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Host hybridization alters specificity of cnidarian–dinoflagellate associations

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ABSTRACT: Scleractinian coral evolution is often characterized by alternating patterns of lineage diversification and fusion, thus leading to reticulate evolution. Although this pattern is hypothesized in many coral lineages, including the *Montastraea annularis* species complex, it is not known what effects cladogenesis and hybridization have on the symbioses between corals and their endosymbiotic dinoflagellates (genus *Symbiodinium*). To explore this, the genetic diversity of *M. faveolata* and *M. annularis* in the Upper Florida Keys, USA, and Exuma Cays, The Bahamas, was examined using a mtDNA intergenic region. The host genotypic data were then analyzed in relation to the diversity of the corals' *Symbiodinium* communities as determined by internal transcribed spacer region 2 (ITS2) and 3 microsatellite markers specific to *Symbiodinium* Clade B. *M. faveolata* and *M. annularis* in the Upper Florida Keys were genetically distinct from one another while these coral species in the Exuma Cays shared mtDNA haplotypes. These findings suggest possible regional differences in the degree of intergressive hybridization between *M. faveolata* and *M. annularis*. When *Symbiodinium* diversity was examined, *Montastraea* spp. from both regions shared *Symbiodinium* ITS2 genotypes; however, host–symbiont specificity was observed using higher resolution microsatellite markers. Specifically, *M. faveolata* and *M. annularis* from the Upper Florida Keys all harbored genetically distinct multilocus Clade B genotypes, whereas these 2 coral species in the Exuma Cays shared Clade B genotypes. Consequently, the degree of fine-scale specificity between *Symbiodinium* Clade B genotypes and *Montastraea* spp. appears to be governed by the degree of genetic distinction, and possibly hybridization, between these host 'species'.

KEY WORDS: Symbiosis · Reticulate evolution · Coral reef · Endosymbiosis · *Montastraea* · *Symbiodinium* · Scleractinia

INTRODUCTION

The evolution of many reef-building (i.e. scleractinian) corals has been shaped by alternations between cladogenesis and hybridization (reviewed in Willis et al. 2006), hypothetically leading to a reticulate pattern of evolution (Veron 1995). This pattern stems from a high potential for interspecies hybridization because the majority of corals reproduce via synchronous broadcast spawning of gametes, followed by external fertilization in the water column (Szmant 1986, Richmond & Hunter 1990). As a result, corals experience few environmental or behavioral prezygotic barriers to interspecies fertilization. For instance, the Caribbean broadcasting species *Acropora cervicornis* and *A. palmata* are known to hybridize regularly, producing apparently sterile offspring (i.e. *A. prolifera*) that are morphologically intermediate between the parent species (van Oppen et al. 2000, Vollmer & Palumbi 2002). Furthermore, strong genetic similarities among Caribbean *Madracis* (Diekmann et al. 2001) and Australian *Platygyra* (Miller & Benzie 1997) may also provide evidence for such phenomena. In the case of closely related Indo-Pacific *Acropora*, widespread hybridization has led to complex evolutionary patterns that have hindered the demarcation of species boundaries

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(reviewed in Willis et al. 2006). Given this, it is not surprising that elucidating coral evolution has been especially challenging when different processes (i.e. isolation versus hybridization) occur contemporaneously in varying regions of the world (Benzie 1999).

One example of a broadcast-spawning scleractinian lineage with an intricate evolutionary history is the *Montastraea annularis* species complex. Members of this lineage occur throughout the Caribbean Sea and western Atlantic Ocean where they are major contributors to coral reef accretion (Goreau 1959). Although debate surrounds the taxonomic status of members within the complex (Knowlton et al. 1992, 1997, Szmant et al. 1997, Medina et al. 1999), 3 morphospecies have been formally described: *M. annularis*, *M. faveolata* and *M. franski*. Notably, recent morphological and molecular genetic studies indicate these 'species' hybridize at a higher frequency in the Bahamas than in Panama (Budd & Pandolfi 2004, Fukami et al. 2004). This has lead to the suggestion that a genetic isolation versus hybridization gradient exists for *Montastraea* spp., resulting in differing 'species' boundaries across the Caribbean (Fukami et al. 2004). However, the frequency of hybridization between these corals at other localities, as well as how it may influence other aspects of their biology, remains to be determined.

One interesting, yet unexplored, question is whether hybridization among scleractinian 'species' affects the intimate relationship they share with endosymbiotic dinoflagellates. Belonging to the genus *Symbiodinium*, these symbionts represent a diverse group of photosynthetic eukaryotic microbes that are associated with numerous protistan and invertebrate hosts (reviewed in Trench 1993, Stat et al. 2006) and which play a pivotal role in the high growth and calcification rates of scleractinians (reviewed in Barnes & Chalker 1990, Trench 1993). Based on molecular genetic evidence, *Symbiodinium* can be divided into 8 major clades (i.e. A to H; reviewed in Coffroth & Santos 2005), each of which can contain numerous 'types' (defined by the sequences of their internal transcribed spacer [ITS] regions) and with many 'types' proposed to be distinct species (LaJeunesse 2001, Sampayo et al. 2009, but see Pochon et al. 2007, Correa & Baker 2009). In terms of their symbiotic biology, certain 'types', known as 'specialists', associate with only a single host species whereas others, referred to as 'generalists', form symbioses with more than a single host species (reviewed in Stat et al. 2006). Conversely, many hosts harbor only a single *Symbiodinium* 'type' throughout their geographic and depth ranges, while some are capable of symbioses with multiple *Symbiodinium* lineages either occurring separately or simultaneously (e.g. LaJeunesse 2002, Goulet 2006, Thornhill et al. 2008). Although a considerable body of work on *Symbiodinium* diversity and distributions has accumulated, little research has attempted to integrate such data into the genetics of the host populations from which they were acquired (but see Barshis et al. 2010, Bongaerts et al. 2010).

