showed similar shared and unique forereef and back reef sequences types, with greater overall sequence diversity (Fig. 7 and S6). No difference in statistical outcome was observed between the individual sequence and consensus methods with the greater F_{ST} of the consensus method ($F_{ST} = 0.221$) probably caused by the reduced number of sequence types within that analysis (Hedrick 1999).

Discussion

Back reef and forereef P. lobata *exhibit variability in protein response*

The significant source colony and transplant effects for all four biomarkers demonstrate that coral response is both a function of genotypic and environmental influences. The most consistent differences observed were the elevated levels of ubiquitin-conjugated proteins in back reef vs. forereef source colonies. Although similar trends were observed for all markers, three of the four (hsp70, MnSOD, and 4HNE) showed substantial variability in response to the different characteristics of the fore and back reef environments (e.g. temperature, salinity, light, flow; Fig. 2, S1–S5, Supporting Information). A high degree of variability in some of the markers has been observed previously for field-collected corals (van Oppen & Gates 2006) making it difficult to draw any substantial conclusions from these three

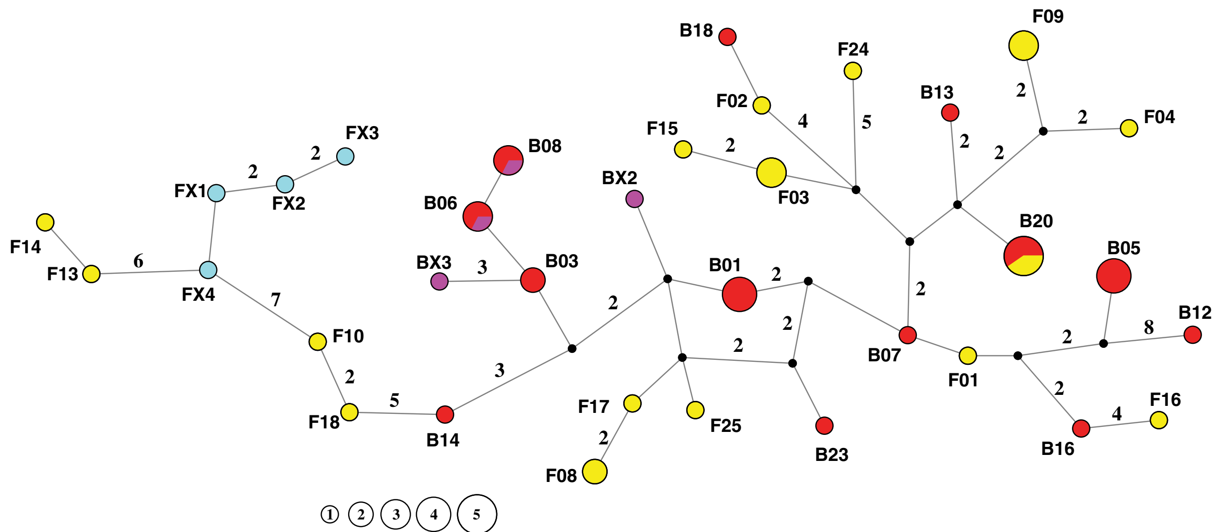

Fig. 7 Maximum parsimony network of ITS consensus sequences. Colour scheme is the same as in Fig. 5 excluding dark blue. Individual sequence names are shown in black with a B or F for back reef and forereef individuals, respectively, followed by colony number, Xs denote the source colonies used in the RTE. Lines between nodes represent single substitutions, gaps and/or indels; multiple substitutions are denoted using single black digits located next to each line. Gaps and/or indels were weighted as single changes regardless of length. Diameter of circle at each node is proportional to the number of clones with identical sequences.

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markers. Thus, the remainder of the discussion will focus on the ubiquitin-conjugate data.

Information regarding the relative contribution of genotypic and environmental influence on ubiquitin-conjugate response may be gained by looking at the consistency of significant effects for each factor. A significant effect of source colony origin was found for all within-day tests while an effect of transplant destination was only significant for two of the five days tested with an opposing direction of difference on day two than day five (Fig. 4). Additionally, the magnitude of average differences between ubiquitin-conjugate levels based on source colony origin was much greater than that based on transplant destination (~790% average difference between origins vs. ~41% between destinations). Taken as a whole, the influence of source colony origin on the ubiquitin response appears stronger than that of transplant destination. Additionally, the lack of increased ubiquitin-conjugates in forereef cross transplants suggests limitations to the capacity for phenotypic plasticity which could be due to long-term acclimatization or fixed genetic constraints on the phenotypic response.

An important factor to consider is that the five day duration of the RTE may not have been long enough to adequately allow full acclimatization of protein responses to new environmental conditions. Other studies on coral stress response have observed strong physiological changes on the scale of hours to days (Brown *et al.* 2002a; Richier *et al.* 2005; Putnam & Edmunds 2009), yet the dynamics of coral acclimatization remain poorly understood (Jokiel 2004; Edmunds & Gates 2008). Evidence from a concurrent, 6-month transplant study using the same *P. lobata* source colonies and RTE design observed a similar combination of genotypic and environmental influences on growth responses over a longer experimental duration (Smith *et al.* 2007).

