A peer-reviewed version of this preprint was published in PeerJ on 13 May 2014.

Green EA, Davies SW, Matz MV, Medina M. 2014. Quantifying cryptic *Symbiodinium* diversity within *Orbicella faveolata* and *Orbicella franksi* at the Flower Garden Banks, Gulf of Mexico. PeerJ 2:e386 https://doi.org/10.7717/peerj.386

Next-generation sequencing reveals cryptic *Symbiodinium* diversity within *Orbicella faveolata* and *Orbicella franksi* at the Flower Garden Banks, Gulf of Mexico

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Abstract

The genetic composition of the resident *Symbiodinium* endosymbionts appears to strongly modulate the physiological performance of reef-building corals. Here, we used deep amplicon sequencing to quantitatively assess *Symbiodinium* genetic diversity for the two mountainous star corals, *Orbicella franksi* and *Orbicella faveolata*, from two reefs separated by 19 kilometers of deep water. We aimed to determine if symbiont diversity is largely partitioned with respect to coral host species or geographic location. Our results demonstrate that across the two reefs both coral species contained only *Symbiodinium* identifiable as clade B type B1, represented by five distinct haplotypes. Three of these haplotypes have not been previously described and may be endemic to the Flower Garden Banks. No consistent differences in symbiont composition were detected between the two coral species. However, significant quantitative differences were observed between the east and west banks for two of the five haplotypes. These results highlight the need for consistent molecular genotyping techniques to assess local community assemblages of *Symbiodinium*-host relationships, which could be largely irrespective of host genetic background. This deep-sequencing approach used to sensitively characterize cryptic genetic diversity of *Symbiodinium* will potentially contribute to the understanding of physiological variations among coral populations.

Key words: next-generation sequencing (NGS), Flower Garden Banks, Caribbean, *Symbiodinium*, *Orbicella faveolata*, *Orbicella franksi*, ITS-2, amplicon sequencing, OTU

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Introduction

The symbiotic relationship between scleractinian corals and dinoflagellate algae in the genus *Symbiodinium* is well known, but there is still much to understand about the establishment and plasticity of this complex symbiosis. Knowledge of *Symbiodinium* taxonomic diversity has increased over the last two decades with advancing molecular genotyping techniques detecting novel haplotypes within each of the nine accepted clades (Coffroth & Santos 2005; Pochon & Gates 2010). Some of these haplotypes may impart different physiological benefits and evidence suggests that *Symbiodinium* infection modulates overall health and response mechanisms of coral hosts (Rowan et al. 1997; Sampayo et al. 2008; Voolstra et al. 2009).

Symbiodinium provide hosts with photosynthetic products critical for metabolic processes and calcification (Muscatine & Cernichiari 1969; Muscatine et al. 1984; Trench 1987). A severely broken symbiosis will lead to a bleaching event where the brown algal cells are expelled resulting in a white coloration of the coral (Glynn 1993; Hoegh-Guldberg 1999; Hoegh-Guldberg & Smith 1989). Dependent on the severity and duration of this broken relationship, the coral host may or may not recover (Lang et al. 1992; Marshall & Baird 2000). Understanding the flexibility of symbiosis between corals capable of housing a mixed infection (Douglas 1998; LaJeunesse et al. 2003) versus corals with strict specificity for one symbiont type (Diekmann 2002; Sampayo et al. 2007) will allow us to understand the ability of corals to survive different environmental stressors.

Currently it is still challenging to gain a comprehensive understanding of endosymbiont distributions on a global scale, yet such knowledge is critical in the assessment of coral reef resilience. It is therefore essential to detect diversity at the subspecies level in a consistent and quantitative manner. As a consequence we are in need to develop detection methods that allow for a consistent quantitative detection of symbiont species for example across a diverse set of host species. The *Orbicella annularis* species complex has been shown to typically host a mixed population of several *Symbiodinium* species (Rowan & Knowlton 1995; Rowan et al. 1997). However, little is known about the functional and genetic diversity of these *Symbiodinium* species and how their presence correlates with host physiology (Baker 2003; Knowlton & Rohwer 2003). *Symbiodinium* species have shown varying photosynthetic efficiency and saturation points suggesting coral host physiology is at least partially dependent on symbiotic interactions (Baums et al. 2010; DeSalvo et al. 2010; Fitt & Warner 1995; Warner et al. 1996).

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The specific physiological contributions of *Symbiodinium* spp. to the host require more investigation, but general attributes for species in clades A-C have been proposed. Clades A and B have been more commonly found in high irradiance environments (Rowan et al. 1997; Toller et al. 2001), clade A members have been show to provide increased UV protection (Reynolds et al. 2008), and members of clade C, the most diverse *Symbiodinium* lineage are thought to enhance host calcification rates (Cantin et al. 2009; LaJeunesse 2005).

To close this gap we should strive to consistently detect *Symbiodinium* taxonomic diversity across numerous diverse sites and host species. This will achieve an expansion in the investigation of coral physiology and will add new means of detection precision. Experiments prior to 1993 heavily relied on coarse resolution genotyping techniques likely unable to detect all *Symbiodinium* species in a mixed infection (Loram et al. 2007; Thornhill et al. 2006). Molecular techniques utilized for the past two decades paired with growing databases of commonly used phylogenetic markers have provided support for various hosts to house mixed *Symbiodinium* populations and detect unique genetic haplotypes previously underestimated likely due to coarse genotyping techniques (Baird et al. 2007; Baker & Romanski 2007; Fay & Weber 2012; LaJeunesse 2002; Rowan et al. 1997). Use of next-generation sequencing (NGS) platforms has gained popularity as a cost effective, high throughput method capable of detecting low frequency strains of *Symbiodinium* within mixed symbiotic communities (Kenkel et al. 2013; Quigley KM 'unpublished data'). Detecting these novel haplotypes in mixed communities can help to enhance our understanding of the role that *Symbiodinium* physiology holds for their hosts and how they may define geographical distributions of *Symbiodinium* species (Jones & Berkelmans 2010; Mieog et al. 2009). In order to more accurately evaluate not only the biogeographic distributions of different coral-algal symbioses but also the ability of coral hosts to survive increasingly stressful environmental conditions, an accurate quantitative assessment of *Symbiodinium* diversity is imperative.

Here we use deep amplicon sequencing (Roche 454 GS FLX platform) of the internal transcribed spacer (ITS-2) nuclear ribosomal DNA to assess species diversity of *Symbiodinium* within the endangered Caribbean *Orbicella annularis* species complex (IUCN 2011), formerly known as a member of the genus *Montastraea* (Budd et al. 2012). To investigate whether *Symbiodinium*-host relationships are more variable between genetically distinct host species or their geographic locations, we assess *Symbiodinium* diversity in two genetically distinct host

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species (*O. faveolata* and *O. franksi*). Both species are known to equally flourish on the east and west banks of the Flower Garden Banks National Marine Sanctuary (FGBNMS), Gulf of Mexico, two geographical locations that experience similar environmental conditions.

