

# Functional diversity in coral–dinoflagellate symbiosis

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Symbioses are widespread in nature and occur along a continuum from parasitism to mutualism. Coral–dinoflagellate symbioses are defined as mutualistic because both partners receive benefit from the association via the exchange of nutrients. This successful interaction underpins the growth and formation of coral reefs. The symbiotic dinoflagellate genus *Symbiodinium* is genetically diverse containing eight divergent lineages (clades A–H). Corals predominantly associate with clade C *Symbiodinium* and to a lesser extent with clades A, B, D, F, and G. Variation in the function and interactive physiology of different coral–dinoflagellate assemblages is virtually unexplored but is an important consideration when developing the contextual framework of factors that contribute to coral reef resilience. In this study, we present evidence that clade A *Symbiodinium* are functionally less beneficial to corals than the dominant clade C *Symbiodinium* and may represent parasitic rather than mutualistic symbionts. Our hypothesis is supported by (i) a significant correlation between the presence of *Symbiodinium* clade A and health-compromised coral; (ii) a phylogeny and genetic diversity within *Symbiodinium* that suggests a different evolutionary trajectory for clade A compared with the other dominant *Symbiodinium* lineages; and (iii) a significantly lower amount of carbon fixed and released by clade A in the presence of a coral synthetic host factor as compared with the dominant coral symbiont lineage, clade C. Collectively, these data suggest that along the symbiotic continuum the interaction between clade A *Symbiodinium* and corals may be closer to parasitism than mutualism.

Cnidaria | *Symbiodinium* | disease | mutualistic | parasitic

The term symbiosis is inclusive of a variety of interactions that occur along a continuum that includes mutualistic, commensal, and parasitic associations (1). In commensal and mutualistic symbioses one or both partners benefit from the association, respectively, which contrasts with parasitic interactions, where one partner benefits whereas the other is harmed by the relationship (1, 2). The most well known examples of mutualistic symbioses in marine ecosystems are those between corals and photosynthetic dinoflagellates belonging to the genus *Symbiodinium*. Here, inorganic waste metabolites from the animal host are exchanged for organic nutrients fixed by dinoflagellate photosynthesis, primarily in the form of glycerol, that serve as respiratory substrates in the animal host that support the growth and formation of coral reefs (3, 4). Other common mutualisms include interactions between plants with mycorrhizal fungi and nitrogen-fixing bacteria (5, 6).

The dinoflagellate genus *Symbiodinium* is divided into eight divergent lineages, referred to as clades A–H, with each containing many subclade types based on rDNA (7–13). The diversity within the genus can be appreciated by the large number of taxa that form symbiotic interactions with *Symbiodinium*, including marine invertebrates from four phyla (Cnidaria: corals, jellyfish, anemones, zoanthids; Mollusca: snails and clams; Platyhelminthes: flatworms; Porifera: sponges) and the single-celled protist Foraminifera (14). Furthermore, an early phylogenetic comparison of partial 18S rDNA revealed that within the genus *Symbiodinium* the genetic diversity is equivalent to order-level taxonomic differences seen in other dinoflagellate groups (15).

The genetic diversity within *Symbiodinium* is likely to correlate with an equally diverse range of physiological properties in the host–symbiont assemblages. For example, juvenile coral hosts harboring clade C *Symbiodinium* have been shown to grow two to three times faster than juveniles harboring clade D (16). Clade D, however, has been shown to have a higher tolerance to thermal stress than clade C, suggesting corals harboring clade D are more resilient to coral bleaching events (17). These data point to differences in the function of these symbiotic interactions that reflect not only the type of *Symbiodinium* present but also the environment and developmental stage of the coral.

Understanding the functional diversity and physiological thresholds of coral–dinoflagellate symbioses is critical to predicting the fate of corals under the threat of global climate change and the increasing incidence of coral bleaching events and disease outbreaks (18, 19). In this study, we challenge the assumption that clade A and C *Symbiodinium* form equally mutualistic symbioses with corals by (i) evaluating the health state of corals that harbor clade A and C *Symbiodinium*, (ii) evaluating the phylogeny and diversity of *Symbiodinium* relative to free-living dinoflagellate groups, and (iii) investigating *in vitro* carbon fixation and release by *Symbiodinium* clades A and C.

