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Cryptic diversity hides host and habitat specialization in a gorgonian-algal symbiosis

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Abstract

Shallow water anthozoans, the major builders of modern coral reefs, enhance their metabolic and calcification rates with algal symbionts. Controversy exists over whether these anthozoan–algae associations are flexible over the lifetimes of individual hosts, promoting acclimative plasticity, or are closely linked, such that hosts and symbionts co-evolve across generations. Given the diversity of algal symbionts and the morphological plasticity of many host species, cryptic variation within either partner could potentially confound studies of anthozoan-algal associations. Here, we used ribosomal, organelle and nuclear sequences, along with microsatellite variation, to study the relationship between lineages of a common Caribbean gorgonian and its algal symbionts. The gorgonian *Eunicea flexuosa* is a broadcast spawner, composed of two recently diverged, genetically distinct lineages largely segregated by depth. We sampled colonies of the two lineages across depth gradients at three Caribbean locations. We find that each host lineage is associated with a unique *Symbiodinium* B1/184 phylotype. This relationship between host and symbiont is maintained when host colonies are reciprocally transplanted, although cases of within phylotype switching were also observed. Even when the phylotypes of both partners are present at intermediate depths, the specificity between host and symbiont lineages remained absolute. Unrecognized cryptic diversity may mask host-symbiont specificity and change the inference of evolutionary processes in mutualistic associations. Symbiotic specificity thus likely contributes to the ecological divergence of the two partners, generating species diversity within coral reefs.

Keywords: broadcast spawner, co-evolution, depth, ecological speciation, *Symbiodinium*

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Introduction

An obligate association between cnidarians and unicellular algae of the genus *Symbiodinium* forms the foundation of coral reefs, the most diverse marine ecosystem. In these nutrient-poor environments, algae provide organic compounds and energy to their coral hosts, while the coral provides recycled inorganic nutrients and access to a stable light environment for the alga (Muscatine *et al.* 1981, 1984; Colombo-Pallotta *et al.* 2010). This endosymbiosis is physiologically controlled by both partners (Muscatine *et al.* 1983; Davy *et al.* 2012). At the genomic level, the coral's functional genome is uniquely shaped by different *Symbiodinium* species (DeSalvo *et al.* 2010). At the extreme, incompatible symbionts activate immune responses in their hosts, even in coral species that associate with multiple distantly related phylotypes (Voolstra *et al.* 2009). The synchronized efforts of both partners affect their growth and thermal tolerance (Little *et al.* 2004; Abrego *et al.* 2008), so that *Symbiodinium* can facilitate coral acclimation to heterogeneous environments (Chen *et al.* 2005;

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Sampayo *et al.* 2007) and even to warmer oceans (Rowan *et al.* 1997; Baker 1999; Rowan 2004; Berkelmans & van Oppen 2006; Sampayo *et al.* 2008).

Given the functional and phylogenetic diversity within *Symbiodinium,* corals may acclimate through symbiont acquisition. Once known as a single species, nine major clades of *Symbiodinium* are now recognized, with intercladal genetic distances at rDNA markers that far exceed those among bird families (Rowan & Powers 1992; Rowan & Knowlton 1995; Coffroth & Santos 2005; LaJeunesse *et al.* 2010; Pochon & Gates 2010). Lineages within these clades are often segregated by habitat (usually depth) (Rowan & Knowlton 1995; Sampayo *et al.* 2007) and geography (LaJeunesse *et al.* 2010). Corals can exploit functional differences among these *Symbiodinium* lineages either via facultative associations that may vary over a host's lifetime or species-specific coupling, which results in co-evolution of corals and dinoflagellates.

At deep evolutionary scales (i.e. family level), corals may associate with more than one highly divergent lineage of dinoflagellates in the genus *Symbiodinium,* with some coral species harbouring multiple symbiont phylotypes within a single colony (Rowan & Knowlton 1995; Little *et al.* 2004). At first glance, then, corals may seem flexible in their associations with *Symbiodinium* and able to interact with any symbiont type. The uptake of multiple genotypes of *Symbiodinium* is notorious early in coral development, when many *Symbiodinium* species can be found within a developing polyp (Poland *et al.* 2013). However, while background or transient symbiont phylotypes may exist (Mieog *et al.* 2007; LaJeunesse *et al.* 2009; Silverstein *et al.* 2012), most adult anthozoan colonies host a single-dominant algal type (Goulet & Coffroth 2003; Thornhill *et al.* 2009; Finney *et al.* 2010; Pettay *et al.* 2011), opening the possibility for co-evolution between partners (LaJeunesse *et al.* 2004; LaJeunesse 2005; Finney *et al.* 2010; Thornhill *et al.* 2013, 2014). At microevolutionary scales, symbiont types may associate with individual anthozoan species and even intraspecific phylogeographical lineages (Santos *et al.* 2003b; LaJeunesse 2005; Goulet 2006; Finney *et al.* 2010; Pinzon & LaJeunesse 2011). These close evolutionary associations are particularly pronounced in shallow water Caribbean gorgonians, which associate almost exclusively with *Symbiodinium* in clade B (LaJeunesse 2002; Santos *et al.* 2004; Goulet *et al.* 2008). Recent work has verified that Clade B (as other clades) contains many cryptic species, with a high degree of host-symbiont specificity (Finney *et al.* 2010).

