REPORT

Reef location has a greater impact than coral bleaching severity on the microbiome of *Pocillopora acuta*

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Abstract Coral reefs are increasingly threatened by heat stress events leading to coral bleaching. In 2016, a mass bleaching event affected large parts of the Great Barrier Reef (GBR). Whilst bleaching severity and coral mortality are usually monitored throughout major bleaching events, other health indicators, such as changes in microbial partners, are rarely assessed. We examined the impact of the 2016 bleaching event on the composition of the microbial communities in the coral *Pocillopora acuta* at Havannah Island Pandora reef, separated by 12 km on the inshore central GBR. Corals experienced moderate heat stress (3.6 and 5.3 degree heating weeks), inducing major bleaching (30–60%) at the coral community level. Samples were partitioned according to Symbiodiniaceae densities into three bleaching severity categories (mild, moderate, and severe). Whilst Symbiodiniaceae densities were similar at both reef locations, sequencing of the Symbiodiniaceae ITS2 and prokaryotic 16S rRNA genes revealed that microbial communities were significantly different between reefs, but not according to bleaching severity. Symbiodiniaceae composition was dominated by the genus *Cladocopium* with low abundances of *Durusdinium* detected in moderately and severely bleached colonies at both sites, despite site-specific ITS2 profiles. Bacterial communities were dominated by Proteobacteria and were almost entirely lacking the common Pocilloporid associate *Endozoicomonas* regardless of bleaching severity. Strikingly, only 11.2% of the bacterial Amplicon Sequencing Variants (ASVs) were shared between sites. This reef specificity was driven by 165 ASVs, mainly from the family Rhodobacteraceae. Comparison with previous studies suggests that the moderate heat stress experienced on the central GBR in 2016 caused the near-complete absence of *Endozoicomonas*. Symbiodiniaceae and bacteria (particularly Rhodobacteraceae) can be vertically transmitted in *P. acuta*, and larval propagation can be spatially restricted for this brooding species. Our results demonstrate that, unlike bleaching severity, location-specific factors and species-specific life history traits might have been paramount in shaping the *P. acuta* microbiome.

Keywords Coral bleaching · Coral microbiome · *Pocillopora* · Symbiodiniaceae · *Endozoicomonas* · Great Barrier Reef

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Introduction

Reef-building corals are symbiotic animals, often referred to as "holobionts" composed of an animal (cnidarian) host, eukaryotic algae of the family Symbiodiniaceae as well as other protists, fungi, prokaryotes and viruses (Zilber-Rosenberg and Rosenberg 2008). The fine-tuned relationships between the various partners ensure the health of the holobiont under optimal environmental conditions (Rosenberg et al. 2007; Bourne et al. 2016; Vanwonterghem and Webster 2020). One of the best characterised marine symbioses occurs between the cnidarian host and the Symbiodiniaceae, whose taxonomy has only recently been resolved (LaJeunesse et al. 2018). Whilst the coral provides carbon and nitrogen to sustain the growth of photosynthetic Symbiodiniaceae, the algal symbiont transfers photosynthates and essential nutrients to the host to support daily metabolic energy demands (Muscatine and Porter 1977). In parallel, the presence and role of coral-associated prokaryotic communities have been increasingly investigated over the last two decades (see Bourne et al. 2016 for an extensive review), revealing that reef-building corals harbour host-specific, diverse and distinct prokaryotic communities in their surface mucus layer, tissues and skeleton (Rohwer et al. 2002; Koren and Rosenberg 2006; Pernice et al. 2020; Pogoreutz et al. 2021). Bacteria have been frequently implicated in coral fitness, with shifts in microbial communities occurring concomitantly with disease or more generally declining health (Bourne et al. 2009, 2013; Glasl et al. 2016). Within the coral tissues, prokaryotes play key roles in nitrogen cycling (Lema et al. 2012, 2016; Ceh et al. 2013; Cardini et al. 2015; Messer et al. 2017) and have been linked to carbon and sulphur cycling (Raina et al. 2009; Robbins et al. 2019). In addition, whilst the coral host can acquire essential nutrients heterotrophically or from the Symbiodiniaceae, it may also benefit from bacterial biosynthesis of the compounds it cannot synthesise itself, including antimicrobial compounds, amino acids and vitamins (Raina et al. 2016; Robbins et al. 2019; Modolon et al. 2020). The complex relationships within the coral holobiont illustrate how symbiotic partners efficiently cooperate under optimal environmental conditions (Voolstra and Ziegler 2020), but also how fragile the holobiont can be upon stress, and in particular thermal stress. Many corals live close to their upper thermal limit (Hoegh-Guldberg 1999) and during periods of heat stress, the symbiosis between the host and Symbiodiniaceae breaks down as a result of shifting metabolism, and an accumulation of reactive oxygen and nitrogen species within the tissue (Jones et al. 1998; Suggett and Smith 2020; Rädecker et al. 2021). Whilst the ultimate trigger of dysbiosis has not yet been elucidated, it

results in the expulsion of the dense Symbiodiniaceae community into the environment, leaving behind the transparent coral tissue and white calcareous skeleton, a phenomenon referred to as coral bleaching (Glynn 1983; Lesser 1997).

Extreme or prolonged thermal stress can cause death of the coral colony within weeks of the peak heat stress (Hughes et al. 2017). Conversely, bleaching can be followed by survival of the colony and recovery of the symbiotic partners after moderate heat stress, if temperatures cool below normal summer maximum thresholds (Brown 1997). Changes in the Symbiodiniaceae community composition within the coral host are often implicated in the survival of corals following bleaching. This can result from "background" thermal-tolerant Symbiodiniaceae genotypes (typically from the *Durusdinium* genus) becoming dominant (Berkelmans and Van Oppen 2006), or from the uptake of more thermal-tolerant Symbiodiniaceae from the environment (Boulotte et al. 2016). Bacterial communities have also been reported to shift in response to bleaching events, but these shifts can have deleterious outcomes such as increases in potentially pathogenic *Vibrio* populations and bacterial virulence genes (Bourne et al. 2008; Littman et al. 2011; Ziegler et al. 2017).

The spatial footprint of extreme thermal anomalies and the severity of coral bleaching has increased at a faster rate than predicted only a decade ago (Hoegh-Guldberg et al. 2007; Hughes et al. 2017, 2019). Over the last 30 years, coral reefs have experienced three global mass bleaching events in 1998, 2010 and 2015–2017 (Eakin et al. 2019), which impacted large areas of coral reefs worldwide, including the largest coral reef ecosystem, Australia's Great Barrier Reef (GBR). Additional bleaching events have occurred on the GBR in 2002, and 2020 (Berkelmans et al. 2004; Hughes et al. 2017, 2019) but the 2016 bleaching event was particularly widespread as only 8.9% of the 1,156 reefs surveyed escaped any form of bleaching (Hughes et al. 2017). In addition, the percentage of reefs experiencing extreme levels of bleaching was four times higher than in 1998 and 2002. The levels of accumulated heat stress during bleaching are often measured using the National Oceanic and Atmospheric Administration (NOAA) Degree Heating Week product (DHW; °C-week) which can be defined as the accumulation of temperature anomalies exceeding the threshold set by the historical climatological maximum of the monthly mean sea surface temperature (SST) over a rolling 12-week period (Skirving et al. 2020). This remote sensing tool provides predictive forecasting as heat waves develop, and hindcasting comparisons of heat stress levels across thermal ranges. In summer 2016, 4 °C-weeks resulted in 30–40% of bleached corals along the GBR (Hughes et al. 2017), regarded as a severe level of bleaching during past events on the GBR in

1998 and 2002 (Hughes et al. 2018). Whilst the Southern GBR reefs escaped high levels of bleaching, the Central GBR reefs generally experienced moderate bleaching (between ∼ 2.5 and 5 °C-weeks).

