

THE DIVERSITY OF SYMBIODINIACEAE HOSTED BY *Palythoa tuberculosa* FOUND AT THE EDGE OF THE SOUTH CHINA SEA

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Abstract: The Symbiodiniaceae genera composition hosted by the zooxanthellate zoantharian *Palythoa tuberculosa* is well documented in some parts of the Indo-Pacific region. Nevertheless, there are limited studies when it comes to the South China Sea (SCS), and no reports from the northern half of this region are available. This study examined Symbiodiniaceae diversity hosted by *P. tuberculosa* around Dongsha Atoll, in the north of the SCS. Samples of *P. tuberculosa* were collected inside and outside of the atoll. Phylogenetic analyses were conducted using sequences of the conservative internal transcribed spacer region 2 of rDNA (ITS2) and the hypervariable non-coding region of the chloroplast psbA gene (psbA^{ncr}). *Cladocopium* was the dominant Symbiodiniaceae genus found in Dongsha, with only one *Durusdinium*-hosting *P. tuberculosa* colony found within the atoll. psbA^{ncr} results showed three *Cladocopium* lineages previously recorded in Okinawa (lineages 1, 2 and 3 *sensu* Noda *et al.*, 2017). An experiment was set up outside the atoll to compare temperature fluctuations caused by internal waves, and the host-symbiont relationships found on spurs (n = 27, depth = 5.95 ± 0.23 m) and within grooves (n = 27, depth = 8.11 ± 0.47 m). Our results showed that there were no significant differences of *Cladocopium* lineages among the *P. tuberculosa* found at spurs and grooves despite significant differences in water temperature (p < 0.001). Our results show that *P. tuberculosa* is flexible with *Cladocopium* lineages hosted, and that the internal wave regime at Dongsha Atoll apparently did not affect the host-symbiont relationship, at least in the spurs and grooves.

Keywords: Symbiodiniaceae, *Palythoa tuberculosa*, Dongsha Atoll, spurs and grooves

Introduction

Recently, the Symbiodiniaceae family (previously genus *Symbiodinium*) has been reevaluated because of its high level of diversity, which is higher than previously thought (LaJeunesse *et al.*, 2018). In addition, the application of hypervariable non-coding plastid minicircle region sequences of the chloroplast psbA gene (psbA^{ncr}) as a DNA marker has improved the resolution of delineating lineages to a finer level besides using the more conservative internal transcribed spacer region 2 (ITS2) of nuclear ribosomal DNA (Moore *et al.*, 2003; LaJeunesse & Thornhill, 2011). Many studies have shown that psbA^{ncr} can discern different lineages of Symbiodiniaceae, even when differences were not detected between ITS2 sequences, and in some cases, the physical distance between specimens may be as little as

200 m (Noda *et al.*, 2017; Reimer *et al.*, 2017a; Wee *et al.*, 2019). Hence, ecological studies of the host-Symbiodiniaceae (henceforth known as holobiont) relationship are now possible at finer genetic and geographic scales than previously possible.

Palythoa tuberculosa (Esper, 1805) (Anthozoa: Hexacorallia: Zoantharia) is a ubiquitous sessile marine cnidarian, which can be found in temperate to tropical waters of the Indo-Pacific Ocean (Burnett, 2002; Reimer *et al.*, 2008; Reimer & Todd, 2009; Irei *et al.*, 2011; Reimer *et al.*, 2017b; 2017c). Aside from having a wide distribution, *P. tuberculosa* is present in marine environments beyond the tolerance limits of most zooxanthellate hexacorals, such as near estuaries, on acidified reefs, in intertidal zones, and in relatively hot areas (Inoue *et al.*, 2013; Yang *et al.*, 2013; Reimer *et al.*, 2015; Reimer

et al., 2017a). Their physiological resilience is due to their highly plastic colony form, a lack of calcium carbonate skeletal structure, thick and highly developed coenenchyme, and their active heterotrophic ability (Reimer, 1971; Fabricius & Metzner, 2004; Reimer & Todd, 2009; Ong *et al.*, 2012). Furthermore, *P. tuberculosa* is one of the easiest zoantharians to identify in the field (Reimer, 2010) and they host symbiotic dinoflagellate algae of the family Symbiodiniaceae (Dinophyceae: Suesiales) (Burnett, 2002; Ono *et al.*, 2008). The high resilience and wide distribution of *P. tuberculosa* have encouraged many studies on Symbiodiniaceae diversity in various environments and geographical areas (Burnett, 2002; Reimer *et al.*, 2013; Noda *et al.*, 2017; Reimer *et al.*, 2017a; Wee *et al.*, 2019). Hence, they are often utilized as a model to study the physiology and ecology of this relatively understudied zoantharian group (Shiroma & Reimer, 2010; Polak *et al.*, 2011; Ong *et al.*, 2012; Noda *et al.*, 2017).