Methods

Locations

The Flower Garden Banks (FGB) is a National Marine Sanctuary established in 1992 and situated 185 kilometers off the coast of Texas (27°54' N, 93°35'W and 27°53'N, 93°49'W for east and west localities, respectively) in the Gulf of Mexico (Fig. 1). The east and west banks are separated by 19 kilometers. Flower Garden Banks are the most northern coral reefs in the Gulf of Mexico making it an important location to understand limits of latitudinal distributions of coral species (Schmahl et al. 2008). Twenty-four shallow-water (<50 meters) coral species reside at the east and west FGB (Schmahl et al. 2008). Compared to other Caribbean reefs, the FGB have less species diversity, but has been found to have much higher coral cover ranging between 50% and 70% (Precht et al. 2005). In addition, the FGB is a uniquely deep reef starting at 17 meters and extending beyond 45 meters (Schmahl et al. 2008). Annual average temperatures range between 18°C and 30°C providing a unique opportunity to study corals exposed to their thermal minimums (Schmahl et al. 2008). The remote location of the FGB protects these reefs from most anthropogenic stressors; both land based and recreational, likely contributing to high coral cover and near pristine conditions.

Coral Collections

A total of 197 1cm *x* 1cm coral fragments were collected from the outer edge of two *Orbicella* colonies at both the east and west FGB in August 2011 (*O. faveolata*, n=96) and August 2012 (*O. franksi*, n=101) with approximately n=50 per species per bank. Coral tissue was preserved in 96% ethanol and stored at room temperature. Sample depth ranged from 21 to 23 meters.

Laboratory Procedures and Host Genotyping

FGB holobiont DNA was isolated following the phenol-chloroform protocol described in Davies et al. (2013). One hundred ninety-three coral hosts were successfully amplified at nine microsatellite loci (Davies et al. 2013). STRUCTURE (v2.3.4) output (q-score) (Falush et al. 2003; Falush et al. 2007; Hubisz et al. 2009; Pritchard et al. 2000) was used to identify non-hybrid coral colonies. Hybrids from the *O. annularis* species complex have been reported in literature (Budd & Pandolfi 2004; Fukami et al. 2004; Szmant et al. 1997). Only individuals with

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greater than 80% posterior probability of belonging to one of the two major STRUCTURE derived clusters were retained (73 samples of *O. faveolata* and 101 samples of *O. franksi*) (Foster et al. 2012). Sixty of these, fifteen colonies of *O. faveolata* and fifteen colonies of *O. franksi* from both east and west FGB, were chosen for *Symbiodinium* ITS-2 genotyping. To look for genetic structure among coral populations between the two locations (east and west banks), an admixture model was run starting with a uniform alpha for degree of admixture, uncorrelated allele frequencies for five simulations, a burn-in of 300,000 steps and 10^6 Markov-Chain Monte Carlo (MCMC) iterations. STRUCTURE results were then used as input to run STRUCTURE HARVESTER to select the optimal number of clusters (K) (Earl & vonHoldt 2012; Evanno et al. 2005). Using CLUMPP (Jakobsson & Rosenberg 2007), output files from STRUCTURE HARVESTER were used to combine the results of replicated runs by computing weighted averages followed by plotting the results using DISTRUCT (Rosenberg 2004). To assess within species differentiation each species was analyzed separately in STRUCTURE applying the same parameters for all analyses (Foster et al. 2012). An analysis of molecular variance (AMOVA) was implemented in GenAlEx (version 6.5) to assess genetic differentiation by computing pairwise F_{ST} for species and sites (Peakall & Smouse 2012).

Amplification of ITS-2 for 454 sequencing

ITS-2 was amplified in each of the sixty individual hosts and submitted for deep amplicon sequencing in January 2013 using *Symbiodinium* specific ITS-2 primers, ITS-Dino-forward (5'- GTGAATTGCAGAACTCCGTG-3') (Pochon et al. 2001) and its2rev2-reverse (5'-CCTCCGCTTACTTATATGCTT-3') (Stat et al. 2009). The target amplicon was approximately 300 base pairs long. Each 30 µL PCR reaction contained 13.3 µL of water, 3.0 µL 10 *x ExTaq* HS buffer, 0.2 mM dNTP, 0.75 U *ExTaq* HS polymerase (Takara Biotechnology), 0.375 U *Pfu* polymerase (Agilent Technologies), 0.2 µM final primer concentration and 50 ng of DNA template (Kenkel et al. 2013; Quigley, KM 'unpublished data'). A DNA Engine Tetrad 2 Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used for all amplifications. Individuals were amplified to approximately the same intensity in order to prevent over or under representation of PCR products. The following PCR protocol was used: 20 cycles of 94°C for five minutes, 95°C for 40 seconds, 59°C for two minutes, 72°C for one minute and final extensions of 72°C for five minutes. Additional cycles were added to individuals to obtain the same uniform intensity and the final cycle number was recorded. Individuals that had not amplified by 35 cycles were

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repeated using a lower starting template (20 ng/µL) to reduce the inhibition by contaminants. PCR product intensity of all individuals was determined on one two percent agarose gel. All individuals amplified by 34 cycles except one west FGB *O. faveolata* and one east FGB *O. faveolata* which were removed from the analysis.

PCR products were cleaned using GeneJET PCR purification kits (Fermentas Life Sciences). Six individuals were randomly selected and run on one two percent agarose gel to ensure sufficient DNA quantities remained post clean-up. Possible modification for future protocol use would include quantifying DNA post PCR clean-up and diluting DNA to equal concentrations prior to assigning barcodes.

