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SYMBIOSIS ECOLOGY OF TWO CARIBBEAN ACROPORID CORALS

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ABSTRACT

The elkhorn coral *Acropora palmata* and the staghorn coral *A. cervicornis* are two common species of Caribbean reef-building coral. The typical depth distribution of *A. palmata* is much narrower (0-6m or less) than *A. cervicornis* (0-20m or more). We examined the genetic diversity of zooxanthellae from these two species using restriction fragment length polymorphisms (RFLPs) in large subunit ribosomal RNA (rRNA) genes. In shallow (<3m) waters in Panamá, the two coral species host indistinguishable *Symbiodinium*. However, in deeper waters (>15m), where *A. palmata* does not occur, *A. cervicornis* hosts different *Symbiodinium* that are only distantly-related to those found in shallow water. Similar patterns are found in the Bahamas. The shallow- and deep-water symbiont populations in *A. cervicornis* are closely-related to those previously documented in symbiosis with *Montastraea annularis* and *M. faveolata* on the same reefs. Taken together, these data suggest that different algal symbionts enable coral hosts to live competitively in different photic habitats, and that the photic requirements of different algal taxa are phylogenetically conserved features of their biology.

Although *Acropora cervicornis* hosts two different taxa of zooxanthellae, individual colonies have not been found to contain both taxa of zooxanthellae simultaneously. This is in contrast to *Montastraea* spp., whose colonies can frequently contain two or more symbiont taxa. We hypothesize that symbiont diversity in individual coral-algal symbioses are the outcome of numerous interacting factors which may include the degree of taxonomic (host-symbiont) specificity; the local environmental conditions; the mode and frequency of symbiont acquisition; and the extent of intracolony symbiont circulation. Differences in symbiont diversity may help explain the differences in the depth distributions of these two species of *Acropora* on Caribbean reefs.

INTRODUCTION

Zooxanthellae are unicellular endosymbiotic algae found in many hundreds of marine invertebrate species (Taylor 1984, Trench 1987). In hermatypic (reef-building) corals their phototrophic contribution to the energy budget of their hosts and their role in enhancing calcification are generally considered critical in explaining the ecologically dominant role corals play in shallow tropical seas (Muscatine and Porter 1977, but see also Marshall 1996). The vast majority of zooxanthellae were originally thought to be members of a single pandemic species of dinoflagellate, *Symbiodinium microadriaticum* Freudenthal, but over the last two decades a variety of independent evidence has shown that zooxanthellae are an extraordinarily diverse assemblage (Schoenberg and Trench 1980a,b; Fitt et al., 1981; Blank and Trench 1985; Rowan and Powers 1991b). Reflecting this, zooxanthellae have recently been described as belonging to at least 7 genera in 4 orders of dinoflagellates (Banaszak et al. 1993). The greatest recorded diversity of zooxanthellae is found in the genus *Symbiodinium*, which contains some taxa separated by genetic distances greater than those separating some species of non-symbiotic dinoflagellates belonging to different orders (Rowan and Powers 1992). The known phylogeny of this genus, inferred from partial sequences of the nuclear genes encoding small subunit rRNA, consists of three major clades, designated "A", "B" and "C" (Rowan and Powers 1991b).

The prevailing paradigm explaining the high diversity of symbiotic dinoflagellates is that their associations with marine invertebrates exhibit a high degree of taxonomic specificity (Schoenberg and Trench 1976, Trench 1992). This paradigm is useful in explaining patterns of association at higher taxonomic ranks: for example, all stony corals host zooxanthellae belonging to the genus *Symbiodinium* (Trench 1988). However, the view that one type of host contains one type of symbiont is not always supported at lower taxonomic levels. For example, some "polymorphic" species of Caribbean reef-building corals in the genus *Montastraea* host multiple taxa of *Symbiodinium* (Rowan and Knowlton 1995).