P. tuberculosa reportedly exists in large numbers around Dongsha Atoll (20°41'15" N; 116°48'42" E) (Reimer *et al.*, 2017b). The atoll is 45 km in diameter and is situated on the northern edge of the South China Sea. The nearest land from the atoll is approximately 250 km away (Chou, 2013). A research station, Dongsha Atoll Research Station (DARS), is located at the inner west island of the atoll (Figure 1). Water temperature inside the atoll is 1 to 2°C warmer than the outside (Chou, 2013). However, the outer atoll temperature has wide daily fluctuations of up to almost 8°C due to the influence of internal waves, which bring nutrient-rich cold water from the deep open ocean to the forereef via upwelling (Chang *et al.*, 2006; Wang *et al.*, 2007).

In a previous study, *P. tuberculosa* has been reported from both the spurs and grooves of the forereefs of Dongsha Atoll (Reimer *et al.*, 2017b). This shows that this zoantharian can survive the periodic upwelling of cold-water internal waves. In this study, we aim to examine if the resilience of the holobiont is due to association

with certain Symbiodiniaceae. We examined if there is any difference of the Symbiodiniaceae at the spurs and grooves due to the channeling of internal waves and differing environments. Real time logging of the water temperature and light intensity at the spurs and grooves have been conducted to compare the two sites.

Materials and Methods

Three survey trips were conducted at the atoll from November 27 to December 8, 2017, April 20 to May 4, 2018, and June 6 to June 13, 2019. The first survey mainly focused on the inner atoll, the second survey was at the northwest side of the outer atoll (Figure 1), and the final survey involved the retrieval of data loggers installed at the inner atoll. In the 2017 trip, five *P. tuberculosa* specimens were collected by snorkeling at the southeast coast of the island (20°41'48.17" N, 116°43'59.20" E). HOBO Pendant® Temperature/Light 64K Data Loggers (OneTemp, Richmond, Adelaide, Australia) were deployed at the area. Approximately, 2 cm² of *P. tuberculosa* specimens were collected with a knife. The specimens were preserved individually on land and stored in absolute ethanol (99.5%) and sent back to the University of the Ryukyus (UoR) in Japan for analyses.

The 2018 trip was designed to focus on *P. tuberculosa* in spurs and grooves at the northwest outer atoll (20°46'17.46" N; 116°46'03.42" E). Six sets of adjacent spurs (depth = 5.95±0.23 m) and grooves (8.11±0.47 m) were chosen (Figure 2), each tagged with a HOBO Pendant® Temperature/Light 64K Data Logger. The loggers were set at 10-minute logging intervals from April 20 to April 30, 2018. Five *P. tuberculosa* colonies near each logger were collected at each spur and groove, where a total of 60 specimens were collected. Specimens were fixed in absolute ethanol (99.5%) and sent to UoR for analyses. Genomic DNA of the specimens were extracted using the Qiagen DNeasy Blood and Tissue extraction kit (Qiagen, Tokyo, Japan). Symbiodiniaceae DNA was amplified via polymerase chain reaction (PCR) using two sets of primers targeting DNA regions

in different organelles: the conservative ITS2 of nuclear ribosomal DNA and the hypervariable non-coding region of *psbA^{ncr}*. The ITS2 region was amplified using the primers zITSf (5'-CCG GTG AAT TAT TCG GAC TGACGC AGT-3') and ITS4 (5'-TCC TCC GCT TAT TGATAT GC-3') (White *et al.*, 1990; Rowan & Powers, 1991; Hunter *et al.*, 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 were composed of 0.5-1.0 µl of genomic DNA, 10.0 µl of HotStarTaq Plus Master Mix (Qiagen, Tokyo, Japan), 1.0 µl of each primer (10 pmol), 1.0 µl MgCl₂ (25 mmol), 0.5 µl Bovine Serum Albumin (20 mg/ml), 1.5 µl Coral Load and topped up to the maximum of 20 µl with autoclaved reverse-osmosis water. Thermocycle conditions were modified from Noda *et al.* (2017). For amplification of ITS2, the cycle began at 95.0 °C for five minutes, followed by 35 cycles of denaturation at 94.0 °C for 30 s, annealing at 51.0 °C for 45 s, and elongation at 72.0 °C for 2 min before a final extension at 72.0 °C for 10 min. For *psbA^{ncr}*, the cycle began at 95.0 °C for 5 min, followed by 40 cycles of 94.0 °C for 10 s, 55.0 °C for 30 s, and 72.0 °C for 2 min before a final extension at 72.0 °C for 10 min. The products were sent for sequencing in both directions (Fasmac, Kanagawa, Japan).