## Results

***Symbiodinium* Genotypes and Coral Health.** *Symbiodinium* belonging to clades A and C were recovered from 43 colonies of *Acropora cytherea*. Sixteen of 17 healthy *A. cytherea* colonies (Fig. 1a) harbored *Symbiodinium* belonging to clade C and one, to clade A. Corals displaying abnormal phenotypes (Fig. 1b) contained either clade A ( $n = 5$ ) or clade C ( $n = 11$ ), whereas corals showing evidence of disease (Fig. 1c) contained either clade A ( $n = 5$ ), clade C ( $n = 5$ ), or clade A and C ( $n = 1$ ). There was a significant association between the symbiont clade and the observed health state of colonies (likelihood ratio test;  $8.924$ ,  $df = 2$ ,  $P = 0.012$ ), with corals hosting clade A symbionts showing a significantly higher incidence of disease or abnormal phenotypes than those hosting clade C (Fisher Exact Test;  $P = 0.015$ ).

**Genetic Diversity and Phylogenetic Analyses.** The phylogenetic analyses using maximum-likelihood, maximum-parsimony, and neighbor-joining resulted in trees with very similar topologies (maximum-likelihood shown in Fig. 2). Among the dinoflagellates, the genus *Symbiodinium* forms a well supported monophyletic group with clades A and E as a sister grouping to clades B, C, and D.

Corrected maximum-likelihood pairwise sequence comparisons of *Symbiodinium* clades A, B, C, D, and E and the dinoflagellates *Gymnodinium beii*, *Gymnodinium simplex*, *Pollarella glacialis*, *Thoracosphaera heimii*, and *Cachonina hallii* are available in supporting information (SI) Table S1. The distances

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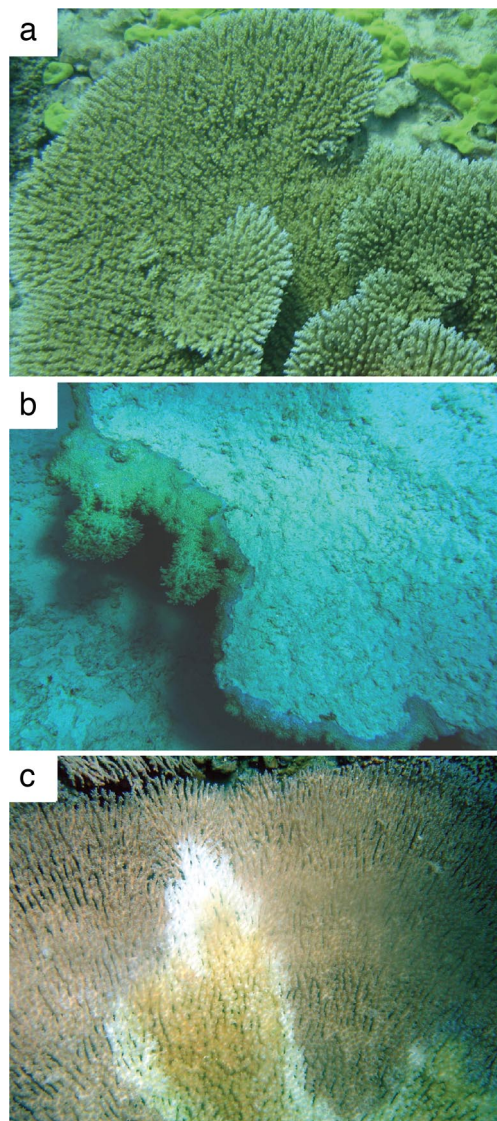
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**Fig. 1.** Colonies of *A. cytherea* sampled from Papahānaumokuākea (Northwestern Hawaiian Islands Marine National Monument) with healthy specimens (a), abnormal morphology showing evidence of past tissue loss and blue pigmentation (b), and active tissue loss phenotypes (c).

between *Symbiodinium* clade A and *G. beii*, *G. simplex*, and *P. glacialis* (0.061, 0.064, and 0.081 respectively) are less than those between clade A and the *Symbiodinium* clades B, C, D, and E (0.115, 0.115, 0.099 and 0.107, respectively). Similarly, the distances between clade E *Symbiodinium* and *G. beii* and *G. simplex* (0.091 and 0.088, respectively) are less than the distances between clade E and clades B, C, and D (0.102, 0.115, and 0.100, respectively). Further, the pairwise sequence diversity within clade C (0.051) and between different *Symbiodinium* clades is greater than the genetic distance between dinoflagellates that are in different orders (e.g., 0.036 between *T. heimii* order Thoracosphaerales and *C. hallii* order Peridinales).