Fine scale genetic markers, such microsatellites, provide the ability to test for co-evolution at the population level (Santos *et al.* 2003b, 2004). Genetic variation and structure in host populations have been correlated with host reproductive traits (i.e., brooding vs. spawning, larval duration, (Kahng *et al.* 2011)), while the major factors that affect *Symbiodinium* distribution include limited dispersal (Santos *et al.* 2003b; Magalon *et al.* 2006; Thornhill *et al.* 2009; LaJeunesse *et al.* 2010) and, in particular, light environment (Rowan *et al.* 1997; Finney *et al.* 2010). Selection pressures that affect the distribution of one or both partners can shape co-evolution; however, reciprocal evolutionary change can only be detected when the genetic structure of both partners are examined across relevant spatial scales.

Eunicea flexuosa is a broadcast spawning gorgonian with larvae that acquire their symbionts horizontally from the water column (Beiring & Lasker 2000; Coffroth *et al.* 2001; Kim *et al.* 2004). Thus, relative to brooding corals with vertical transmission, *E. flexuosa* maintains the potential to shuffle its symbionts across generations. A microsatellite-based analysis of *E. flexuosa* and *Symbiodinium* population structure by Wirshing *et al.* (2013) found that, contrary to previous studies in Caribbean gorgonians (Santos *et al.* 2003b; Coffroth & Santos 2005; Andras *et al.* 2009; Kirk *et al.* 2009), the genetic structure of host and symbiont populations were not correlated. However, the sampling design of this study did not consider depth as a potential structuring force. Cryptic host diversity and habitat (depth) thus offer unexplored alternative explanations for the genetic variation of *Symbiodinium* found in *E. flexuosa*.

Here, we elucidate variation in *Symbiodinium* associated with the recently diverged shallow and deep lineages of *E. flexuosa* using four different types of molecular markers. *Eunicea flexuosa* provides an excellent system for disentangling the roles of host specificity, habitat and geography in partitioning *Symbiodinium* diversity because: (i) *E. flexuosa* is composed of a sympatric pair of recently diverged shallow and deep lineages (Prada *et al.* 2008; Prada & Hellberg 2013); (ii) these host lineages occasionally co-occur at intermediate depths (Prada & Hellberg 2013); and (iii) *E. flexuosa* lineages are widespread across the Caribbean and the shallow/deep distribution varies geographically (Bayer 1961; Prada & Hellberg 2013).

We first analysed *Symbiodinium* variation at higher taxonomic levels using ribosomal ITS2 and chloroplast sequences, along with nuclear sequences to elucidate within clade B variation. We then used microsatellites to resolve population subdivision within *Symbiodinium* lineages at geographical scales. We also tested the stability of the host–symbiont relationships with a reciprocal transplant experiment. If coral–algal associations are mainly driven by host species identity (Finney *et al.* 2010; LaJeunesse *et al.* 2010), then colonies in reciprocal transplants will rarely switch symbiont types, and host

colonies will remain true to symbiont types, even when occurring outside their usual depth.

Materials and methods

Sampling and sequence data

Colonies were sampled at three locations: Puerto Rico, Bahamas and Curaçao [for full descriptions of these locations see (Prada & Hellberg 2013)]. These locations span regions where the distributions of depth-segregated cryptic species within *Eunicea flexuosa* have been previously investigated (Prada & Hellberg 2013). At each location, we sampled adult colonies (>50 cm in height) at two depths, as earlier work suggested that deep (>20 m) and shallow (<5 m) populations are genetically differentiated across the Caribbean (Prada *et al.* 2008; Prada & Hellberg 2013). At each depth, we collected tissue from between 16 and 38 colonies. To avoid sampling clones, collected colonies were at least 5 m apart. Multilocus genotypes confirmed that all colonies came from unique genets.