Despite the profound impact of bleaching events on coral reefs and the crucial role of microbes for coral health, several aspects of the response of microbial communities to coral bleaching are under-studied. For instance, it is well-documented that different coral colonies of a given species, exposed to similar levels of thermal stress at the same location can exhibit varying degrees of bleaching (Swain et al. 2016; Green et al. 2019); however, we do not know if this mosaic of bleaching phenotypes observed at the colony-level is reflected in the microbiome. To address this question, we used the emerging model species *Pocillopora acuta*, a reef-building coral with well characterised ecology, reproduction and microbiome (Torda et al. 2013a; Schmidt-Roach et al. 2014a, b; Nakajima et al. 2018; Damjanovic et al. 2020b; Ros et al. 2021). Previous studies have revealed that the microbiomes of *Pocillopora* species are generally stable under changing environmental conditions (Pogoreutz et al. 2018; Epstein et al. 2019), with species such as *P. acuta* often hosting site-specific microbiomes (Torres et al. 2020), perhaps due to the brooding reproductive strategy of the host (Torda et al. 2013a). In this study, we characterised the impacts of a range of bleaching severities on the *P. acuta* microbiome within two reefs of the Palm Island archipelago on the Central GBR, following the 2016 mass bleaching event.

Material and methods

Temperature data

Seawater temperature measurements were retrieved for Pandora Reef (18°48′54.9″S 146°25′52.7″E) and Havannah Island (18°50′22.6″S 146°32′02.1″E) from the Australian Institute of Marine Science (AIMS) Sea Water Temperature Observing System temperature logger program (AIMS 2017). These two reefs (hereafter referred to as "Havannah" and "Pandora") are located 12 km apart in the GBR Marine Park within the Palm Island Group (Fig. 1). Data for each site were collected from July 2015 to December 2016, capturing the warming rate and accumulation of heat stress during the 2016 bleaching event. Remote sensing SST data and DHW thermal stress index data across the entire GBR were collected from the satellite observations from the NOAA Coral Reef Watch Version 3.1 for the 2016 summer heat wave (Skirving et al. 2020). Maps of site locations, community bleaching severity and spatial footprint of thermal heat stress (NOAA DHW) were generated using QGIS v. 3.12.

In-water community coral bleaching surveys

To assess whether corals of Havannah and Pandora reefs had experienced similar levels of bleaching at the community level, in-water bleaching surveys of the reef communities around the two sites were conducted at two depths (2 m and 5 m) on the 4th and 6th of April 2016, respectively. Five 10 × 1 m belt transects were used with observers capturing 1 m × 1 m square continuous images along each transect with an Olympus TG-4 camera in a housing using an Inon UWL-H100 28 mm Wide Conversion lens and a Coral Watch colour card (Siebeck et al. 2006) for colour correction and white balancing at the start of each transect. Observers counted each coral colony > 5 cm in diameter within each 1 m × 1 m image, recorded genus level identification and assigned a categorical bleaching score for each individual hard and soft coral colony, based upon the proportion of living coral tissue visibly bleached white. Individual colony bleaching severity was determined using a categorical bleaching score previously established (Gleason 1993; Marshall and Baird 2000). The following individual colony categories were used, based on the total area of the living tissue visibly bleached white: (1) No bleaching or (2) Pale; (3) 1–50% tissue bleached white; (4) 51–99% bleached white; (5) 100% bleached white with or without intense fluorescence; (6) partial or complete mortality of the colony (recently dead regions of tissue). Survey methods used to assess reef community bleaching severity aligned with and contributed to the 2016 Australian National Bleaching Taskforce GBR Survey response (Hughes et al. 2017). Accordlingly, bleaching severity categories at the reef community level were based on the percentage of corals bleached as follows: no bleaching (0% bleached corals); minor bleaching (1–10%), moderate (10–30%); major (30–60%); severe (> 60%) (Berkelmans et al. 2004) (Supplementary Material).

Coral sample collection

Coral fragments were collected from 22 colonies at Pandora on the 4th April 2016 and from 25 colonies at Havannah on the 12th April 2016 at the end of the summer peak heat stress (Fig. 2) using GBRMPA permit G16/38488.1. Colonies were randomly selected, and all samples were collected at 5 m depth. A small branch from each colony (> 5 cm) was cut using sterilised stainless steel bone cutters, snap-frozen in liquid nitrogen, transported to AIMS and stored at − 80 °C until further processing.
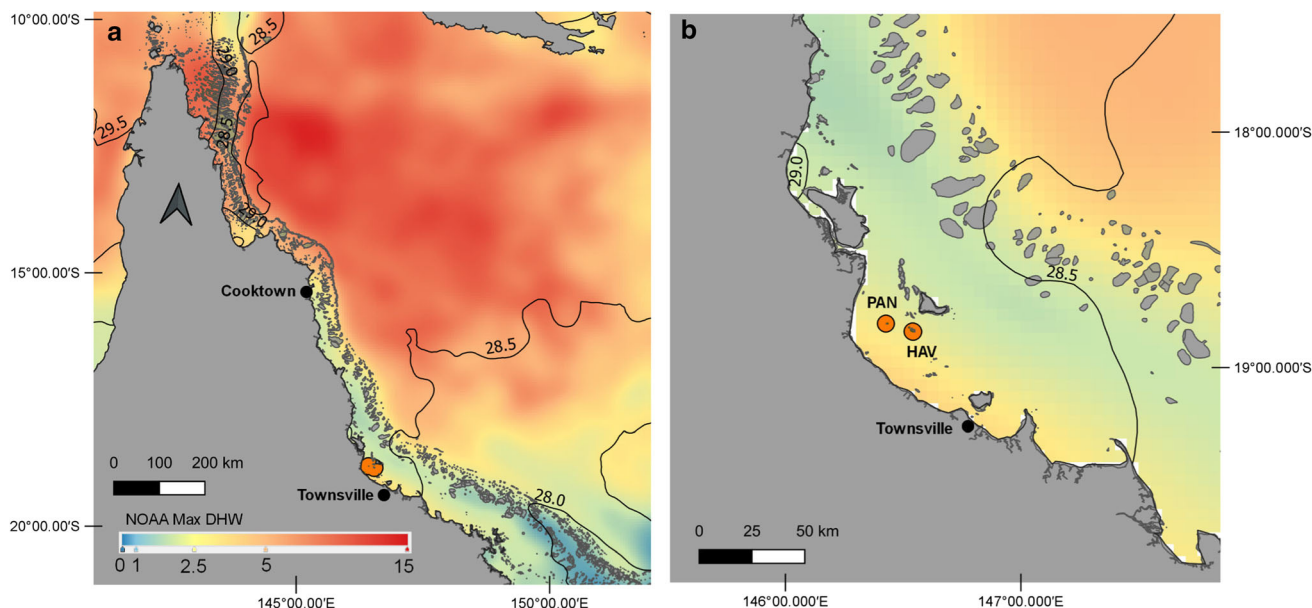
Fig. 1 Spatial footprint of the maximum annual accumulated thermal stress in 2016 measured using the NOAA Coral Reef Watch v3.1 degree heating week (DHW) product including the latitudinal variation in Maximum Monthly Mean (MMM) SST climatology (black isolines); orange dot represents results of in-water bleaching surveys at Havannah Island Reef (HAV) and Pandora Reef (PAN). Coloured scale represents max DHW (**a**) throughout the Great Barrier Reef and (**b**) surrounding the sampled reef locations

Tissue sample preparation and Symbiodiniaceae density

Frozen samples were thawed on ice, rinsed in 0.02 μm filtered seawater (FSW) and placed into sterile zip-lock bags. A high-pressure air gun was used to remove the coral tissue from the calcium carbonate skeleton into 10–15 mL of 0.02 μm FSW. The coral blastate was decanted into a 50 mL Falcon tube and its volume topped to 15 mL for all samples with 0.02 μm FSW, prior to vortexing using a bench top homogeniser (Bio-Gen PRO200, PRO Scientific, USA). Aliquots of 2 mL each were kept at − 20 °C for DNA extraction whilst a 1 mL aliquot was preserved in 2% formaldehyde for subsequent Symbiodiniaceae cell counts using a Neubauer haemocytometer (Fitt et al. 2000). Cell counts were achieved by adding 7 μL of the preserved aliquot onto the haemocytometer and conducting at least four replicate counts to achieve a coefficient of variance of < 10%.

