Genotypic diversity and spatial–temporal distribution of *Symbiodinium* clones in an abundant reef coral

DANIEL T. PETTAY, DREW C. WHAM, JORGE H. PINZÓN and TODD C. LAJEUNESSE
Department of Biology, The Pennsylvania State University, 208 Mueller Laboratory, University Park, PA 16802, USA

Abstract

Genetic data are rapidly advancing our understanding of various biological systems including the ecology and evolution of coral–algal symbioses. The fine-scale interactions between individual genotypes of host and symbiont remain largely unstudied and constitute a major gap in knowledge. By applying microsatellite markers developed for both host and symbiont, we investigated the intracolony diversity, prevalence and stability of *Symbiodinium glynni* (type D1) multilocus genotypes in association with dense populations of *Pocillopora* at two sites in the Gulf of California. The genetic diversity and allelic frequencies in reef populations of *S. glynni* remained stable over 3 years. Common clone genotypes persisted over this period, and no temporal population subdivision (φPT = 0.021 and φST = 0.003) was detected. Collections from circular plots showed no statistical correlation between related *Pocillopora* individuals and their associations with particular *S. glynni* genotypes, with no spatial structuring or clonal aggregation across a reef for the symbiont. From permanent linear transects, samples were analysed from multiple locations within a colony and some were resampled approximately 1 year later. Many of these multisampled colonies (approximately 53%) were dominated by a single *S. glynni* genotype and tended to associate with the same symbiont genotype(s) over time, while colony ramets often possessed unrelated symbiont genotypes. In contrast to the species level, associations between genotypes of *Pocillopora* and *S. glynni* are apparently more flexible over space and time. The abundance of sexually recombinant genotypes of *S. glynni* combined with greater flexibility might provide adaptive mechanisms for these symbioses to evolve rapidly to changes in environmental conditions and allow particular symbiont genotypes to spread through a host population.

Keywords: clade D, clonality, dinoflagellates, eastern Pacific, Gulf of California, *Pocillopora*, population genetics, spatial structure, *Symbiodinium glynni*, zooxanthellae

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Introduction

Mutualistic associations between cnidarians and endosymbiotic dinoflagellates, genus *Symbiodinium*, are arguably the most prevalent and ecologically important photosynthetic symbioses in shallow warm water marine environments (Trench 1993). Advanced knowledge of the diversity, physiology, ecology and evolution of dinoflagellate symbionts has accumulated rapidly from investigations utilizing genetic markers. Initial analyses of conserved ribosomal DNA sequences recognized that the genus comprised deeply divergent lineages (e.g. clades A, B, C sensu Rowan and Powers 1992). Subsequent data from less conserved, rapidly evolving, markers has revealed a high diversity of independently evolving lineages within most clade subgroupings (e.g. LaJeunesse 2002; LaJeunesse et al. 2003, 2010a; Pochon et al. 2007; Sampayo et al. 2009; Finney et al. 2010). Each improvement in genetic resolution provides new insights necessary for deducing ecological and evolutionary processes underpinning cnidarian–dinoflagellate symbioses.

The stability and/or fidelity of host–symbiont associations over space and time represent long-standing
questions within the field. Answers to these questions may explain how reef corals adapt to major shifts in climate and how the symbioses upon which they depend might respond to current trends in sea surface warming (Donner et al. 2005; Berkelmans & Van Oppen 2006; LaJeunesse et al. 2010b). Data from a range of genetic markers indicate that a single symbiont tends to dominate a coral colony (e.g. Loh et al. 2001; LaJeunesse 2002; Chen et al. 2005; Coffroth and Santos 2005; Goulet 2006; Sampayo et al. 2009; although see Rowan et al. 1997). Furthermore, most host species exhibit associations with a particular symbiont, or a select subset, in a given geographical location and environment (e.g. LaJeunesse 2002; Sampayo et al. 2007; Stat et al. 2009; Finney et al. 2010; LaJeunesse et al. 2010a). The spatial-temporal persistence of a particular association depends on factors related to host identity, seasonal variation and stress history (Toller et al. 2001; Chen et al. 2005; Thornhill et al. 2006; Sampayo et al. 2008; LaJeunesse et al. 2009). For example, exposure to severe thermal stress may initiate the opportunistic rise of rare and/or heterologous Symbiodinium; however, the creation of unusual partner combinations is often short lived once external environmental conditions return to normal (Thornhill et al. 2006; Jones et al. 2008; LaJeunesse et al. 2009, 2010b). Therefore, flexibility in most cnidarian-dinoflagellate associations appears highly regulated, at least at the species level of diversity (Sampayo et al. 2009).

Microsatellite markers for genotyping individuals provide the next level of genetic resolution enabling the study of interindividual diversity, distribution and gene flow. The use of microsatellites on Symbiodinium has begun to generate new revelations in understanding the dynamics between host and symbiont individuals and populations (Santos et al. 2004; Thornhill et al. 2009; Andras et al. 2011). As with other microorganisms, distinct genotypes within a species of Symbiodinium may exhibit variation in physiology and give some lineages an adaptive advantage when subjected to selective processes created by a changing climate (Bell et al. 2006; LaJeunesse et al. 2010b). Therefore, the diversity and stability of Symbiodinium clones within a colony and on a reef may dictate the adaptive potential of these symbioses to environmental change.

Recent analyses using microsatellite markers confirm the early work of Goulet & Coffroth (2003a,b) that each host sampled usually contains a single detectable multilocus genotype (Santos et al. 2003; Pettay & LaJeunesse 2007, 2009; Thornhill et al. 2009; Kirk et al. 2009; Pinzón et al. 2010; Andras et al. 2011; Wham et al. 2011; but see Magalon et al. 2006; Howells et al. 2009). Because most of these investigations were based on the analyses of single samples, taken at one point in time, it remains unclear to what extent and for how long whole colonies are dominated by a particular individual symbiont genotype. Further data are clearly needed to test the generality of these observations.

