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**ABSTRACT**

Scleractinian corals form a mutualistic symbiosis with photosynthetic dinoflagellates in the genus *Symbiodinium*. However, the spatial variability of *Symbiodinium* across habitat types and environmental regimes is poorly understood at large scales, yet is essential for determining patterns of distribution and potential resistance to future stress events. To investigate symbiont distribution in a dominant reef-building coral, colonies of *Montipora capitata* were sampled across Kāne‘ohe Bay, O‘ahu, Hawai‘i, USA for symbiont community analysis. A total of 707 colonies were tagged and sampled from reefs across Kāne‘ohe Bay at different reef habitats and depths. Symbiont dominance and the relative ratio of clade C to D in each sample were recovered by quantitative PCR. Clade distribution was significantly associated with depth wherein clade D dominated corals in shallow environments and clade C was more prevalent at depths > 1.29 m. Colony color morph had a similar pattern where orange morphs dominated the shallow environment and brown morphs dominated depths > 3.64 m, suggesting a potential interactive effect between *Symbiodinium* and colony color morph in need of further investigation. No significant spatial patterns existed across reefs, regions of the bay or between patch and fringing reef types. This work reveals that essential symbioses in *M. capitata* throughout Kāne‘ohe Bay result from depth-related abiotic conditions.

**INTRODUCTION**

Coral reefs are among the most biologically diverse ecosystems on the planet, providing essential services such as shoreline protection, 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 (*Symbiodinium* spp.). Through this symbiosis, the coral host gains the majority of its required nutrients as photosynthate from the symbiont, allowing for coral growth (Baker 2003, Berkelmans & Van Oppen 2006).

Scleractinian corals associate with a diverse array of symbionts.Nine clades (A-I) have been described in *Symbiodinium* based on the internal transcribed spacer (ITS) region on nuclear ribosomal DNA (Pochon & Gates 2010). Different symbiont types within each clade have characteristic levels of stress-tolerance and physiological optima (Boulotte et al. 2016). Clade D symbionts have shown higher levels of thermal tolerance and photoprotection, but growth rates and photosynthetic efficiency of clade D-dominated corals are often depressed relative to corals associating with other symbiont clades (Little et al. 2004, Rowan 2004). Conversely, many types of symbionts in clade C tend to be less thermally tolerant, yet are better able to supply photosynthate to the coral host’s tissues (Cantin et al. 2009). Clade D, therefore, potentially functions more as an opportunistic symbiont that dominates and stressful environments (Toller et al. 2001, Baker 2004, Cantin et al. 2009, Cooper et al. 2011, Stat et al. 2013).

Most coral species associate with a single symbiont clade; some even with a single type within a clade (LaJeunesse et al. 2004, Goulet 2006). Other species are able to host multiple clades concurrently (Van Oppen et al. 2001, Rowan 2004). While colonies harboring multiple clades are typically dominated by one clade with background populations of others (Silverstein et al. 2012), the presence of heterogeneous mixtures of multiple symbionts suggests a potential for symbiont shuffling (change in relative abundance of *in hospite* *Symbiodinium*) or switching (uptake of novel *Symbiodinium*) in response to changing environmental conditions (Rowan et al. 1995, Mieog et al. 2007, Jones et al. 2008, Boulotte et al. 2016).

Because symbioses play a vital role in coral survivorship, the factors shaping the distribution and dominance of symbionts must be readily understood. Little is known about the environmental factors controlling the spatial distribution of *Symbiodinium* within a species though evidence suggests that patterns may be due to factors such as irradiance and thermal stress (Rowan et al. 1995, Abrego et al. 2009, Stat et al. 2015). *Symbiodinium* of clade D (D1) have shown thermal tolerance not observed in clade C (C1b-c) and dominated in areas that experienced thermal stress (Baker et al. 2016). This is consistent with previous correlative studies of symbiont distribution and sea surface temperatures (Tonk et al. 2013). Similarly, clade C symbionts (C1c) in the Gulf of California performed better in low light at depth than did clade D symbionts (D1) which were more successful in shallow, high light environments, suggesting irradiance plays a vital role in niche-partitioning of *Symbiodinium* (Iglesias-Prieto et al. 2004, Sampayo et al. 2007, Bongaerts et al. 2015).

