J. Phycol. **37,** 900–912 (2001)

GENETIC COMPARISONS OF FRESHLY ISOLATED VERSUS CULTURED SYMBIOTIC DINOFLAGELLATES: IMPLICATIONS FOR EXTRAPOLATING TO THE INTACT SYMBIOSIS[1]

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Zooxanthellae, algal symbionts in divergent marine invertebrate hosts, are a genetically heterogeneous group. All species descriptions and most physiological and infectivity studies of zooxanthellae have been conducted using cultured material. However, few studies have attempted to quantify the representation of cultures isolated from cnidarians to the *in hospite* zooxanthella populations of the individual host or host species from which they were established. RFLPs of small subunit (18S) rDNA, internal transcribed spacer (ITS)-rDNA sequence data, and microsatellite analyses were conducted to assess the relatedness between cultured zooxanthellae and the *in hospite* populations of the individual host or host species from which they were isolated. RFLP data demonstrated that cultures may represent either the numerically dominant symbiont or ones present in lower number. ITS-rDNA sequences from zooxanthella cultures were disconcordant with ITS-rDNA sequences identified from *in hospite* zooxanthellae of the same host species, and microsatellites present in *in hospite* zooxanthella populations were absent from the corresponding cultures. Finally, reexamination of the literature revealed examples of zooxanthella cultures being nonrepresentative of *in hospite* populations. These data suggest that, in most cases, cultures are a subset of the original *in hospite* population. Factors such as failing to homogenize bulk cultures before transfer, growth medium used, and the picking of single motile cells may contribute to many zooxanthella cultures being nonrepresentative.

Key index words: **culture; internal transcribed spacer; microsatellites, small subunit rDNA genes; *Symbiodinium*; symbiosis; symbiotic dinoflagellates; zooxanthellae**

Abbreviations: **ITS, internal transcribed spacer**

Associations between invertebrates and symbiotic dinoflagellates, commonly referred to as zooxanthellae, are taxonomically widespread in marine environments. These associations involve various and numerous host species, including foraminiferans, sponges, scleractinian corals, sea anemones, octocorals, zoanthids, hydrocorals, and mollusks (Glynn 1996). Zooxanthellae have been found to play a significant role in their host's nutrition and physiology. In some associations, enough photosynthetically fixed carbon may be translocated to the host to meet their respiratory demands (Falkowski et al. 1984, Muscatine et al. 1984), and the presence of zooxanthellae may also facilitate the assimilation of dissolved inorganic nitrogen (e.g. Kawaguti 1953, Burris 1983, Ambariyanto and Hoegh-Guldberg 1996) and conservation of nitrogen (Lewis and Smith 1971) by the host. In scleractinian corals, calcification rate is also influenced by the presence of zooxanthellae (Pearse and Muscatine 1971, Barnes and Chalker 1990).

It was once thought that all invertebrates harbored a single species of symbiotic dinoflagellate, *Symbiodinium microadriaticum* Freudenthal (Taylor 1974). However, infectivity, ultrastructural, behavioral, and molecular studies on cultured and freshly isolated and *in hospite* algae have demonstrated that zooxanthellae comprise a heterogeneous group of many strains and species (Kinzie and Chee 1979, Schoenberg and Trench 1980a,b,c, Fitt et al. 1981, Chang and Trench 1982, Fitt and Trench 1983, Blank and Trench 1985, Trench 1987, Trench and Blank 1987, Blank et al. 1988, Rowan 1991, Rowan and Powers 1991a,b, Rowan and Powers 1992, Sadler et al. 1992, Banaszak et al. 1993, McNally et al. 1994, Crafts and Tuliszewski 1995, Rowan and Knowlton 1995, Rowan et al. 1996, Baker et al. 1997, Baillie et al. 1998, Hill and Wilcox 1998, Loh et al. 1998, Rowan 1998, Baker 1999, Belda-Baillie et al. 1999, Carlos et al. 1999, 2000, Baillie et al. 2000a,b, Banaszak et al. 2000, Darius et al. 2000, LaJeunesse and Trench 2000, Kinzie et al. 2001). Trench (1993) listed 10 species of *Symbiodinium* and 14 other species of symbiotic dinoflagellate isolated from a range of hosts. All these species descriptions and most physiological and infectivity studies have been conducted using cultured material. For example, Schoenberg and Trench (1980a,b,c) were among the first to use cultured zooxanthellae from a variety of host species to establish that differences in *Symbiodinium* existed. In most studies, it was assumed that the cultured material represented the numerically dominant symbiont harbored by the individual host or host species from which it was isolated. However, attempts to verify this have only been conducted in a few studies (Schoenberg and Trench 1980a, Stochaj and Grossman 1997, Carlos et al. 2000).

In many cases, cultured zooxanthellae are isolated from a single individual or small number of individuals of a particular host species. This is problematic because different individuals of the same host species can harbor distinctly different symbionts (Rowan and Knowlton 1995, Baker and Rowan 1997, Baker et al. 1997, Goulet and Coffroth 1997, Rowan et al. 1997, Darius et al. 1998, Baker 1999, Belda-Baillie et al. 1999,

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Carlos et al. 1999, 2000, Goulet 1999, Baillie et al. 2000a,b, Darius et al. 2000, LaJeunesse and Trench 2000, Coffroth et al. 2001), and in some cases different zooxanthellae may be present in specific regions of an individual host (Rowan and Knowlton 1995, Rowan et al. 1997, Baker 1999). Additionally, microorganismal culturing is highly selective (Wintzingerode et al. 1997), resulting in the proliferation of some lineages at the expense of others. It has been recognized that culture conditions may select one genotype of *Symbiodinium* over another (Rowan 1998). In mollusks that harbor zooxanthellae, some of the numerically dominant symbionts of a host are unculturable, whereas less numerous ones proliferate (Rowan et al. 1996, Carlos et al. 2000). However, no studies have quantified the relationship between cultures and *in hospite* zooxanthella populations from a variety of cnidarian hosts using DNA techniques. The purpose of this study was to quantify this relationship using several techniques that measure different levels of genetic diversity. In many cases, zooxanthella cultures isolated from cnidarian hosts were a subset of the original *in hospite* population. These data have important implications for past, present, and future use of zooxanthella cultures and the extrapolation of *in vitro* results to the intact symbiotic system.

MATERIALS AND METHODS

Florida Aiptasia pallida *zooxanthella cultures versus* in hospite *populations: RFLP analysis of small subunit (18S) rDNA.* Zooxanthella cultures were established from four *Aiptasia pallida* anemones, obtained from Long Key, Florida (Table 1). Oral disks were severed from each anemone, placed in 0.22 μm filtered seawater, and cut into two equal pieces. One piece was preserved in 95% ethanol for later molecular analysis. The other half was macerated in f/2 medium (Guillard and Ryther 1962) using a tissue grinder and filtered through nylon meshes (125, 74, and 20 μm) to remove larger animal debris and mucus. Zooxanthellae were collected and washed several times in f/2 medium by centrifugation at 700*g* for 5 min. Zooxanthellae were brought into bulk culture by inoculating several drops of a dense suspension into 75 mL of f/2 media. Cultures were maintained at a constant temperature of 29° C, irradiance level of 80 μmol photons$\cdot m^{-2} \cdot s^{-1}$, and photoperiod of 12:12-h light:dark. To prevent prokaryotic organisms from overrunning the newly established cultures, the cultures were split into two sets. One set received antibiotic treatment (Polne-Fuller 1991), whereas the other set received no treatment. This split resulted in a total of eight cultures. Before culture transfers, including the split into two sets, cells were suspended using a Pasteur pipette and 10 μL transferred to 75 mL of fresh f/2 medium. Transfers were conducted on a monthly basis. After transfers, cells from each month-old culture were preserved in 95% ethanol. Total nucleic acids from cultures and intact host tissue, which contained the original *in hospite* population, were extracted and quantified in 0.7% tris-borate (TBE) agarose gels according to the methods of Coffroth et al. (1992). Zooxanthella small subunit (18S) rDNA was amplified by PCR using the primers ss5 (5′-GGT TGATCCTGCCAGTAGTCATATGCTTG-3′) and ss3z (5′-AGCA CTGCGTCAGTCCGAATAATTCACCGG-3′) according to Rowan and Powers (1991b). PCR products were digested with *Taq* I restriction enzyme to generate RFLPs (Rowan and Powers 1991b). RFLP analysis of 18S-rDNA PCR products separated zooxanthellae into several large clades (i.e. *Symbiodinium* clades A, B, C [Rowan and Powers 1991a,b], and E [R. Rowan, University of Guam, personal communication; Genbank accessions AF238261 and AF238262]), with each clade probably comprised of many species (Rowan 1998). Digestion products were separated on 2% TBE agarose gels.

The presence of low concentrations of *Symbiodinium* clade B was determined with the *Symbiodinium* clade B-biased 18S-rDNA primers, BSPECUP and BSPECDN (Coffroth, Santos, and Goulet, unpublished data; sequence available upon request), used under the following reaction conditions: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 0.3 μM of each primer, 1 U *Taq* polymerase, and 15–30 ng of template DNA in a total volume of 25 μL. PCR cycling conditions were as follows: initial denaturing period of 2 min at 94° C followed by 29 cycles of 94° C for 1 min, 59° C for 1 min, and 72° C for 2 min. A final extension cycle of 5 min was also added. Reactions were carried out in a PTC-100 thermocycler (MJ Research Inc., Watertown, MA). PCR products generated with the primer set BSPECUP/BSPECDN were also digested with *Taq* I and separated as above.

Quantification of the relative abundance of *Symbiodinium* clades A and B was determined by RFLPs of PCR products generated from a dilution series of synthetic mixtures of *Symbiodinium* clade A and *Symbiodinium* clade B DNA (Rowan et al. 1997). Culture RFLPs were visually compared with these standards to estimate zooxanthella cell number at the start of the experimental period. Comparisons between previous results from the same culture allowed each culture's pedigree to be followed over the 1-year experimental period.

