

# Genome-wide survey of single-nucleotide polymorphisms reveals fine-scale population structure and signs of selection in the threatened Caribbean elkhorn coral, *Acropora palmata* (#18713)

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# Genome-wide survey of single-nucleotide polymorphisms reveals fine-scale population structure and signs of selection in the threatened Caribbean elkhorn coral, *Acropora palmata*

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The advent of next-generation sequencing tools has made it possible to conduct fine-scale surveys of population differentiation and genome-wide scans for signatures of selection in non-model organisms. Such surveys are of particular importance in sharply declining coral species, since knowledge of population boundaries and signs of local adaptation can inform restoration and conservation efforts. Here, genome-wide surveys of single-nucleotide polymorphisms in the threatened Caribbean elkhorn coral, *Acropora palmata*, reveal fine-scale population structure and place the major barrier to gene flow that separates the eastern and western Caribbean populations between the Bahamas and Puerto Rico. The exact location of this break had been subject to discussion because two previous studies based on microsatellite data had come to differing conclusions. We investigate this contradiction by analyzing an extended set of 12 microsatellite markers including the five previously employed and discovered that one of the original microsatellite loci is apparently under selection. Exclusion of this locus reconciles the results from the SNP and the microsatellite datasets. Scans for outlier loci in the SNP data detected 12 candidate loci under positive selection. Together, these results suggest that restoration of populations should use local sources and utilize existing functional variation among populations in *ex situ* crossing experiments to improve stress resistance of this species.

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# Abstract

The advent of next-generation sequencing tools has made it possible to conduct fine-scale surveys of population differentiation and genome-wide scans for signatures of selection in non-model organisms. Such surveys are of particular importance in sharply declining coral species, since knowledge of population boundaries and signs of local adaptation can inform restoration and conservation efforts. Here, genome-wide surveys of single-nucleotide polymorphisms in the threatened Caribbean elkhorn coral, *Acropora palmata*, reveal fine-scale population structure and place the major barrier to gene flow that separates the eastern and western Caribbean populations between the Bahamas and Puerto Rico. The exact location of this break had been subject to discussion because two previous studies based on microsatellite data had come to differing conclusions. We investigate this contradiction by analyzing an extended set of 12 microsatellite markers including the five previously employed and discovered that one of the original microsatellite loci is apparently under selection. Exclusion of this locus reconciles the results from the SNP and the microsatellite datasets. Scans for outlier loci in the SNP data detected 12 candidate loci under positive selection. Together, these results suggest that restoration of populations should use local sources and utilize existing functional variation among populations in *ex situ* crossing experiments to improve stress resistance of this species.

# Introduction

There is an ongoing debate about the importance of local recruitment and barriers to gene flow in marine species. Many marine species reproduce via planktonic larvae and strong ocean currents have the potential to carry propagules over long distances. However, genetic evidence has revealed a high degree of self-recruitment in a range of species with planktonic larval duration being a poor predictor of genetic structure (Selkoe & Toonen 2011). The development of cheap genome-scale genotyping is poised to open a new chapter in this discussion (Peterson et al. 2012a; Toonen et al. 2013; Wang et al. 2012). American eels for example show panmixia in their central breeding ground in the North Atlantic but single nucleotide polymorphism (SNP) genotyping of adults along the Eastern seaboard revealed local differentiation (Gagnaire et al. 2012). Thus, a well-mixed pool of larvae sorted into environmental niches resulting in a structured adult population.

Resolving significant population genetic structure indicative of predominantly local recruitment is of particular significance for *in situ* restoration efforts targeting declining populations of reef-building corals (Baums 2008 - 2011; Epstein et al. 2001; Griffin et al. 2015; Griffin et al. 2012; Rinkevich 2006; Schopmeyer et al. 2012). Restoration genetic best practices suggest that propagules should not be moved among genetically distinct populations to avoid outbreeding depression (Baums 2008 - 2011) and this approach has been adopted by some permitting agencies (e.g. the Florida Keys National Marine Sanctuary). On the other hand, crosses between genotypes from different populations may show heterosis with respect to environmental stressors as seen in the hybrid *Acropora prolifera* (Fogarty 2012) and therefore might be worth exploring in an *ex situ* setting.

Diversity within functional regions of the genome that may be under selection (those regions that code for proteins or regulate transcription of genes), are not commonly surveyed even though it is these regions of the genome that are of interest to conservation managers who want to understand how much capacity there is in a species to adapt to changing conditions (Becks et al. 2010). Statistical methods have been developed that allow scanning of SNP loci for signatures of selection. Despite the risk of generating false positive results (Vilas et al. 2012), these methods yield candidate loci that should be substantiated by further testing to be of functional significance. The same methods can be used to scan microsatellite loci for signatures of selection, however, power is often limited by the small number of assayed loci.

SNPs are ubiquitous throughout the genome, located in coding and non-coding regions, and each locus has a maximum of four alleles (the four bases). This is in contrast to microsatellite loci that consist of tandem repeats, in which allelic variation is determined by the number of tandem repeats and thus can be large. The limited number of alleles at each SNP locus requires a larger number of loci to be assayed to achieve the same power of detecting population genetic structure as a panel of microsatellite loci (Morin et al. 2009; Ryman et al. 2006). The advent of reduced representation sequencing methods have made it possible to develop and assay a large number of SNP loci at a reasonable cost (Altshuler et al. 2000; Hoffberg et al. 2016). Recently, Genotyping by Sequencing (GBS) data including 4,764 SNPs in *A. cervicornis* identified population structure within the Florida Reef tract (Drury et al. 2016b; Willing et al. 2012). Other flavors of reduced representation sequencing methods (Toonen et al. 2013; Wang et al. 2012) have yielded information on population structure, loci under selection and genetic diversity in reef building corals (Drury et al. 2016b; Howells et al. 2016a)

*Acropora palmata* is one of a few Caribbean coral species whose population genetic structure has been thoroughly investigated on local and range-wide scales (Baums et al. 2014a; Baums et al. 2005b; Baums et al. 2006a). A range-wide survey of *A. palmata* population genetic structure using five coral specific polymorphic microsatellite markers showed that *A. palmata* stands are structured into two long-separated populations (Baums et al. 2005a). While most reefs are self-recruiting, *A. palmata* stands are not inbred and harbor high genetic diversity at microsatellite loci (Baums et al. 2005b). Bio-physical modeling identified a transient feature in the Mona Passage important in restricting present-day gene flow between the eastern and western population (Baums et al. 2006b). However, it is unclear whether the eastern and western populations differentiated initially due to selection. Subsequent denser sampling of *A. palmata* along the Antilles Island Arc raised the possibility of a hybrid zone across Puerto Rico rather than a clear-cut break between the eastern and western Caribbean at the Mona Passage (Mège et al. 2014).

The east-west population divide or possible finer scales of population differentiation were tested in this study by developing a large number of SNP markers to obtain a more comprehensive estimate of genetic differentiation across the genome and compare them to a set of microsatellite loci (Baums et al. 2009). The second goal was to screen SNP loci for signatures of selection. We developed genome-wide SNPs and assayed them in archived samples from two



regions in the western *A. palmata* population (Bahamas and Florida) and two regions in the eastern population (Puerto Rico and the U.S. Virgin Islands (USVI)). We then compared the results to population structure derived from ten and eleven microsatellite loci.

# Materials & Methods

## SAMPLE COLLECTION

Colonies of *A. palmata* were collected between 2002 and 2010 and previously genotyped (Baums et al. 2014a; Baums et al. 2005b). Unique genets were selected from our database for a total of 24 samples from each of four regions; the Bahamas, Florida, Puerto Rico and the US Virgin Islands (USVI). The goal was to have eight samples from three different reefs within each region, however this was not always possible either due to a small sample sizes from a particular reef or low clonal diversity of a reef. In those cases, we selected additional unique genets from nearby reefs. See Table 1A for detailed sample information.

We used an extended set of samples to compare the population genetic structure ascertained via microsatellite genotyping to the SNP results. This extended set of samples included 260 samples from six regions; Belize, Florida, Puerto Rico, the USVI, and Curacao (Table 1B).