To disentangle the roles of host species identity and depth, we also sampled 40 colonies from intermediate depths (12–15 m) where the *E. flexuosa* lineages co-occur. In Puerto Rico, we also reciprocally transplanted coral colonies to native and foreign depths (see above; 38 per depth, 152 total fragments) and resampled them after 18 months. Fitness across depths for these colonies was assessed previously (Prada & Hellberg 2013). We preserved all samples in 95% ethanol at −20 °C. We extracted genomic DNA from these samples using either a 2X CTAB method (Coffroth *et al.* 1992) or a QIAGEN DNeasy Kit following the manufacture's protocols.

Host genotypes were scored for one mtDNA marker, *msh*, and three nuclear markers: *inositol-3-phosphatase* (*i3p*), *elongation factor 1 alpha* (*ef1a*) and *calcium transporter 2* (*ct2*). These multilocus genotypes have been used previously to discern the two depth-based cryptic *E. flexuosa* lineages (Prada & Hellberg 2013).

To determine which *Symbiodinium* clade was found in *E. flexuosa*, we amplified the ribosomal ITS2 and the hypervariable region of the chloroplast 23s-rDNA following earlier protocols [(LaJeunesse 2002) for ITS2; (Santos *et al.* 2003a) for cp23s-rDNA]. We sequenced 224 bases of the ITS2 (see below). The cp23s-rDNA amplicons were sized in polyacrylamide gels using a metric ruler with DNA ladders as size references, and alleles were named based on established clade and fragment length nomenclature (Santos *et al.* 2003a).

To identify cryptic lineages within *Symbiodinium* B1/184, the predominant symbiont type, we used DNA sequences from two chloroplast genes and one nuclear locus. We amplified and sequenced samples for the two chloroplast markers following previous protocols [(Moore *et al.* 2003) for *psb*A; (Santos *et al.* 2002) for cp23s-rDNA]. We also sequenced a subset of samples (42) from all populations (three locations and two depths) using the flanking region of the microsatellite B7SYM15, following previous procedures (Pettay & LaJeunesse 2007). Amplicons were directly sequenced in both directions (except B7SYM15, forward only) in an ABI 3100 using BigDye chemistry v 3.1 and both amplification primers. *Symbiodinium* is haploid, so no allele phasing was required. We edited and assembled all sequences using Geneious v4.5.5 (Drummond *et al.* 2009).

To further characterize genetic variation across sampling location in *Symbiodinium* B1/184, we genotyped all colonies at four microsatellite markers: B7SYM15, B7SYM34, B7SYM36 (Pettay & LaJeunesse 2007) and GV2 100 (Andras *et al.* 2009). To test for host–symbiont associations following a change in environment, we genotyped post-transplanted colonies at the B7SYM15, B7SYM34 and B7SYM36 loci. Primers and PCR conditions for these loci were carried out according to conditions in previous studies (Pettay & LaJeunesse 2007; Andras *et al.* 2009). Fragments were amplified with fluorescently labelled primers and visualized on a 7% polyacrylamide gel in a LI-COR NEN® Global IR2 DNA Sequencer (Santos *et al.* 2003a,b). Microsatellites were scored by eye relative to 50–350 bp size standards (LI-COR Biotechnology Division).

Data analysis

To identify the patterns of associations between *Symbiodinium* within clade variants and *Eunicea flexuosa,* we built a phylogeny based on the ribosomal ITS2. To root the genealogy, we included sequence data from a recent description for *Symbiodinium* B1 (LaJeunesse *et al.* 2012) (AF333510, AF333511 and AF333512) and also from the widely used culture 704 (JN558059). We estimated the model of molecular evolution using MrAIC v. 1.4.5 (Nylander 2004). We used Bayesian analyses to estimate each gene tree (Mr. Bayes v. 3.1) and estimated nodal support using posterior probabilities (Huelsenbeck & Ronquist 2001). We also constructed parsimony haplotype networks for the chloroplast markers cp23s and *psb*A and the nuclear B7SYM15 using the Templeton *et al.* (1992) algorithm implemented in tcs 1.21 (Clement *et al.* 2000). Each network was constructed with a confidence level set at 95% and excluding gaps.