Overall, 'species' of the *Montastraea annularis* complex are among the most flexible scleractinians known when it comes to their *Symbiodinium* complements; symbioses with both 'specialist' and 'generalist' ITS 'types' from Clades A, B, C and D have been reported across these corals' depth and geographic ranges (Toller et al. 2001, LaJeunesse 2002, Thornhill et al. 2006a). Despite this, Thornhill et al. (2009) observed species-specific *Symbiodinium* Clade B genotypes, identified via microsatellites, associated with *M. annularis* and *M. faveolata* from the Upper Florida Keys, USA. In contrast, *Symbiodinium* Clade B microsatellite genotypes were shared between these *Montastraea* species in the Exuma Cays, The Bahamas (Thornhill et al. 2009). Such observations led to the hypothesis that specificity in the *Symbiodinium* Clade B associations of *M. annularis* and *M. faveolata* is inversely correlated to the degree of hybridization between the 2 hosts (Thornhill et al. 2009). If correct, *M. annularis* and *M. faveolata* in the Florida Keys are hypothesized to be genetically distinct, whereas the same 2 'species' should be genetically similar in the Exuma Cays (following Fukami et al. 2004). In the present study, these predictions were tested in the Florida Keys and Exuma Cays by examining the genetic diversity and structure of *M. annularis* and *M. faveolata* in the context of their corresponding population-level *Symbiodinium* Clade B diversity.

METHODS

Study sites and collection of *Montastraea faveolata* and *M. annularis*. *Montastraea faveolata* and *M. annularis* were sampled from 5 western Atlantic Ocean locations, including 3 reefs in the Upper Florida Keys and 2 reefs in the Exuma Cays (Fig. 1). In the Florida Keys, these sampling locations were the inshore Admiral Patch Reef (ADM; 1 to 2 m depth, 25.045° N, 80.395° W), offshore Little Grecian Reef (LG; 3 to 4 m depth, 25.119° N, 80.302° W) and the deeper water Alligator Reef (AG; 12 m depth, 24.842° N, 80.624° W). Similarly, the inshore North Norman's Patch (NP; 2 to 4 m depth, 23.791° N, 76.137° W) and deeper water South Perry Reef (SP; 12 to 15 m depth, 23.775° N, 76.090° W) were sampled in the Exuma Cays.

On each reef, 6 colonies of both *Montastraea faveolata* and *M. annularis*, each separated by approximately 4 to 50 m from the nearest conspecific individual, were sampled by SCUBA or snorkel diving (a total of

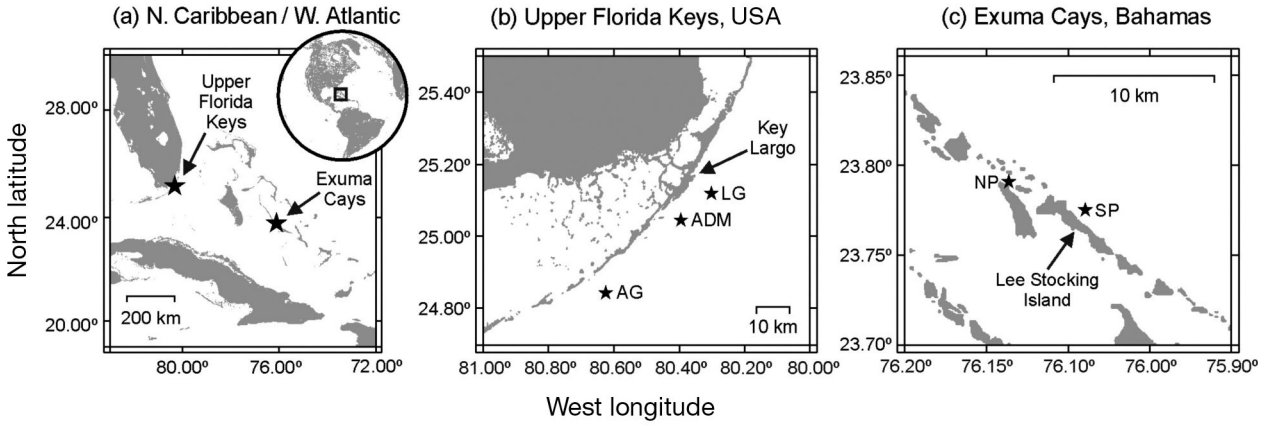

Fig. 1. *Montastraea faveolata* and *M. annularis*. (a) Regional and (b,c) local collection sites. Reef abbreviations for the Upper Florida Keys are: LG , Little Grecian Reef; ADM, Admiral Patch Reef; AG, Alligator Reef. Abbreviations for the Exuma Cays are: NP, North Norman's Patch Reef; SP, South Perry Reef. Figure is adapted from Thornhill et al. (2009)

30 colonies for *M. faveolata* and 30 for *M. annularis*). Colonies were specifically selected based on colony features and corallite morphology characteristic of either *M. faveolata* or *M. annularis* as described by Knowlton et al. (1992). While selecting morphologically distinct colonies helps ensure correct identification of each 'species', this approach potentially underestimates the degree of genetic similarity between *Montastraea* species. Therefore, presented results should be considered minimum measures of genetic similarity within a 'species' for a region. Approximately 10 cm^2 fragments were removed by means of a hammer and chisel, and care was taken to ensure that the same relative position (i.e. unshaded colony tops) was sampled from each colony. Upon collection, fragments were placed in labeled plastic bags filled with seawater and immediately transported to the laboratory in an insulated cooler for processing.