Coral skeletons were bleached and dried to standardise the tissue parameters to surface area of each individual coral branch. To estimate surface area, each coral branch was dipped twice into 60 °C paraffin wax following an established protocol (Veal et al. 2010). Briefly, coral skeletons were dipped once in paraffin wax to seal the porous skeleton and weighed. Corals were then dipped a second time to estimate the surface area of the coral branch. PVC cylinders across the full-size range of coral branches were also double-dipped to fit a > 99%

confidence linear curve to standardise paraffin weight gain to a known surface area.

Pocillopora acuta colony bleaching severity

P. acuta colonies were categorised for statistical analysis of the microbial communities based on Symbiodiniaceae density counts. Bleaching categories were compared to the healthiest colony within the sample set: (1) Mild bleaching (0–30% decrease in symbiont cell density); (2) Moderate (30–70% decrease in cell density) and (3) Severe (> 70% decrease in symbiont cell density).

DNA extraction and host species identification

The MOBIO Power Plant Pro DNA Isolation kit was used to extract DNA from each sample (50 mg of pelleted tissue homogenate) including two bead-beating steps of 40 s at 4 ms^{-1} (MP Biomedicals FastPrep). To further confirm species identification, we conducted molecular typing of the samples following previously established methodology (Torda et al. 2013b) with minor modifications as follows: the BIO-X-ACT Taq Polymerase from Bioline was used at 0.08 U/μL in reactions containing 1 X buffer, 1 mM DNTPs (0.25 mM each), 3 mM MgCl2, 0.5 μL of Hi-Specificity additive, 0.5 μM of each primer (Pdam-F AAGAAGATTCGGGCTCGTTT and Pdam-R CGCCTCCTCTACCAAGACAG), and 2 μL of 1:100 diluted DNA in a total 10 μL PCR reaction. Cycling started
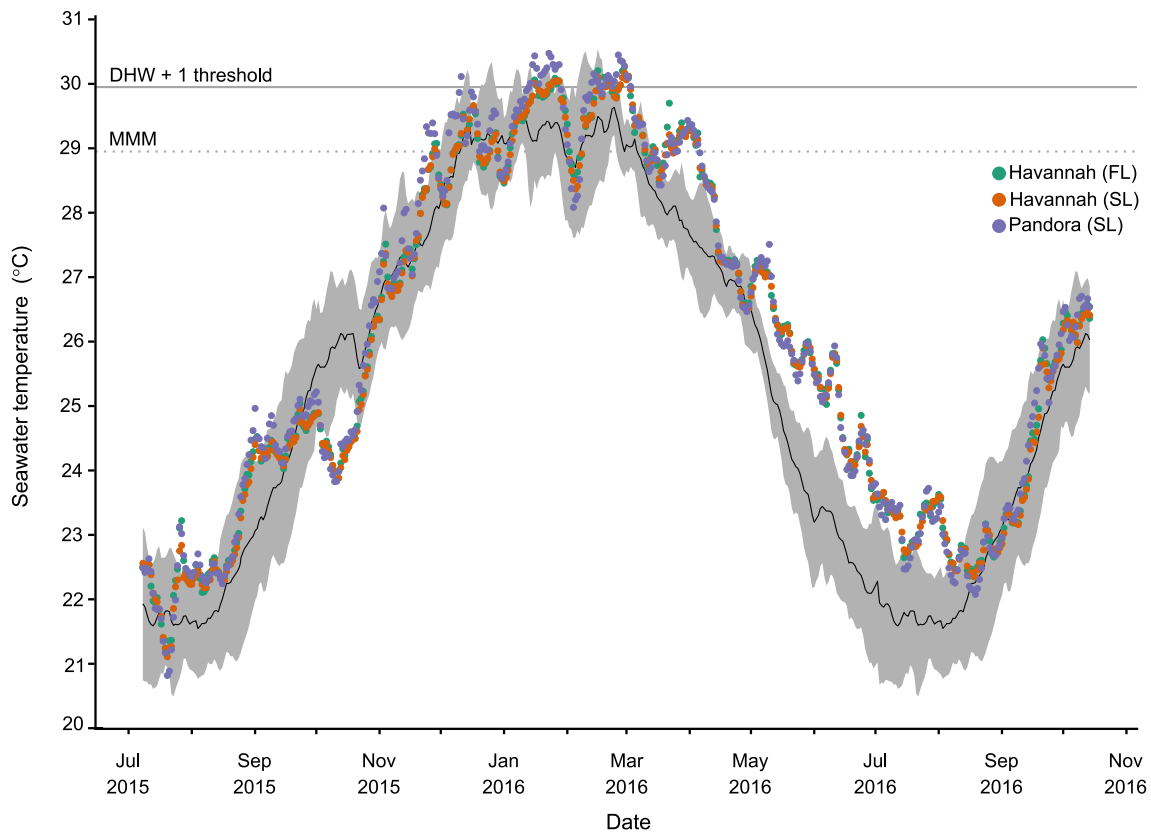
Fig. 2 Daily average water temperature from July 2015 to October 2016 at each sampling site. Slope data (SL) were collected at 5.6 m at Pandora and 6.0 m at Havannah; reef flat (FL) data were collected at 2.0 m (Havannah only). The solid black line represents in-water climatology from Pandora reef SL based on continuous seawater temperature data from 2007 to 2012 as daily average. Grey fill represents daily standard deviation. Reference lines represent 5 km pixel centred on Pandora reef from the NOAA CRW v3.1 Maximum Monthly Mean (MMM) Sea Surface Temperature climatology and the upper thermal limit 1 °C above the MMM value (Degree Heating Weeks + 1 Threshold) at which point daily hotspot values begin to accumulate Degree Heating Weeks (DHW) as an indicator of thermal stress for coral reefs

with 5 min at 95 °C, followed by 32 cycles of 30 s at 94 °C, 60 s at 58 °C and 60 s at 72 °C, and a long extension of 10 min at 72 °C. PCR products were then subjected to Alu1 restriction enzyme digestion without purification for 1 h at 37 °C, using 5 μL of PCR product with 0.25 μL (2.5 U) of Alu1 enzyme and 1 μL of 10 × NE CutSmart Buffer (New England BioLabs) in a 10 μL reaction. Five μL of the digested products were run on 4% TBE agarose gels (using SFR Super Fine Resolution Low Melt Agarose, VWR) for 2 h at 150 Volts and 4 °C. Banding patterns were scored manually and independently by two individuals and confirmed that all samples included in this study were *P. acuta*, as per Torda et al. (2013b).

DNA sequencing of microbial taxonomic markers

Sequencing was performed using Illumina MiSeq 300 bp paired-end chemistry at the Australian Centre for Ecogenomics (ACE) at the University of Queensland. The ITS2 region was amplified using primers ITS2_F (GTGAATTGCAGAACTCCGTG) and ITS2_R (CCTCCGCTTACTTATATGCTT) (Pochon et al. 2001). Each PCR reaction contained 12.5 μl of Q5 hot start high-fidelity 2 × master mix (M0494L), 1.25 μl of each primer (10 μM) and water to reach 25 μL total volume. Cycling conditions were 98 °C for 2 min, 98 °C for 10 s, 25 cycles of 55 °C for 30 s, 72 °C for 30 s and 72 °C for 2 min. Primers 27F (AGAGTTTGATCMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG) were used for 16S rRNA gene amplification (Suzuki and Giovanni 1996; Marchioro et al. 2020). PCR reactions contained 1X PCR buffer, 1 mM dNTPs, 3 mM $MgCl_2$, 0.5 μL of Hi-Specificity additive, 0.4 μM of each primer, $0.08U.μl^{-1}$ of Q5 High-Fidelity DNA polymerase and 2 μl of DNA template for a total of 10 μl of PCR reaction. PCR cycling was conducted with 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 45 s at 53 °C and 60 s at 72 °C, and an extension of 10 min at 72 °C. Three samples (SA8730 from Pandora mild bleaching, SA8686 from Pandora severe bleaching and SA8815 from Havannah mild bleaching) resulted in poor

ITS2 rDNA sequencing but presented otherwise high numbers of 16S rRNA gene reads and sensible Symbiodiniaeae densities. Therefore, considering the relatively low sample size, these sample were included in all 16S rRNA gene sequence analyses. *Symbiodiniaceae* ITS2 profiling generated an average of 3,680 reads per sample, with a minimum of 1,357 reads and a maximum of 239,319 reads, and 16S rRNA gene sequencing generated between 10,303 and 86,995 reads, with an average of 36,354.