The dominant shallow-water coral in the tropical and subtropical eastern Pacific, Pocillopora type 1 (sensu Pinzón & LaJeunesse 2011), exhibits high specificity for Symbiodinium glynni (type D1 in clade D; sensu LaJeunesse et al. 2008; Pinzón & LaJeunesse 2011) and/or type C1b-c in clade C (LaJeunesse et al. 2008, 2010b). The ecology and physiology of these coral-dinoflagellate symbioses have been extensively studied because colonies with S. glynni are particularly resistant to bleaching and mortality, which likely explains the regional dominance of Pocillopora type 1 (Glynn et al. 2001; Reyes-Bonilla et al. 2002; Iglesias-Prieto et al. 2003; Baker et al. 2004; Chávez-Romo & Reyes-Bonilla 2007; LaJeunesse et al. 2007, 2008, 2010b; Pettay 2011; Pinzón & LaJeunesse 2011). S. glynni has therefore emerged as a model organism to investigate how a thermally tolerant symbiont may influence the future viability of coral communities on a warming planet (LaJeunesse et al. 2010b).

In this study, we utilized nine microsatellite markers to examine the genotypic diversity, degree of clonality, fine-scale spatial distributions and temporal stability of S. glynni populations present within and among colonies of Pocillopora type 1 at two reef locations in the Gulf of California near La Paz, Mexico. It is hypothesized that the entrainment of symbionts in the vertically transmitting life cycle of the host limits the capacity for change in symbiont genotype. To test for stability and/or flexibility between host and symbiont genotypes, host genotypes were also acquired to examine their influence on the distributions of S. glynni clonal lines. The analyses conducted here were designed to fill gaps in knowledge that impede our understanding of the specificity and stability of these associations and reveal patterns for inferring ecological processes important to the evolution of Symbiodinium and their co-evolution with cnidarians.

Materials and methods

Sample collection

Samples were collected from two sites separated by 10 km, Isla Gaviotas (ISLG) and Punta Galeras (PGAL), at depths ranging from two to ten metres in the Gulf of California (GoC) near La Paz, Mexico (Fig. 1). Branch fragments (approximately 2 cm²) from colonies of Pocillopora type 1 (sensu Pinzón & LaJeunesse 2011) were collected by SCUBA and preserved in a high salt, 20% DMSO buffer (Seutin et al. 1991) and stored at −20 °C.
until DNA extraction. In May 2006, colonies exhibiting different morphologies (representing different host genotypes) were sampled from three 25-m linear transects (142 colonies total) established at each site. At this time, three colonies at each site were sampled intensively at the tops and bottoms of branches from the north, east, south, west and centre of the colony. To examine more broadly the genotypic diversity of S. glynni populations within a single colony, we returned in May of 2007 to sample from 10 colonies in triplicate that were being monitored at each site (totaling 20 colonies and 60 samples). Three independent samples from separate regions, and approximately equidistant from each other, were collected from the outer portions of each colony (Fig. 1). In August 2008, these collections were conducted again, but this time attention was paid to the orientation of the colony, with single samples acquired from the north, east, south and west sides of 10 colonies from PGAL. Nine of the ten colonies from 2008 were then resampled using the same orientation 9 months later in May 2009.

Lastly, a random sampling strategy was employed in May 2009 to examine the spatial distribution of symbiont clones across a host population and the correlation between host and symbiont genotypes. Twenty colonies were sampled from three 20-m-diameter circular plots (>30 m apart) positioned over the original linear transects established in 2006 (Fig. 1; Baums et al. 2006). Prior to sampling, random coordinates were generated for each plot with a precision of one degree and fifty centimetres. From the centre point of each plot, a compass and measuring tape were used to locate each sampling point, and a small branch from the central portion of each colony was removed. If no colony was found at a particular position, then the next set of coordinates was used until twenty colonies were sampled from each plot.

DNA extraction and symbiont identification

Nucleic acid extractions were conducted using a modified Promega Wizard genomic DNA extraction protocol (LaJeunesse et al. 2003). The dominant resident
symbiont was identified by denaturing gradient gel electrophoresis (DGGE) fingerprinting of the partial 5.8S and internal transcribed spacer (ITS) region 2 (LaJeunesse 2002). The region was amplified using a touch-down thermal cycle profile with the primers TS2clamp and TSinforn2 (LaJeunesse & Trench 2000), and the PCR products resolved on denaturing gels (45–80% of 7 m urea and 40% formamide) using a CScientific system (Del Mar, CA, USA) for 16 h at 115 volts. Samples where S. glynni (D1) was detected were utilized for this study.

Microsatellite analysis

Nine independently sorting polymorphic microsatellite loci originally developed for clade D *Symbiodinium* were utilized to examine the clonal diversity and spatial structure of *S. glynni* (D1Sym11, D1Sym14, D1Sym17, D1Sym34, D1Sym67, D1Sym77a, D1Sym77b, D1Sym87 and D1Sym92; Pettay & LaJeunesse 2009; Wham et al. 2011). Each locus was amplified in separate 10-μL reaction volumes containing 2.5 mM Mg-free Buffer (2.5 mM MgCl2), 1× Mg-free Buffer (2.5 mM MgCl2), 1 μM of the forward and reverse primers and approximately 50 ng of DNA template. PCR amplification conditions consisted of an initial denaturing step of 94 °C for 2 min, 32 cycles of 94 °C for 15 s, annealing temperature (T_a, see Supporting information Table S1) for 15 s, 72 °C for 15 s and a final extension of 72 °C for 5 min. Following amplification, fragment sizes were analysed on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a 500-bp standard (LIZ-labelled) at the Pennsylvania State University Genomics Core Facility. Fragments sizes were visually analysed using GeneMarker v.1.51 (SoftGenetics, State College, PA, USA). The presence of multiple peaks was interpreted to indicate that a sample contained more than one haploid genotype. Multiple alleles at a particular locus were scored when a second clear peak was found in the expected size range and was at least one-third the size of the dominant peak. This method was used to prevent the inclusion of microsatellite stutter peaks, which do not represent true alleles. Based on tests using artificial mixtures of single genotypes of differing concentrations, all loci show the ability to resolve the numerically dominant *S. glynni* genotype based on fragment peak height (Supporting information Fig. S1). Therefore, there appears to be little to no bias in fragment amplification based on size (i.e. Havryliuk et al. 2008), and the dominant MLG is represented by the dominant fragment peaks, which were used for statistical analyses. The occasional exclusion of small ambiguous peaks may occasionally cause a low-background genotype to be missed; however, this conservative approach prevents the wrong overestimation of genetic diversity (Anderson et al. 2000). In repeatedly sampled colonies where there were mixtures of two or more symbiont genotypes, the most likely identities of each individual genotype were determined by comparing replicate samples where a single MLG was present and comparing relative peak heights between alleles at loci with multiple peaks. However, the true identity of ‘background’ MLGs may not be completely reliable and must be interpreted cautiously.