## LIBRARY PREPARATION

Coral tissue samples were extracted from ethanol preserved samples using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) with the following modifications. Time of incubation in the extraction buffer was increased to 16-20 hours and two 100 µl elutions were performed, the second of which was kept for library production as this fraction contained the high molecular weight DNA. Extracted DNA was then treated with 0.01 mg of RNase A (10 mg/ml, Amresco Solon, OH). Extraction concentrations ranging from 500 ng to 6 µg were double-digested with 10 units of each of the restriction enzymes MluCI (^AATT) and NlaIII (CATG^) (New England Biolabs, Ipswich, MA) following the protocol described by Peterson et al. (2012a). Digestions were purified using 1.5X Ampure beads (Beckman Coulter Inc, Brea, CA) and quantified on a Qubit® fluorometer (Life Technologies, Carlsbad, CA). Digested DNA was standardized to 100 ng for each sample before adaptor ligation. Samples were identified with eight 6-bp indices on the NlaIII (rare-cutter) P1 adapter (Supplementary Table 1). Samples were pooled into 12

libraries and then size selected in the range of 200-800 bp on a Pippin-Prep (Sage Science, Beverly, MA). Next, Illumina flow-cell annealing sequences, unique multiplexing indices and sequencing primer annealing regions were added through PCR amplification to the MluCL cut end (See (Peterson et al. 2012b), Protocol S1, Figure 1). The libraries were enriched with 12 amplification cycles in four separate PCR reactions for each library containing 10 µl of Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, Ipswich, MA), 2 µl of each amplification primer, 1 µl of library DNA and 5 µl of water (total 20 µl). Samples were pooled into four libraries each containing 24 samples (Table 2). Each library was sequenced on one lane of Illumina HiSeq 2000 sequencer (paired-end, 2x150 bp) at the Pennsylvania State Genomics Core Facility. There were two libraries sequenced on each chip.

#### RAW SEQUENCE FILTERING

Raw sequence reads were filtered using the process\_radtags in the pipeline STACKS 1.21 (Catchen et al. 2013; Catchen et al. 2011). Barcodes and the RAD-Tag cut sites were identified to de-multiplex the pooled data into individual samples. Reads were discarded that had low quality (with an average raw phred score <10 within a 15-base pair sliding-window), adapter contamination, and uncalled bases. Since all indices differed by at least 2 bp, it was possible to correct and retain any index that differed by a single bp from an expected index.

#### ASSEMBLY

Processed sequences were then aligned to the *Acropora digitifera* genome (V1.0) (Shinzato et al. 2011) with BOWTIE2 (Langmead & Salzberg 2012) within the GALAXY (Bedoya-Reina et al. 2013; Blankenberg et al. 2014) framework using end-end read alignment settings in order to remove symbiont and other associated microorganisms. After alignment, paired-end sequencing BAM files were assembled in the ref\_map.pl pipeline in STACKS 1.30 with the following parameters. Each paired-end sequencing set was run separately through STACKS to compare results (designated Read1 and Read2). The number of raw reads required to report a stack was m=5. The number of mismatches allowed between loci when building the catalog was n=4. SNPs with a log-likelihood of less than -10 were removed as reads with poor log-likelihoods tend to have sequencing error and/or low coverage. Two of the barcodes (TCGAT and CGATC) had a very low amount of sequence reads across all four populations, all Illumina lanes were affected, and those samples were removed before assembly in STACKS.

# GENOME COVERAGE

BEDTOOLS (Quinlan & Hall 2010) was used to create a histogram of genome coverage for each sample from the BOWTIE2 BAM format alignment files. All positions with a depth of coverage greater to or equal to 20 were combined into a single bin in the histogram. Regions were averaged (excluding samples with barcodes TCGAT and CGATC) and a cumulative distribution of sequencing coverage was then plotted in SIGMAPLOT v12.

# POPULATION GENETIC STATISTICS

More stringent filtering was implemented in the Populations module in STACKS 1.30  where a locus had to be present in at least 60% of the individuals within a population and had to be present in all four populations in order to be processed for  $F_{ST}$  calculations and outlier analysis. A minor allele frequency (MAF) cutoff of 0.05 was applied. A  $p$ -value correction was applied to  $F_{ST}$  scores, so that if a  $F_{ST}$  score is not significantly different from 0 (according to Fisher's Exact Test) the value was set to 0. Additionally, only one random SNP from any RAD locus was written to the STRUCTURE export file in order to prevent linked loci from being processed. Read 1 and Read 2 STRUCTURE export files were combined and duplicate loci removed randomly between reads. An Analysis of Molecular Variance (AMOVA) corrected  $F_{ST}$  ( $p$ -value<0.05) was calculated within STACKS.

# CLUSTERING ANALYSES

Clustering analyses were performed in the program STRUCTURE 2.3.4 (Falush et al. 2003; Hubisz et al. 2009) using the admixture model with correlated allele frequencies. The analysis included the following parameters: 100,000 burn-in iterations and 1,000,000 Markov chain Monte Carlo repetitions, with and without a population prior, for a total of three replicates for each value of K. K values ranged from 2 to 5. The most likely value for K was determined by CLUMPAK (KOPELMAN ET AL. 2015B) BEST K which uses  $LN(PR(X|K))$  to identify the K for which  $PR(K=K)$  is the highest as described in STRUCTURE's manual section 5.1. Results of the three structure runs were merged with CLUMPAK (Kopelman et al. 2015a).

Previously genotyped samples at 10 and 11 (n=260) microsatellite markers (181, 182, 192, 207, 0585, 0513, 2637, 007, 9253, 5047, with and without locus 166) (Baums et al. 2009; Baums et al. 2005a) were also analyzed with STRUCTURE 2.3.4 (Falush et al. 2003; Hubisz et al. 2009) using the admixture model with correlated allele frequencies (See Table 1 for sample

information). The analysis included the following parameters; 100,000 burn-in iterations and 1,000,000 MCMC repetitions, with and without a population prior, for a total of 3 replicates for each value of K. K values tested ranged from 2 to 7.

# MANTEL TESTS

Data on temperature, salinity, dissolved oxygen (ml/l), and phosphates was downloaded from the World Ocean Atlas 2013 (WOA13 V2). Silicates and nitrates were not used as there was not sufficient data for all locations. For the Bahamas, Puerto Rico, and the USVI the geographic center point among several sampling sites was used because reefs were further apart than in Florida. For all data, the statistical mean of the annual average of years 1955-2012 and depths of 0-10 m was used. Grid sizes were 1/4° for temperature and salinity, and 1° for dissolved oxygen (ml/l), and phosphates (μmol/l) (Supplemental Table 2). SPSS V22 was used to calculate a dissimilarity matrix expressed as the Euclidean distances between regions based on the above environmental data. GenAlEx v6.501 (Peakall & Smouse 2006) was used to calculate a pairwise geographic distance matrix between the four regions and to perform a Mantel multi-comparison test between the geographic distance matrix,  $F_{ST}$  pairwise matrix between regions from STACKS, and the environmental dissimilarity matrix.

# OUTLIER ANALYSIS

Two independent methods were used to identify putative loci under selection. The first program used was LOSITAN (Antao et al. 2008) which utilizes the method of Beaumont and Nichols (1996) to identify loci under selection based on the joint distributions of expected heterozygosity and  $F_{ST}$  under an island model of migration. The following settings were used for the SNP and the microsatellite datasets. The neutral mean setting was selected in which during an initial run (100,000 simulations), a candidate subset of selected loci (outside the 95 % confidence interval) were identified and removed. Then the distribution of neutral  $F_{ST}$  was computed using 100,000 simulations and a bisection approximation algorithm (Antao et al. 2008), with the following options, force mean  $F_{ST}$ , infinite alleles mutation model, and a confidence interval 0.99. A FDR < 0.1 correction for multiple testing was applied. Loci outside the upper and lower confidence areas were identified as candidates affected by positive and balancing selection, respectively. All populations were analyzed together. The positive outlier loci were blasted against the NCBI nr, UniProt, and Trembl databases with parameters of

expected value = 0.00001, gap opening penalty = 11, gap extension penalty = 1, length of initial exact match (word size) = 6 and scoring matrix = BLOSUM62 using BLASTX 2.2.32+ (Altschul et al. 1997).

