Variation in *Symbiodinium* ITS2 Sequence Assemblages among Coral Colonies

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Abstract

Endosymbiotic dinoflagellates in the genus *Symbiodinium* are fundamentally important to the biology of scleractinian corals, as well as to a variety of other marine organisms. The genus *Symbiodinium* is genetically and functionally diverse and the taxonomic nature of the union between *Symbiodinium* and corals is implicated as a key trait determining the environmental tolerance of the symbiosis. Surprisingly, the question of how *Symbiodinium* diversity partitions within a species across spatial scales of meters to kilometers has received little attention, but is important to understanding the intrinsic biological scope of a given coral population and adaptations to the local environment. Here we address this gap by describing the *Symbiodinium* ITS2 sequence assemblages recovered from colonies of the reef building coral *Montipora capitata* sampled across Kane‘ohe Bay, Hawai‘i. A total of 52 corals were sampled in a nested design of Coral Colony(Site(Region)) reflecting spatial scales of meters to kilometers. A diversity of *Symbiodinium* ITS2 sequences was recovered with the majority of variance partitioning at the level of the Coral Colony. To confirm this result, the *Symbiodinium* ITS2 sequence diversity in six *M. capitata* colonies were analyzed in much greater depth with 35 to 55 clones per colony. The ITS2 sequences and quantitative composition recovered from these colonies varied significantly, indicating that each coral hosted a different assemblage of *Symbiodinium*. The diversity of *Symbiodinium* ITS2 sequence assemblages retrieved from individual colonies of *M. capitata* here highlights the problems inherent in interpreting multi-copy and intra-genomically variable molecular markers, and serves as a context for discussing the utility and biological relevance of assigning species names based on *Symbiodinium* ITS2 genotyping.

Introduction

Coral reefs are biologically diverse ecosystems providing habitat for a wide range of marine organisms. The growth of corals and their ability to form the calcium carbonate substrates reflect their endosymbioses with photosynthetic dinoflagellates belonging to the genus *Symbiodinium* [1]. Nine divergent lineages, clades A–I, have been described in *Symbiodinium* based on nuclear ribosomal DNA (rDNA) and chloroplast 23S rDNA [2] with each clade containing multiple genetic varieties often resolved using the internal transcribed spacer (ITS) regions [e.g. 3–6].

*Symbiodinium* diversity is partitioned by a variety of factors including biogeographical barriers, host species, colony depth, irradiance, and host symbiont transmission strategy [7–10]. Biogeographic patterns in *Symbiodinium* are evident between reefs in different oceans (Pacific versus Atlantic) [9], among reefs within an ocean (e.g. Pacific reefs in Japan and the Great Barrier Reef Australia) [11,12], and from reefs across a latitudinal gradient (e.g. eastern Australia coastline) [12,13]. The same coral species from inshore and offshore reefs within the same reef complex (e.g. in the central Great Barrier Reef or in Panama) can also associate with different *Symbiodinium* [12,14], as can colonies of the same species from the same reef environment [5,10,14,15]. Fidelity in the association between some coral species and *Symbiodinium* has lead to a degree of co-evolution resulting in host-symbiont specificity [9,16]. For example, the ITS2 *Symbiodinium* genotype C42 associates with *Pocillopora* and C31 with *Montipora* [9]. Attributed to levels of irradiation, *Symbiodinium* in corals such as *Montastraea* spp. and *Amphiprion pharaoensis* in Panama [9,17] and *Pocillopora damicornis* in the Great Barrier Reef [18] partition as a function of depth and/or location on individual colonies [8]. Host symbiont acquisition strategy also affects *Symbiodinium* assemblages with hosts that acquire their symbionts from the environment (horizontal symbiont acquisition strategy) primarily associating with a similar pool of symbionts, and those that acquire their symbionts from the parent colony (vertical symbiont acquisition strategy) harboring their own unique suite of symbionts specific to a host genus [9,10]. Understanding the factors that affect distribution and specificity patterns in coral-dinoflagellate symbioses and the physiological

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range of host-symbiont combinations is important for understanding how corals will respond to environmental change. In this regard, functional variability in isolated Symbiodinium types and specific coral-Symbiodinium symbioses have been correlated with numerous factors. Variation in the photophysiology of Symbiodinium [17,19,20], growth rate of coral colonies [21], symbiont carbon fixation and translocation to the host [22,23], symbiont thermal tolerance [24], and host disease susceptibility [22] all provide evidence for range thresholds in physiological performance of different host-symbiont assemblages as a response to the environment. As coral bleaching and disease are predicted to impact coral reef ecosystems in the future and have recently increased in severity and occurrence [25,26], the different host-symbiont combinations that can occur and the environmental tolerances of those symbioses will provide the framework for predicting future shifts in coral reef communities.

The number of unique Symbiodinium that reside in individual coral hosts is an area of ongoing debate [27,28]. Heterogeneous mixtures of Symbiodinium have been identified in a variety of host species e.g. [7,8,15,18], and more sensitive molecular techniques such as quantitative real time PCR have enabled the detection of Symbiodinium clades in low abundance [29–31]. However, the number of Symbiodinium species and their occurrence among marine hosts remains a central issue that is highly relevant to our understanding of the capacity of coral-algal symbiosis and reef ecosystems to adapt with changes in the environment [32]. The nuclear internal transcribed spacer region 2 (ITS2) is currently most often utilized to resolve Symbiodinium diversity within the phylogenetic clades A–I e.g. [2,5,12,16,18,33], and is being promoted as a species level marker [9,30,34]. However, the multi-copy nature and intra-genomic variability of the ITS2 [35,36] often results in the isolation of more than one ITS2 sequence type from an individual Symbiodinium cell, and this interpretational complexity combined with low genetic divergence among ITS2 sequences [e.g. 9] makes the application of this marker in species assignment problematic [16,37].