Statistical analysis

**Clonal diversity and structure.** Basic measurements of clonal diversity and spatial organization, along with spatial structure analyses, were calculated using GenClone (v. 2.0, Arnaud-Haond & Belkhir 2007). The descriptive statistics included the number of samples (N), number of genotypes (G), clonal richness (R), clonal heterogeneity as the Simpson complement (D^s), clonal evenness as the Simpson evenness (V) and the Pareto distribution of clonal membership. Clonal richness is calculated as R = (G−1)/(N−1) and ranges from zero to one, with a value of one indicating all samples are distinct MLGs (i.e. no clones). The Simpson complement, which equals one minus the Simpson index (λ), describes the probability that two random individuals within a sampling are distinct MLGs. Simpson evenness describes the distribution of MLGs with respect to their abundance and ranges from zero to one, with one indicating all MLGs are in equal abundance. For clonal organisms, the distribution of ramets into clonal size classes, or the number of samples with identical MLGs, follows the Pareto, or power law, distribution (Arnaud-Haond et al. 2007). The slope of the regression (β line) is a function of both the evenness and richness of a sample set and is a way to graphically represent the heterogeneity of clone abundance. Descriptions of these indices, their derivation and benefits are reviewed in Arnaud-Haond et al. (2007).

Spatial analyses of clonal structure were conducted for all circular plots using GenClone (version 2.0) to obtain a clonal aggregation index (A_c), an edge effect (E_o), the clonal subrange and a kinship coefficient (F_o) for each distance class (r) for spatial autocorrelation at both the ramet and genet level. Additionally, a multivariate approach to spatial autocorrelation, whereby multiple genetic loci are assessed simultaneously, was conducted using GenAlEx (version 6.4, Peakall & Smouse 2006) and was run using the multiple pops option. This option not only analyses each plot individually but also groups all plots on the same reef together, providing increased sample size and statistical
power, and is useful when common processes contributing to spatial structure are believed to be occurring at each location (Peakall & Smouse 2006), like would be expected from multiple circular plots on a single. For these analyses, replicate MLGs were included in the data set, making it a spatial analysis at the ramet level. The significance of the autocorrelation analyses was tested using a permutation method, as implement by GenClone (1000 permutations) and GenAlEx (10,000 permutations).

Temporal stability of reef populations. To examine whether populations of S. glynni change in genotypic diversity, composition or structure over time, allelic frequencies and the degree of differentiation between populations collected in 2006 (linear transects) with those collected in 2009 (circular plots) were calculated for each site using GenAlEx. For the calculation of allele frequencies and genetic similarity, duplicate MLGs were removed. These allelic frequencies were also used to calculate haploid genetic diversity (\(h\)), which gives an indication that two individuals drawn at random will be genetically different, and information index (I), which is a measure of allelic diversity (Peakall & Smouse 2006). Population differentiation between time points was assessed using an analysis of molecular variance (AMOVA) for both sites. Using these time point populations for each reef, AMOVAS along with a permutation procedure were performed to test for significant difference in genetic diversity between populations (Excoffier et al. 1992). The AMOVA produces variance components along with \(\Phi\)-statistics (\(F\)-statistic analogues), which partition genetic variation at different hierarchical levels (Excoffier et al. 1992). The significance of the genetic variance and \(\Phi\)-statistics was then tested using 10,000 permutations.

Correlation between host and symbiont genotypes. Pocillopora transmit their symbionts vertically from mother to offspring (Glynn et al. 1991; Chávez-Romo & Reyes-Bonilla 2007), which may lead to a correlation between host and symbiont genotypes. To test whether a statistical relationship exists, Mantel tests were performed on genetic distance–based matrices of pairwise comparisons between microsatellite MLGs of both Pocillopora and S. glynni individuals from the circular plots. The Mantel tests were conducted using GenAlEx; the statistical significance of the correlation coefficient between the two matrices was tested using 9999 random permutations with an \(\alpha = 0.05\), and a null hypothesis that no significant relationship exists between the host and symbiont genetic distances. Two mantel tests were conducted, one per reef, using the combined data from the three circular plots with each combination of host and symbiont genotype represented only once (i.e. holobiont clones were removed). The Pocillopora data were acquired from the analysis of six microsatellite loci from Pinzón (2011).

Results

Symbiont identification

The microsatellite analyses targeted a Symbiodinium whose genome is characterized by a single numerically dominant ITS variant inferred from ITS2-DGGE fingerprinting (type D1; sensu Thornhill et al. 2007; Sampayo et al. 2009; LaJeunesse et al. 2010a). A total of 458 samples were analysed to determine symbiont identity. While in most of the samples analysed by ITS2-DGGE detected only S. glynni, Cl1b-c, a clade C Pocillopora-specific type was present in a small proportion of samples (approximately 10%). Genetic comparisons with this and other clade D Symbiodinium and its apparent specificity for Pocillopora indicate that Symbiodinium glynni is a distinct operational taxonomic unit (LaJeunesse et al. 2010a; Wham et al. 2011; T.C. LaJeunesse Unpublished data).

