**ABSTRACT**

As environmental conditions continue to change, the mutualistic symbiosis between Scleractinian corals and photosynthetic dinoflagellates of the genus *Symbiodinium* becomes increasingly critical. Divergent clades in the genus *Symbiodinium* possess physiologically functional optima lending to potential variability in environmental stress tolerance. Remarkably, the spatial variability of *Symbiodinium* among different habitat types and environmental regimes is poorly understood at large scales, yet is essential for understanding stress-response and potential recovery in coral populations. To investigate the potential distribution among *Montipora capitata*, coral colonies were tagged and sampled at reefs across Kāne’ohe Bay for quantitative PCR analysis of the symbiont community based on internal transcribed spacer (ITS2) sequences.

**INTRODUCTION**

Coral reef habitats are among the most biologically diverse ecosystems on the planet. They provide essential services such as protecting the shoreline, serving as vital habitat for fish and other organisms and functioning as a tourist destination with economic value (Barbier et al. 2011). Calcification by hermatypic stony corals in the order Scleractinia builds critical habitat, made possible by the formation of a mutualistic endosymbiosis with photosynthetic dinoflagellates in the genus *Symbiodinium*. Through this symbiosis, the coral host gains the majority of its required nutrients as photosynthate from the symbiont, which allows for coral growth and survival (Baker 2003, Berkelmans & Van Oppen 2006).

Scleractinian corals are known to associate with a diverse array of *Symbiodinium*.Nine clades (A-I) have been described in *Symbiodinium* based on the internal transcribed spacer (ITS) region on nuclear ribosomal DNA (Pochon & Gates 2010). Though *Symbiodinium* can occupy nine clades, four clades (A-D) comprise the majority of symbionts found in corals (Van Oppen et al. 2001). Clades A and B tend to be most common in Atlantic corals, while corals in the Pacific largely harbor clades C and D (LaJeunesse et al. 2004, Jones et al. 2008).

Each symbiont clade has characteristic levels of stress-tolerance and physiological optima (Boulotte et al. 2016). Clade D has shown higher levels of thermal tolerance and photoprotection, but growth rates and photosynthetic efficiency of clade D-dominated corals are often depressed (Little et al. 2004, Rowan 2004). Conversely, clade C *Symbiodinium* tend to be less tolerant of thermal stress, yet are better able to supply photosynthate to the coral host’s tissues (Cantin et al. 2009). Thus, clade D potentially functions more as an opportunistic symbiont that dominates as a response to recent stress anomalies (Toller et al. 2001, Baker 2004, Cantin et al. 2009, Stat et al. 2013).

The majority of coral species tend to associate with a single symbiont clade (LaJeunesse et al. 2004, Goulet 2006), but other species are able to host multiple clades concurrently (Van Oppen et al. 2001, Rowan 2004). Colonies harboring multiple clades are typically dominated by one clade over the other, yet the presence of heterogeneous mixtures of multiple symbionts suggests a potential for symbiont shuffling or switching in response to changing environmental conditions (Rowan et al. 1995, Jones et al. 2008).

Little is known about the environmental factors controlling the spatial distribution of *Symbiodinium*. Evidence suggests that variability may be due to factors such as depth, irradiance and thermal stress, though few studies have investigated this (Rowan et al. 1995, Abrego et al. 2009). Research has recently begun exploring the patterns of symbiont association among host species, particularly when considering differences in habitat. *Acropora millepora* was found to associate with both *Symbiodinium* clades C and D on the Great Barrier Reef, with D proving more common in corals exposed to poor water quality (Cooper et al. 2011). *Acroporids* in American Samoa were more likely to associate with clade D if occupying habitats with a history of higher temperatures (Oliver & Palumbi 2009). Patterns are suggested for differences between inshore versus offshore reefs, across latitudinal gradients and even within the same reef environment, yet extensive studies of these phenomena are absent.