Once genealogical patterns had been uncovered, we analysed hierarchical genetic subdivision within the two major *Symbiodinium* lineages using microsatellite data. To apportion genetic subdivision among host

genotype, depth and geography, we used Analysis of Molecular Variance (AMOVA) as implemented in GENODIVE (Meirmans & Van Tienderen 2004). We performed 100 000 permutations using standard F-statistics. We defined populations by host's genotype, depth and geography. To further characterize geographical differentiation within the *Symbiodinium* group designated B1/B184, we used a Bayesian clustering approach implemented by STRUCTURE (Pritchard *et al.* 2000). Because multiple algal genotypes were found in 44 of the host colonies, we analysed the data in two ways. We first generated all the multilocus combinations that could possibly be generated for each colony (full data set). Alternatively, we eliminated all colonies with ambiguous genotypes (short data set).

We used the admixture model in STRUCTURE without information of the origin of each individual. For each STRUCTURE analysis, we set up a burn-in of one million steps followed by 5 million iterations and 10 replicates per run. We ran STRUCTURE using a variety of K's (number of inferred populations) from the minimum (1) to the maximum (6) and then used the Evanno method (Evanno *et al.* 2005) as implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2011) to infer the number of populations (K's) present in the data set. We processed replicates with best K (either 2 or 5) in CLUMMP (Jakobsson & Rosenberg 2007) using the default parameters. To generate figures, we used DISTRUCT 1.1 (Rosenberg 2004). To test for host–symbiont associations between depths, we ran STRUCTURE assuming the most likely K (=2) for samples collected in Puerto Rico.

Results

Shallow and deep lineages of Eunicea flexuosa *associate with Symbiodinium B1/184*

Both ITS2 and chloroplast data suggest that all *Eunicea flexuosa* colonies harbour *Symbiodinium* phylotype B1/B184, regardless of the host's genotype, depth or geography (Fig. 1). Of the 183 colonies sampled, no colony contained any *Symbiodinium* from other clades or any other clade B types. We re-covered a single ITS2 haplotype for all individuals, but multiple haplotypes for *cp23s*, *psbA* and *SYM15* (Fig. 2). Genetic variation within these three loci revealed differences in algal composition between host colonies sampled in shallow and deep habitats (Fig. 2). Three haplotypes were re-covered from analysis of *SYM15*. These divided into two distinct lineages, which sorted by depth and matched the shallow and deep host lineages. *psbA* also had three haplotypes: one found solely in shallow hosts, another solely in deep hosts, with a third found mainly in deep hosts but also in shallow colonies from the Bahamas. *cp23s* contained four haplotypes: two of high frequency that sorted by depth and host, and two endemic ones restricted to Bahamas Shallow and Curaçao Deep, respectively (Fig. 1B).

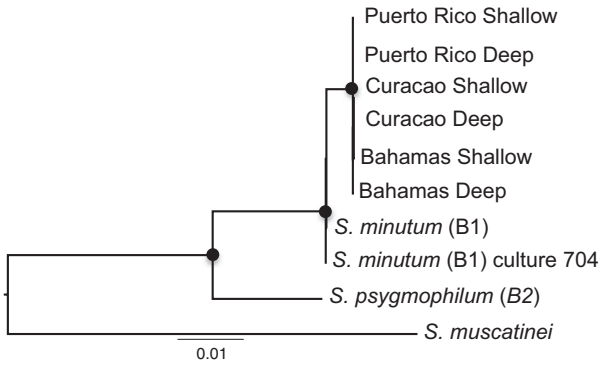

Fig. 1 *Symbiodinium* Clade B phylogeny based on ITS2 from colonies of *Eunicea flexuosa* sampled across depths and locations across the Caribbean. Black circles indicate posterior probability > 0.9.