Nucleic acid extraction, PCR and DNA sequencing. Each sample was split into 2 fragments. Tissue was removed from one fragment using a recirculating Waterpik (Water Pik) with 0.45 µm filtered seawater and the 'blastate' pulsed for 1 to 4 s with a tissue homogenizer (Polytron Kinematica Tissue Homogenizer™, Brinkmann Instruments) to disperse mucopolysaccharides. *Symbiodinium* cells for molecular analyses were concentrated from the saltwater 'blastate' via centrifugation in 50 ml tubes at 2000 to 3000 × *g* for 5 min, preserved in DMSO buffer (20% DMSO and 0.25 M EDTA in NaCl-saturated water, Seutin et al. 1991) and nucleic acids subsequently extracted as described by Thornhill et al. (2006a). The data collection methods for the internal transcribed spacer region 2 (ITS2)-PCR, DGGE and microsatellite amplifications of *Symbiodinium* Clade B populations were provided in Thornhill et al. (2009).

The second fragment was placed directly into DMSO buffer and genomic DNA was extracted from the holobiont (i.e. host and *Symbiodinium*) using the DNeasy Tissue Kit (QIAGEN) according to the manufacturer's instructions. To examine the genetic diversity of *Montastraea* spp., a number of loci targeting the nuclear genome, including 4 microsatellites (Severance et al. 2004a,b) and 3 sequence-characterized regions derived from amplified fragment length polymorphisms (AFLP) (Fukami et al. 2004), were tested. These microsatellite and AFLP markers, however, failed to consistently amplify across all samples on numerous attempts and under modified (e.g. lower primer annealing temperatures and/or varying $MgCl_2$ concentrations) PCR conditions. For this reason, as well as to avoid potential misinterpretations of characteristics such as null alleles and heterozygote deficiencies, the limited data derived from these markers are not presented here. As an alternative, an 891 bp intergenic region of noncoding mtDNA was targeted (Fukami et al. 2004). This noncoding mtDNA region is flanked by the cytochrome oxidase subunit I (COI) and $tRNA^{MET}$ genes and appears to have originated via a duplication of COI (Fukami & Knowlton 2005). In contrast to the nuclear markers, the intergenic region of the mtDNA was readily amplified from all samples (a ~900 bp amplicon) using the host-specific primers 'MCN1f' (5′-GAGCTGGGCTTCTTTAGAGTG-3′) and 'MCN1r' (5′-GTGAGACTCGAACTCACTTTTC-3′) (Fukami et al. 2004). Amplification was performed with the following thermocycling conditions: initial denaturation, 3 min at 94°C; 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s; followed by a final extension of 10 min at 72°C. To ensure that these primers were specific to the coral host, control PCRs were performed on DNA extracted

from 5 *Symbiodinium* cultures representing ITS2 'types' from Clades A through F. These included cultures 292 ('type' A3), 141 ('type' B2), 152 ('type' C1), A001 ('type' D1a) and 133 ('type' F2) (ITS 'type' nomenclature sensu LaJeunesse 2001, 2002). In all cases, the MCN1f/MCN1r primers failed to generate a product from these cultures, indicating amplicons resulting from holobiont genomic DNA were of *Montastraea* spp., and not *Symbiodinium*, origin.

Amplification success was verified by 1× sodium borate agarose gel electrophoresis and amplicons subsequently purified using a QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Products were bidirectionally sequenced using the MCN1f/MCN1r primer set by Geneway Research (Hayward). The resulting chromatograms were checked in Chromas v. 2.33 (Technelysium Pty) and sequences deposited in GenBank (www.ncbi.nlm.nih. gov) under accession numbers HM162916 to HM162925.

Molecular analyses. *Symbiodinium* ITS2 and multilocus microsatellite genotype data reported by Thornhill et al. (2009) were reanalyzed to evaluate the hypothesis that specificity in the *Symbiodinium* Clade B associations of *Montastraea annularis* and *M. faveolata* are correlated to the degree of genetic differentiation between these 2 coral species. Pairwise tests for *Symbiodinium* population differentiation, as accessed by Φ_{PT} values (a haploid equivalent to F_{ST}), were conducted by randomizing genotypes between pairs of populations (host–reef combinations) for both regions using GenAlEx v. 6.2 (Peakall & Smouse 2006). Significance of the pairwise tests was assessed by 9999 permutations and with $\alpha = 0.01$. Additionally, the number of genetically differentiated populations (k) as well as the affiliation of samples (n = 3 to 4 per host colony; see Thornhill et al. 2009) with similar multilocus genotypes to those populations were determined by Bayesian clustering analyses implemented in Structure v. 2.2 (Pritchard et al. 2000) using the default admixture model with correlated allele frequencies. Twenty replicate runs for k values between 1 (no population differentiation) and 18 (the maximum number of distinct *Symbiodinium* Clade B multilocus genotypes sampled in any region) were performed, with each replicate run for 1×10^6 iterations following an initial burn-in of 1×10^5 iterations.