*Montipora capitata* is a dominant reef-building species inKāne’ohe Bay that can harbor both symbiont clades C and D (Stat et al. 2011, Cunning et al. 2016). The associations between the coral host and its symbiont community have proven relatively stable over time (Cunning et al. 2016) but the factors driving the distribution of clades C and D in *M. capitata* across different environmental regimes are still poorly understood. Two distinct color morphs (brown and orange) are represented by this species and while the development of these color morphs is unknown studies suggest that color development in coral results from fluorescent proteins (Matz et al. 1999, Lukyanov et al. 2000). The brown color morph of this species around O’ahu is known to possess a particular endosymbiosis with *Symbiodinium* of clade C (LaJeunesse et al. 2004). *M. capitata* is commonly found in a variety of habitat types in Kāne’ohe Bay, thus providing an excellent study species to explore the factors driving the relative abundance of multiple symbionts in a dominant reef-building coral.

Kāne’ohe Bay is a unique environment with a strong history of disturbance (Jokiel 1991). Both from environmental and anthropogenic influences, these anomalies are potential drivers of symbiont distribution (Cooper et al. 2011, Stat et al. 2011). The patch and fringing reef systems of Kāne’ohe Bay are quite shallow; some sections of the reef tops can be exposed during low tides (Bahr et al. 2015). Shallow depths and gradients of oceanic influence and freshwater input throughout the bay pose negative implications for stress, which has been observed in successive bleaching events in 2014 and 2015. Despite its seemingly intolerable physiognomies, there exists high coral coverage and rapid recovery rates in Kāne’ohe Bay. This study aimed to characterize the spatial patterns of *Symbiodinium* clades C and D to investigate the potential stress-response of the Kāne’ohe Bay population of *M. capitata* in light of recent bleaching events.

**MATERIALS AND METHODS**

*Study Design and Location*

Individual colonies of *Montipora capitata* were tagged and sampled to measure the spatial variability of *Symbiodinium* clades C and D found in colonies from different habitats. All corals were sampled from Kāne’ohe Bay, located on the east side of O’ahu, Hawai’i, USA. Corals were tagged with medium-sized yellow cattle tags throughout the bay for continued monitoring and future studies. Colonies at five patch reefs and three fringing reefs were tagged in each of the northern, central and southern regions of the bay with an additional submerged patch reef south of the Hawai’i Institute of Marine Biology on Moku o Lo’e. At each patch reef, 30 colonies were tagged; 10 colonies each from windward slope, top and leeward slope with depth recorded using a depth gauge. Given the lack of leeward slope on fringing reefs, 20 colonies were tagged at each site; 10 colonies each from the top and slope. At the tops of the patch reefs and the fringe sites most colonies were between 0 and 1 meter depth. Along the windward and leeward slopes, colonies were tagged randomly at a depth from 1 meter to 13 meters. Reefs lacking sufficient colonies from depths greater than 5 meters were re-visited and five additional colonies were sampled. Depth was later adjusted according to differences in mean sea level using NOAA’s daily tide tables for Moku o Lo’e, Kāne’ohe Bay at 6-minute intervals. In total, 16 patch reefs and 9 fringing reefs were sampled across Kāne’ohe Bay resulting in a sample size of 707 colonies (Fig 1). Tagging, photographing and sampling of colonies took place between 7 June 2016 and 12 August 2016.

*Sample Collection and Processing*

Ten weights with attached floats were randomly thrown from the surface across a distance of approximately 20 meters on each reef area (top and both slopes). The closest colony of *M. capitata* in proximityto each float was tagged and sampled. Each sample consisted of a small branch fragment (~4-5cm) taken from the tip of a branch located at the top of the colony. *In situ* photographs with an included scale bar and color standard were taken of each colony to later be used for size assessment and color assignment of each colony (Fig. 2). Each coral fragment was subsampled for a tissue biopsy shortly after collection (never greater than 1.5 hours), which was placed in 500μL DNA buffer (5M NaCl, 0.5M EDTA) with 1% sodium dodecyl sulfate (SDS). The remaining fragment was immediately frozen in liquid nitrogen and archived at -80°C in the laboratory. DNA was extracted from each sample biopsy following a modified CTAB-chloroform protocol (dx.doi.org/10.17504/protocols.io.dyq7vv).