As part of a survey of zooxanthella diversity in Caribbean corals, we documented symbiont diversity in two common species of *Acropora* from Panamá and the Bahamas. The elkhorn coral *Acropora palmata* is generally found at shallow depths (<5m) on the upper fore-reef or reef-crest; whilst the staghorn coral *A. cervicornis* exhibits a wider depth distribution that can extend from the surface to deep water (>30m). We used restriction fragment length polymorphisms (RFLPs) in large subunit ribosomal RNA (rRNA) genes as molecular markers for the identification of the otherwise cryptic taxa of zooxanthellae contained within these two coral species. The validity of this approach has been demonstrated using small subunit rRNA genes (Rowan and Powers 1991a,b; Rowan and Knowlton, 1995; Wilcox 1995). Study of the more variable large subunit (Hillis and Dixon 1991) should allow the identification of more closely-related *Symbiodinium* within each of the three major clades.

Studies of the environmental factors influencing symbiont distributions are fundamental to our growing appreciation of the role of polymorphic symbiosis in coral ecology (Buddemeier and Fautin 1993). A better understanding of the patterns and processes underlying zooxanthella distributions across different coral species would provide powerful tools for the investigation of phenomena as diverse as coral reef bleaching, larval ecology and coral zonation.

MATERIALS AND METHODS

Sample Collection and Preservation

Samples of *Acropora cervicornis* were collected from depths of 1-16m from Aguadargana (Sail Rock) and Korbisky reefs in the San Blas Islands, Panamá (N=29); and from depths of 3-28m from Norman's Pond Cay and Rock Cay Wall reefs, near Lee Stocking Island in the Bahamas (N=3). Samples of *A. palmata* were collected from a depth of 1-2m from Aguadargana reef, Panamá (N=10); and from depths of 2-3m from the *A. palmata* reef just off Lee Stocking Island, Bahamas (N=10).

Acropora cervicornis samples were generally snapped off by hand 5-8cm from the branch tip, and the distal (non-pigmented) end discarded. Sampled colonies were ≥6m apart and only one sample was removed per colony. Cores of *Acropora palmata*, approximately 1cm in diameter and 0.5cm in depth, were taken from the well-lit "topside" (N=10) and shaded "underside" (N=10) surfaces using a hammer and a hollow steel punch. Branches growing at right angles to the downwelling light were preferred,

and cores were taken well away (≥20cm) from the growing margin. Sampled colonies were ≥3m apart and only one pair of cores was taken from any one colony.

Samples were transported to the surface in labelled polythene ziplock bags before being transferred to wide-mouthed 5ml vials containing saline DMSO buffer [20% (v/v) dimethyl sulfoxide (DMSO), 0.25M EDTA, saturated NaCl] as a chemical DNA preservant (Seutin et al. 1991).

DNA Extraction

Coral samples were airbrush-blasted with modified ZB (Rowan and Knowlton 1995) to remove all traces of tissue. The resulting slurry, together with the saline DMSO solution in which it was stored (containing leached pigments and tissue fragments), was centrifuged for 10 minutes at ~1600g. All steps were carried out at 0-4°C. The pellet (containing coral tissue and zooxanthellae) was resuspended in 1ml DNAB (Rowan and Powers 1991a) containing 1% SDS. Total nucleic acid was then obtained using established protocols (Rowan and Powers 1991a, Rowan and Knowlton 1995).

Isolation, Amplification and Characterization of Zooxanthella DNA

Large subunit rRNA genes were PCR-amplified from the genomic DNA samples using the conserved primers 24D15F1 (5'-TTAAGCATATAAGTAAGCGGAGGA-3') and 24D23R1 (5'-CTCCTTGGTCCGTGTTTCAAGACG-3'). These conserved primers were designed to simultaneously amplify large subunit rRNA genes from both corals and zooxanthellae. The amplified region in corals is approximately 800bp in length, compared to about 600bp in zooxanthellae (Baker, unpubl. data). This difference occurs because the D1 and D2 regions of large subunit rRNA vary significantly in size between phylogenetically different groups of organisms (Hassouna 1984, Hillis and Dixon 1991). This approach represents a novel strategy to avoid contamination from the coral host, since coral- and zooxanthella-derived PCR products can be easily separated by agarose gel electrophoresis. This method does not use phylogenetically-biased primers (which might not amplify previously undocumented symbiont taxa); nor does it involve repeated centrifugation of cell and tissue slurries (which might bias the analysis against less dense symbiont taxa).