Both directions of the ITS2 sequences were checked and aligned manually to form a consensus sequence using BioEdit (Hall, 1999). No consensus sequence was produced with *psbA^{ncr}* as the hypervariable region made consensus impossible (Hawkins *et al.*, 2016; Noda *et al.*, 2017; Kunihiro & Reimer, 2018). The compiled sequences of ITS2, forward *psbA^{ncr}* and reverse *psbA^{ncr}* were aligned separately in MEGA X (Kumar *et al.*, 2018). Archived GenBank sequences (11 sequences) of *Cladocopium*, *Durusdinium* and *Effrenium* were obtained and included in the ITS2 alignment as references. The references for forward *psbA^{ncr}*

sequences were obtained from Noda *et al.* (2017), which were identified into four lineages: lineage 1 to 4. In total, 57 (597bp length), 38 (251bp), and 51 (174bp) sequences were used in the alignments of ITS2, forward *psbA^{ncr}* and reverse *psbA^{ncr}*, respectively.

Maximum likelihood (ML) and Bayesian inference (BI) phylogenetic trees were constructed based on the alignments of ITS2 and *psbA^{ncr}*. The best substitution methods for ML and BI were chosen based on the Neighbour Joining (NJ) tree reference under the automatic model selection of MEGA. Kimura-2 and Juke-Cantor model with 1000 bootstraps at uniform rates were utilized to construct ML trees of ITS2 and *psbA^{ncr}* (Hasegawa *et al.*, 1985). The MrBayes version 3.2.6 x64 (Huelsenbeck & Ronquist, 2001) software was used to construct BI phylogenetic trees of ITS2 (chain length = 5,000,000; burn-in = 1,250,000) and *psbA^{ncr}* (chain length = 15,000,000; burn-in = 3,750,000).

Data from the HOBO loggers were extracted and averaged based on the inner atoll and outer atoll (spurs and grooves) grouping. Daily average temperature differences of all three categories were calculated. Light intensity data at night with values equal to 0 lux were removed for all categories. Friedman tests were conducted for average temperature (°C) and light intensity (lux) data, and post-hoc Wilcoxon pairwise tests with Holm correction were conducted for results that were significantly different ($p < 0.05$).

Results

Based on ITS2 (Figure 3), all *P. tuberculosa* samples at the spurs and grooves ($n = 53$), and most of the inner atoll ($n = 3/4$), were found to host of *Cladocopium zooxanthellae* (C1) (ML = 99%, BI = 0.97). Only one colony at the inner atoll hosted the *Durusdinium* symbiont (ML = 99%, BI = 1.00). The *psbA^{ncr}* region examination was restricted to only *Cladocopium* symbionts (Figure 4) and based on the *psbA^{ncr}* forward sequences (Figure 4a), three *Cladocopium*

lineages were found, all of which had been previously recorded in southern Japan (Noda *et al.*, 2017). Two lineages were dominant: lineage 1 (n = 24; ML = 61%, BI = 1.00) and lineage 2 (n = 12; ML = 51%, BI = 1.00) *sensu* Noda *et al.* (2017) (Noda *et al.*, 2017). Furthermore, there was also a small number of lineage 3 (n = 2; ML = 65%, BI = 0.97) *sensu* Noda *et al.* (2017) (Noda *et al.*, 2017). Additionally, reverse sequences all agreed with the forward sequences for lineage 1 (n = 28; ML = 99%, BI = 1.00), lineage 2 (n =

20; ML = 65%, BI = 0.99) and lineage 3 (n = 3; ML = 99%, BI = 1.00) (Figure 4b). There were 54 *P. tuberculosa* colonies hosting *Cladocopium* identified based on forward and reverse *psbA^{ncr}* sequences, with 27 each from the spurs and grooves (Figure 5). Lineage 1 was the dominant lineage at both spurs (n = 14) and grooves (n = 17), followed by lineage 2 (spurs = 12, grooves = 8), and finally, lineage 3 (spurs = 1, grooves = 2).