In order to further investigate the partitioning of Symbiodinium in corals and the utility of the ITS2 marker in describing Symbiodinium diversity, we set out to investigate the Symbiodinium communities in colonies of Montipora capitata at similar depths over a spatial scale of meters to kilometers in Kāne‘ohe Bay, O‘ahu Island, Hawai‘i. As M. capitata exhibits vertical transmission of its symbionts, we also set out to examine whether patterns of Symbiodinium ITS2 diversity map onto the M. capitata atpβ and nad5 genotypes. The data reveal that Symbiodinium ITS2 diversity is different among colonies of M. capitata and does not reflect host genotype. These data highlight both the complexity of the Symbiodinium ITS2 sequence diversity in corals, and are used as a framework to discuss the problems inherent in using this marker to designate species in the genus Symbiodinium.

**Methods**

**Ethics Statement**

This study was conducted under the research guidelines of the University of Hawaii Executive Policy E5.211 and corals collected under the State of Hawaii Special Activity Permit number 2007-02 issued to the Hawaii Institute of Marine Biology.

**Sample collection and sites**

The sampling for this study was conducted in June 2007 in Kāne‘ohe Bay, on the island of O‘ahu. 52 colonies of Montipora capitata (brown branching morph) were sampled from one location at the same relative position on each colony (upper region) using a hammer and chisel at a depth of 1–2 m from three sites nested in three regions of the bay (sites 1–9; Figure 1) that lie on a northerly environmental gradient from nearshore to offshore. Region 1 was located near the Kāne‘ohe Stream mouth (Sites 1–3), Region 2 in the centre of the bay (Sites 4–6), and Region 3, near the outer barrier reef (Sites 7–9). Latitudinal and longitudinal coordinates for Sites 1–9 are 21.24.902N and 157.46.826W, 21.25.271N and 157.47.255W, 21.25.574N and 157.47.336W, 21.26.039N and 157.47.255W, 21.25.574N and 157.47.336W, 21.26.039N and 157.47.255W, 21.25.574N and 157.47.336W, 21.26.039N and 157.47.255W.

Figure 1. Location of corals sampled in study. Location of study in Hawai‘i (1a) and sites in Kāne‘ohe Bay, O‘ahu (1b). Six colonies of Montipora capitata were sampled at a depth of 1–2 m from each of the nine sites (except Site 9 where only 4 colonies were sampled). Region 1 is shaded in blue, region 2 in green, and region 3 in yellow.

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157.47.497W, 21.26.200N and 157.47.518W, 21.26.256 and 157.47.440W, 21.27.026N and 157.47.503W, 21.26.929N and 157.47.762W, 21.27.112N and 157.47.820W, respectively. Six *M. capitata* colonies were sampled from Sites 1–9. Two samples from Site 9 failed to amplify in PCR, reducing the sample number at that site to four.

**DNA extraction**

For extraction of nucleic acids, the coral fragments (~5 mm² of tissue from verrucae and surrounding corallites including entire polyps) were removed from each colony and stored at -4°C in 400 µl of DNA extraction buffer [50% (v/v) guanidium isothiocyanate; 50 mM Tris pH 7.6; 10 mM EDTA; 4.2% (w/v) Sarkosyl; 2.1% (v/v) β-mercaptoethanol] at the time of collection, until processed (up to 2 weeks). The coral samples in DNA extraction buffer were then incubated at 72°C for 10 min and centrifuged at 16,000 g for 5 min. The supernatant was mixed with an equal volume of 100% isopropanol to precipitate the DNA and chilled at -20°C overnight. The precipitated DNA was pelleted by centrifugation at 16,000 g for 15 min, and washed in 70% ethanol before resuspension and storage in Tris Buffer (0.1 M pH 8).

**PCR, cloning, and sequencing of Symbiodinium**

The *Symbiodinium* partial 5.8S, ITS2, and partial 28S region was amplified in PCR using the forward 5’-GTGAATTTGCG-GAACCTCGTG 3’ and reverse 3’-TTATATGCTT 3’ primers [38]. The products of these amplifications are identified to be from here as *Symbiodinium* ITS2 sequences. Each 25 µl PCR reaction contained 1 µl of DNA template, 2.5 µl of 10x ImmoBuffer, 0.1 µl IMMOBASE™ Hot-Start DNA Polymerase (Bioline, MA), 3 mM MgCl₂, 0.5 µl of 10 mM total dNTPs (2.5 mM each), 5 pmol each primer, and deionized sterile water to volume. PCR was performed on a BioRad iCycler™ using the following conditions: 95°C for 7 min, followed by 35 cycles of 45 s at 95°C, 45 s at 52°C, and 45 s at 72°C, with a final extension at 72°C for 5 min. PCR amplicons were purified using the QIAquick® PCR Purification Kit (Qiagen, CA), ligated into the pGEM®-T Easy vector (Promega, WI), transformed into a-select gold efficiency competent cells (Bioline, MA), and grown overnight on selective LB media (ampicillin 50 µg/ml, 0.1 mM IPTG, 50 µg/ml X-gal). Positive clones were grown overnight in Circlecrog® (MP Biomedicals, CA) and plasmids purified using the Perfectprep® Plasmid Isolation Kit (Eppendorf, Hamburg). Clones from PCR products (3 clones from 1 coral colony, 5 clones from each of 36 coral colonies, 6 from each of 15 coral colonies, and 7 from each of 2 coral colonies) were sequenced in both directions using BigDye Terminators (PerkinElmer, MA) on an ABI-3100 automated sequencer at the University of Hawai’i. Additional clones were sequenced from two colonies sampled from each region (six colonies in total, 35–55 clones per colony). Sequences were inspected, aligned, and edited using MacVector® 8.0.2 software. *Symbiodinium* ITS2 sequences used for downstream analyses were edited as described in Stat et al. [16]. For all analyses, *Symbiodinium* ITS2 was categorized by clade (C or D) [34], ITS2 secondary structure (folding), and ITS2 sequence. The secondary structure of all ITS2 sequences were estimated using 4SALi and the ITS2 database website [39–42] using published *Symbiodinium* ITS2 structures as templates [16,36,43].