Amplified fragments were digested with the restriction enzymes *TaqI*, *HhaI* and *Alw26I*, which were chosen for their ability to distinguish the three major clades of zooxanthellae, based on analysis of reference sequences (archived in Genbank U63483-U63485) for each of these clades.

PCR products were TA-cloned, purified and cycle-sequenced using T7/SP6 primers that border the cloned insert. Cycle-sequenced product from 2 replicate clones was sequenced in both directions on an Applied Biosystems 377 DNA sequencer. Typical reads were 450-500 nucleotides in length.

RESULTS

Amplification of Coral and Zooxanthella DNA

Zooxanthella large subunit (24S) rRNA genes were successfully amplified from all samples attempted. Surprisingly, however, the equivalent regions in large subunit *Acropora* genes were not amplified using this strategy. Failure of these coral genes to amplify is unlikely to be a result of primer-template mismatch, because the primers used are complementary to highly conserved regions of the large subunit rRNA gene. Neither lowering the stringency of the PCR nor designing "staggered" primers along this conserved region result in amplification. It is possible that the D1 and/or D2 regions of these coral species exhibit complex secondary structure or are the sites of large insertions that foil the PCR. Similar problems have been encountered when amplifying large subunit rRNA genes in certain other coral species (Baker unpubl. data).

Our inability to amplify *Acropora* genes is unlikely to have affected our survey of *Symbiodinium*, because: (1) zooxanthella genes from all *Acropora* samples amplified successfully; (2) analysis of a subset of these samples with small subunit rRNA primers (Rowan and Powers 1991a) confirmed our zooxanthella identifications; and (3) our surveys of other coral species successfully amplified symbionts from all of the three known clades of *Symbiodinium*.

Distribution of Zooxanthella Genotypes

Acropora palmata

Restriction enzyme analysis of PCR-amplified zooxanthella DNA from *Acropora palmata* documented only one RFLP in all samples (N=20), regardless of whether they originated from the upper or lower surface of a colony, or from Panamá or the Bahamas (Figure 1).