Bioinformatics

ITS2 raw reads were submitted to the SymPortal platform (Hume et al. 2019) for remote analysis (https://symportal.org/). This approach allowed us to retain intragenomic information and identify Defining Intragenomic Variants (DIVs) as well as ITS2 profiles. ITS2 profiles are built using the presence as well as the abundance of the DIVs identified. The name of each profile reflects the proportion of each DIV within. As an example, profile C1d.C1.C42.2 would be mostly composed of C1d-type sequences, with C1-type sequences and C42.2-type sequences present in decreasing proportions. Absolute abundances were used for subsequent analysis. Visualisation of 16S rRNA gene read quality using Fastqc as well as the demux plug-in of QIIME2 (Bolyen et al. 2019) revealed that reverse reads were of poor quality and hence only forward reads were retained and analysed following the QIIME2 pipeline. The DADA2 plug-in was used to remove primers and reads shorter than 200 bp, dereplicate reads, truncate high-quality reads (quality score > 30) to 200 bp and filter out chimaeras and singletons. The feature-classifier plug-in was used to train a naïve-Bayes classifier using the Silva database 132 (Quast et al. 2012) based on the 27F-519R primer pair region. The Amplicon Sequencing Variants (ASVs) table was filtered to remove all eukaryotic, mitochondrial and chloroplast ASVs from the dataset.

Statistical analysis of DNA sequencing data

ITS2 sequencing data recovered from the SymPortal platform were transformed into relative abundances and fourth-root transformed using PRIMER. A Bray–Curtis dissimilarity matrix was built prior to testing the effect of bleaching severity and location using PERMANOVA (10,000 permutations). The number of reads originating from 16S rRNA gene sequencing was visualised as rarefaction curves using the package vegan in R (Oksanen et al. 2017; R Development Core Team 2017) to confirm sufficient sequencing. Data were rarefied to the sample with the lowest number of reads (10,303) to calculate richness and diversity indices in R. Richness data were log10-transformed to meet homogeneity of variances and

the normality of residuals assumption, prior to performing ANOVA to test the effect of bleaching severity and location (Tables S1-S5). Shannon diversity index data were normal and homogeneous, and no transformation was necessary prior to ANOVA testing (Tables S6-S9). Statistical testing of the effect of bleaching severity and location on richness and Shannon diversity index was conducted with ANOVA. The effects of bleaching severity and reef were assessed on the overall bacterial communities (ASVs) with PERMDISP and PERMANOVA (10,000 permutations) in PRIMER on a Bray–Curtis dissimilarity matrix of a square-root transformed rarefied and non-rarefied dataset. Since rarefaction did not change the outcome of the PERMANOVA, all reads were retained in the final results to avoid biases of the rarefaction process (McMurdie and Holmes 2014). Similarly, as visualisation of the beta-diversity on a Principal Coordinate Analysis plot on PRIMER was identical on rarefied and non-rarefied data, the non-rarefied data were retained for Fig. 3. Based on initial statistical results, a Venn diagram was built to assess the percentage of ASVs shared between reefs, and a SIMPER analysis was conducted on the non-rarefied Bray–Curtis dissimilarity matrix to identify the ASVs explaining 20% of the cumulative variation between the two reefs. DESeq2 was used to identify ASVs that were significantly different between Havannah and Pandora reefs. Finally, a PERMANOVA was conducted with 10,000 permutations using ITS2 profiles as a factor. All data wrangling and visualisation were performed in R using the package tidyverse and ggplot2, respectively (Wickham and Chang 2015; Wickham et al. 2019).

Results

Thermal stress and coral bleaching in 2016 at Havannah and Pandora

Levels of thermal stress at the central GBR study sites in 2016 were considered moderate (between 2.5 and 5 °C-weeks) with severe levels of heat stress exceeding 6 °C-weeks restricted to the sector located North of Cooktown (Fig. 1a). Historical summer maximum temperatures are similar (using NOAA MMM CoralTemp v3.1; Skirving et al. 2020) at Havannah and Pandora reefs (28.86 °C and 28.95 °C, respectively). Pandora accumulated higher levels of heat stress up to 5.3 °C-weeks at the time of sampling, compared to 3.6 °C-weeks at Havannah, which corresponded to moderate heat stress (Fig. 1b). In-water survey further revealed that this level of stress induced major bleaching at the community level for corals living in both Havannah and Pandora reefs, with 30–60% of hard and soft corals bleached (Fig. 1b). Frequency of severe individual
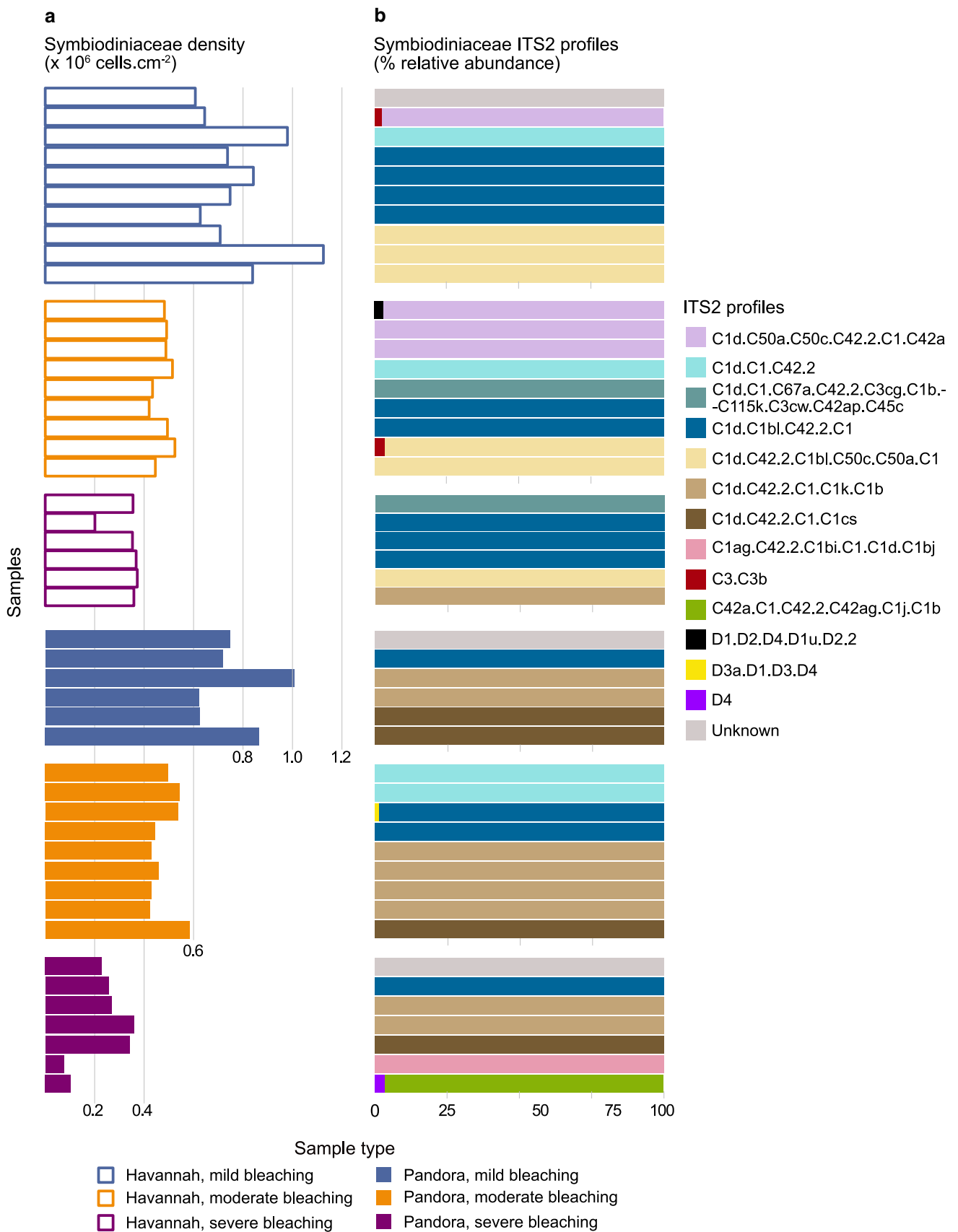
a

