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Specificity is rarely absolute in coral–algal symbiosis: implications for coral response to climate change

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Some reef-building corals have been shown to respond to environmental change by shifting the composition of their algal symbiont (genus *Symbiodinium*) communities. These shifts have been proposed as a potential mechanism by which corals might survive climate stressors, such as increased temperatures. Conventional molecular methods suggest this adaptive capacity may not be widespread because few (~25%) coral species have been found to associate with multiple *Symbiodinium* clades. However, these methods can fail to detect low abundance symbionts (typically less than 10–20% of the total algal symbiont community). To determine whether additional *Symbiodinium* clades are present, but are not detected using conventional techniques, we applied a high-resolution, real-time PCR assay to survey *Symbiodinium* (in clades A–D) from 39 species of phylogenetically and geographically diverse scleractinian corals. This survey included 26 coral species thought to be restricted to hosting a single *Symbiodinium* clade ('symbiotic specialists'). We detected at least two *Symbiodinium* clades (C and D) in at least one sample of all 39 coral species tested; all four *Symbiodinium* clades were detected in over half (54%) of the 26 symbiotic specialist coral species. Furthermore, on average, 68 per cent of all sampled colonies within a given coral species hosted two or more symbiont clades. We conclude that the ability to associate with multiple symbiont clades is common in scleractinian (stony) corals, and that, in coral–algal symbiosis, 'specificity' and 'flexibility' are relative terms: specificity is rarely absolute. The potential for reef corals to adapt or acclimatize to environmental change via symbiont community shifts may therefore be more phylogenetically widespread than has previously been assumed.

Keywords: biodiversity; climate change; real-time PCR; coral reef; *Symbiodinium*; thermotolerance

1. INTRODUCTION

The high productivity and diversity of coral reefs is largely due to the mutualistic symbiosis between corals and single-celled dinoflagellate algae in the genus *Symbiodinium*. Although morphologically cryptic, *Symbiodinium* are genetically diverse, with nine sub-generic clades (named A–I) currently recognized [1]. Reef-building corals most commonly associate with *Symbiodinium* in clades A–D [2], although members of clades F and G have also been reported [3,4] from these hosts. Within these *Symbiodinium* clades, over 400 distinct internal-transcribed spacer-2 (ITS-2) rDNA 'types' have been identified based on sequence differences and denaturing gradient gel electrophoresis (DGGE) profiles [5–9], but it is not yet clear how many of these types represent distinct species [10,11]. The distribution of some *Symbiodinium* variants has been correlated with different environmental conditions, usually temperature or light [2,12–20], for physiology studies (see earlier studies [21–24]). Physiological variability among *Symbiodinium* variants likely contributes to the ability of a coral host to thrive in a variety of environmental conditions, such as gradients in depth, latitude, irradiance and temperature [20,25–28]. *Symbiodinium* clade D has attracted particular interest in this regard because it includes opportunistic variants (e.g. ITS-2; types D1 and D1a) that can associate with hosts under conditions, such as temperature anomalies, that are typically sub-optimal for many members of other clades (reviewed by Baker [2] and Stat & Gates [29]).

Models predict that reef ecosystems throughout much of the tropics will decrease in coral cover, diversity and/or undergo phase shifts in the coming decades [30–33]. An assumption of these models is that corals will be unable to compensate for increasing temperatures over relatively short timescales. The ability to associate with diverse symbionts has been suggested as one mechanism by which corals might be able to respond rapidly to environmental change [17,19,25,26,34–37]. However, it has been argued that symbiont-mediated acclimatization is not feasible for most coral species based on perceptions that (i) many coral taxa are 'symbiotic specialists' that are restricted

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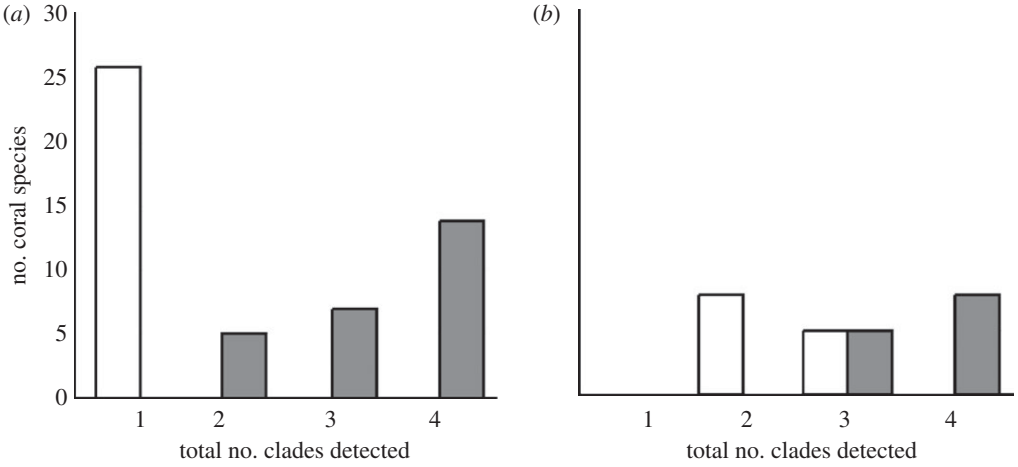