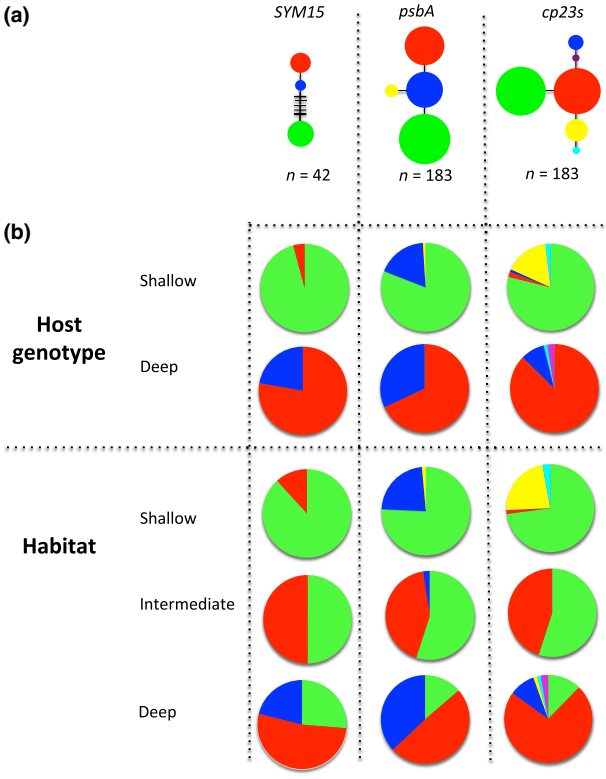

Fig. 2 Genetic diversity in *Symbiodinium* clade B1/B184 within *E. flexuosa* across the Caribbean. A) Haplotype networks for the flanking region of the microsatellite *SYM15* and the chloroplast *psbA* and *cp23S*. B) Distribution of haplotypes by host genotype or depth across the Caribbean for each locus. Circles are drawn proportional to the number of individuals contained and colors signify the different haplotypes.

Symbiodinium *diversity corresponds to host lineage irrespective of depth*

The AMOVA suggested that all factors (host lineage, depth and geography) significantly influenced symbiont type, with host and depth accounting for a larger proportion of the variance than geography. In a hierarchical analysis with geography, host lineage explained 93% of the variation, while depth accounted for only 64% (Table 1), with contributions from geography becoming insignificant. Genetic variation thus is primarily divided by host lineage.

To further disentangle the role of host lineage and depth in predicting the symbiont lineage, we sampled colonies at an intermediate depth in Puerto Rico, where the two host lineages co-occur. Our results suggest that the influence of host lineage is stronger than depth: for the 40 colonies, we sampled from intermediate depth, and we found an absolute host–algal lineage association (Fig. 3). In addition, two colonies mismatched to habitats for host lineage (i.e. *Eunicea flexuosa* Shallow corals found in deep environments) were also mismatched for their symbiont lineage. In Curaçao, for example, where about half of the colonies in deep environments are *E. flexuosa* Shallow, these depth-mismatched colonies hosted *Symbiodinium* B1/B184 Shallow symbiont. This host–symbiont association was also evident in Puerto Rico.

Symbiodinium *B1/184 host-depth associated lineages segregate further geographically*

Bayesian clustering of microsatellite variation suggests two major gentic clusters of symbionts that largely correspond to the host shallow and deep lineages, respectively (Fig. 4). Both the short and the long data sets provided similar results; we present results from the full data set in the main text and results from the short data set as supplementary material (Fig. S1, Supporting information). When K was increased to five (K with highest likelihood), geographical differentiation within each shallow/deep symbiont clusters was revealed (Fig. 4). This geographical structure within *Symbiodinium* contrasts with that of the host, where both deep and shallow lineages are apparently connected across the Caribbean (Prada & Hellberg 2013).

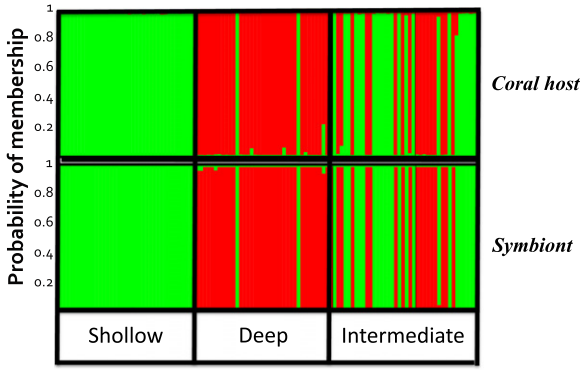

Fig. 3 STRUCTURE results (K = 2) for host and *Symbiodinium* sampled at shallow, deep and intermediate sites in Puerto Rico.

Host–Symbiodinium *associations were temporally stable*

Of the 26 colonies for which pre- and post-transplantation *Symbiodinium* genotypic data were available, three (of 15) switched from Deep to Shallow B1/B184 ($P = 0.22$, Fisher's exact test two tails) and two (of 11) from Shallow to Deep ($P = 0.24$, Fisher's exact test two tails). We also observed three (out of 26) switching to a different symbiont in the same lineage. *Symbiodinium* genotypes thus are generally temporally stable over a timescale of 18 months, yet not absolutely so. This within-lineage symbiont change may be due to within-colony variation in symbiont types, resulting from sampling different parts of the colony before and after transplantation, or a change in the frequencies of types already present in the colony prior to transplantation.