To examine the spatial and 'species' distributions of mtDNA genetic variation in *Montastraea* spp., the noncoding intergenic sequences were first aligned manually with MacClade v. 4.06 (Sinauer & Associates). The numbers of polymorphic and parsimony informative sites as well as haplotype and nucleotide diversities were quantified using DnaSP v. 4.06 (Rozas et al. 2003). Percentages of adenine and thymine (A-T) and guanine and cytosine (G-C) content were calculated using BioEdit v. 7.0.9 (Hall 1999). To test for genetic differentiation between *M. faveolata* and *M. annularis* harboring *Symbiodinium* Clade B populations within each region, the nearest-neighbor statistic, *Snn*, (Hudson 2000) was estimated and significance assessed by 1000 permutations with $\alpha = 0.01$ in DnaSP v. 4.06. Networks were also constructed separately for the *Montastraea* species haplotypes from Florida and the Bahamas with TCS v. 1.21 (Clement et al. 2000) using the default settings to visualize their relationships. Lastly, the networks were overlaid onto the Structure analyses (see above) to correlate the particular *Symbiodinium* Clade B multilocus genotypes/population hosted by a given colony relative to its specific *Montastraea* spp. mtDNA haplotype.

RESULTS

Genetic diversity and symbiotic specificity of *Symbiodinium*

Thornhill et al. (2009) presented the spatial and temporal distribution of *Symbiodinium* genotypes from the *Montastraea* spp. corals examined in the present study. Herein we summarized and reanalyzed these data in the context of host–symbiont specificity. At the resolu-

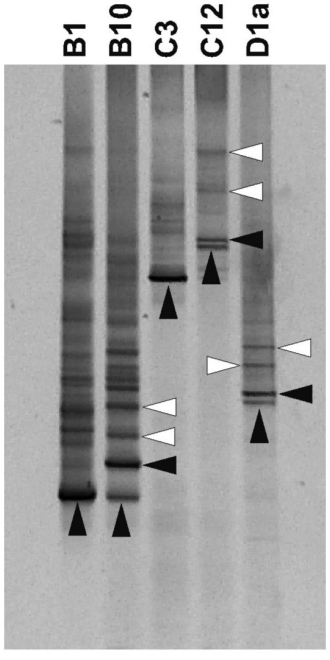

Fig. 2. *Symbiodinium*. Example PCR-DGGE profiles of the ITS2 region for *Symbiodinium* 'types' B1, B10, C3, C12 and D1a. Primary diagnostic bands are indicated for each *Symbiodinium* 'type' (▲). Fainter bands in each profile represent heteroduplexes (Δ; see LaJeunesse 2002), rare ITS2 intragenomic variants (see Thornhill et al. 2007) or other methodological artifacts (see Thornhill et al. 2010). Profiles are presented as inverse images

tion provided by ITS2 PCR-DGGE (see Thornhill et al. 2006a, LaJeunesse et al. 2009), a total of 5 different *Symbiodinium* ITS2 'types' were detected among the 60 colonies of *M. faveolata* and *M. annularis* we sampled. These included ITS2 'types' B1, B10, C3, C12 and D1a (Fig. 2). Two of these 'types' (i.e. B1 in both regions and C12 in the Exuma Cays) were recovered from both host species, whereas the other 'types' were found only in either *M. faveolata* (i.e. D1a at NP) or *M. annularis* (i.e. B10 at LG; C3 at ADM and AG). However, previous work on these tagged colonies detected additional host–*Symbiodinium* combinations, including B10 in *M. faveolata* (at LG) and D1a in *M. annularis* (at ADM and LG) (Thornhill et al. 2006a). Thus, at the ITS2 level, these 5 *Symbiodinium* 'types' appear to form relatively nonspecific symbioses with members of the *M. annularis* species complex.

The majority (50 of 60 colonies, 83.3%) of *Montastraea* spp. examined associated with ITS2 'types' within *Symbiodinium* Clade B (i.e. B1 or B10). For these, 3 microsatellite loci specific to *Symbiodinium* Clade B were amplified to further investigate host–symbiont pairings at finer genetic scales. Twenty-four unique multilocus genotypes were detected among the *Montastraea* species colonies. Importantly, while colonies were either immediately adjacent, or in close proximity, to each other on the same reefs in Florida, no multilocus genotypes were shared among *M. faveolata* and *M. annularis* (Thornhill et al. 2009). The genetic isolation and fixed genotypic differences of *Symbiodinium* Clade B populations from *M. faveolata* and *M. annularis* in the Florida Keys were also evident in the Bayesian clustering analyses (Fig. 3a). In this case, although some reefs contained multiple inferred clusters, they were not shared between the host 'species' (Fig. 3a). Furthermore, all pairwise comparisons of *Symbiodinium* Clade B population differentiation from the different host 'species' were significant in the Florida Keys (Table 1). By contrast, 2 multilocus genotypes of *Symbiodinium* Clade B were shared between reefs as well as host 'species' for *M. faveolata* and *M. annularis* in the Exuma Cays (Fig. 3b). Given this, certain pairwise comparisons of population differentiation across the host 'species' were nonsignificant (Table 2). Taken together, *Symbiodinium* Clade B populations appear to be consistently host-specific in Florida, whereas such a pattern is less apparent for both *M. faveolata* and *M. annularis* in the Bahamas (Fig. 3).

Genetic diversity and symbiotic specificity of *Montastraea faveolata* and *M. annularis*

A total of 919 bp of mitochondrial noncoding sequence data were successfully obtained for all (100%) of the *Montastraea faveolata* and *M. annularis* colonies examined in this study (n = 60). Of these, 100% of the nucleotide positions could be unambiguously aligned. This aligned data set is publicly available from www.auburn.edu/~santosr/sequencedatasets.htm.