Octocoral zooxanthella cultures versus in hospite *populations: internal transcribed spacer (ITS)-rDNA sequences and analyses.* The relatedness between cultured zooxanthellae and *in hospite* populations of the host species from which they originated was determined by establishing cultures from the Caribbean gorgonians *Plexaura kuna*, *P. flexuosa*, and *Pseudopterogorgia elisabethae* and the Indo-Pacific soft coral *Sinularia* sp. (Table 1) in the same manner as above except for no antibiotic treatment. Total nucleic acids were extracted and quantified from cultures, as well as *in hospite* populations, as described above. Samples were screened by 18S-rDNA RFLP analysis to determine clade affinity and PCR-template quality. The ribosomal ITS-rDNA region was PCR amplified using the primers ZITSUPM13 (5′-CACGACG TTGTAAAACGACCCGGTGAATTATTCGGACTGACGCAGT GCT-3′) and ZITSDNM13 (5′-GGATAACAATTTCACACAGGCT GTTTAGTTCCTTTTCCTCCGC-3′), designed by the first author for conserved regions of the 3′ and 5′ ends of the zooxanthellar 18S and 28S-rDNA genes, respectively. Amplifications were performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 0.3 μM of each primer, 1 U *Taq* polymerase, and 15–30 ng of template DNA in a total volume of 25 μL. Reactions were carried out in a PTC-100 thermocycler (MJ Research Inc.). PCR conditions were as follows: initial denaturing period of 2 min at 94° C, 35 cycles consisting of 94° C for 30 s, 60° C for 30 s, and 72° C for 45 s, and a final extension period of 7 min. PCR products were polyethylene glycol precipitated (Glenn et al. 1999) and 2% agarose gel purified in 1× modified Tris-acetate-EDTA buffer (0.04 M Tris-acetate, 0.0001 M EDTA final concentration). After purification, DNA samples were quantified on 2% TBE agarose gels.

The first 19 and 20 5′-nucleotides of ZITSUPM13 and ZITSDNM13, respectively, allowed DNA nucleotide sequencing of the approximately 750-base pair (bp) PCR products using 5′-IRD800 fluorescent-labeled M13 primers (LI-COR Biotechnology Division, Lincoln, NE). Sequencing reactions were performed with the SequiTherm EXCEL™ II DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI) according to the supplier's recommendations. Cycle sequencing products were sequenced completely in both directions with a LI-COR Gene ReadIR™ 4200 automatic DNA sequencer using 5.5% Long Ranger acrylamide (FMC Bioproducts, Rockland, ME) gels.

Forward and reverse ITS-rDNA sequences for each sample were aligned using Sequencher™ 3.0.1 (Gene Codes Corp, Ann Arbor, MI) and ambiguities corrected by comparison with the reverse sequence or coded using the IUPAC ambiguity codes. Consensus sequences of 657 bp were aligned using ClustalX (Thompson et al. 1997), and phylogenetic trees were obtained with PAUP*4.0 (Swofford 2000). The ITS-rDNA sequence of the symbiotic dinoflagellate cultured from the scler-

TABLE 1. List of host organisms, culture names, collection locations, and zooxanthella clades included in this study.

Host name	Corresponding culture(s) name(s)	Collection location	Zooxanthella clade(s)[a]
Aiptasia pallida individual 1	FLAp#1, FLAp#1 10AB	Long Key, Florida	A + B
Aiptasia pallida individual 2	FLAp#2, FLAp#2 10AB	Long Key, Florida	A + B
Aiptasia pallida individual 3	FLAp#3, FLAp#3 10AB	Long Key, Florida	A + B
Aiptasia pallida individual 4	FLAp#4, FLAp#4 10AB	Long Key, Florida	A + B
Plexaura kuna	Pk13	Northern Florida Keys	B
P. kuna[b]	Pk702	San Blas Islands, Republic of Panama	B
P. kuna[b]	Pk704	San Blas Islands, Republic of Panama	B
P. kuna[b]	Pk706	San Blas Islands, Republic of Panama	B
Plexaura flexuosa	PurPflex	Tennessee Reef, Florida Keys	B
Pseudopterogorgia elisabethae	SSPe	San Salvador, Bahamas	B
Sinularia sp.	Sin	Guam, Indo-Pacific	C

[a] Clade designation based on 18S-rDNA RFLP (Rowan and Powers 1991b).
[b] Cultures for which there are no corresponding *in hospite* samples.

actinian coral *Montipora verrucosa* (Hawaii) was used as the outgroup. The symbiont cultured from *M. verrucosa* is described as *Symbiodinium kawagutii* (Trench and Blank 1987) and belongs to *Symbiodinium* clade C (Carlos et al. 1999, Banaszak et al. 2000, S. R. Santos, personal observation). Gaps were treated as a fifth base and weighted by length. For example, a gap of four nucleotides was assigned values of 0.25 for each of the four positions. To determine the best-fit model of DNA evolution, the PAUP*4.0 Nexus file was run in Modeltest v3.0 (Posada and Crandall 1998) before calculating sequence distance values. ITS-rDNA sequences were deposited in GenBank under accession numbers AF360550–AF360564 and AF360567–AF360578.

The null hypothesis that zooxanthella cultures were most closely related to the *in hospite* populations of the host species from which they originated was tested by using a constraint tree constructed in PAUP*4.0. In three cases, direct comparisons were made between cultures and *in hospite* zooxanthella populations from the same host individual (Fig. 1). Sequences obtained from the *in hospite* zooxanthella populations of several additional Caribbean gorgonian species were also included in the analysis to increase sample diversity. Maximum parsimony trees, with and without constraints, were constructed using the heuristic search option. When multiple best-fit trees were found, these trees were used to construct a single consensus tree. Statistical testing between trees was done using a Templeton's (Wilcoxon signed-ranks) test in PAUP*4.0. The PAUP*4.0 Nexus file, which contains the aligned sequences used in the analysis and the constraint tree, was deposited in TreeBASE (http://www.herbaria.harvard.edu/treebase/index.html) under study accession number S586 and matrix accession number M883.

Caribbean gorgonian zooxanthella cultures versus in hospite *populations: microsatellite amplifications.* The zooxanthella cultures, as well as the *in hospite* populations, from *Plexaura flexuosa* and *Pseudopterogorgia elisabethae* (Table 1) were further screened with microsatellite primer pairs (Table 2) designed for the *in hospite* zooxanthella populations of adult *Plexaura kuna* colonies. Freshly isolated zooxanthellae from *P. kuna* colonies, collected from around the Caribbean, were used in constructing the microsatellite library. Briefly, the microsatellite library was constructed in the following manner. Host tissue was gently macerated in zooxanthellae isolation buffer (ZB) (Rowan and Powers 1991b) and zooxanthellae collected by centrifugation at 700*g* for 5 min. Zooxanthella cells were washed several times in ZB and collected by centrifugation as above. To further separate host materials and other contaminates from the zooxanthella cells, zooxanthellae were centrifuged through a Percoll gradient (Stochaj and Grossman 1997) and washed several additional times in ZB. Light microscopy examination of the zooxanthella cells after centrifugation and washing revealed an absence of contamination from the freshly isolated zooxanthellae. Zooxanthella DNA was extracted according to the methods of Coffroth et al. (1992). This DNA was used to construct the microsatellite library following the enrichment protocol of Ciofi and Bruford (1998).

Zooxanthella culture and *in hospite* DNA screened in the microsatellite analysis were extracted and quantified as described above. Microsatellite amplifications were performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 0.3 μM of each primer, 1 U *Taq* polymerase, and 15–30 ng of template DNA in a total volume of 10 μL. Reactions were carried out in a PTC-100 thermocycler (MJ Research Inc.). After an initial denaturing period of 3 min at 94° C, 39 cycles of PCR were performed consisting of 94° C for 45 s, 60° C for 45 s, and 72° C for 30 s, with a final extension period of 5 min. PCR products were separated in 2% TBE agarose gels. Microsatellite data generated with the primer sets were treated in a nontraditional manner. Rather than examining size variation at a given locus and scoring allelic frequencies, microsatellite loci were used as molecular markers and alleles at the locus under amplification were scored as present or absent. To test for reproducibility, microsatellite amplifications were conducted three times per sample from independent DNA extractions of whole tissue or algal cells.

Zooxanthella cultures versus in hospite *populations: literature reexamination.* Total nucleic acids were extracted and quantified as described above from zooxanthella cultures established from the following organisms: *Mastigias* sp., *Pocillopora damicornis*, and *Tridacna gigas*. These cultures represent symbiotic dinoflagellates isolated from a jellyfish, scleractinian coral, and mollusk, respectively, and were kindly provided by Dr. Robert A. Kinzie III of the Hawaii Institute of Marine Biology. These isolates have been in culture for 2–10 years and were brought into bulk culture with zooxanthellae from a single host. Zooxanthella 18S-rDNA was amplified by PCR using the primers ss5/ss3z, and products were digested with *Taq* I and separated in 2% TBE agarose gels as described above. These data, along with other 18S-rDNA data presented here, were compared with results published in Banaszak et al. (2000). Comparisons were made between zooxanthella cultures isolated from similar or identical organisms by laboratories using different culturing methodologies. Additionally, a detailed reexamination of the literature over the last 60 years was conducted to assess factors that can contribute to the isolation of nonrepresentative zooxanthella cultures.