Symbiodiniaceae density
(x 10^6 cells.cm^{-2})

b

Symbiodiniaceae ITS2 profiles
(% relative abundance)

ITS2 profiles

- C1d.C50a.C50c.C42.2.C1.C42a
- C1d.C1.C42.2
- C1d.C1.C67a.C42.2.C3cg.C1b.-
  -C115k.C3cw.C42ap.C45c
- C1d.C1bl.C42.2.C1
- C1d.C42.2.C1bl.C50c.C50a.C1
- C1d.C42.2.C1.C1k.C1b
- C1d.C42.2.C1.C1cs
- C1ag.C42.2.C1bi.C1.C1d.C1bj
- C3.C3b
- C42a.C1.C42.2.C42ag.C1j.C1b
- D1.D2.D4.D1u.D2.2
- D3a.D1.D3.D4
- D4
- Unknown

Samples

Sample type

Havannah, mild bleaching   Pandora, mild bleaching
Havannah, moderate bleaching   Pandora, moderate bleaching
Havannah, severe bleaching   Pandora, severe bleaching

◄ **Fig. 3** Characterisation of the Symbiodiniaceae communities in *P. acuta* at Havannah and Pandora reefs across bleaching severities. Each bar in panel **a** represents the same sample in panel **b**. (**a**) Symbiodiniaceae cell density ($\times\ 10^6$ cells.cm^{-2}). (**b**) ITS2 profiles. Relative abundance is expressed as a percentage. Each horizontal bar represents a sample

colony bleaching (colony bleaching category 5; 100% white) was low within the bleached communities at both reefs (0–1.0% and 9.3–17.9% of the hard and soft corals within the belt transects, at Pandora and Havannah, respectively) (Supplementary Material). Recent bleaching-related mortality (colony bleaching category 6) was also low at both study sites (0% and 0.3–1.2% mortality of hard and soft corals at Pandora and Havannah, respectively).

Maximum temperatures at both reef locations exceeded the historical maximum summer climatology by more than 1 °C for prolonged periods in January, February and March, with an extended summer period throughout the entire month of March, when temperatures typically begin to cool (Fig. 2). Maximum temperature anomalies were recorded on the slope of Pandora reef (Fig. 2).

Symbiodiniaceae communities

Symbiodiniaceae densities were used to attribute bleaching severity categories to individual colonies (see Methods section, Supplementary text and Fig. S1). Symbiodiniaceae densities (Fig. 3a) ranged between 0.2 and 1.1 million cells.cm^{-2} in Havannah-sourced corals and between 0.07 and 1 million cells.cm^{-2} in Pandora-sourced corals. Severe bleaching corresponded with colonies exhibiting < 0.4 million cells.cm^{-2} densities, moderate bleaching to colonies with densities comprised between 0.4 and 0.6 million cells.cm^{-2}, whilst mild bleaching corresponded with corals presenting > 0.6 million cells.cm^{-2} Symbiodiniaceae densities.

The bleaching category assignments resulted in a total of 10 and 6 mildly bleached corals from Havannah and Pandora, respectively, 9 moderately bleached samples from both locations, as well as 6 and 7 severely bleached samples from Havannah and Pandora, respectively. These categories are those used in the remainder of this article unless otherwise stated.

Symbiodiniaceae communities were represented by 15 distinct profiles across Havannah and Pandora, including 10 containing the genus *Cladocopium,* three *Durusdinium* and two *Symbiodinium* (Fig. 3b). The two *Symbiodinium* (A-dominated profiles) were found at 0.1% and 0.5% in a single moderately bleached Pandora sample and we hereafter concentrate on the 13 remaining ITS2 profiles.

Symbiodiniaceae from the *Cladocopium* C1d-type dominated the communities at both reefs. However, 75% of the profiles were specific to a reef, with 5 found exclusively in Pandora and 5 exclusively in Havannah. Only 3 profiles (all in the genus *Cladocopium*) were shared between reefs. Consistent with these observations, location had a significant effect on the distribution of the Symbiodiniaceae profiles (PERMANOVA, $F_{1,38} = 3.7916$, $p = 0.0027$, Table S10), but bleaching severity did not and there was no interaction between the two factors (PERMANOVA, Table S10). Furthermore, bleaching severity did not significantly affect Symbiodiniaceae communities at each reef independently (PERMANOVA, Tables S11-S12).

The vast majority of samples only harboured a single profile, with only 5 samples harbouring a second profile (including all 3 *Durusdinium* profiles), all at a relative abundance of 1–4% within a sample. The mildly bleached corals only harboured *Cladocopium* profiles at Pandora, and the two *Durusdinium* profiles found at that location were symbionts of a moderately bleached coral and a severely bleached coral (1.2% and 3.7%, respectively). At Havannah, the only *Durusdinium* profile was found in a moderately bleached coral at 3% relative abundance.

The most common profile at Havannah (C1d.C1bl.C42.2.C1) was found in 4 mildly bleached corals, as well as 3 severely bleached and two moderately bleached corals. Conversely, the most common profile at Pandora (C1d.C42.2.C1.C1k.C1b) was found in 4 moderately bleached samples, two mildly bleached and two severely bleached corals.

Bacterial communities

A total of 5,584 ASVs (all belonging to bacterial phyla) were found in Havannah samples and 4,154 in Pandora samples. Richness and diversity (assessed with the Shannon index) of the bacterial communities were not significantly different between reefs or across bleaching severity categories, and there was no significant interaction between the location and bleaching severity factors (Fig. 4 and Tables S13-S14). Within each reef, richness was not statistically affected by bleaching severity (Tables S15-16), and nor was diversity (Tables S17-S18).

The bacterial communities of *P. acuta* were dominated by Proteobacteria, in particular Alpha- and Gammaproteobacteria (Fig. 5a). Representatives of the phyla Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes and Spirochaetes were also found at abundances > 1%. The most abundant families at Havannah were the Rhodobacteraceae (with an average of 27.4–39.9% across the different bleaching categories), Marinobacteraceae (13.4–17.5%), Sphingomonadaceae (7.3–10.1%), Pseudomonadaceae (4.3–6.0%) and

Cyanobiaceae (3.1–6.2%). Microbial communities of *P. acuta* at Pandora also exhibited high relative abundances of Rhodobacteraceae (11.1–27.4%), Marinobacteraceae (13.8–24.3%), Cyanobiaceae (7.3–14.9%) and Sphingomonadaceae (1.2–4.2%), as well as high proportions of Alcanivoracaceae (2.9–4.0%). The family Endoizoicomonadaceae which comprises the *Endozoicomonas* genus was found at low abundances only (< 1%), at both sites.

Bacterial communities of *P. acuta* partitioned according to reef (PERMANOVA, $F_{1,41} = 4.744$, $p = 0.0001$, Table S19), but not bleaching severity and no interaction was found between these two factors (PERMANOVA, Table S19) (Fig. 5b). When analysed separately, microbial communities were not significantly impacted by bleaching severity at Havannah nor at Pandora (PERMANOVA, Tables S20-S21, Fig. 5c, d). The combination of PCO1 and PCO2 explained 24.1% of the total variability at Havannah and 41.1% at Pandora. Dispersion of the samples did not significantly differ between sampling sites (PERMDISP, Table S22), nor bleaching severity at each of the reefs (PERMDISP, Tables S23-S24). Finally, Symbiodiniaceae ITS2 profiles did not have a significant effect on the prokaryotic communities of both reefs (PERMANOVA, Tables S25-S26).

