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Effects of temperature, salinity, and depth on Symbiodiniaceae lineages hosted by *Palythoa tuberculosa* near a river mouth

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ABSTRACT: The diversity of symbiotic dinoflagellate Symbiodiniaceae hosted by anthozoans is known to be driven by the environment where the hosts are found. This study examined how environmental variations (<1 to 100s m) can influence the diversity of Symbiodiniaceae within the zoantharian *Palythoa tuberculosa*. We monitored the dominant Symbiodiniaceae lineages within tagged *P. tuberculosa* colonies near the Hija River mouth and adjacent coastal reefs at Mizugama, Okinawa, Japan, between July 2016 and April 2018. Seven sites were chosen based on depth and distance from the river mouth, with 5 tagged colonies at each site. Water parameters of tidal pools (TPs) at the river mouth, especially temperature, were significantly different from other sites. Surprisingly, *P. tuberculosa* colonies at TPs were more resilient to bleaching during summers compared to colonies at other shallow sites. We observed different *Cladocopium* psbA^{ncr} lineages hosted by the tagged *P. tuberculosa*, with TPs colonies usually hosting one *Cladocopium* lineage (designated as lineage 4). Colonies from the deep sites and other shallow sites hosted mostly *Cladocopium* lineages 1 (generalist) and 2 (riverine specialist). Throughout the study period, the shallow colonies (included TPs) recorded higher rates of switching dominant *Cladocopium* lineages (mostly to lineage 1), whereas most deep colonies did not switch their dominant *Cladocopium* lineages. Our results show that *Cladocopium* lineage 1, previously reported as a generalist lineage in terms of environmental parameters, could also be an opportunist lineage during periods of host stress. Our study confirms that *Cladocopium* lineage flexibility likely helps the resilience of *P. tuberculosa* in such variable environments.

KEY WORDS: Okinawa · Zoantharian · Anthozoa · Bleaching · Zooxanthellae

1. INTRODUCTION

Coral reefs near river mouths face constant stress and have lower resilience due to discharges of freshwater. Studies have shown freshwater or low salinity seawater discharges from river mouths can contain soil, nutrients, and pollutants, which can influence the composition of benthic organisms especially zooxanthellate hard coral (West & van Woesik 2001, Golbuu et al. 2008, 2011, Tarya et al. 2018). Furthermore, if the river mouth is directly in contact with the open ocean without any physical barriers, a narrow transitional zone is present where the fresh and marine waters meet (Fabricius et al. 2014). The fresh–marine waters at the transitional zone are distinct in clarity and environmental quality, with the zone extending

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from the river mouth being heavily influenced by the volume of discharge, especially during heavy rainfall and rainy/wet seasons (Fabricius et al. 2014). This creates a boundary of significant benthic community changes at river mouths due to these stark environmental differences (West & van Woesik 2001).

Aside from affecting the general composition of the benthic community, river discharges also influence the physiology of sessile benthic organisms (Lough 2007, Mellin et al. 2019). Sessile benthic organisms found at river mouths often have wide tolerances to environmental fluctuations in salinity, turbidity, temperatures, and nutrient levels in order to survive (Yang et al. 2013, Moura et al. 2016). Hence, river mouths can provide a natural laboratory to study the physiology of benthic organisms living within river systems, transitional zones, and the ocean, particularly for sessile benthic organisms that can be found across more than one of these ecosystems.

Studies on marine benthic communities near river systems in Okinawa in subtropical southern Japan have noted the common presence of a zoantharian, *Palythoa tuberculosa* (Anthozoa: Hexacorallia: Zoantharia) (West & van Woesik 2001, Yang et al. 2013, Noda et al. 2017). *P. tuberculosa* is a ubiquitous generalist in the Indo-Pacific Ocean, and is found from the intertidal zone down to the depth of 50 m (Reimer 1971, Hibino et al. 2014, Wee et al. 2015, Risi & Macdonald 2016, Reimer et al. 2017a). It is often used as a model zoantharian in research (Shiroma & Reimer 2010, Hirose et al. 2011, Ong et al. 2013, Yang et al. 2013, Parkinson et al. 2016), as it is one of the easiest zoantharian species to identify in the field (Reimer 2010). *P. tuberculosa* forms symbiotic relationships with dinoflagellate Symbiodiniaceae (Burnett 2002, Reimer et al. 2006, LaJeunesse et al. 2018).

Symbiodiniaceae form symbiotic relationships with various marine organisms, including many reef-associated anthozoans (Tonk et al. 2013, LaJeunesse et al. 2018). Hosts provide physical protection and resources for the symbiont to photosynthesize, and in return the host receives metabolites from the symbiont. However, there is a narrow range of ideal water parameters (temperature, salinity, turbidity, pH) under which most of these symbiotic relationships exist (Rogers & Davis 2006, LaJeunesse et al. 2010, Graham & Sanders 2016, Suggett et al. 2017), and slight deviations can cause the breakdown of the symbiosis ('bleaching'). Some host species form relationships with different genera and species of Symbiodiniaceae that may have different ranges of environmental tolerances. Such flexibility, although often limited (Cunning et al. 2015, Baker et al. 2018), can provide some host species with mechanisms to possibly adjust to environmental changes (Baker 2003, Núñez-Pons et al. 2017, Cunning et al. 2018). Thus, accurate identification of Symbiodiniaceae is critical to help predict the adaptability and resilience of the holobiont.

The most accurate method to identify the genera and species/genotypes of Symbiodiniaceae is to use genetic approaches. Many studies have utilized sequences of the internal transcribed spacer 2 region of ribosomal DNA (ITS2) (LaJeunesse 2001, Stat et al. 2011, Franklin et al. 2012, Smith et al. 2017, Tan et al. 2020). Additionally, more recently developed DNA marker regions can delineate Symbiodiniaceae at very high resolution, such as the non-coding region of the psbA plastid minicircle ($psbA^{ncr}$) (Moore et al. 2003, LaJeunesse & Thornhill 2011), and this marker has recently been utilized to examine the symbionts of *P. tuberculosa* in the Indo-Pacific (Noda et al. 2017, Reimer et al. 2017b, Wee et al. 2019). Notably, a recent study used $psbA^{ncr}$ sequences to examine the diversity of Symbiodiniaceae in *P. tuberculosa* on reefs of Okinawa Main Island (Noda et al. 2017).

Noda et al. (2017) recorded 4 Symbiodiniaceae *Cladocopium* lineages hosted by *P. tuberculosa* in Okinawa based on $psbA^{ncr}$ sequences. Lineage 1 was proposed to be a generalist found across different environments as it was documented at all study locations around the island; lineage 2 was restricted to reefs near river mouths and was hence inferred to be a riverine specialist; lineage 3 was restricted in distribution to the northwest of Okinawa Island; and the ecological niche of lineage 4 was could not be determined due to low numbers in the study. Of all the locations examined in their study, *P. tuberculosa* colonies at the Mizugama coastline were shown to harbor the highest number of Symbiodiniaceae *Cladocopium* $psbA^{ncr}$ lineages around Okinawa Island (lineages 1, 2, and 4) (Noda et al. 2017).

Another recent study (Wee et al. 2019) on Symbiodiniaceae hosted by *P. tuberculosa* at a carbon dioxide bubble vent found that environmental variation can influence the symbiont lineage hosted at small geographical scales (approximately 200 m). The results of these studies raise the question of whether the environmental variation at river mouths such as at Mizugama can drive the diversity of Symbiodiniaceae hosted by *P. tuberculosa.*

The coast of Mizugama is a reclaimed seawall adjacent to the Hija River (Masucci & Reimer 2019). The Hija River is one of the most polluted rivers on Okinawa Island, with endocrine disruptors, lead:cadmium ratio, and uranium levels considered borderline

under WHO and Japanese water safety guidelines (Kawahata et al. 2004, Ramos et al. 2004, Mochizuki et al. 2015). Several studies have found high abundances of *P. tuberculosa* at Mizugama, especially near the mouth of the Hija River (Shiroma & Reimer 2010, Obuchi & Reimer 2011, Yang et al. 2013, Parkinson et al. 2016). Furthermore, West & van Woesik (2001) found the presence of a significant transition boundary for benthic cover (change in coral composition) just outside the Hija River mouth at the reef edge. Taken together, these results suggest that the effects of river discharge are in the immediate area within and adjacent to the river mouth, which in turn may affect the Symbiodiniaceae diversity in *P. tuberculosa* colonies at small geographical distances.

Thus, the Hija River and the adjacent coral reef at Mizugama coast provide an ideal location to study small-scale environmental variations across distances from the river mouth and across water depths, and how they influence the diversity of symbiotic Symbiodiniaceae hosted by a single species of host. In this study, these distances, hereafter referred to as 'small-scale', represent 200 to 500 m horizontally and <10 m in vertical water depth. We focused on the host species *P. tuberculosa* and determined the dominant Symbiodiniaceae across different environments. In this study, we therefore aimed to (1) examine the genotypic diversity of Symbiodiniaceae hosted by *P. tuberculosa* at different sites within Mizugama coast (distances from the river mouth and across different depths); (2) record the bleaching state of the hosts before, during, and after the summer 2016 and 2017 mass bleaching event; (3) determine the relation between Symbiodiniaceae diversity and bleaching state of *P. tuberculosa*; and finally (4) examine if there was intra-colonial (hereafter referred to as 'microenvironment') Symbiodiniaceae diversity in *P. tuberculosa*. Our main study (Expt 1) was designed to answer the first 3 objectives, while the last objective was conducted by an auxiliary study (Expt 2).