Figure 1. Frequency distributions of *Symbiodinium* diversity in scleractinian corals identified (in previous studies) using conventional (e.g. DGGE, RFLP, SSCP) versus high-sensitivity (RT-PCR) molecular techniques (in this study). Host species were designated as (*a*) 'symbiotic specialists' or (*b*) 'symbiotic generalists' based on previous symbiont detections using conventional techniques (see electronic supplementary material, table S1). Conventional molecular techniques greatly overestimate the number of specific host species. White bars, conventional methods; grey bars, RT-PCR.

to hosting a single symbiont taxon and (ii) most scleractinian corals do not associate with thermo- and/or stress-tolerant algae, such as some members of *Symbiodinium* clade D [2,6,9,30,33,38–46]. Perceived symbiont specificity has even been used as a criterion for recognizing both symbiont 'species' [4] and cryptic coral species [47]. However, these perceptions were based on studies using conventional molecular techniques such as analysis of restriction fragment length polymorphisms (RFLPs), single-stranded conformational polymorphisms (SSCPs) and DGGEs of nuclear ribosomal DNA (figure 1; electronic supplementary material, table S1). These methods are typically effective at detecting only the dominant, or most abundant, taxa in a (potentially) diverse symbiont community. Thornhill and co-workers [48] estimated that symbiont taxa representing less than 5–10% of the total symbiont community are not detected using DGGE, whereas LaJeunesse *et al.* [49] found that detection limits depended on symbiont type and molecular marker. For example, using ITS-2, detections of some *Symbiodinium* (e.g. C1b-c) occur even when these variants comprise just 0.3 per cent of the total community, whereas detections of other *Symbiodinium* (e.g. D1) occur only when variants represent at least 10–20% of the total symbiont community. Using the ITS-1 region marker, however, *Symbiodinium* D1 was not detected until it comprised at least 50 to 90 per cent of the total community, whereas *Symbiodinium* C1b-c remained detectable at 0.3 per cent [49]. These studies indicate that *Symbiodinium* variants that are not dominant (i.e. most abundant) community members may not be detected using conventional molecular techniques [44], even though they may be numerically abundant. For example, in a typical scleractinian coral containing 1–2 million symbionts per square centimetre of coral tissue [50], a *Symbiodinium* taxon representing just 1 per cent of the community would still represent 10 000–20 000 cells per square centimetre of coral tissue, but would not necessarily be detected using DGGE [49].

High-resolution techniques, such as real-time PCR (RT-PCR), have detection thresholds that are at least 100–1000 times more sensitive than conventional techniques [45]. These techniques have recently revealed the presence of *Symbiodinium* clades at low abundance, as well as their dynamics within select coral species [45,46,51–56]. These findings support the idea that stony corals may be more flexible in their associations ('symbiotic generalists') than has been indicated using conventional molecular methods. However, it has remained unclear whether the relatively few host taxa surveyed using high-sensitivity techniques are representative of stony coral–*Symbiodinium* associations in general [45,46].

In this study, 'dominant' symbionts will refer to symbionts that have been routinely detected using conventional molecular techniques (which may include more than one clade for a given coral species), and 'background' symbionts will refer to symbionts which are newly detected using high-sensitivity techniques. As they have not been previously detected with conventional techniques, they are likely present in corals at lower abundances.

Here, we assayed multiple individuals ($n = 4–12$) of 39 geographically widespread and phylogenetically diverse coral species for the presence of *Symbiodinium* in clades A–D. This study provides (i) the most comprehensive RT-PCR survey of symbiont diversity in corals to date and (ii) baseline data for symbiont community ecology studies.

2. MATERIAL AND METHODS

(a) *DNA extraction and RT-PCR assay*

RT-PCR assays were used to determine the presence/absence of *Symbiodinium* in clades A–D in a total of 321 samples from 39 reef coral species, representing 28 genera and 14 families. Based on previous studies using conventional molecular techniques to detect *Symbiodinium* in coral hosts (through November 2009), 26 of these coral species were designated 'symbiotic specialists' (defined here as species previously reported to host only a single *Symbiodinium* clade), whereas 13 were 'symbiotic generalists' (defined here as species previously documented to host multiple *Symbiodinium* clades). Samples were collected from various locations worldwide between 1995 and 2009 (electronic supplementary material, table S1). Previous *Symbiodinium* detections reported between September 2006 and November 2009 are listed in the electronic supplementary material, table S1. Detections prior to 2006 are

summarized in Goulet [41], and are included in this analysis but not enumerated here. Papers published since November 2009, using conventional molecular techniques as well as RT-PCR, may have found multiple *Symbiodinium* clades in some of these species [4,57], but were not included in this analysis.