New 30 µL PCR reactions were performed to attach A and B Rapid adaptors specific for 454 GS FLX. The adaptors designs were as follows: reverse barcoded primer sequence (A-Rapid primer+unique barcode+its2rev2 primer) and forward B-rapid primer (B-Rapid primer+ITS-Dino) (Fig. S1). Each reaction contained 50 ng of cleaned PCR product,17.6 µL water, 0.2 mM dNTP, 3 µL 10 *x ExTaq* HS buffer, 0.75 U *ExTaq* HS polymerase (Takara Biotechnology), 0.375 U *Pfu* polymerase (Agilent Technologies), 50 ng of PCR product, 0.33 µM of 454 B-Rapid ITS2-forward (5'-CCTATCCCCTGTGTGCCTTGAGAGACGHC+GTGAATTGCAGAACTCCGTG-3') and 0.33 µM of 454 A-Rapid ITS2 adaptor with unique barcode (5'-CCATCTCATCCCTGCGTGTCTCCGACGACT+**TGTAGCGC**+CCTCCGCTTACTTATATGCTT-3') (Kenkel et al. 2013; Quigley, KM 'unpublished data'). PCR was performed on a DNA Engine Tetrad 2 Thermal Cycler (Bio-Rad, Hercules,CA,USA) under the following conditions: 95°C for five minutes, four cycles of 95°C for 30 seconds, 59°C for 30 seconds, 72°C for one minute followed by incubation at 72°C for five minutes. Samples were verified on one two percent agarose gel and pooled based on band intensity. Pools were ethanol precipitated. Three to five micrograms of the cleaned product was run on a one percent SYBR Green (Invitrogen) stained gel. The target band was excised using a blue-light box and soaked in 25 µL of milli-Q water overnight at 4°C. The supernatant was submitted and sequenced at the University of Texas-Austin Genome Sequencing and Analysis Facility (GSAF) aiming to obtain two thousand reads per sample.

Bioinformatics

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Uniquely barcoded individual reads were extracted and trimmed with custom Perl scripts (Data S1) to remove adaptors, barcodes and low quality reads (Kenkel et al. 2013; Quigley, KM 'unpublished data'). All reads with lengths less than 290 base pairs were removed. The clustering algorithm *usearch* was used to cluster reads into operational taxonomic units (OTUs) (Edgar 2010). Reads were mapped to OTUs using SHRIMP2 (David et al. 2011). Of 153 OTUs identified, only five OTUs had a median count exceeding one (i.e., were detected in more than half of all samples) and were retained. These OTUs were used as queries for BLASTn (Altschul et al. 1990) and were aligned between each other using Clustal Omega online server version 1.2.0 (Goujon et al. 2010; McWilliam et al. 2013; Sievers et al. 2011). Alignments were examined and manually trimmed using SeaView version 4.4.2 (Gouy et al. 2010).

Statistical Analysis

R Studio v 3.0.2 (R Developmental Core Team 2013) was used for all statistical analyses (Data S1). To generate variance-stabilized data for the principal component analysis (PCA), 'DESeq' package (Anders & Huber 2010) was used. The total number of reads mapping to the five reference OTUs was used as a sample size factor for each individual and variance-stabilizing transformation was performed using empirical dispersion estimates (function estimateDispersions, options sharingMode="gene-est-only"). The principal component analysis was performed using the library 'vegan' (Oksanen et al. 2013). The differences in OTU representation among species and sites were estimated jointly for all OTUs based on raw counts data using Poisson-lognormal generalized linear mixed model, following the methodology developed for quantitative PCR data (Matz et al. 2013). The model included fixed effects of OTU, OTU:species, OTU:site, and OTU:species:site, plus the scalar random effect of sample. The model was fitted using MCMCglmm function (Hadfield 2010). The results were extracted and visualized using HPDplotBygeneBygroup function from the MCMC qpcr package (Matz et al. 2013).

Results

STRUCTURE analysis detected genetic differences between the two coral species, but no divergence between locations for either of them (Fig. 2). Output files from STRUCTURE HARVESTER showed a delta K of two for all analyses except the independent analysis of *Orbicella faveolata* (n=73) which showed a delta K of three (Fig. S2). This result was confirmed by AMOVA analysis (Table 1). AMOVA results comparing F_{ST} between species and sites

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showed no significant genetic differentiation between the two host species collected at each site (Table 1).

ITS-2 sequencing yielded 170,349 raw reads for 58 individuals, averaging 2,937 reads per individual (Table 2). After removing all reads shorter than 290bp, 122,867 reads representing 20,260 unique sequences remained. Clustering the unique sequences yielded 153 OTUs. Mapping the original filtered reads to these 153 OTUs revealed that only five of the OTUs were detected in more than half of all coral individuals sequenced. Only these five OTUs, hereafter referred to as haplotypes, were analyzed further. Haplotype II was by far the most dominant accounting for 94% of all reads (Fig. 3). Generalized linear mixed modeling analysis revealed that haplotypes IV and V were significantly ($P_{MCMC}<0.001$) diminished at the west bank; moreover, haplotype V was significantly ($P_{MCMC}=0.002$) more diminished in *O. faveolata* than in *O. franksi* (Fig. 4 and Table 3).

All the five haplotypes best matched *Symbiodinium* clade B type B1 (JN 558059.1) (Pochon et al. 2012), recently identified as *Symbiodinium minutum* (AF 333511.1) (LaJeunesse et al. 2012). After trimming, haplotypes I and II matched B1 (JN 558059.1, AF333511.1) (LaJeunesse et al. 2012; Pochon et al. 2012) with 100% identity, whereas the remaining three haplotypes did not find an exact match in the database (Fig. S3 (Gouy et al. 2010)). Haplotype III differs from B1(LaJeunesse et al. 2012; Pochon et al. 2012) by a 13 base pair deletion. Haplotype IV differs from B1 (JN 558059.1, AF333511.1) (LaJeunesse et al. 2012; Pochon et al. 2012 by a ten base pair insertion. Haplotype V differs from B1 (JN 558059.1, AF333511.1) (LaJeunesse et al. 2012; Pochon et al. 2012) by a nine base pair deletion. These indels do not occur in homopolymer repeats and likely are not the result of sequencing error (Margulies et al. 2005).

The first component (PC1) from the PCA explained 40.83% of the variation and principle component two (PC2) explained 22.63% of the variation. Retaining the first two components meets Kaiser's criterion (Kaiser 1960), defined as all components with a standard deviation greater than one, and explain 63.46% of the variation. The samples were visibly partitioned with respect to the sampling locality along PC1 (Fig. 5).

Discussion

Host genotyping significance

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Nine recently developed microsatellite markers (Davies et al. 2013) were used for host genotyping to distinguish the two host species, *Orbicella faveolata* and *O. franksi*, since this species complex has been shown to hybridize (Budd & Pandolfi 2004; Fukami et al. 2004; Szmant et al. 1997). Multiple analyses were conducted to confirm that the two host species in this study do not include individuals showing evidence of recent introgression (Fig. 2, Table 1). Species in the *Orbicella annularis* complex vary considerably morphologically however genetically these differences are not as pronounced making species identification in the *Orbicella annularis* species complex challenging (Fukami et al. 2004). Multiple efforts were devoted to host genotyping to ensure the selected individuals were not potential hybrids as demonstrated by the advanced detection limits from these recently developed nine loci (Davies et al. 2013). Continued use of these nine loci, which contribute to eight previously developed microsatellite loci (Lopez et al. 1999; Severance et al. 2004), and continued efforts in high resolution marker development will advance detection limits to confidently assign species in the *O. annularis* species complex and ultimately lead to a better understanding of host connectivity patterns (Davies, S. unpublished data).