Because of a lower pairwise genetic distance between clade A and *G. beii*, *G. simplex*, and *P. glacialis* than between clade A and other *Symbiodinium* lineages, the Shimodaira–Hasegawa test was used. There was a significant difference in tree topologies when forcing *Symbiodinium* into a paraphyletic grouping by constraining clades A and clade E with the outgroup *G. simplex* [ $\delta = L_1 - L_2 = -18.57092$ ;  $P = 0.026$ ; log-likelihood ( $L_1$ ) = 2866.68434].

**Carbon Fixation and Release.** Freshly isolated symbionts (FIS) belonging to clade A and C incubated in filtered sea water (FSW) fixed similar amounts of carbon (mean value of  $1.0168\text{E-}12$  and  $1.1698\text{E-}12$ , respectively; Fig. 3a). In contrast, clade C FIS incubated in synthetic host factor (SHF) fixed approximately four times more carbon than clade A FIS (mean value of  $3.7344\text{E-}12$  and  $1.0290\text{E-}12$ , respectively;  $t$  test =  $-2.8032$ ,  $P = 0.020$ ; Fig. 3b). Similarly, clade C FIS incubated in both FSW and SHF released significantly greater amounts of carbon into the medium than clade A (FSW mean value of  $2.9924\text{E-}13$  and  $5.0654\text{E-}14$ , respectively;  $t$  test =  $-3.3052$ ,  $P = 0.008$ ; SHF mean value of  $1.56878\text{E-}12$  and  $6.68978\text{E-}14$ , respectively;  $t$  test =  $-2.984$ ,  $P = 0.014$ ; Fig. 3c and d).

For clade A, there was no significant difference between the amount of carbon fixed in FSW or SHF or in the amount released in FSW or SHF. In contrast, for clade C, there was a significant difference in the amount of carbon fixed in FSW to SHF ( $t$  test =  $-2.5834$ ,  $P = 0.027$ ) and the amount released in FSW to SHF ( $t$  test =  $-2.4959$ ,  $P = 0.032$ ).