DNA extraction and purification methods were based on Baker *et al.* [58]. The RT-PCR assays and analyses followed Correa *et al.* [46] and had similar specificities, efficiencies and sensitivities to the assays described therein. (For more detailed information on RT-PCR assay methodology, see electronic supplementary material.)

(b) *Contamination tests*

Previous studies of symbiont diversity in scleractinian corals using RT-PCR have considered any *Symbiodinium* detected to be *in hospite* (i.e. residing in a symbiosome) [4,45,46,51,59]. However, it is also possible that RT-PCR could detect symbionts that are merely (i) surface contaminants (e.g. *Symbiodinium* that are free-living or recently expelled from other hosts) or (ii) gut contaminants (i.e. recently ingested food items). To assess the extent to which RT-PCR detections reflect potential false positives for *Symbiodinium in hospite*, we analysed samples of the azooxanthellate coral, *Tubastrea coccinea* ($n = 13$ total), using the methods described earlier, as a 'biological negative control'. Any positive amplifications for *Symbiodinium* from azooxanthellate samples must therefore have been derived from recently ingested algal symbionts or *Symbiodinium* cells adhered to the surface of coral polyps. Symbiont detections from azooxanthellate coral can be considered estimates of the relative proportion of RT-PCR detections of background symbiont populations from zooxanthellate (symbiotic) corals that may actually be 'contaminant' *Symbiodinium*. (For methodology details, see electronic supplementary material.)

Given the extreme sensitivity of RT-PCR, we also assessed any potential contamination resulting from the laboratory protocol of Baker *et al.* [58] in paired, randomly selected coral samples ($n = 9$). This extraction protocol uses an airbrush to blast tissue from coral skeletons using an EDTA-based (DNA) buffer solution into a plastic bag, after which the bag is rinsed vigorously in running fresh water between each sample. The bag is replaced after approximately 5–10 samples. Although no contamination has been detected for this protocol using conventional, less-sensitive techniques, such as DGGE, we investigated whether the highly sensitive RT-PCR technique might detect residual *Symbiodinium* cells remaining in collection bags after rinsing between samples. (For methodology details, see electronic supplementary material.)

(c) *SYBR assay specificity*

The SYBR green RT-PCR assays used in this study were fully validated in Correa *et al.* [46]. However, SYBR green RT-PCR assays have been suggested to be potentially less specific to their target sequences than other RT-PCR assays, for example, those conducted with Taqman probes. We therefore conducted an extra specificity test of the SYBR green assays used in this study (for more methodology details, see electronic supplementary material).

(d) Symbiodinium *diversity analyses within-host species*

Within a particular host species, the percentages of colonies containing one, two, three or four symbiont clades were calculated (electronic supplementary material, table S4) and the average number of colonies ($\pm$s.d.) across all species (electronic supplementary material, table S3). These data were then grouped as follows (i) all symbiotic generalist coral species; (ii) all symbiotic specialist coral species; and (iii) all coral species and are presented in the electronic supplementary material, table S3.

(e) *Total* Symbiodinium *community diversity analyses*

For each coral species, we calculated a Shannon's diversity index for the total *Symbiodinium* community detected, based on the combined positive symbiont detections from all sampled colonies. Shannon's *t*-tests [60] were then used to make pairwise comparisons of the Shannon's diversity indices (total *Symbiodinium* diversity) between all 39 coral species. After the Bonferroni correction, the *p*-value for these multiple tests of significance was set at 3.37×10^{-5}. This approach was also used to compare the total *Symbiodinium* diversity identified from Caribbean versus Indo-Pacific corals, and from symbiotic generalists versus symbiotic specialists. The Bonferroni correction was not applicable to these latter two tests, and therefore a *p*-value of 0.05 was used. All Shannon's diversity indices and Shannon's *t*-tests were calculated using the biodiversity calculator [61].

ANOVAs of linear regressions (performed in Microsoft Excel) confirmed that Shannon's diversity index results were not biased by the number of geographic regions or the number of coral colonies analysed for a given coral species. *p*-Values greater than 0.5 indicated that an independent variable (e.g. number of geographic regions) was not significant in explaining the Shannon's diversity index obtained for a given coral species. This same approach was also used to confirm that the number of colonies analysed for a given coral species did not bias comparisons of total *Symbiodinium* diversity among coral species.