Allelic and genotypic diversity and temporal structure from linear transects and circular plots

The total number of alleles per locus acquired from the linear transects (2006) ranged from one to eight (PGAL) and two to five (ISLG). Similarly, in 2009, one to six (PGAL) and two to six (ISLG) alleles per locus were obtained from the circular plot (Table 1). The effective number of alleles (\(A_e\)) per locus ranged from 1.00 to 4.80 (PGAL06), 1.32 to 3.00 (ISLG06), 1.00 to 3.83 (PGAL09) and 1.66 to 4.65 (ISLG09) (Table 1). Allele frequencies at each locus ranged from 0.028 to 0.778 (PGAL06), 0.032 to 1.000 (PGAL09), 0.048 to 0.762 (ISLG06) and 0.045 to 0.727 (ISLG09) (Supporting information Table S1). Allele frequencies remained similar, and the dominance of a particular allele(s) persisted, in most instances, over the 3-year period. Similarly, the temporal population differentiation for each site as calculated by an AMOVA indicated that the population at each reef was temporally stable and undifferentiated (\(\Phi_{PT} = 0.021, P = 0.150\) for ISLG and \(\Phi_{PT} = -0.003, p = 0.501\) for PGAL; Supporting information Table S2). A small number of alleles were unique to each sampling time (10 alleles at PGAL; 9 alleles at ISLG) and usually occurred at low frequencies (<0.100). The exception was two alleles from ISLG (181 at D1Sym14 & 260 at D1Sym87), which had allele frequencies >0.200 in 1 year and was not found in the other. The mean haploid diversity (\(H\)) was 0.56 for ISLG and 0.50 for PGAL, and the information index (I) was 0.98 for ISLG and
Table 1 Description of microsatellite loci used in this study and their diversity according to sampling date and location including allele size range, number of alleles and $A_e = \text{effective alleles}$

<table>
<thead>
<tr>
<th>Locus</th>
<th>ISLG 2006</th>
<th>ISLG 2009</th>
<th>PGAL 2006</th>
<th>PGAL 2009</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Size range (bp)</td>
<td>Number of alleles</td>
<td>$A_e$</td>
<td>Size range (bp)</td>
</tr>
<tr>
<td>D1Sym11</td>
<td>151–159</td>
<td>3</td>
<td>1.62</td>
<td>153–159</td>
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<tr>
<td>D1Sym14</td>
<td>179–183</td>
<td>3</td>
<td>2.38</td>
<td>179–185</td>
</tr>
<tr>
<td>D1Sym17</td>
<td>145–149</td>
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<tr>
<td>D1Sym34</td>
<td>388–404</td>
<td>5</td>
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<td>388–400</td>
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<tr>
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<tr>
<td>D1Sym77b</td>
<td>190–193</td>
<td>3</td>
<td>1.76</td>
<td>181–193</td>
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<tr>
<td>D1Sym87</td>
<td>244–256</td>
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<td>D1Sym92</td>
<td>124–128</td>
<td>2</td>
<td>1.32</td>
<td>124–128</td>
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0.95 for PGAL (Table 2). The probability of identity ($P_{ID}$) was calculated using allele frequency data at each locus and combining both the linear transects and circular plots for each site. The probability that two samples with the same MLGs may not have originated from the same clone lineage was $1.09 \times 10^{-6}$ for ISLG to $3.1 \times 10^{-6}$ for PGAL.

 Alleles were amplified for all samples at all nine loci except for D1Sym17, D1Sym77a and D1Sym77b. The D1Sym17 locus did not amplify in just two instances perhaps because of DNA quality. The frequencies of nonamplifiable alleles for D1Sym77a and D1Sym77b were high in samples of good DNA quality and ranged from 0.132 to 0.333 (77a) and 0.245 to 0.714 (77b) for a particular transect or plot and appear to be the result of accumulated sequence differences in the flanking regions of D1Sym77 that inhibit the proper amplification of divergent alleles (i.e. null alleles). For this reason, these null alleles were coded as a distinct allele and included in the data for subsequent analyses.

Table 2 provides a summary of allelic and genotypic diversity, along with measures of clonality, clonal distribution and size, for each transect and circular plot. Excluding the within-colony sampling, 85 different genotypes were differentiated among 204 MLGs obtained from the ISLG and PGAL transects and circular plots. Pairwise genetic distance between individual symbiont genotypes within each site indicate that most are genotypically divergent (up to nine alleles) from each other (Fig. 2). The number of distinct genotypes per transect/plot ranged from 6 to 16 (Table 2). Many genotypes of S. glynni were found more than once but were often restricted to a particular transect/plot. The maximum clone size found during a sampling time was seven and eighteen for PGAL and ISLG, respectively. The clone recovered in the highest frequency at ISLG in 2006 was also the most abundantly sampled clone on that same site in 2009 (12 times in 2006, 18 in 2009). This pattern was also observed at PGAL where a particular clone was found at high regularity during both sampling efforts (seven times in 2006, seven in 2009). A total of six (6.1%, ISLG) and thirteen (12.3%, PGAL) clones were shared between the 3-year sampling times, while nine distinct clones were shared between sites (4.4%).

Genotypic richness ($R$) for both sites at each sampling time was 0.49 and 0.38 for ISLG and 0.63 and 0.58 for PGAL in 2006 and 2009, respectively. Between individual transects and/or plots $R$ was variable and ranged from 0.29 to 0.93 (Table 2). Clonal heterogeneity ($D^*$) per reef was 0.92 and 0.87 for ISLG and 0.97 and 0.96 for PGAL in 2006 and 2009, respectively. Values of $D^*$ per transect and/or plots were variable and ranged from 0.79 to 0.98 (Table 2). Evenness ($V$) was highly variable per transect and/or plot ranging from 0 (only one clone) to 0.78, with $V$ per reef equal to 0.76 and 0.71 for ISLG and 0.86 and 0.86 for PGAL in 2006 and 2009 (Table 2). Evenness as measured by the Pareto distribution was also highly variable with $\beta$ ranging from 0.42 to 1.26. In general, the distribution of clonal membership was more skewed by the dominance of a few clones in ISLG than in PGAL. While the estimates of $R$, $D^*$ and $V$ within a reef in a particular year give an indication of relative clonality and evenness between transects or plots, these measures are particularly sensitive to sampling strategy, which differed between 2006 and 2009 (Arnaud-Haond et al. 2007).