2. MATERIALS AND METHODS

2.1. Expt 1: Symbiodiniaceae diversity at different locations near the Hija River mouth

The study was conducted between July 27, 2016 and April 9, 2018, at the Hija River mouth and along the adjacent Mizugama coast. The Mizugama area consists of reclaimed land on the southwest coast of Okinawa Island and its neighboring coral reef (Fig. 1). At the north end of Mizugama, the 350 m wide Hija River mouth enters the sea. The shallow coral reef of Mizugama is exposed during low tides, and the reef edge steadily drops off to 10–20 m depths. In this study, 4 locations based on distance from the Hija River mouth were selected: (1) tidal pools behind a river mouth seawall (TPs); (2) the coral reef edge where the river mouth meets the ocean (0 m from river mouth); (3) a coral reef 500 m south of the river mouth; and (4) a coral reef 1000 m from the river mouth. Two depths (2 m [shallow] and 10 m [deep]) were examined at all locations except TPs, where the depth was 1.5 m. Thus, in total we investigated 7 sites: tidal pools (TPs), shallow and deep 0 m (0S and 0D), shallow and deep 500 m (500S and 500D), and shallow and deep 1000 m (1000S and 1000D).

All 7 sites had high numbers of *Palythoa tuberculosa* colonies. At each site, 5 *P. tuberculosa* colonies were tagged, with each colony given a unique number (TPs-1 to -5, 0D-1 to -5, 0S-1 to -5, etc.). At each site we also placed an Onset HOBO Pendant® Temperature/Light 64K data logger to collect surrounding seawater temperature and relative light levels. All loggers were set to record temperature and light intensity at an interval of 1 h (60 min). The loggers were cleaned during surveys at each site and replaced as needed.

We surveyed each site every 2 mo from July 27, 2016 to April 9, 2018, except during July to September each year when we increased survey frequencies to monthly in order to record the bleaching state of the tagged colonies during the bleaching events of summer 2016 and 2017. Occasionally surveys could not be conducted due to typhoons, and thus we conducted a total of 17 surveys within the study period.

During each survey, various water parameters (sea surface temperature, pH, salinity, conductivity, and turbidity) were recorded using a WQC-24 multiple water parameter probe (DKK-TOA) at each of the 4 locations (TPs, 0, 500, 1000 m) at a depth of 0.5 to 1 m. No water parameters were recorded for the deep sites in this study, as the receiver/interface of the multiple water parameter probe was not waterproof, and the probe cable was not long enough to extend to 10 m depths. The 35 tagged *P. tuberculosa* colonies were photographed with the Coral Colour Reference Card (Siebeck et al. 2006) using a camera with underwater housing (Olympus TG-3). After photography, approximately 2 cm^2 of tissue from each colony was extracted with a knife. Tissue specimens were fixed individually and stored in 99% molecular grade ethanol until molecular analysis.

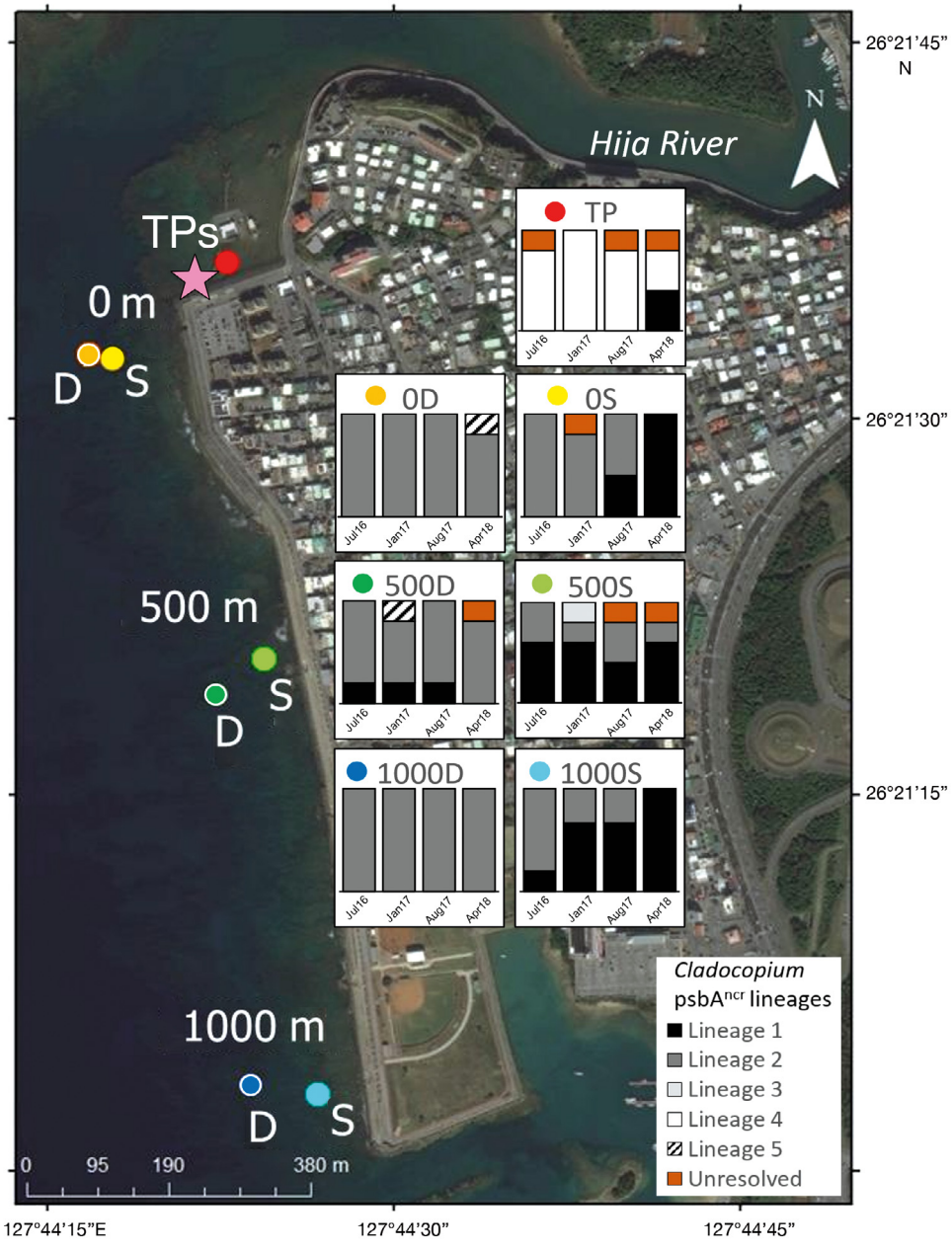


Fig. 1. Study sites at Mizugama and the dominant Symbiodiniaceae *Cladocopium* psbA^{ncr} lineage in each colony at each site based on surveys. The study sites are indicated by the colored markers. Two depths were set up at the 0, 500, and 1000 m locations: 2 m (S) and 10 m (D). The bar graphs next to each location represent the dominant *Cladocopium* (Symbiodiniaceae) psbA^{ncr} lineages based on surveys: Jul16 (July 27, 2016), Jan17 (January 30, 2017), Aug17 (August 18, 2017), Apr18 (April 9, 2018) (dates from left to right in inset charts). (☆) Location where colonies were chosen for analysis of the intracolonial variation of Symbiodiniaceae

2.2. Molecular analyses

Four surveys were chosen for molecular analyses: 2 from bleaching summer (July 27, 2016 [Jul16]; August 17, 2017 [Aug17]), and 2 from non-bleaching winter and spring (January 30, 2017 [Jan17]; April 9, 2018 [Apr18]). In total, we conducted molecular identification of Symbiodiniaceae in 140 specimens (35 tagged colonies × 4 surveys). Specimens from the surveys were selected for genomic DNA extraction utilizing a DNeasy Blood and Tissue extraction kit according to the manufacturer's instructions (Qiagen). Two different target DNA regions of Symbiodiniaceae were amplified via direct Sanger sequencing of polymerase chain reaction (PCR) products: the internal transcribed spacer 2 region of nuclear

ribosomal DNA (ITS2) and the hypervariable non-coding region of the plastid minicircle (psbA^{ncr}).

The ITS2 region was amplified using the primers zITSf (5′-CCG GTG AAT TAT TCG GAC TGA CGC AGT-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990, Rowan & Powers 1992, Hunter 1997), while psbA^{ncr} was amplified using the primers 7.4-Forw (5′-GCA TGA AAG AAA TGC ACA CAA CTT CCC-3′) and 7.8-Rev (5′-GGT TCT CTT ATT CCA TCA ATA TCT ACT G-3′) (Moore et al. 2003, LaJeunesse & Thornhill 2011). The PCR mixes (20 µl) were composed of 0.5 to 1.0 µl of genomic DNA, 10.0 µl of HotStarTaq Plus Master Mix (Qiagen), 1.0 µl of each primer (10 pmol), 1.0 µl $MgCl_2$ (25 mmol), 0.5 µl bovine serum albumin (20 mg ml^{-1}), 1.5 µl CoralLoad PCR buffer (Qiagen), and autoclaved Milli-Q water to fill the rest of the volume. Thermocycle conditions were modified slightly from Noda et al. (2017): ITS2: 95.0°C for 5 min; 35 cycles of 94.0°C for 30 s, 51.0°C for 45 s, and 72.0°C for 2 min; with a final extension at 72.0°C for 10 min; and for psbA^{ncr}: 95.0°C for 5 min; 40 cycles of 94.0°C for 10 s, 55.0°C for 30 s, and 72.0°C for 2 min; with a final extension at 72.0°C for 10 min. The products were sent to Fasmac (Kanagawa, Japan) for direct sequencing in both directions.

2.3. Phylogenetic analyses

The Symbiodiniaceae nucleotide sequences of ITS2 (n = 70) and psbA^{ncr} (n = 134) obtained were compiled, inspected, and edited (cleaned) manually using Bioedit (Hall 1999) into 3 separate datasets: ITS2, and psbA^{ncr} forward and reverse sequences. The psbA^{ncr} hypervariable region forward and reverse reads were non-overlapping, hence the 2 alignment sets were utilized to maximize identity resolution (Hawkins et al. 2016, Noda et al. 2017, Kunihiro & Reimer 2018). The compiled sequences were aligned separately using the MUSCLE package within Molecular Evolutionary Genetic Analysis version 10 (MEGA X) (Kumar et al. 2018). Each data set was inspected manually, and primer region and uneven ends were removed. Sequences which had 'double peaks' or mixed chromatogram signals over both regions were removed from phylogenetic analyses and designated as unresolved (Fig. 1).