### Discussion

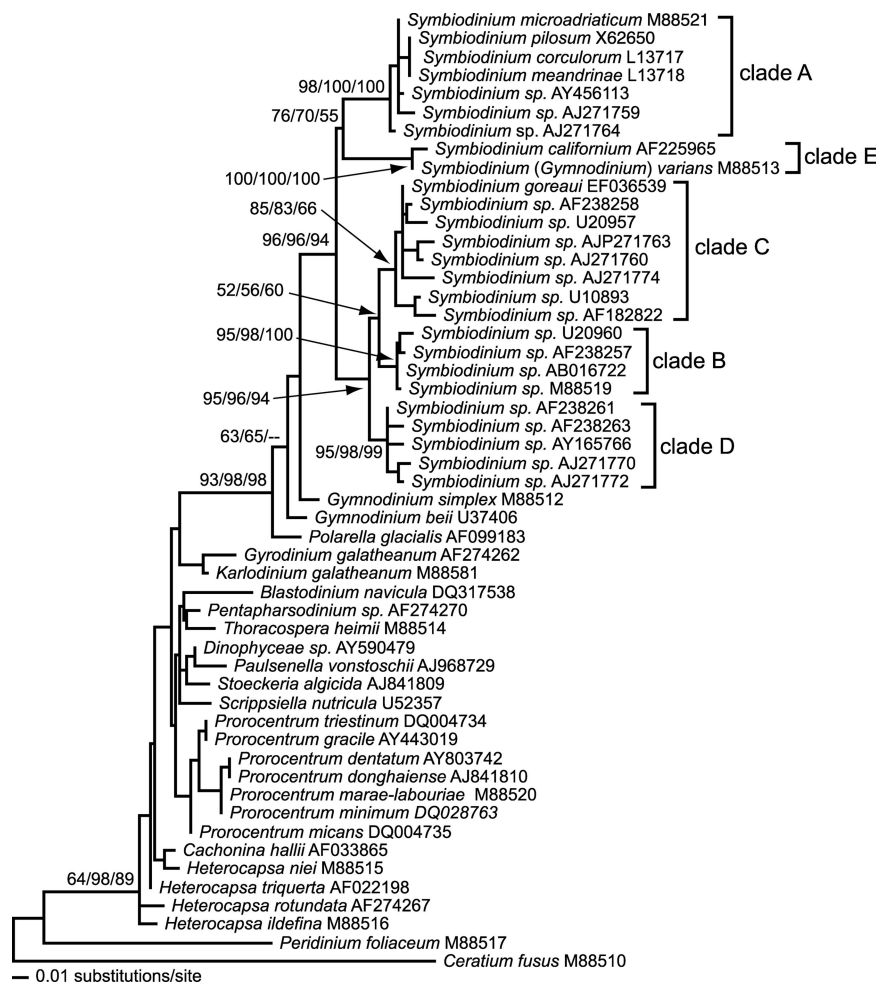
The efficiency of mutualistic symbioses depends on the host genotype, symbiont genotype, and the environment (1, 2, 20). Coral–dinoflagellate symbioses are defined as mutualistic; however, here we provide evidence that the symbiosis between a Pacific coral and the *Symbiodinium* clade A lineage appears as a reduction in the health state of the coral that may result from the host not receiving sufficient nutritional input from this symbiont lineage.

Clade A *Symbiodinium* are rarely reported in coral hosts; however, this group has been described as fast growing and opportunistic because it is found in corals recovering from bleaching events (21, 22). Here we show that *A. cytherea* from Hawaii harboring clade A exhibit suboptimal health states and an increased incidence of disease as compared with corals sampled on the same reef harboring *Symbiodinium* clade C. Although the incidence of coral disease in Hawaii is low, mass coral mortality caused by disease is widespread in the Atlantic Caribbean, especially within the genus *Acropora* (23–26). Interestingly, these corals also harbor *Symbiodinium* belonging to clade A as the dominant symbiont type (27, 28).

The relationship between health-compromised corals and clade A symbionts may reflect a directly harmful trait of the interaction between the coral host and these symbionts or the proliferation of opportunistic symbionts in health-compromised corals. Our *in vitro* carbon fixation experiments suggest that clade A symbionts may not provide as much carbon to the host as clade C symbionts and thus may not meet the host's nutritional requirements. In corals that have a high autotrophic dependence, such a scenario would certainly be reflected in a reduction of fitness that could ultimately render the host more susceptible to disease. Conversely, for the symbionts, the capacity to retain carbon would increase fitness and account for the high *in situ* growth rates reported for this group.

The evidence presented here does not allow us to infer that there is a direct negative effect of hosting clade A symbionts for the animal host (1, 29, 30). Thus there is no direct evidence that the interaction between clade A symbionts and corals belonging to the genus *Acropora* is parasitic. However, it is generally believed that horizontal transmission of symbionts promotes the evolution of parasitism (31–36). *Acropora* acquires its symbionts via horizontal transmission (37), and the competition between different lineages of *Symbiodinium* found within *Acropora* (clade A and C co-occurring) may be driving clade A *Symbiodinium* toward parasitism (34–36, 38–40). Furthermore, as health-compromised corals persist in nature, this phenotype would promote an increase in abundance of clade A *Symbiodinium*. Interestingly, in the symbiosis between the scyphozoan *Cassiopea*