The STACKS exported GENEPOP dataset was also reformatted with PGDSPIDER version 2.0.5.2 (Lischer & Excoffier 2012) to a GESTE file. The method of Foll and Gaggiotti (2008) was performed using BAYESCAN 2.0 (<http://www-leca.ujf-grenoble.fr/logiciels.html>). For each locus, the probability of being under selection was inferred using the Bayes factor (BF). Based on Jeffreys' (1961) (Jeffreys 1961) scale of evidence, a log10 BF of 1.5–2.0 is interpreted as “strong evidence” of selection. For our analysis, the estimation of model parameters was set as 20 pilot runs of 5,000 iterations each, followed by 50,000 iterations.

## Results

### Summary statistics

Illumina sequencing of the RAD libraries generated 49.3 million reads per pool of eight samples, averaging 6.2 million 150 bp reads per individual prior to quality filtering. After quality filtering, 4.99 million (81%) reads per individual were retained on average (Table 2). Pools had similar numbers of reads after processing (mean = 39.9 million per pool, SD = 4.95 million, one-way ANOVA,  $F = 2.638$ ,  $p > 0.1$ ). The average % GC content for Read 1 and 2 was 41.7 and 39.6, respectively. The percentage of polymorphic sites per region varied little among populations, from 0.150 to 0.173 % (Table 3). The observed heterozygosity in variant sites was 22% on average. Overall  $F_{IS}$  values, when considering all sites with a minor allele frequency cutoff  $a \geq 0.05$ , were close to 0 and hence provided no evidence of inbreeding (Table 3). However, when only considering variant positions within the region of Florida,  $F_{IS}$  values were negative ( $F_{IS} = -0.0086$ ), indicating an excess of heterozygosity. Using the two paired-end read sets as replicates, a one-way ANOVA was performed for each variable (Table 4). Populations were found to be similar for all summary statistics. Alignment of *A. palmata* SNPs to the published *A. digitifera* genome indicated that on average, 2.5% percent of the *A. digitifera* genome had sequence coverage at a stack depth of 5 (Fig 1). All four populations produced similar sequence coverage.

# Population genetics

A total of 390 SNPS were identified after filtering and including a minor allele frequency cutoff  $a \geq 0.05$  (Table 3). This included 219 for Read1 and 176 for Read2 from the paired-end sequencing (5 SNPs were identical between reads and only considered once). Analysis of Molecular Variance (AMOVA) revealed patterns of genetic differentiation among populations (Table 4). This was also evident when the 307 SNPs (analysis included only one SNP per 150 bp locus) after combining Read1 and Read2, were subjected to a multi-locus clustering analysis in STRUCTURE. Individuals from Florida clustered first, followed by the Bahamas at K=3. Puerto Rico and the USVI were not distinguishable until K=4, (Fig 2). CLUMPAK BEST K (Kopelman et al. 2015b) indicated that K=3 was the most likely K-value regardless of whether the sampling region was used as a prior.

To compare to the SNP analysis, microsatellite data from samples collected in six regions were analyzed in STRUCTURE using the sampling region as a prior. At K=2, a western (including Belize, Florida, Bahamas and Puerto Rico) and an eastern cluster (including the USVI and Curacao) was evident (Fig 3A). At K=3, an isolation-by-distance like pattern was apparent in the western cluster (Fig 3B). K=4 was the most likely K-value based on 11 microsatellite markers (Kopelman et al. 2015b) which grouped Florida and Belize as one cluster, and Puerto Rico and the Bahamas as the second, with the USVI as the third and Curacao as an admixed fourth cluster (Fig 3C).

According to the outlier analysis in Lositan, locus 166 was identified as a potential outlier and thus possibly under selection. It was therefore excluded from the analysis in STRUCTURE. This resulted in more comparable results to the SNP analysis with the most likely K-value being 3 (Kopelman et al. 2015b). Again, the first separation was between a western and an eastern cluster, however this time Puerto Rico assigned to the eastern cluster with an isolation-by-distance like pattern appearing between the west and east (Fig 3D). At the most likely K of 3, Curacao now formed a separate cluster. At K=4, the Bahamas started to separate from the remainder of the western regions similar to what was observed in the SNP clustering analysis (Fig 3E).

## Environmental drivers of population structure

A Mantel test showed a significant positive relationship in the SNP dataset between pairwise  $F_{ST}$  values and geographic distance ( $R^2 = 0.65$ ,  $p = 0.05$ ) consistent with the microsatellite results (10 loci) from the Florida, Bahamas, Puerto Rico, and Curacao samples only (Fig4C, Fig4D). Correlations between environmental factors including average temperature, salinity, dissolved oxygen, and pairwise  $F_{ST}$  values or geographic distance were not significant (Fig4A, Fig4B). However, it should be noted that the environmental data had a resolution of  $\frac{1}{4}$  to 1 degree latitude whereas the genetic data was collected on much smaller spatial scales (the reefs in each region are on average 66, 10, 106, and 68 km apart for the Bahamas, Florida, Puerto Rico and the U.S. Virgin Islands, respectively)(Supplemental Table 2). Therefore, landscape genetic approaches that may reveal environmental drivers of population differentiation (Manel et al. 2003) must await higher resolution environmental data.

## Loci under selection

BAYESCAN and LOSITAN identified 2 and 13 SNPs (Supplementary Table 3) that showed signs of positive selection when including all four populations, one of which was identified by both programs (a total of 12 unique loci identified between both programs). Outliers accounted for 3.3% of the total SNPs, consistent with other studies in which  $F_{ST}$  outlier loci have represented a substantial fraction of the total loci investigated (2-10%) (Nosil et al. 2009). Annotation of the candidate loci proved difficult as only 23% produced significant hits when queried against the NCBI NR database, Uniprot, and TrEMBL; with two of the hits being annotated as unconventional myosin-IXb isoform X7 and tyrosine-protein kinase transmembrane receptor ROR1-like. Screening of the microsatellite loci identified locus 166 as an outlier under positive selection, yet no annotation information of this locus is currently available.

## Discussion

### Comparison with previous *Acropora* gene flow studies

The previous range-wide survey of *A. palmata* population genetic structure using five, presumed neutrally evolving microsatellite markers showed that while most reefs are self-recruiting, *A. palmata* stands are not inbred and harbor high microsatellite genetic diversity (Baums et al. 2005). Furthermore, *A. palmata* stands were structured into two long-separated

populations, one in the eastern and one in the western Caribbean (Baums et al. 2005). Here, we report that genome-wide SNPs ( $MAF \geq 0.05$ ) resolved further population structure in the endangered reef-building coral, *A. palmata* from Florida to the USVI compared to previous microsatellite-based analyses.

It was recently suggested that the East-West divide of *A. palmata* lies not in the Mona Passage (Baums et al. 2005b; Baums et al. 2006b) but rather to the east of Puerto Rico (Mège et al. 2014). The 307 SNPs analyzed here confirm earlier findings that Puerto Rico and the USVI regions are more similar to each other than Puerto Rico is to either the Bahamas or Florida without imposing any priors in a STRUCTURE analysis ( $MAF \geq 0.05$ ). However, it is not always possible to determine, with confidence, the correct clustering solution that accurately reflects genetic population structure when there is an underlying isolation by distance pattern (Frantz et al. 2009). We show here that there is significant isolation by geographic distance from Florida to the USVI when using presumably neutrally evolving SNP and microsatellite loci. Interestingly, inclusion of microsatellite locus 166, flagged as being an outlier locus, obscured this isolation by distance pattern. Therefore, locus 166 is a strong candidate for a locus under selection (or it is linked to a locus under selection) and its functional significance might prove a fruitful subject for future studies.

An east-west Caribbean divide was also evident in the corals *Orbicella annularis* (Foster et al. 2012) and *Acropora cervicornis* (Vollmer & Palumbi 2007). An additional barrier to gene flow in *A. palmata* was reported by Porto-Hannes et al. (2014) between Venezuela and the Mesoamerican Barrier Reef System utilizing four of the microsatellites markers.