**PCR and sequencing of Montipora capitata genes**

To determine whether *Symbiodinium* ITS2 composition is a factor of host lineage, the host *Montipora capitata* colonies were genotyped using both the mitochondrial NADH dehydrogenase 3’ intron (nad5) and the nuclear ATP synthase subunit beta intron (atpsβ). *M. capitata* nad5 was amplified with primer pair ND515a/ND5_700E: 5’ YTCGCGGATGCGATGGAG 3’ and ND5_157R 5’ GGCGGCAGG-GCGCCGGGGGTGCCTGCTG 3’ as outlined in Concepcion et al. [44], and atpsβ was amplified with a primer pair redesigned from Garman et al. [45] to be specific for *M. capitata* (F: 5’ TGATT-GTGTCGTAGTTATGCTT 3’ and R: 5’ CCGGCGACCGG-CGCCCCGGGATGTAGTTATGCTT 3’). [46]. For both markers, each 25 µl PCR contained 1 µl of DNA template, 2.5 µl of 10x ImmoBuffer, 0.1 µl IMMOBASE™ Hot-Start DNA Polymerase (Bioline Inc.), 3 mM MgCl₂, 0.5 µl of 10 mM total dNTPs (2.5 mM each), 13 pmol each primer, and deionized sterile water to volume. PCR amplification was performed on a BioRad iCycler™ as follows: 95°C for 7 min, followed by 35 cycles at 95°C for 30 s, 53°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. All successfully amplified PCR products were “cleaned” with 0.75 units of Exonuclease I: 0.5 units of Shrimp Alkaline Phosphatase (Exo:SAP) per 7.5 µl PCR product at 37°C for 60 min, followed by deactivation at 80°C for 10 min prior to being cycle-seqenced in both directions using Big Dye Terminators (Applied Biosystems) and run on an ABI-3130XL automated DNA sequencer. *atpsβ* alignments were confirmed by eye and trimmed to 252 bp. Since computational phasing of diploid nuclear loci can be more accurate than cloning in separating alleles from heterozygous individuals [47], gametic phases for *atpsβ* were inferred using PHASE [48,49] as implemented in DNAsP [50].

**Statistical parsimony networks**

Statistical parsimony networks of *Symbiodinium* ITS2 sequences were constructed using the software TCS 1.21 [51]. The cladogram estimation was performed under a 95% connection limit and gaps were treated as a 5th state with the alignment edited so that each indel was considered a single mutation.

**Analysis of spatial partitioning in Symbiodinium and Montipora**

We set out to determine the spatial scale(s) at which *Montipora capitata* and *Symbiodinium* composition partition across Kāne‘ohe Bay: meters (Coral Colony), 10’s of meters (Site), and 100’s to 1000’s of meters (Region). Due to the sampling design, Sites are nested within Regions, denoted as Site(R), and *M. capitata* colonies are nested within Sites, denoted as Colony(S,R). We used the PERMANOVA+1.0.2 software add-on for PRIMER 6 [52] to run three-level hierarchical analyses of molecular variance (AMOVA) [53] to test for spatial structuring. PERMANOVA+ was run using Type I sums of squares, unrestricted permutation of raw data, and significance was determined by permutation test (10,000 permutations) of the pseudo-F statistic. Post hoc pairwise comparisons were conducted among Regions, Sites, and Colonies using an alpha of 0.05 while controlling the family-wise false discovery rate at or below 0.05 [54]. Φ statistics (analogous to Wright’s [55] F-statistics) were calculated from the PERMANOVA+ output following Excoffier et al. [53]. Φ ranges from 0 to 1, where 0 indicates that genetic composition among samples is identical and 1 indicates that at least one sample is completely differentiated and fixed for a single unique genetic sequence or type. We used PERMANOVA+ because the standard AMOVA software, ARLEQUIN 3.1 [56], cannot run analyses on data sets with more than two hierarchical spatial levels with non-diploid data. PERMANOVA+ was not developed with AMOVA in mind, consequently, some calculations were required prior to running the following analysis. Prior to analysis, the AMOVA matrices of genetic distance were generated in ARLEQUIN 3.1, the square root of each distance was taken, and the matrices were imported to
PERMANOVA+ For Symbiodinium ITS2 and M. capitata atpββ sequences, the simple pairwise genetic distance was used. For Symbiodinium ITS2 secondary structure, the average simple pairwise genetic distance among sequences coding for each folding group was used. For Symbiodinium ITS2 clades, because sequence divergence has no impact on the analysis of two categories (clade C or D), the only possible distances were zero or one.

AMOVA uses certain statistical terms and notations that carry accepted biological meanings based on loci with either two biparentally inherited alleles or one maternally inherited haplotype per individual. Symbiodinium ITS2 is a multi-copy intra-genomically variable marker and we are drawing sequences from multiple individuals of Symbiodinium, therefore we incorporate this assumption into our AMOVA analysis. We thereby negate any traditional biological inferences, such as the inbreeding coefficient \( F_{IS} \), that are calculated when each sequence represents a single haplotype or one of two alleles [55]. The lowest level of inference that can be made here for Symbiodinium is the variation in ITS2 sequences within Col- onies(S|R), denoted as \( \Phi_{CSR2} \). \( \Phi_{CSR2} \) carries biological meaning, just not that of \( F_{IS} \). In the interest of clarity, we similarly avoid other standard AMOVA notation laden with biological implications such as \( \Phi_{CT} \), \( \Phi_{SC} \), and \( \Phi_{ST} \) [53] in order to focus on the statistical inference of AMOVA in ITS2. If there is a significant difference in the ITS2 composition detected by the AMOVA, this implies that the Symbiodinium assemblages are partitioned, regardless of the actual number of individuals represented.