Within-colony clone diversity and stability

The total number of alleles per colony ranged from nine to twenty, with the number of loci differentiating clones within a colony ranging from one to eight. Many colonies (0.53) appeared to host homogeneous clonal
Table 2: Summary statistics for 2006 linear transects and 2009 circular plots for Isla Gaviotas and Punta Galeras in the Gulf of California. The statistics are as follows: number of samples (N), number of genotypes (G), clonal richness (R), information index (I), haploid diversity (H), clonal heterogeneity as Simpson complement (D*), clonal evenness as Simpson evenness (V), Pareto distribution slope (β) and curve fit (r^2) and significance (P), largest size class of clones (MAX), clonal subrange (CS), aggregation index (A_c) and the edge effect (E_e). Numbers in parentheses represent standard error for I and H, while they represent P-values for A_c and E_e. Dashes represent values that could not be calculated.

<table>
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<tr>
<th>No.</th>
<th>Freq. of D</th>
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<th>G</th>
<th>R</th>
<th>I</th>
<th>H</th>
<th>D*</th>
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<td>0.203 (0.021)</td>
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<td>0.918</td>
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<td>–</td>
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<td>8</td>
<td>0.036 (0.218)</td>
<td>-0.008 (0.269)</td>
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<td>53</td>
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<td>0.984 (0.183)</td>
<td>0.514 (0.088)</td>
<td>0.971</td>
<td>0.856</td>
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<td>0.99</td>
<td>0.004</td>
<td>7</td>
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populations of *S. glynni* despite being sampled multiple times (Fig. 3). The frequency of colonies with two or more MLGs ranged between 0.07 and 0.70 depending on the site and the within-colony sampling strategy employed (Table 3). Colonies sampled only once tended to have a single *S. glynni* genotype (0.87). More genotypes per colony were found when colonies were sampled multiple times as different regions/branches of some colonies possessed different *S. glynni* genotypes. Colonies sampled at three separate positions revealed a similar diversity to those sampled at ten places (Table 3; Supporting information Fig. S2). A lower frequency of colonies with mixed symbiont genotypes was observed when genotypes that differed at only a single locus were removed (Table 3).

The estimated number of *S. glynni* genotypes ranged from one to six in colonies sampled multiple times (average 1.80). Colonies at PGAL consistently possessed a higher incidence of *S. glynni* populations comprising multiple genotypes, whereas colonies at ISLG tended to be homogenous for one genotype (Table 3). The presence of a certain clone or mixture of clones in a particular area of the colony did not correspond to their orientation in the colony (data not shown). Clonal identity and diversity were identical in almost all cases (n = 59 of 60) at the apex and base of a branch, indicating that irradiance has little influence on genotype distribution and sampling a single branch multiple times yielded no additional genotypic diversity.

Colonies sampled over 9 months on their north, south, east and west sides (Fig. 3) suggested that genotype diversity of *S. glynni* populations exhibited relative temporal stability at the colony level, but may undergo dynamic changes across regions of a colony. Most branches maintained the same symbiont clone(s) over time, with the frequency of a stable genotype(s) equalling 0.67 (north), 0.89 (east), 0.78 (south) and 0.67 (west). For entire colonies, two-thirds experienced a change in clone diversity in at least one location (e.g. north side, west side) within the colony, but generally involved the displacement of one clone by another already present in another region of the colony (Fig. 3). However, in three of the nine colonies (33%), a new

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**Fig. 2** The range and distribution of genetic distances (number of distinct alleles) based on pairwise comparisons among *S. glynni* multilocus genotypes characterized from Isla Gaviotas and Punta Galeras (after the removal of duplicated genotypes).

**Fig. 3** Examples of *S. glynni* genotype diversity, distribution and temporal stability in colonies of *Pocillopora* type 1. (a) Most colonies were dominated by a single clone genotype. (b) In some cases, colonies harboured multiple (but rarely more than three) codominant clones partitioned to different areas of the colony with some zones of overlap. Particular clones may persist temporally in a host as the dominant genotype(s), while some may be displaced over time as new ones appear. Examples are shown of (c) a single clone and (d) a mixture of two clones that remained stable over time. (e) Another example showing a shift in dominance from two to just one genotype over time (top) and the perceived replacement of dominant clones by a ‘new’ genotype (right, red) after 9 months.
A genotype was found that had not been detected in the colony 9 months earlier (Fig. 3).

**Spatial analyses**

The multivariate spatial autocorrelation analyses at the ramet level indicated a lack of spatial structure at the scale measured (approximately 20 m) for all plots at both ISLG and PGAL (Fig. 4 and Supporting information Fig. S3). While an occasional distance class displayed significant correlation (five for ISLG plots and three for PGAL plots), the results were often related to distance classes with low sample sizes, and there appeared no general trends in the correlation coefficients (r) for any plot. Similarly, when the data from the three plots were combined per site, there was no indication of spatial structure for either reef (ISLG or PGAL), with no significant distance classes for ISLG and only two significant for PGAL (Supporting information Fig. S3). Spatial autocorrelation analyses at both the ramet and genet level for each plot using GenClone also indicated a lack of spatial structure for all plots (data not shown).

Clonal aggregation ($A_c$) for each plot ranged from −0.19 to 0.20 and was nonsignificant except for ISLG #3 ($R = 0.20, P = 0.021$), suggesting that symbiont clones are not spatially restricted. The maximum distance between clones within plots ranged from 8.3 to 13 m and was highest at ISLG #1 and PGAL #3. No significant edge effect ($E_e$) was found for all plots except ISLG #2 ($R = 0.48, P = 0.006$) and ranged from −0.01 to 0.48. The general lack of significance for $E_e$ indicates the sampling scheme was adequate, and there is a low probability of sampling-induced bias on measures such as genotypic richness (Arnaud-Haond & Belkhir 2007).