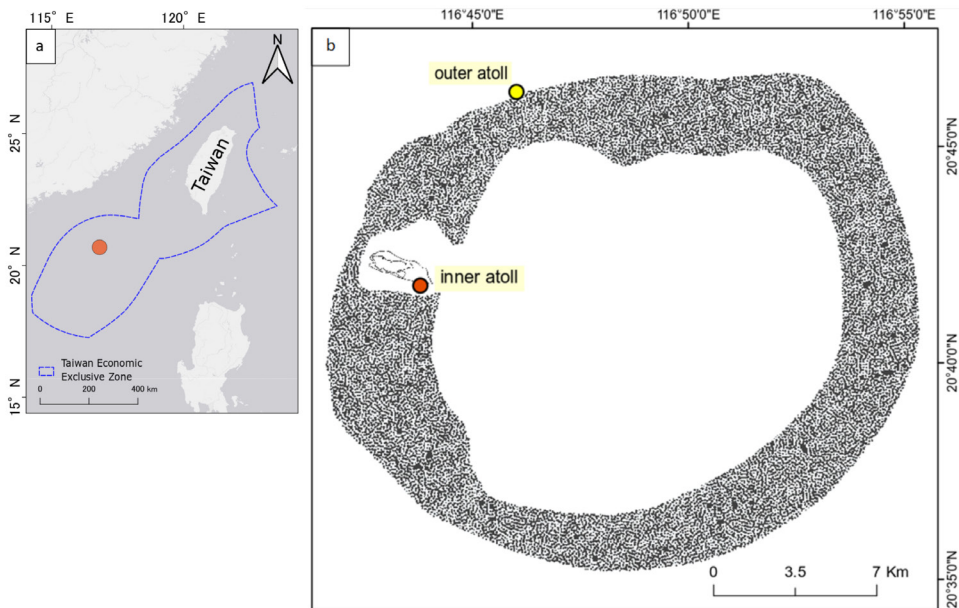


Figure 1: Map of (a) the location of Dongsha Atoll (indicated by orange dot), with the blue dotted line representing the Exclusive Economic Zone (EEZ) of Taiwan (Flanders Marine Institute, 2018), and (b) the sampling sites at the atoll; inner atoll (2017) and outer atoll (2018), the dotted marking represents the submerged atoll.