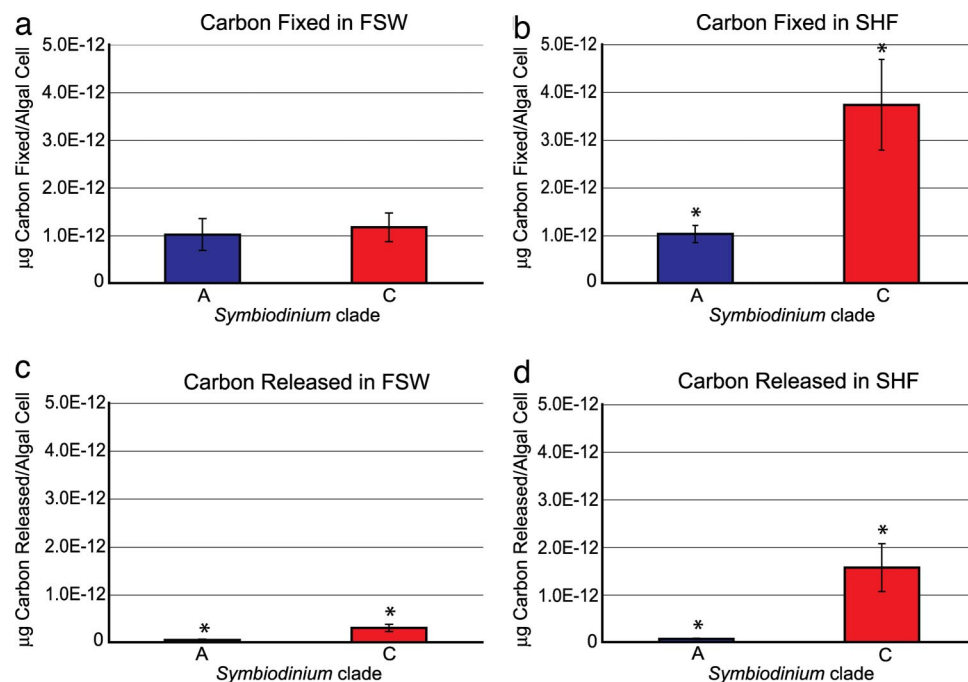
**Fig. 2.** Maximum-likelihood phylogeny inferred by using partial nuclear SSU rDNA. Numerals at nodes indicate bootstrap analyses for maximum-likelihood (100 replicates)/maximum-parsimony (1,000 replicates)/neighbor-joining (1,000 replicates).

and *Symbiodinium*, a shift from mutualism to parasitism was inferred from a decoupling of the symbiosis from vertical symbiont acquisition to horizontal acquisition that resulted in a reduction in fitness of the host (41). In the *Cassiopea* that acquired symbionts from the environment, there was a reduction in host growth rate and a proliferation leading to higher densities of *Symbiodinium*.

An alternative interpretation is that mutualism has never been achieved between clade A *Symbiodinium* and corals. The phylogenetic positioning of clades A and E is either basal (8, 10, 13, 42–47) or sister to the other *Symbiodinium* clades (this study and refs. 8, 10, 13, and 42–47). This positioning suggests that clade A (and E, or both) was the first group to initiate symbiosis with invertebrates, that they are divergent lineages on a different evolutionary trajectory to other *Symbiodinium* clades that evolved from the ancestral dinoflagellate that first formed symbiosis with invertebrates, or that a free-living state is ancestral within the genus and a symbiotic lifestyle evolved independently in the two sister lineages. The transition from free-living to mutualism in clade A may not be a completely evolved trait, whereas the sister grouping or more derived *Symbiodinium* clades (especially the dominant coral symbiont clade C) may have successfully achieved mutualism. It is generally believed that parasitism is the ancestral state in the evolution of endosymbiotic mutualisms and occurs during the process of endocytosis, recognition, and the adaptive evolution of both partners (1, 48–51). Clade A and clade C *Symbiodinium* may represent

symbiont lineages characterizing these two steps in the symbiosis continuum. The phylogeny presented here places clade A *Symbiodinium* as sister taxa along with clade E to clades B, C, and D. Clade E *Symbiodinium* is extremely rare and has only been identified in the temperate soft-bodied anemone *Anthopleura elegantissima* (10). The rarity and absence of this *Symbiodinium* lineage suggests that it does not form symbioses readily, it is going extinct, or it is an opportunistic dinoflagellate that is more abundant as a free-living entity. In terms of the latter, it is important to note that the diversity of *Symbiodinium* in the ocean environment is not well characterized (8, 52, 53).