Reference sequences were obtained from GenBank to include in each alignment. The ITS2 references were previously recorded *Cladocopium* C1-related sequences (GenBank accession numbers DQ480631, DQ480639, DQ889741, DQ889743) and a *Durusdinium* sequence (EU333712) hosted by *P. tuberculosa* from southern Japan and Singapore. Furthermore, sequences from Symbiodiniaceae hosted by other host species (AB207184, AF333516, AB704033, KU535564, MH243738, MH243740) were also included for reference. Additional forward psbA^{ncr} references (268 bp) included in the alignment were from a previous study on Symbiodiniaceae hosted by *P. tuberculosa* in Okinawa (MF593404–MF593453) (Noda et al. 2017). Finally, additional reverse psbA^{ncr} references (155 bp) were obtained from a dataset of symbionts in *P. tuberculosa* in the South China Sea (Wee et al. 2020). Novel sequences (forward: MW517360–MW517427; reverse: MW517428–MW517547) were deposited in NCBI GenBank (Supplementary Fasta psbA_For, psbA_Rev).

Best fit substitution models for ML analyses of all the datasets were obtained by running NJ tree reference under the automatic model selection function in MEGA X. The phylogenetic trees of the aligned sequences were constructed using MEGA X for maximum likelihood (ML), neighbor-joining (NJ), maximum parsimony (MP), and MrBayes (Huelsenbeck & Ronquist 2001) for Bayesian inference (BI) methods. Construction of ML, NJ, and MP phylogenetic trees were generated with the Kimura-2 model for the ITS2 alignment, and the Juke-Cantor model for both psbA^{ncr} alignments with 1000 bootstraps (Hasegawa et al. 1985). A BI tree for each alignment was constructed based on the models from the output in MEGA X with parameters set for each alignment (chain length = 15 000 000; burn-in < 3 750 000). The net genetic distances between psbA^{ncr} lineages were calculated using the Juke-Cantor model via MEGA X.

2.4. Data analyses

Numerical and ordinal data obtained in this study were compiled and analyzed in the R console (V 3.6.1) with the Rstudio graphical user interface (R Core Team 2019, RStudio Team 2019). Significance levels for all analyses were set at $p < 0.05$.

Seawater parameter data (pH, turbidity, temperature, salinity, and conductivity) were grouped based on survey and location (shallow sites). Normality tests (Shapiro-Wilks test) were conducted and followed by tests of homogeneity for normally (Levene's test) and not normally (Fligner-Killeen test) distributed variables. Pearson's rho correlation tests were conducted among all water parameters. Comparisons of significant differences of water parameters among locations and surveys were conducted using 1-way ANOVA (normal, homoscedasticity),

Kruskal-Wallis (non-normal, homoscedasticity), and Brown-Forsythe (non-normal, heteroscedasticity). The data were normalized and transformed with the Euclidean matrix format. A principal component analysis (PCA) was conducted based on the transformed dataset. Permutational multivariate analysis of variance (PERMANOVA) and pairwise post-hoc tests were conducted for the factors, locations, and surveys separately (Oksanen et al. 2015).

HOBO logger data were collected between July 27, 2016 and April 9, 2018. The loggers were compiled and grouped based on depth; namely tidal pools (TPs), shallow (S), and deep (D). A Kolmogorov-Smirnov test of normality was conducted for this dataset, and a Shapiro-Wilks test was limited to a dataset of less than 5000 points to prevent unintentional bias. A Kruskal-Wallis test was conducted on the dataset, followed by a Wilcoxon signed rank test with Holm's correction as a post-hoc test.

The bleaching state of each tagged *P. tuberculosa* colony was determined each survey by using a standard Coral Colour Reference Card (Siebeck et al. 2006). Coral color scores (CCS) were given to each tagged colony following adapted methods described by Parkinson et al. (2016). CCS ranged from '1' (white, fully bleached) to '6' (darkest color, not bleached at all) based on the color saturation and brightness of the colonies relative to the reference card (Siebeck et al. 2006; see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m667p043_supp.pdf). The CCS data were categorized based on location, depth, and survey. Location and depth factors were concatenated to form sites. A normality test (Shapiro-Wilk's test) was conducted for the dataset and showed it was not normally distributed ($p < 0.05$). A non-parametric 2-factor test (Scheirer-Ray-Hare test) was conducted on the CCS, along with a Dunn post-hoc test with Holm corrections if there were significant differences within each factor. Furthermore, the depth and location interaction were tested using the Brown-Forsythe test (onewaytests) with a pairwise Wilcoxon post-hoc test to examine the difference in CCS among sites (depth × location). The CCS of shallow colonies were compared with water parameter data (pH, turbidity, temperature, salinity, and conductivity) via Pearson's correlation with Holm's adjustment. The factors driving the CCS of tagged shallow colonies were determined with decision-making multivariate regression trees (MRT; Mellin et al. 2019). The predictors in these analyses were surveys, locations, and water parameter data. The nodes of the MRT defined the CCS of the colonies based on the spatial–environmental drivers. Groupings of the mean score were generated at the leaves based on the most likely predictors.

2.5. Expt 2: Intracolonial variation of Symbiodiniaceae

Eight large *P. tuberculosa* colonies with large vertical coverage (spanning >1 m in height) at the Hija River mouth near the TPs site were selected (Fig. 1). Colonies were chosen based on the potential different parts of the colonies being exposed to different environments at very small physical distances (<1 m; microenvironments). In detail, colonies were selected that had at least 2 of the criteria below in order to examine microenvironmental differences: (1) part of the colony had an exposed top during spring low tide (colonies I, II III, V, VI, VIII), (2) part of the colony was shadowed by adjacent boulders (colonies I, II, III, IV, V, VI, VIII), and (3) part of the colony was submerged in water all the time (colonies I, II, III, IV, V, VI, VIII). HOBO Pendant® Temperature/Light 64K data loggers were secured on the top (loggers I-t, II-t, III-t, IV-t, V-t, VI-t, VII-t, VIII-t) and bottom (I-b, II-b, III-b, IV-b, V-b, VI-b, VII-b, VIII-b) parts of the colonies to record temperature and light intensity experienced by the colonies between August 11, 2017 and October 17, 2017 (68 d). Large colonies had extra loggers at the middle microenvironment (II-m, VI-m) in order to obtain more detailed transitional data. The relative sea level depth of each logger was measured on January 15, 2018 at noon (±30 min), when the tide level was between the range of 0.82 to 0.87 m. The measurements obtained were corrected with the 'Tide and Current Predictor' (Pentcheff 2018) to calculate actual sea level height of the loggers placed at each microenvironment of the tagged colonies.

Collections of tissue from tagged *P. tuberculosa* were conducted on September 16, October 18, and November 3, 2017. A 3 cm^2 area was collected near the loggers (n = 18) and also at a random spot on each colony (n = 8). Collected specimens were individually fixed in 99.5% ethanol. Specimens were brought to the laboratory, and then subsequently molecularly analyzed via sequencing and alignment of the $psbA^{ncr}$ forward sequence following the procedures described in Sections 2.2 & 2.3 (note ITS2 was not sequenced in this experiment). ML and NJ phylogenetic trees were constructed for these alignments.

The temperature (maximum, minimum, average, standard deviation) and light intensity (maximum, average, standard deviation) data of each microenvironment was compiled. The microenvironment data

(7 variables as for Expt 2) of each logger were correlated (Pearson's correlation) with the corrected sea level depth (SLD: 85 cm based on January 15, 2018) at which loggers were anchored. Additionally, microenvironmental data were averaged according to each part of the colonies (top, middle, bottom, n = 3). Mann-Whitney *U* tests were conducted to compare means between parts of the colonies and the microenvironments (maximum, minimum, average of temperature, light intensity), and the corrected SLD as described above, using the R console (3.6) in RStudio (1.2.1335).

3. RESULTS

3.1. Surface water parameters

The mean and standard deviation of water parameters, namely pH, turbidity (mg l^{-1}), temperature (°C), salinity (ppt), and conductivity (S m^{-1}), were categorized by distance from river mouth/location and survey (Table 1). Tests of normality (Shapiro-Wilks test) and homogeneity of variance (Levene's test and Fligner-Killeen test) showed pH was normal with homogenized variance based on both location and survey, temperature and turbidity were not normally distributed but homogenized in variance when grouped by location, and the rest of the factors were not normal and had no equal variance among groups. Comparisons between water parameters demonstrated conductivity had a strong positive correlation with salinity (Pearson: r = 0.80, p < 0.001) and a negative correlation with temperature (Pearson: r = –0.54, p < 0.001) (Fig. 2). Independent permutation comparisons within each factor showed significant differences in the water parameters among locations (PERMANOVA: R^2 = 0.085, df = 3, p = 0.047) and surveys (PERMANOVA: R^2 = 0.396, df = 16, p = 0.002). However, a post-hoc test showed no significant difference within each factor (pairwise.adonis: p > 0.05).

PCA revealed that distance from the river mouth drove the variation in water parameters; particularly pH, turbidity, salinity, and conductivity (Fig. 3). pH was significantly different among locations (ANOVA: F = 3.909, df = 3, p = 0.013), especially between TPs (pH 8.26 ± 0.09) and 1000 m (pH 8.36 ± 0.10) (Tukey's HSD: p = 0.012). Conductivity (Brown-Forsythe: F = 4.919, df = 3, p = 0.006) had a similar pattern, with TPs (4.71 ± 0.21 S m^{-1}) having lower conductivity than 1000 m (4.86 ± 0.06 S m^{-1}) (pairwise Wilcoxon: p = 0.023). Salinity (Brown-Forsythe: F = 2.998, df = 3, p = 0.048) demonstrated significant differences between 1000 m (32.4 ± 0.2 ppt) and TPs (31.1 ± 1.5 ppt; pairwise Wilcoxon: p = 0.002) and 500 m (32.0 ± 0.4 ppt; pairwise Wilcoxon: p = 0.018). Turbidity (Kruskal-Wallis: χ^2 = 8.051, df = 3, p = 0.045) showed significant differences but the post-hoc test did not detect any differences between locations (pairwise Wilcoxon: p > 0.05), while temperature (Kruskal-Wallis: χ^2 = 0.525, df = 3, p = 0.913) did not show any significant difference among locations.