The total number of SNPs ( $n=307$ ) retained for population genetic analysis was lower than expected. This was due to a 10-fold increase in the number of fragments retrieved from the genome digest using the enzymes MluCI ( $\wedge$ AATT) and NlaIII (CATG $\wedge$ ) compared to what was predicted from an in-silico restriction of an incomplete draft genome of *A. palmata* (Baums, unpublished). A larger set of SNP loci may reveal additional finer scale structure across the Caribbean in *A. palmata*. However, model based clustering methods of 905,561 SNPs failed to reveal population structure in *A. digitifera* collected from the Ryukyu Archipelago of Japan, although a principle component analysis clustered the 122 samples into 4 groups identified as Okinawa, Kerama, Yaeyama-North, and Yaeyama-South, respectively (Shinzato et al. 2015). Low coverage, 5X in this study, is also a concern. Yet in the coral



*Platygyra daedalea*, 5x coverage was sufficient to assign samples to two distinct clusters based on their geographic origin, the Persian Gulf or Sea of Oman and was consistent with their 20x coverage data set (Howells et al. 2016b).

### Genetic diversity indices in *A. palmata*

Several factors could account for negative  $F_{IS}$  values including negative assortative mating, if a species is outcrossed and lacks selfed progeny or there is a selection pressure that favors the most heterozygous individuals. Of our samples, 49 out of 96 were ramets of larger genets. *A. palmata* colonies fragment frequently; the branches regrow into new colonies resulting in stands of genetically identical colonies (Baums et al. 2006a). [Note that samples included here all represented distinct genets]. Asexual reproduction could explain the excess of heterozygosity in *A. palmata* within the Florida region (see (Balloux et al. 2003; Carlon 1999; Delmotte et al. 2002). Excess heterozygosity has been observed in other clonal organisms. For example, significant negative  $F_{IS}$  values in a partially clonal but self-incompatible wild cherry tree was explained in part by asexual reproduction (Stoeckel et al. 2006).

The nucleotide diversity  $\pi$ , describes the degree of nucleotide polymorphism in a population and can be calculated based on variant sites only or on variant and non-variant sites combined. In *A. cervicornis*, nucleotide diversity based on variant sites only ranged from 0.239–0.44, with all means of reefs in Florida being higher than the Dominican Republic. (Drury et al. 2016b). However, in *A. palmata* we find that Florida is the least genetically diverse region when comparing variant sites only (0.203, Table 3), as would be expected in a marginal environment (Arnaud-Haond et al. 2006; Baums 2008; Baums et al. 2014b; Cahill & Levinton 2016; Eckert et al. 2008). In *Acropora austera* populations in the south-west Indian Ocean, nucleotide diversity ranged from 0.007 to 0.022, with lower estimates in the south than north (Macdonald et al. 2011). The nucleotide diversity estimate for *A. cervicornis*, including variant and non-variant SNP sites was 0.09 (Drury et al. 2016a). In the sea anemones, *Aiptasia* and *Nematostella* (Cnidaria) a genome-wide estimate of nucleotide diversity was 0.004 SNPs/bp surveyed (Bellis et al. 2016) and 0.0065 SNPs/bp (Putnam et al. 2007), respectively. In a survey of transcriptome derived SNPs in three gorgonian species synonymous nucleotide diversity ranged from 0.012 – 0.020 (Romiguier et al. 2014). Average pairwise nucleotide diversity in other metazoans include *Caenorhabditis elegans* (~0.001 SNPs/bp, (Swan et al. 2002)), *Drosophila*

*pseudoobscura* [ $\sim 0.002$  SNPs/bp (Kulathinal et al. 2009), and *homo sapiens* [ $7.51 \times 10^{-4}$  SNPs/bp (Sachidanandam et al. 2001)].

Our estimates of nucleotide diversity (including variant and non-variant sites) was 0.0004 SNPs/bp for all populations, an order of magnitude lower than in other cnidarians. Based on a survey of 374 individual transcriptome derived SNPs from 76 non-model animal species, the level of nucleotide diversity found in *A. palmata* is well below that predicted for a long-lived species, with small propagule size and large adult size (Romiguier et al. 2014). This low nucleotide diversity could be due to either a relatively small long-term effective population size, a severe bottleneck associated with a selective sweep (Ellegren & Galtier 2016) or the small number of SNPs included in this study (Fischer et al. 2017).

Allelic richness of microsatellite data correlates better with genome-wide estimates of genetic diversity based on SNPs than heterozygosity (Fischer et al. 2017) and allelic richness is more sensitive to recent population bottlenecks than heterozygosity (Allendorf 1986). Average microsatellite-based allelic richness in 14 Indo-Pacific *Acropora* corals was 4.96 overall and 6.21 in the five geographically widespread species (calculated based on Table 6 in Richards & Oppen 2012) which compares favorably with an average allelic richness of 8.49 in *A. palmata* found here. Thus, allelic richness of microsatellite loci remains high in Caribbean *A. palmata* despite recent population declines and the documented loss of alleles in Florida (Williams et al. 2014).

Future studies should include several thousand SNPs assayed in samples from across the species range to provide conclusive data on the impact of recent population declines on overall genetic diversity in *A. palmata*.

# **Genes under positive selection**

One of the SNP loci identified as being under positive selection was annotated as a *tyrosine-protein kinase transmembrane receptor ROR1-like*. ROR receptor protein is associated with the nervous system in the fruit fly *Drosophila* (Wilson et al. 1993), nematode *C. elegans* (Francis et al. 2005), and sea slug *Aplysia californica* (McKay et al. 2001). Functional analysis of *cam-1*, a gene that encodes for a ROR kinase in *C. elegans*, demonstrated roles in both the orientation of polarity in asymmetric cell division and axon outgrowth, and the ability to guide

migrating cells (Forrester et al. 1999). The role of *ROR1* receptors in Cnidaria is unknown although studies in *Hydra* suggest a function in regulating cell specification and tissue morphogenesis (Bertrand et al. 2014; Krishnapati & Ghaskadbi 2014; Lange et al. 2014).

Another SNP identified as being under positive selection was located in the gene annotated as unconventional *myosin-IXb isoform X7*, a Rho GTPase-activating protein (RhoGAP) that is essential for coordinating the activity of Rho GTPases. Invertebrates are thought to contain a single myosin class IX gene (the exception is *Drosophila* which has none) whereas most vertebrates have two with fishes having four (Liao et al. 2010). In general, Rho GTPases control the assembly and organization of the actin cytoskeleton which includes many functions such as cell adhesion, contraction and spreading, migration, morphogenesis, and phagocytosis. Little is known about the function of myosin-IX in invertebrates. However, a recent study in which *Orbicella faveolata* were exposed to immune challenges identified Unconventional myosin-IXb as a transcript that was significantly correlated with melanin protein activity (Fuess et al. 2016). In humans, Myosin-IXb is highly expressed in tissues of the immune system such as the lymph nodes, thymus, and spleen and also in immune cells like dendritic cells, macrophages and CD4 + T cells (Wirth et al. 1996). Myosin-IXb knockout mice showed impaired recruitment of monocytes and macrophages when exposed to a chemoattractant demonstrating that Myosin-IXb has an important function in innate immune responses *in vivo* (Hanley et al. 2010). Because statistical screens for loci under selection carry a high rate of false positive results, further experimental evidence is necessary before these loci can be considered targets of selection.

# **Restoration implications**

Restoration efforts should proceed under the assumption that *A. palmata* harbors a significant amount of population structure requiring close matches of collection and outplant sites. Hybridization of *A. palmata* from different regions may or may not result in heterosis depending on compatibility, but would be worth pursuing in an *ex situ* setting to enable close monitoring of offspring performance under elevated temperatures (van Oppen et al. 2015). With respect to the sharply declining Florida colonies, these findings underline the need to manage and restore Florida's *A. palmata* as an isolated, genotypically depleted population (Williams et al. 2014).