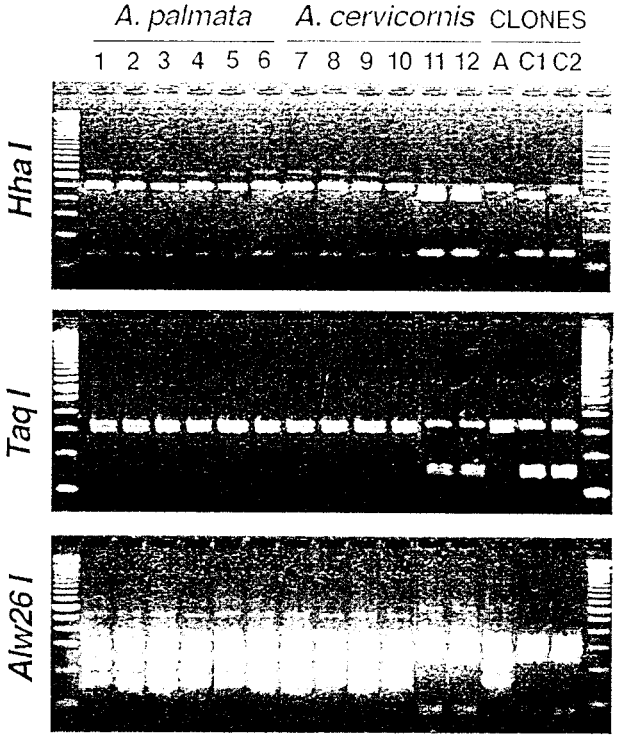

Fig.1: Zooxanthella RFLP diversity in *Acropora palmata* and *A. cervicornis* from Panamá. Gels show a 600bp region of large subunit rDNA cut with the restriction enzymes *HhaI*, *TaqI* and *Alw26I*. Lanes 1-6 show paired samples from each of three colonies of *A. palmata* from 2m depth. Each pair represents samples from the topside and underside of the same colony; all zooxanthellae are indistinguishable. Lanes 7-12 show 6 colonies of *A. cervicornis* taken from depths of 2m, 4m, 6m, 10m, 16m and 17m respectively; zooxanthella RFLP genotypes show zonation with depth. The final three lanes show RFLP genotypes of cloned genes. Note that lanes 11 and 12 (deep *A. cervicornis*) are composite RFLPs of clone genotypes C1 and C2 (last two lanes). End lanes are 100bp DNA ladders.

The sequence obtained from these PCR products was the same in each case (within accuracy limits determined by

replicate sequencing as >99.5%) and showed concordance with the RFLPs. This sequence has been archived (Genbank U63480). Based on its close resemblance to a reference sequence (Genbank U63483), it was identified as a *Symbiodinium* belonging to clade "A" (Rowan and Powers 1991b).

Because we observe no RFLP or sequence variation, we conclude that *A. palmata* probably hosts only one type "A" zooxanthella taxon. We recognize that a community of very closely-related symbionts might be cryptic to our study of large subunit rRNA genes. However, because percent sequence divergence in zooxanthella large subunit rRNA genes is approximately 4 times higher than small subunit genes (Baker and Rowan, unpubl. data) this conclusion is stronger than similar conclusions based on the small subunit (Rowan and Powers 1991a,b; Rowan and Knowlton 1995; Wilcox 1995).

Acropora cervicornis

Zooxanthellae from *Acropora cervicornis* in Panamá showed three RFLPs distributed as a function of colony depth (χ^2=15.998; df=5; P=0.0069). See Figure 2. The shallowest of these RFLPs ("A" in Figure 1), which occurred exclusively at depths <9m (N=17), was identical to that found in *A. palmata* (clade "A" *Symbiodinium*). Sequences for this RFLP were also identical (Genbank U63483). The deeper RFLPs in *A. cervicornis* ("C1" and "C2" in Figure 1) always (N=7) occurred together in the same relative proportions ("C2" slightly less than "C1"). This composite RFLP occurred at greater depths with increasing frequency (Figure 2). Sequences for these RFLPs have been archived (Genbank U63481-63482). Based on their resemblance to a reference sequence (Genbank U63485) these sequences were both identified as *Symbiodinium* belonging to clade "C". The same pattern was found in the Bahamas with more limited sampling (one sample from shallow (3m) waters containing "A" and two samples from deep (27-28m) waters containing "C1+C2").

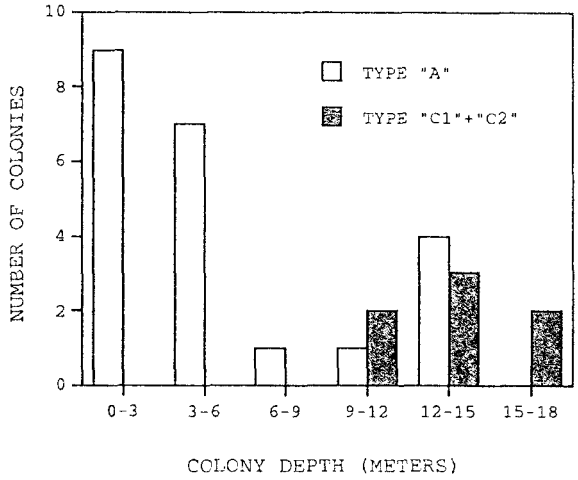

Fig. 2: Depth distribution of zooxanthella taxa in *Acropora cervicornis* from the San Blas, Panamá. Shallow-water colonies host zooxanthellae with one RFLP genotype "A"; deeper colonies host zooxanthellae with two RFLP "C" genotypes in constant proportions (χ^2=15.998; df=5; P=0.0069). See Figure 1.