Table 1 Analysis of molecular variance partitioning genetic subdivision within the two *Symbiodinium* lineages among host lineage, depths and geography. Significant values ($P < 0.05$) are in bold

	*psb*A		cp23s		Overall	
	FST	% Variance	FST	% Variance	FST	% Variance
Host lineage	**0.611**	61.1	**0.692**	69.2	**0.652**	65.2
Depth	**0.497**	49.7	**0.58**	58	**0.539**	53.9
Geography	**0.512**	51.2	**0.293**	29.3	**0.41**	41
Host lineage – Geography						
Host lineage	**0.663**	66.3	**0.929**	92.9	**0.786**	78.6
Geography	0.297	29.7	0.014	−1.4	0.153	15.3
Depth – Geography						
Depth	**0.399**	39.9	**0.64**	64	**0.51**	51
Geography	0.353	35.3	0.033	3.3	0.205	20.5

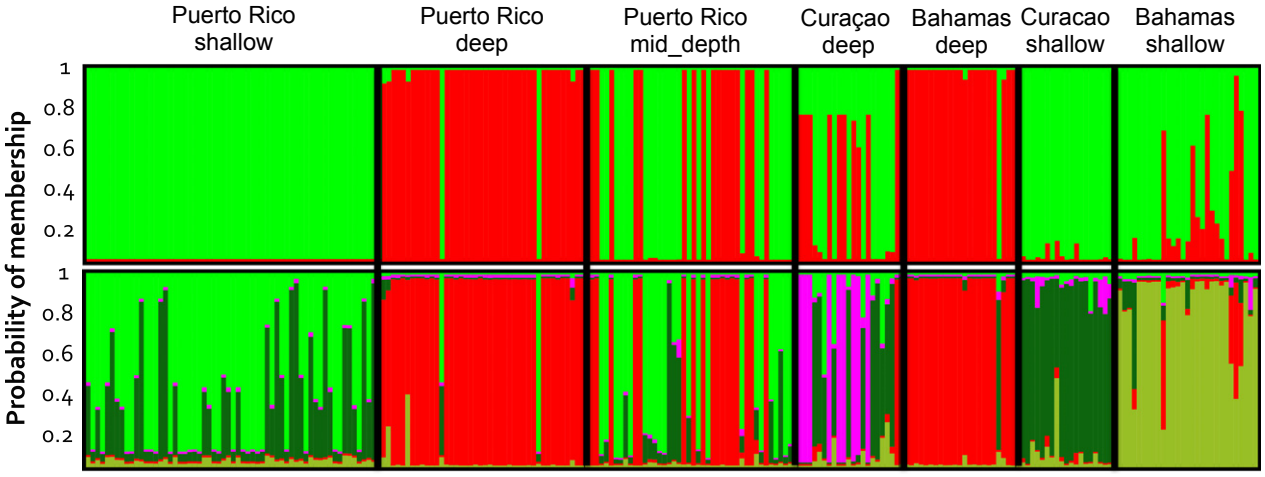

Fig. 4 Bayesian clustering for all microsatellites and all samples combined for *Symbiodinium* B1/184. Top panel shows K = 2 as suggested by the Evanno method and lower panel shows K = 5, K with the highest likelihood.

Discussion

We found two closely related, yet distinct lineages of *Symbiodinium* clade B that associate tightly with distinct lineages of their host *Eunicea flexuosa*. Although both host coral and their corresponding symbiont types are associated with different depths, surveys of hosts at intermediate depths and transplant experiments demonstrate that the effect of host identity is stronger than that of depth. Divergence within each *Symbiodinium* lineage largely follows a geographical pattern of segregation. The tight association between octocorals and their *Symbiodinium* species seen here only becomes evident when cryptic host species are recognized, revealing a holobiont (coral + symbiotic algae) unit critical for understanding adaptation at each depth or geographical locale.

Cryptic species and co-evolution

The abundance of cryptic species among both host and symbionts has led to the recognition that 'ecological specialists' may dominate coral–*Symbiodinium* symbioses (Finney *et al.* 2010). The discovery of correlated cryptic diversity in this octocoral and *Symbiodinium* association echoes co-evolutionary studies in terrestrial systems, in which the use of populations of both partners, along with multiple molecular markers analysed within a phylogenetic framework, have been critical to delineating symbiotic relationships. For example, in cases in which the co-evolutionary status of fig wasp interactions have been questioned, finer scale genetic variation has been critical to understand the one-to-one interaction of fig trees and their pollinating wasps (Kobmoo *et al.* 2010). Similarly, ecotypic morphological variation in yucca plants drove the evolution of two species of pollinator moths, so that once plant cryptic variation is accounted for, the pattern of co-evolution is clear in an otherwise widespread and promiscuous plant species (Godsoe *et al.* 2008). Perhaps more relevant, given the tight physiological interaction reflected in coral–symbiont associations, are yeast-termite (Prillinger *et al.* 1996) and legume-rhizobium (Heath *et al.* 2012) mutualisms. In both cases, revealing cryptic variation in the yeast and bacterial symbionts has exposed dozens of species that specialize on a single host and in some cases even particular habitats within those hosts (Prillinger *et al.* 1996; Heath *et al.* 2012). In rhizobium, the genomes of both partners work in concert, and genetic variation within either species generate fitness differences to the plant (Heath *et al.* 2012). In a similar way, we are starting to understand how the functional genome of corals changes in predictable ways depending on the types of *Symbiodinium* they host (DeSalvo *et al.* 2010). Cryptic diversity present in the lineages of *Eunicea flexuosa* and *Symbiodinium* B1/B184 may be an ecologically relevant association, with fitness costs to both for mismatching and genomic coordination that has evolved over many generations.