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427

# Figure legends

**Figure 1** A Cumulative distribution of sequencing coverage of Bowtie2 aligned reads to the *A. digitifera* using Bedtools. On average 2.5% percent of the *A. digitifera* genome had sequence coverage at a stack depth of 5.

**Figure 2** Bayesian cluster analysis with STRUCTURE. Reefs within regions 1-4 sorted by latitude: Florida, Bahamas, Puerto Rico, US Virgin Islands. Analysis of 307 SNPs (analysis included only one SNP per locus) after combining Read1 and Read2. Panels K=2 (A), and K = 3 (B), K=4 (C). The most probable K was 3 (B) for the minor allele frequency corrected SNPs based on the mean estimated log probability of the data at a given K (3 replicate runs per K, +/- 1 standard deviation).

**Figure 3** Bayesian cluster analysis with STRUCTURE. Panels (A-C). Analysis of 11 microsatellites with the most probable K being 4. Panels (D-E). Exclusion of locus 166 that was identified as an outlier resulted in an analysis of 10 microsatellites with the most probable K being 3.

**Figure 4** MANTEL matrix correlation test between genetic and geographic distances, and environmental parameters as calculated by a dissimilarity matrix expressed as the Euclidean distances between regions based on measured environmental data. *Acropora palmata* samples from four regions (Florida, Bahamas, Puerto Rico and USVI) were genotyped with 307 SNP (a, c) or 10 neutral microsatellite markers (d). Panel (a)  $y = 0.0107x + 0.0104$ ,  $R^2 = 0.6104$ ,  $p = 0.09$ . Panel (b)  $y = 0.002x + 0.4175$ ,  $R^2 = 0.1012$ ,  $p = 0.21$ . Panel (c)  $y = 0.000007x + 0.0098$ .  $R^2 = 0.6483$ ,  $p\text{-value} = 0.05$ . Panel (d)  $y = 0.000007x + 0.0027$ .  $R^2 = 0.69$ ,  $p = 0.04$ .

**Supplemental Figure** MANTEL matrix correlation test between genetic and geographic distances. *Acropora palmata* samples from four regions (Florida, Bahamas, Puerto Rico and USVI) were genotyped with 11 microsatellite markers, including the 166 outlier locus.  $y = 5E-06x + 0.0137$ .  $R^2 = 0.1147$ ,  $p\text{-value} = 0.1$ .

# Tables

**Table 1** *Acropora palmata* colonies included in SNP analysis. Samples were obtained from four regions in the Caribbean and 3 – 6 reefs per region. Given are latitude and longitude in decimal degrees.

**Table 2** RAD-tag sequencing summary table of *Acropora palmata* samples.

**Table 3** Summary statistics for Read 1 and Read 2 combined. % PL = percent polymorphic loci, Obs Hom = observed homozygosity, Obs Het = observed heterozygosity, StdErr = standard error, Exp = expected.  $F_{IS}$  calculations with and without minor allele frequency restrictions. Calculated by STACKS 1.30.

**Table 4** Pairwise  $F_{ST}$  calculated from STACKS 1.3. Read 1 and 2 combined (duplicated stacks between reads removed,  $MAF \geq 0.05$ ) calculated on the AMOVA corrected (p-value  $< 0.05$ )  $F_{ST}$  measurements. Considered were loci present in all populations.

**Supplementary Table 1** DD-Rad sequencing. There were 12 pools with 8 unique barcodes in each. The Database ID is a unique identifier for each coral specimen. Given is also the total number of ramets for each genet that was included in the RAD sequencing. The indices are short DNA sequences that uniquely identify products in the final libraries.

**Supplemental Table 2** GPS coordinates in decimal degrees for the World Ocean Atlas 2013 (WOA13 V2) environmental data averaged for a region.

**Supplementary Table 3** Outlier SNPs identified by programs LOSITAN and BAYESCAN. Stacks locus\_bp is the STACKS program locus ID with the SNP location basepair after the underscore. Read category indicates whether the outlier SNP was found in read 1 or 2 or the paired-end sequencing run. Digitifera scaffold identifies the scaffold where the Stacks locus aligned to, followed by the basepair location in the next column. S start= sequence start. S end = Sequence end.

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**Table 1** *Acropora palmata* colonies included in SNP analysis. Samples were obtained from four regions in the Caribbean and 3 – 6 reefs per region. Given are latitude and longitude in decimal degrees.

A)

| <i>Region</i>      | <i>Reef</i>          | <i>Count of Samples</i> | <i>Latitude</i> | <i>Longitude</i> |
|--------------------|----------------------|-------------------------|-----------------|------------------|
| <i>Florida</i>     | Sand Island          | 6                       | 25.018093       | -80.368472       |
|                    | French               | 8                       | 25.03393        | -80.34941        |
|                    | Little Grecian       | 1                       | 25.118433       | -80.31715        |
|                    | Horseshoe            | 1                       | 25.139467       | -80.29435        |
|                    | Elbow                | 8                       | 25.143628       | -80.257927       |
| <i>Bahamas</i>     | Little Ragged Island | 1                       | 22.15375        | -75.687208       |
|                    | Adelaine Cay         | 8                       | 22.173372       | -75.703016       |
|                    | Elkhorn Cay          | 2                       | 22.328253       | -75.783228       |
|                    | Johnson Cay          | 3                       | 22.33312        | -75.77892        |
|                    | Nairn Cay            | 8                       | 22.35199        | -75.79612        |
|                    | Middle Beach         | 2                       | 23.781199       | -76.10391        |
| <i>Puerto Rico</i> | San Cristobal        | 8                       | 17.56493        | -67.04515        |
|                    | Rincon               | 6                       | 18.21007        | -67.15849        |
|                    | Tres Palmas          | 2                       | 18.350133       | -67.266333       |
|                    | La Cordillera        | 8                       | 18.368522       | -65.571678       |
| <i>USVI</i>        | Tague Bay            | 8                       | 17.763867       | 64.613397        |
|                    | Hawksnest Bay        | 8                       | 18.347183       | -64.780775       |
|                    | Johnsons Reef        | 8                       | 18.361733       | -64.7743         |
| <i>Grand Total</i> |                      | 96                      |                 |                  |

B)

| <i>Region</i>  | <i>Reef</i>    | <i>Count of Samples</i> | <i>Latitude</i> | <i>Longitude</i> |
|----------------|----------------|-------------------------|-----------------|------------------|
| <i>Florida</i> | Horseshoe      | 1                       | 25.1395         | -80.294          |
|                | Little Grecian | 1                       | 25.1184         | -80.317          |
|                | Sand Island    | 6                       | 25.0179         | -80.369          |
|                | Western Sambo  | 6                       | 24.4799         | -81.719          |
|                | Rock Key       | 4                       | 24.456          | -81.86           |
|                | Dry Tortugas   | 1                       | 24.6209         | -82.868          |
|                | Marker 3       | 1                       | 25.3733         | -80.16           |
|                | Boomerang Reef | 1                       | 25.3525         | -80.179          |
|                | Carysfort      | 4                       | 25.2219         | -80.211          |
| <i>Bahamas</i> | Great Iguana   | 19                      | 26.7075         | -77.154          |
|                | Middle Beach   | 2                       | 23.7812         | -76.104          |