There is strong support for a monophyletic grouping of *Symbiodinium*; however, the lower genetic distances between clade A *Symbiodinium* and the *Symbiodinium* outgroups *Gymnodinium* sp. and *P. glacialis* (*G. simplex* being free-living) relative to other *Symbiodinium* clades suggests a nonconstant molecular clock within the genus. DNA evolution rates are known to be variable between mutualists and nonmutualists of the same organism (54). Furthermore, faster DNA evolution rates have been identified in aphid bacterial endosymbionts (genus *Buchnera*) than in free-living relatives (55), and in fungi found in mutualisms with liverworts (56). Indeed, clade A *Symbiodinium* has been shown to have a slower rate of DNA evolution than other *Symbiodinium* clades (57), which is consistent with rates expected of a nonmutualistic lineage. Also, the bootstrap support for the *Symbiodinium* outgroups presented here and in another comprehensive phylogenetic study of the



**Fig. 3.** Mean values of carbon expressed as  $\mu\text{g}/\text{algal cell}$  fixed (a and b) and released (c and d) in the presence of FSW or SHF by *Symbiodinium* clade A (blue histograms) and clade C (red histograms). Bars in the histograms represent the standard error and an asterisk denotes values that are significantly different within a treatment.

genus in relation to free-living pelagic symbionts using partial large subunit rDNA (58), both show very little support for the *Gymnodinium* species. Clearly, further analysis of these dinoflagellates is required to better understand the evolution of symbiosis within the grouping.

Genetic diversity within the genus *Symbiodinium* has previously been reported as comparable to that seen within different orders of dinoflagellates (15). Here, we show that this order-level genetic diversity actually exists within a single lineage of *Symbiodinium*, clade C. Such high levels of genetic divergence within the *Symbiodinium* genus supports the idea that the group contains members with highly diverse functions and physiologies, some of which may provide them with the capacity to form symbiotic interactions with coral. Based on the data presented here, a conservative approach would classify clade lineages of *Symbiodinium* into different families when comparing the amount of pairwise sequence diversity between other dinoflagellates. Parasitic and mutualistic symbiont lineages have been shown to group together in other interactions (59–62), and different genetic varieties of the same symbiont can be either mutualistic or harmful to the host. For example, the interactions between the actinomycete fungi *Frankia* and *Casuarina* plant species can be both beneficial or antagonistic (63), mycorrhizal-plant interactions can be mutualistic or antagonistic (64), and the bacterial group *Vibrionaceae* that contains both free-living and symbiotic varieties that form symbioses with marine hosts is either cooperative or parasitic (65).

The amount of diversity in clade A and clade C is very different (58) with clade C containing many more types (21, 58). An opportunistic symbiotic lifestyle in clade A could potentially explain the discrepancy in diversity within these clades. If clade A exists primarily in the free-living environment and only occasionally infects a host colony, then diversification within the clade may be restricted because of the need to occupy two compartments, the host and the ocean. Clade C *Symbiodinium* (and to a lesser extent other clade lineages) associate with a diverse range of hosts, many that use vertical transmission of

symbionts, providing them with more, highly specialized environments (coral and other host organisms) that would promote the evolution of multiple specialized types. This mechanism has been suggested as a factor that differentiates the genetically diverse genus *Symbiodinium* compared with other less diverse pelagic symbionts (58).

Further evidence that supports clade A *Symbiodinium* as adapted more toward a free-living dinoflagellate comes from the history of culturing *Symbiodinium*. Clade A *Symbiodinium* is easily cultured and outcompetes other clades even when the starting culture obtained from a host species contains predominantly clade C and undetectable clade A symbionts (8, 27). In addition, clade A and B *Symbiodinium* have been cultured from the ocean environment with a group of isolates within clade A suggested to be nonsymbiotic (53). In contrast, clade C is extremely difficult to culture and may represent an obligate symbiont adapted to survive only in the highly specialized environment of the animal host cell.