(f) *Clade-level* Symbiodinium *diversity ordination analyses*

We used non-metric multidimensional scaling (NMDS) with Bray–Curtis index dissimilarities to visually represent configurations of the relative abundances (i.e. the number of times a particular clade was detected, normalized to the host sample size) of *Symbiodinium* in clades A–D hosted by different coral species (R v. 2.9.1 [62], Vegan library). Standard error ellipses delineate the standard error with respect to the relative abundances of symbiont clades within (i) symbiotic specialist and symbiotic generalist coral taxa and (ii) Indo-Pacific and Caribbean regions, such that the centre of the ellipse corresponds to the mean relative abundance. Because NMDS is a visual analytical tool and does not analyse statistically significance between groups, analysis of similarity (ANOSIM) was also performed in order to determine whether symbiont communities were significantly different between (i) symbiotic specialist versus symbiotic generalist categories of coral species and between (ii) Indo-Pacific and Caribbean corals (R v. 2.9.1 [62], Vegan library).

3. RESULTS

(a) *RT-PCR results*

Of the coral species examined here, 38 of 39 were found to associate with a *Symbiodinium* clade that had not previously been reported using conventional techniques (electronic supplementary material, table S1). *Pocillopora damicornis*, which was already known to host clades A, C and D, was the exception, as it was not found to associate with clade B in this study. (In subsequent studies, this host species has also been found in association with clade B with DGGE analysis [4], R. Cunning and A.C.B., manuscript in

preparation). In total, 78 previously unreported, clade-level, coral-symbiont detections are reported here (electronic supplementary material, table S1). We found that all 39 coral taxa contained *Symbiodinium* in clades C and D, in at least one of the colonies analysed ($n = 4$–12 colonies per host taxon; electronic supplementary material, table S1).

Of the 26 coral taxa thought to be symbiont specialists, 14 (54%) associated with all four symbiont clades (A + B + C + D) in one or more samples, whereas 21 (81%) hosted at least three clades (either A + C + D or B + C + D) in one or more samples. Of the 13 coral taxa considered to be symbiotic generalists, eight (62%) hosted all four clades in one or more samples, and all (100%) associated with at least three clades in one or more samples. Based on these RT-PCR assays, none of the symbiotic generalist species associated with only two clades.

Here, clade A was detected for the first time in samples from Bermuda (*Madracis mirabilis*, $n = 6$), and clade D was detected for the first time in the Galapagos (*Pavona gigantea*, $n = 1$; *Pavona clavus*, $n = 4$; *Pocillopora damicornis*, $n = 3$; electronic supplementary material, table S1).

(b) *Within-host species* Symbiodinium *diversity comparisons*

In any given coral species, most individual colonies (mean 68% of all colonies of a given coral species) hosted more than one *Symbiodinium* clade. In 23 per cent ($n = 9$ of 39) of the coral species examined, every colony analysed hosted multiple clades. On average, for a given coral species, 40 per cent of the colonies analysed hosted two clades, whereas 24 per cent hosted three clades.

(c) *Between-host species diversity comparisons*

The *Symbiodinium* community diversity of each coral species was calculated using the Shannon diversity index. Values ranged from 0.52 (*Hydnophora exesa*) to 1.33 (*Colpophyllia natans*), but low sample sizes precluded some calculations (electronic supplementary material, table S2). Pairwise two-tailed Shannon's *t*-tests for all coral taxa (electronic supplementary material, figure S1*a*) showed that the relative diversity of coral–*Symbiodinium* associations (i) differed among species and (ii) followed a gradient of overall symbiont diversity (for a more detailed description of results, see electronic supplementary material).

Although some coral species contained *Symbiodinium* communities that were significantly more diverse than others (electronic supplementary material, figure S1*a*), most host species examined in this study were equivalent in terms of their total *Symbiodinium* diversity (electronic supplementary material, figure S1*b*). Pairwise comparisons of *Symbiodinium* revealed that all coral species contained symbiont community diversity equivalent to at least 50 per cent of the other 38 coral species (electronic supplementary material, figure S1*b*), including taxa previously considered to be symbiotic generalists and symbiotic specialists. When considered as groups, there were no differences in overall diversity between symbiotic specialists and symbiotic generalists (Shannon's *t*-test, $p = 0.06$).

