

# Intraspecific diversity and ecological zonation in coral–algal symbiosis

(zooxanthellae/reefs)

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**ABSTRACT** All reef-building corals are obligately associated with photosynthetic microalgal endosymbionts called zooxanthellae. Zooxanthella taxonomy has emphasized differences between species of hosts, but the possibility of ecologically significant zooxanthella diversity within hosts has been the subject of speculation for decades. Analysis of two dominant Caribbean corals showed that each associates with three taxa of zooxanthellae that exhibit zonation with depth—the primary environmental gradient for light-dependent marine organisms. Some colonies apparently host two taxa of symbionts in proportions that can vary across the colony. This common occurrence of polymorphic, habitat-specific symbioses challenges conventional understanding of the units of biodiversity but also illuminates many distinctive aspects of marine animal–algal associations. Habitat specificity provides ecological explanations for the previously documented poor concordance between host and symbiont phylogenies and the otherwise surprising lack of direct, maternal transmission of symbionts in many species of hosts. Polymorphic symbioses may underlie the conspicuous and enigmatic variability characteristic of responses to environmental stress (e.g., coral “bleaching”) and contribute importantly to the phenomenon of photoadaptation.

*Montastraea annularis* sensu lato is the predominant reef-building coral of the Caribbean Sea (1). In shallow to intermediate depths, it consists of three “sibling” species that are morphologically and genetically distinct (2): *M. annularis* (Ellis and Solander, 1786) sensu stricto plus the recently resurrected *M. faveolata* (Ellis and Solander, 1786) and *M. franksi* (Gregory, 1895) (3). Like all other reef-building corals, these species are obligately associated with symbiotic dinoflagellates that, as far as is known, belong to the genus *Symbiodinium* (4). Diversity among these microalgae (4–7), commonly referred to as zooxanthellae, can be recognized in some instances by restriction fragment length polymorphism (RFLP) in small ribosomal subunit RNA (ssRNA) genes (8, 9). We used this method to assess zooxanthella diversity in these corals on Panamanian reefs at depths of 0–14 m.<sup>¶</sup>

## MATERIALS AND METHODS

Samples were collected from apparently healthy coral colonies at Salar-1 (June 1992) and Aguadargana (April 1992 and January 1993) reefs (3) in San Blas, Panama. These two sites are protected and semi-exposed, respectively. At the depths sampled, they consist of mixed species assemblages in which the three sibling species overlap in distribution and are easily identified by their characteristic colony morphologies (2, 3). Sampled colonies were in open, unshaded areas; they were otherwise selected haphazardly across the sampled depth

range, without regard to colony color. Conspecific samples were taken from colonies separated by at least 5 m. Samples were frozen on dry ice and stored at  $-70^{\circ}\text{C}$ . After thawing, zooxanthella DNA was isolated (9) from 5–10 cm<sup>2</sup> of tissue; MgSO<sub>4</sub> was omitted from the zooxanthella isolation buffer.

For most samples from *M. annularis* (data reported in Figs. 1a and 3 and in Table 1), nuclear ssRNA genes were amplified with “universal” PCR primers ss5 and ss3 in 30- to 50- $\mu\text{l}$  reaction mixtures (9) by using 30 cycles of the profile  $94^{\circ}\text{C}$  (45 s),  $56^{\circ}\text{C}$  (45 s),  $72^{\circ}\text{C}$  (2 min; 8 min on the last cycle). These conditions should amplify essentially the entire ssRNA-encoding sequence from most (perhaps all) eukaryotic nuclei. Samples from *M. faveolata* and *M. franksi* were analyzed similarly, except that phylogenetically biased (“zooxanthella-specific”) PCR primers ss5Z and ss3Z (9) were also used, to avoid host DNA that contaminated many of these samples (see ref. 9). Phylogenetically biased primers were also used for 11 colonies of *M. annularis* from which multiple samples were analyzed. Sequence data from 11 zooxanthella ssRNA genes obtained from *M. annularis* with “universal” primers (those reported in Table 1), from one *M. annularis* ssRNA gene (unpublished data), and from other zooxanthella and cnidarian ssRNA genes (9) indicate that ss5Z and ss3Z will amplify *Symbiodinium*-like, but not cnidarian, genes (note that ss5Z and ss3Z are “nested” relative to ss5 and ss3). The primers ss5Z and ss3Z were used together and, to reduce any bias against “unknown” zooxanthella ssRNA genes, in combination with ss3 and ss5 (see ref. 9); independent analyses of every sample with all primer combinations (ss5Z plus ss3Z, ss5 plus ss3Z, and ss5Z plus ss3) were consistent.