*Montipora capitata* is a dominant reef-building species inKāne‘ohe Bay that can harbor both clade C and D symbionts; specifically associating with types C31 and D1a (Stat et al. 2011, Cunning et al. 2016). The factors driving the distribution of these clades in *M. capitata* across different environmental regimes are not well described. In Kāne‘ohe Bay, *M. capitata* occurs as two distinct color morphs (brown and orange), and while the cause of these color morphs is unknown, studies suggest that fluorescent protein-like proteins play a role in coral color development (Matz et al. 1999, Lukyanov et al. 2000). It is unclear what functional differences exist among *M. capitata* color morphs, however around O‘ahu the brown and orange color morphs were suggested to possess a specific endosymbiosis with *Symbiodinium* of clades C and D respectively (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 anthropogenic and environmental disturbance (Smith et al. 1981, Jokiel 1991) which may drive symbiont distributions (Cooper et al. 2011, Stat et al. 2011). Higher temperatures exist in the bay due to restricted circulation, causing corals to exist in conditions projected not to be experienced for another century (Bahr et al. 2015). The tops of the shallow patch and fringing reef systems of Kāne‘ohe Bay can be exposed during low tides (Bahr et al. 2015), likely contributing to thermal stress as well. Despite being comparatively shallow relative to other reef systems, habitats on a single reef can be considerably different due to dramatic turbidity increases with depth. Though Kāne‘ohe Bay is a unique environment, there exists high coral coverage and rapid recovery rates from bleaching events (Bahr et al. 2015, Cunning et al. 2016). This study was designed to characterize the spatial variability of *Symbiodinium* clades C and D to investigate the variability in symbioses among the Kāne‘ohe Bay population of *M. capitata*.

**MATERIALS AND METHODS**

*Study Design and Location*

Individual colonies of *Montipora capitata* in Kāne‘ohe Bay were tagged for continued monitoring and sampled to measure the spatial variability of *Symbiodinium* clades C and D among corals in different habitats. Colonies at five patch reefs and three fringing reefs were tagged in each of the northern, central and southern regions of the bay, along a gradient of decreasing oceanic influence, with an additional submerged patch reef south of the Hawai‘i Institute of Marine Biology on Moku o Lo‘e (Fig. 4). 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 ≤ 1 m depth. Along the windward and leeward slopes, colonies were tagged randomly at 1 - 13 m depth. In total, 16 patch reefs and 9 fringing reefs were sampled across Kāne’ohe Bay resulting in a sample size of 707 colonies. 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 m 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 - 5 cm) 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 assess size and color morph of each colony (Fig. 1). Each coral fragment was subsampled for a tissue biopsy shortly after collection (never greater than 1.5 hours) and placed in 500 μL 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 resulted from clade-level primers and probes targeting specific actin loci in *Symbiodinium* clades C and D (Cunning & Baker 2013). All samples were assayed with primers of both clades C and D in duplicate 10 μL 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 (Cunning et al. 2016). 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 dissimilarity 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 (Goslee & Urban 2007). 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 tested the effects of depth and location (bay region, reef type and reef ID) 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 (Lenth 2016). The spatial autocorrelation of the interaction between 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 (Lenth 2016). 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 R v.3.2.2 (R Core Team 2016).

**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 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. 2). However, when clade D was present in a colony, it was almost always in abundance > 80 %, demonstrating that presence of clade D often indicates a D-dominated colony (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).