Similar to the comparisons of Shannon's diversity indices, the NMDS biplot compares the composition of the *Symbiodnium* communities present within each host species. However, this NMDS analysis additionally accounts for symbiont clade identity, rather than only the number of clades detected and relative evenness of those clades, as with Shannon's values. Furthermore, the biplot (figure 2) illustrates similarities between coral host species based on the relative abundance of each of the symbiont clades A–D, rather than determining statistical significance between species having more or less diverse communities, as with Shannon's *t*-tests. The relatively low stress of the NMDS ordination (0.1) indicates that the distribution of coral species based on the composition of their symbiont communities is well represented by the two axes of the biplot. The biplot reveals that coral species from the Indo-Pacific region are more closely associated with symbionts from clades C and D than the five Caribbean coral species included in this study, which are more closely associated with clades A and B (figure 2). In agreement with the hierarchical geographic diversity analyses performed using Shannon diversity index values, ANOSIM confirmed that the *Symbiodinium* communities hosted by Indo-Pacific versus Caribbean coral species host are significantly different from one another ($p = 0.001$, $r^2 = 0.49$; figure 2). ANOSIM further indicated that symbiont communities are not significantly different between symbiotic specialist and symbiotic generalist coral groups ($p = 0.73$, $r^2 = 0.009$; figure 2).

(d) *SYBR assay specificity*

All of the 16 RT-PCR amplifications from nine randomly selected coral samples selected for direct sequencing were specific to their target *Symbiodinium* clade, based on BLASTn searches of the NCBI nr database (GenBank accession numbers: clade A: AF427466 ($n = 4$), EU074862 ($n = 1$); clade B: DQ200710 ($n = 2$); clade C: EF372040 ($n = 2$), EF372052 ($n = 1$), FJ851421 ($n = 1$), AY903353, ($n = 1$), EF372067 ($n = 1$) and; clade D: AB248879 ($n = 3$)).

(e) *Contamination tests*

Out of 52 assays in which contaminant *Symbiodinium* could have been detected from azooxanthellate corals (13 *T. coccinea* samples × four symbiont clade assays per sample), only one of the azooxanthellate *T. coccinea* samples (1.9%) amplified for one clade (C) of *Symbiodinium*. Out of nine paired 'positive' and 'negative' controls analysed for potential contamination during the extraction protocol, 14 positive *Symbiodinium* clade detections were generated from the positive extraction controls (out of 36 possible detections: nine samples × four clades), but only one negative extraction control sample amplified for a single clade (C) of *Symbiodinium* (one detection out of 14 possible detections = 7.1%). Overall, we detected the presence of novel clades within this dataset 249 times. If extraction and biological contamination accounted for 2 per cent and 7 per cent of these detections, respectively, then we can estimate the number of false positives as 22 of 249 (9% of total), indicating that the vast majority (91%) of the novel coral–*Symbiodinium* associations we report do indeed represent *Symbiodinium in hospite* (see also electronic supplementary material for discussion of these results).

4. DISCUSSION

(a) *Diversity in coral–algal symbiosis*

RT-PCR analysis reveals that the majority of the 39 reef coral species examined associate with a higher diversity of