Failing to recognize cryptic species in either host or symbiont may lead to an underestimation of diversity, an overestimation of the ecological and physiological ranges of individual species (i.e., one generalist instead of many specialists), and a mistaken view of how species acclimate or adapt to changing conditions. Wirshing *et al.* (2013), for example, did not consider cryptic species within *E. flexuosa* and *Symbiodinium*, leading to their conclusion of a flexible association between *E. flexuosa* and *Symbiodinium* B1/B184 and geographical variation within *E. flexuosa*. They made no note of the depth of their sampled colonies, opening the possibility that geography could be confounded with cryptic species identity. This seems likely in Panama (their most divergent population), where both lineages of *E. flexuosa* co-occur in shallow areas (Prada & Hellberg 2013). Bocas del Toro

(Panama) is an atypical Caribbean location in which local sedimentation often draws organisms typical of deep water into shallow depths. Black corals, for example, can be observed as shallow as 5 m, whereas they occur >20 m elsewhere in the Caribbean (Opresko & Sanchez 2005). Wirshing *et al.* (2013) also note null alleles for all loci (except Plf67) in their Panama samples. Such a pattern would be expected if the microsatellites amplify well for the shallow lineage (for which the primers were developed), but fail for colonies of the divergent deep lineage, which are at high frequency in shallow areas in Panama. Thus, when reconsidered in the light of cryptic host species, *E. flexuosa's* symbiont associations are like those of other Caribbean gorgonians, in which different *Symbiodinium* phylotypes associate with different octocoral species (Santos *et al.* 2004).

Co-evolutionary patterns of divergence are not restricted to octocorals. Recent work in several brooding corals, including *Agaricia* (Bongaerts *et al.* 2013), *Seriatopora* (Bongaerts *et al.* 2010) and *Madracis* (Frade *et al.* 2008), suggests that species or genetically differentiated populations have co-evolved with their algal symbionts. Specific coral–symbiont associations seem more prevalent in brooding scleractinians in which *Symbiodinium* are transmitted vertically. The *Symbiodinium* – *E. flexuosa* tight association is remarkable and similar to that in *Orbicella* species (Thornhill *et al.* 2014) because specialization proceeded in a broadcasting species in the absence of direct vertical transmission and in a system in which larvae can travel large distances (>10 km) (Prada & Hellberg 2013; Wirshing *et al.* 2013) and only acquire symbionts once the larvae have settled and metamorphosed into a polyp (Poland *et al.* 2013).

The coral–symbiont association need not be viewed as a strict dichotomy of flexibility or species specificity. While specificity is common in octocorals (Santos *et al.* 2004; Goulet 2006), some scleractinian corals acquire various *Symbiodinium* phylotypes over ecological scales (Rowan *et al.* 1997; LaJeunesse *et al.* 2009). However, such apparent flexibility contains finer specificity (Finney *et al.* 2010). *Orbicella faveolata*, for example, associates with *Symbiodinium* from clades A, B, C and D, but only with specific phylotypes within those four clades, especially within clade C (Thornhill *et al.* 2014). Such mixed patterns of flexibility at macroevolutionary timescales and specificity at microevolutionary scales beg for more studies to understand the evolution of symbiosis in anthozoans.