*Symbiont Community Analysis*

Quantitative PCR (qPCR) was used to analyze the symbiont community of each collected sample. Sequences of *Symbiodinium* clades C and D resulted from clade-level primers and probes based on amplification of the internal transcribed spacer (ITS2) region (Cunning & Baker 2012). All samples were assayed with primers of both clades C and D in duplicate 10μL reactions for 40 cycles on a StepOnePlus platform (Applied Biosystems). Parameters were set at a fluorescence threshold of 0.01 and a baseline interval of cycles 15-22. The StepOnePlus software produced the target symbiont ratio of clade C to D in each sample, normalized for fluorescence intensity and locus gene copy number. Symbiont clades detected in only one qPCR reaction were not considered as present in a colony. The proportion of clade C-dominance was calculated from the clade C to D ratio by the formula [(C:D)/(C:D+1)]. The resulting proportion of clade D-dominance was then calculated by the formula 1-[(C:D)/(C:D+1)]. Based on the proportion values of clades C and D, the dominant symbiont type was determined. If a colony harbored both symbiont clades, designated as a heterogeneous mixture, the clade present in higher proportion was noted as CD or DC accordingly.

*Data Analysis*

The differences in proportion of clades C and D present in colonies dominated by each clade and color morph were investigated using Chi-Squared analyses. Chi-Squared tests were then used to assess differences in dominant symbiont clade, colony color morph and heterogeneous symbiont mixtures between bay areas (northern, central and southern), reef types (patch vs. fringe) and each individually sampled reef. Bray-Curtis coefficient of similarity metrics were used to calculate differences in the dominant symbiont and color morph compositions of each reef, which were tested for spatial autocorrelation using Mantel Tests. To estimate the probability of occurrence of dominant symbiont and color morph as a function of depth, logistic regressions of generalized linear models were used. Depth was corrected for differences in mean sea level using daily tide tables for Moku o Lo’e, Kāne’ohe Bay at 6-minute intervals. Two-Way ANOVA tests were used to investigate the interactive effects of depth and location on the dominant symbiont and color morph. Spatial autocorrelation of dominant symbiont and color morph was tested using Mantel Tests after a MANCOVA adjusted for the influence of depth. The spatial autocorrelation of the interaction of both dominant symbiont and color morph was tested using a Mantel Test after a Multinomial Logistic Regression was performed to discount the influence of depth on the spatial distribution of the interaction. A final Chi-Squared analysis was run on the interaction of colony color morph and dominant symbiont as a function of location (i.e. bay area). All analyses were performed in RStudio v.3.2.2.

**RESULTS**

*Symbiont Community Composition*

Quantitative PCR on 707 colonies of *Montipora capitata* detected *Symbiodinium* clades C and D present both in heterogeneous mixtures and as a single clade exclusively. Across all samples, 53% contained clade C only, 1.2% contained clade D only and 45.8% contained a mixture of both clades C and D. The dominant symbiont across all samples was clade C, being the dominant symbiont in 61% of colonies. In 86.6% of colonies dominated by clade C *Symbiodinium*, clade C was the only symbiont present. In striking dissimilarity, only 3.3% of clade D-dominated colonies harbored clade D *Symbiodinium* exclusively (Fig. 3). A significant relationship between color morph and dominant symbiont clade was observed wherein C-dominance was observed in 89% of brown colonies and 41% of orange colonies (p < 0.001). Clade D, when present in a colony, was almost always in abundance greater than 80%, demonstrating that presence of clade D often indicates a D-dominated colony (Fig. 4).

*Spatial Distribution*

There was a slight relationship between a colony’s color morph and the area of the bay it occupied (p < 0.05); reefs were more heavily dominated by the orange color morph in the northern bay than in the central and southern regions of the bay. This relationship was not different for each reef type (p = 0.29). No significant effects on symbiont-dominance resulted from location within the bay (p = 0.14) or reef type (p = 0.37) alone. When considering the interaction of depth and reef type, a significant effect was had on both dominant symbiont (p < 0.01) and colony color morph (p < 0.05). When eliminating the influence of the submerged reef south of HIMB, bay area proved to be more influential on symbiont distribution, yet the relationship was still insignificant (p=0.06). The interaction between color morph and dominant symbiont clade was significantly related to the area of the bay when adjusting for the influence of colony depth (p < 0.01; Fig. 5). Brown colonies dominated by clade D were more abundant in the southern and central bay areas than they were in the northern region, though this pertains to an insignificant number of colonies (n = 33).