Smith *et al.* (2007) found that back reef source corals had higher survivorship and elevated growth when compared with forereef source corals (genotypic influences) while corals from both origins showed elevated growth in the back reef vs. the forereef transplant destinations (environmental influence). The findings of Smith *et al.* (2007) agree with those of the current study demonstrating limitations to phenotypic plasticity based on source colony origin; a finding that has also been found previously for the congeneric *P. attenuata* (Raymundo 2001).

However, a potential contradiction exists between the greater levels of ubiquitin conjugates in back reef source colonies observed in this study and the increased growth and survival of back reef source colonies observed by Smith *et al.* (2007). Increases in ubiquitin-conjugated proteins have been shown to correlate with increased exposure to toxic pollutants (Yum 2006; Downs & Downs 2007) and high temperatures in corals (Brown *et al.* 2002b), and both warm and cold thermal stresses in other marine organisms (Hofmann & Somero 1995, 1996b; Place *et al.* 2004; Teranishi & Stillman 2007; Dutton & Hofmann 2008). It would follow then, that the elevated concentrations of ubiquitin conjugates observed in back reef source corals indicate higher stress levels caused by the dynamic fluctuations in environmental conditions of the back reef site. In contrast, the increased growth rates of back reef source colonies and transplants observed in the previous study (Smith *et al.* 2007) indicate that the conditions of the back reef environment are also conducive to growth and survival.

Beneficial aspects of exposure to fluctuating temperatures such as increases in growth (Spigarelli *et al.* 1982; Wang *et al.* 2007; Les *et al.* 2009) and thermal tolerance limits (Feldmeth *et al.* 1974; Hines *et al.* 1980; Feminella & Matthews 1984) have been observed for a variety of aquatic organisms. Furthermore, nonlethal stresses have also been shown to elicit large ubiquitin responses both seasonally and over tidal cycles in naturally occurring populations of inter and subtidal marine mussels (Hofmann & Somero 1995, 1996a; b) with contradictory responses mirroring opposing range distributions (Dutton & Hofmann 2008). Thus, an alternative hypothesis for the increased ubiquitin response of back reef source corals is that it may be temporally variable, illustrating their capacity to survive the daily extremes of the back reef without a corresponding reduction in other fitness characteristics such as growth and survival. Future work will concentrate on determining the upper limits of ubiquitin response in forereef corals in hopes of elucidating the extent of source population influence and the potential of the ubiquitin-response to limit range distributions.

Back reef and forereef Symbiodinium *populations are genetically indistinguishable*

Research over the past few decades has revealed that certain genotypic combinations of coral host and algal endosymbiont can exhibit different physiological characteristics such as differential growth (Little *et al.* 2004), thermal tolerance and bleaching resistance (Rowan *et al.* 1997; Baker *et al.* 2004; Rowan 2004; Jones *et al.* 2008), and susceptibility to disease (Stat *et al.* 2008b). Recently, it has also been shown that the same type of *Symbiodinium* can elicit both increased and decreased thermal tolerance depending on the host genetic background (Abrego *et al.* 2008). Even the degree of mutualism within the cnidarian–dinoflagellate symbiosis has been called into question, with an association bordering on parasitism found in a particular Hawaiian coral (Stat

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et al. 2008b). To further add to this complexity is an inherent difficulty in characterizing the diversity of the genus *Symbiodinium*. Genetic diversity within this genus can be as great as order-level differences seen in other dinoflagellate groups (Rowan & Powers 1992). Additionally, much of the molecular characterization of *Symbiodinium* diversity is based on nuclear ribosomal gene sequences which, because of their multicopy nature, can exhibit large levels of intragenomic variation (Hillis & Dixon 1991).

The data generated during this study give us the unique opportunity to examine the question of *Symbiodinium* intragenomic variability within a sampling strategy that spans two thermally distinct regions within one host species over a relatively short distance (<5 km). The resulting comparison showed that all individuals sampled contained a majority of sequence types identical to ITS2 type C15, in addition to four other closely related variants following screening for artefact and psuedogenes. These results suggest that the back reef and forereef *Symbiodinium* populations are genetically very similar and largely comprised of the single ITS2 type C15, a type common in *Porites* species across the Pacific (LaJeunesse *et al.* 2003). While it is possible that cryptic genetic diversity may be present within a single ITS2 type (Santos *et al.* 2004), the presence of such diversity within Clade C *Symbiodinium* and any associated physiological significance remains unknown.

The apparent lack of symbiont genetic diversity in these coral populations could be caused by the particular symbiont transmission strategy of *P. lobata*. *P. lobata* is among the corals found to transmit their symbionts directly from parent to offspring (vertical/maternal transmission) rather than acquire them anew each generation from the surrounding environment (horizontal transmission; Richmond & Hunter 1990). Coral species with a vertical transmission strategy have been found to often associate with a single distinct type or genus-specific lineage of *Symbiodinium* (Stat *et al.* 2008a). While limiting the flexibility to associate with a variety of symbionts, this strategy can prove advantageous in conserving effective host–symbiont associations over multiple generations (Douglas 1998).