**Relationships between host and symbiont genotypes**

While several specific MLGs of host and symbiont were found together multiple times (seven at ISLG and one at PGAL), mantel tests indicated there was no correlation between closely related host associating with closely related symbionts at either ISLG ($R = 0.058, P = 0.198$) or PGAL ($R = -0.045, P = 0.203$). Identical Pocillopora genotypes (ramets) were often found to harbour different genotypes of *S. glynni* (up to fifteen),

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**Table 3** Within-colony diversity of *S. glynni* MLGs in *Pocillopora* from the Gulf of California. Frequency of branches and colonies where multiple genotypes were detected is based on sampling strategy, or number of samples per colony, for each reef, with an average for both reefs together. The total number of branches (i.e. samples) and colonies sampled are indicated by $n_{samp}$ and $n_{col}$, respectively. Frequencies mixtures were calculated for genotypic differences based on only one locus and for those with alleles differing at two or more loci.

<table>
<thead>
<tr>
<th>Sampling strategy</th>
<th>Location</th>
<th>$n_{samp}$</th>
<th>Samples w/mixtures</th>
<th>Samples w/mixtures (≥2 loci)</th>
<th>$n_{col}$</th>
<th>Colonies w/mixtures</th>
<th>Colonies w/mixtures (≥2 loci)</th>
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<tr>
<td>Single</td>
<td>ISLG</td>
<td>43</td>
<td>0.069</td>
<td>0.047</td>
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<td></td>
<td>36</td>
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<td>0.143</td>
<td>43</td>
<td>0.069</td>
<td>0.047</td>
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<td></td>
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<td>0.131</td>
<td>0.101</td>
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<td>0.047</td>
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<tr>
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<td>0.233</td>
<td>0.167</td>
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<tr>
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<td>0.550</td>
<td>0.350</td>
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<tr>
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<tr>
<td></td>
<td></td>
<td>3</td>
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<td>0.000</td>
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<tr>
<td></td>
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<td>60</td>
<td>0.067</td>
<td>0.333</td>
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<td>0.333</td>
<td>0.333</td>
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</table>

*Five branches of each colony were sampled, but each branch was sampled at both the top and bottom effectively making these colonies sampled ten times.

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and, conversely, host genets (up to seven) frequently harboured the same *S. glynni* genotype (Figs 4 and 5). However, host ramets found close to each other, probably the result fragmentation, often associated with the same symbiont genotype. One widespread *Pocillopora* genet comprising 43 colonies distributed across 90 m of nearshore habitat at ISLG associated with fifteen different *S. glynni* genotypes (Fig. 5). Pairwise genetic distance between these fifteen individual symbiont genotypes indicates that most are genotypically divergent and likely originate from sexual recombination (i.e. not a part of the same clonal lineage) (Fig. 5 inset).

**Discussion**

A population genetics approach offers the genetic resolution critical to understanding biological processes important to cnidarian–dinoflagellate symbioses. The stress tolerant and ecologically dominant symbiosis between *S. glynni* and colonies of the *Pocillopora* type 1 is widespread throughout the eastern Pacific (LaJeunesse et al. 2010b; Pinzón & LaJeunesse 2011). With population genetic markers available for both partners, this symbiosis is uniquely suited for investigating fundamental biological attributes concerning symbiont population dynamics within and between host colonies and populations.

High genotypic diversity and population stability of *S. glynni* in the GoC

The populations of *S. glynni* found at PGAL and ISLG comprise numerous clonal lineages (i.e. genotypes), many of which are the products of sexual recombination rather than somatic mutation and clonal evolution (Fig. 2; LaJeunesse 2001; Santos & Coffroth 2003). While many genotypes were observed only once, others were common, locally widespread and persistent through time. Given the relatively small sampling effort, the ninety-six distinct genotypes characterized represent a small subset of the actual genetic variation probably present in the local *S. glynni* population. The total number of distinct genotypes that exist in the Gulf of California and across the entire eastern Pacific must be enormous. The high diversity of *S. glynni* genotypes is in many ways analogous to that found in bloom-forming planktonic species (Rynearson & Armburst 2005; Rynearson et al. 2006); however, the distribution of *S. glynni* genotypes is fundamentally different than in ‘free-living’ taxa. Instead, a majority of symbiont genotypes are discreetly partitioned among colonies of the host population (see Discussion below).

Among various dinoflagellate and diatom populations, there is little temporal differentiation in genetic composition for periods ranging from days to several years (Evans et al. 2005; Rynearson & Armburst 2005; Rynearson et al. 2006; Casteleyn et al. 2009; Nagai et al. 2009; however see Godhe & Härmström 2010). Despite their ecological differences from other microalgae, allelic frequencies of *S. glynni* reef populations also remained stable over time and highlight the similarities in the population dynamics between the two vastly different lifestyles (Supporting information Table S2). In contrast to free-living microalgal populations where clonal lineages are apparently short lived, genotypes of *S. glynni* can persist for several years, and possibly longer, owing to the stable conditions within their host. The average life expectancy and persistence of *Symbiodinium* genotypes within colonies and across host populations may vary considerably and is likely influenced by various historical factors involving selective and/or ran-
dom processes. A particular genotype can occupy a host for many years and continuously generate propagules through mitotic division (Goulet and Coffroth 2003a), thus enhancing the clone’s longevity and dispersal potential. The combination of sexual and clonal propagation influences both population dynamics and generational turnover and leads to overlapping generations that may influence evolutionary rates among lineages of Symbiodinium.