RESULTS AND DISCUSSION

Florida Aiptasia pallida *zooxanthella cultures versus* in hospite *populations: RFLP analysis of 18S-rDNA.* According to RFLPs of PCR products generated with the 18S-rDNA dinoflagellate-biased primers ss5/ss3z, the *in hospite* zooxanthellae of the four *Aiptasia pallida* anemones from Long Key, Florida appeared to be comprised largely of a *Symbiodinium* clade A RFLP pattern (Fig. 2a). However, a faint *Symbiodinium* clade B RFLP pattern was also detected from all four anemones. The presence of low con-

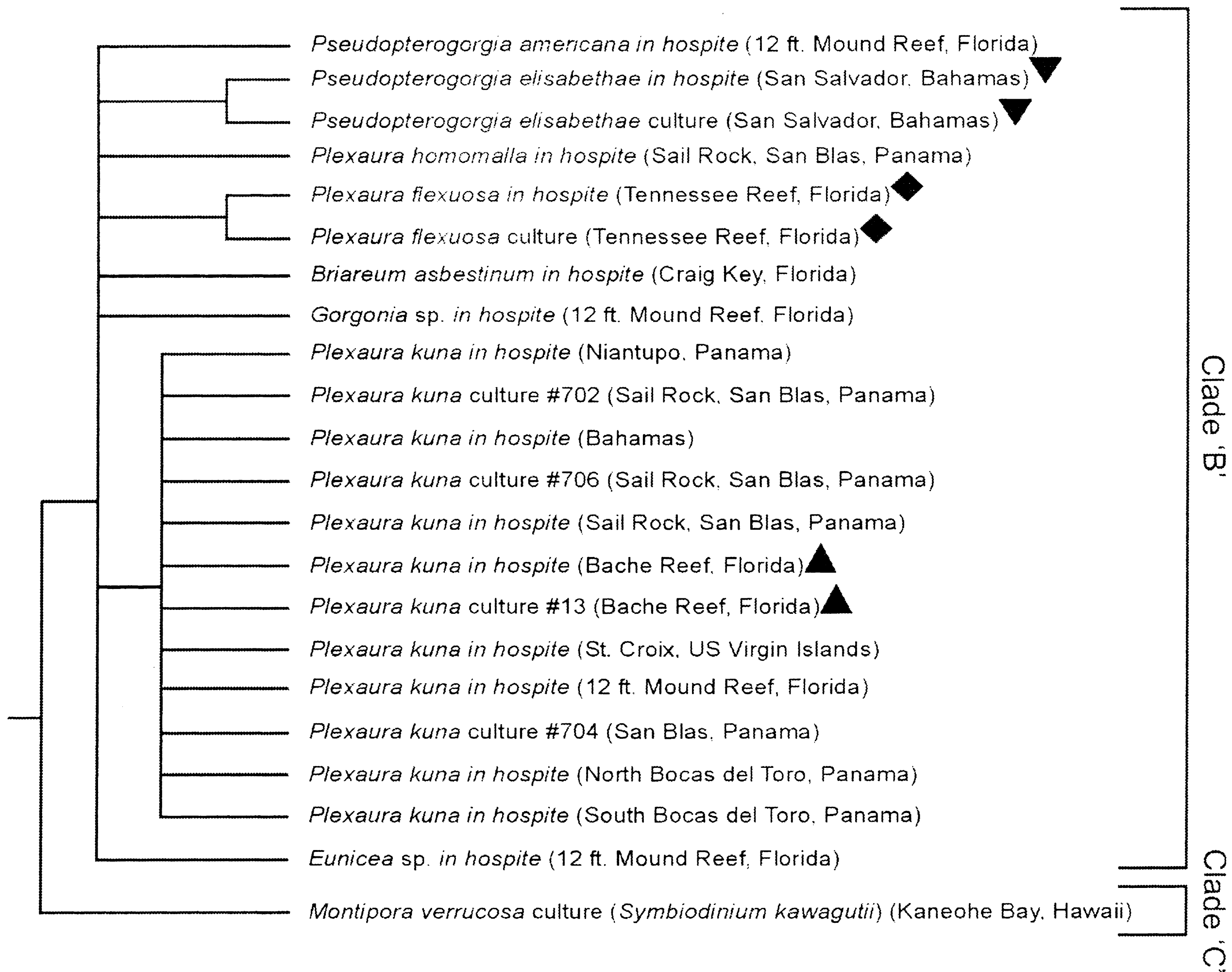

FIG. 1. PAUP*4.0-constructed constraint tree representing the null hypothesis that zooxanthellae cultures were most closely related to the *in hospite* populations of the Caribbean gorgonian host species from which they originated. Geographic origins of samples are stated in parentheses. Pairs of identical symbols following host name and geographic origin represent culture and *in hospite* zooxanthellae population from the same host individual.

centrations of *Symbiodinium* clade B was confirmed by RFLPs of the *Symbiodinium* clade B-biased 18S-rDNA primer (BSPECUP/BSPECDN) products (Fig. 2b). By comparing the RFLPs generated from these *Symbiodinium* clade B-biased 18S-rDNA products to amplifications and RFLPs from a dilution series using the same primers, it was estimated that *in hospite Symbiodinium* clade B were present at approximately 1 cell of *Symbiodinium* clade B per 1000 cells of *Symbiodinium* clade A (data not shown) at the start of culturing. Within a month of isolation, *Symbiodinium* clade B could easily be detected in most of the eight cultures started from *A. pallida* using the 18S-rDNA zooxanthellae-biased primers ss5/ss3z (Fig. 2c). This represents as much as a 10-fold increase in the number of *Symbiodinium* clade B cells relative to *Symbiodinium* clade A cells under culture conditions and compared with what was originally present in the host tissue. Additionally, the increase in *Symbiodinium* clade B relative to *Symbiodinium* clade A was not constant (Fig. 2c), demonstrating that individual cultures respond differently. After 1 year in culture, *Symbiodinium* clade B dominated four of the eight cultures, whereas *Symbiodinium* clade A dominated a single culture (Table 3). Surprisingly, three cultures remained as approximately equally mixed populations of *Symbiodinium* clades A and B over the 1-year experimental period (Table 3).

TABLE 2. Primer sequences and approximate allele size of dinucleotide microsatellites developed for *Plexaura kuna in hospite* zooxanthella populations.

Primer name	Primer nucleotide sequence	Allele size (bp)
GA2.8UP	5′-TCGACTCTTGGCGCAAAATG-3′	155
GA2.8DN	5′-GGGATGAAACACTGGGATAATCCAG-3′	
GA4.84UP	5′-GATCAAACTCTTGTGATGAC-3′	170
GA4.84DN	5′-GTCAGATTGTCATCAAAGACTGC-3′	
CA6.10UP	5′-GGTCCAAGCTGGAAGCAGC-3′	119
CA6.10DN	5′-CCTACAATAGACAGCAACCTAGGAC-3′	

"CA" or "GA" in primer name designates type of dinucleotide repeat.

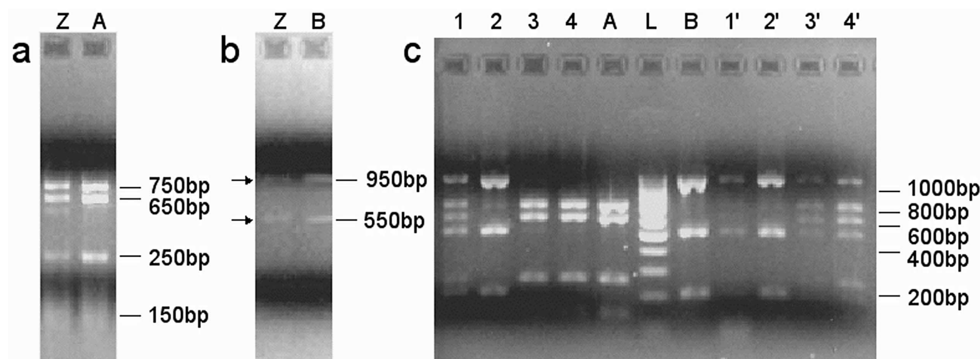

FIG. 2. 18S-rDNA RFLPs obtained from Florida *Aiptasia pallida* (FLAp) anemones. (a) Representative *Taq* I-generated RFLP from amplification with primers ss5 and ss3z (Rowan and Powers 1991b) of *in hospite* zooxanthellae. (b) Representative *Taq* I-generated RFLP from amplification with *Symbiodinium* clade B-biased primers BSPECUP and BSPECDN for same sample as a. Arrows indicate position of RFLP product. (c) *Taq* I-generated RFLPs from amplifications with primers ss5 and ss3z (Rowan and Powers 1991b) of *A. pallida* cultures, with and without antibiotic treatment, approximately 1 month following isolation. Z, *in hospite* zooxanthellae; A, *Symbiodinium* clade A RFLP standard; B, *Symbiodinium* clade B RFLP standard; L, 100-bp DNA size ladder; 1–4, FLAp cultures 1 to 4 without antibiotic treatment; 1′–4′, FLAp cultures 1 to 4 with antibiotic treatment. See text and Table 1 for additional details.

To test if *Symbiodinium* clades A and B segregated spatially, samples of cells from various areas of nonhomogenized mixed population flasks were collected. These areas included three spots 120 degrees apart from each other on the bottom of the flask and along the sides of the flask at the air–medium interface. This sampling revealed that *Symbiodinium* clades A and B inhabited different parts of the flask. *Symbiodinium* clade B appeared to prefer the air–medium interface, whereas spots of *Symbiodinium* clade A or mixes of *Symbiodinium* clades A and B were found on the bottom of the flask. This phenomenon was observed to occur in all three flasks that remained as mixed populations after 1 year (data not shown). The fact that zooxanthellae of different clades segregate themselves suggests one way in which cultures may become nonrepresentative. Cultures are typically transferred at monthly intervals by scrapping a small patch of cells from the bottom of one flask and passing it into new growth media. If the culture is a mixture of zooxanthella clades that has segregated spatially, the patch of cells that is transferred could result in a nonrepresentative culture via a founder effect.

One unexpected outcome of the antibiotic treatment of the Florida *A. pallida* zooxanthella cultures is the suggestion that this treatment may influence which zooxanthellae will dominate a culture. Of the four cultures that received antibiotic treatment, 75% were dominated by *Symbiodinium* clade B, which is in contrast to the nontreated cultures (25% being dominated by *Symbiodinium* clade B) (Table 3). Statistically, this result is not significant (chi square test of independence, $P > 0.1$). However, an effect of antibiotic treatment warrants mention. It has been observed that diatoms, which are eukaryotic organisms, can be eliminated from zooxanthella cultures during a 1-month antibiotic treatment (S. R. Santos, personal observation). The treatment used is a combination of 10 antibiotics developed for and used specifically in the production of axenic cultures of *Symbiodinium* (Polne-Fuller 1991). Polne-Fuller (1991) noted that the *Symbiodinium* cultures tested were resistant to the antibiotic treatment. This is consistent with observations that no zooxanthella cultures have been lost during or after treatment with this antibiotic cocktail (S. R. Santos, personal observation). However, given the observed effect on diatoms, it is possible that in mixed populations, some zooxanthella types may be adversely affected by this treatment. This may lead to their eventual loss from the culture without the recognition that they were originally present. Given these observations, antibiotic treatment may act as a selective force during *Symbiodinium* culturing. This possibility deserves further investigation.