Mitochondrial DNA is notoriously invariable among scleractinian corals (Medina et al. 1999, Shearer et al. 2002, Hellberg 2006). Despite this, nucleotide sequence diversity was detected among the sampled *Montastraea faveolata* and *M. annularis*. Specifically, a total of 11 positions (1.2%) were found to be variable; of these, 4 (0.4%) were parsimony-informative. Total nucleotide diversity (π) was 0.00131 ± 0.00013 (±SD) and sequences were adenine and thymine (A-T) rich (63.8%) compared with mean guanine and cytosine (G-C) content (36.2%). Also, *M. franski* colonies were sampled wherever they were available to ensure the reported mtDNA variation did not result from inadvertent missampling of this *Montastraea* species. In all cases, *M. franski* could be readily distinguished from other *Montastraea* species by specific host mtDNA haplotypes, *Symbiodinium* associations (i.e. 'types' C3 or C12 with *M. franski* and 'type' B1 in other *Montastraea* species) and/or morphology (results not shown).

Ten different noncoding mtDNA haplotypes were detected among the 60 *Montastraea faveolata* and *M. annularis* (Table 3), for a haplotype diversity (*h*) of 0.783 ± 0.031 (±SD). In the context of each host 'species', a total of 8 haplotypes were recovered from *M. faveolata* (designated A, B, E, F, G, H, I and J), of which 2 (i.e. F and G) and 4 (i.e. A, E, I and J) occurred exclusively in the Florida Keys and Exuma Cays, respectively (Table 3, Fig. 3). The remaining 2 haplotypes (i.e. B and H) were found in *M. faveolata* of both regions (Fig. 3). Similar patterns were identified among *M. annularis*; 4 total haplotypes were detected (i.e. A, C, D and E), one (i.e. C) of which was exclusive to the Florida Keys, none occurred exclusively in the Exuma Cays and 3 (i.e. A, D and E) were shared between regions. Perhaps most notably, no haplotypes were shared between host 'species' in the Upper Florida Keys (Table 3). This genetic differentiation between Florida *M. faveolata* and *M. annularis* was reflected statistically by a significant nearest-neighbor comparison between the 2 species ($Snn = 0.93750$, $p < 0.0001$). In contrast, the sharing of 2 haplotypes (i.e. A and E) among the different host morphospecies in the Exuma Cays (Table 3) led to a nonsignificant nearest-neighbor comparison for *M. faveolata* and *M. annularis* ($Snn = 0.70667$, $p > 0.01$).

To further explore the relationships among *Montastraea faveolata* and *M. annularis* noncoding mtDNA haplotypes, statistical parsimony (TCS) analyses were conducted for the Florida Keys and Exuma Cays populations. As noted above, no haplotypes were shared

between host species in the Florida Keys. Instead, haplotype B was recovered from the majority (n = 15) of *M. faveolata* while haplotype A accounted for the majority (n = 14) of *M. annularis* (Fig. 3a). Three additional haplotyes were also found in *M. faveolata* (i.e. F, G and H) and *M. annularis* (i.e. C, D and E) in the Florida Keys, albeit at low (n = 1 or 2) frequencies. When the *Symbiodinium* Clade B multilocus genotypes associated with specific colonies are overlaid onto the network, correlations between symbiont diversity and distributions and host 'species' noncoding mtDNA haplotypes are readily apparent (Fig. 3a). For example, 4 *Symbiodinium* clusters associated with LG *Montastraea* species. One of these clusters associated exclusively with *M. annularis* (halpotype A), whereas the remaining 3 clusters were specific to *M. faveolata*