In light of these results, further analyses were conducted to identify the bacterial groups responsible for this site-specific pattern. Only 11.2% of ASVs were shared between reefs (Venn diagram, not shown) and 32 ASVs explained 20% of the variability between reefs according to SIMPER analysis (Table 1). A total of 165 ASVs exhibited significantly different abundances between reefs (Supplementary Material). The taxonomy of 29 of these ASVs was not resolved at the family level but the remaining 136 ASVs were members of 62 families. Of particular interest were families Rhodobacteraceae, Sphingomonadaceae, Pirellulaceae, Marinobacteraceae and Cyanobiaceae (Fig. 6). The family containing the highest number of significantly different ASVs was Rhodobacteraceae with 13 ASVs, representing 9.6% of the differentially abundant ASVs taxonomically resolved at the family level, followed by Sphingomonadaceae with 8 ASVs and Pirellulaceae with 7 ASVs (5.8% and 5.1%, respectively) (Fig. 6a, b). When considering relative abundances, the 13 Rhobacteraceae ASVs together represented 19.4% of the bacterial communities at Havannah and 4.0% at Pandora, whilst the 8 Sphingomonadaceae ASVs represented 7% of the communities at Havannah and 1.8% of Pandora. The 6 significantly different Marinobacteraceae ASVs and the 4 significantly different Cyanobiaceae ASVs showed the opposite pattern: they respectively, represented 15.4% and 10.3% of the communities at Pandora but only 4.8% and 3.8% at Havannah. Finally, two *Endozoicomonas* ASVs were significantly different between reefs, and their combined mean relative abundances amounted to 0.2% at Havannah but to a negligible quantity at Pandora (< 0.0001%).

Discussion

Coral-associated microbial communities underpin the fitness of their host and have been shown to shift in response to environmental disturbances and geographic location.

Fig. 4 Diversity of the bacterial communities in *P. acuta* at Havannah and Pandora reefs across bleaching severity. (a) Richness expressed as the mean number of ASVs (± standard error). (b) Shannon index expressed as mean (± standard error). Data were rarefied with all samples containing 10,303 ASVs for both panels
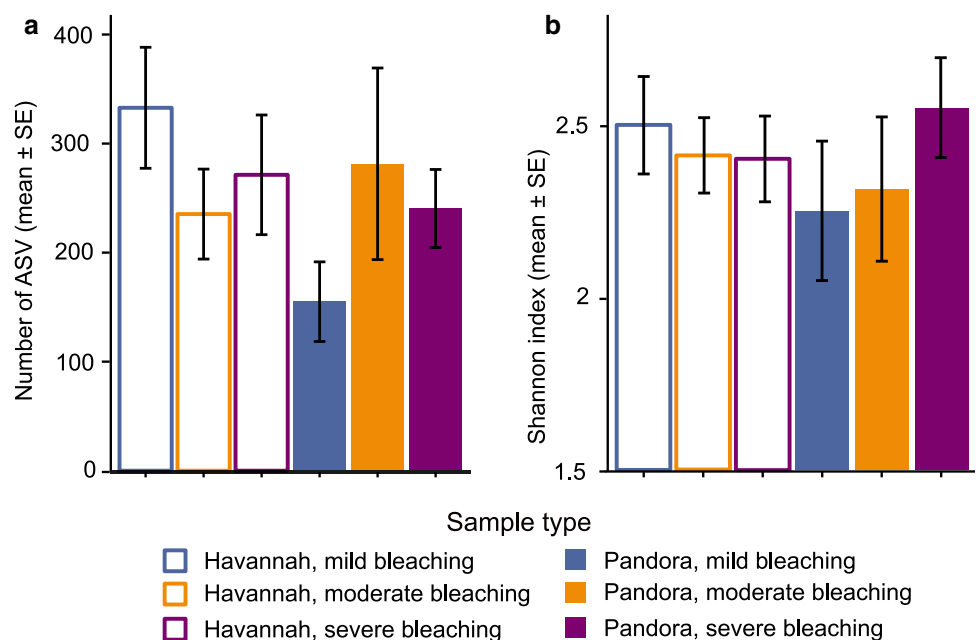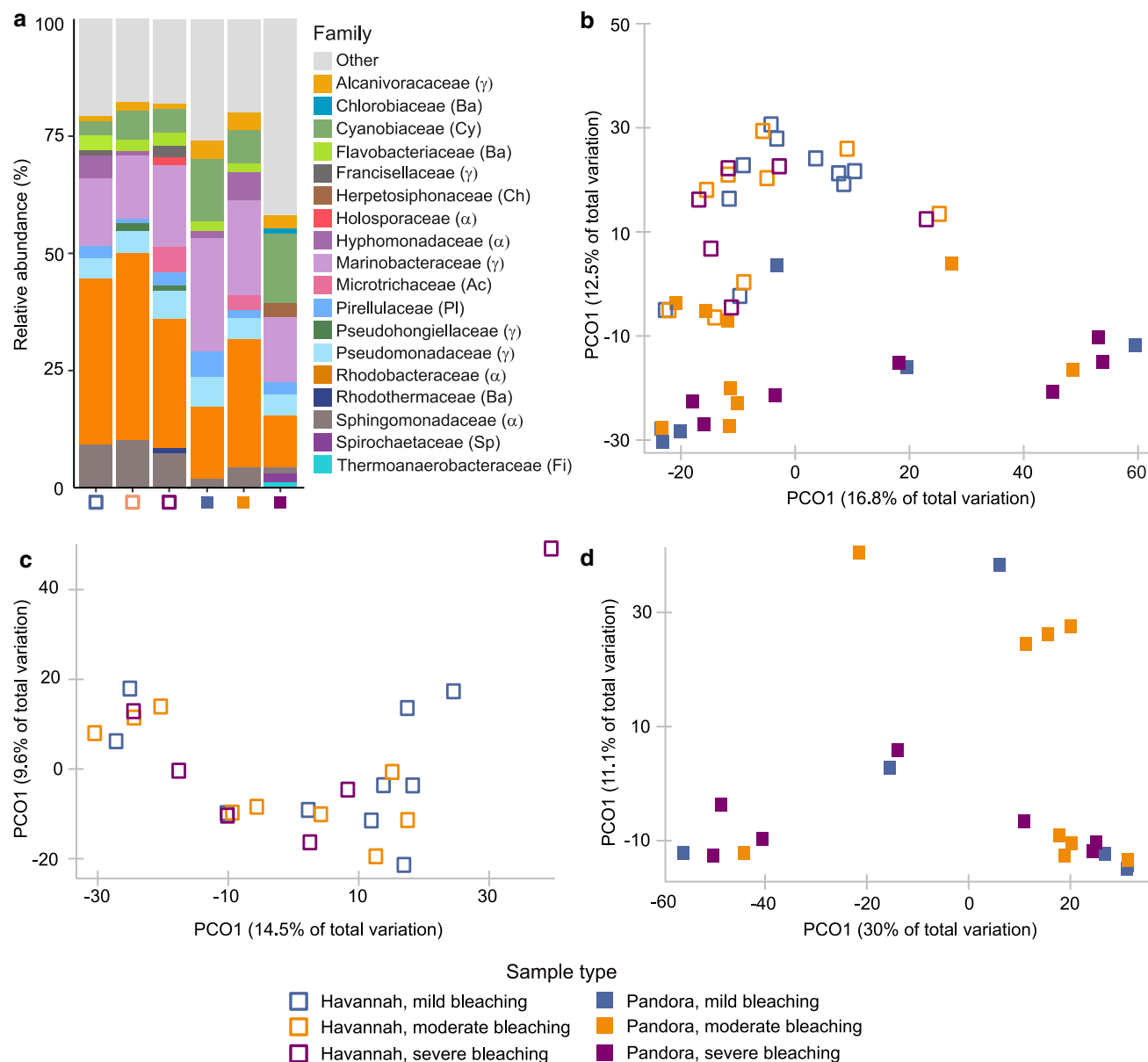
Fig. 5 Characterisation of the bacterial communities of *P. acuta* at Havannah and Pandora reefs across bleaching categories. (**a**) Mean relative abundance at the family level for families representing > 1% of the total community. Ac: Actinobacteria, Ba: Bacteroidetes, Ch:

Chloroflexi, Cy: Cyanobacteria, Fi: Firmicutes, Pl: Planctomycetes, Sp: Spirochetes, α: Alphaproteobacteria, γ: Gammaproteobacteria. Distribution of the samples on Principal Coordinates Analysis for both reefs (**b**), Havannah only (**c**), and Pandora only (**d**)

Here, we show that the composition of both Symbiodiniaceae and prokaryotic communities associated with *P. acuta* did not respond to bleaching severity during the 2016 global mass bleaching event. Rather, microbial communities differed substantially between two inshore reefs located only 12 km apart on the GBR, highlighting the importance of fine-scale sampling when investigating the consequences of large-scale heat stress events on host-associated microbes.