| <i>Region</i>      | <i>Reef</i>          | <i>Count of Samples</i> | <i>Latitude</i> | <i>Longitude</i> |
|--------------------|----------------------|-------------------------|-----------------|------------------|
|                    | Charlies Beach       | 1                       | 23.7808         | -76.104          |
|                    | Black Bouy           | 1                       | 23.8022         | -76.146          |
|                    | Bock Cay             | 1                       | 23.8075         | -76.16           |
|                    | Little Darby         | 2                       | 23.8474         | -76.209          |
|                    | Rocky Dundas         | 1                       | 24.2788         | -76.539          |
|                    | Halls Pond           | 2                       | 24.3539         | -76.57           |
|                    | LSI                  | 3                       | 23.7691         | -76.096          |
|                    | Little Ragged Island | 1                       | 22.1538         | -75.687          |
|                    | Adelaine Cay         | 1                       | 22.1734         | -75.703          |
|                    | Johnson Cay          | 1                       | 22.3331         | -75.779          |
|                    | Nairn Cay            | 4                       | 22.352          | -75.796          |
| <i>Puerto Rico</i> | San Cristobal        | 14                      | 17.5649         | -67.045          |
|                    | Rincon               | 24                      | 18.2101         | -67.159          |
|                    | Aurora               | 3                       | 17.9425         | -66.871          |
|                    | Paraguera            | 1                       | 17.997          | -67.052          |
| <i>USVI</i>        | Hawksnest Bay        | 6                       | 18.3472         | -64.781          |
|                    | Johnsons Reef        | 12                      | 18.3617         | -64.774          |
|                    | Haulover Bay         | 13                      | 18.3489         | -64.677          |
|                    | Buck Island          | 14                      | 18.2774         | -64.894          |
|                    | Flat Key             | 4                       | 18.317          | -64.989          |
|                    | Hans Lollik          | 4                       | 18.4019         | -64.906          |
|                    | Sapphire             | 6                       | 18.3333         | -64.85           |
|                    | Botany               | 3                       | 18.3572         | -65.036          |
| <i>Belize</i>      | unknown              | 3                       |                 |                  |
|                    | Bugle Caye           | 1                       |                 |                  |
|                    | Curlew               | 5                       | -68.896         | -88.083          |
|                    | Gladden              | 1                       | 16.4401         | -88.192          |
|                    | Glovers Atoll        | 3                       |                 |                  |
|                    | GSTF1                | 5                       | 16.5499         | -88.05           |
|                    | GSTF12               | 7                       | 16.5499         | -88.05           |
|                    | LarksCaye            | 1                       |                 |                  |
|                    | Laughing Bird Caye   | 4                       | 16.4367         | -88.199          |
|                    | Loggerhead           | 2                       |                 |                  |
|                    | Sandbores            | 3                       | 16.7791         | -88.118          |

| <i>Region</i>  | <i>Reef</i>   | <i>Count of Samples</i> | <i>Latitude</i> | <i>Longitude</i> |
|----------------|---------------|-------------------------|-----------------|------------------|
|                | Carrie Bow    | 13                      | 16.8021         | -88.082          |
| <i>Curacao</i> | Blue Bay      | 7                       | 12.1352         | -68.99           |
|                | Boka Patrick  | 8                       | 12.2873         | -69.043          |
|                | Directors Bay | 2                       | 12.0664         | -68.8603         |
|                | East Point    | 4                       | 12.0407         | -68.783          |
|                | PuntuPicu     | 9                       | 12.0831         | -68.896          |
|                | Red Bay       | 2                       | 12.1355         | -68.99           |
|                | Sea Aquarium  | 9                       | 12.0838         | -68.896          |
|                | Water Factory | 3                       | 12.1085         | -68.9528         |

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**Table 2** RAD-tag sequencing summary table of *Acropora palmata* samples.

| <i>Region</i> |             | <i>Pool</i> | <i>Coral colonies</i> | <i>Lane</i> | <i>Total Reads</i> | <i>Retained Reads after processing</i> | <i>Average number of retained sequence reads per sample</i> | <i>Standard Deviation</i> |
|---------------|-------------|-------------|-----------------------|-------------|--------------------|----------------------------------------|-------------------------------------------------------------|---------------------------|
| <i>West</i>   | Bahamas     | B1          | 8                     | 2           | 50,900,230         | 41,199,646                             | 5,149,956                                                   | 1,915,875                 |
|               |             | B2          | 8                     | 2           | 56,097,984         | 45,237,633                             | 5,654,704                                                   | 1,853,265                 |
|               |             | B3          | 8                     | 2           | 58,379,852         | 47,706,860                             | 5,963,358                                                   | 2,734,261                 |
|               | Florida     | F1          | 8                     | 1           | 50,925,548         | 39,750,070                             | 4,968,759                                                   | 1,681,820                 |
|               |             | F2          | 8                     | 1           | 48,752,776         | 42,036,153                             | 5,254,519                                                   | 4,422,737                 |
|               |             | F3          | 8                     | 1           | 49,942,322         | 38,611,895                             | 4,826,487                                                   | 2,518,097                 |
| <i>East</i>   | Puerto Rico | P1          | 8                     | 1           | 43,979,338         | 36,237,997                             | 4,529,750                                                   | 4,166,551                 |
|               |             | P2          | 8                     | 1           | 55,267,402         | 47,235,081                             | 5,904,385                                                   | 4,096,287                 |
|               |             | P3          | 8                     | 1           | 47,324,190         | 34,835,445                             | 4,354,431                                                   | 3,117,707                 |
|               | USVI        | U1          | 8                     | 2           | 40,616,766         | 33,170,324                             | 4,146,291                                                   | 2,187,597                 |
|               |             | U2          | 8                     | 2           | 43,215,386         | 34,291,498                             | 4,286,437                                                   | 1,187,166                 |
|               |             | U3          | 8                     | 2           | 45,849,098         | 38,439,719                             | 4,804,965                                                   | 1,555,938                 |
|               |             | Grand Total | 96                    |             | 591,250,892        | 478,752,321                            |                                                             |                           |



744 **Table 3** Summary statistics for Read 1 and Read 2 combined. % PL = percent polymorphic loci,  
 745 Obs Hom = observed homozygosity, Obs Het = observed heterozygosity, StdErr = standard  
 746 error, Exp = expected.  $F_{IS}$  calculations with and without minor allele frequency restrictions.  
 747 Calculated by STACKS 1.30.

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|                                     |                                | Bahamas | Florida        | Puerto Rico | USVI    |
|-------------------------------------|--------------------------------|---------|----------------|-------------|---------|
| All positions:<br>variant and fixed | Total Sites                    | 200425  | 200425         | 200425      | 200425  |
|                                     | Variant Sites                  | 390     | 390            | 390         | 390     |
|                                     | Private Alleles                | 2       | 1              | 0           | 2       |
|                                     | % PL                           | 0.1732  | 0.1497         | 0.1694      | 0.1668  |
|                                     | Fis                            | 0.00005 | 0              | 0           | 0.00005 |
|                                     | Nucleotide diversity ( $\pi$ ) | 0.0004  | 0.0004         | 0.0004      | 0.0004  |
| Variant positions only              | Obs Hom                        | 0.7728  | 0.7874         | 0.7791      | 0.7815  |
|                                     | Std Err                        | 0.0164  | 0.0164         | 0.0154      | 0.0154  |
|                                     | Obs Het                        | 0.2273  | 0.2126         | 0.2210      | 0.2186  |
|                                     | Std Err                        | 0.0164  | 0.0164         | 0.0154      | 0.0154  |
|                                     | Exp Hom                        | 0.7832  | 0.8050         | 0.7919      | 0.7916  |
|                                     | Exp Het                        | 0.2169  | 0.1951         | 0.2081      | 0.2085  |
|                                     | Fis                            | 0.02235 | <b>-0.0086</b> | 0.0035      | 0.02065 |
|                                     | Nucleotide diversity ( $\pi$ ) | 0.2254  | 0.2034         | 0.2174      | 0.21705 |

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**Table 4** Pairwise  $F_{ST}$  calculated from STACKS 1.3. Read 1 and 2 combined (duplicated stacks between reads removed,  $MAF \geq 0.05$ ) calculated on the AMOVA corrected (p-value  $< 0.05$ )  $F_{ST}$  measurements. Considered were loci present in all populations.