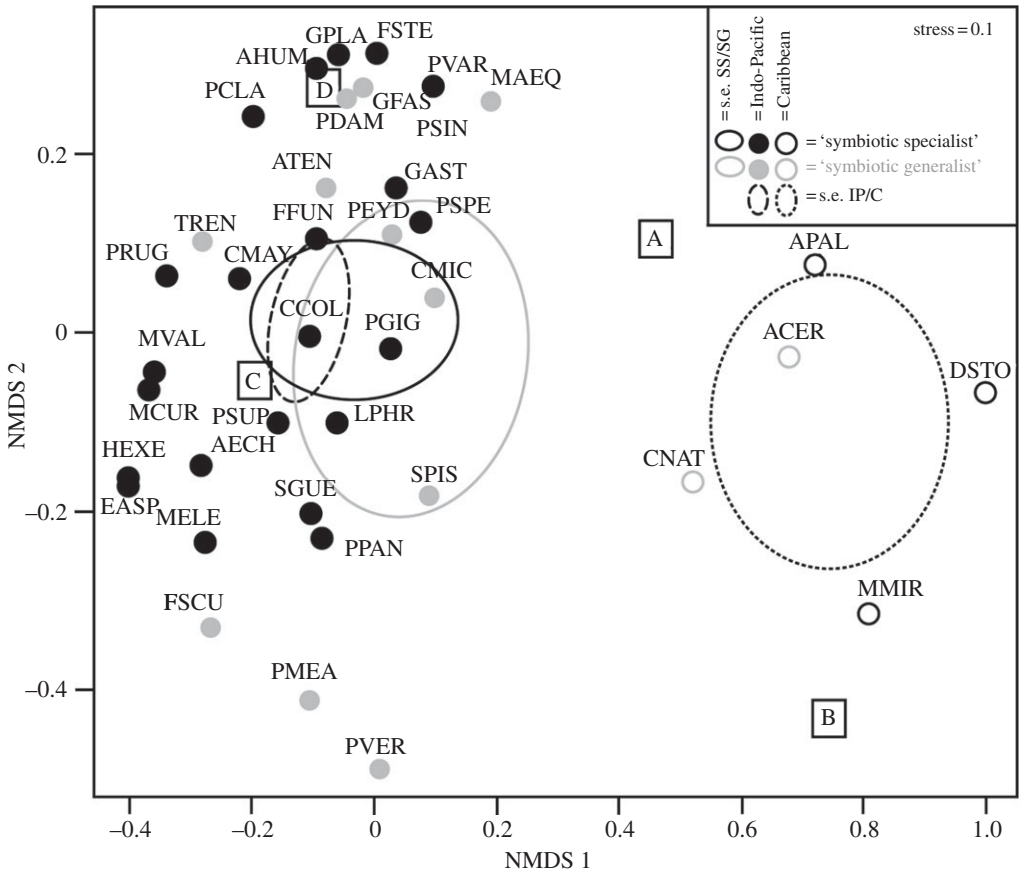

Figure 2. Non-metric multidimensional scaling biplot showing configuration of the relative abundances of *Symbiodinium* clades within different coral host species. Boxed letters A, B, C and D represent each clade's designated position within the biplot, around which the species scores of each coral were oriented. Most Indo-Pacific coral species cluster between clades C and D, whereas Caribbean coral species are more closely associated with *Symbiodinium* clades A and B. Open circles indicate Caribbean coral species, and full circles indicate Indo-Pacific coral species. Grey markers refer to symbiotic generalist species, whereas black markers refer to symbiotic specialist species. Solid ellipses in either grey or black indicate standard error (s.e.) of the symbiotic generalist (SG) and symbiotic specialist (SS) species groups, respectively, and long or short dashed-line ellipses indicate s.e. of the Indo-Pacific (IP) and Caribbean (C) coral groups, respectively. Coral species are denoted by a four-letter species code where the first letter represents the genus, and the remaining three letters represent the first three letters of the species name: *Acanthastrea echinata*, AECH; *Acropora cervicornis*, ACER; *Acropora humilis*, AHUM; *Acropora palmata*, APAL; *Acropora tenuis*, ATEN; *Coeloseris mayeri*, CMAY; *Colpophyllia natans*, CNAT; *Coscinaraea columna*, CCOL; *Cyphastrea micropthalma*, CMIC; *Dichocoenia stokesii*, DSTO; *Echinophyllia aspera*, EASP; *Favia stelligera*, FSTE; *Fungia fungites*, FFUN; *Fungia scutaria*, FSCU; *Galaxea astreata*, GAST; *Galaxea fascicularis*, GFAS; *Gardineroseris planulata*, GPLA; *Hydnophora exesa*, HEXE; *Leptoria phrygia*, LPHR; *Madracis mirabilis*, MMIR; *Montastraea curta*, MCUR; *Montastraea valenciennesi*, MVAL; *Montipora aequituberculata*, MAEQ; *Mycedium elephantotus*, MELE; *Pachyseris rugosa*, PRUG; *Pachyseris speciosa*, PSPE; *Pavona clavus*, PCLA; *Pavona gigantea*, PGIG; *Pavona varians*, PVAR; *Platygyra sinensis*, PSIN; *Plesiastrea versipora*, PVER; *Pocillopora damicornis*, PDAM; *Pocillopora eydouxi*, PEYD; *Pocillopora meandrina*, PMEA; *Porites panamensis*, PPAN; *Psammocora superficialis*, PSUP; *Stylocoeniella guentheri*, SGUE; *Stylophora pistillata*, SPIS; *Turbinaria reniformis*, TREN.

Symbiodinium clades than has been previously recorded using conventional molecular techniques. Overall, our results are still a conservative estimation of the true diversity of symbionts in coral species because (i) we focused on detecting cryptic *Symbiodinium* diversity in symbiotic specialists, and included fewer symbiotic generalist species; (ii) the RT-PCR assays used detect only the most commonly observed symbionts of scleractinian corals (*Symbiodinium* in clades A–D), yet clades F and G have also been reported in a few scleractinian coral species [3,4], and might therefore be detected from these coral species if appropriate assays were developed; (iii) many coral species are known to associate with multiple *Symbiodinium* types within a single clade [63,64], and it is likely that the development of RT-PCR assays specific to *Symbiodinium* sub-clades would detect additional within-clade diversity; (iv) the samples analysed in this study originate from few of the full range of habitats and regions in which each host species may be found. Analysis of additional samples for each coral species may reveal additional symbiotic associations from spatial or temporal niches not sampled in this study; and (v) individual coral colonies can contain spatially heterogeneous distributions of *Symbiodinium* clades [12,65], but we sampled each colony only once. This study identifies high *Symbiodinium* diversity within 39 coral species using a relatively conservative approach, and it is therefore likely that additional diversity would be found if RT-PCR were to be expanded to other *Symbiodinium* clades and applied to other hosts, across their full biogeographic and environmental ranges [2,44]. Therefore, although corals clearly exhibit selectivity in their associations [5,41,42,66], strict host specificity to a particular symbiont or clade does not appear to be the case for any coral species assayed in this study, even corals thought to be highly selective.