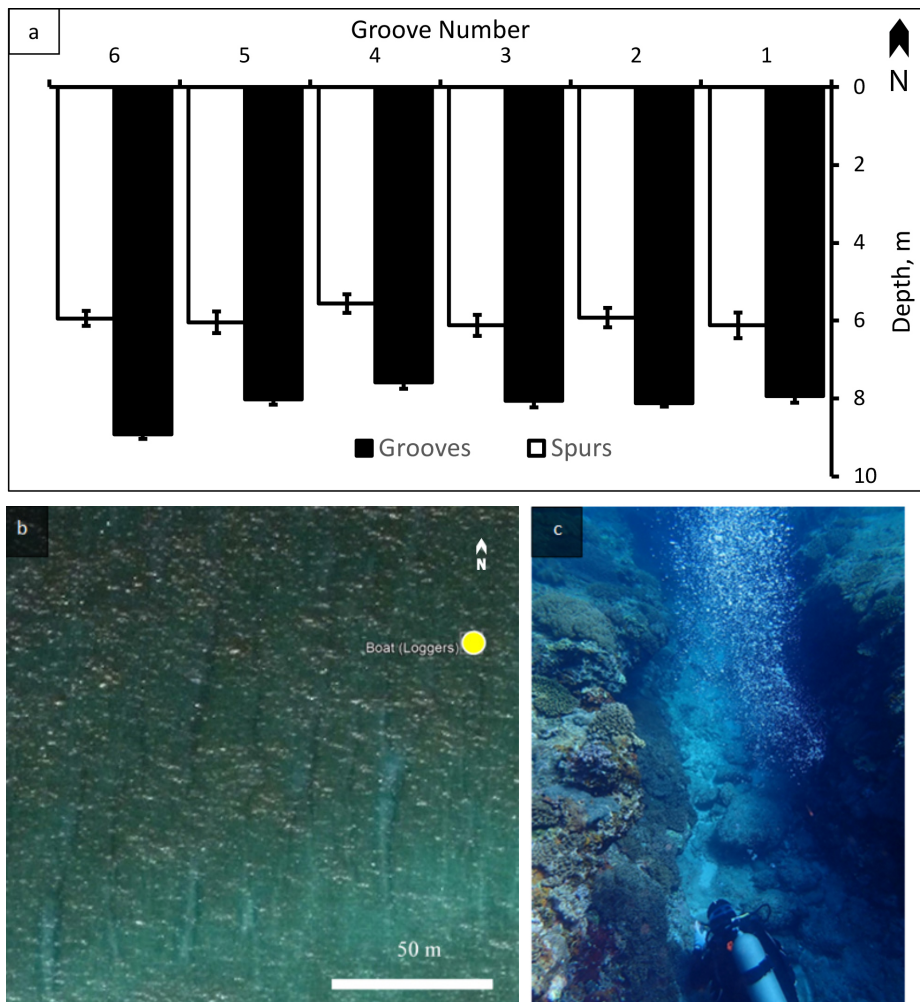


Figure 2: Study design of the spurs and grooves. (a) Average depths of *Palythoa tuberculosa* colonies sampled at the spurs (white) and grooves (black). The error bars represent standard deviations. (b) Aerial photograph of the spurs and grooves study site. The yellow dot represents the first groove tagged, and subsequent spurs and grooves were chosen westward (to the left) adjacent to the previous set. (c) Photograph of a researcher working in a groove, with spurs on each side.

The seawater temperature range was between 23.292°C (grooves: April 22) to 29.152°C (inner atoll: April 30) (Figure 6a). The daily range of temperature fluctuation was between Δ 0.976°C (Inner atoll: April 20) to Δ 4.124°C (inner atoll: April 30). There were significant differences (Friedman: $X^2 = 388.21$, $df = 2$, $p < 0.001$) in temperature among the inner atoll (26.924 \pm 0.749°C), spurs (25.364 \pm 0.666°C) and grooves (25.211 \pm 0.633°C). Post-hoc test

(Wilcoxon pairwise test with Holm correction) showed that there were significant differences between all categories, especially the inner and outer atoll sites ($p < 0.001$). On the other hand, light intensity during the day ranged between 5.4 (grooves: April 26) to 39955.8 lux (inner atoll: April 27) (Figure 6b). There were significant differences (Friedman: $X^2 = 62.01$, $df = 2$, $p < 0.001$) in light intensity among the inner atoll (average = 7861.11 lux), spurs

(average = 3940.300 lux) and grooves (average = 3464.87 lux). Post-hoc test (Wilcoxon with Holm correction) showed that the inner atoll had significantly higher light intensity compared to

the outer atoll sites ($p < 0.001$), whereas there was no significant difference between spurs and grooves in terms of light intensity ($p = 0.117$).