Monotypic symbiont population at FGB

In this study, deep amplicon sequencing was used to detect *Symbiodinium* species diversity within *O. faveolata* and *O. franksi* at east and west FGB using ITS-2. Both *Orbicella* species hosted clade B type B1, the most prevalent *Symbiodinium* type within the Caribbean (Baker 2003; LaJeunesse 2002; LaJeunesse et al. 2003). Interestingly other assessments of *Symbiodinium* diversity in *Orbicella* species throughout the Caribbean have shown mixed populations of species ranging from clade A to clade D (Rowan & Knowlton 1995; Rowan et al. 1997; Thornhill et al. 2006; Toller et al. 2001). A variety of environmental factors have been proposed to explain *Symbiodinium* distributions, including but not limited to depth, irradiance levels, latitudinal location and temperature. Our results for FGB *Oribicella* species show an exclusive specificity for *Symbiodinium* clade B, which parallels findings of fewer mixed infections in corals from deeper environments (LaJeunesse 2002). Corals from the FGB likely experience lower thermal minimums relative to the rest of the Caribbean (Schmahl et al. 2008; Thornhill et al. 2008) and these corals represent the northernmost latitudinal reef in the Gulf of Mexico (LaJeunesse & Trench 2000). However, we acknowledge use of faster evolving loci, such as microsatellites, may reveal more fine scale genetic diversity within ITS-2 clade B

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between the two collected coral host species and geographic locations (Finney et al. 2010; Pettay & LaJeunesse 2007; Santos et al. 2004).

Symbiodinium variation between two geographic locations

We present results for a comprehensive genotype analysis of both host *Orbicella* species and resident *Symbiodinium*. Our results showed little genetic divergence between the two coral host species *O. faveolata* and *O. franksi* and a monotypic *Symbiodinium* population of only clade B type B1. Previous studies have shown strong genetic structuring in *Symbiodinium* communities and in host species across different habitat types (Bongaerts et al. 2010). As a consequence, we hypothesize the monotypic *Symbiodinium* species seen at the FGB for *O. faveolata* and *O. franksi* do not show more diverse populations because of the lack of genetic divergence at the host level and the similar environmental conditions at both banks.

Furthermore, our results did show that within *Symbiodinium* type B1 haplotypes IV and V were significantly diminished at the west FGB. Additionally, haplotype V was significantly more diminished in *O. faveolata* compared to *O. franksi*. This result is interesting since the east and west FGB are only separated by 19 kilometers and experience similar environmental conditions (Schmahl et al. 2008). Previous studies have shown strong genetic partitioning of host and symbionts across habitats (Bongaerts et al. 2010) suggesting that *Symbiodinium* genotype affects host physiology (DeSalvo et al. 2010). Though physiological contributions of host and *Symbiodinium* populations were outside the scope of this study, we do show the significance of accurately detecting low frequency *Symbiodinium* genotypes to contribute to understanding the distributions of local community assemblages and how *Symbiodinium* genotypes affect host physiology.

Potential roles of mesophotic reefs

The roles of mesophotic reefs, reefs between 30 and 150 meters (Lesser et al. 2010), remain understudied. Previous studies suggest mesophotic reefs may supply host larvae for shallow water reef systems (Lesser et al. 2009). There is increasing interest to investigate possible connectivity patterns between shallow and deep reefs to understand the roles and ecology of deep ranging hosts and *Symbiodinium* genotypes from mesophotic coral ecosystems (Kahng et al. 2014; Lesser et al. 2009; Lesser et al. 2010). The FGB are one example of an understudied mesophotic reef, likely due to its isolated location and depth. However, the FGB has reduced anthropogenic influences, fewer recorded bleaching events and minimal total cover

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loss relative to other Caribbean reefs since monitoring began in the 1970s (Hickerson & Schmahl 2005). This presents a unique location for future studies to assess species diversity, correlate environmental factors with *Symbiodinium* distributions and investigate roles of mesophotic reefs. The pristine and undisturbed conditions at the FGB may suggest the unique host-algal genotype combinations seen at the FGB between *Symbiodinium minutum* and coral hosts *O. faveolata* and *O. franksi* may be combinations that have been maintained over many generations. Their potential roles for shallow water reefs and connectivity patterns to other Caribbean reefs are an area of future research.

Plasticity of symbiosis

Two mechanisms have been postulated to explain the plasticity of symbiosis between host and symbiont termed "shuffling" and "switching". "Shuffling" is a change in the existing proportions of a mixed *Symbiodinium* infection whereby a dominant symbiont type may become reduced while a background, or cryptic, symbiont type becomes increasingly prevalent (Berkelmans & van Oppen 2006; Fay & Weber 2012; LaJeunesse et al. 2009; Rowan et al. 1997; Silverstein et al. 2012; Stat et al. 2006). "Switching" is when new exogenous *Symbiodinium* are acquired as the dominant type, also known as an "open" symbiotic system (Baker 2001; Buddemeier & Fautin 1993). In order to assess whether corals "switch" or "shuffle", we must consistently and confidently detect cryptic *Symbiodinium* diversity. Use of a quantitative molecular genotyping approaches with high sensitivity will allow us to assess distribution patterns of *Symbiodinium*-host relationships ranging from global scales over regional to individual reef scales. By doing so, it will also become more feasible to examine changes in *Symbiodinium* composition over time and detect species shuffling as well as potential horizontal uptake with more fine spatio-temporal resolution. This presents an anticipative future for contributing to cumulative databases of *Symbiodinium* types.

Using deep amplicon sequencing to detect species diversity

Multiple efforts were made to avoid including PCR and sequencing errors (Kenkel et al. 2013; Quigley, KM 'unpublished data'). The two-step barcode approach reduces PCR bias by using as few cycles as possible (Berry et al. 2011). By annealing unique barcodes to each individual we pooled up to thirty individuals making this protocol high-throughput with reduced cost. We pooled equal representations of each individual after assigning barcodes to increase the likelihood of equal coverage across individuals. Quigley, KM ('unpublished data') verified

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sensitivity down to 0.1% with an increased target minimum coverage of 10,000 reads per individual. This protocol utilizes one set of barcoded primers that allows the detection of fine scale proportions of *Symbiodinium* diversity within all clades. An additional advantage of this technique is no a priori knowledge of *Symbiodinium* species diversity is required. There is an initial upfront cost associated with barcoded primers, however this method will become increasingly more high-throughput and cost effective as Illumina releases more tags and read lengths increase. We can now investigate *Symbiodinium* diversity by multiplexing multiple loci into a single Illumina lane. This method appears to be high-throughput, cost effective and reproducible capable of detecting low frequency species in a sample with a mixed *Symbiodinium* population (Kenkel et al. 2013; Quigley, KM 'unpublished data'). Future studies can apply this method to investigate other members of the coral holobiont (Rohwer et al. 2002), such as other algae, fungi, protists, bacteria, archaea, viruses.