For surveys, only pH (ANOVA: F = 3.389, df = 16, p < 0.001), temperature (Brown-Forsythe: F = 218.750, df = 16, p < 0.001), and conductivity (Brown-Forsythe: F = 3.807, df = 16, p = 0.019) showed significant differ-

Table 1. Average (SD) of the water parameters (pH, turbidity [mg l^{-1}], temperature [°C], conductivity [S m^{-1}], and salinity [ppt]) of the shallow sites based on (a) location (tidal pools [TPs], 0, 500, and 1000 m), and (b) survey. Surveys with Symbiodiniaceae of tagged *P. tuberculosa* colonies identified using molecular approach are given in **bold**. Dates given as d/mo/yr

	pH	Turbidity	Temperature	Conductivity	Salinity
a) Location					
TPs	8.26 (0.09)	6.5 (7.3)	27.5 (3.3)	4.10 (0.21)	31.1 (1.4)
0 m	8.28 (0.09)	14.4 (25.6)	27.2 (3.3)	4.80 (0.14)	31.5 (2.2)
500 m	8.29 (0.09)	5.0 (5.2)	27.6 (3.3)	4.83 (0.08)	32.0 (0.4)
1000 m	8.36 (0.10)	22.3 (38.1)	27.8 (35)	4.86 (0.06)	32.3 (0.2)
Average	8.30 (0.10)	12.1 (23.7)	27.6 (3.3)	4.76 (0.32)	31.8 (1.4)
Min.–Max.	8.12–8.56	0.0–140.0	20.7–33.5	4.08–4.95	23.6–32.9
b) Survey					
27/7/16	**8.38 (0.13)**	**9.0 (9.7)**	**30.7 (0.7)**	**4.5 (0.335)**	**28.6 (4.3)**
9/8/16	8.30 (0.07)	10.8 (9.6)	29.7 (0.6)	4.787 (0.006)	31.9 (0.5)
23/8/16	8.34 (0.08)	11.0 (11.7)	30.1 (0.5)	4.685 (0.080)	31.5 (0.5)
10/9/16	8.30 (0.03)	9.3 (3.7)	29.3 (0.1)	4.703 (0.161)	31.3 (1.2)
23/9/16	8.38 (0.04)	7.3 (12.1)	28.8 (0.3)	4.850 (0.022)	32.4 (0.1)
10/10/16	8.21 (0.05)	12.2 (7.8)	28.3 (0.1)	4.858 (0.040)	32.4 (0.2)
13/11/16	8.44 (0.06)	5.8 (1.5)	26.1 (0.2)	4.875 (0.047)	32.2 (0.3)
6/12/16	8.25 (0.10)	6.0 (4.9)	25.9 (0.1)	4.925 (0.006)	32.5 (0.1)
30/1/17	**8.25 (0.05)**	**14.9 (8.8)**	**22.6 (0.1)**	**4.883 (0.034)**	**31.8 (0.3)**
24/3/17	8.17 (0.01)	9.1 (3.5)	20.8 (0.1)	4.918 (0.059)	32.0 (0.4)
22/5/17	8.30 (0.11)	2.4 (2.8)	25.0 (0.7)	4.883 (0.033)	32.1 (0.3)
5/7/17	8.31 (0.13)	5.7 (2.1)	30.2 (1.1)	4.695 (0.044)	31.4 (0.4)
18/8/17	**8.35 (0.06)**	**27.0 (48.7)**	**32.9 (0.4)**	**4.703 (0.127)**	**32.0 (1.1)**
30/8/17	8.20 (0.07)	3.3 (3.7)	30.2 (0.1)	4.768 (0.107)	32.0 (0.8)
20/9/17	8.25 (0.08)	2.1 (2.9)	29.0 (0.2)	4.773 (0.105)	31.9 (0.7)
2/11/17	8.27 (0.11)	2.2 (2.2)	26.6 (0.4)	4.760 (0.146)	31.4 (1.2)
9/4/18	**8.34 (0.02)**	**65.6 (68.3)**	**22.8 (0.3)**	**4.923 (0.042)**	**32.4 (0.1)**

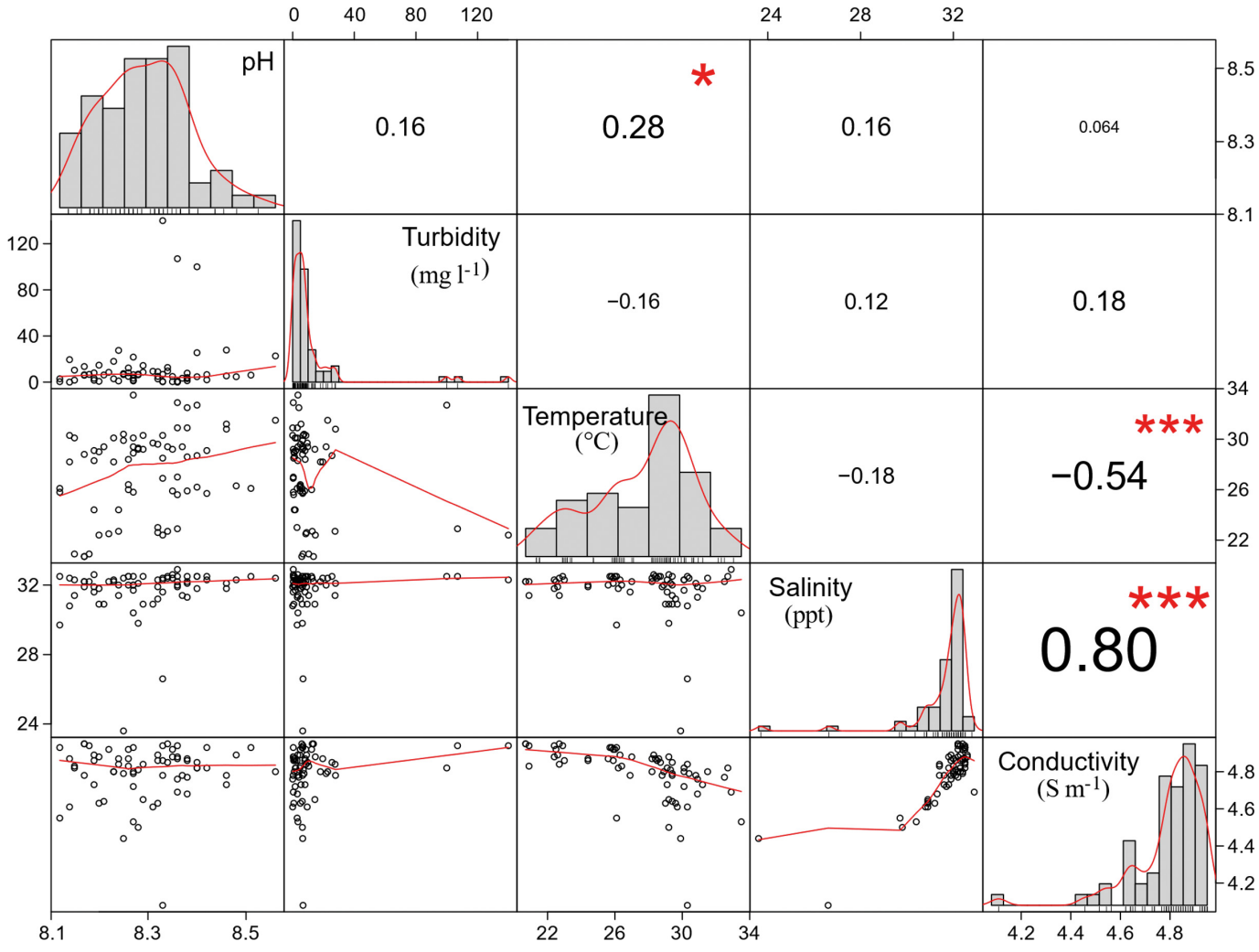


Fig. 2. Pearson's correlation of the water parameters at the shallow sites. The diagonal bar graphs represent the histogram distributions of pH, turbidity, temperature, salinity, and conductivity. The scatterplots below the diagonal represent the correlations between the water parameters while the Pearson's correlation values (represented in number, and in size of font) among all parameters are given above the diagonal. *p < 0.05; **p < 0.01; ***p < 0.001

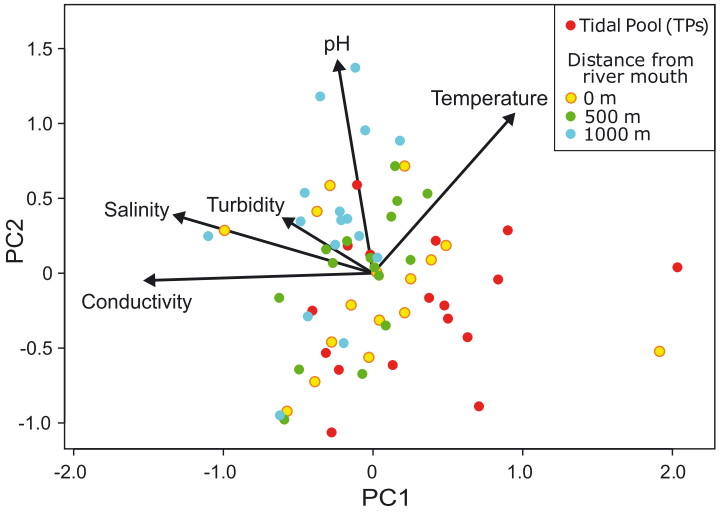


Fig. 3. Principle component analysis (PCA) of the distribution of the water parameters (pH, temperature, turbidity, salinity, conductivity) among the shallow locations: tidal pools (TPs), and 0, 500, and 1000 m from the Hija River mouth. PC1 (42.29%) and PC2 (25.56%) represent 67.85% of the total eigenvalues (5.00)