Amplified DNAs were digested with *Taq* I and with *Dpn* II [New England Biolabs; *Dpn* II restriction endonuclease is an isoschizomer of *Sau*3AI, which was used previously (9)] and electrophoresed in 2.5% NuSieve/1% SeaKem agarose (FMC) gels stained with ethidium bromide. As before (8), small PCR products of unknown origin (10) occurred (reproducibly) in a few samples but did not obscure RFLP genotypes. RFLP genotypes of cloned ssRNA genes (below) were run as standards. They were obtained as above, by using  $\approx 0.1$  ng of purified bacteriophage DNA as PCR template. For samples presumed to contain two genotypes, their relative abundance was estimated by proxy from the relative abundance of the two types of ssRNA genes. This was determined by visual comparison with standards that were obtained by mixing two cloned ssRNA genes in molar ratios ranging from 1:8 to 8:1 (in 2-fold steps) prior to PCR amplification.

For sequencing, zooxanthella ssRNA genes were amplified from 10 corals (*M. annularis*) that exhibited only one zooxan-

Abbreviations: RFLP, restriction fragment length polymorphism; ssRNA, small ribosomal subunit RNA.

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<sup>¶</sup>The sequences reported in this paper have been deposited in the GenBank database (accession nos. U20952–U20962.)

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thella RFLP genotype (*A*, *B*, or *C*) and from 1 coral that exhibited genotype *C\** (a mixture of type *C* and *D* genes; see Fig. 1*a*), by using "universal" PCR primers (see above), and cloned into the vector M13mp18 (9). Clone genotypes were verified by *Taq* I digestion (see above). One clone from each coral was sequenced in two regions, including the variable domains V2 and V4 (11), by using conserved primers. These cloned genes also provided RFLP standards (see above). To confirm the presence of two ssRNA genotypes in some samples, zooxanthella ssRNA genes were amplified and cloned, and the genotypes of 18 independent clones from each sample were determined (all as above) by using about 0.1% of one bacteriophage plaque as a PCR template.

## RESULTS

We observed four zooxanthella genotypes that, as explained below, imply that conspecific *Montastraea* colonies associate with three different species of symbionts, either one or two at a time.

Zooxanthellae from both *M. annularis* (Fig. 1*a*) and *M. faveolata* (Fig. 1*b*) included three familiar (8, 9) RFLP genotypes designated *A*–*C* (lanes 1–3, respectively). *M. franksi* samples contained only genotype *C* (data not shown), but this host was not widely sampled, due to its rarity in shallow water (2, 3, 12). Comparisons to cloned ssRNA genes showed that each of these RFLPs is explained by one ssRNA gene, whereas a fourth (uncommon) genotype *C\** (Fig. 1*a*, lane 4) represents two distinct genes, *C* and *D* (Fig. 1*a*, lanes 7 and 8). Substantial sequence differences among genotypes *A*–*C* (Table 1; in contrast, *C* and *D* from *C\** differ by only four nucleotides) justify the conclusion that they identify three distinct taxa of dinoflagellates (8, 9). Given the presumed specificity of scleractinian corals for *Symbiodinium* (4) and the sequence similarities to *Symbiodinium* ssRNA genes from morphologically characterized, cultured material (8, 9) (Table 1), these can be regarded as three species [or groups of related species (13)] of *Symbiodinium*.