Depth proved to be the significant driving factor for symbiont-dominance (p < 0.001) and color morph (p < 0.001) among colonies of *M. capitata*. The probability of a colony harboring C as the dominant symbiont clade was higher at depths greater than 1 meter, while clade D dominated shallow colonies. A higher probability of orange-dominance was observed in colonies at depths shallower than 4 meters, where dominance shifted to the brown color morph. When considering the influence of depth on the interaction of colony color morph and dominant symbiont, brown colonies were always more likely to be dominated by clade C *Symbiodinium,* consistent with results showing a higher likelihood of a colony being brown at depth and a dominance of clade C at depth. Orange colonies, however, were more likely to be dominated by clade D at depths less than 2.75 meters and clade C at depths greater than 2.75 meters (p < 0.001; Fig 6).

**DISCUSSION**

Symbiont association in *Montipora capitata* across Kāne’ohe Bay showed a strong depth-dependent distribution with shallow colonies being dominated by clade D and colonies at depths greater than 1 meter being dominated by clade C (Fig 6). This was observed among all reefs throughout the bay, indicating that the factors driving symbiont dominance exist along a vertical depth gradient rather than a horizontal spatial distribution. No significant spatial differences were observed among reefs, reef types or regions of the bay, consistent with the lack of spatial variation across sites and regions found in previous reports (Stat et al. 2011). Depth partitioning is consistent with observations that habitat depth influences bathymetric zonation of coral symbionts between shallow, high irradiance environments and deep, low irradiance environments (Finney et al. 2010).

Quantitative PCR (qPCR) revealed that the symbiont composition of sample colonies (n = 707) existed as either heterogeneous mixtures of clades C and D or as one clade exclusively. The presence of symbiont mixtures in a coral suggests a potential for either symbiont shuffling or switching to lessen the impacts of shifting environmental conditions (Mieog et al. 2007). Under stressful conditions (i.e. thermal anomalies), corals have been shown to use clade D symbionts to supplement heterotrophy despite the limited supply of nutrients that these symbionts provide (Stat et al. 2008). Mixtures could also be more advantageous for recovery from stress events. In *M. capitata*, the presence of clade D may serve to support the coral host during stress (Baker 2003) while the presence of clade C may aid in reversion once a shift back to more idealistic conditions occurs; however, a study of symbiont association over time showed no switching between clades C and D in this species (Cunning et al. 2016).

Clade C symbionts tend to be ideal given the more efficient carbon-delivery they provide to the coral host (Little et al. 2004) while clade D symbionts tend to be opportunistic and functionally serve as a resilient symbiont (Cantin et al. 2009). In the 707 sample colonies, clade C was overwhelmingly widespread; found in 698 colonies, while clade D was found in 331 colonies. However, when clade D was present, its abundance was typically greater than clade C (Fig 4). Of the nine colonies that exclusively harbored clade D, five colonies showed amplification of clade C in one of the technical qPCR replicates and all displayed high cycle thresholds (≥ 35), suggesting poor amplification and a likelihood that clade C was present but amplified further back in the reaction than the 40 cycles evaluated. It may be that all *M. capitata* colonies host some clade C *Symbiodinium* but the levels of association were below the detection threshold of qPCR.

Physical conditions in Kāne’ohe Bay can be quite variable. Current data shows insignificant variation among bay regions in terms of daily mean temperature (Ritson-Williams & Gates 2016b). These comparable thermal regimes on a horizontal spatial scale are consistent with the findings that symbiont distribution is relatively similar across the bay. Thermal variation on a vertical scale, given current knowledge of tolerance in coral symbionts, is a candidate for monitoring at reefs in each region of the bay to determine their likelihood of contributing to variability in *Symbiodinium*.