Coral host populations show significant differentiation

The pattern of genetic subdivision between back reef and forereef populations of *P. lobata* suggest the presence of a physical or environmental barrier to gene flow between these two populations. There is a growing body of evidence suggesting that small-scale heterogeneity in coral population structure is not uncommon. Some studies have found only regional isolation of coral populations with panmictic patterns at within-region spatial scales (<100 km; Ridgway *et al.* 2001; Baums *et al.* 2005; Ridgway *et al.* 2008), while many others have discovered population subdivision at smaller spatial scales of cross-shelf (<35 km; Mackenzie *et al.* 2004; Nishikawa 2008), within-island (<10 km; Magalon *et al.* 2005) and within-reef (<5 km; Ayre & Hughes 2000; Underwood *et al.* 2007, 2009; Miller & Ayre 2008) distances. Subdivision between within-island habitat types has also been shown previously by Sherman *et al.* (2006) between lagunal and reef slope populations of *Pocillopora damicornis*, with 83% of genetic variation attributable to habitat type. The genetic subdivision of forereef and back reef populations of *P. lobata* despite their close proximity (<5 km) found in our study, demonstrate maintenance of some type of reduction in gene-flow between the two environments either via physical or environmental barriers.

One possible explanation for maintenance of this differentiation could be that forereef and back reef genotypes constitute different cryptic species. While massive *Porites* can be difficult to identify based on skeletal and morphological characteristics, every attempt was made to accurately identify the samples prior to inclusion. Also, each mtDNA and ITS sequence used within this study was compared with a recent, comprehensive molecular phylogeny of *Porites* (Forsman *et al.* 2009) and all were found to occur within the monophyletic Clade I, a clade containing all other *P. lobata* samples used in the phylogeny. *P. lutea*, the most closely related species morphologically occurs in the region, is readily distinguishable genetically from *P. lobata* and found within an entirely different clade from all samples used in this study (Forsman *et al.* 2009). Additionally, the presence of intermediate and shared genotypes in both the mtDNA and ITS markers (Figs 6 and 7) as well as a lack of a discernable pattern in the network constructions, argue against cryptic species driving the observed pattern of population differentiation. Review of the recent literature suggests that such small-scale population structure in reef-building corals is more common than previously thought (Severance & Karl 2006; Whitaker 2006; Underwood *et al.* 2007, 2009; Miller & Ayre 2008). The results presented here suggest that differences in habitat characteristics may be a driving force behind the maintenance of such small-scale population subdivision.

Conclusions

While the specific mechanisms of increased performance of back reef vs. forereef corals remain elusive, our findings and those of previous work (Smith *et al.* 2007) demonstrate clear physiological response differences between these back reef and forereef *P. lobata* populations. The RTE of these corals showed a

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combination of environmental and genetic influences on physiological response, suggesting that the thermal history of these corals plays a large part in their reaction to novel environmental stresses. The lack of differentiation in symbiont community structure and fine-scale genetic subdivision of the cnidarian host indicates a host-driven influence behind these response differences. While acclimatization potential exists in this group of corals, the strong influence of source colony origin suggests that certain populations may be severely limited in their capacity to respond to drastic environmental changes; a result with important implications in a future of rapid climate change.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 (a) Three-way repeated measures ANOVA (Source colony origin, transplant destination, and source colony individual) for protein biomarker concentrations (ubiquitin conjugates, hsp70, MnSOD, and 4HNE) repeated over the five day course of the experiment. (b) Three-way ANOVA for ubiquitin conjugate concentrations analyzed for each day separately

Table S2 Summary of unique sequences generated for use in this study for each gene fragment under and their corresponding accession numbers. Sequence labels indicate site (B for back reef, F for forereef), an X indicates colonies that were used in the RTE, colony number, followed by clone number

Fig. S1 Temperatures (°C) in the back reef (black line) and forereef (yellow line) recorded every 30 minutes continuously from 2-November-2006 to 21-May-2007 using Water Temp Pro® temperature loggers (Onset Computer Corp.).

Fig. S2 Salinity measurements taken every 30 minutes in the back reef (black line) and forereef (yellow line) from 21 March 2006 to 1 April 2006.

Fig. S3 Dissolved oxygen (% saturation) measurements taken every 30 min in the back reef (black line) and forereef (yellow line) from 21 March 2006 to 1 April 2006.

Fig. S4 Relative light intensity (Lumens/sf) measurements taken every 30 min in the back reef (black line) and forereef (yellow line) from 21 March 2006 to 1 April 2006.

Fig. S5 $Si(OH)_4$ concentration (μM) of water samples taken every four hours for 48 hours starting at midnight on 31 March 2006 and ending at midnight 2 April 2006.

Fig. S6 Median-joining network construction of entire ITS cloned sequence data set. Mutations are shown in red on the branches (numbers refer to positions in the alignment). The smallest red circles denote unsampled intermediate sequence types. Next size circles and larger are proportional to the number of individual sequences with that sequence type. Red circles are Back Reef samples, Yellow circles are Forereef samples.

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