**Diversity Indices**

“True diversity”, \( D \), [57] was calculated using the Shannon and Weaver [58] diversity index, \( H' \), as follows,

\[
D = \exp(H')
\]

\[
H' = -\sum_{i=1}^{S} p_i \ln(p_i)
\]

where \( p \) is the proportion of ITS2 sequence \( i \) out of \( s \) sequences in the sample. True diversity represents the effective number of elements, which in this case is the effective number of ITS2 sequences [57]. Coverage estimates of clone libraries were calculated using the equation:

\[
C = \left( 1 - \left( \frac{n}{N} \right) \right) \times 100
\]

where \( n \) is the number of unique Symbiodinium ITS2 sequences and \( N \) is the total number of clones sequenced from the library [59]. Rarefaction analyses [60,61] were performed using Analytic Rarefaction v2 [62].

**Results**

**Symbiodinium identified in Montipora capitata from Kāne‘ohe Bay**

A total of 275 Symbiodinium ITS2 sequences belonging to clades C and D were recovered from the colonies of *M. capitata*. Seventeen different Symbiodinium ITS2 sequences were identified; 14 in clade C and 3 in clade D (Table 1). In addition to the previously published ITS2 sequences C3, C17, C17.2, C21, C31, D1, and D1a [5,16,33,34,63], nine novel clade C sequences and one novel clade D sequence were recovered (C3.14, C21.6, C21.11, C21.16, C31.1, C31.5, C31.6, C31.9, C31.10; and D1.6, accession numbers HQ630872-HQ630881). Statistical parsimony analysis resolved single networks for Symbiodinium ITS2 sequences in clade C and D (Figure 2). Conformational changes to the ITS2 secondary structures occur within stems I and II for sequences in clade C and in stem II for sequences in clade D (Figure 2, Figure S1). Five putative ITS2 folding structures were identified for sequences in clade C; Group A contains C3 and C5.14, Group B contains C17, C21, C21.6, C21.11, and C21.16, Group C contains C17.2, Group D contains C31.9 and C31.10, and Group E contains C31, C31.1, C31.5, C31.6 (Figure 2). Two folding structures were identified in clade D; Group F contains D1a, and Group G contains D1 and D1.6.

**Spatial structure and diversity of Symbiodinium in Kāne‘ohe Bay**

We set out to determine if there is any partitioning of Symbiodinium composition at the nested scales of Region, Site(R), and host Coral Colony(S | R) using AMOVA. In most analyses, data organized by clade, secondary structure group, or ITS2 sequence gave concordant results (Table 2), therefore we present the analyses of ITS2 sequences and note when differences occurred in secondary structure and clade analyses from here forward. Spatial partitioning of Symbiodinium ITS2 sequence composition was detected at the scales of Site(S) (\( P<0.01 \) and Colony(S | R) (\( P<0.01 \); Table 2). The greatest structuring in ITS2 composition occurred among Coastal Colonies(S | R) (\( \Phi_{CSR2} = 0.87 \)), as opposed to Sites(R) (\( \Phi_{CSR} = 0.27 \)). Because there was no spatial structure in ITS2 by Region, Regions were pooled for post hoc pairwise comparisons of ITS2 among all Sites and Colonies(S).

Zero of 36 pairwise comparisons among Sites and 42 of 126 comparisons among Colonies(S) indicated statistically significant differences in Symbiodinium ITS2 sequence composition when controlling the family-wise false discovery rate, but there was no apparent spatial pattern to these differences. Among pairwise comparisons of Colonies(S), grouping the sequences by clade resulted in the detection of fewer statistically significant differences (33 of 42) than when grouping by secondary structure (42 of 42).

As results from the hierarchical AMOVA indicate that the majority of the spatial structure in Symbiodinium ITS2 composition within *M. capitata* in Kāne‘ohe Bay occurs at the scale of Coral Colony, we sequenced additional clones from two colonies haphazardly selected from each Region (6 colonies with a total of 35–55 clones per colony) to further explore inter-colony Symbiodi- nium sequence diversity. Symbiodinium from clade C was recovered from four colonies, clade D from one colony, and clades C and D from one colony (Figure 3). The number of sequence types recovered from each colony varied from two in Colony 1 to nine in Colony 9. The “true diversity” of Symbiodinium ITS2 within each colony was also variable (Colony 1: \( D = 1.9 \); 9: \( D = 5 \); 25: \( D = 2.2 \); 31: \( D = 2.6 \), 44: \( D = 5.3 \); 49: \( D = 1.9 \)). AMOVA-based pairwise comparisons of ITS2 sequences in the six colonies indicate that the clone libraries from each colony are different from one another with the exception of those from Colonies 1 and 25 (Table 3). Despite the fact that all clones from Colonies 9, 31, 44, and 49 are from clade C, they represent unique non-random distributions of Symbiodinium ITS2 sequences. The coverage estimates indicated that the obtained sequences covered a high percentage of the diversity in each clone library (\( C = 94\% \), 83\%, 94\%, 94\%, 84\% and 95\% for Colonies 1, 9, 25, 31, 44, and 49 respectively), and are supported by rarefaction curves reaching an asymptote for libraries from four colonies (1, 25, 31, 49), and approaching an asymptote for the remaining two (9, 44; Figure 4). For Colonies 9 and 44, additional sequencing would have recovered minimally more diversity that would not have affected the result. Therefore, given that: 1) the hierarchical AMOVA indicated...
Table 1. *Symbiodinium* ITS2 sequences and *Montipora capitata* ATP synthetase subunit β genotypes for colonies sampled in Kane‘ohe Bay, Hawaii.