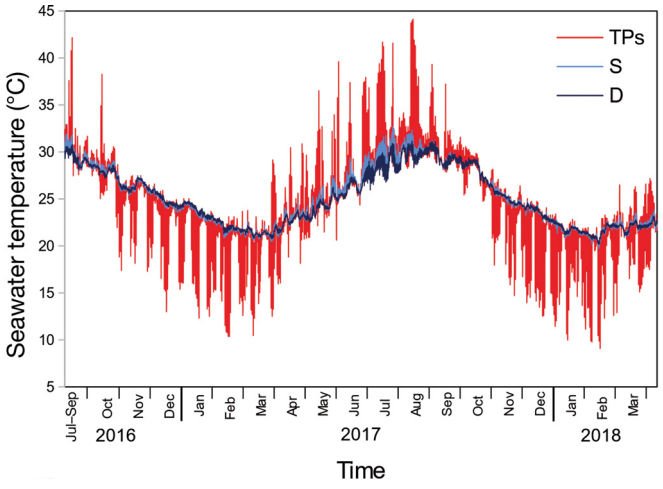


Fig. 4. Seawater temperature as recorded by HOBO Pendant loggers at tidal pools (TPs), shallow (S), and deep (D) sites between July 2016 and April 2018

ences. There were significant differences in pH between July 7, 2016 (pH 8.38 ± 0.13), October 10, 2016 (pH 8.21 ± 0.05), November 13, 2016 (pH 8.44 ± 0.06), March 24, 2017 (pH 8.17 ± 0.01), and August 30, 2017 (pH 8.20 ± 0.07) (Tukey's HSD: $p < 0.05$). Due to the low number of replicates ($n = 4$), a post-hoc test did not detect any significant differences between surveys for temperature and conductivity (pairwise Wilcoxon: $p = 1.000$).

In summary, pH, conductivity, and salinity showed significant differences among locations, especially between TPs and 1000 m away from river mouth. For the different surveys, pH, conductivity, and temperature changed significantly, demonstrating that these parameters were influenced by both the distance from river mouth and survey timing (seasonality).

3.2. Continuous recording of seawater temperature

HOBO Pendant® logger results showed that the highest and the lowest temperatures were recorded at the TPs site: 44.0°C (August 19, 2017) and 9.1°C (February 13, 2018), respectively (Fig. 4). The temperature ranges of the shallow (19.6 to 33.0°C) and deep (19.9 to 32.7°C) sites were much narrower than that of TPs. The Kruskal-Wallis test showed significant differences among depths ($\chi^2 = 27.201$, df = 2, $p < 0.01$). TPs (24.4 ± 3.8°C) was significantly different (Wilcoxon test: $p < 0.01$) from shallow (25.0 ± 3.1°C) and deep (24.9 ± 2.9°C) sites. However, no significant differences were detected between shallow and deep sites (Wilcoxon test: $p = 0.222$).

3.3. *Palythoa tuberculosa* CCS

The CCS of each *Palythoa tuberculosa* colony was averaged based on the location (TPs, 0, 500, 1000 m), depth (shallow and deep) and survey (Fig. 5a). Two colonies from the shallow site 1000 m away from river mouth (1000S) were completely bleached by September 2016 (CCS = 1.00) and had died (colonies disintegrated) by October 10, 2016.

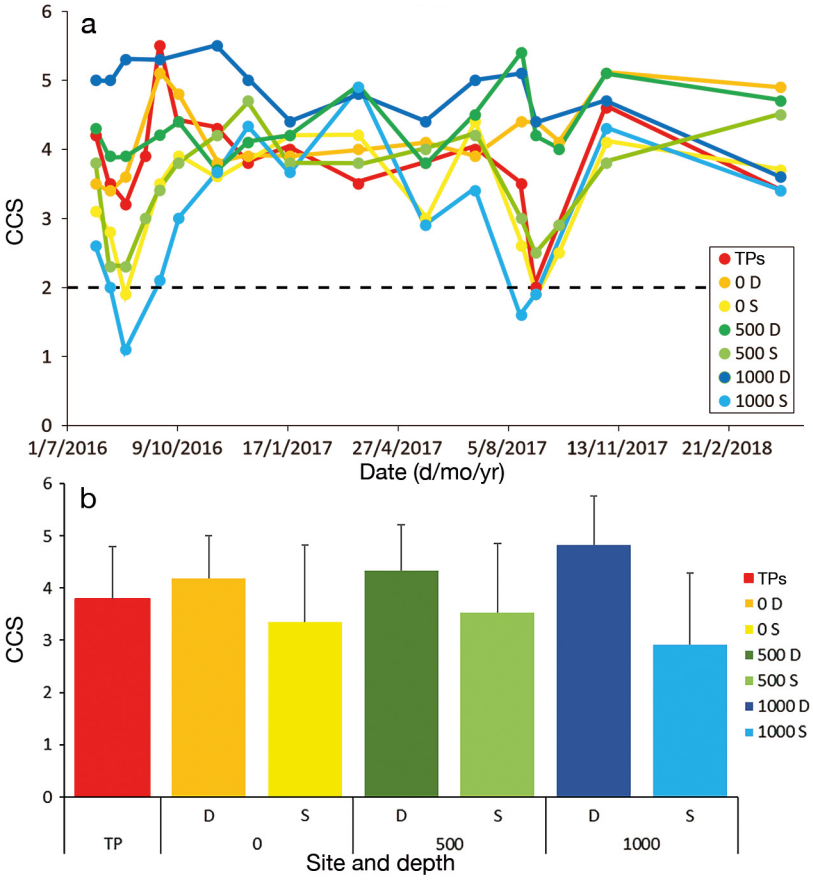


Fig. 5. Coral color score (CCS) of the tagged *Palythoa tuberculosa* at tidal pools (TPs), 0 m shallow (0S) and deep (0D), 500 m shallow (500S) and deep (500D), and 1000 m shallow (1000S) and deep (1000D) sites. (a) CCS changes of tagged *P. tuberculosa* at each site between July 2016 and April 2018; (dashed line) survival threshold of *P. tuberculosa* as determined by Hibino et al. (2013). (b) Average CCS of tagged *P. tuberculosa* colonies throughout the survey period. Error bars represent the SD for each respective site and depth

Two factorial non-parametric analyses (Scheirer-Ray-Hare test) showed that differences in CCS among the groups were significant for factor interactions between location and depth (sites) (H = 15.924, df = 2, p < 0.001) and surveys and depths (H = 56.43, df = 31, p = 0.003). No significant difference was detected between factor interactions between location and survey (H = 43.408, df = 45, p = 0.540). Regarding the average CCS of colonies based on site (location × depth, Fig. 5b), *P. tuberculosa* at 1000S (2.896 ± 1.391 SD) had the lowest CCS, while colonies at 1000D (4.821 ± 0.937) had the highest score. The median score was at TPs (3.794 ± 1.001). A pairwise post-hoc test (Wilcoxon test) among the sites showed significant differences in almost all site comparisons, except between TPs and 0S (p = 0.082), 0D (p = 0.082), and 500S (p = 0.657); between 0S and 500S (p = 0.657) and 1000S (p = 0.437); between 500S and 1000S (p = 0.079); and between 0D and 500D (p = 0.657).

Averaged CCS among tagged colonies at TPs (3.794 ± 1.001), 0 m (3.763 ± 1.259), 500 m (3.919 ± 1.196), and 1000 m (3.992 ± 1.865) showed no significant differences (Scheirer-Ray-Hare: H = 1.344, df = 3, p = 0.719). On the other hand, significant differences were detected among the depths (H = 93.168, df = 2, p < 0.001) TPs (3.794 ± 1.001), shallow (3.308 ± 1.411), and deep (4.428 ± 0.913) (Dunn test: p < 0.05). Finally, the highest CCS by survey was recorded on November 2, 2017 (4.500 ± 0.875), while the lowest was recorded on August 23, 2016 (3.04. ± 1.602). Average CCS were significantly different among the surveys (Scheirer-Ray-Hare: H = 4 8.487, df = 16, p < 0.001). The post-hoc test (Wilcoxon test) showed that the CCS of tagged colonies was significantly higher on November 2, 2017 than on August 9, 2016 (3.271 ± 1.426, Z = −4.004, p = 0.008), August 23, 2016 (3.043 ± 1.601, Z = −4.278, p = 0.003), August 30, 2017 (3.106 ± 1.374, Z = −4.085, p = 0.006), and September 20, 2017 (3.280 ± 1.217, Z = −3.555, p < 0.050). The CCS of tagged colonies on August 23, 2016 was also significantly lower that for July 6, 2017 (4.318 ± 0.873, Z = −3.653, p = 0.034).