TABLE 3. 18S-rDNA RFLP clade identification of Florida *Aiptasia pallida* zooxanthella cultures initially and 1 year after isolation.

	Initial	1 Year after isolation	
		No antibiotics	Antibiotic treatment
FLAp #1	A + (B)	A + B	A + B
FLAp #2	A + (B)	B	B
FLAp #3	A + (B)	A + B	B
FLAp #4	A + (B)	A	B

FLAp, Florida *A. pallida*. Letter in parentheses indicates clade present at low concentrations.

The discovery that *A. pallida* anemones from Long Key, Florida predominately harbor *Symbiodinium* clade A is also surprising. Several studies (Rowan and Powers 1991a, Hill and Wilcox 1998, Baker 1999, Banaszak et al. 2000) have examined by RFLP analysis either the *in hospite* or cultured zooxanthellae obtained from *A. pallida* in the Caribbean and have listed the symbiont harbored by this anemone as belonging to *Symbiodinium* clade B. A total of 80 *A. pallida* anemones have been collected from three locations in the Middle Florida Keys and all contained either *Symbiodinium* clade A only or *Symbiodinium* clades A and B in various ratios (G. E.

May, University of Buffalo, personal communication). This suggests that the association of *A. pallida* with zooxanthellae belonging to *Symbiodinium* clade A is fairly widespread and common in the Middle Florida Keys. This is in sharp contrast to the finding that *A. pallida* in Bermuda contain only *Symbiodinium* clade B zooxanthellae ($n = 7$; G. E. May, University of Buffalo, personal communication) and the literature listed above. This finding has several possible explanations. First, small sample sizes from limited geographical areas of *in hospite* zooxanthellae, along with the tendency of *A. pallida* zooxanthella cultures to favor the proliferation of *Symbiodinium* clade B (this study), could contribute to the misrepresentation of *Symbiodinium* clade B as being the predominant symbiont of this anemone. Second, only one species of *Aiptasia* anemone is recognized throughout the Caribbean, *A. pallida* (= *A. tagetes* sensu Verrill; Sterrer 1986). It is possible that the reason for this difference in the symbiotic compliment is that the *Aiptasia* anemones present in the Middle Florida Keys and those in Bermuda are cryptic species with specific symbiont preferences. Finally, differences in geography, namely the latitudinal difference between the Florida Keys and Bermuda, may account for the observed associations. Latitudinal differences in zooxanthella complements have been observed in the scleractinian coral *Plesiastrea versipora* (Baker 1999, Rodriguez-Lanetty et al. 2001) and the anemone *Anthopleura elegantissima* (LaJeunesse and Trench 2000) and will probably become more common as sampling becomes more widespread. Work into some of these possible explanations is presently underway.

Octocoral zooxanthella cultures versus in hospite *populations: ITS-rDNA sequences and analyses.* The ITS-rDNA region in zooxanthellae, as in other eukaryotic organisms, contains two ITS (i.e. ITS1 and ITS2) and an intervening 5.8S-rDNA region. PCR amplification using the above primers generates a single product from all samples of zooxanthellae screened to date, and this product does not vary in length on 2% TBE agarose gels (data not shown). In *Symbiodinium* clade B, the average G + C content of the entire region is approximately 50% and one short (4 bp) insertion/deletion (indel) in ITS2 was common to several of the sequences. This indel was found in the zooxanthella sequences obtained from *Plexaura kuna* culture 702, *P. flexuosa* culture, and *Briareum asbestinum in hospite* zooxanthellae. Interestingly, this 4-bp indel was also found in the ITS-rDNA sequence of zooxanthellae cultured from the anemone *Aiptasia pulchella* (Hawaii) (data not shown). Because indels of this type typically represent a shared history for a group (Prather and Jansen 1998), these data suggest that these zooxanthellae from geographically distant areas form a closely related group. This pattern of closely related or identical zooxanthella taxa from distant geographical areas and phylogenetically diverse hosts has been reported for *Symbiodinium* ITS-rDNA (Baillie et al. 2000b) and other nuclear rDNAs (Baker and Rowan 1997, Baker et al. 1997, Baker 1999, Carlos et al. 1999, Goulet 1999, Banaszak et al. 2000, Darius et al. 2000) and appears to be a common feature of invertebrate-*Symbiodinium* symbioses.

Alignment and analysis of the ITS-rDNA regions revealed a striking conservation of sequence among *Symbiodinium* clade B. Modeltest v3.0 analysis determined the Hasegawa et al. (1985) (HKY85) model as being the best-fit model of DNA evolution for the ITS regions of *Symbiodinium* clade B ($P < 0.000001$). HKY85 distance values within the zooxanthellae examined ranged from 0 to 2.8% divergence by pairwise comparison. For the three direct comparisons, the degree of genetic variation between zooxanthella cultures and the *in hospite* populations from which they originated were 0%, 0.2%, and 1.7% for *Pseudopterogorgia elisabethae*, *Plexaura kuna*, and *P. flexuosa*, respectively. The lack of sequence divergence in ITS-rDNA reported here for *Symbiodinium* clade B is also seen in *Symbiodinium* clade A (Belda-Baillie et al. 1999, Baillie et al. 2000b), symbiotic with *Tridacna* and other giant bivalves, and scleractinian *Symbiodinium* clade C from the western Indo-Pacific (M. Hidaka and M. Hirose, University of the Ryukyus, personal communication). This was unexpected because many microalgal species and populations possess highly variable and divergent ITS-rDNA sequences. For example, in the agarophyte genera *Gracilaria* and *Gracilariopsis*, there is as much variation among individuals of a population as there is between individuals of geographically separate populations (Goff et al. 1994). Furthermore, Manhart et al. (1995) found 57 point mutations and three indels in 257 bp of ITS1 in the diatoms *Pseudo-nitzschia pungens* and *P. mutiseries*. However, ITS-rDNA sequence differences found between cultured and *in hospite* zooxanthellae of the Pacific *Symbiodinium* clade C-harboring soft coral *Sinularia* sp. differed by approximately 18.8%. This result suggests that some zooxanthellae within the same clade possess highly divergent ITS-rDNA sequences. In dinoflagellates that produce calcareous cysts, D'Onofrio et al. (1999) found that *Scrippsiella* species and species isolates differed from each other from 13% to 36.6% and different genera ranged from 6% to 77% in the ITS-rDNA region. The high level of sequence difference observed between the *in hospite* zooxanthellae and culture obtained from *Sinularia* sp. are in agreement with D'Onofrio et al. (1999) and suggests that the culture belongs to a different species or genus than the numerically dominant symbiont *in hospite* (see below).

The maximum parsimony consensus tree shows two major groups of *Symbiodinium* clade B (Fig. 3). Both groups contain representatives of cultures and *in hospite* zooxanthella populations. Templeton's test revealed that the consensus tree (Fig. 3) differed significantly compared with the constraint tree (Fig. 1) (17 steps longer, $n = 16$, $z = -2.62$, $P < 0.0088$), resulting in the rejection of the null hypothesis that the zooxanthella cultures were most closely related to the *in hospite* populations of the gorgonian host species from which they originated. For example, the grouping of *Plexaura kuna* culture 702/*P. flexuosa* culture/*Briareum asbestinum in*

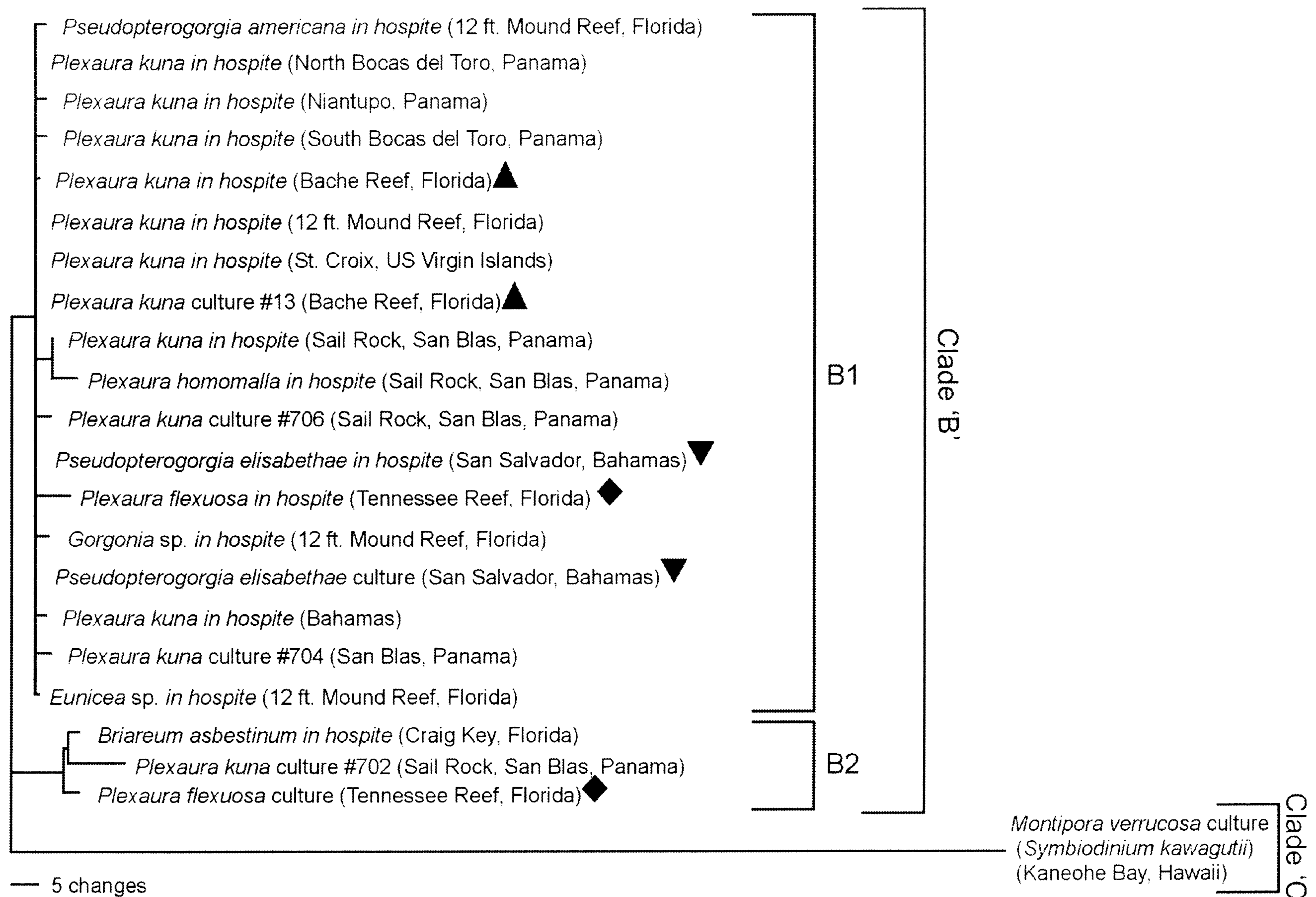

FIG. 3. Maximum parsimony strict consensus tree resolved from a heuristic search in PAUP*4.0 of ITS-rDNA nucleotide sequences from Caribbean gorgonian *in hospite* and cultured zooxanthellae. Geographic origins of samples are stated in parentheses. Pairs of identical symbols following host name and geographic origin represent culture and *in hospite* zooxanthella population from the same host individual.