Limitations of deep amplicon sequencing

While the sensitivity of using deep amplicon sequencing to detect species diversity offers many advantages caution should be applied, as deep amplicon sequencing does not detect functional versus non-functional haplotypes. Our study identified three unique B1 ITS-2 types. Given the abundance of these haplotypes across both species and geographic locations, we believe that these haplotypes are natural sequences likely specific for the FGB. However, we carefully hypothesize that these haplotypes might be prospective pseudogenes maintained in the populations (Thornhill et al. 2007). It is unlikely that these indels result from sequencing errors since they are not in homopolymer repeats (Margulies et al. 2005). We acknowledge this protocol does not overcome the use of a multi-copy marker undergoing concerted evolution such is the case of ribosomal sequences (Koch et al. 2003; Thornhill et al. 2007). Given unknown whole and partial genome duplication events in *Symbiodinium* some of these reference haplotypes could potentially come from the same genome (Hou & Lin 2009). Empirical analyses may predict copy numbers but do not provide conclusive results for inter versus intra-genomic haplotypes. Future users should use caution when assigning haplotypes within *Symbiodinium* clades to reference sequences to avoid over estimating species diversity.

Conclusions

In our study we apply deep amplicon sequencing to assess *Symbiodinium* diversity at the remote Flower Garden Banks. Results show coral hosts *Orbicella faveolata* and *O. franksi*

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uniquely harbor *Symbiodinium* type B1, however three possible endemic haplotypes were also detected. Two of these haplotypes were significantly diminished at the west FGB, one of which was also significantly diminished more in *O. faveolata* compared to *O. franksi*. Future work using faster evolving loci, such as microsatellites developed for *Symbiodinium*, may show variations between host species or geographic locations within clade B lineages. Continued use of deep amplicon sequencing, not only with ITS-2 but with additional loci, to assess *Symbiodinium* species diversity within multiple hosts will generate a better understanding of these complex community assemblages.

Acknowledgements

We acknowledge personnel at the FGBNMS (E. Hickerson & G.P. Schmahl) for permits (FGBNMS-2009-005-A2, A3) and boat time. We also acknowledge Michele Weber and Anke Kleuter for assistance editing and their expertise in *Symbiodinium* genetic diversity, Bishoy Kamel for bioinformatics support and Dr. Scott Hunicke-Smith and staff at the Genomics Sequencing and Analysis Facility at University of Texas at Austin for efficiently sequencing our submission and providing technical support.

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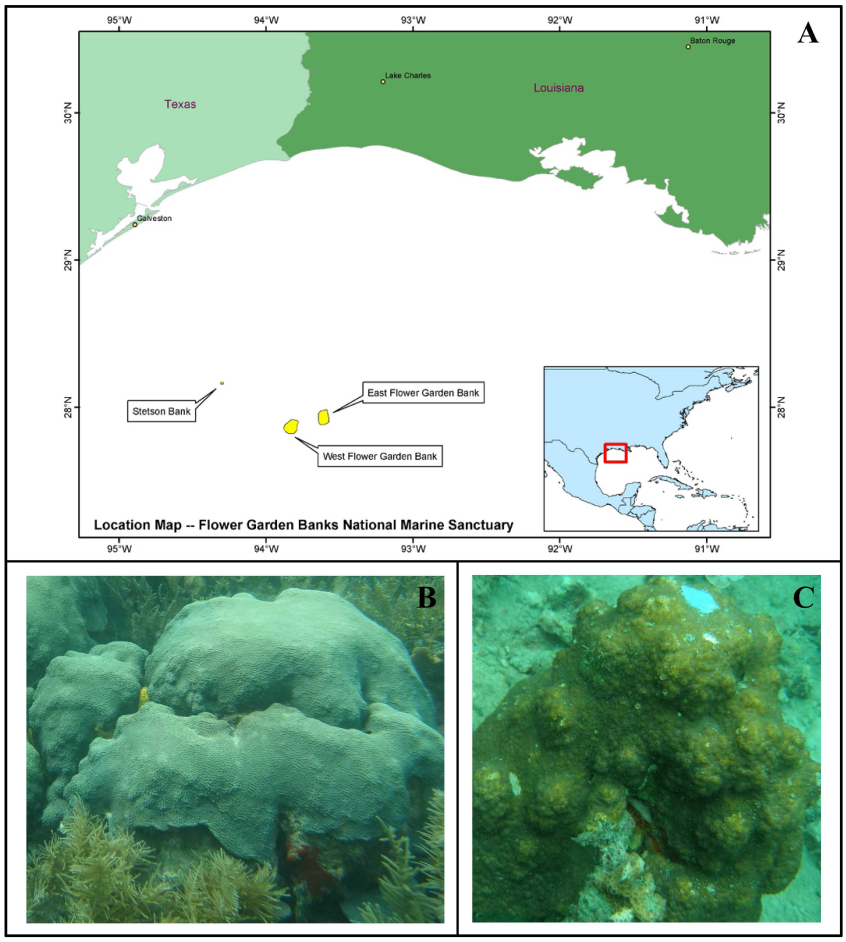

Figure 1: The general location of the Flower Garden Banks and pictures of coral species. A. Location of Flower Garden Banks National Marine Sanctuary, Gulf of Mexico (27°54' N, 93°35'W for east Flower Garden Banks and 27°53'N, 93°49'W west Flower Garden Banks) Credit: USGS (http://pubs.usgs.gov/of/2003/of03-002/html/FGB_figs.htm) **B.** *Orbicella faveolata* from Panama, Credit: Mónica Medina **C.** *Orbicella franksi* from Panama, Credit: Mónica Medina