Finally, these data indicate sufficient gene flow maintains a single population of S. glynni among adjacent reefs separated by at least 10 km (Fig. 1; Supporting information Table S2). ‘Free-living’ bloom-forming dinoflagellates and diatoms are capable of long-distance dispersal and migration (Evans et al. 2005; Rynearson & Armbrust 2005; Rynearson et al. 2006; Nagai et al. 2007, 2009; Casteleyn et al. 2009; Godhe & Härnström 2010; Lowe et al. 2010). The extent to which symbionts disperse is likely dependent on multiple intrinsic and extrinsic factors. Ultimately, further analyses are required to evaluate gene flow and dispersal among populations of S. glynni and examine the influence of host dispersal on symbiont populations.

Symbiont genotypic diversity and spatial/temporal stability inside a host colony

Are colonies typically dominated by a single Symbiodinium genotype and to what extent are these combinations stable? The available data from multilocus genotyping suggest most colonies associate with a single stable dominant genotype (Goulet & Coffroth 2003a,b; Santos et al. 2003; Pettay & LaJeunesse 2007; Kirk et al. 2009; Thornhill et al. 2009; Pinzón et al. 2010). However, others suggest a majority of colonies harbour extensive genotypic diversity (Apprill & Gates 2007; Magalon et al. 2006; Howells et al. 2009). An analysis of multiple replicate samples from individual colonies using nine optimized loci tested against isoclonal cell lines (Pettay & LaJeunesse 2009; Wham et al. 2011) were initiated to resolve these competing, but not necessarily exclusive possibilities.

The majority of samples (i.e. branches) contained only one detectable genotype (Table 3). However, when additional samples from different sides/locations on a colony were analysed, additional genotypes were recovered (approximately 47% of colonies sampled three or more times; Fig. 3, Supporting information Fig. S2, Table 3). The co-existence of multiple genotypes is therefore considerably greater once the whole colony is considered, but far less than indicated by previous analyses of Pocillopora symbionts (Magalon et al. 2006). When multiple genotypes did occur in a colony, each typically inhabited/dominated separate regions and unrelated to irradiance levels (Fig. 3). In those colonies where five branches were subsampled (Fig. 1), the base of a branch, deep inside the colony, had the same genotype found at the apex of the branch exposed to full sunlight in nearly all branches (59 of 60). This within branch homogeneity and finding the same symbiont genotype in nearby branches suggests that some colonies are a patchwork of a small number of S. glynni genotypes with areas dominated by one genotype and the occasional overlap in distribution of two or more
co-occurring genotypes (Fig. 3). While some physiological differences probably exist among S. glynni genotypes (an assumption that requires testing), there was no indication that a particular genotype favoured a particular side/location of the colony (e.g. north vs. south) or zone of the branch canopy (upper vs. lower), suggesting that external environmental factors have a limited influence on S. glynni genotype distribution at the scale of the colony.

Temporal stability of a resident symbiont genotype may be a common feature for coral–Symbiodinium associations (Goulet & Coffroth 2003a,b; LaJeunesse 2005; Kirk et al. 2005; Thornhill et al. 2009). Colonies of the Montastraea annularis species complex sampled repeatedly over time and at different locations within the colony were found to host mostly one genotype of Symbiodinium B1 from seasonal samplings over 4 years (Thornhill et al. 2009). A majority (67–89%) of S. glynni genotypes sampled from a particular colony orientation (north, south, east or west) remained stable when resampled after 9 months (Fig. 3). Many of the observed temporal ‘changes’ in symbiont identity involved the replacement of one genotype by another already present within the colony. Finding of a novel genotype 9 months later suggests the possibility that the introduction and spread of a distinct S. glynni genotype may occur rapidly. However, it was not possible to determine whether instances of a ‘new’ genotype represented an introduction or one simply missed during the initial sampling effort.

No correlation between host and symbiont genotypes

The vertical transmission of symbionts in the life cycle of Pocillopora could lead to the co-evolution between host and symbiont multilocus lineages (Glynn et al. 1991; Chávez-Romo & Reyes-Bonilla 2007; Hirose et al. 2008). Monophyletic ‘species’ lineages of Symbiodinium specific to Pocillopora do exist and indicate that the mode of symbiont transmission influences the diversification of symbiont lineages (LaJeunesse 2005; Pinzón & LaJeunesse 2011). Genetic analyses were conducted for both host and symbiont to test whether such patterns exist at the finest levels of genetic diversity. In contrast to species diversity, relatedness among S. glynni genotypes did not correlate with that of its Pocillopora host (Figs 4 and 5). While symbiont populations are relatively stable and homogenous across most colonies, there is some degree of flexibility and interchangeability between host and symbiont multilocus lineages.

The examination of multiple ramets of a host genet offers a way to examine the frequency and/or propensity for S. glynni genotypes to shift in dominance within a colony either through the acquisition of new genotypes or from the differential growth of background populations. The most notable of these was the large host genet comprising 43 clone mates distributed over ninety metres of nearshore habitat at ISLG (Pinzón 2011). It associated with fifteen genetically distinct S. glynni genotypes (Fig. 5) and suggests that switching may occur between clonal lineages of host and symbiont with some regularity over the host’s lifetime (Figs 4 and 5). When and why shifts in the dominant symbiont genotype occur is unknown, but many colonies frequently harbour two or three, and sometimes as many as six, unrelated S. glynni genotypes (Figs 3 and 5). Such patterns are consistent with the explanation that associations between host and symbiont genotypes are dynamic over space and time. This variability, or flexibility, in genotypic combinations contrasts with the highly specific associations exhibited at the level of species where Pocillopora type 1 associates with only two Symbiodinium spp. in the region (LaJeunesse et al. 2008, 2010b; Pinzón & LaJeunesse 2011). How distributions of various genotypes are influenced by long-standing regional environmental conditions is currently unknown. Populations of Pocillopora with S. glynni are distributed across a broad latitudinal range where average annual temperatures and seasonal variation in water clarity differ considerably. If significant physiological variation exists among genotypes of S. glynni, then these strong environmental gradients may affect the genetic composition and diversity among populations of S. glynni (Pettay 2011).