In this study we also show that clade A *Symbiodinium* releases very little carbon that can be used for host nutrition, the defining factor in coral-dinoflagellate mutualism (4), and there is no increase in the amount of carbon fixed in the presence of SHF. This finding is in contrast to the common coral symbiont, clade C, which both increases carbon fixation and release in the presence of SHF. Recently, Loram *et al.* (66) showed functional variability in the symbiosis between the giant sea anemone, *Condylactis gigantea*, and clades A and B *Symbiodinium*. In this association, clade A was shown to be more beneficial to the animal host than clade B. Considering the diversity and biology of the these host taxa, *Symbiodinium* belonging to clade A is likely to have a different interaction with soft-bodied anthozoans than with the Scleractinia. The most obvious differences between the two interactions are the formation of the calcium carbonate skeleton in the Scleractinia and the persistence of anemones when devoid of their dinoflagellate symbionts to rely on heterotrophy to survive, which is not possible for coral (67).

Taken together, we suggest that *Symbiodinium* clade A is less beneficial to corals than other *Symbiodinium* lineages and may be more representative of a parasitic than a mutualistic symbiont, whereby the animal host does not receive sufficient nutritional input from the dinoflagellate symbiont, a circumstance that ultimately renders the coral more susceptible to disease and mortality.

## Methods

**Symbiodinium Genotypes and Coral Health.** Colonies of *A. cytherea* (1–2 m in size) were sampled from French Frigate Shoals in the Northwestern Hawaiian Islands Marine National Monument, Papahānaumokuākea, during September 2005, May 2006 and September 2007. Approximately 2 cm<sup>2</sup> of coral was removed from each colony with a hammer and chisel and stored at –20°C in DMSO preservation buffer (68). Each coral sampled was photographed and categorized as (i) healthy (Fig. 1a), (ii) displaying abnormal phenotypes consisting of pronounced blue pigmentation and evidence of past tissue loss (Fig. 1b), or (iii) suffering from a disease causing active tissue loss (Fig. 1c).

DNA from the stored coral samples was extracted by using a modified cetyltrimethylammonium bromide (CTAB) protocol (69). Briefly, the tissue was incubated in 500 µl of CTAB buffer [100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (wt/vol) CTAB, 2% (vol/vol) 2-mercaptoethanol, 1% (wt/vol) polyvinylpyrrolidone, pH 8.0] at 65°C for 30 min. An equal amount of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the contents of the tube were mixed and centrifuged at 15,000 × g for 20 min. The aqueous phase was removed, and an equal volume of chloroform/isoamyl alcohol (24:1) was added, thoroughly mixed, and centrifuged at 15,000 × g again for 20 min. The DNA was precipitated from the resulting aqueous phase by the addition of 1/2 volume of 5 M NaCl and an equal volume of isopropanol and incubated for 1 h at –80°C. The DNA was pelleted by centrifugation at 15,000 × g for 30 min, washed twice with 70% ethanol, and resuspended in sterile water.

The nuclear small subunit (SSU) rDNA was amplified from DNAs extracted from *Symbiodinium* by using the primers ss5z and ss3z (70). The PCR products were digested by using *TaqI* (New England Biolabs) for 2 h at 65°C, and the clade of *Symbiodinium* present in each *A. cytherea* colony was identified based on the patterns of restriction fragment length polymorphisms (RFLPs) in the digests visualized on 2% agarose gels under UV illumination.

**Genetic Diversity and Phylogenetic Analyses.** *Symbiodinium* and other dinoflagellate nuclear SSU rDNA sequences were obtained from GenBank. The analysis is restricted to only five clades of *Symbiodinium*, which represents available SSU rDNA data for the genus. A concatenated alignment of 459-nt characters consisting of two regions within the SSU rDNA was constructed and manually edited by using the software MacVector 8.0.2. The DNA alignment was tested against 56 models of DNA substitution in the program PAUP\*, version 4.0b10, and the best-fit model was selected by using the program Modeltest, version 3.7 (71). The Hierarchical Likelihood Ratio Tests and the Akaike Information Criterion both selected the TrN+I+G as the best fit model of DNA evolution with stationary base frequencies of A = 0.2659, C = 0.1978, G = 0.2422, and T = 0.2941, proportion of invariable sites = 0.2485, and a gamma distribution shape parameter of 0.6227. Pairwise distances and phylogenies were inferred from the alignment by using PAUP\*, version 4.0b10 (72). For corrected maximum-likelihood pairwise genetic distances, the distance showing the greatest amount of genetic diversity when comparing a *Symbiodinium* clade was used. The maximum-likelihood phylogeny was constructed by using the results from Modeltest, and the Kimura two-parameter model (73) was used to infer the neighbor-joining tree. For maximum-parsimony, the minimum