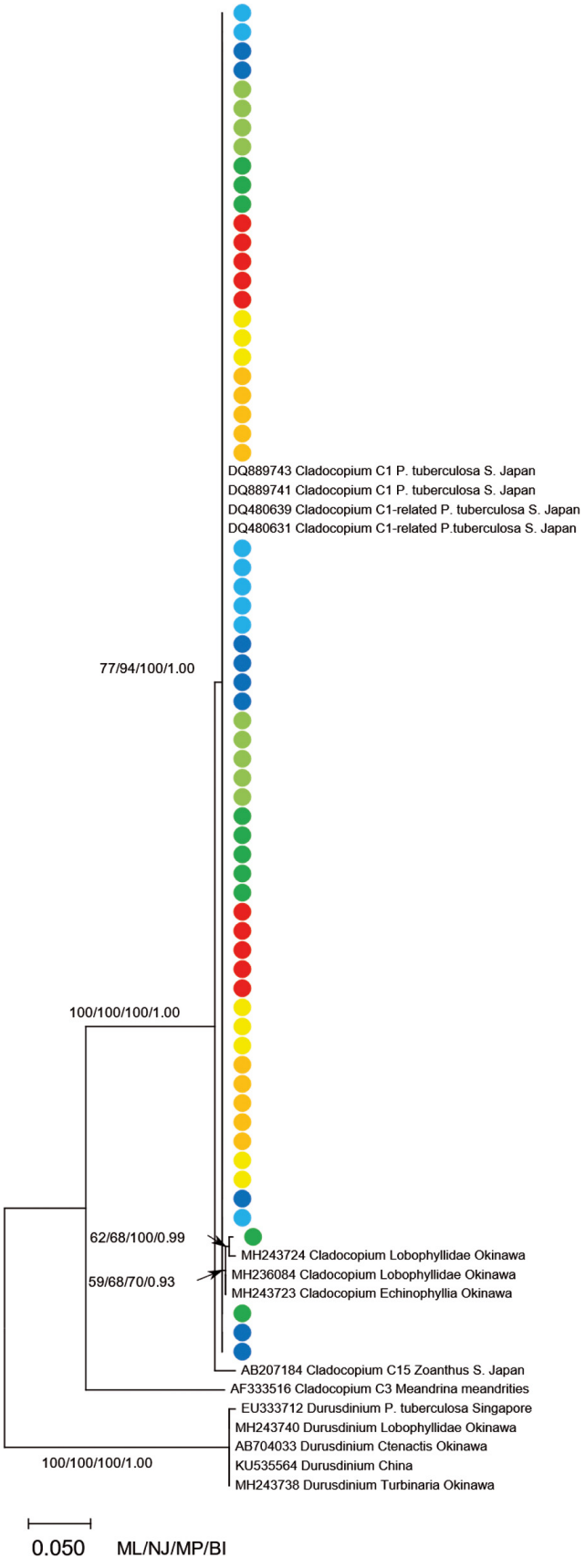
Aside from sites (location × depth) and surveys, the CCS of shallow water colonies varied among different water parameters/environment, with predictions made on the data using MRT. Of the water parameters recorded (temperature, pH, salinity, conductivity, turbidity), the CCS of the shallow colonies only showed a significant, but weakly negative, correlation with temperature (Pearson: r = −0.314, p < 0.001). However, the MRT model showed survey, location, and turbidity were the main predictors for the CCS of shallow colonies (Fig. S2 in the Supplement). From the model, there were 6 CCS score classes for the bleaching state of the tagged *P. tuberculosa* colonies. In general, 55% of the colonies had a CCS higher than 4.00. The tagged colonies bleached more (mean CCS = 2.45) in summer (surveys: August 9, 2016; August 23, 2016; August 18, 2017; August 30, 2017; September 20, 2017) than in the non-summer period (mean CCS = 3.86). In addition, TPs colonies bleached less than the other shallow locations, especially during summer. To a lesser degree, turbidity also affected the CCS of tagged colonies, as colonies in clearer waters (<0.8 nephelometric turbidity units [NTU]) had higher CCS.

3.4. Symbiodiniaceae identification

Phylogenetic identification of the dominant Symbiodiniaceae based on ITS2 sequences was conducted for only 2 periods, summer 2016 (July 27, 2016) and winter 2017 (January 30, 2017). The results showed that all tagged *P. tuberculosa* colonies hosted *Cladocopium* (Fig. 6: ML = 100%, NJ = 100%, MP = 100%, BI = 1.00). Almost all (n = 64) *Cladocopium* sequences in this study were identical (100%) and clustered within a C1/C1-related subclade (ML = 77%, NJ = 94%, MP = 100%, BI = 1.00). A sequence from 1 specimen collected in winter 2017 (500D-2-Jan17) was in a separate moderately well supported cluster (ML = 62%, NJ = 68%, MP = 100%, BI = 0.99) within the C1/C1-related cluster and the rest of the specimens (Fig. 6).

For the psbA^{ncr} region, 134 specimens from 35 tagged host colonies were collected. Of these, we successfully identified psbA^{ncr} lineages of Symbiodiniaceae from 127 specimens. Six sequences were omitted from analyses due to multiple peaks in their chromatograms: 1 each in summer 2016 (TPs-1-Jul16) and winter 2017 (0S-1-Jan17), 2 in summer 2017 (TPs-5-Aug17, 500S-4-Aug17), and 3 in spring 2018 (TPs-1-Apr18, 500D-4-Apr18, 500S-4-Apr18).

The *Cladocopium* sequences in this study clustered into 5 distinct psbA^{ncr} lineages based on the phylogenetic analyses (Fig. 7, Table S1 in the Supplement). Most tagged *P. tuberculosa* hosted lineage 2 *Cladocopium* (sensu Noda et al. 2017) (Forward: ML = 89%, NJ = 79%, MP = 100%, BI = 1.00; Reverse: ML = 59%, NJ = 35%, MP = 100%, BI = -). The next most abundant lineages were lineage 1 (ML = 100%, NJ = 99%, MP = 100%, BI = 1.00; ML = 71%, NJ = 67%, MP = 50%, BI = 0.83) and lineage 4 (ML = 100%, NJ = 100%, MP = 100%, BI = 1.00;



ML = 99%, NJ = 100%, MP = 100%, BI = 1.00) (Fig. 7). There were 3 *Cladocopium* that could only be identified via reverse psbA^{ncr} sequences, 2 from novel lineage 5 (ML = 99%, NJ = 99%, MP = 100%, BI = 1.00), and 1 from lineage 3 (ML = 75%, NJ = 41%, MP = 100%, BI = 1.00). The net genetic distances between the psbA^{ncr} lineages are given in Table S2 in the Supplement.

The identified *Cladocopium* lineages were then grouped based on site (location × depth) (Fig. 1, Table S1). Tagged *P. tuberculosa* at TPs hosted predominantly lineage 4 throughout the study. However, 2 colonies at TPs (TPs-2 and TPs-3) switched to dominantly hosting lineage 1 between summer 2017 and spring 2018. At the shallow sites 0 and 1000 m from the river mouth, all tagged colonies switched their dominant *Cladocopium* from lineage 2 to lineage 1 by the end of the study (2 colonies died in summer 2016: 1000S-2 and 1000S-4), while at 500 m, lineage 1 dominated throughout the study. There was 1 *P. tuberculosa* colony at the shallow 500 m site (500S-2) that switched the dominant *Cladocopium* from lineage 1 in summer 2016 to lineage 3 in winter 2017, and finally to lineage 2 in summer 2018 (Table S1).

For deeper colonies, the dominant *Cladocopium* lineages hosted were relatively more stable. Tagged *P. tuberculosa* at 0 m from the river mouth (0D) constantly hosted lineage 2 except for colony 0D-4, which switched to novel lineage 5 in spring 2018 (0D-4-Apr18). The site 500 m from the river mouth also had novel lineage 5 (500D-2-Jan17) in 1 of the tagged *P. tuberculosa* colonies in winter 2017 that then switched its dominant *Cladocopium* back to lineage 2 in subsequent surveys. Aside from these cases, lineage 2 was the dominant *Cladocopium* lineage at deep sites, with an additional 3 colonies hosting lineage 1 and 1 specimen having an unresolved dominant *Cladocopium* identity. Tagged colonies at the deep 1000 m site were the most stable, with all colonies hosting lineage 2 and showing no changes in *Cladocopium* lineage throughout the study.

Fig. 6. Phylogeny of Symbiodiniaceae from *Palythoa tuberculosa* at Mizugama. Maximum likelihood (ML) tree of the internal transcribed spacer 2 of ribosomal DNA (ITS2) of nuclear ribosomal DNA from Symbiodiniaceae. Colored dots on the tree represent each specimen found at tidal pools (TPs: red), 0 m shallow (0S: yellow) and deep (0D: orange), 500 m shallow (500S: light green) and deep (500D: dark green), and 1000 m shallow (1000S: light blue) and deep (1000D: dark blue). Values at the branches represent ML, neighbor joining (NJ) and maximum parsimony (MP) bootstrap supports, and Bayesian inference (BI) posterior probability