| Region | Site | Colony | *Symbiodinium* ITS2 sequence(s) | *Montipora capitata* genotype |
|--------|------|--------|-------------------------------|-----------------------------|
| 1      | 1    | 1*     | D1α, D1a                     | C                           |
| 1      | 2    | C21α, C31α, C21.16α | D                           |
| 3      | D1a  | M                                 |
| 4      | C31α, C17.2, C21.6 | E                           |
| 5      | C31α, C17.2, C21.1 | A                           |
| 6      | C31α, C17.2 | Q                           |
| 7      | C31α, C17.2 | H                           |
| 8      | D1α, D1α | A                      |
| 9*     | C21α, C21.11α | I                           |
| 10     | D1α, C17.6 | R                           |
| 11     | C17.2α, C21α | M                          |
| 12     | D1α, D1.6 | L                           |
| 13     | C31α, C21α, C17.7α, C21.11α | H                           |
| 14     | C21α, C17.7α, C21.6α | C                       |
| 15     | C17.2α, C31.5 | E                           |
| 16     | C31α, C17.2, C31.5 | H                           |
| 17     | C17.2α, C21.5, C31.14α, C31α | A                       |
| 18     | C31α, C17.2 | V                           |
| 2      | 4    | 19     | C17.2α, C31.5, C31.10 | H                           |
| 20     | C17.2α, C31.5, C21.11α | J                           |
| 21     | C31α, C31α, C17.2α, C17.2α | F                           |
| 22     | C21α, C17.2α | P                           |
| 23     | C31α, C17.2α, C21.16α | A                           |
| 24     | C31α, C21α | H                           |
| 5      | 25*  | D1α, D1α | P                           |
| 26     | D1α, D1α | T                           |
| 27     | C31α | N                           |
| 28     | D1α, C21α, D1α | H                           |
| 29     | D1α, D1α | G                           |
| 30     | C21α, C21.6α, C17.2α | J                           |
| 6      | 31*  | C21.11α, C21α | H                           |
| 32     | C31α, C21α | H                           |
| 33     | C17.2α, C17.2 | S                           |
| 34     | C17.2α, C31α, C17.2α, C31.10α | N                           |
| 35     | C31α, C17.2α | M                           |
| 36     | C21α, C31α, C17.2α | J                           |
| 3      | 7    | 37     | C17.2α, C31.10α, D1α | A                           |
| 38     | C31α | A                           |
| 39     | C31α, C21α, C31.1α | F                           |
| 40     | C21α, C17.2α, C31α | B                           |
| 41     | C31α, C21.11α, C31.11α | O                           |
| 42     | C31α | U                           |
| 8      | 43*  | C21α, C17.2α, C21.6α, C31.1α, C31.6 | K                           |
| 44*    | C31α, C31α, C21α, C21.11α | I                           |
| 45     | C21α, C31α, C17.2 | C                           |
| 46     | C21α, C31α, C21α | K                           |

Table 1. Cont.

| Region | Site | Colony | *Symbiodinium* ITS2 sequence(s) | *Montipora capitata* genotype |
|--------|------|--------|-------------------------------|-----------------------------|
|        |      | 47     | D1α4, D1α3 | H                           |
|        |      | 48     | C31α1, C21.11α, C31.1α | W                           |
| 4      | 49*  | C31α1, C17.2α | M                           |
|        |      | 50     | C31α1, C31.5α, C31.9α | A                           |
|        |      | 51     | D1α2, D1αα, C31α | A                           |
|        |      | 52     | C21α1, C31α | I                           |

* denotes corals where 35–55 *Symbiodinium* ITS2 sequences were recovered. Only the first 5 sequences identified from these colonies are presented in the table. Superscript numerals indicate the frequency of that sequence in the colony. doi:10.1371/journal.pone.0015854.t001

Coral Colony as the level at which most variation in *Symbiodinium* ITS2 sequence composition occurs, and 2) pairwise comparisons of the six colonies with increased clone sampling indicates variation in ITS2 composition between colonies, we conclude that the *Symbiodinium* assemblage in *Montipora capitata* from Kaneohe Bay is mostly partitioned at the level of Coral Colony.

The *Symbiodinium* ITS2 composition in *Montipora capitata* in Kane‘ohe Bay from all colonies (3–7 clones from 52 colonies) compared to the six colonies with additional clones (35–55 clones from 6 colonies) was assessed to determine whether a similar sequence diversity (not distribution) could be recovered using these two approaches. Of the 17 *Symbiodinium* ITS2 sequences identified in *M. capitata* from Kane‘ohe Bay, 13 were recovered from the six colonies with increased clone sequencing (Figure 5). The four that were not identified (C21.6, C21.16, C31.6, and D1.6) represent rare or low frequency in the grouped sequences. The true diversity of *Symbiodinium* ITS2 sequences was the same for all colonies sampled in the Bay grouped and the six colonies grouped (D = 7.2). A high coverage of sequences from the clone libraries pooled for the two groupings was achieved (C = 93% and 95% for all colonies and six colonies respectively) and is further supported by rarefaction analyses (Figure 6). There was also no significant difference in the *Symbiodinium* ITS2 sequence composition between the groups using AMOVA (Φ = -0.07, P = 0.487). These data suggest that the total *Symbiodinium* sequence diversity (not distribution) present in shallow water *M. capitata* in Kane‘ohe Bay can be recovered with either sequencing a few clones from many coral colonies or by sequencing a large number of clones from a few coral colonies.

Spatial Structure of *Montipora capitata* in Kane‘ohe Bay

All corals sampled in this study had the same host haplotype, which was identical to accession HQ630861-HQ630871 of *Montipora capitata* from NCBI [44]. Because there was no sequence variation among samples, this marker is not discussed any further.