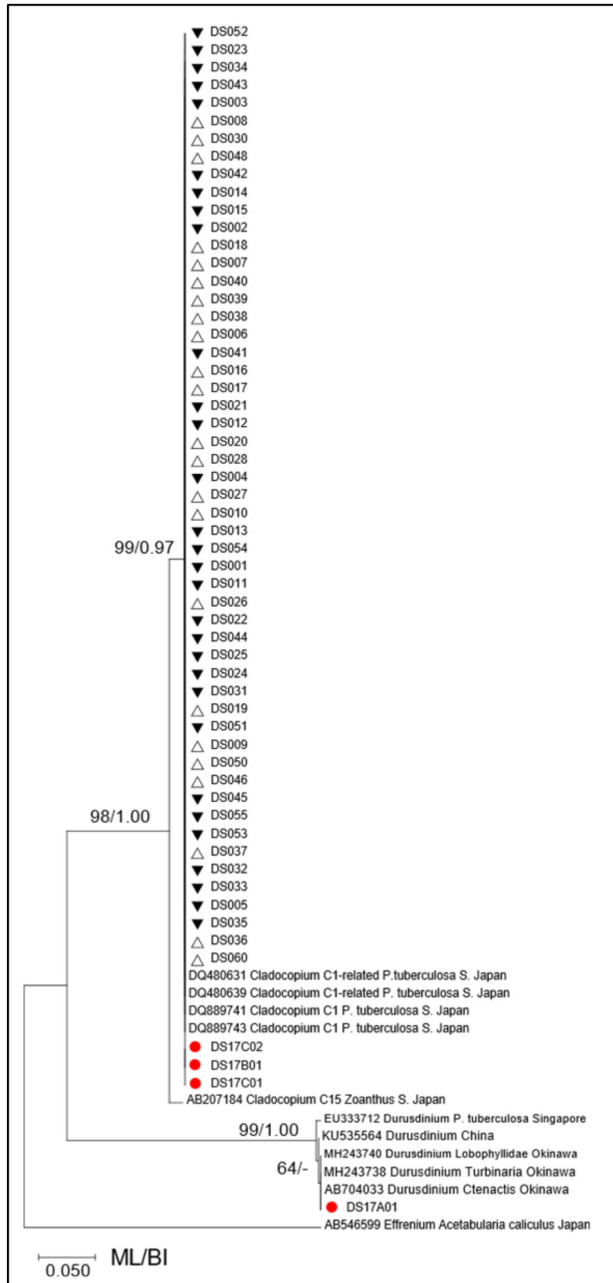


Figure 3: Phylogeny of Symbiodiniaceae hosted by *P. tuberculosa* found at Dongsha Atoll based on ITS2 region represented by maximum likelihood (ML) tree with ML bootstrap support and Bayesian inference (BI) posterior probability. Red dot = inner atoll; white upper triangle = spurs; black lower triangle = grooves.