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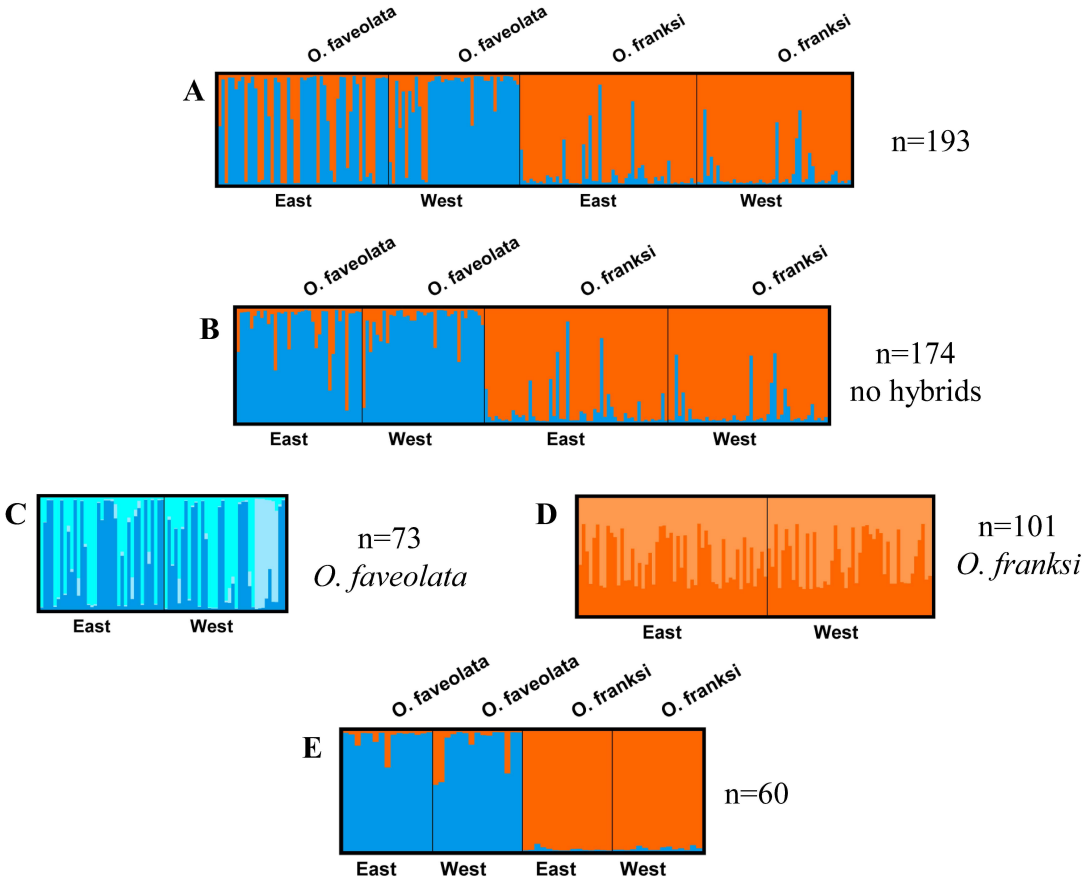

Figure 2: DISTRUCT plots of all STRUCTURE analyses. DISTRUCT plots from STRUCTURE for K=2 except where noted A. All samples from *Orbicella faveolata* and *Orbicella franksi* in east and west Flower Garden Banks National Marine Sanctuary, Gulf of Mexico (n=193) **B.** Same as A but potential hybrids removed (n=174) **C.** *Orbicella faveolata* only with potential hybrids removed (n=73, K=3) and **D.** *Orbicella franksi* only with potential hybrids removed (n=101) **E.** The selected 60 *Orbicella faveolata* (n=30) and *Orbicella franksi* (n=30)

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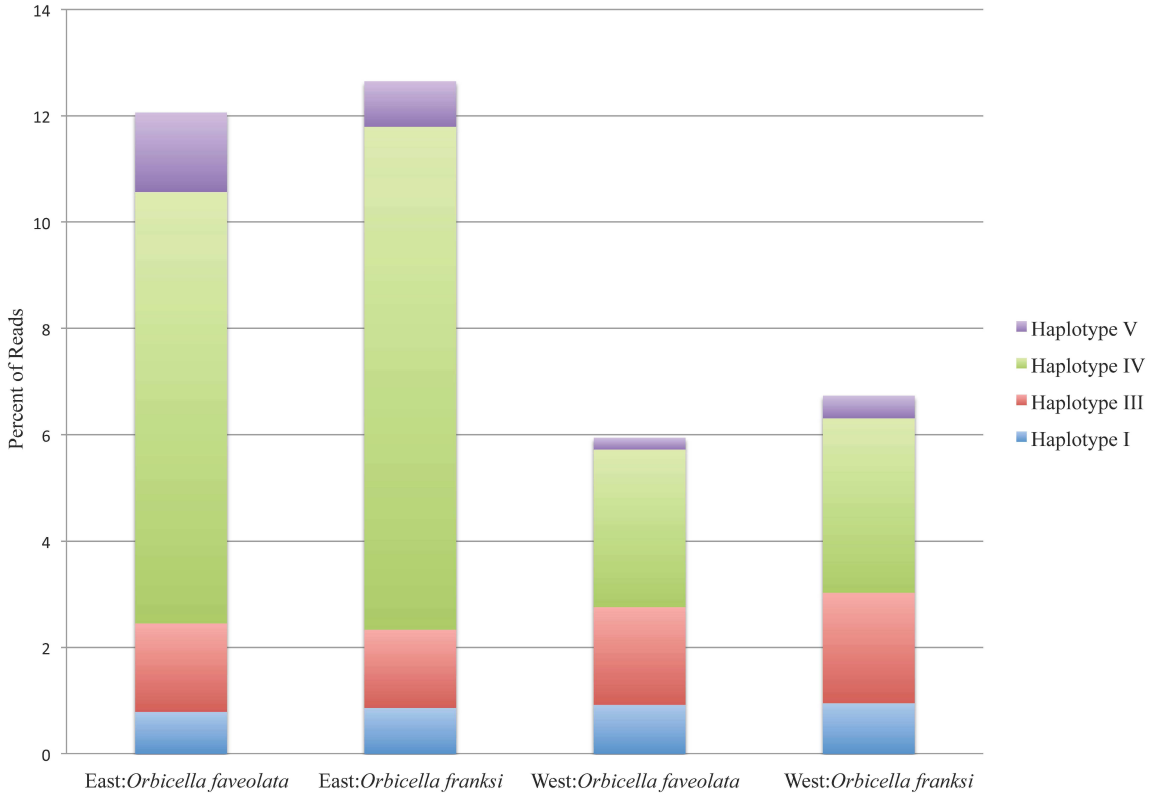

Figure 3: The percentage of reads for the four minor haplotypes by geographic location and species. Percentage of minor *Symbiodinium* B1 haplotypes by geographic location and coral species. Only haplotypes I, III, IV, V are shown. The dominant *Symbiodinium* B1 haplotype II, used 93.26% across all individuals, is not shown. (East: *Orbicella faveolata* = 27,121 sequences, East: *Orbicella franksi* = 40,078 sequences, West: *Orbicella faveolata* = 26,143 sequences, West: *Orbicella franksi* = 27,376 sequences)