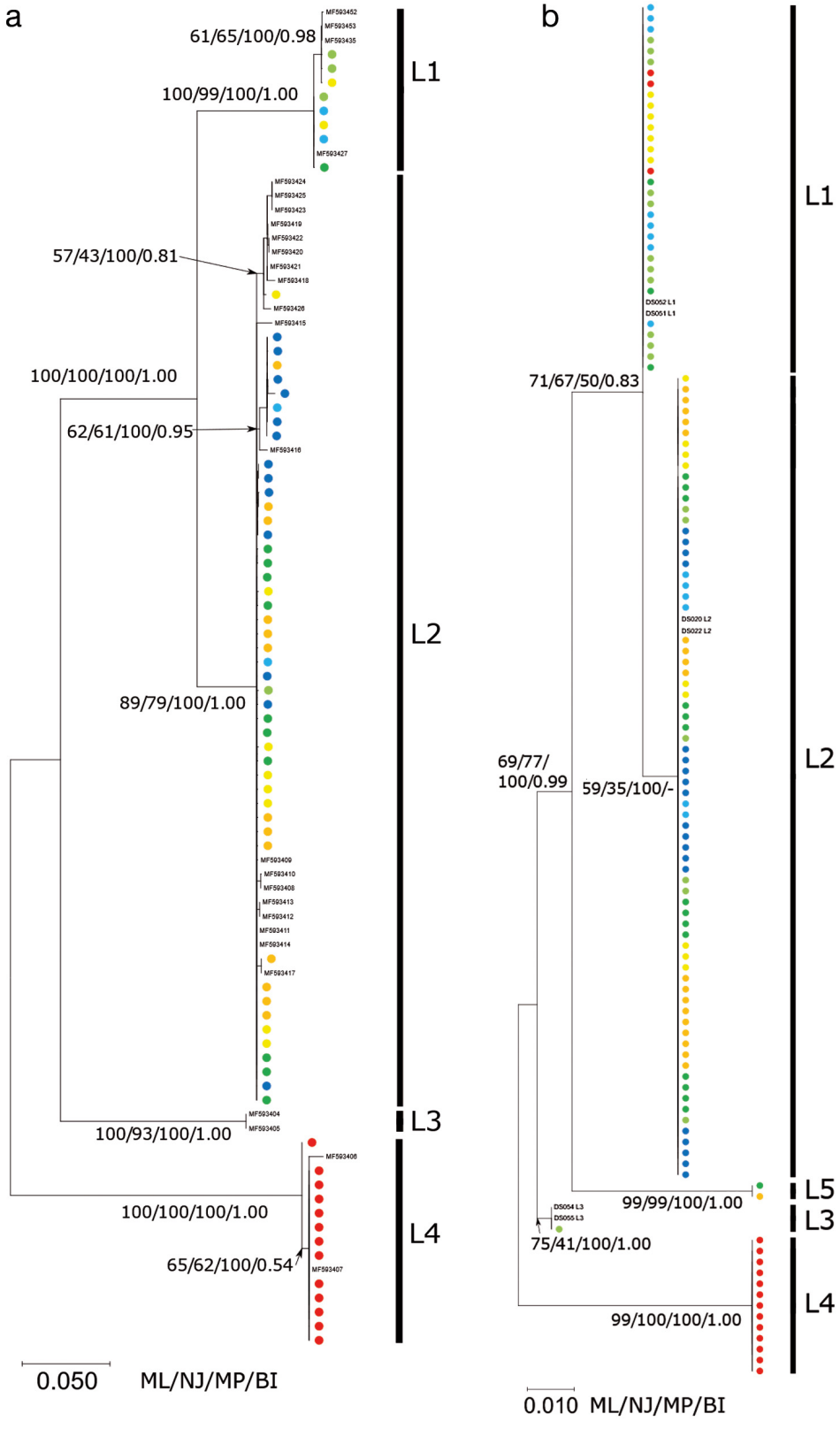


Fig. 7. Phylogeny of *Cladocopium* psbA^{ncr} lineages from *Palythoa tuberculosa* at Mizugama. Maximum Likelihood (ML) tree of (a) forward, and (b) reverse sequences of *Cladocopium* chloroplast psbA noncoding region (psbA^{ncr}) with reference from Noda et al. (2017) of the same region. See Fig. 6 legend for definitions of colors and abbreviations

In summary, shallow colonies switched their dominant symbiont at relatively higher frequencies (n = 13 observed dominant lineage-switching events) compared to TPs (n = 2 observed dominant lineage-switching events) and deep colonies (n = 4 observed dominant lineage-switching events). Unresolved Symbiodiniaceae identities were also higher in shallow and TPs (n = 3 unresolved each) colonies as compared to deep colonies (n = 1 unresolved). Furthermore, shallow colonies also had a higher prevalence of lineage 1 *Cladocopium* (48%) than TPs (10%) and deep colonies (5%), and the rate of switching dominant *Cladocopium* lineages increased at the end of the study. We observed an increase in total prevalence of lineage 1 from the start (14.29%) to the end of the study (39.39%), and a reduction in total prevalence of lineage 2 from 71.43 to 42.42% within the same period.

3.5. Intracolonial diversity of Symbiodiniaceae

Corrected SLD of tagged colony locations showed significant positive correlations with the maximum (Pearson: r = 0.600, p = 0.010) and standard deviation (Pearson: r = 0.730, p < 0.001) for temperature, and the standard deviation (Pearson: r = 0.500, p = 0.040) for light intensity. In contrast, the minimum temperature was negatively correlated with SLD (Pearson: r = −0.590, p = 0.010). No significant correlations between the SLD and average temperature (Pearson: r = 0.290, p = 0.270), light intensity (Pearson: r = 0.420, p = 0.100), or maximum light intensity (Pearson: r = 0.090, p < 0.740) were observed.

Comparisons of environmental data between the top and bottom locations of *P. tuberculosa* colonies showed the SLD of the tops (−22.25 ± 19.52 cm) was shallower (Mann-Whitney: U = 5.5, p = 0.011) than the bottoms (−52.86 ± 15.24 cm). Only 2 environmental data parameters showed significant differences between tops and bottoms; maximum (Mann-Whitney: U = 7.5, p = 0.020) and standard deviations (Mann-Whitney: U = 9.0, p = 0.029) of temperature. The tops of colonies had higher maximum temperatures (tops vs. bottoms: 43.72 ± 2.10°C vs. 38.83 ± 4.35°C) and wider standard deviations (1.38°C vs. 1.14°C) compared to the bottoms of colonies.

We were able to obtain Symbiodiniaceae psbA^{ncr} sequences from 15 of the 18 specimens collected (Table 2, Fig. S3 in the Supplement). From the resulting sequences, 3 *Cladocopium* psbA^{ncr} lineages were observed: 13 specimens clustered as lineage 1 (ML = 100%, NJ = 100%), 1 specimen from V-b was lineage 4 (ML = 98%, NL = 94%), and 1 novel lineage 6 specimen from II-m was recorded (ML = 100%, NJ = 100%). The intracolonial results showed that some colonies (n = 2/8) presented intracolonial variation of Symbiodiniaceae lineages.

Table 2. Coordinates, adjusted sea level depth (SLD, cm), temperature (°C) and light intensity (Lux) records for all tagged *Palythoa tuberculosa* colonies analyzed for intracolony variation of Symbiodiniaceae in Expt 2. T_Avg: average temperature; T_Max: maximum temperature; T_Min: minimum temperature; T_SD: SD of temperature; LI_Avg: average light intensity; LI_Max: maximum light intensity; LI_SD: SD of light intensity. Parts of colonies that had dominant *Cladocopium* lineages other than lineage 1 are given in **bold**

Code	Col.	Section	Coordinate	SLD	T_Avg	T_Max	T_Min	T_SD	LI_Avg	LI_Max	LI_SD	*Cladocopium* lineages
I-b	I	bottom	26° 21′ 36.5″ N, 127° 44′ 22.3″ E	−40	29.88	39.95	25.90	1.190	3547	253512	12942	1
I-t	I	top	26° 21′ 36.5″ N, 127° 44′ 22.3″ E	−20	29.92	45.70	25.31	1.463	11300	330668	31057	1
II-b	II	bottom	26° 21′ 35.6″ N, 127° 44′ 21.8″ E	−30	29.60	42.28	25.12	1.130	3287	220445	15124	1
II-m	**II**	**middle**	**26° 21′ 35.6″ N, 127° 44′ 21.8″ E**	**−18**	**29.84**	**44.82**	**25.22**	**1.488**	**12560**	**264535**	**30438**	**6**
II-t	II	top	26° 21′ 35.6″ N, 127° 44′ 21.8″ E	10	29.95	44.70	25.61	1.444	5910	264535	23802	1
III-b	III	bottom	26° 21′ 35.8″ N, 127° 44′ 21.6″ E	−70	29.62	33.22	25.61	1.032	2188	209423	8803	1
III-t	III	top	26° 21′ 35.8″ N, 127° 44′ 21.6″ E	−22	29.72	39.27	25.80	1.190	903	63378	2976	1
IV-b	IV	bottom	26° 21′ 36.4″ N, 127° 44′ 22.3″ E	−55	29.66	33.32	26.00	1.077	66	7577	308	1
IV-t	IV	top	26° 21′ 36.4″ N, 127° 44′ 22.3″ E	−38	29.14	42.28	25.31	1.178	924	132267	7019	1
V-b	**V**	**bottom**	**26° 21′ 37.3″ N, 127° 44′ 22.5″ E**	**−70**	**29.86**	**44.33**	**26.97**	**1.218**	**8403**	**330668**	**22486**	**4**
V-t	V	top	26° 21′ 37.3″ N, 127° 44′ 22.5″ E	−46	29.81	43.60	27.86	1.121	2440	330668	12624	1
VI-b	VI	bottom	26° 21′ 35.6″ N, 127° 44′ 22.0″ E	−45	29.81	41.34	25.80	1.207	6577	187379	20916	1
VI-m	VI	middle	26° 21′ 35.6″ N, 127° 44′ 22.0″ E	−30	29.75	42.87	25.61	1.284	973	187379	7498	1
VI-t	VI	top	26° 21′ 35.6″ N, 127° 44′ 22.0″ E	0	30.05	45.45	24.35	2.035	15565	253512	37249	1
VII-t	VII	top	26° 21′ 36.9″ N, 127° 44′ 22.5″ E	−22	29.89	44.08	25.51	1.348	6270	242490	19856	–
VIII-b	VIII	bottom	26° 21′ 35.9″ N, 127° 44′ 22.1″ E	−60	29.91	37.38	27.96	1.096	4441	231468	13007	1
VIII-t	VIII	top	26° 21′ 35.9″ N, 127° 44′ 22.1″ E	−40	30.01	44.70	25.51	1.251	8845	330668	21521	–

4. DISCUSSION

This study was conducted for 21 mo between July 2016 and April 2018, with 17 surveys sampling tagged *Palythoa tuberculosa* colonies. From these collected specimens, we examined 4 time points (out of 17 surveys) for molecular identification of dominant Symbiodiniaceae: 2 bleaching time points (summer: Jul16, Aug17), and 2 non-bleaching time points (winter and spring: Jan17, Apr18). Choosing these time points allowed comparison of host–symbiont relationships for the tagged colonies during, between, and after bleaching events. Furthermore, the choice of a single host may limit the host contribution in determining the symbiotic relationship in different small-scale environments (Tonk et al. 2013), thereby allowing examination of the specific Symbiodiniaceae niche.