Four polymorphic sites with no indels in the region aligned for atpB accounted for 11 unique alleles (Genbank accession numbers HQ630861-HQ630871) and 23 unique single-locus genotypes among our coral host samples (host genotype A–W, Table 1). We set out to determine if there is any partitioning of *Montipora capitata* atpB composition at the nested scales of Region, Site(R), and Colony(S(R)) using AMOVA. As we expected, there was no partitioning of *M. capitata* by Region (Φ_C17 = 0.01, P = 0.34) or Site(Region) (Φ_SC = 0.04, P = 0.21). There was, however, a significant difference among Colonies(S(R)) (Φ_IS = 0.46, P<0.001).
We tested whether *Symbiodinium* composition is related to the host coral genotype using AMOVA based on host genotypes represented in more than one colony (11 genotypes, 40 colonies). There is no indication that *Symbiodinium* ITS2 sequence composition is related to *M. capitata*’s *atpB* genotype \((W = 20.14, P = 0.91)\).

**Discussion**

**Spatial partitioning of Symbiodinium in Montipora capitata across Kane’ohe Bay**

The absence of Symbiodinium community structure in *Montipora capitata* among Regions in Kane’ohe Bay contrasts with the partitioning of *Symbiodinium* in corals between oceans, reefs at

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**Figure 2. Symbiodinium ITS2 statistical parsimony networks for clade C and D inferred from sequences recovered from colonies of Montipora capitata sampled across Kane’ohe Bay, Hawaii.** Open boxes indicate a single mutational step. Letters a – g indicate ITS2 secondary structures and dashed lines on networks separate sequences grouped by folds.

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different latitudes, inner and outer lagoonal environments, and on a single reef as a function of depth [e.g. 5,7,12,13,14,17,18]. Differences between sites within Kane‘ohe Bay in the Symbiodinium community of M. capitata were evident primarily as a dominance of either clade C or D (colonies at Sites 2 and 5 contained more clade D than other sites). Garren et al. [14] reported that an increase in clade D Symbiodinium abundance in the Montastraea annularis species complex on Panamanian reefs was attributed to increased levels of suspended solids present in inner lagoonal environments relative to the outer lagoonal environment where clade C was dominant. Some symbionts in clade D Symbiodinium appear to be associated with corals that are exposed to “stressful” environmental conditions (e.g. elevated sea surface temperature and increased sedimentation) [64,65]. Similarly here, Site 2 is close to the outlet of the Kane‘ohe Bay Stream and has low salinity (Palmer et al. unpubl. data), which may represent a stressful environment for corals at this site. However, Symbiodinium clade D was also more abundant than other clades at site 5, which is situated approximately 3 km from the stream outlet where there is no indication of environmental stressors (temperature, salinity, sedimentation) that are harmful to corals (Palmer et al. unpubl. data). Even though the presence of Symbiodinium clade D is mostly attributed to factors causing a more stressful environment, its occurrence may not be strictly correlated with such factors as has been shown over regional scales with temperature anomalies [66]. Also, the scale at which Symbiodinium diversity is recorded and the spatial scale at which environmental factors are measured may influence results investigating correlations between clade D Symbiodinium and stressful environments.

Table 2. Differences in Symbiodinium diversities among Colony(Site(Region)), categorized by clade, secondary structure, and ITS2 sequence, analyzed by AMOVA.

|                | df | Clade |                  |                  | ITS2 Secondary Structure |                  | ITS2 Sequence |                  |
|----------------|----|-------|------------------|------------------|--------------------------|------------------|---------------|------------------|
|                |    | P     |                  |                  | Phi                       |                  | P             |                  |
| Region         | 2  | -0.134| 0.905            | -0.126           | 0.925                     | -0.024           | 0.905         |
| Site(Region)   | 6  | 0.267*| 0.010*           | 0.268*           | 0.009*                    | 0.271*           | 0.009*        |
| Colony((Site)Region) | 43 | 0.918*| 0.000*           | 0.855*           | 0.000*                    | 0.870*           | 0.000*        |

Significant values (P < 0.05) are indicated with an asterisk.

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Figure 3. Symbiodinium identified in Montipora capitata colonies from Kane‘ohe Bay, Hawai‘i. The Symbiodinium clades identified per region are displayed as pie charts in 3a. The frequency of Symbiodinium ITS2 sequences per region is displayed as bar graphs in 3b. The total frequency of ITS2 sequences per region is calculated from 3–7 clone sequences from each colony of M. capitata sampled in that region. The frequency of Symbiodinium ITS2 sequences for six colonies of M. capitata in which 35–55 clones were analyzed is displayed as bar graphs in 3c. Boxed numerals indicate groupings of colonies with significantly different Symbiodinium ITS2 composition.

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Spatial partitioning of *Symbiodinium* diversity in *M. capitata* across Kāne‘ohe Bay was most evident at the level of Colony(S/R). It is noteworthy that here, one coral sample was collected from a uniform location on each coral colony to allow for comparison of *Symbiodinium* assemblages among coral colonies. This strategy was adopted to minimize the sampling impact on the 52 coral colonies and to make the analytical work feasible in terms of cost and effort. However, it is possible that samples taken from multiple locations on the same coral colony might resolve spatial heterogeneity of *Symbiodinium* assemblage in *Montipora capitata* colonies, as has been demonstrated in *Montastraea* spp. from the Caribbean [7,67].