hospite (group B2 in Fig. 3) represents zooxanthellae from three host species. Culture 702 was originally isolated from a juvenile *P. kuna* polyp from the San Blas Islands, Panama, whereas the *P. flexuosa* culture was isolated from an adult colony in Florida. Both cultures possessed ITS-rDNA sequences more closely related to the *in hospite* symbionts of *B. asbestinum* found in Florida than to other culture isolates or *in hospite* populations from the same host species. The data suggest that these cultured zooxanthellae may be present in *P. kuna* and *P. flexuosa* as a subset of the population or may be transient residents of the adult host. Again, it also suggests that recently diverged types of zooxanthellae are geographically widespread (see above).

It should be noted that the sequence data used in these analyses were generated from direct sequencing of PCR-generated products. This strategy was chosen over traditional cloning and sequencing because preliminary studies suggested that in *Symbiodinium* clade B, within-individual variation in ITS-rDNA sequences of an isoclonal cell line can be as great as differences between cell lines (S. R. Santos, unpublished data). Thus, direct sequencing of PCR-generated products was used because it results in sequence data that are representative of the average DNA sequence (Hillis et al. 1996) and should represent the numerically dominant symbiont (Baillie et al. 2000b).

Caribbean gorgonian zooxanthella cultures versus in hospite *populations: microsatellite amplifications.* Seven microsatellite loci have been identified from *in hospite* zooxanthella populations of adult *Plexaura kuna* colonies. The three microsatellite primer pairs reported in this study produced amplification products from approximately 60%–98% of the 126 colonies screened (S. R. Santos, unpublished data). In addition, the three microsatellite primer pairs were found to amplify *in hospite Symbiodinium* clade B from other host species, including the two other Caribbean gorgonians included in this study (Fig. 4). In one case, allelic size variation was observed between the *in hospite* zooxanthellae of *P. kuna* and *P. flexuosa* (Fig. 4b). The zooxanthella allele present in *P. flexuosa* has been observed to occur in *P. kuna* zooxanthella populations *in hospite* (S. R. Santos, unpublished data). However, only one microsatellite allele was amplified from a zooxanthella culture (Fig. 4c). This culture, isolated from *Pseudopterogorgia elisabethae*, possessed the same allele as the original *in hospite* population (Fig. 4c and data not shown). Microsatel-

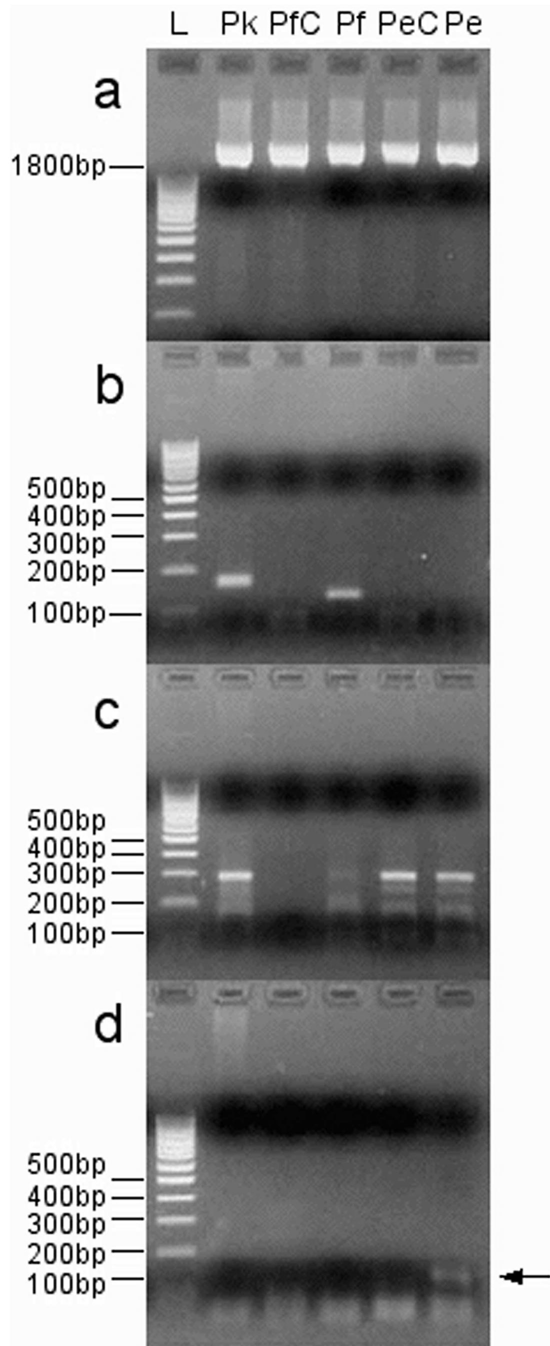

FIG. 4. Microsatellite PCR amplifications of Caribbean gorgonian *in hospite* and cultured zooxanthellae. (a) 18S-rDNA amplification with primers ss5 and ss3z (Rowan and Powers 1991b). (b) Amplification of microsatellite locus GA2.8. (c) Amplification of microsatellite locus GA4.84. (d) Amplification of microsatellite locus CA6.10; arrow indicates position of PCR product. L, 100 bp DNA size ladder; Pk, *Plexaura kuna in hospite* zooxanthellae; PfC, *P. flexuosa* zooxanthella culture; Pf, *P. flexuosa in hospite* zooxanthellae; PeC, *Pseudopterogorgia elisabethae* zooxanthella culture; Pe, *P. elisabethae in hospite* zooxanthellae. See text for additional details.

lite screening of the remaining gorgonian *Symbiodinium* clade B cultures presented in this study (Table 1) with the same primers failed to produce amplification.

The pattern of presence or absence of a locus between the original *in hospite* zooxanthella populations and the resulting cultures suggests that a microsatellite primer set is specific to a particular zooxanthella type. For example, in Figure 4b, the *in hospite* zooxanthellae of *P. flexuosa* and *P. kuna* possessed a locus that was absent in the other screened samples. However, in Figure 4c, the *in hospite* zooxanthellae of *P. elisabethae*, the culture obtained from *P. elisabethae*, and the *in hospite* population of *P. kuna* possessed a locus that the *in hospite* and cultured zooxanthellae of *P. flexuosa* did not. Finally, in Figure 4d, the *in hospite* zooxanthellae of *P. elisabethae* possessed that locus (allele of approximately 120 bp, indicated by arrow), whereas all the other samples did not. It is important to reiterate at this point that all samples were screened by 18S-rDNA amplification (Fig. 4a) before microsatellite amplifications to assess PCR-template quality. Furthermore, to test for reproducibility, amplifications were conducted three times per sample from independent DNA extractions of whole tissue or algal cells under stringent PCR conditions (see Materials and Methods). In addition, tests of the microsatellite primers on DNA isolated from aposymbiotic *P. kuna* planulae demonstrated that the loci are not part of the host genome (data not shown). Thus, the data suggests that these zooxanthella cultures represent a subset of the original *in hospite* zooxanthella population and that these hosts are capable of harboring heterogeneous populations of zooxanthellae.

Zooxanthella cultures versus in hospite *populations: literature reexamination.* The culturing of zooxanthellae has a long history, dating back to at least the 1940s (Kawaguti 1944). The ground-breaking work of McLaughlin and Zahl (McLaughlin and Zahl 1957, 1959, 1962a,b, Zahl and McLaughlin 1957, 1959) and Freudenthal (1962) demonstrated, by using axenic cultures, that zooxanthellae were in fact members of the dinoflagellates by eliciting motile forms from vegetative cells. However, these early researchers worked from the assumption that all zooxanthellae belonged to a single pandemic species, *Symbiodinium microadriaticum* Freudenthal (Taylor 1974), and that an individual host harbored a population of this symbiont. Recent evidence from a number of investigators has demonstrated that the ideas of a single pandemic species and homogenous zooxanthella populations in a single individual are no longer valid. Reexamination of the literature of the last several decades reveals that many zooxanthella cultures are or have the potential to be a subset of the original *in hospite* populations.

The evidence that many zooxanthella cultures are a subset of the original *in hospite* population is most easily seen at the level of 18S-rDNA RFLPs. Following the publication of Rowan and Powers (1991a), this technique became the method of choice due to its specificity and simplicity and has been expanded to include

28S-rDNA (Baker et al. 1997, Hill and Wilcox 1998, Baker 1999). Even with the recognition that RFLP data are limited in the level of diversity that it reveals (Dowling et al. 1996), it remains a staple for studies in invertebrate-dinoflagellate symbioses. Until recently, there has not been a large-scale 18S-rDNA RFLP survey and comparison of cultured zooxanthellae published in the literature. The work of Banaszak et al. (2000), along with this study, provide an opportunity to examine the question of zooxanthella culture nonrepresentation with data commonly seen in the field.