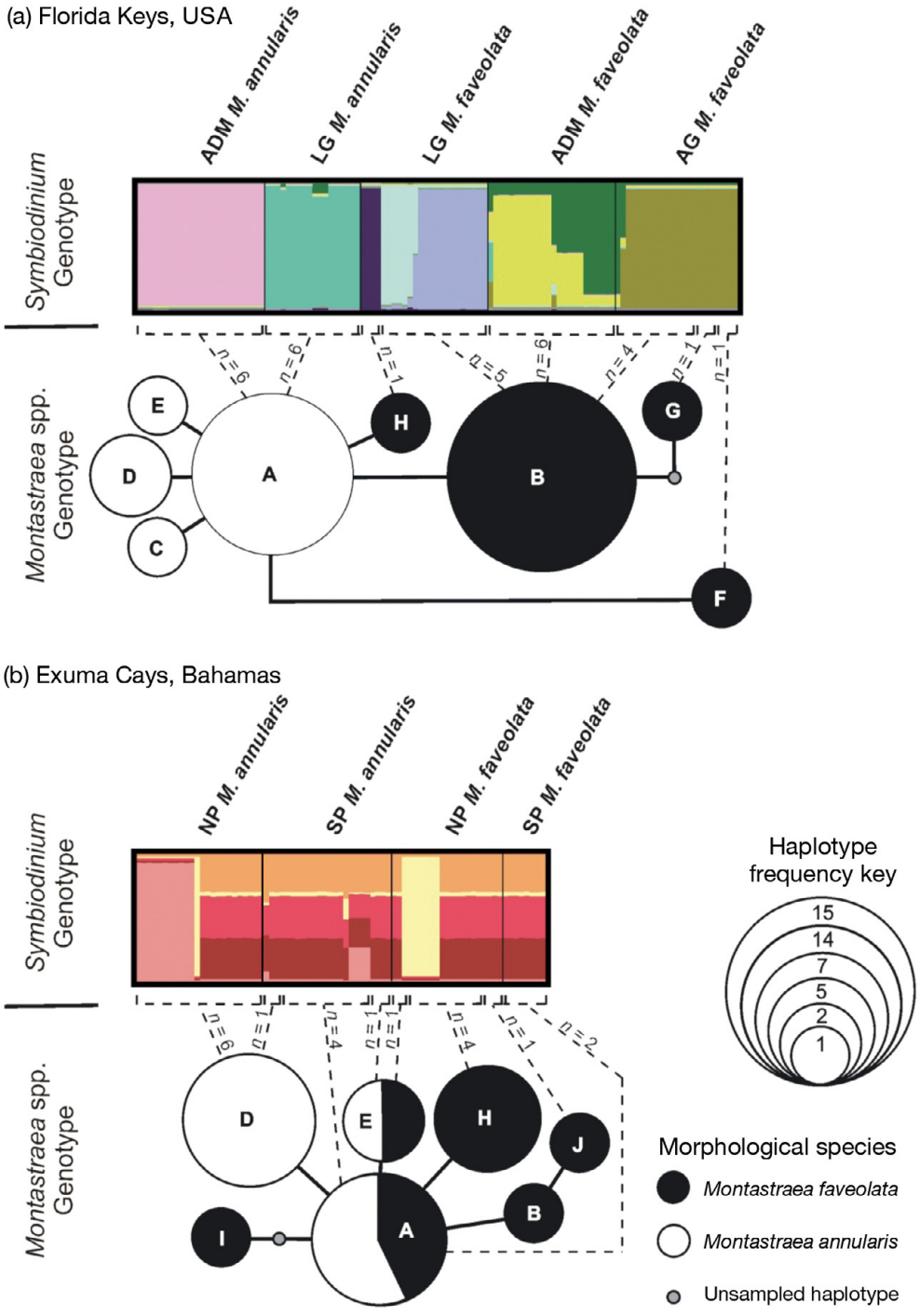

Table 1. *Symbiodinium*. Pairwise comparisons of Φ_{PT} values (a haploid equivalent to F_{ST}) among *Symbiodinium* Clade B populations originating from *Montastraea faveolata* and *M. annularis* in the Upper Florida Keys. See Fig. 1 for reef abbreviations. *p < 0.0001

Reef and host species	LG *M. faveolata*	ADM *M. faveolata*	AG *M. faveolata*	LG *M. annularis*	ADM *M. annularis*
LG *M. faveolata*		0.669*	0.758*	0.774*	0.707*
ADM *M. faveolata*			0.682*	0.798*	0.876*
AG *M. faveolata*				0.905*	0.942*
LG *M. annularis*					0.912*
ADM *M. annularis*					

Table 2. *Symbiodinium*. Pairwise comparisons of Φ_{PT} values (a haploid equivalent to F_{ST}) among *Symbiodinium* Clade B populations originating from *Montastraea faveolata* and *M. annularis* in the Exuma Cays. See Fig. 1 for reef abbreviations. *p < 0.01, **p < 0.0001; ns: not significant, p > 0.01

Reef and host species	NP *M. faveolata*	SP *M. faveolata*	NP *M. annularis*	SP *M. annularis*
NP *M. faveolata*		0.190 (ns)	0.335**	0.203*
SP *M. faveolata*			0.273 (ns)	0.029 (ns)
NP *M. annularis*				0.201**
SP *M. annularis*				

(i.e. 2 clusters with halplotype B and a third cluster with haplotype H) (Fig. 3a).

Compared with the genetic differentiation of *Montastraea* species in the Florida Keys, the network analysis clearly showed the 2 haplotypes (i.e. A and E) were shared between the morphospecies *Montastraea faveolata* and *M. annularis* in the Exuma Cays (Fig. 3b). Specifically, 3 colonies of *M. faveolata* and 4 colonies of *M. annularis* shared haplotype A (the most common *M. annularis* haplotype in the Upper Florida Keys), while one colony of *M. faveolata* and one colony of *M. annularis* shared haplotype E. The remaining Exuma Cay haplotypes were recovered in only 1 of the 2 host species. For *M. faveolata*, these included haplotypes B (n = 1; note the difference in abundance relative to Upper Florida Keys specimens), J (n = 1), H (n = 5) and I (n = 1), whereas the remaining *M. annularis* belonged to a single haplotype, D (n = 7). In this case, similar or identical *Symbiodinium* Clade B multilocus genotypes/populations were common among *M. faveolata* and *M. annularis*, including those with the shared host mtDNA haplotypes of A and E (Fig. 3b).

DISCUSSION

Hybridization among *Montastraea* 'species'

The results presented demonstrate that *Montastraea faveolata* and *M. annularis* in the Upper Florida Keys are genetically structured at the level of both host mtDNA haplotypes and their resident *Symbiodinium* Clade B populations. In contrast, an absence of genetic structure between *M. faveolata* and *M. annularis* in the Exuma Cays was due to mtDNA haplotypes being shared between them, and it is notable that *Symbiodinium* Clade B multilocus genotypes were also common between the host 'species' at these sites. Thus, this pattern is consistent with the hypothesis proposed by Thornhill et al. (2009) in that the fine-scale genotypic specificity of *Symbiodinium* Clade B associating with *Montastraea* is linked to the degree of genetic differentiation among the host 'species'. Although one potential explanation of the host mtDNA data is simply incomplete lineage sorting among *Montastraea* spe-