F value was incorporated as the character-state optimization and gaps in the sequence were treated as a fifth base. Midpoint grouping was used in the maximum-likelihood and neighbor-joining trees, and *Peridinium foliaceum* and *Ceratium fusus* were used as outgroups in the maximum-parsimony tree. The validity of node placement within each phylogeny was tested by using bootstrap analyses (74) for 1,000 replicates for neighbor-joining and maximum-parsimony trees and 100 replicates for maximum-likelihood analysis.

**Carbon Fixation and Release.** FIS were obtained from the jellyfish *Cassiopeia* sp. (*n* = 6) collected from the Hilton Hawaiian Village lagoon in Waikiki and from the coral *Pocillopora damicornis* (*n* = 6) collected from the reef surrounding the Hawaiian Institute of Marine Biology in Kaneohe Bay, Oahu. *Symbiodinium* were isolated from *Cassiopeia* sp. by homogenizing tentacles in 2 ml of FSW and from *P. damicornis* by blasting the tissue off the skeleton with a dental water pick. The FIS were collected by centrifugation at 5,000 × g at 24°C for 5 min, and the FIS pellets were washed free of host tissues by repeated resuspension in FSW and centrifugation (×3). The final FIS pellets were resuspended in 1.5 ml of FSW, and the number of cells were assessed with a Neubauer haemocytometer and a light microscope (four per sample) and expressed per ml. The clade of *Symbiodinium* harbored by each host was confirmed by using SSU rDNA RFLP as described above.

The total carbon fixed and released by FIS incubated in FSW and a SHF was assessed by using NaH<sup>14</sup>CO<sub>3</sub>. Six replicate experiments for each treatment, for each clade were performed with FIS isolated from a different animal. FIS (200 µl at 4 × 10<sup>6</sup> cells/ml) were mixed with an equal volume of FSW or SHF and incubated in the light (300 µmol quanta·m<sup>–2</sup>·s<sup>–1</sup>) for 1 h at 26°C in the presence of 1 µCi of NaH<sup>14</sup>CO<sub>3</sub> (added in 10 µl of MilliQ water before the 1-h incubation). The SHF consisted of 1.24 µM aspartic acid, 15.15 µM glutamic acid, 3.61 µM serine, 0.92 µM histidine, 7.13 µM glycine, 2.11 µM arginine, 3.13 µM taurine, 10.41 µM alanine, 0.97 µM tyrosine, 1.73 µM methionine, 5.93 µM valine, 2.32 µM phenylalanine, 2.51 µM isoleucine, 2.43 µM leucine, and 0.64 µM asparagine, dissolved in distilled water with pH 8.3 and adjusted to a salinity 33 parts per thousand (75).

After incubation, the tubes were mixed by vortexing, and two 50-µl samples were removed to assess total <sup>14</sup>C fixed. FIS were then pelleted at 5,000 × g for 5 min, and two 50-µl samples of the supernatant were placed in scintillation vials to calculate the amount of carbon released by the FIS. The unincorporated <sup>14</sup>C was removed from the samples by acidification by adding 100 µl of 0.1 M HCl to each scintillation vial and incubating it at room temperature for 1 h. Biodegradable counting scintillant (BCS) (4 ml; Amersham Biosciences) was added to each scintillation vial, and the amount of radioactivity was assessed with a Beckman LS 3801 scintillation counter. The total <sup>14</sup>C fixed and released was calculated and expressed as counts per FIS and as a ratio of counts fixed and released relative to the FSW control. An estimate of the absolute amount of <sup>14</sup>C fixed or released was calculated by using the specific activity of the label (NaH<sup>14</sup>CO<sub>3</sub>) expressed in cpm/fmol and the following equation [cpm × (cpm/fmol<sup>–1</sup>) × 10].

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