Banaszak et al. (2000) examined 27 symbiotic dinoflagellate cultures isolated from a number of hosts and geographical locations. The authors state that there were several discrepancies between what they had in culture compared with what was found and reported *in hospite*. In particular, they note that the symbiont cultured from *Pocillopora damicornis* (Hawaii) conformed to *Symbiodinium* clade B and two independent culture isolations from *Meandrina meandrites* (Jamaica) resulted in one *Symbiodinium* clade A culture and one *Symbiodinium* clade C culture. Banaszak et al. (2000) concluded that *P. damicornis* and *M. meandrites* must harbor two (*Symbiodinium* clades B and C, with clade C being the dominant *in hospite* symbiont; see Rowan and Powers 1991a) and three (*Symbiodinium* clades A, B, and C; *Symbiodinium* clade B can be found *in hospite*; see Baker and Rowan 1997) clades of *Symbiodinium*, respectively. This conclusion is consistent with the idea that symbiotic hosts harbor heterogeneous populations of zooxanthellae. Comparisons between the zooxanthella cultures of Banaszak et al. (2000) and this study (Table 4) for identical or closely related hosts from the same large geographical area reveal a number of discrepancies. Accepting the facts that most symbiotic hosts can associate with more than one type of zooxanthellae and that many hosts naturally harbor heterogeneous zooxanthella populations, at least two other factors may account for these discrepancies.

The first large-scale attempts to culture and characterize zooxanthellae from a number of host species are the studies of Schoenberg and Trench (1980a,b,c). The authors attempted to culture zooxanthellae from a total of 70 host species, of which only 17 (≈25%) produced viable cultures in the growth medium ASP-8A (Ahles 1967). This inability to culture zooxanthellae from all the attempted hosts was the first published indication that zooxanthellae from different hosts do not possess identical culturing requirements. Many different growth media have been formulated and used over the years (Table 5) in an effort to obtain cultured zooxanthellae from hosts, maintain the cultures after isolation, or for experimental studies. However, the use of different growth media brings its own set of problems. For example, in a situation where a heterogeneous population of zooxanthellae is present at isolation, some types may proliferate in a particular medium, whereas other types do not. Banaszak et al. (2000) lists two of three zooxanthella cultures obtained from three species of Caribbean gorgonians as belonging to *Symbiodinium* clade A (Table 4). This is in contrast to adult Caribbean gorgonian zooxanthella cultures presented in this study, all of which were *Symbiodinium* clade B. One explanation for this discrepancy may be the difference in growth media used at culture isolation. Many adult Caribbean gorgonians harbor *Symbiodinium* clade B (Goulet 1999), whereas newly settled aposymbiotic polyps rapidly acquire either *Symbiodinium* clades A or B or both (Coffroth et al. 2001) simultaneously from the environment. By the time juvenile gorgonians reach 10 cm in height, only *Symbiodinium* clade B can be detected in the host (Coffroth et al. 2001). It may be possible that juvenile and adult Caribbean gorgonians harbor extremely low levels of *Symbiodinium* clade A as part of their symbiont population or *Symbiodinium* clade A may be present as a contaminant on the host or within its digestive system. These rare genotypes may escape detection by current molecular methods and become apparent after culturing with certain growth media. These possibilities deserve further investigation.

In the experiments reported here, zooxanthellae were brought into bulk culture before analysis. This is a common method by which symbiotic dinoflagellates are brought into culture (McLaughlin and Zahl 1959, Kinzie 1974, Kinzie and Chee 1979, Schoenberg and Trench 1980a, Lesser and Shick 1990, Lee et al. 1995,

TABLE 4. Discrepancies in zooxanthella culture 18S-rDNA RFLP data from Banaszak et al. (2000) and this study compared with *in hospite* zooxanthella populations of various invertebrates.

Host species	Banaszak et al. (2000) 18S-rDNA RFLP	This study 18S-rDNA RFLP	*In hospite* 18S-rDNA RFLP
Mastigias sp.	A (Belau)	C (Palau)	n.d.
Caribbean *Aiptasia* anemones	B (Puerto Rico)	A; A and B; B (Middle Florida Keys)	A[e], A and B[e], B[a,b]
Pocillopora damicornis	B (Hawaii)	B (Hawaii)	C[b]
Class Alcyonacea (gorgonians)	A (Caribbean) (2 out of 3 cultures)	B (Caribbean)	B[b–e]
Tridacna gigas	A(GBR, Australia)	A (Indo-Pacific)	A and C[f]

n.d., no data available. Geographic origins of individual hosts are in parentheses.
[a] Baker (1999).
[b] Rowan and Powers (1991a).
[c] Goulet (1999).
[d] Coffroth et al. (2001).
[e] This study.
[f] Rowan et al. (1996), Belda-Baillie et al. (1999), Carlos et al. (1999), Baillie et al. (2000a,b), Carlos et al. (2000).

Table 5. Historical list of growth media used in zooxanthellae culturing.

Name of medium	Formulation by	Used by
ASP-8A	Ahles (1967)	Schoenberg and Trench (1980a)
		Fitt et al. (1981)
		Colley and Trench (1983)
		Chang et al. (1983)
		Fitt and Trench (1983)
		Trench and Fisher (1983)
		Fitt (1984)
		Blank (1987)
		Trench and Blank (1987)
		Blank and Trench (1988)
		Blank and Huss (1989)
		Rogerson et al. (1989)
		Lesser and Shick (1990)
		Markell et al. (1992)
		Iglesias-Prieto et al. (1992)
		Markell and Trench (1993)
		Iglesias-Prieto et al. (1993)
		Iglesias-Prieto and Trench (1994)
		Trench and Thinh (1995)
		Iglesias-Prieto and Trench (1997)
		Stochaj and Grossman (1997)
		Wilcox (1998)
		Warner et al. (1999)
		Banaszak et al. (2000)
EMS	Watanabe et al. (1988)	Carlos et al. (1999)
K	Keller et al. (1987)	Carlos et al. (1999)
		Carlos et al. (2000)
M series	McLaughlin and Zahl (1959)	McLaughlin and Zahl(1959)
		Kinzie (1974)
CSI	McLaughlin and Zahl (1959)	McLaughlin and Zahl (1959)
f/2	Guillard and Ryther (1962)	Kinzie et al. (1984)
		Crafts and Tuliszewski (1995)
		Rowan et al. (1996)
		Baillie et al. (1998)
		Belda-Baillie et al. (1999)
		Kinzie et al. (2001)
		This study
ES	Provasoli (1968)	Kinzie and Chee (1979)
		Lerch and Cook (1984)
		Steen (1987)
ISM	Lee et al. (1980)	Lee et al. (1995)

Stochaj and Grossman 1997, Baillie et al. 1998, Wilcox 1998, Belda-Baillie et al. 1999, Carlos et al. 1999, 2000) before establishing isoclonal lines of cells. However, in several of these studies, including the more recent ones, only motile cells were used in the establishment of isoclonal cell lines. This could prove to be problematic because different zooxanthellae possess characteristic patterns of motility (Fitt et al. 1981, Fitt and Trench 1983, Crafts and Tuliszewski 1995). This investigator bias toward motile cells could result in the establishment of nonrepresentative cultures from heterogeneous bulk cultures. Before isoclonal lines of zooxanthellae are established from bulk cultures (either by picking single cells with micropipets or by serial dilutions), zooxanthella cultures should be homogenized to ensure that biases are minimized.

As was stated earlier, all zooxanthella species descriptions and most physiological and infectivity studies have been conducted using cultured material under the assumption that a culture represented the dominant symbiont harbored by that individual host or host species. However, the data presented in this study suggest that in many cases, cultures are not representative of the *in hospite* zooxanthella population present in an individual host or host species. This nonrepresentation results from the fact that symbiotic hosts can associate with more than one type of zooxanthellae and that many hosts naturally harbor heterogeneous zooxanthella populations. The selective nature of microorganismal culturing, such as which growth medium is used or the manner in which cultures are established, compounds this. Despite this, zooxanthella cultures should continue to remain a vital part of studies exploring invertebrate-dinoflagellate symbioses. Cultures offer a glimpse of the diversity present in wild zooxanthella populations and act as sources of cells and zooxanthella DNA free of host materials. Furthermore, proper species descriptions from cultures should be considered valid because they represent the characterization of a living entity. However, if zooxanthella cultures are used in other types of studies, efforts should be made to determine their relationship to populations *in hospite*.

CONCLUSIONS

These data suggest that many, if not all, symbiotic hosts harbor heterogeneous populations of zooxanthellae. This heterogeneity may be manifested as a mixture of two or more zooxanthella clades (in the case of Florida *A. pallida* anemones) or different genetic lineages of the same clade (Caribbean gorgonians and an Indo-Pacific soft coral) within a single host individual. With the recent application of molecular genetic techniques to the study of invertebrate-dinoflagellate symbioses, the discovery that a host can harbor heterogeneous populations of zooxanthellae is becoming quite common (Rowan and Knowlton 1995, Baker et al. 1997, Baker and Rowan 1997, Goulet and Coffroth 1997, Rowan et al. 1997, Darius et al. 1998, 2000, Hill and Wilcox 1998, Baker 1999, Carlos et al. 1999, 2000, Baillie et al. 2000a,b, LaJeunesse and Trench 2000, Coffroth et al. 2001). The results presented here demonstrate that cultured zooxanthellae may not represent the dominant symbiont within an individual host or host species. These data demonstrate that if a mixture of zooxanthellae is originally present in a host, cultures that result may represent either the dominant symbiont or ones present in lower numbers. For this reason, the idea that a zooxanthella culture is “the” symbiont of a particular host should be discarded. We suggest that in most cases, cultures represent “a” symbiont that is

capable of forming a symbiotic relationship with that host. In the future, investigators who use cultured zooxanthellae in experiments should quantify the relationship and representation of cultures to *in hospite* populations before use in experiments to ensure that the results of their study can be reasonably extrapolated to the intact symbiotic system.

We thank the Kuna Nation and the Republic of Panama, along with the Florida Keys National Marine Sanctuary, for permission to collect and export samples from Panama and Florida, respectively. We thank the staff and scientists of the Smithsonian Tropical Research Institute (STRI) and the Keys Marine Laboratory (KML) for technical and logistical support. We also thank Dr. Robert A. Kinzie III for kindly providing access to his zooxanthella culture collection and comments made on an earlier draft of the manuscript; R. Rowan for access to unpublished data and comments made on the manuscript; L. Bright, D. Brancato, and J. Weaver for culturing assistance; T. L. Snell for assistance in the field; and the Aquarium of Niagara (Niagara Falls, NY) for access to synthetic seawater. This research was supported by an NSF Minority Graduate Fellowship (to S.R.S.) and NSF OCE-95-30057 and OCE-99-07319 (to M.A.C.).