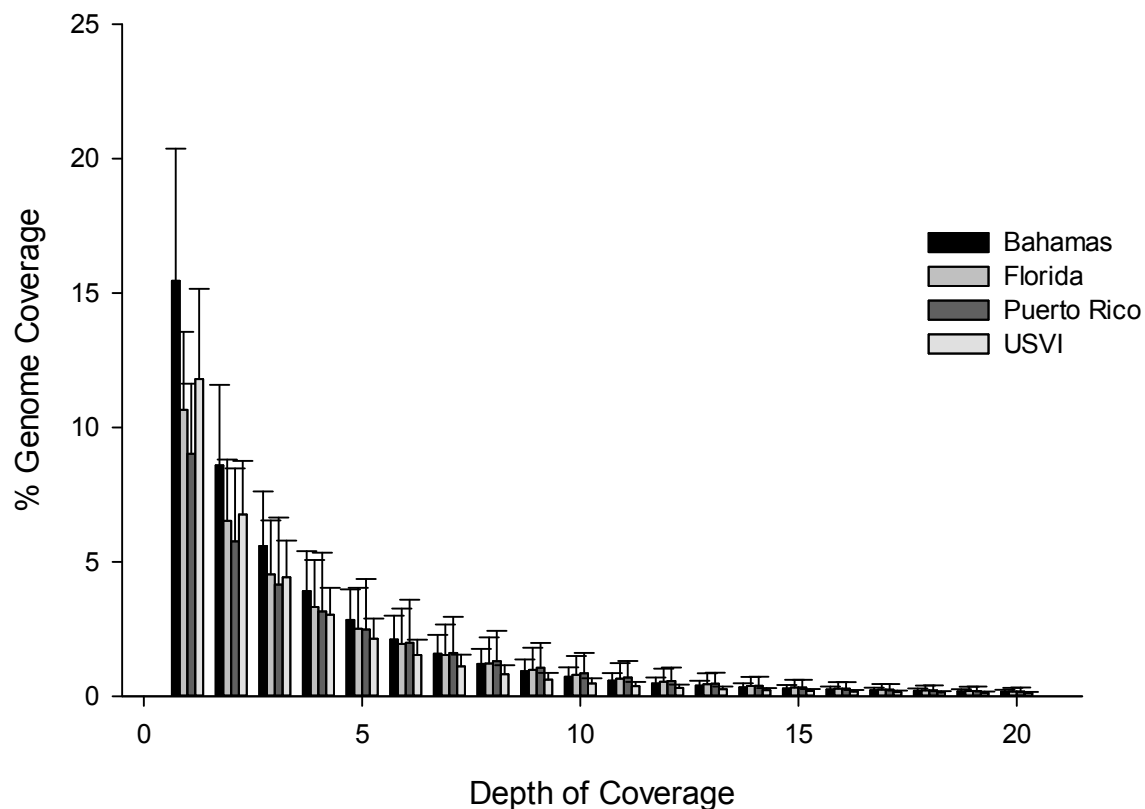
A)

|                    | <i>Bahamas</i> | <i>Florida</i> | <i>Puerto Rico</i> | <i>USVI</i> |
|--------------------|----------------|----------------|--------------------|-------------|
| <i>Bahamas</i>     |                |                |                    |             |
| <i>Florida</i>     | 0.018          |                |                    |             |
| <i>Puerto Rico</i> | 0.013          | 0.022          |                    |             |
| <i>USVI</i>        | 0.018          | 0.022          | 0.009              |             |

B)

|             | Belize | Florida | Bahamas | Puerto Rico | USVI   | Curacao |
|-------------|--------|---------|---------|-------------|--------|---------|
| Belize      |        |         |         |             |        |         |
| Florida     | 0.0040 |         |         |             |        |         |
| Bahamas     | 0.0115 | 0.0097  |         |             |        |         |
| Puerto Rico | 0.0206 | 0.0153  | 0.0063  |             |        |         |
| USVI        | 0.0206 | 0.0174  | 0.0098  | 0.0037      |        |         |
| Curacao     | 0.0240 | 0.0138  | 0.0181  | 0.0173      | 0.0208 |         |

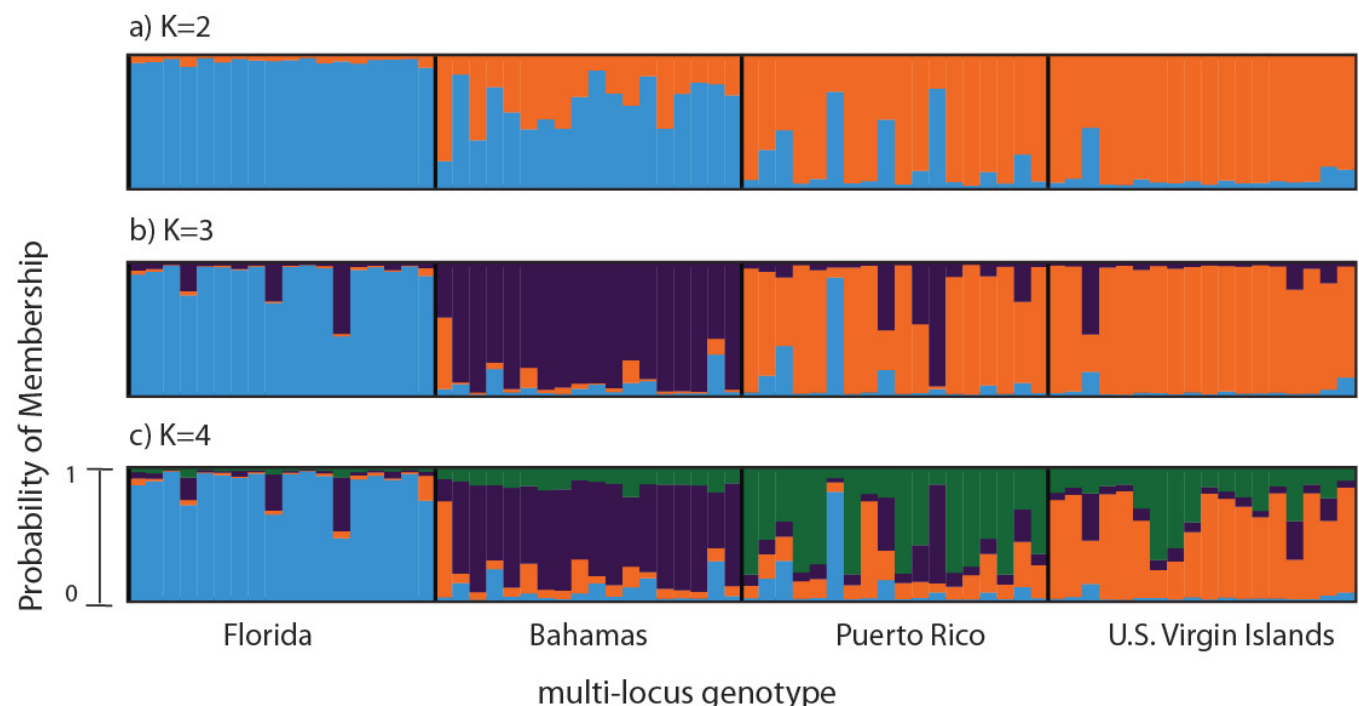
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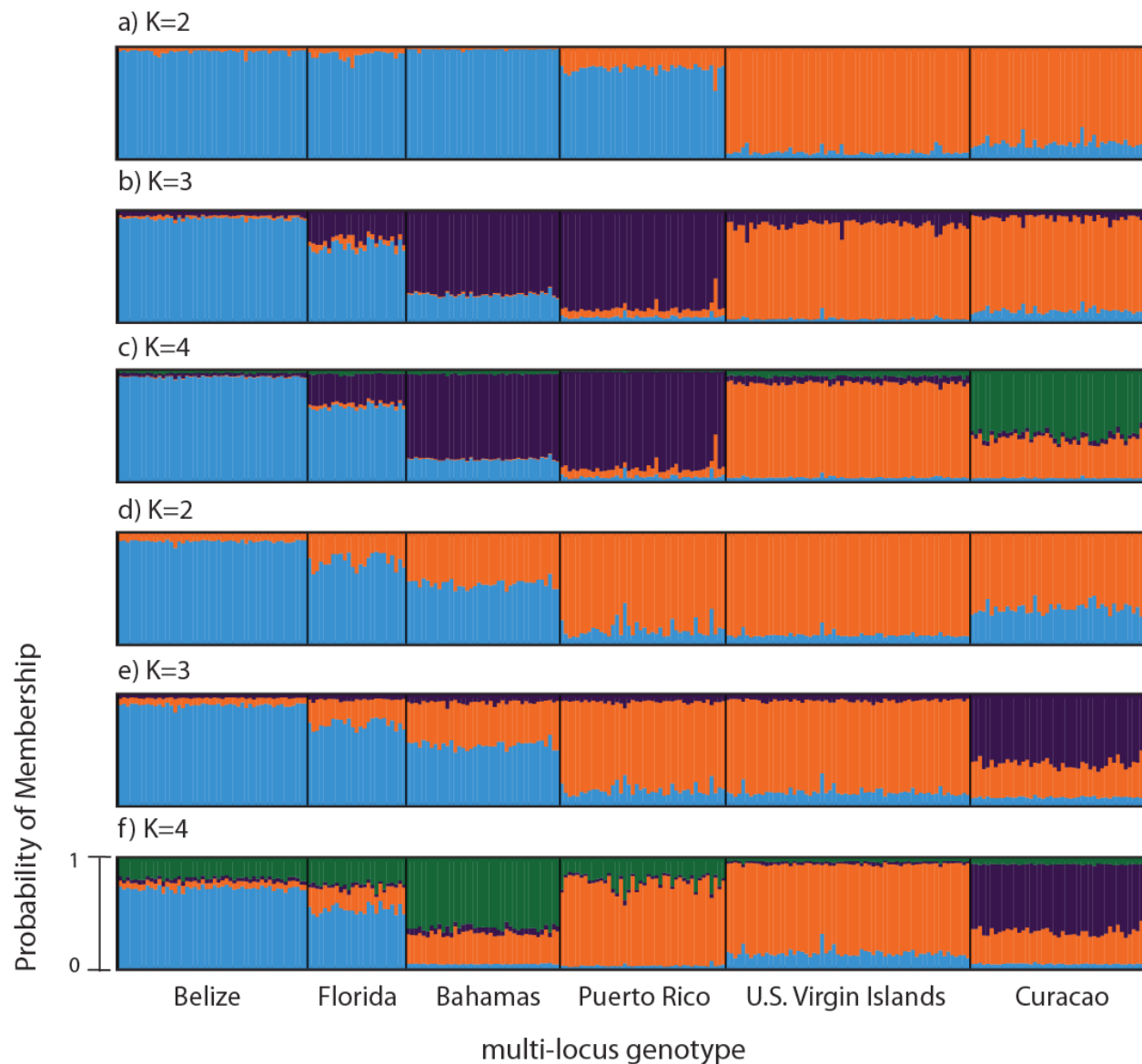
**Figure 1** A Cumulative distribution of sequencing coverage of aligned reads to the *A. digitifera* genome using Bedtools. On average, 2.5% percent of the *A. digitifera* genome had sequence coverage at a stack depth of 5.