Fig. 3. *Symbiodinium* Clade B multilocus microsatellite and *Montastraea* spp. mtDNA genotypes as depicted by Structure and TCS analyses, respectively, for the (a) Upper Florida Keys and (b) Exuma Cays. For the Structure analyses (upper sections of panels a and b), results are presented as a graphical summary derived from 3 microsatellite loci specific to *Symbiodinium* Clade B. For (a) the Upper Florida Keys $k = 8$, whereas for (b) the Exuma Cays $k = 5$. Each sample (n = 3 or 4 per host colony) is represented by a vertical line broken into segments to represent the estimated proportions of that sample's genome originating from each of the inferred clusters, and each cluster is represented by a different color. Sampling localities are separated by vertical black lines; reef and host species are noted above the graph (see Fig. 1 for reef abbreviations). For the TCS analyses (lower sections of panels a and b), *Montastraea* spp. haplotype networks based on 921 bp of mtDNA are depicted using the 95% connectivity level. Sampled haplotypes are indicated by circles; missing or unsampled haplotypes are indicated by grey dots. Each branch (solid lines) indicates a single mutational difference and circle size is proportional to observed haplotype frequency (see legend, lower right). Haplotypes are shaded according to the morphological species of host from which the sample was collected (see legend, lower right). The clusters of *Symbiodinium* genotypes associated with each coral mtDNA haplotype are indicated by dotted lines connecting the TCS and Structure analyses. The number of coral colonies per haplotype associated with each *Symbiodinium* cluster is indicated along each dotted line. Coral colonies not linked to the Structure analyses by a dotted line harbored only Clade C *Symbiodinium* (i.e. ITS2 'type' C3 in the Upper Florida Keys and ITS2 'type' C12 in the Exuma Cays) within the detection limits for ITS2 PCR-DGGE (see Thornhill et al. 2006a, LaJeunesse et al. 2009)

Table 3. *Montastraea annularis* and *M. faveolata*. Distribution of mtDNA intergenic region haplotypes among host species, reefs and colonies. See Fig. 1 for reef abbreviations

Montastraea spp. mtDNA haplotype	Coral morphological species	Reef and colony nos.	GenBank accession nos.
A	*M. annularis*	ADM 1–6, LG 1–6; AG 1, 6; SP 2–4, 6	HM162916
A	*M. faveolata*	SP 1–3	HM162916
B	*M. faveolata*	ADM 1–6; LG 1–3, 5, 6; AG 2, 4–6; SP 5	HM162917
C	*M. annularis*	AG 5	HM162918
D	*M. annularis*	AG 2, 4; NP 1–6; SP 5	HM162919
E	*M. annularis*	AG 3; SP 1	HM162920
E	*M. faveolata*	NP 3	HM162920
F	*M. faveolata*	AG 3	HM162921
G	*M. faveolata*	AG 1	HM162922
H	*M. faveolata*	LG 4; NP 1, 2, 4, 6; SP 6	HM162923
I	*M. faveolata*	SP 4	HM162924
J	*M. faveolata*	NP 5	HM162925

cies in the Exuma Cays, this possibility seems unlikely considering the distinctly different pattern observed in the Upper Florida Keys and elsewhere (Budd & Pandolfi 2004, Fukami et al. 2004). Rather, a likelier scenario is that regional differences in interspecies hybridization (either in the geologic past or contemporarily) underlie these observations. Geologic evidence, for instance, suggests that introgressive hybridization occurred in the Bahamas during the sea level and climatic fluctuations of the late Pleistocene (Budd & Pandolfi 2004). If this is the case, some type of environmental, prezygotic and/or postzygotic barriers to interspecies reproduction must have been present historically (and/or is present today) in these coral lineages from the Upper Florida Keys (but not in the Exuma Cays), at least among the colonies and reefs investigated in the present study.

Previous reproductive studies have documented *Montastraea faveolata* and *M. annularis* as spawning synchronously and sympatrically in both the Florida Keys and the Exuma Cays, suggesting environmental barriers to hybridization are limited (Szmant et al. 1997, Levitan et al. 2004, see also Knowlton et al. 1997). Furthermore, experimental crossings of *M. faveolata* and *M. annularis* have sometimes, but not always, resulted in planula larvae, demonstrating an incompleteness of prezygotic barriers (e.g. Knowlton et al. 1997, Szmant et al. 1997, Levitan et al. 2004). However, such experiments in *Montastraea* spp. have been conducted in the absence of conspecific sperm or eggs and it is possible that interspecies fertilization declines precipitously when intra- and interspecies gametes compete for fertilization opportunities, as would be the typical case during a spawning event. Although postzygotic barriers to hybridization have not been evaluated thus far in members of the *M. annularis* species complex, they may also play an important role in determining the success of coral zygotes and larvae of hybrid origin. For example, the Indo-Pacific species *Acropora hyacinthus* and *A. cytherea* are sympatric, spawn synchronously and are highly cross-fertile (Márquez et al. 2002). Despite this seemingly large potential for hybridization, *A. hyacinthus* and *A. cytherea* remain statistically distinguishable genetic lineages, signifying infrequent introgressive hybridization in nature due to either pre- or postzygotic reproductive barriers (Márquez et al. 2002). In the case of *Montastraea* spp., the factors ultimately influencing hybridization or incomplete lineage sorting in some regions and divergence in others remain unknown. However, the data presented here, combined with previous work (Budd & Pandolfi 2004, Fukami et al. 2004), suggest the 'species' boundaries between *M. faveolata* and *M. annularis* are weaker in the Exuma Cays than in other regions of the Caribbean Sea.

When considering the *Montastraea* genetic data, it is important to recognize the relatively slow rate at which mtDNA generally evolves in scleractinian corals (Shearer et al. 2002, Hellberg 2006), including the 'species' examined here (Medina et al. 1999). For instance, Fukami & Knowlton (2005) found just 25 variable characters from the entire mitochondrial genomes (~16.2 kbp) of the 3 members of the *M. annularis* species complex (notably, 16 of these characters came from the specimen of *M. franksi* that was sequenced). Based on this level of genetic variability and their split from other *Montastraea* at 3 to 4 million years ago, they estimated *Montastraea* mtDNA diverges at a maximum rate of only 0.03 to 0.04% per 1 million years (Fukami & Knowlton 2005). Furthermore, fossil evidence indicates that *M. annularis* and *M. cavernosa* diverged approximately 24 million years ago and yet these congeners differ by merely 2.4% in COI mtDNA (Medina et al. 1999). Given this remarkably low level of mtDNA divergence, any variation, even the difference of a single base pair, has the potential to be significant in an evolutionary context. As a result, it is entirely possible (and in fact likely) that much higher levels of genetic differentiation would be measured from the nuclear genomes of these *Montastraea* species. While it would have been ideal to evaluate this possibility with a suite of nuclear loci, candidates identified from the published literature and screened here

proved problematic among our samples, thus making confident detection of genotypes unreliable, especially among potential heterozygotes. The specific reasons (e.g. primer design or primers–template mismatch) surrounding the issues with these markers require further investigation.