*Spatial Distribution*

No significant differences in symbiont-dominance resulted between regions of the bay (p = 0.14) or reef types (p = 0.37) alone. However, the effect of reef type on both symbiont-dominance and colony color morph also depended on the influence of depth (p < 0.01, p < 0.05). When eliminating the influence of the submerged reef south of the Hawai‘i Institute of Marine Biology, 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. 4). Brown colonies dominated by clade D were more abundant in the southern (n = 18) and central (n = 10) bay areas than they were in the northern (n = 5) region, though this results from a trivial 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 > 1.29 m, while clade D dominated colonies at depths < 1.29 m. A higher probability of orange-dominance was observed in colonies at depths < 3.64 m, whereas brown color morphs dominated at depths > 3.64 m. Brown colonies were more likely to be dominated by clade C *Symbiodinium* across all depths, but orange colonies were more likely to be dominated by clade D at depths < 2.75 m and clade C at depths > 2.75 m (p < 0.001; Fig. 5).

**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 deeper colonies being dominated by clade C (Fig. 5). This distribution was observed across all sampled reefs, indicating that the factors driving symbiont dominance in Kāne‘ohe Bay exist along a depth gradient rather than a latitudinal geographic distribution. No significant spatial differences were observed among reefs, reef types or regions of the bay, consistent with the lack of spatial variation in symbiont dominance previously observed across sites and regions (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 (Frade, de Jonghe, et al. 2008, Finney et al. 2010).

Quantitative PCR (qPCR) revealed the symbiont composition of sample colonies (n = 707) existed as either heterogeneous mixtures of multiple clades or as one clade exclusively, suggesting a potential for either symbiont shuffling or switching to lessen the impacts of changing environmental conditions (Mieog et al. 2007). Clade C symbionts, given efficient carbon-delivery to the coral host, tend to dominate in more idyllic environments (Little et al. 2004, Cantin et al. 2009). Conversely, clade D symbionts tend to be less beneficial to the coral host in terms of nutrient-supply, yet may be functionally important as an opportunistic symbiont in stressful conditions (Stat et al. 2008, Cantin et al. 2009, Cunning & Baker 2013). In the 707 sample colonies, clade C was present in 98.7 % of the colonies, while clade D was found in 46.8 % of the colonies. However, when clade D was present, it typically dominated the symbiont community (Fig. 3). Of the nine colonies that exclusively harbored clade D, five colonies showed amplification of clade C in one of the technical qPCR replicates, suggesting likelihood that clade C was present in low abundance. Therefore, all *M. capitata* colonies potentially host some clade C *Symbiodinium* but the levels of association may sometimes exist below the detection threshold of qPCR.

*M. capitata* also exists as two distinct color morphologies in Kāne‘ohe Bay (Fig. 1) that exhibited a similar depth distribution (Fig. 5). While this division in color has previously correlated with differences in symbiont community composition (LaJeunesse et al. 2004), color differences in this species are understudied overall. Work on other coral species suggests color results from phenotypic plasticity in fluorescent protein-like proteins (Kelmanson & Matz 2003) and may be indicative of physiological function. For example, at depth, brown morphs of *Porites astreoides* were observed more frequently than green morphs, suggesting acclimation to low-light environments (Gleason 1993). This phenomenon may be pertinent to *M. capitata*,which exhibited a comparable pattern in which brown morphs were found more commonly at depth where light intensity was often reduced and orange morphs were found in shallow environments probably exposed to higher light intensity. Consequently, it is hypothesized that orange morphs may serve a photoprotective purpose (Salih et al. 2000) contributing to the dominance of this color morph in shallow depths, but further investigation is needed.