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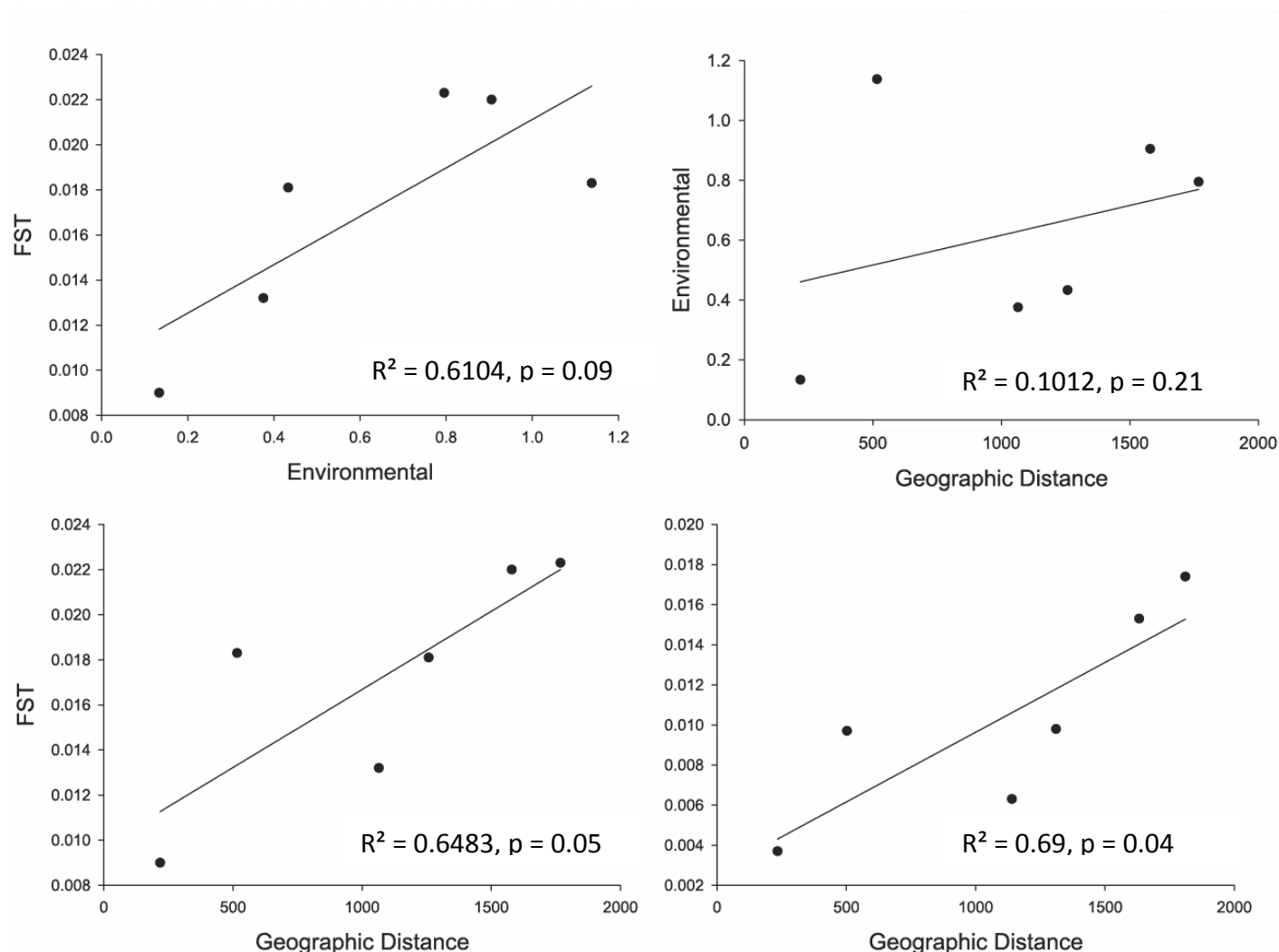


**Figure 2** Bayesian cluster analysis of 307 *Acropora palmata* SNP loci from four regions (Pritchard et al. 2000). Reefs within the regions (Florida, Bahamas, Puerto Rico, US Virgin Islands) are sorted by latitude: Only one SNP per locus were included, after combining Read1 and Read2. Panels show the combined results of 3 replicate run per K.  $K=2$  (a), and  $K=3$  (b),  $K=4$  (c). The most probable K was 3 (B) for the minor allele frequency corrected SNPs based on the mean estimated log probability of the data at a given K.



**Figure 3** Bayesian cluster analysis of 11 (a-c) and 10 (d-f) *Acropora palmata* microsatellite loci (Pritchard et al. 2000). Analysis of 11 microsatellites with the most probable K being 4. Panels (D-E). Exclusion of locus 166 that was identified as an outlier resulted in an analysis of 10 microsatellites with the most probable K being 3.

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**Figure 4** MANTEL matrix correlation test between genetic and geographic distances, and environmental parameters as calculated by a dissimilarity matrix expressed as the Euclidean distances between regions based on measured environmental data. *Acropora palmata* samples from four regions (Florida, Bahamas, Puerto Rico and USVI) were genotyped with 307 SNP (a, c) or 10 neutral microsatellite markers (d). Panel (a)  $y = 0.0107x + 0.0104$ . Panel (b)  $y = 0.002x + 0.4175$ . Panel (c)  $y = 0.000007x + 0.0098$ . Panel (d)  $y = 0.000007x + 0.0027$ .

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