Specificity in cnidarian–*Symbiodinium* associations

The proximate processes driving specificity remain unknown in most cnidarian–*Symbiodinium* relationships, including *Montastraea faveolata* and *M. annularis* sampled here in the Upper Florida Keys. Despite this, recent research has provided considerable insight into some of the mechanisms underlying recognition and specificity of host–symbiont pairings in these systems. For those cnidarians acquiring their *Symbiodinium* from the environment anew at each generation (i.e. horizontal acquisition), locally available *Symbiodinium* diversity provides an initial limit on the number of potential host–symbiont combinations. Initial infection is often nonspecific followed by a winnowing process leading to specificity in hours to months (e.g. Coffroth et al. 2001, Little et al. 2004, Rodriguez-Lanetty et al. 2006a, Thornhill et al. 2006b). Once *Symbiodinium* cells enter the cnidarian gastrovascular cavity, the prephagocytic recognition process for a number of corals involves interactions between *Symbiodinium* cell surface glycans and host lectins (Wood-Charlson et al. 2006, Kvennefors et al. 2008). Following this initial recognition, *Symbiodinium* cells are phagocitized and housed within intracellular vacuoles of the host gastroderm (e.g. Wakefield & Kempf 2001) where they apparently prevent lysosomal maturation that would otherwise lead to their digestion (Fitt & Trench 1983, Chen et al. 2003). During this time, certain coral– dinoflagellate pairings fail to maintain a suitable and stable symbiosis owing to either an incompatibility between symbiotic partners or an inappropriate microhabitat (e.g. temperature and/or light regimes) for either the coral host or the dinoflagellate endosymbiont. In host–symbiont combinations that fail to form sustained symbioses, postphagocytic winnowing involves host cell apoptosis, proteolysis and immune system responses (Dunn & Weis 2009, Voolstra et al. 2009). In contrast, there are minimal to relatively minor postphagocytic changes in host gene expression of pairings that lead to a successful symbiosis, suggesting that *Symbiodinium* inhabit their hosts by either escaping detection or active inhibition of the host response (Barneah et al. 2006, Rodriguez-Lanetty et al. 2006b, Voolstra et al. 2009). Finally, competition within a host among competent *Symbiodinium* 'types' may also play a role in determining the dominant endosymbiont (Fitt 1985, Coffroth et al. 2001, Little et al. 2004, Thornhill et al. 2006b). Thus, although a variety of phenomena are ultimately responsible for host–symbiont specificity, it appears that the overall process is relaxed when hybridization and introgression occur between coral species, as is apparently the case for *Montastraea* species in the Exuma Cays.

The *Symbiodinium* multilocus microsatellite genotypes detected here exhibited local and regional endemism, with no genotypes shared between the Upper Florida Keys and Exuma Cays (Thornhill et al. 2009). This finding was consistent with other studies (Santos et al. 2003, Howells et al. 2009) indicating that endemism may be a common characteristic of *Symbiodinium* populations. Unfortunately, the presence of endemic *Symbiodinium* populations hinders definitive determination of the relative influence of host versus symbiont genotype on symbiotic specificity in *Montastraea* spp. One alternative interpretation for our data, for instance, is that *Symbiodinium* genotypes from the Upper Florida Keys are more host specific than are their Exuma Cays counterparts, regardless of coral hybridization patterns. Although the correlation between host genetic distinctiveness and endosymbiont genotype argues against this possibility and for coral genotype and hybridization involvement in host–symbiont specificity, the complexity of these symbioses and endemic nature of *Symbiodinium* populations leaves possible such alternative interpretations.

Much clearly remains to be discovered regarding the specificity and/or flexibility of these dynamic and fascinating symbioses. Our results represent a modest first step towards documenting the fine-scale genotypic specificity and complexity of cnidarian–*Symbiodinium* relationships. These findings are noteworthy since commonly used molecular markers (e.g. ribosomal genes and their spacers) provide a much 'coarser' resolution of *Symbiodinium* genetic diversity, thereby suggesting flexible symbioses for both *Montastraea faveolata* and *M. annularis* (Toller et al. 2001, LaJeunesse 2002, Thornhill et al. 2006a). Instead, application of fine-scale symbiont microsatellites, in combination with host mtDNA markers, revealed strong specificity as well as potential mechanisms (i.e. hybridization and introgression) affecting the intimacy of these relationships. Further investigation of invertebrate–*Symbiodinium* specificity is of particular interest in predicting how such symbioses will respond to climate change. For example, stressors like high temperature and light levels can disrupt coral–algal symbioses, often leading to host mortality and ecosystem shifts (e.g. Hoegh-Guldberg 1999, Sampayo et al. 2008). Therefore, it is important to better determine whether, and under what circumstances, such events drive surviving coral and

Symbiodinium populations into novel host–symbiont combinations (Goulet et al. 2008, Jones et al. 2008, LaJeunesse et al. 2009, Thornhill et al. 2009). Hybridization provides one potential mechanism that may relax symbiotic specificity and increase the numbers of viable host–*Symbiodinium* associations in the future.

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