No single colony, from either Panamá or the Bahamas, was found containing zooxanthellae with all three RFLPs ("A" and "C1+C2"). At intermediate depths (10-14m) in Panamá, samples (1-2cm branch segments) contained either the "A" RFLP genotype or the composite "C1+C2" genotype, but never both simultaneously (N=8). To confirm this, two to five replicate samples were taken from different parts (branch tip, mid-branch, base etc.) from each of 5 different colonies in the intermediate (9-15m) depth range. They showed no intracolony variation in their zooxanthella RFLP genotype. In contrast, single samples of *Montastraea annularis* and *M. faveolata* (representing as little as 1cm^2 tissue) frequently contain multiple symbiont taxa when removed from colonies at intermediate depths (Rowan and Knowlton 1995).

Composite genotypes consisting of multiple RFLPs ("C1+C2") could represent multiple taxa of zooxanthellae within one host, or one species of dinoflagellate with multiple different large subunit rRNA genes (Rowan and Knowlton 1995). We favor the latter interpretation for *A. cervicornis* because: (1) the two RFLPs do not occur independently of one another; (2) one of the RFLPs ("C2") has never been found in isolation in other coral species (Baker unpubl. data); (3) all samples show the same C1:C2 ratio; and (4) the sequence divergence between C1 and C2 is small (<1%).

Despite concerted evolution (Arnheim 1983), multiple different rRNA genes have been previously recorded in dinoflagellates in both small and large subunit rDNA (Scholin et al., 1993, Zardoya et al., 1996). We hypothesize that the composite clade "C" RFLP represents a single taxon of zooxanthella with two different large subunit rRNA genes. To date, this zooxanthella taxon has not been found in any other scleractinian hosts (Baker unpubl. data). The high sequence similarity between C1 and C2 and the fact that the few sequence dissimilarities between them occur in variable regions suggest that both genes may still be functional and that "C2" may have resulted from a recent duplication event.

DISCUSSION

Acropora cervicornis occurs over a wide (0-30m or more) depth range, and hosts at least two different taxa of zooxanthellae that occur predictably as a function of depth. *Acropora palmata* has a shallow depth distribution (0-5m or less), and hosts only one taxon of zooxanthella. The shallow-water ("high light") zooxanthellae hosted by *A. cervicornis* are indistinguishable from those hosted by *A. palmata*, but the deep-water ("low light") zooxanthellae hosted by *A. cervicornis* are only distantly-related.

We did not find any deep-water ("low light") zooxanthellae in symbiosis with *A. palmata*. One interpretation of this is that the light levels available to zooxanthellae on the undersides of *A. palmata* in Panamá are not low enough to favor this association. Measurements of upwelling irradiance (PAR) on the undersides of *A. palmata* at 1-2m depth in Panamá are approximately equal to the highest irradiance at which *A. cervicornis* hosts deep-water zooxanthellae at the same site (Baker, unpubl. data). Accordingly, there may be no reason to expect to find deep-water zooxanthellae in the colonies of *A. palmata* sampled in this study. However, colonies of *A. palmata* are rare at depths >2m in Panamá. This may indicate that incident irradiance levels on the undersides of colonies at greater depths are insufficient for the "high light" zooxanthellae found in *A. palmata*. This supports the conclusion that *A. palmata* is not able to host a second "low-light" symbiont taxon.

Our findings suggest that the ability to host a diverse symbiont population enables corals to exist competitively in a broad range of habitats. The genetic similarity between the habitat-specific zooxanthella taxa found in *Montastraea* spp. (Rowan and Knowlton 1995) and those found in *Acropora* supports this. A unified theory of the symbiosis ecology of reef-building corals must explain why different taxa of zooxanthellae occur where they do; the observation that particular symbiont

taxa consistently show similar distributions considerably simplifies that task.