Ahles, M. D. 1967. *Some Aspects of the Morphology and Physiology of* Symbiodinium microadriaticum. Ph.D. dissertation. Fordham University, New York, 173 p.

Ambariyanto & Hoegh-Guldberg, O. 1996. Nutrient enrichment and the ultrastructure of zooxanthellae from the giant clam *Tridacna maxima*. *Mar. Biol.* 125:359–63.

Baillie, B. K., Monje, V. A., Silvestre, V., Sison, M. & Belda-Baillie, C. A. 1998. Allozyme electrophoresis as a tool for distinguishing different zooxanthellae symbiotic with giant clams. *Proc. R. Soc. Lond. B* 256:1949–56.

Baillie, B. K., Belda-Baillie, C. A., Silvestre, V., Sison, M., Gomez, A. V., Gomez, E. D. & Monje, V. 2000a. Genetic variation in *Symbiodinium* isolates from giant clams based on random-amplified-polymorphic DNA (RAPD) patterns. *Mar. Biol.* 136:829–36.

Baillie, B. K., Belda-Baillie, C. A. & Maruyama, T. 2000b. Conspecificity and Indo-Pacific distribution of *Symbiodinium* genotypes (Dinophyceae) from giant clams. *J. Phycol.* 36:1153–61.

Baker, A. C. & Rowan, R. 1997. Diversity of symbiotic dinoflagellates (zooxanthellae) in scleractinian corals of the Caribbean and Eastern Pacific. *Proc. 8th Int. Coral Reef Symp.* 2:1301–6.

Baker, A. C., Rowan, R. & Knowlton, N. 1997. Symbiosis ecology of two Caribbean Acroporid corals. *Proc. 8th Int. Coral Reef Symp.* 2:1295–300.

Baker, A. C. 1999. *The Symbiosis Ecology of Reef-Building Corals.* Ph.D. dissertation, University of Miami, Florida, 120 pp.

Banaszak, A. T., Iglesias-Prieto, R. & Trench, R. K. 1993. *Scrippsiella velellae* sp. nov. (Peridiniales) and *Gloeodinium viscum* sp. nov. (Phytodiniales), dinoflagellate symbionts of two hydrozoans (Cnidarians). *J. Phycol.* 29:517–28.

Banaszak, A. T., LaJeunesse, T. C. & Trench, R. K. 2000. The synthesis of microsporine-like amino acids (MAAs) by cultured, symbiotic dinoflagellates. *J. Exp. Mar. Biol. Ecol.* 249:219–33.

Barnes, D. J. & Chalker, B. E. 1990. Calcification and photosynthesis in reef-building corals and algae. *In* Dubinsky, Z. [Ed.] *Ecosystems of the World 25: Coral Reefs.* Elsevier, Amsterdam, pp. 109–31.

Belda-Baillie, C. A., Sison, M., Silvstre, V., Villamor, K., Monje, V., Gomez, E. D. & Baillie, B. K. 1999. Evidence for changing symbiotic algae in juvenile tridacnids. *J. Exp. Mar. Biol. Ecol.* 241:207–21.

Blank, R. J. & Trench, R. K. 1985. Speciation and symbiotic dinoflagellates. *Science* 229:656–8.

Blank, R. J. 1987. Cell architecture of the dinoflagellate *Symbiodinium* sp. inhabiting the Hawaiian stony coral *Montipora verrucosa*. *Mar. Biol.* 94:143–55.

Blank, R. J., Huss, V. A. R. & Kersten, W. 1988. Base composition of DNA from symbiotic dinoflagellates: a tool for phylogenetic classification. *Arch. Microbiol.* 149:515–20.

Blank, R. J. & Trench, R. K. 1988. Immunogold localization of ribulose-1,5-bisphosphate carboxylase-oxygenase in *Symbiodinium kawagutii* Trench et Blank, an endosymbiotic dinoflagellate. *Endocyt. Cell. Res.* 5:75–82.

Blank, R. J. & Huss, V. A. R. 1989. DNA divergency and speciation in *Symbiodinium* (Dinophyceae). *Pl. Syst. Evol.* 163:153–63.

Burris, R. H. 1983. Uptake and assimilation of $^{15}NH_4$ by a variety of corals. *Mar. Biol.* 75:151–5.

Carlos, A. A., Baillie, B. K., Kawachi, M. & Maruyama, T. 1999. Phylogenetic position of *Symbiodinium* (Dinophyceae) isolates from Tridacnids (Bivalvia), Cardiids (Bivalvia), a sponge (Porifera), a soft coral (Anthozoa), and a free-living strain. *J. Phycol.* 35:1054–62.

Carlos, A. A., Baillie, B. K. & Maruyama, T. 2000. Diversity of dinoflagellate symbionts (zooxanthellae) in a host individual. *Mar. Ecol. Prog. Ser.* 195:93–100.

Chang, S. S. & Trench, R. K. 1982. Peridinin-chlorophyll *a* proteins from the symbiotic dinoflagellate *Symbiodinium* (=*Gymnodinium*) *microadriaticum*, Freudenthal *Proc. R. Soc. Lond. B* 215:191–210.

Chang, S. S., Prezelin, B. B. & Trench, R. K. 1983. Mechanisms of photoadaptation in three strains of the symbiotic dinoflagellate *Symbiodinium microadriaticum*. *Mar. Biol.* 76:219–29.

Ciofi, C. & Bruford, M. W. 1998. Isolation and characterization of microsatellite loci in the Komodo dragon *Varanus komodoensis*. *Mol. Ecol.* 7:133–5.

Coffroth, M. A., Lasker, H. R., Diamond, M. E., Bruenn, J. A. & Bermingham, E. 1992. DNA fingerprinting of a gorgonian coral: a method for detecting clonal structure in a vegetative species. *Mar. Biol.* 114:317–25.

Coffroth, M. A., Santos, S. R. & Goulet, T. L. 2001. Early ontogenetic expression of specificity in a cnidarian-algal symbiosis. *Mar. Ecol. Prog. Ser.* (in press).

Colley, N. J. & Trench, R. K. 1983. Selectivity in phagocytosis and persistence of symbiotic algae by the scyphistoma stage of the jellyfish *Cassiopeia xamachana*. *Proc. R. Soc. Lond. B* 219:61–82.

Crafts, C. B. & Tuliszewski, J. R. 1995. Motility rhythms in cultured zooxanthellae isolated from the scyphomedusa *Linuche unguiculata*. *Bull. Mar. Sci.* 56:822–5.

Darius, H. T., Dauga, C., Grimont, P. A. D., Chungue, E. & Martin, P. M. V. 1998. Diversity in symbiotic dinoflagellates (Pyrrhophyta) from seven scleractinian coral species: restriction enzyme analysis of small subunit ribosomal RNA genes. *J. Euk. Microbiol.* 45:619–27.

Darius, H. T., Martin, P. M. V., Grimont, P. A. D. & Dauga, C. 2000. Small subunit rDNA sequence analysis of symbiotic dinoflagellates from seven scleractinian corals in a Tahitian lagoon. *J. Phycol.* 36:951–9.

D'Onofrio, G., Marino, D., Bianco, L., Busico, E. & Montresor, M. 1999. Toward an assessment of the taxonomy of Dinoflagellates that produce calcareous cysts (Calciodinelloideae, Dinophyceae): a morphological and molecular approach. *J. Phycol.* 35:1063–78.

Dowling, T. E., Moritz, C., Palmer, J. D. & Rieseberg, L. H. 1996. Nucleic acids III. Analysis of fragments and restriction sites. *In* Hillis, D. M., Moritz, C. & Mable, B. K.. [Eds.] *Molecular Systematics*, 2nd ed. Sinauer Associates, Massachusetts, pp. 249–320.

Falkowski, P. G., Dubinsky, Z., Muscatine, L. & Porter, J. W. 1984. Light and the bioenergetics of a symbiotic coral. *Bioscience* 34:705–9.

Fitt, W. K., Chang, S. S. & Trench, R. K. 1981. Motility patterns of different strains of the symbiotic dinoflagellate *Symbiodinium* (=*Gymnodinium*) *microadriaticum* (Freudenthal) in culture. *Bull. Mar. Sci.* 31:436–43.

Fitt, W. K. & Trench R. K. 1983. The relation of diel patterns of cell division to diel patterns of motility in the symbiotic dinoflagellate *Symbiodinium microadriaticum* Freudenthal in culture. *New Phytol.* 94:421–32.

Fitt, W. K. 1984. The role of chemosensory behavior of *Symbiodinium microadriaticum*, intermediate hosts, and host behavior in the infection of coelenterates and molluscs with zooxanthellae. *Mar. Biol.* 81:9–17.

Freudenthal, H. D. 1962. *Symbiodinium* gen. Nov. and *Symbiodinium microadriaticum* sp. nov., a zooxanthella: taxonomy, life cycle and morphology. *J. Protozool.* 9:45–52.

Glenn, T. C., Stephan, W. & Braun, M. J. 1999. Effects of a population bottleneck on whooping crane mitochondrial DNA variation. *Conserv. Biol.* 13:1097–107.

Glynn, P. W. 1996. Coral reef bleaching: facts, hypotheses and implications. *Glob. Change Biol.* 2:495–509.

Goff, L. J., Moon, D. A. & Coleman, A. W. 1994. Molecular delineation of species and species relationships in the red algal *Agarophytes, Gracilariopsis* and *Gracilaria* (Gracilariales). *J. Phycol.* 30:521–37.

Goulet, T. L. & Coffroth, M. A. 1997. A within colony comparison of zooxanthellae genotypes in the Caribbean gorgonian *Plexaura kuna*. *Proc. 8th Int. Coral Reef Symp.* 2:1331–4.

Goulet, T. L. 1999. *Temporal and Spatial Stability of Zooxanthellae in Octocorals.* Ph.D. dissertation. State University of New York at Buffalo, New York, 101 pp.