Many samples exhibited apparent mixtures of two RFLP genotypes, as evidenced by comparison to the RFLPs obtained by mixing two cloned ssRNA genes. Genotypes *B* and *C* commonly occurred together in *M. annularis* (33% of samples;

Table 1. Sequence differences among zooxanthella ssRNA genes

Zooxanthellae from other hosts	Zooxanthellae from <i>M. annularis</i>			
	A	B	C	D
<i>S. microadriaticum</i>	0	31–34	28–31	32
<i>Symbiodinium</i> sp. 8	29	3–5	14–17	18
Consensus <i>C</i>	26	13–15	2–5	6

Partial sequences (474 nucleotide positions) from *M. annularis* zooxanthellae of genotypes *A* ( $n = 2$ ), *B* ( $n = 4$ ), *C* ( $n = 4$ ), and *D* ( $n = 1$ ) were aligned with homologous sequences from cultured *Symbiodinium microadriaticum* and *Symbiodinium* sp. 8 [previously shown to represent genotypes *A* and *B*, respectively (13)] and with a consensus genotype *C* sequence that represents uncultured zooxanthellae from nine other host species (8). The numbers (or ranges) of observed nucleotide substitutions in these alignments are given, with a 2-nucleotide deletion in type *A* genes scored as a single difference.

examples in Fig. 1*a*, lanes 9–11), and *A* and *C* commonly occurred together in *M. faveolata* (27% of samples; examples in Fig. 1*b*, lanes 7–10). Mixtures ranged from mostly *B* or *A* to mostly *C*, as judged by visual comparisons with standards (Fig. 1*a*, lanes 12–14; Fig. 1*b*, lanes 11–13). Several observations argue against the interpretation that these more complex RFLPs are simply the result of incomplete digestions of one genotype. Patterns were always reproducible, qualitatively and quantitatively, in repeated analyses (data not shown); analyses of such samples with different restriction enzymes were consistent, qualitatively and quantitatively (Fig. 1); RFLP analyses of individual PCR products (obtained by cloning) directly confirmed the presence of both genotypes in all of six samples tested (three each of *B/C* and *A/C*; data not shown).

The presence of two zooxanthella genotypes in one sample could represent two taxa of zooxanthellae within one host (14) or one species of dinoflagellate with two different ssRNA genes (15). For mixtures of genotypes *B* and *C* and of genotypes *A* and *C*, we favor the former interpretation because each genotype was also found in isolation in these and other (8) host species and because ssRNA sequence dissimilarities (Table 1) imply substantial phylogenetic divergence between them (8, 13). Using the same reasoning, we do not yet suggest that the two genes of genotype *C\** (*C* and *D*; Fig. 1*a*; Table 1) represent