The discovery that *Acropora cervicornis* hosts two taxa of *Symbiodinium* at different depths demonstrates that intraspecific diversity and ecological zonation in coral-algal symbiosis is not confined to the genus *Montastraea* (Rowan and Knowlton 1995). Depth zonation in both genera support the notion that light is the primary environmental gradient underlying the intraspecific distributions of *Symbiodinium* in the Caribbean. This implies that different taxa of zooxanthellae occupy different photic niches on the reef. The same conclusion has been reached from studies of cultured zooxanthellae that show that different taxa vary in their photoacclimatory abilities (Chang et al. 1983, Iglesias-Prieto and Trench 1994).

Two major differences distinguish the symbioses found in *Acropora cervicornis* from those found in the genus *Montastraea*. First, although they host similar symbiont taxa, the transition depth at which algal zonation occurs in *Acropora cervicornis* is much deeper (9-15m) than in *Montastraea* spp. (3-7m) from the same sites (Rowan and Knowlton 1995). Secondly (and perhaps not independently), we found no evidence that single colonies of *A. cervicornis* host multiple taxa of zooxanthellae simultaneously, whereas colonies of *Montastraea* have been found containing at least three distinct symbiont taxa (Rowan unpubl. data). One interpretation of these data is that *A. cervicornis* actually represents two cryptic species which show partially overlapping depth distributions and different symbiont specificities. However, since the taxonomy of *A. cervicornis* is currently relatively undisputed, and since there is precedence for intraspecific zooxanthella diversity in corals (Rowan and Knowlton 1995), this possibility is probably unlikely.

Why do some coral-algal symbioses (for example, *A. cervicornis*) exhibit polymorphisms, whilst others (for example *A. palmata*) do not? Similarly, why do some polymorphic coral-algal symbioses also exhibit those polymorphisms within single colonies (*Montastraea* spp.), whilst other polymorphic symbioses (*A. cervicornis*) do not? We hypothesize that the characteristics of individual coral-algal symbioses are the outcome of numerous interacting factors which may include: (1) the degree of taxonomic (host-symbiont) specificity; (2) the local environmental conditions experienced by the association; (3) the mode and frequency of symbiont acquisition; and (4) the extent of symbiont circulation within the colony.

Taxonomic specificity

It has long been recognized that microalgal-invertebrate symbioses exhibit specificity (Trench 1988, 1992, 1993). The scleractinian corals are no exception to this. However, molecular surveys of zooxanthella diversity in Caribbean *Montastraea* (Rowan and Knowlton 1995) and *Acropora* (this study) demonstrate that whilst specificity in some cases is restrictive enough to limit some coral species to associating with a single taxon of zooxanthella (for example *Acropora palmata*, this study), specificity in other species is demonstrably less restrictive, resulting in stable and predictable polymorphic symbioses. The exact mechanisms which mediate specificity are still poorly understood (but see Trench 1988); nevertheless, it is becoming clear that the *degree* of specificity varies from one coral species to another.

Environmental factors

Acropora cervicornis (this study), *Montastraea annularis* and *Montastraea faveolata* (Rowan and Knowlton 1995) all host multiple taxa of zooxanthellae, and all exhibit zonation of those different taxa with depth. Zonation suggests that different symbiont taxa partition the light gradient within the host due to intrinsic differences in their photoacclimatory ability. To date, no examples of zonation across other environmental gradients have been documented. Nevertheless, emergent patterns from existing data suggest that, within the constraints dictated by taxonomic (host-symbiont) specificity, and other limitations (see below), environmental parameters can determine the relative abundance of different symbiont taxa within the host.

Symbiont acquisition

Although significant work has been done on symbiont acquisition and rejection in a variety of marine invertebrates, most notably *Aiptasia* and *Cassiopeia* (e.g.: Schoenberg and Trench 1980c, Colley and Trench 1983), it is not known whether acquisition of zooxanthellae by scleractinian corals occurs uniquely at the larval stage, or occurs continuously throughout the life of a coral colony.