Increasingly severe bleaching did not induce drastic shifts in Symbiodiniaceae communities

Most *P. acuta* samples comprised a single Symbiodiniaceae ITS2 profile, consistent with previous reports (Damjanovic et al. 2020a). The Symbiodiniaceae community composition was dominated by *Cladocopium* and the most abundant profiles were C1d-dominant and C42-dominant, which was also consistent with *P. acuta* colonies from nearby Orpheus Island (Epstein et al. 2019). The most abundant profile at Havannah was C1d.C1bl.C42.2.C1,

Table 1 Taxonomy (family level) of the (32) top Amplicon Sequencing Variants explaining 20% of the variation between Havannah and Pandora as identified via SIMPER analysis

Family	% variation explained	Number of ASV
Rhodobacteraceae	5.03	6
Marinobacteraceae	4.76	4
Sphingomonadaceae	2.34	5
Cyanobiaceae	1.98	4
Others (8)	6.02	13
Total	*20.13*	*32*

Italics indicates total

which is closely related to the most dominant ITS2 profile of *P. acuta* collected at nearby Magnetic Island (Damjanovic et al. 2020b).

Three Symbiodiniaceae profiles in the thermally tolerant genus *Durusdinium* were detected, all restricted to moderately and severely bleached samples. The two *Durusdinium* profiles detected at Pandora were 3 times more abundant in the severely bleached sample than in the moderately bleached sample. Whilst our small sample size precludes any strong conclusion, these findings constitute a low-lying signal of symbiont shuffling or shifting in corals having experienced heat stress, as was previously found in multiple species, including *Pocillopora damicornis* (LaJeunesse et al. 2009; Boulotte et al. 2016). As samples for the current study were collected only during the bleaching event (as opposed to prior or after), it remains uncertain whether these represent new genotypes acquired through symbiont shifting or if they were present prior to the bleaching event and if so, at which proportion. However, *Durusdinium* were completely absent from 10 *P. acuta* colonies sampled every month over the course of 8 months at Orpheus Island, except for a single colony in July 2016 (Epstein et al. 2019). It is therefore more likely that the *Durusdinium* detected in the present study represent newly acquired genotypes in response to heat stress.

In corals, the rare Symbiodiniaceae biosphere has been described as any genotype found at relative abundances lower than 1% (Boulotte et al. 2016). Here, we show that *P. acuta* experiencing 3 to 5 DHW can host heat-tolerant Symbiodiniaceae at relative abundances more than threefold higher than the rare biosphere threshold. Whilst rare taxa are often overlooked in microbial ecology studies, they have been shown to play crucial roles in natural ecosystems, including during environmental changes (Shade et al. 2014; Chen et al. 2020). Increased relative abundance of *Durusdinium* after a bleaching event has been linked to increased thermal tolerance of the coral holobiont (Berkelmans and Van Oppen 2006; Silverstein et al. 2015). Therefore, whilst changes in Symbiodiniaceae

communities can come at the cost of other physiological traits, such as reduced growth, photophysiology and calcification (Little et al. 2004; Cantin et al. 2009; Pettay et al. 2015), it is possible that the colonies hosting *Durusdinium* Symbiodiniaceae may increase their tolerance to future bleaching events following major disturbance events such as the 2016 mass bleaching. Given that Symbiodiniaceae from the rare biosphere can shift up to 18 months post-bleaching (Boulotte et al. 2016), our findings call for greater and repeated temporal sampling of bleached colonies.

Near-complete lack of Endozoicomonas regardless of bleaching severity

Our results corroborate previous findings that heat stress does not impact the overall bacterial community composition of *P. acuta* (Epstein et al. 2019). The bacterial communities associated with *Pocillopora verrucosa* were also highly stable throughout bleaching induced by excessive dissolved organic carbon (Pogoreutz et al. 2018) and upon transplantation of coral colonies across environmental gradients (Ziegler et al. 2019). Nine families (namely Alcanivoracaceae, Chlorobiaceae, Flavobacteraceae, Francisellaceae, Hyphomonadaceae, Pseudomonadaceae, Rhodobacteraceae, Rhodothermaceae and Sphingomonadaceae) dominated the *P. acuta* microbial communities, in accordance with previous studies investigating *P. acuta* collected at Orpheus Island within the Palm Island archipelago throughout 2016 and at Magnetic Island in March 2017 (Epstein et al. 2019; Damjanovic et al. 2020b). However, it is important to note that DNA sequencing detects genetic information regardless of the viability or activity state of the cells, and that bacterial composition does not capture how these microbes are functionally contributing to the holobiont under stress.

The major deviation from previous reports of the *Pocillopora*-associated bacterial community was the very low relative abundance of *Endozoicomonas* reported here (< 1%) (Pogoreutz et al. 2018; Epstein et al. 2019; Ziegler et al. 2019; Voolstra and Ziegler 2020). Rarefaction curves indicated sufficient sequencing depth and read numbers were comparable with previous studies at nearby locations (Epstein et al. 2019). In addition, the primers used here can detect coral-associated *Endozoicomonas* (Glasl et al. 2019; Marchioro et al. 2020). Therefore, the reduced representation of *Endozoicomonas* may have been due to the fact that the onset of coral bleaching represents a tipping point for this bacterial species in *P. acuta*. Indeed, all corals from the present study exhibited some level of bleaching, with 3.5–5.6 °C-weeks and an absence of *Endozoicomonas* regardless of bleaching severity, whereas *P. acuta*, which experienced 2 °C-weeks and no bleaching within the same