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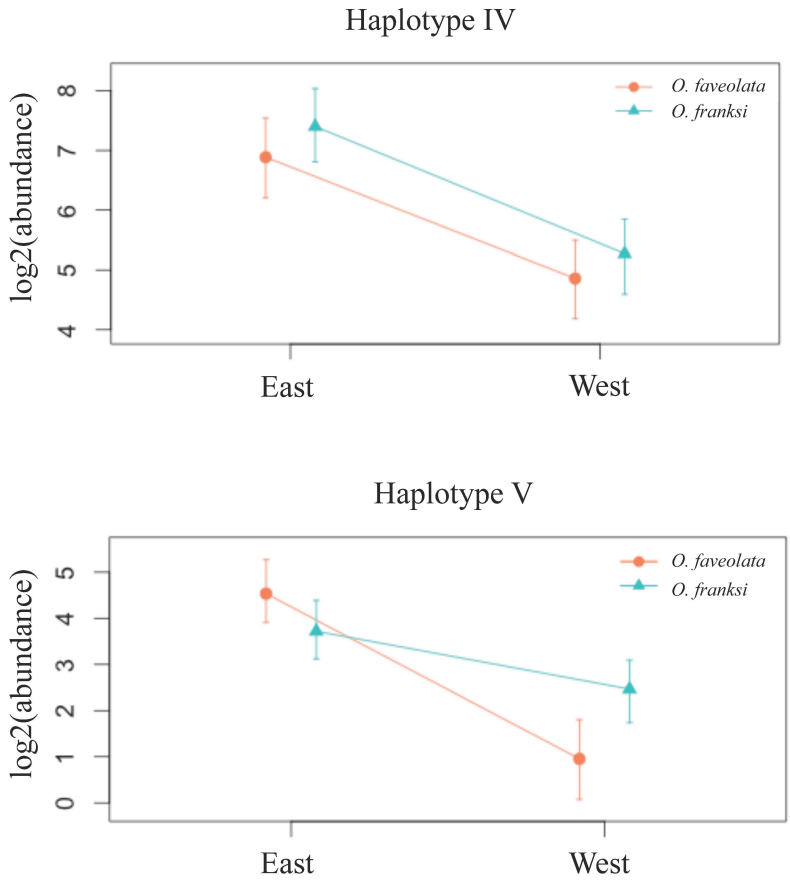

Figure 4: Distribution of abundance of two haplotypes significant by site. Distribution of abundance (log2 transformed) of *Symbiodinium* type B1 haplotypes IV and V in east and west Flower Garden Banks, Gulf of Mexico from the Poisson-lognormal model. Circles indicate *Orbicella faveolata*. Triangles indicate *Orbicella franksi*. Haplotypes I, II and III did not have significant effects, not shown.

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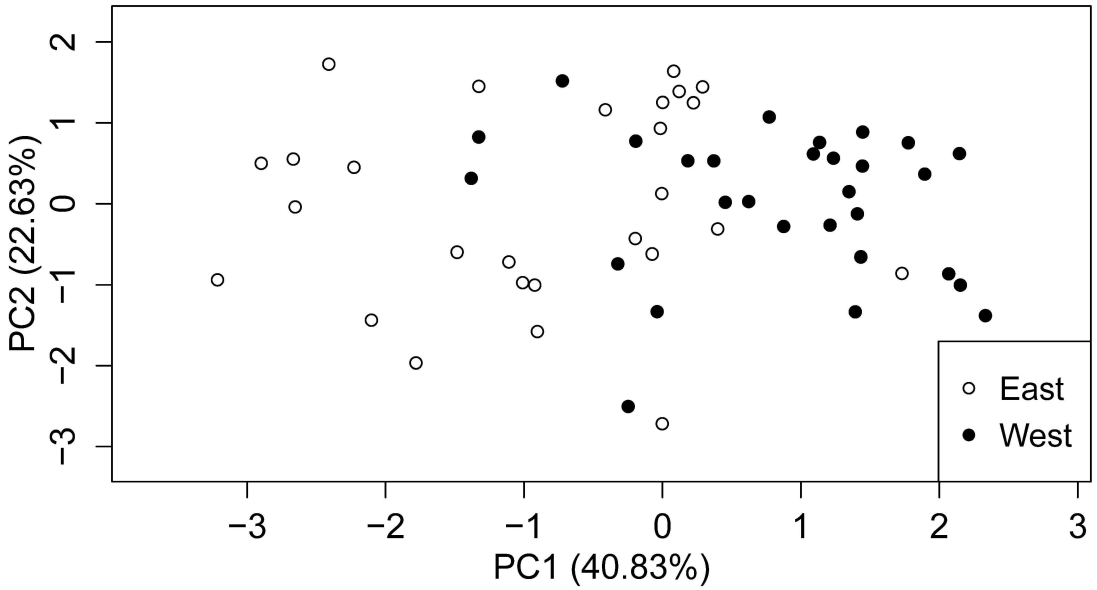

Figure 5: Principle Components Analysis (PCA) showing the first two principle components. PCA plot of variance stabilized transformed (VST) data from a count data set showing partitioning of samples by geographic location. Principle component 1 (PC1) explains 40.83% of the variation and principle component 2 (PC2) explains 22.63% of the variation (n=56).

	F_{ST}	*p-value*
O. faveolata vs *O. franksi*	0.069	0.001
O. franksi East vs West	0	0.529
O. faveolata East vs West	0.009	0.016

Table 1: Analysis of Molecular Variance (AMOVA) Fixation index (F_{ST}) values. Analysis of Molecular Variance (AMOVA) Fixation index (F_{ST}) values showing no genetic differentiation among *Orbicella faveolata* and *Orbicella franksi*, among *Orbicella faveolata* within the two geographic locations or among *Orbicella franksi* within the two geographic locations.

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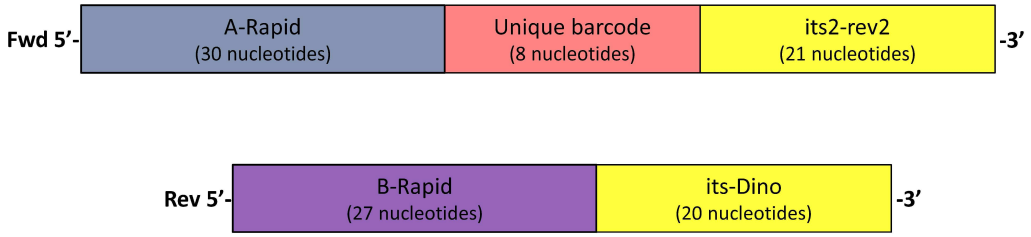
	Raw Read Number	Trimmed Reads	Mapped Reads	Mapping Efficiency
East	95,478	68,670	68,637	100%
West	74,871	54,197	54,175	100%
O. faveolata	74,840	53,938	53,913	100%
O. franksi	95,509	68,929	68,899	100%
TOTAL	170,349	122,867	122,812	100%

Table 2: The sequencing coverage and mapping efficiency by geographic location and species. Summary of sequence coverage ITS-2 amplicon sequencing of Flower Garden Banks, Gulf of Mexico. Individuals are sorted by geographic location and species using the 454 GS FLX platform.

	posterior mean	lower 95% CI	upper 95% CI	effective sample size	*p-value* MCMC
Haplotype IV:West	-1.407299	-2.0212	-0.698313	1059.1	<0.001
Haplotype V:West	-2.486064	-3.26447	-1.698435	719.1	<0.001
Haplotype V:West:*O.franksi*	1.611213	0.650416	2.66628	811.4	<0.001

Table 3: The significant Markov Chain Monte Carlo Generalized Linear Model results. Only showing significant results from Poisson-lognormal Generalized Linear Models (GLMs). Haplotypes IV and V are significantly diminished at the west bank compared to the east bank (P_{MCMC}<0.001). Haplotype V is also significantly more diminished in *Orbicella faveolata* than in *Orbicella franksi* (P_{MCMC}=0.002).