Larval acquisition of zooxanthellae includes both maternal transmission of symbionts from the parent colony, and infection of gametes or larvae from the environment. The acquisition event in both cases could involve either single or multiple algal cells. If no new symbionts can be acquired after the larval stage, different colonies of "polymorphic" coral species (see above) could contain different symbionts as a consequence of their larval history. Individual colonies might host only one symbiont genotype, although that genotype could vary between different colonies (e.g. *Acropora cervicornis*).

Alternatively, if symbiont acquisition is not a unique, single-cell event, but instead involves either multiple symbionts (of different genotypes) or multiple events (larval and adult), then individual colonies of polymorphic coral species will be more likely to also exhibit zooxanthella diversity at the colony level (e.g. *Montastraea annularis* and *M. faveolata*).

Patterns of symbiosis polymorphism might also be influenced by mode of coral reproduction (broadcast spawning vs. brooding), since this could affect the method of symbiont acquisition (environmental vs. maternal). Coral species that acquire their zooxanthellae from the environment may be preadapted for greater symbiotic diversity because the pool of potential symbionts is greater on the reef than in the parent colony. However, reproductive mode cannot by itself account for patterns of polymorphic coral symbiosis. For example, *Acropora palmata*, *A. cervicornis*, *Montastraea annularis* and *M. faveolata* are all broadcast spawners that acquire their zooxanthellae from the environment (Szmant 1986, 1991). However, *A. palmata* is only found in association with one taxon of symbiont (this study), whilst the other three species host multiple symbiont taxa (Rowan and Knowlton 1995, this study). Furthermore, the two species of *Montastraea* show this multiple association even at the colony level, whereas *A. cervicornis* does not (Rowan unpubl. data).

Symbiont circulation

Patterns of intracolony symbiont diversity in scleractinian corals may also be influenced by the transport of zooxanthellae within the coelenteric circulation of the host. Gladfelter (1983) first demonstrated the connectivity of the canals of the scleractinian gastrovascular system. She noted the presence of free zooxanthellae within these canals, as well as the attachment of zooxanthellae to the canal wall. If zooxanthellae circulate in such a way that they encounter substantial photic variation, this could select for a single zooxanthella taxon best suited to that range of variation, rather than multiple taxa that subdivide the light gradient. More photic variation will be experienced by zooxanthellae in those coral species that have high rates of fluid circulation; and in branching, as opposed to massive, coral hosts. *Acropora cervicornis* is a branching coral species that has extremely high rates of fluid circulation, in contrast to *Montastraea annularis* (*sensu lato*), which is massive

and has slow circulation rates (Gladfelter 1986). This may explain why colonies of *Montastraea* host multiple symbiont taxa but colonies of *A. cervicornis* host only one.

If, as appears to be the case, individual colonies of *A. cervicornis* can only host one taxon of zooxanthella (either only "A" or only "C"), it might be expected that each of these two symbioses will have different depth optima depending on the particular photosynthetic characteristics of the algae involved (Iglesias-Prieto and Trench 1994). This might lead to a bimodal depth distribution for *A. cervicornis*, with high colony abundance in both shallow (all "A") and deep (all "C") waters, but relatively few colonies in between (where neither "A" nor "C" is well-suited). In contrast, polymorphic species that are able to host multiple zooxanthella taxa at the colony level might be expected to show more even depth distributions (e.g.: *Montastraea*). These patterns have yet to be tested. Additionally, colonies transplanted from one depth to another (shallow to deep, or vice versa) may not survive unless they are able to adjust their symbiont populations. This may be more difficult for colonies hosting a single symbiont taxon than for colonies which host multiple taxa, especially if there is no zooxanthella acquisition during the adult stage (see above). Reef restoration attempts might benefit from considerations of this kind.

Surveys of symbiont diversity and its distribution within and between different species of scleractinian coral are critical to our growing appreciation of polymorphic symbiosis on coral reefs. Discovering whether symbiotic flexibility is an important factor determining: (1) coral abundance, zonation and depth range on reefs; (2) resistance of corals to periods of environmental stress; and (3) biogeographic patterns of coral species distribution, are major objectives in ecological studies of coral-algal symbiosis.

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