Depth, photobiology and ecological speciation

Diversity in *Symbiodinium* has often been associated with habitat heterogeneity. In a landmark study, Rowan *et al.* (1997) showed how subtle changes in light availability within single coral colonies can segregate *Symbiodinium* genetic variation. More recent work suggests that depth, often correlated with light, underlies *Symbiodinium* variation across habitats (Sampayo *et al.* 2008; Kirk *et al.* 2009; Finney *et al.* 2010; Lesser *et al.* 2010). In these heterogeneous environments, different host–symbiont combinations cause differences in survivorship and growth for the holobiont (Berkelmans & van Oppen 2006; Sampayo *et al.* 2007). Segregation in *Symbiodinium* types in *Eunicea flexuosa* is associated with a transition in depth, raising the likelihood that different host–symbiont combinations perform better at their native depths (Sampayo *et al.* 2007).

Studies of the photobiology of *Symbiodinium* suggest that different species have different capacities and perform better at different light conditions, such those that vary with depth (Iglesias-Prieto & Trench 1994). B1 species (as in *E. flexuosa*), for example, show different biophysical properties, such active fluorescence and photosystem-specific signatures (Robison & Warner 2006; Hennige *et al.* 2008, 2011). Moreover, *Symbiodinium* species segregated by depth have different light optima and enhanced capacity (i.e., photosystem pressure) at native depths, even if these depth-segregated species are reciprocally transplanted (Iglesias-Prieto *et al.* 2004). It is thus likely that the enhanced survivorship of *E. flexuosa* to each habitat is partly due to its tight association with a specific *Symbiodinium* type.

In *E. flexuosa,* the formation and maintenance of the two lineages appear to have been promoted by immigrant inviability (Prada & Hellberg 2013), which occurs when locally adapted populations migrate to suboptimal environments, increasing mortality of these maladapted individuals (Nosil *et al.* 2005). For both *E. flexuosa* and *Symbiodinium,* their partner becomes another 'environment'. Immigrant inviability can thus act in two ways. First, it can act against individuals with the 'wrong' host–symbiont coupling. Next, even for pairs with the 'right' host–symbiont coupling, inviability will sort holobionts by depth. Unlike brooders, in which most host–symbiont specialization has been observed, *E. flexuosa* acquires its *Symbiodinium* horizontally (from the surrounding environment upon settlement, rather than via its parent), increasing the potential for immigrant inviability.

Geographical variation and phylogeography

Symbiodinium in *Eunicea flexuosa* shows patterns of geographical differentiation within each shallow and deep lineage, along with the segregation by host species and depth. Geographical differentiation is pervasive in *Symbiodinium*, and most studies have reported geographical differentiation often (as here) at scales smaller than their host (Andras *et al.* 2011). For example, Andras *et al.* (2011) found that populations of *Symbiodinium* are similar between Puerto Rico and the Bahamas but

different from those in Curaçao. We found the same pattern for *Symbiodinium* associated with *E. flexuosa* Deep, but a full segregation across localities for the shallow lineage. The higher partition across geography by the shallow lineage suggests that even at small depth contours, physical and biological processes vary in the Caribbean, uniquely altering connectivity patterns of populations at different depths.

Conclusion

The high specificity between genetically distinct populations of *Symbiodinium* with different *Eunicea flexuosa* lineages suggests a pattern of co-evolution such that this coupling defines the fitness of the holobiont in different environments. Other symbiotic or parasitic partners, such as bacteria, fungi and viruses, may further shape survivorship and reproduction of the holobiont. These species interactions may influence evolutionary patterns and, when coupled with ecological factors such depth, may play a role in speciation. During ecological speciation, species interactions may reinforce prezygotic isolation by preventing the establishment of maladapted coral–symbiont–depth combinations, each of these combinations being a potential link at which immigrant inviability can operate. Knowlton (1993) highlighted the vast diversity of sibling species in the sea. We are now beginning to understand how marine diversity segregated along ecological factors like depth creates complexes of codiversifying species and increases diversity in the sea.

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C.P., M.A.C. and M.E.H. conceived the project. C.P. and D.M.B. did transplant experiment, collections and DNA sequencing. D.J.V., S.E.M., S.A.F. and M.A.C. did cp23s analysis and microsatellite genotyping. C.P. did all analysis. C.P. and M.E.H. wrote the manuscript. All authors commented and agreed on the manuscript.

Data accessibility

Sequence data is available from GenBank. Accession nos for *Eunicea flexuosa* are: KC310499–KC310687 and KC333998–KC335131. Accession nos for *Symbiodinium* B1/B184 are: ITS2 (KJ780833 – KJ780838), cp23s (KJ780839 – KJ781021) and psbA (KJ781022 – KJ781204). The short and the long microsatellite data sets along with alignments for all sequences are available through Dryad (doi:10.5061/dryad.ns34t).

Supporting information

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

Fig. S1 Bayesian clustering for the short dataset for all microsatellites for *Symbiodinium* B1/184.