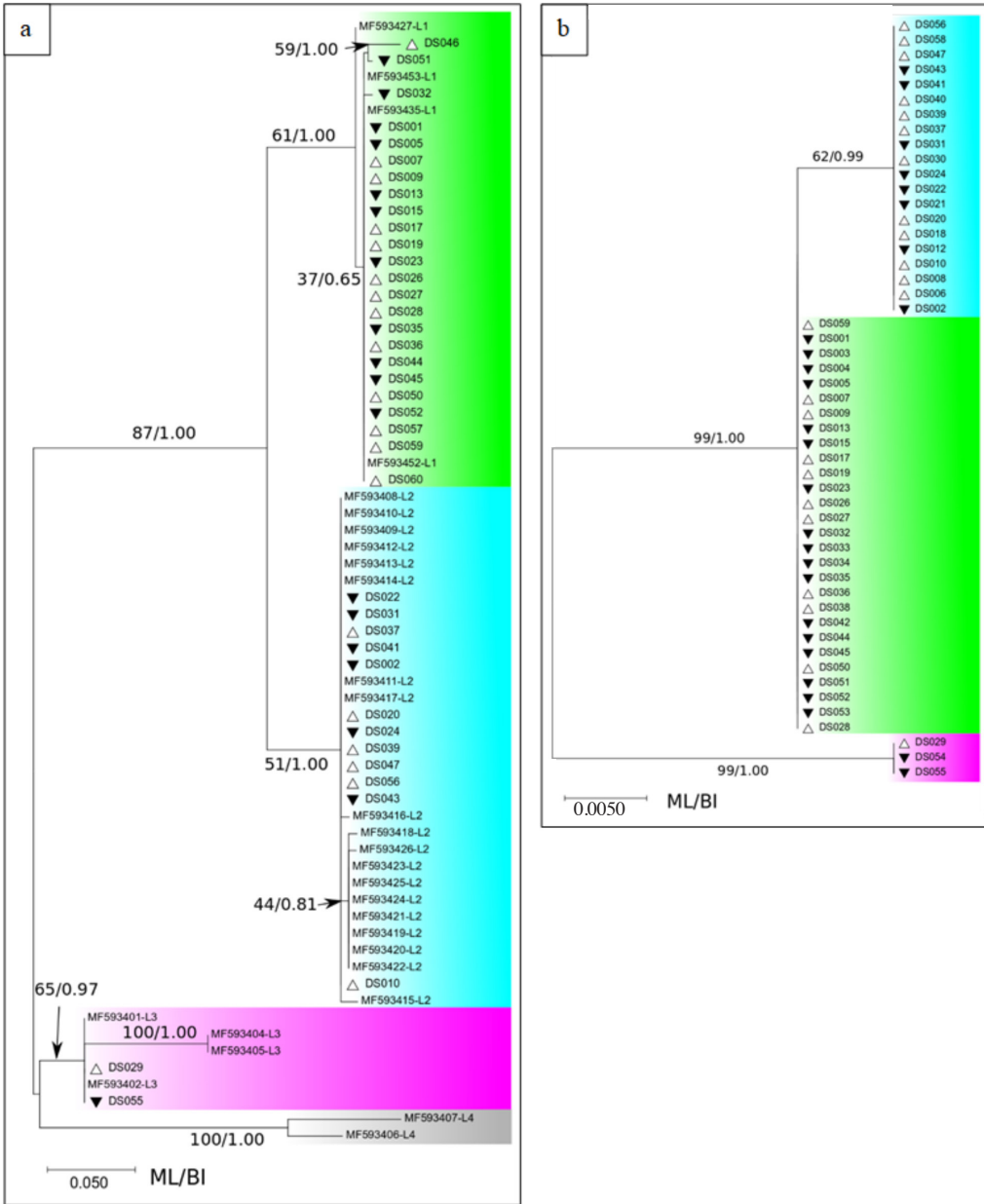


Figure 4: Phylogeny of *Cladocopium psbA^{ncr}* lineages hosted by *P. tuberculosis* found at Dongsha Atoll based on (a) forward *psbA^{ncr}* and (b) reverse *psbA^{ncr}* sequences represented by maximum likelihood (ML) phylogenies with ML bootstrap support and Bayesian inference (BI) posterior probability. White upper triangle = spurs; black lower triangle = grooves. Green = lineage 1; blue = lineage 2; purple = lineage 3; grey = lineage 4.

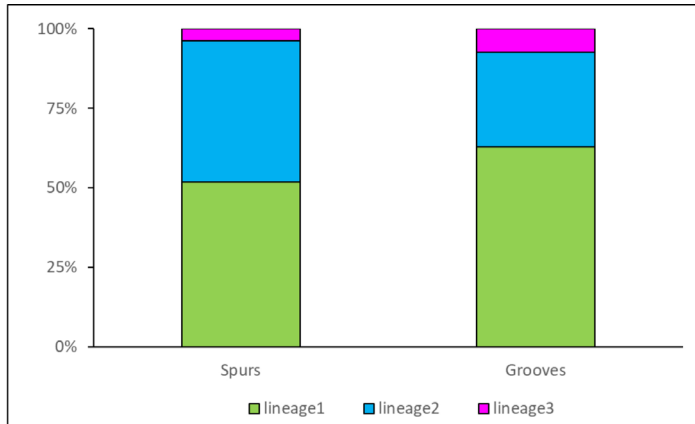


Figure 5: *Cladocopium psbA^{ncr}* lineage composition of spurs (n = 27) and grooves (n = 27). Green = lineage 1; cyan = lineage 2; purple = lineage 3.