In this study, the ITS2 and psbA^{ncr} sequences of Symbiodiniaceae hosted by *P. tuberculosa* specimens were directly sequenced. Hence, the sequences incorporated into the phylogenetic identification represent the dominant Symbiodiniaceae for each host colony. Changes in Symbiodiniaceae across our time points represent the switching of dominant Symbiodiniaceae in each respective host colony and, therefore, any switching of Symbiodiniaceae genotype represents the switching of dominant Symbiodiniaceae in hosts (Jones et al. 2008). We did not examine the presence of minority/background Symbiodiniaceae lineages hosted by the tagged *P. tuberculosa*, which are known to be present in many other zooxanthellate anthozoans hosts (Cunning et al. 2017, Hume et al. 2019), as this was not the focus of the current work.

Our results demonstrate that there are apparent fine-scale habitat differences in the dominant Symbiodiniaceae hosted by *P. tuberculosa*, specifically with regards to the water parameters temperature, pH, and salinity, and to different depths. Additionally, our observations demonstrate occasional dominant Symbiodiniaceae lineage switching in colonies over time (Cunning et al. 2015, Boulotte et al. 2016). These observations provide a possible explanation for how *P. tuberculosa* can survive across a wide range of environments via hosting specific Symbiodiniaceae under various conditions. As suggested in other recent research (Noda et al. 2017, Reimer et al. 2017b, Wee et al. 2019), fine-scale environmental differences at a level not usually considered in field surveys appear to be important drivers of Symbiodiniaceae diversity.

The Hija River mouth and the adjacent Mizugama coast have wide environmental parameter variations across small-scale geographical distances (200 to 500 m horizontal distances, 8 m vertical water depths). The effect of the Hija River discharge was limited to the direct boundary of the river mouth with the ocean, and this can be seen from the rather gradual changes in water parameters (pH, salinity, conductivity), with significant differences only seen between TPs and the site 1000 m from the river mouth. These gradual changes in water parameters contrasted with a previous study (West & van Woesik 2001) that noted a sharp transitional zone of benthic composition related to the substantial change in water parameters at the river mouth caused by the river discharge. The limited extension of the river discharge influence on the adjacent reef was probably due to the comparatively small size of the Hija River and the deflection by coastal currents that push the seawater inland and north (Tarya et al. 2018). In contrast, continuous seawater temperature recording (HOBO loggers) at TPs showed wider daily and annual variation compared to our examined shallow and deep points. This seawater temperature variation is likely due to the intertidal nature of TPs, with exposure to the open air during extreme tides in winter and summer (Shah et al. 2010). In addition, the TPs site is somewhat isolated from the open sea by a shallow rock wall at the river mouth that possibly causes some pooling of the river water. Further support for the uniqueness of the TPs site can be seen in the statistically different water parameter measurements between TPs and the remaining shallow sites.

In this study, all Symbiodiniaceae hosted by *P. tuberculosa* at Mizugama belonged to the genus *Cladocopium*. *Cladocopium* is common in the Indo-Pacific region, and thus far the only recorded genus hosted by *P. tuberculosa* at this latitude and further north (Reimer et al. 2006, Noda et al. 2017, Wee et al. 2019). In our main experiment, we recorded 5 dominant psbA^{ncr} lineages of *Cladocopium*, 4 previously recorded lineages and 1 novel lineage (Noda et al. 2017, Wee et al. 2019). *Cladocopium* hosted by *P. tuberculosa* are dominated by the putative riverine specialist lineage 2 between 0 and 1000 km from the Hija River mouth at both depths (Noda et al. 2017). Lineage 4, which was documented in low numbers by Noda et al. (2017), was the dominant *Cladocopium* lineage at the TPs location, and may be a shallow water or variable environment specialist. Putative generalist lineage 1, initially sporadically present in summer 2016 at shallow sites, later increased in prevalence as the dominant Symbiodiniaceae hosted in *P. tuberculosa* as the study continued across bleaching events in the summers of 2016 and 2017. Finally, there were 2 *Cladocopium* lineages that ap-

peared in low numbers, lineage 3 and novel lineage 5. Novel lineage 5 was found at deeper depths and during the cold season at 0 and 500 m from the river mouth during separate surveys. The distribution of Symbiodiniaceae based on the location of hosts at different distances from the river mouth and at different depths indicates possible adaptation to small-scale environments.

Between summer 2016 and spring 2018, the switching of dominant *Cladocopium* lineages was documented in several tagged *P. tuberculosa* colonies. Most of the dominant lineage switching happened between summer and winter. The switching of the dominant *Cladocopium* lineages in this study coincided with the bleaching state (CCS) of *P. tuberculosa* host colonies. Tagged colonies from the TPs, shallow 0 m, and shallow 1000 m sites bleached the most during summers, especially in summer 2017 (CCS < 2.00). Between August and September 2017, tagged colonies were severely bleached due to the extremely high temperatures, especially at TPs (HOBO Pendant: 44.13°C on August 27, 2017). Furthermore, at the site worst affected by bleaching, shallow 1000 m, 2 tagged *P. tuberculosa* colonies had died by September 2016. During summer 2016, colonies at the shallow 1000 m site had a consistent CCS of <2.00, which is considered the threshold CCS for *P. tuberculosa* before colony death (Hibino et al. 2013).

Thus, as *P. tuberculosa* bleached in the summers of 2016 and 2017, it is possible hosts switched their dominant Symbiodiniaceae due to abnormally high seawater temperatures. The most common dominant lineage switching observed was from the riverine specialist lineage 2 to the generalist lineage 1. Switching of dominant *Cladocopium* lineage was more prevalent at shallow sites, and particularly at the 0 and 1000 m sites, where all colonies had switched their dominant *Cladocopium* from lineage 2 to lineage 1 by spring 2018. On the other hand, colonies at deep sites showed little to no change in dominant Symbiodiniaceae lineage throughout the study, and this was also reflected by the consistently high CCS (>3.4).

The bleaching state of tagged shallow *P. tuberculosa* colonies was shown to be affected by 3 major drivers of water parameter fluctuation: seasonality, location, and turbidity. During the summers of 2016 and 2017, *P. tuberculosa* colonies were bleached (CCS < 2.90) due to high summer seawater temperatures (Figs. 4 & 5, Table 1, Table S1). During this time period, global mass bleaching events were reported, with as much as 90% of shallow coral reefs around the world affected by the increased sea surface temperatures (Kayanne et al. 2017, Frade et al. 2018, Hughes et al. 2018, Monroe et al. 2018). *P. tuberculosa* was severely affected, as would be expected, as *Palythoa* spp. have been reported to be among the first anthozoans to bleach during heat stress (Williams & Bunkley-Williams 1988, 1990).

Aside from seasonal variation, there was also a clear difference in the resilience of holobionts towards bleaching based on location. Regardless of temperature variation brought by seasonal change, TPs colonies did not bleach as much as colonies at other locations, even though they were consistently exposed to harsher environments. This was most evident when considering temperature variation at TPs, where colonies were exposed to 35.05°C annual variation, compared to a maximum of 13.44°C annual variation for tagged colonies at the shallow sites. The resilience of colonies at TPs could be due to association with resilient Symbiodiniaceae, in this case with *Cladocopium* lineage 4. The association with this possibly thermotolerant lineage may have allowed the *P. tuberculosa* holobiont to perform better in the harsh intertidal environment. Similar results are known from many studies on various host species, where associations of niche Symbiodiniaceae have been demonstrated to improve the performance of holobionts living in marginal environments (Baker 2003, LaJeunesse et al. 2014, D'Angelo et al. 2015, Hume et al. 2015).

Cladocopium lineage 1 is apparently not only an environmental generalist found across a wide range of environments in Okinawa (Noda et al. 2017, Wee et al. 2019), but it may also be opportunistic. After bleaching of tagged colonies was observed at shallow sites, we observed an increase in *Cladocopium* lineage 1 prevalence as the dominant Symbiodiniaceae hosted by *P. tuberculosa*, and thus lineage 1 emerged as a 'winner' of these bleaching events. Even though *Cladocopium* lineage 4 resisted bleaching in summer 2016, the warmer 2017 summer caused TPs colonies to bleach. Other shallow colonies that had CCS < 2.00 also switched their dominant *Cladocopium* lineage to lineage 1 after recovering from the summer 2017 bleaching event. All of these results indicate that *Cladocopium* lineage 1 is opportunistic, and it appears to thrive in comparatively marginal environments on coral reefs in Okinawa (Grupstra et al. 2017, Wee et al. 2019).

We observed different *Cladocopium* psbA^{ncr} lineages hosted by *P. tuberculosa* at different sites (location × depth) at the Hija River mouth and along the Mizugama coastline. This, coupled with significant differences in seawater temperature and small but consistent small-scale environmental shifts in water

parameters (pH, salinity, and conductivity), appeared to influence the dominant *Cladocopium* lineages of *P. tuberculosa*. This flexibility likely helps the resilience of *P. tuberculosa* in such variable environments. Furthermore, we also observed a low frequency of intracolonial variation in dominant *Cladocopium* lineages in very shallow and large *P. tuberculosa* colonies, and hypothesize that microenvironmental differences in different areas of colonies may be the cause of such differences. Such intracolonial Symbiodiniaceae variation has also been recorded in scleractinian corals, particularly shallow colonies (Kemp et al. 2015), and thus our results agree with these previous studies (Kennedy et al. 2016). Nevertheless, future studies with higher numbers of replicates are required to more robustly ascertain the microenvironmental drivers of intracolonial Symbiodiniaceae variation. Both previous and current studies have shown that the hypervariable psbA^{ncr} can distinguish higher resolution Symbiodiniaceae diversity in line with a changing environment (i.e. temperature) (Noda et al. 2017, Reimer et al. 2017b, Wee et al. 2019). Thus, analyses of psbA^{ncr} sequences can provide opportunities to study the influence of small-scale and microenvironmental variation on the diversity of symbionts and the corresponding resilience of holobionts.

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