Based on RT-PCR, the 39 scleractinian coral species examined here fall along a continuum in terms of the frequency with which they host multiple *Symbiodinium* clades, with all species tested being flexible to some degree. The total *Symbiodinium* clade diversity detected using RT-PCR was similar for most coral species (i.e. for a given coral species, Shannon's diversity index was statistically indistinguishable from the diversity indices for over half of the other 38 coral species examined; electronic supplementary material, figure S1*b*). Furthermore, the total *Symbiodinium* diversity among corals previously thought to be symbiotic generalists compared with symbiotic specialists (as respective groups) was not statistically different (Shannon's *t*-test: $p = 0.06$; ANOSIM of NMDS ordination: $p = 0.73$, $r^2 = 0.009$). Therefore, classifying corals species as either 'specific' or 'flexible' in their associations with *Symbiodinium* is an artificial dichotomy. Instead, a gradient of specificity exists over which scleractinian coral species vary in the frequency with which they (i) are dominated by a particular *Symbiodinium* clade/type; and (ii) contain background populations of additional *Symbiodinium* clades/types (electronic supplementary material, figure S1*a*,*b* and table S1).

Furthermore, colonies hosting multiple symbiont clades represent the majority (average: 68%) of individual colonies analysed for a given species. This indicates that the ability of coral colonies to host multiple clades is common within populations, and not limited to a few unusual individuals within a coral species. Therefore, within a particular species, many colonies have the potential to benefit from associating with multiple symbiotic partners.

(b) *High symbiotic diversity realized using sensitive molecular tools in other systems*

Many metazoan species harbour symbiotic microbes, and recent studies from a variety of systems have also reported that microbial symbiont community diversity is greater than previously thought. For example, using RT-PCR, Olson *et al.* [67] detected and quantified a greater diversity of nitrogen-fixing bacteria in close association with the corals *Montipora capitata* and *Montipora flabellata* than had not been previously observed using conventional techniques. These findings may partly explain how corals are able to thrive in low-nutrient environments. Furthermore, there was a strong positive correlation between the density of *Symbiodinium* cells in a coral sample and the number of gene copies detected from nitrogen-fixing bacteria, suggesting a relationship between coral-associated microbes and *Symbiodinium* communities. Flexibility in associations between coral and its symbiotic bacterial communities has also been proposed as a potential mechanism by which corals could rapidly acclimate to environmental changes [68]. In other systems, symbiotic bacterial diversity associated with newly discovered, whale bone-eating polychaete worms, *Osedax* spp., was analysed using RT-PCR [69] and fluorescence *in situ* hybridization [70]. This approach showed differences in bacterial communities harboured by distinct species of *Osedax*, suggesting that *Osedax* species may occupy different ecological niches. Using another high-resolution technique (454-pyrosequencing), fungus-growing (attine) ants have also been shown to associate with more than one strain of antibiotic-producing *Pseudonocardia* bacteria, contradicting previous assumptions that a single ant nest hosts a single strain of *Pseudonocardia* [71].

High-sensitivity molecular techniques are revealing that mixed microbial symbiont communities are common in many host taxa. Additionally, studies have also shown that these symbiotic relationships can exhibit flexibility between multiple partners. Redundant partners may confer the same *type*, although not necessarily the same *degree*, of benefit, but may prevent the host from experiencing an entirely aposymbiotic state during environmental stress [72]. For example, environmentally mediated associations exist between bark beetles and species of 'cool' or 'warm' fungal partners, which increase or decrease in abundance in response to temperature, and serve to expand the beetles' environmental range [72]. Similarly, pea aphids associate with two species of bacterial symbionts, one of which confers thermal tolerance to the aphid [73], although evidence suggests that it is an inferior symbiont under normal conditions [74]. The diverse coral–algal associations reported here therefore follows a general trend in microbial ecology—the recognition of more diverse and flexible symbiotic associations.