Photoprotection in coral serves to aid in survival in stressful environments (Jones & Hoegh-Guldberg 2001). Several mechanisms for photoacclimation have been suggested including adjustments of photosynthetic pigments to avoid oxidative stress from excess light energy (Titlyanov 1981). Non-photochemical quenching (NPQ), the dissipation of excess energy from excited-state chlorophylls, is facilitated by the xanthophyll cycle in which carotenoid pigments adopt harvesting or protective forms depending on environmental conditions (Müller et al. 2001) and may contribute to adaptations among scleractinian corals in habitats with different light intensities. Given the significant association observed between symbiont clade and color morph in *M. capitata*, there exists a potential for NPQ to play a role in persistence of this species in Kāne‘ohe Bay. Theoretically, clade D symbionts or orange color morphs found in shallow environments would have a higher capacity for NPQ, though no studies have yet investigated this.

Colony color and its symbiont community exhibited similar distributions evident of similar responses to abiotic conditions (Fig. 5). Brown colonies were always more likely to be clade C-dominated whereas orange colonies shifted from clade D-dominance to clade C-dominance at 2.75 m. This relationship is consistent with symbiont association patterns observed in previous reports (LaJeunesse et al. 2004). Which agent is causing the other, however, remains undetermined. Previous studies have suggested that the presence of certain fluorescence proteins coupled with a concentration of specific symbionts determines the coloration of a colony (Dove et al. 2006) while other studies determined depth-related abiotic conditions shape color morph (Frade, Englebert, et al. 2008). We argue the latter proves more influential in shaping distributional patterns of bothsymbiont association and colony color morph in *M. capitata* across Kāne‘ohe Bay.

Physical conditions in Kāne‘ohe Bay are observably variable within a narrow depth-range. This contrasts geographic distributional data (Ritson-Williams & Gates 2016a, Ritson-Williams & Gates 2016b) showing insignificant variation among bay regions in terms of daily mean temperature and sedimentation (Fig. S1). However, light data (Ritson-Williams & Gates 2016c) recovered highest measurements in the southern region of the bay (Fig. S2), which may contribute to the greater number of brown colonies harboring clade D in this region (Fig. S3) supporting the notion that clade D symbionts are possibly tolerant to high light environments (Finney et al. 2010).

Symbiont association in *M. capitata* switched from a dominance of clade D to clade C at a transition depth of 2.75 m. As Kāne‘ohe Bay is quite turbid, this shallow 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. Light attenuation with increasing depth in the photic zone has been considered the major environmental gradient influencing coral symbioses, driving photosynthetic responses result by means of photoinhibition and photoprotection in corals dominated by clades C and D respectively (Salih et al. 2000, Veron 2000, Rowan 2004). Such a response variance validates the hypothesis that different clades of *Symbiodinium* adapt to particular light intensities (Iglesias-Prieto et al. 2004) and supports the concept that habitat partitioning of the symbiont community composition exists along depth-mediated light gradients (Iglesias-Prieto & Trench 1994) whether these be total irradiance or spectral niches as some studies suggest (Frade, Englebert, et al. 2008).

As demonstrated by qPCR analyses of *M. capitata* colonies across Kāne‘ohe Bay, we showed that the spatial variability of *Symbiodinium* occurs most strongly as a function of depth. No significant spatial distributional 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 m) at low tide and are probably exposed to high temperatures and irradiance during summer months. Corals associating with clade D dominate this highly variable environment. Because clade C and D symbionts have different physiological tolerances, depth-mediated distributional patterns may serve as an adaptive mechanism to changing environmental conditions.

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**FIGURE CAPTIONS**

Fig. 1. *Montipora capitata* colonies of both color morphs: orange (left) and brown (right). Photo credit: Raphael Ritson-Williams

Fig. 2. Proportion of occurrence of *Symbiodinium* clades C and D in *Montipora capitata* colonies per dominant symbiont clade

Fig. 3. Percentage of clade D in all colonies of *Montipora capitata*. Bar colors indicate colony color morph

Fig. 4. Latitudinal geographic distribution of *Symbiodinium* and color morph in *Montipora capitata* across Kāne’ohe Bay, O’ahu, Hawai’i, USA

Fig. 5. (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