Light intensity declines as depth in the photic zone increases and has induced differences in photosynthetic responses by means of photoinhibition and photoprotection in corals dominated by clades C and D respectively (Salih et al. 2000, Rowan 2004). Response variance validates the hypothesis that different clades of *Symbiodinium* adapt to particular light intensities (Iglesias-Prieto et al. 2004). The putative implication of light intensity on *Symbiodinium* variability supports the notion that habitat partitioning of the symbiont community composition exists along depth-mediated light gradients (Iglesias-Prieto & Trench 1994). A transition depth exists between 1 and 2 meters, at which *M. capitata* association switches from a dominance of clade D to clade C. As Kāne’ohe Bay is quite turbid, this shallow depth threshold suggests that depth stratification of light intensity might be a common driver of distribution among symbiont association, though the threshold depth of transition would depend on local abiotic conditions.

Throughout Hawai’i, *M. capitata* exists as two distinct color morphologies (Fig 2). This division in color has been observed before and was correlated with differences in symbiont communities (LaJeunesse et al. 2004). Research investigating the production of color in this species is understudied, yet previous work on other coral species suggests an exploitation of phenotypic plasticity in fluorescent proteins (Kelmanson & Matz 2003). Similarly to *M. capitata*, *Lobophyllia hemprichii* can exist as an orange morph which has been proven to result from a change in color due to light irradiation levels stimulating green fluorescent proteins (Oswald et al. 2007). Consequently, it is hypothesized that orange morphs may serve a photoprotective purpose, contributing to the dominance of this color morph in shallow depths observed in *M. capitata*, though further investigation is needed.

Studies suggest that coral color may be indicative of physiological function. At shallow depths, the green morph of *Porites astreoides* was observed more frequently than the brown morph, possibly indicative of light tolerance (Gleason 1993). Another study investigating sedimentation influence on *P. astreoides* noted brown colonies more efficiently shed sediment than did green colonies, ultimately preventing sediment-induced mortality (Gleason 1998). Correspondingly, a study on sedimentation influence on *Symbiodinium* revealed that clade C symbionts were found in higher sedimentation areas (Garren et al. 2006). Such phenomena may be pertinent to *M. capitata*,which exhibit a comparable pattern in which brown morphs and clade C were found at depth where sedimentation is often greater and light intensity is often reduced.

As demonstrated by quantitative PCR analysis of *M. capitata* fragments from colonies across Kāne’ohe Bay, we showed that the spatial variability of *Symbiodinium* occurs most strongly as a function of depth. No significant spatial patterns arose from different reefs, reef types or areas of the bay when considering the dominance of one clade over another. Portions of the reefs in Kāne’ohe Bay can be quite shallow (< 0.5 meters) at low tide and are probably exposed to high temperatures during summer months. Corals associating with clade D and often existing as an orange color morph dominate this highly fluctuating environment. Because clades C and D have different physiological tolerances, evidence suggests functional differences among colony color and depth has shown to be the strongest driver symbiont distribution, biotic and abiotic conditions along a depth gradient should be targeted for future investigations, which may assist in conservation strategies and understanding resistance among coral in changing environmental conditions.

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**FIGURE CAPTIONS**

Fig. 1. Collection reef locations in Kāne’ohe Bay, O’ahu, Hawai’i, USA

Fig. 2. *Montipora capitata* colonies of both color morphs: orange (left) and brown (right). Photo credit: Raphael Ritson-Williams

Fig. 3. Proportion of occurrence of *Symbiodinium* clades C and D in *Montipora capitata* colonies per dominant symbiont clade.

Fig. 4. Percentage of clade D in all colonies of *Montipora capitata*. Bar colors indicate colony color morph

Fig. 5. Distribution of *Symbiodinium* and color morph in *Montipora capitata* across the northern, central and southern regions of Kāne’ohe Bay, O’ahu, Hawai’i, USA

Fig. 6. (Top) Bars indicate the proportion of clade-dominance in all colonies grouped by 1m depth intervals. Line indicates the probability of clade D-dominance as a function of depth. (Middle) Bars indicate the proportion of occurrence of each color morph in all colonies grouped by 1m depth intervals. Line indicates the probability of occurrence of the orange color morph as a function of depth. (Bottom) Probability of clade D-dominance for all colonies of each color morph as a function of depth