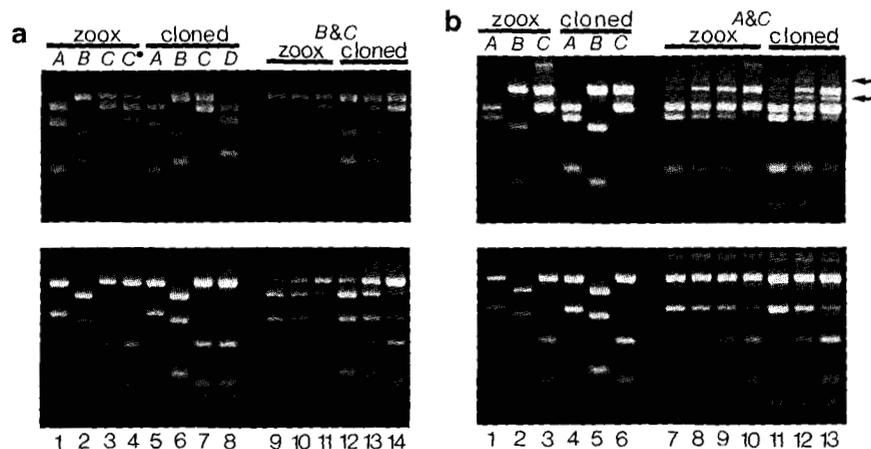


FIG. 1. RFLP genotypes of zooxanthella samples (zoox) from different corals and of cloned ssRNA genes (cloned), determined by *Taq* I (Upper) and *Dpn* II (Lower) digestions of the same preparation. (a) Zooxanthellae and clones from *M. annularis*. Lanes 1–4, genotypes *A*, *B*, *C*, and *C\**, respectively; lanes 5–7, genotypes *A*, *B*, and *C*, respectively, from cloned ssRNA genes, to show that one gene explains these genotypes; lane 8, genotype *D*, cloned from zooxanthella genotype *C\** (both type *C* and type *D* clones were recovered from genotype *C\**); lanes 9–11, zooxanthella samples that appear to contain both genotypes *B* and *C*; lanes 12–14, genotypes from mixing cloned ssRNA genes in molar ratios (type *B*/type *C*) of 8:1, 1:1, and 1:8, respectively, for comparison with zooxanthella data in lanes 9–11. (b) Zooxanthellae from *M. faveolata* compared with clones from *M. annularis*. Lanes 1–3, genotypes *A*, *B*, and *C*, respectively; lanes 4–6, genotypes *A*, *B*, and *C*, respectively, from cloned ssRNA genes; lanes 7–10, zooxanthella samples that appear to contain both genotypes *A* and *C*; lanes 11–13, genotypes from mixing cloned ssRNA genes in molar ratios (type *A*/type *C*) of 8:1, 1:1, and 1:8, respectively, for comparison with zooxanthella data in lanes 7–10. "Extra" bands in these mixtures (e.g., arrows), which are not apparent when type *A* and *C* genes are amplified separately, are probably digestion-resistant, heteroduplex PCR products.

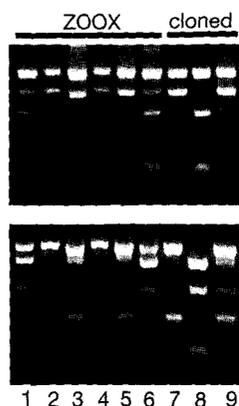


FIG. 2. RFLP genotypes of zooxanthellae (ZOOX) from six samples (lanes 1–6) collected from one (apparent) colony of *M. annularis*, compared with cloned ssRNA genes (cloned) of type C (lane 7), type B (lane 8), and a mixture (8:1) of types C and B (lane 9). The same preparations were digested with *Taq* I (Upper) and with *Dpn* II (Lower). All samples contained genotype C; genotype B is not convincingly detected (lanes 2 and 4), present in lesser amounts (lanes 1, 3, and 5), or predominant (lane 6). Four other corals (data not shown) exhibited a similar mosaic pattern, whereas only genotype C was detected in six others. All colonies came from depths of 8–10 m. Each coral was sampled at six locations, roughly equidistant along the colony perimeter.

different symbionts [data from two *Pocillopora* species from Hawaii present an analogous situation (8, 9)].

The ability of a single species of coral to host more than one type of zooxanthella suggested that symbiont populations

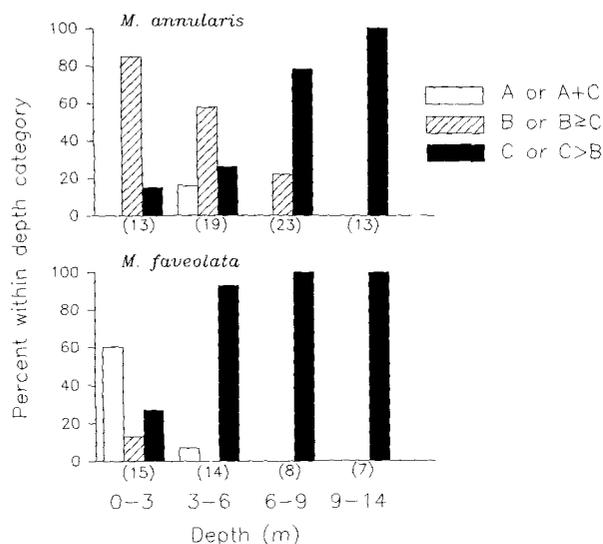


FIG. 3. Relative occurrences of different zooxanthella genotypes in single samples from different colonies of *M. annularis* and *M. faveolata* at different depths. Samples were scored as containing genotype A or A plus C (open bars), B or B with an equal or lesser amount of C (hatched bars), or C or more C than B (solid bars). These three categories show significant differences in depth distribution (0–6 versus 6–14 m) and, for shallow water (0–6 m), in host distribution ( $P$  value < 0.001,  $\chi^2$  test). In very shallow water (0–3 m), symbioses dominated by genotype C (C, or more C than A or B) were over twice as common in *M. faveolata* as in *M. annularis* (but the difference was not significant;  $P > 0.1$ ,  $\chi^2$  test). Samples containing both genotypes A and B were not observed. *M. franksi* (data not shown) is uncommon in shallow water (2, 3, 12); all 16 samples collected at 6–11 m contained only zooxanthella genotype C. Genotype C\* was observed only twice (both from *M. annularis*), and these samples are included with genotype C. The numbers of samples (corals) analyzed in each of four depth ranges are given in parentheses; collections from the two reefs exhibited the same trends and were pooled.