Supplementary Figure 1: The primer design to uniquely barcode individuals. Rapid-barcode primer design annealed in second PCR to uniquely identify individuals and pool.

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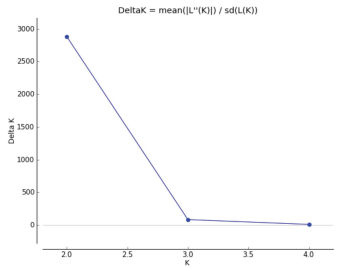
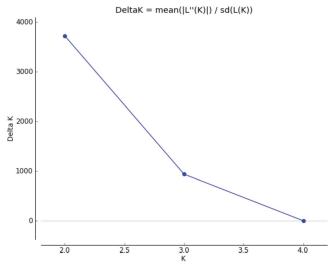
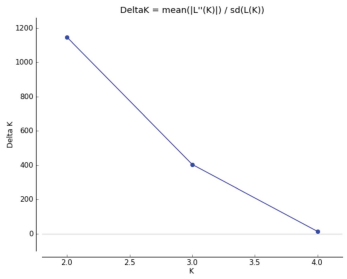


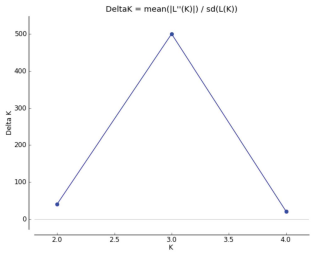
193 individuals



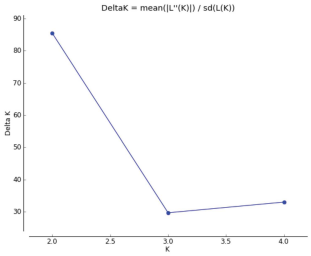
174 individuals



60 selected individuals



Orbicella faveolata n=73



Orbicella franksi n=101

Supplementary Figure 2: The delta K figures from STRUCTURE HARVESTER. Delta K figures from STRUCTURE HARVESTER from STRUCTURE analysis for all collected individuals (n=193), with potential hybrids removed (n=174), the selected 60 individuals, *Orbicella faveolata* (n=73) and *Orbicella franksi* (n=101).

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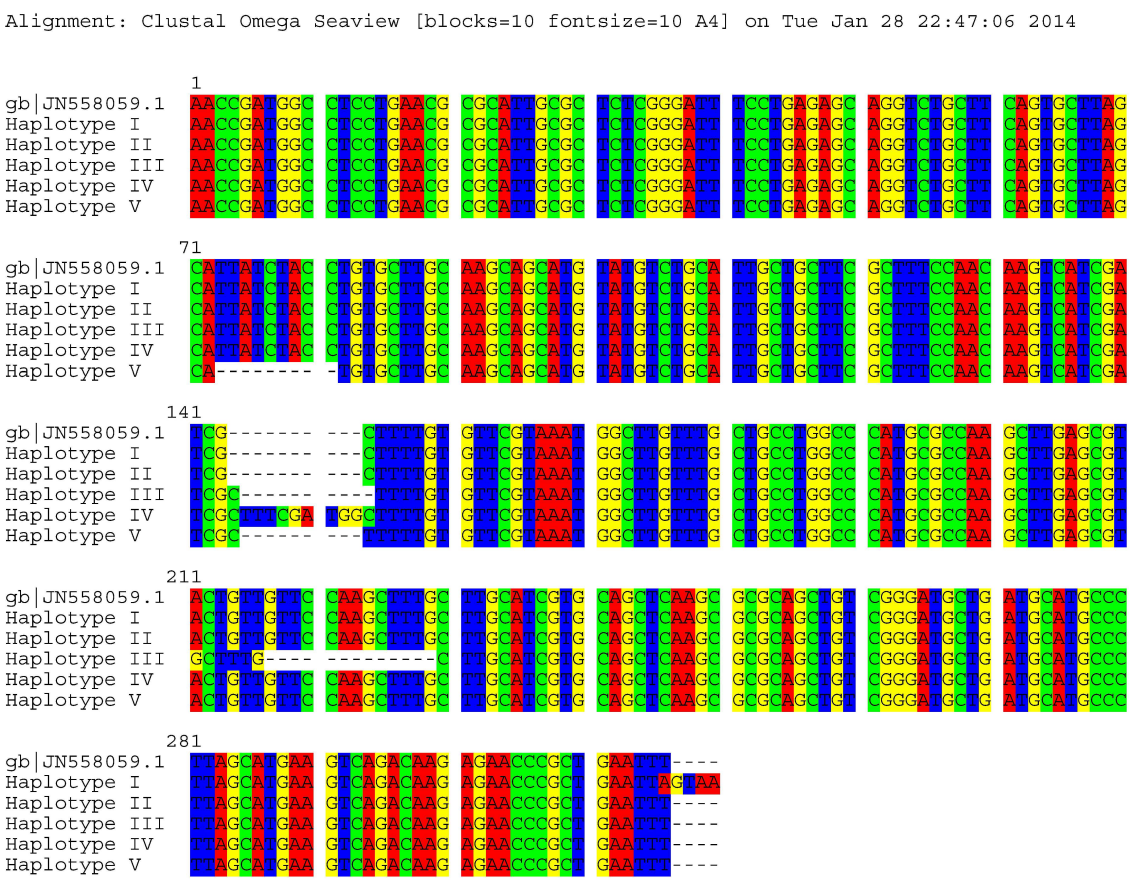

Supplementary Figure 3: The Clustal alignment of five Flower Garden Bank *Symbiodinium* haplotypes and previously published *Symbiodinium* B1. Clustal Omega alignment of five reference haplotypes displayed in SeaView and the previously published *Symbiodinium* B1 (JN 558059.1).

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