Although very few studies examining *Symbiodinium* diversity in corals consider this issue, the complexity of *Symbiodinium* ITS2 assemblages resolved here suggest that it would be a valuable subject to examine in future studies. That said, inter-colony variation in *Symbiodinium* within the same host species has been observed over broad geographic scales (e.g. different latitudes and oceans) [9,11], and as a function of depth on the same reef [e.g. 17,18]. Similarly, variation in *Symbiodinium* within the same host species within the same reef environment has been shown for a few host species [e.g. 15]. However, it has previously been reported that shallow water *M. capitata* (brown morph) around O‘ahu engaged in a highly specific symbiosis with *Symbiodinium* ITS2 C31 [33]. Similarly here, ITS2 C31 was recovered from *M. capitata* colonies with the highest frequency across all Regions (Figure 3b) confirming the prevalence of *Symbiodinium* containing this ITS2 sequence. An unexpectedly high diversity of other *Symbiodinium* ITS2 sequences were also retrieved from *M. capitata* (brown morph) here, including C3, C17, C21, D1, and D1a, with some colonies containing four sub-clade C ITS2 sequences. It is important to note that these ITS2 sequences have previously been described as representing ecologically dominant endosymbionts of corals (i.e. they occupy a distinct ecological niche, either specificity to a host species or biogeographic region and hence interpreted as different species) based on fingerprint profiles of amplified *Symbiodinium* ITS2 using denaturing gradient gel electrophoresis (DGGE) from colonies sampled in nature [5,9,34,63]. This high number of potential endosymbiont “species” within individual coral colonies previously reported to contain a single specific endosymbiont “species” highlights the fact

### Table 3. Φ-values for AMOVA pairwise comparisons of *Symbiodinium* ITS2 sequences among six colonies of *Montipora capitata*.

| Colony  | Colony 9 | Colony 25 | Colony 31 | Colony 44 |
|---------|----------|-----------|-----------|-----------|
| Colony 9 | 0.982*   |           |           |           |
| Colony 25 | 0.041    | 0.904*    |           |           |
| Colony 31 | 0.988*   | 0.085*    | 0.991*    |           |
| Colony 44 | 0.978*   | 0.060*    | 0.901*    | 0.214*    |
| Colony 49 | 0.996*   | 0.550*    | 0.920*    | 0.704*    | 0.349*    |

Statistically significant values (α = 0.05) are indicated with an asterisk. doi:10.1371/journal.pone.0015854.t003

Figure 4. Rarefaction curves of *Symbiodinium* ITS2 sequences recovered from colonies of *Montipora capitata*. Numerals correspond to colony number from Table 1 and Figure 3. doi:10.1371/journal.pone.0015854.g004
Figure 5. Bar graphs of *Symbiodinium* ITS2 sequences pooled from all colonies of *Montipora capitata* sampled across Kane‘ohe Bay (3–7 clones per colony), Hawai‘i, and from six colonies of *M. capitata* in which 35–55 clones were analyzed. doi:10.1371/journal.pone.0015854.g005
that additional sampling, and/or the application of different analytical methods significantly influences the interpretation of the taxonomic nature and composition of Symbiodinium diversity in individual coral colonies and species. In this context, a greater understanding of the spatial scale at which Symbiodinium ITS2 sequences vary (among and within colonies, and among polyps from the same colony), and the extent of intra-genomic variation in individual Symbiodinium cells is needed.

The forces driving differences in Symbiodinium assemblages among the M. capitata colonies described here are unknown, but likely reflect some combination of host-symbiont specificity, environmental, and stochastic processes [68]. Although no evidence of specificity between Symbiodinium ITS2 and host mitochondrial NADH dehydrogenase 5' intron (nad5) and nuclear ATP synthase subunit beta intron (atpsb) genotypes was detected, it is possible that alternate host (or Symbiodinium) markers with different taxonomic resolution might reveal a correlation between host genotype and their endosymbiotic communities.

Interpreting Symbiodinium diversity using ITS2

Identifying heterogeneous Symbiodinium communities is relatively easy at the cladal level because the high level of genetic variation that exists between lineages allows their presence (in high or low abundance) to be determined using sensitive molecular techniques such as Quantitative Real Time PCR [e.g. 29–31]. However, defining the number of sub-clade Symbiodinium present in heterogeneous endosymbiotic communities using a marker like ITS2 is not as straightforward. ITS2 is a multi-copy marker that is intragenomically variable within Symbiodinium [35,36]. In an attempt to overcome these issues, the dominance of an ITS2 sequence amplified in PCR and the accompanying DGGE fingerprint is currently being used to describe the Symbiodinium type present in a sample and delineate species within the genus [e.g. 5,9,12,30,33,63,69]. This methodology and interpretation emphasizes dominance of a sequence in a sample and disregards low abundant sequences (<5–10% in abundance) as intra-genomic variants that are not important [18,29,70]. However, in addition to the dominant sequence type C31, many of the M. capitata colonies in this study associated with multiple Symbiodinium ITS2 sequences that have previously been described as ecologically dominant and representative of independent biological entities (i.e. species). The most extreme examples of this are M. capitata colonies 9 and 44 (Table 1, Figure 3) harboring Symbiodinium ITS2 C3, C17, C21, C31, and other novel types, that collectively encompass almost all of the secondary structures in ITS2 recovered here. As the statistical parsimony network of clade C Symbiodinium depicts a step-wise evolution from the ancestral clade C sequence, ITS2 C3 [9], to the most derived, C31, and as the rDNA is multicopy and is variable in a Symbiodinium genome [35,36], there are three possible biological interpretations of the sequence diversity recovered here that lie at the extremes and at some point along the continuum from intra-genomic to inter-genomic diversity. The first is that every sequence recovered represents an individual Symbiodinium cell type or species (i.e. the highest Symbiodinium diversity possible). The second is that the corals contain a single Symbiodinium cell type or one species that contains intra-genomic variants encompassing all the sequence diversity recovered (C3 to C31; i.e. the lowest Symbiodinium diversity possible). The third, and in our opinion the most likely, is some
combination of possibilities 1 and 2. With the data in hand, it is impossible to distinguish which of these scenarios explains the Symbiodinium sequence diversity in M. capitata reported here. We can say, however, that because the Symbiodinium ITS2 sequence composition among colonies is variable, the Symbiodinium communities in these corals are different. The problems of interpreting exactly what the endosymbiotic ITS2 sequence data from an individual coral means in terms of species diversity are well illustrated when considering the recently nominated species Symbiodinium trenchi and Symbiodinium glynni [30,69]. The species Symbiodinium trenchi is identified using the ITS2 D1a DGGE fingerprint, however, this fingerprint always contains a band that corresponds to the D1 sequence. The D1 sequence can occur independently of D1a, and when D1a is absent, the D1 DGGE fingerprint is used to define the species Symbiodinium glynni. A study by Thornhill et al. [36], however, clearly demonstrates that the D1 and D1a sequences are intra-genomic variants in an isolocal cell line. Therefore, when the D1a ITS2 DGGE fingerprint (with its companion D1 sequence) is detected in an endosymbiotic sample, it is impossible to distinguish whether these sequences represent intra-genomic variants of one cell type, or co-occurring populations of two Symbiodinium species, S. trenchi and S. glynni. Thus, the use of ITS2 sequences that are known to be intra-genomic variants to delineate different species is problematic when assessing the diversity of species in endosymbiotic Symbiodinium communities in corals.