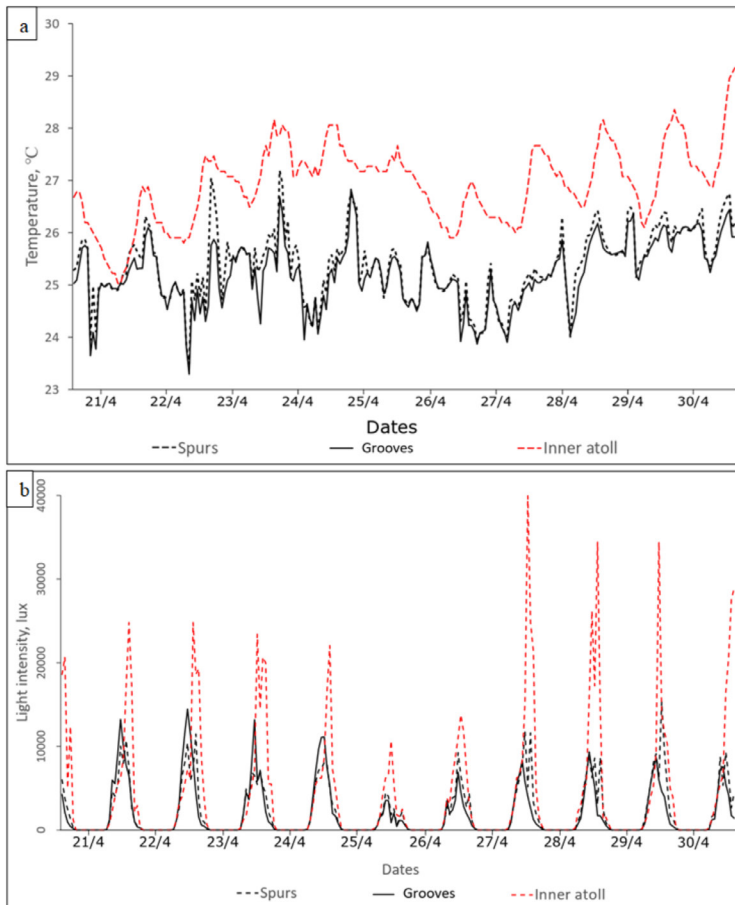


Figure 6: (a) Temperature (°C) and (b) light intensity (Lux) fluctuations at the inner atoll, spurs and grooves of outer Dongsha Atoll from April 20 to 30, 2018. The red dotted line represents the inner atoll, black dotted line at the spurs and the black solid line is the grooves.

Discussion

The *Durusdinium* symbiont was recorded only once at the inner atoll, while *Cladocopium* was predominant throughout the outer grooves and inner atoll. However, no psbA^{ncr} sequences were obtained from the *Cladocopium* specimens in the inner atoll, as amplification of the psbA region proved to be difficult, and thus, we could not speculate on the specific lineage present in these specimens. *Durusdinium* is generally thought to be a relatively thermotolerant genus of Symbiodiniaceae (Bongaerts et al., 2013; Tonk et al., 2013; Yuyama et al., 2016; Silverstein et al., 2017). This was reflected by the seawater temperature recorded at its habitat, which was significantly higher at the inner atoll. Previous records have also shown that the inner atoll is more turbid and warmer than the outer atoll (Chou, 2013). In our study, despite the higher turbidity, the light intensity in the inner atoll was significantly higher than the outer atoll due to the shallow locations where *P. tuberculosa* colonies were sampled (< 2m). Hence, *P. tuberculosa* colonies at the warmer inner atoll might host *Durusdinium* to improve the thermotolerance of the holobiont (Stat et al., 2013). However, due to the limited number of specimens collected from the inner atoll, our understanding of the Symbiodiniaceae in the inner atoll and the conclusions that could be drawn in this study were limited.

At the outer atoll, *Cladocopium* psbA^{ncr} lineages identified were reported in the same host (*P. tuberculosa*) from the Ryukyu Islands (Noda et al., 2017; Wee et al., 2019). Lineage 1 was known as a generalist lineage, while lineage 2 had been observed to be common in low salinity areas around Okinawa main island (Noda et al., 2017; Wee et al., 2019). However, due to low numbers of observations, little was known about lineage 3 (Noda et al., 2017). There was no significant difference in Symbiodiniaceae and *Cladocopium* psbA^{ncr} lineages hosted by *P. tuberculosa* at the spurs and grooves, in contrast with what we initially hypothesized.

At the outer atoll, temperature results showed the spurs were significantly warmer than

the grooves. However, the average differences between the two areas were relatively small (0.153°C). Furthermore, the average daily water fluctuations at both spurs and grooves were less than 4°C, approximately half of what was previously reported at the northwest outer atoll (Wang et al., 2007). On the other hand, there were no significant differences in light intensity between the spurs and grooves. Hence, holobionts at the spurs and grooves appeared to dwell in rather similar environments. Nevertheless, the present dataset covered a relatively short period (10 days), and longer term (e.g. one year) data would be needed for more robust comparison.

Conclusion

P. tuberculosa found at the outer atoll hosted only Symbiodiniaceae from genus *Cladocopium*, with three lineages. However, there was no significant distinction in *Cladocopium* lineage composition between the spurs and grooves. Seawater temperature fluctuations were observed, predominantly due to the upwelling of cold water by internal waves (Wang et al., 2007; Chen et al., 2010). Temperatures between the spurs and grooves were similar in range, but the spurs were slightly but significantly warmer than the grooves, as previously reported (Wang et al., 2007). Our results showed that *P. tuberculosa* was flexible with symbiodinian lineages at the spurs and grooves around Dongsha Atoll, although we observed no conclusive evidence that the internal waves affected the diversity of the host-symbiont relationship in these environments. On the other hand, this study recorded one occurrence of thermo-tolerant Symbiodiniaceae *Durusdinium*-hosting *P. tuberculosa* in the significantly warmer inner area of Dongsha Atoll (Chou, 2013).

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