might also vary within individual coral colonies. *M. annularis* exhibited such intracolony variation in 5 of 11 colonies for which multiple samples were analyzed (Fig. 2). However mixtures of zooxanthella genotypes are interpreted (see above), it follows that zooxanthellae were not genetically uniform within these coral colonies.

In both *M. annularis* and *M. faveolata*, the three taxa of zooxanthellae exhibited conspicuous differences in distribution by depth (Fig. 3). Corals containing genotypes A and B were dominant only at depths less than 6 m, while below 9 m only C was found. Also, the occurrence of the three symbiont genotypes differed significantly between *M. annularis* and *M. faveolata* in shallow water (Fig. 3). These patterns stand regardless of the taxonomic status of the different corals or algal genotypes.

## DISCUSSION

Zooxanthella taxonomy has emphasized differences between, rather than within, species of hosts (4–7). Our ecological study revealed host-species differences (Fig. 3) that are consistent with this view, but also confirmed longstanding speculations (16–18) that a population of conspecific hosts may associate with several taxa of *Symbiodinium*. A discussion of these results should begin by placing them in the context of related work.

Using the same methods as this study, previous surveys recognized the same zooxanthella genotypes A–C but never found evidence of a single host species containing more than one of these major types (8, 9). Are *Montastraea*, then, “atypical” in associating with several species of algae? For several reasons, this question remains open. (i) As noted (8, 9), an analysis of ssRNA genes, especially by RFLPs, will not resolve closely related taxa of zooxanthellae; observing only one genotype in a host species is not good evidence that the host associates with only one species of symbiont. (ii) Smaller sampling efforts in previous studies would have decreased the chances of detecting polymorphisms. In particular, the restricted distributions of some hosts made bathymetric comparisons difficult or impossible. (iii) Of the 17 host species for which five or more individuals were previously sampled (8, 9), only the 6 Caribbean species (5 of which were collected only in shallow water) represent a locale where more than one zooxanthella RFLP genotype occurs commonly (unpublished observations). In summary, given the limitations of zooxanthella taxonomy and the paucity of relevant ecological data, the polymorphism of *Montastraea* symbioses cannot yet be regarded as either typical or atypical of dinoflagellate–invertebrate symbioses. For now, these corals provide a paradigm for a possibly common phenomenon that merits consideration, particularly given the ecological importance of these corals and their extensive use as model systems in many areas of coral biology.

The ability of corals to exist in symbiosis with several species of zooxanthellae would create a variety of forms that greatly transcends the number of one host–one symbiont combinations, challenging the conventional focus on the coral animal species as the fundamental unit of ecological diversity (19). Zonation by depth (Fig. 3) strongly supports the theory that hosting different types of zooxanthellae permits corals to acclimate or adapt to different photic habitats (4, 16–18) and suggests an ecological explanation for the previously documented poor congruence between host and symbiont phylogenies (6, 8). Our findings also establish empirical precedents for suggestions that intraspecific and intracolony variability in the stress-mediated disruption of coral–algal symbioses [coral “bleaching” (20, 21)] is a manifestation of zooxanthella diversity (22–24) and that bleaching could promote adaptive changes in coral–zooxanthella associations (8, 23).

Several processes could promote polymorphism in coral–zooxanthella symbioses. The otherwise surprising (25) absence of direct maternal transmission of symbionts in many hosts (5),

including *M. annularis* (26), could allow widely dispersed juveniles to select locally optimal symbionts (4, 16). Alternatively, different host-symbiont combinations may be under intrinsic (genetic or epigenetic) control, with ecological patterns arising from correlated larval behaviors (27, 28) and/or natural selection after settlement (4). Established symbioses might respond to environmental change by switching partners (23, 29). Polymorphisms in *Montastraea* symbioses offer an opportunity to test such hypotheses.

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