(c) *Implications of mixed symbiont assemblages within hosts*

Intraspecific diversity in coral–algal symbiosis may provide a mechanism by which reef corals can respond to rapid environmental changes. Changes in the composition of symbiont communities within corals might allow corals to optimize aspects of their physiology in response to environmental stressors. It has been suggested, however, that such symbiont shifts can occur only in a minority of coral species, namely those from which symbiont diversity has already been detected [33,41,42,75]. Although reef corals clearly preferentially associate with certain dominant symbiont types [5–9], our findings show that many scleractinian coral species are capable of associating with multiple *Symbiodinium* clades, including members of both clades C and D. While our detection of diverse symbiont communities in corals does not directly address changes in stress tolerance or symbiont shifts over time, these data do show that coral species are not biologically restricted to associating exclusively with particular symbiont clades and/or types. This suggests that corals' cellular mechanisms for recognizing particular strains of *Symbiodinium* [66,76–78] may be less definitive than they appear, perhaps because they are themselves environmentally or ontogenetically mediated. Future work should address the dynamics of background symbiont types over time and during episodes of coral bleaching, or the paling of corals as a result of the loss of symbiotic algae and/or algal pigments. Coral bleaching has been suggested to accelerate alterations in symbiont communities by disturbing an otherwise-stable *Symbiodinium* community [25,35].

Members of *Symbiodinium* in clade D (particularly ITS-2 types D1 and D1a) can be relatively thermotolerant, and coral colonies associating with members of this clade have exhibited increased bleaching resistance ([13,17,19,34,54], but see Abrego *et al.* [18]). However, because we cannot assume that thermotolerance is characteristic of all members of clade D, future work should investigate variability in symbiont physiology within clade D [22,79],

as well as the potential contribution of *Symbiodinium* in other clades to coral survival during acute stress. Background abundances of *Symbiodinium* ITS-2 types D1 or D1a could also be specifically investigated in a wide range of corals using a probe that is custom designed to assay these types.

Symbiodinium in clade C are recognized as common members of reef communities worldwide [9], and it is not surprising that our analysis found members of this clade in every species examined. However, the fact that we also found *Symbiodinium* in clade D present in at least one sample of every host species examined, and across the same broad phyletic diversity of hosts as clade C (14 scleractinian coral families) suggests that members of clade D are also ubiquitous in reef communities and have a cosmopolitan (although frequently low abundance) distribution in hosts (electronic supplementary material, table S1). *Symbiodinium* in clades A and B were also common in our dataset (found in 32 and 24 of the 39 species examined, respectively), although within Indo-Pacific corals they were not as widespread among colonies of a given host species as clades C or D (figure 2).

ANOSIM analysis revealed that the distribution of *Symbiodinium* clades among coral species was not significantly different based on previously designated symbiotic specialist or generalist hosts. However, differences were detected in the clade-level *Symbiodinium* communities found in Caribbean versus Indo-Pacific geographic provinces, which can also be observed in the NMDS biplot (figure 2). Shannon's *t*-tests also indicated that Caribbean symbiont communities have greater clade-level diversity than those hosted by Indo-Pacific corals, agreeing with previous studies conducted using conventional molecular techniques [6]. *Symbiodinium* diversity at the clade level has been correlated with patterns of bleaching severity on reefs over regional scales [80], with regions characterized by more diverse *Symbiodinium*, such as the Caribbean, tending to experience more frequent but less severe bleaching events [80]. In contrast, regions with relatively low *Symbiodinium* diversity, such the Indo-Pacific, tend to experience less frequent but more severe bleaching events [80,81]. The application of high-sensitivity techniques, such as RT-PCR, to understanding microbial community structure in corals may help reveal how low abundance, background symbiont diversity further contributes to patterns of bleaching and recovery. Future surveys of global *Symbiodinium* diversity using high-sensitivity molecular techniques and covering wide geographical and temporal scales will help us understand how changing environmental conditions influence host–symbiont dynamics.

While symbiont identity has been correlated with holobiont physiological tolerance [2,12–14,17–20], the contribution of low abundance symbionts to the overall physiology of the holobiont is still unknown. It is also not yet clear how background symbionts may impact coral survival during disturbances such as coral bleaching. Furthermore, few studies have tracked shifts in symbiont communities over time to understand whether increases in background symbionts, such as opportunistic members of clade D, are long-lived or ephemeral [48,54]. Background *Symbiodinium* may provide functional symbiont redundancy to corals by persisting during stressful conditions when dominant symbionts decline and providing photosynthates to the host during bleaching and recovery, thereby preventing mortality [37,45].

Coral bleaching has become increasingly common on reefs in recent decades due to climate-related stressors such as increases in sea surface temperature [13,30,33]. During bleaching events, corals can suffer high mortality if they do not recover their algal symbionts within weeks to months [82]. Without a mechanism to cope with rapid environmental change, coral bleaching will likely continue to result in widespread declines in coral cover [30,31,33]. Symbiotic flexibility may provide one mechanism by which corals can respond to changing environments, and these results indicate that the potential for this mechanism may be more widespread than previously assumed.

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