That said, defining cryptic Symbiodinium types and their prevalence is fundamentally important when considering endosymbiont shifting/shuffling in corals as a response to changes in the environment [32,64,71]. One solution to the problems encountered in interpreting ITS2 diversity in environmental samples (ie. host organisms) of Symbiodinium is to develop and apply a new marker(s) that has a similar level of resolution to the ITS2, but that exhibits a one to one relationship between sequence type and an individual Symbiodinium cell. In our opinion, the power of applying DGGE of Symbiodinium ITS2 to coral endosymbionts lies in comparing fingerprint patterns among samples to determine whether or not the signatures are the same or different, an approach widely used in the field of microbial ecology. However, the properties of ITS2 as a marker clearly make it a suboptimal choice for species assignment in Symbiodinium.

Endemicty and distribution ranges of Symbiodinium types have mostly been inferred using the ITS2 in studies generally constituting 1–2 colonies per host species [e.g. 5,9,12,63]. The utility of small host sample sizes is to enable a “snapshot” of Symbiodinium diversity from various host species from numerous reef environments. However, replicate sampling of host species on reefs previously targeted in “snapshot” Symbiodinium diversity studies often reveal mixed diversity among endosymbiont communities within a host. For example, Pocillopora damicornis, Stylophora pistillata, Acropora palifera and Gonastrea fasciata have all been shown to associate with a higher diversity of Symbiodinium than originally perceived around Heron Island in the Great Barrier Reef [5,10,18], as was Porites lobata in Hawai‘i [72], and Montastrea franksi and Siderastrea siderea in the Caribbean [73]. Similarly, a Symbiodinium ITS2 sequence previously considered to be Caribbean-specific was reported from Acropora at Johnston Atoll in the central Pacific [16]. Symbiodinium ITS2 C17 and C21 were not previously reported from marine invertebrates hosts in Hawai‘i [33], yet they were all recovered here from increased sampling of one host species, at a single depth, from a single bay. As such, some of the generalized biogeographic and host specificity patterns of Symbiodinium may simply reflect a gross under-sampling of endosymbiont communities in marine invertebrates [9]. The higher Symbiodinium diversity and among colony endosymbiont variation shown here and in the studies described above, shows that some of the biogeographic patterns in Symbiodinium distribution and host specificity do not hold with increased sampling effort. As such, a much greater depth of sampling on a global scale will be required to accurately describe radiation within the genus, understand host specificity and the environmental thresholds of symbioses, and define biogeographic patterns in Symbiodinium diversity.

**Sampling strategy to recover Symbiodinium diversity**

The high sequence diversity of Symbiodinium reported here from colonies of Montipora capitata was recovered by screening a small number of clones from a large number of colonies, or the inverse, screening a large number of clones from a small number of colonies. When additional parameters are included in the experimental design (e.g. sampling, depth, multiple hosts, larger biogeographic region), a greater number of colonies will need to be investigated. Also, we show that there is no standard number of Symbiodinium ITS2 clones that need to be sequenced from all clone libraries to accurately assess endosymbiotic diversity in M. capitata colonies. For some colonies (e.g. Colony 1, 25, 31, 49; Figure 4) Symbiodinium ITS2 diversity can be captured with <10 clone sequences, while for others (e.g. Colony 9 and 44) a higher number of clones need to be sequenced to get an accurate estimation of endosymbiotic ITS2 diversity. Similarly, Stat et al. [16] showed that only Symbiodinium ITS2 C15 was recovered from Porites lobata at Johnston atoll, while a higher sequence diversity (2–7 sequences) was recovered in other coral species at the same location. Therefore the number of coral colonies analyzed and number of clones sequenced per colony will need to be tailored to each study and will reflect some combination of the host species investigated and the environment from which the coral was sampled.

**Conclusion**

Symbiodinium ITS2 sequence assemblages found in M. capitata are variable among individual colonies. The driving force behind these differences is unknown, but likely reflects some combination of host-symbiont specificity, environmental, and stochastic processes. The multi-copy nature and known variability of ITS2 within individual Symbiodinium cells (intra-genomic) make it impossible to distinguish how many independent biological entities these sequence assemblages represent. However, the intricacy of this dataset highlights both the complexity of coral Symbiodinium associations, and innate problems in interpreting ITS2 sequence types that question the assumptions and validity of using the ITS2 to delineate Symbiodinium species.

**Supporting Information**

Figure S1  Symbiodinium ITS2 secondary structures. (DOC)

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**Author Contributions**

Conceived and designed the experiments: MS RDG XP. Performed the experiments: MS XP MT GC LJJC LC DL. Analyzed the data: MS CB GC. Contributed reagents/materials/analysis tools: RDG RT. Wrote the paper: MS CB GC MT RT RDG.
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