Fig. 6 Significant differences within bacterial communities between Havannah and Pandora reefs. (**a**) Mean relative abundances of each significantly different ASV were calculated for each reef, then summed according to families. Data shown for sum > 0.1% relative abundance. Numbers on the side of the plot indicate the number of ASVs represented by the bubble in each family depicted (blank = 1 single ASV). (**b**) Mean relative abundance of the significantly different ASVs within 5 major families (see text). Families are colour-coded with the same colours in both panels
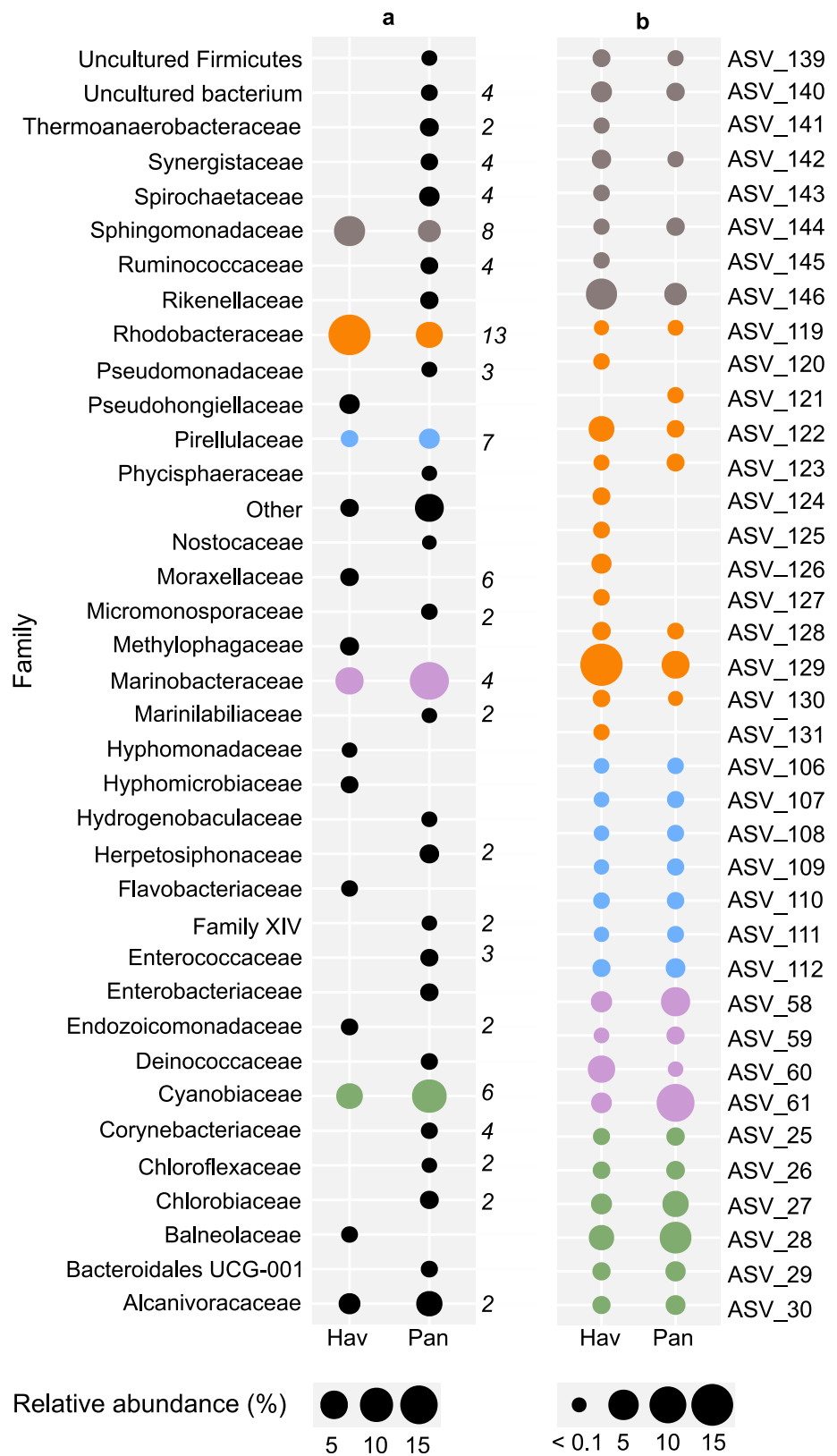
archipelago, still harboured normal levels of *Endozoicomonas* (Epstein et al. 2019).

Supporting this hypothesis is the fact that reduced abundances of *Endozoicomonas* were found in *Acropora*

millepora after a bleaching event on the GBR (Bourne et al. 2008) and that such declines have often been correlated with degraded or stressful environmental conditions across geographic locations. For instance, *A. millepora* living in CO_2 seep-acidified waters of Papua New Guinea presented a 50% reduction in relative abundance of *Endozoicomonas* compared to individuals sampled at a nearby pristine site (Morrow et al. 2015), and unhealthy *Porites astreoides* sampled in Belize displayed decreased abundances of *Endozoicomonas* compared to healthy individuals (Meyer et al. 2014). The consequences of these declines on coral health are unclear, as the functional role of *Endozoicomonas* within corals has remained elusive. However, genomic data and functional prediction tools have suggested that the *Endozoicomonas*-coral association involves amino acids metabolism and nutrient cycling (Morrow et al. 2015; Neave et al. 2017; Robbins et al. 2019). Recent empirical evidence demonstrated that *Endozoicomonas acroporae* isolated from *Acropora sp.* can degrade the sulphur compound dimethylsulphoniopropionate (DMSP) and use it as part of its central carbon metabolism (Tandon et al. 2020). Given that dinoflagellates and the coral host are major producers of DMSP (Broadbent and Jones 2004; Raina et al. 2013), the near-complete absence of *Endozoicomonas* in the present study might have aggravated any nutrient imbalance already at play in the holobiont and further deteriorated the host's health.

The microbial signature of P. acuta is site-specific

Unlike bleaching severity, location strongly constrained both the Symbiodiniaceae and bacterial communities of *P. acuta* living only 12 km apart. A global scale study examining the microbiome of *Pocillopora damicornis* found that geographic location mattered less than thermal regimes in structuring the microbial communities (Brener-Raffalli et al. 2018). However, as noted previously, the present study examined bleached samples and showed differences in microbial communities compared to samples of the same species collected at the same location under milder thermal stress (Epstein et al. 2019).

Whilst it is impossible to rule out that a difference of 1.7 °C-weeks between Havannah and Pandora is responsible for the contrasting microbial community composition, other factors could have played an important structuring role. Indeed, finer scale studies have shown that the microbiome of a single coral species can vary across reef regions (Hernandez-Agreda et al. 2016) or across habitats within a given reef (Glasl et al. 2017). Similarly, *Acropora hyacinthus* and *Acropora spathulata* from Rib Reef and Davies Reef (mid-shelf Great Barrier Reef), separated by 80 km, were found to diverge in the community composition of both Symbiodiniaceae and bacteria (Epstein et al.

2019). Within the Palm Island archipelago, location was an important factor structuring prokaryotic communities of *Acropora* coral species separated by only 40 km (Littman et al. 2009).

Despite the smaller geographic scale discussed in the present study, a combination of ecological and oceanographic factors might explain the strong reef specificity we detected. *P. acuta* is a brooding species, which implies limited dispersal of larvae before settlement, compared to a broadcast spawning species (Torda et al. 2013a). In addition, *P. acuta* exhibits both sexual and asexual reproductive modes (Torda et al. 2013a; Schmidt-Roach et al. 2014b; Gélin et al. 2017; Nakajima et al. 2018). Whilst populations in the Palm Island archipelago preferentially use sexual reproduction, particular sites within the Palm Islands received a greater proportion of recruits than others when examined over a period of two years (Torda et al. 2013a). In other words, self-recruitment can be more prevalent in certain areas of a single reef (Torda et al. 2013a), perhaps due to water currents direction (King and Wolanski 1996; Luick et al. 2007) or differences in wave energy and the frequency or intensity of physical disturbances (Gélin et al. 2017). A lack of connectivity was found in *P. acuta* colonies living 5 km apart in the Philippines (Torres et al. 2020), which is much shorter than the distance between Havannah and Pandora reefs, demonstrating how spatially restricted larval propagation can be for this species.

Crucially, vertical transmission of both Symbiodiniaceae and bacteria occurs in *P. acuta* (Baird et al. 2009; Damjanovic et al. 2020b). In particular, the bacterial family Rhodobacteraceae was one of the two most abundant families shared between parents and offspring of *P. acuta* (Damjanovic et al. 2020b). Therefore, the fact that this family contributed most to site-driven differences in the present study, both in the number of significantly different ASVs and in their relative abundance, strongly suggests that vertical transmission combined with limited larval dispersal played a role in the high reef specificity.

Taken together, our results demonstrate that, unlike bleaching severity, reef site shaped the microbial communities of *P. acuta*. Converging signals suggest that the onset of bleaching might represent a tipping point for the bacterial communities, and that factors such as local oceanic currents and host life history traits are responsible for the detected differences in microbial community composition, both eukaryotic and prokaryotic, between Havannah and Pandora reefs.

Declarations

Conflict of interest The authors declare that there are no conflict of interest.

Data availability The sequencing data generated and analysed in this study can be found in the NCBI Sequence Reads Archive (https://www.ncbi.nlm.nih.gov/sra) under submission PRJNA743719.

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