Future research is required to examine the relative significance of genetic (allelic) and accompanying physiological variation among individual genotypes. The present diversity found within populations of Symbiodinium may contain an unrealized source of diversity permitting the physiological adaptation of corallinoflagellate symbioses within ecological (decadal) time frames (Carroll et al. 2007). For populations of Pocillopora, the capacity for frequent shifts among S. glynni genotypes suggests the potential for rapid replacement of resident genotypes through selective sweeps involving better adapted genotypes (Palys et al. 1997).

Consequences of multiclone infections

What limits the frequency and abundance of multiple Symbiodinium clones inside a colony? The patterns and consequences of multiple-clone infections of disease causing eukaryotes may provide insight into mechanisms regulating coral–algal mutualisms. Plasmodium, the causative agent of malaria, is a haploid eukaryote belonging to Apicomplexa, the sister phylum to Dinoflagellata. Multiple-clone infections of a single Plasmodium spp. are common, with incidence as high as 80%
in some populations (Read & Taylor 2001; Bell et al. 2006; Havrylik & Ferreira 2009). Experimental manipulations show that multiple-clone infections lead to competition for resources, resulting in higher virulence (Taylor et al. 1997; De Roode et al. 2005; Bell et al. 2006). Asymptomatic infections may become symptomatic and potentially dangerous to the host following the second infection of a competing parasite genotype (P. malariae; Bruce et al. 2007; however see Vardo-Zalik & Schall 2008). This increased virulence decreases host fitness and increases parasite transmission (Mackinnon & Schall 2008). To avoid similar processes from occurring within Pocillopora, mechanisms may exist that limit or suppress multiple-clone infections and thereby minimize a competitive arms race for host resources. A rapidly growing Symbiodinium clone that out-competes all other genotypes could negatively affect the growth and reproduction of the host. If multiple-clone infections within a colony lead to instability (i.e. increased resource use at the expense of the host) in the symbiosis, then there may selective advantages for mechanisms that minimize the genotypic diversity within individual colonies. Ultimately, there are numerous biotic and abiotic factors controlling the balance, stability and specificity observed between Symbiodinium populations and their host colony (Trench 1993; Rowan & Knowlton 1995; Douglas 1998; Coffroth et al. 2001). Ongoing research analysing genome expression libraries may provide insight into matters of cellular recognition and population control (e.g. Voolstra et al. 2009).

Conclusions

The application of genetic markers that resolve among individuals ushers a new phase in coral–dinoflagellate symbioses research and discovery. In summary, (i) populations of S. glynni comprised numerous clonal lineages generated by sexual recombination; (ii) a large proportion of these genotypes were found in only colony during one sampling time, while others were common to many colonies, widely distributed (up to 10 km) and persisted in the community for at least 3 years; (iii) the genetic compositions (allele frequencies) of S. glynni populations were stable over 3 years; (iv) a single multilocus genotype was detected in most branches sampled; however, additional genotypes (but rarely more than three) were found in some colonies sampled many times; (v) when present, multiple genotypes within a colony were partitioned spatially and apparently uninfluenced by external irradiance; (vi) many genotypes persisted within a colony over time; and (vii) no correlation was found between host and symbiont multilocus lineages, indicating that individual genotypes of host and symbiont switch through time, providing a mechanism for colonies to acquire physiological variants adapted to local environmental conditions. The numerous observations made from this study should initiate a breadth of questions and inquiry. For example, do the patterns observed here differ substantially when symbionts in host communities are examined from warmer more stable environments? Or, how do these observations relate to coral colonies and other cnidarians with horizontal modes of symbiont acquisition? Continued progress in this emerging area of coral–dinoflagellate symbiosis research will fundamentally advance our ecological and evolutionary understanding of these important partnerships.

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The data presented in this manuscript represent a portion of D.T.P. PhD dissertation on the genetic structuring of populations of clade D Symbiodinium. D.T.P. is interested in the microevolutionary processes that shape the diversity and evolution of coral-algal symbioses and the physiological significance of this diversity with respect to different coral-algal combinations. He is currently a postdoctoral researcher in the laboratory of Dr. M.W. at the University of Delaware, where his research focuses on the effects of ocean acidification on coral-algal symbioses. T.C.L. studies the ecological and co-evolutionary processes important in animal–microbe associations as well as the response of coral-algal partnerships to major changes in climate.

Data accessibility

Sample locations and microsatellite data: DRYAD entry doi:10.5061/dryad.104d5g03.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 An example of how one microsatellite locus (D1Sym87) resolves two S. glynni genotypes at different proportions and demonstrates that there is little preferential amplification based on fragment size.

Fig. S2 Summary plots of S. glynni genotypic diversity within individual Pocillopora colonies based on sampling strategy.

Fig. S3 Correlograms showing the spatial correlation rc at each distance class (1 m) for each circular plot individually and the combined analysis for each reef, 95% confidence interval about the null hypothesis of random distribution of S. glynni clones, and 95% confidence errors bars about rc as determined by bootstrapping.

Table S1 Allele frequencies for each reef according to sample date.

Table S2 Summary of AMOVA examining temporal population differentiation for ISLG and PGAL.

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Supplemental Figure 1. An example of how one microsatellite locus (D1Sym87) resolves two *S. glynni* genotypes at different proportions and demonstrates that there is little preferential amplification based on fragment size. The ratio of the smaller allele to the larger allele is given in the corner of each electropherogram.
Supplemental Figure 2. Summary plots of *S. glynni* genotypic diversity within individual *Pocillopora* colonies based on sampling strategy. (a) Percent of colonies possessing multiple genotypes of *S. glynni* and (b) estimated number of genotypes per colony based on the number times a colony was sampled. *Five branches of each colony were sampled, but these branches were sampled at the tops and bottoms for a total of ten locations genetically analyzed.*
Supplemental Figure 3. Correlograms showing the spatial correlation $r_c$ at each distance class (1 m) for each circular plot individually and the combined analysis for each reef, 95% confidence interval about the null hypothesis of random distribution of $S$. glynni clones, and 95% confidence errors bars about $r_c$ as determined by bootstrapping.