Guillard, R. R. L. & Ryther J. H. 1962. Studies of marine planktonic diatoms. I. *Cycotella nana* hustedl, and *Detonula confervacea* (cleve) gran. *Can. J. Microbiol.* 8:229–39.

Hasegawa, M., Kishino, H. & Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 21:160–74.

Hill, M. & Wilcox, T. 1998. Unusual mode of symbiont repopulation after bleaching in *Anthosigmella varians*: acquisition of different zooxanthellae strains. *Symbiosis* 25:279–89.

Hillis, D. M., Marble, B. K., Larson, A., Davis, S. K. & Zimmer, E. A. 1996. Nucleic acids IV. Sequencing and cloning. *In* Hillis et al. [Eds.] *Molecular Systematics*, 2nd ed. Sinauer Associates, Massachusetts, pp. 332–3.

Iglesias-Prieto, R., Matta, J. L., Robins, W. A. & Trench, R. K. 1992. Photosynthetic response to elevated temperature in the symbiotic dinoflagellate *Symbiodinium microadriaticum* in culture. *Proc. Natl. Acad. Sci. USA* 89:10302–5.

Iglesias-Prieto, R., Govind, N. S. & Trench, R. K. 1993. Isolation and characterization of three membrane-bound chlorophyll-protein complexes from four dinoflagellate species. *Phil. Trans. R. Soc. Lond. B* 340:381–92.

Iglesias-Prieto, R. & Trench, R. K. 1994. Acclimation and adaptation to irradiance in symbiotic dinoflagellates. I. Responses of the photosynthetic unit to changes in photon flux density. *Mar. Ecol. Prog. Ser.* 113:163–75.

Iglesias-Prieto, R. & Trench, R. K. 1997. Acclimation and adaption to irradiance in symbiotic dinoflagellates. II. Response of chlorophyll-protein complexes to different photon-flux densities. *Mar. Biol.* 130:23–33.

Kawaguti, S. 1944. On the physiology of reef corals. VII. Zooxanthellae of the reef corals in *Gymnodinium* sp., dinoflagellate; its culture *in vitro*. *Palao. Trop. Biol. Studies* 2:319–28.

Kawaguti, S. 1953. Ammonium metabolism of the reef corals. *Biol. J. Okayama. Univ.* 1:171–6.

Keller, M. D., Selvin, R. C., Claus, W. & Guillard, R. R. L. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* 23:633–8.

Kinzie R. A. III. 1974. Experimental infection of aposymbiotic gorgonian polyps with zooxanthellae. *J. Exp. Mar. Biol. Ecol.* 15:335–45.

Kinzie R. A. III & Chee, G. S. 1979. The effect of different zooxanthellae on the growth of experimentally reinfected hosts. *Biol. Bull.* 156:315–27.

Kinzie R. A. III, Jokiel, P. L. & York, R. 1984. Effects of light of altered spectral composition on coral zooxanthellae associations and on zooxanthellae *in vitro*. *Mar. Biol.* 78:239–48.

Kinzie R. A. III, Takayama, M., Santos, S. R. & Cofforth, M. A. 2001. The adaptive bleaching hypothesis: experimental tests of critical assumptions. *Biol. Bull.* 200:51–8.

LaJeunesse, T. C. & Trench, R. K. 2000. Biogeography of two species of *Symbiodinium* (Freudenthal) inhabiting the intertidal sea anemone *Anthopleura elegantissima* (Brandt). *Biol. Bull.* 199:126–34.

Lee, J. J., McEnery, M. E. & Garrison, J. R. 1980. Experimental studies of larger foraminifera and their symbionts from the Gulf of Elat. *J. Foramin. Res.* 10:31–47.

Lee, J. J., Wray, C. G. & Lawrence, C. 1995. Could Foraminiferal zooxanthellae be derived from environmental pools contributed to by different coelenterate hosts? *Acta Protozool.* 34:75–85.

Lerch, K. A. & Cook, C. B. 1984. Some effects of photoperiod on the motility rhythm of cultured zooxanthellae. *Bull. Mar. Sci.* 34:477–83.

Lesser, M. P. & Shick, J. M. 1990. Effects of visible and ultraviolet radiation on the ultrastructure of zooxanthellae (*Symbiodinium* sp.) in culture and *in situ*. *Cell Tissue Res.* 261:501–8.

Lewis, D. H. & Smith, D. C. 1971. The autotrophic nutrition of symbiotic marine coelenterates with special references to hermatypic corals. I. Movement of photosynthetic products between the symbionts. *Proc. R. Soc. Lond. B* 178: 111–29.

Loh, W., Carter, D. & Hoegh-Guldberg, O. 1998. Diversity of zooxanthellae from scleractinian corals of One Tree Island (the Great Barrier Reef). *In* Greenwood, J. G. & Hall, N. J. [Eds.] *Proceedings of the Australian Coral Reef Society 75th Anniversary Conference, Heron Island October 1997.* University of Queenland, Brisbane, pp. 87–95.

Manhart, J. R., Fryxell, G. A., Celia Villac, M. & Segura, L. Y. 1995. *Pseudo-nitzschia pungens* and *P. multiseries* (Bacillariophyceae): nuclear ribosomal DNAs and species differences. *J. Phycol.* 31:421–7.

Markell, D. A., Trench, R. A. & Iglesias-Prieto, R. 1992. Macromolecules associated with the cell walls of symbiotic dinoflagellates. *Symbiosis* 12:19–31.

Markell, D. A. & Trench, R. K. 1993. Macromolecules exuded by symbiotic dinoflagellates in culture: amino acid and sugar composition. *J. Phycol.* 29:64–8.

McLaughlin, J. J. A. & Zahl, P. A. 1957. Studies in marine biology. II. *In vitro* culture of zooxanthellae. *Proc. Soc. Exp. Biol. Med.* 95:115.

McLaughlin, J. J. A. & Zahl, P. A. 1959. Axenic zooxanthellae from various invertebrate hosts. *Ann. N. Y. Acad. Sci.* 77:55–72.

McLaughlin, J. J. A. & Zahl, P. A. 1962a. Axenic cultivation of the dinoflagellate symbiont from the coral *Cladocora*. *Arch. Mikrobiol.* 42:40–1.

McLaughlin, J. J. A. & Zahl, P. A. 1962b. Endozoic algae. *In* Lewin, R. A. [Ed.] *The Physiology and Biochemistry of Algae.* Academic Press, New York, pp. 823–6.

McNally, K. L., Govind, N. S., Thome, P. E. & Trench, R. K. 1994. Small-subunit ribosomal DNA sequence analyses and a reconstruction of the inferred phylogeny among symbiotic dinoflagellates (Pyrrophyta). *J. Phycol.* 30:316–29.

Muscatine, L., Falkowski, P. G., Porter, J. W. & Dubinsky, Z. 1984. Fate of photosynthetically-fixed carbon in light and shade-adapted colonies of the symbiotic coral, *Stylophora pistillata*. *Proc. R. Soc. Lond. B* 222:181–202.

Pearse, V. B. & Muscatine, L. 1971. Role of symbiotic algae (zooxanthellae) in coral calcification. *Biol. Bull.* 141:350–63.

Polne-Fuller, M. 1991. A novel technique for preparation of axenic cultures of *Symbiodinium* (Pyrrophyta) through selective digestion by amoebae. *J. Phycol.* 27:552–4.

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–8.

Prather, L. A. & Jansen, R. K. 1998. Phylogeny of Cobaea (Polemonlacaea) based on sequence data from the ITS region of nuclear ribosomal DNA. *Syst. Bot.* 23:57–72.

Provasoli, L. 1968. Media and prospects for the cultivation of marine algae. *In* Watanabe, A. & Hattori, R. [Eds.] *Culture and Collections of Algae.* Proc. U.S.-Japan Conference, Hakone, Japan, pp. 63–95.

Rodriguez-Lanetty, M., Loh, W., Carter, D. & Hoegh-Guldberg, O. 2001. Latitudinal variability in symbiont specificity within the widespread scleractinian coral *Plesiastrea versipora*. *Mar. Biol.* 138:1175–81.

Rogerson, A., Polne-Fuller, M., Trench, R. K. & Gibor, A. 1989. A laboratory-induced association between the marine amoeba *Trichosphaerium* AM-I-7 and the dinoflagellate *Symbiodinium* #8. *Symbiosis* 7:229–41.

Rowan, R. 1991. Molecular systematics of symbiotic algae. *J. Phycol.* 27:661–6.

Rowan, R. & Powers, D. A. 1991a. A molecular genetic identification of zooxanthellae and the evolution of animal-algal symbioses. *Science* 251:1348–51.

Rowan, R. & Powers, D. A. 1991b. Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae). *Mar. Ecol. Prog. Ser.* 71:65–73.

Rowan, R. & Powers, D. A. 1992. Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae). *Proc. Natl. Acad. Sci. USA* 89:3639–43.

Rowan, R. & Knowlton, N. 1995. Intraspecific diversity and ecological zonation in coral algal symbiosis. *Proc. Natl. Acad. Sci. USA* 92:2850–3.

Rowan, R., Whitney, S. M., Fowler, A. & Yellowlees, D. 1996. Rubisco in marine symbiotic dinoflagellates: form II enzymes in eukaryotic oxygenic phototrophs encoded by a nuclear multigene family. *Plant Cell* 8:539–53.

Rowan, R., Knowlton, N., Baker, A. C. & Jara, J. 1997. Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. *Nature* 388:265–9.

Rowan, R. 1998. Diversity and ecology of zooxanthellae on coral reefs. *J. Phycol.* 34:407–17.

Sadler, L. A., McNally, K. L., Govind, N. S., Brunk, C. F. & Trench, R. K. 1992. The nucleotide sequence of the small subunit ribosomal RNA gene from *Symbiodinium pilosum*, a symbiotic dinoflagellate. *Cur. Gene.* 21:409–16.

Schoenberg, D. A. & Trench, R. K. 1980a. Genetic variation in *Symbiodinium* (=*Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. I. Isozyme and soluble protein patterns of axenic cultures of *Symbiodinium microadriaticum*. *Proc. R. Soc. Lond. B* 207:405–27.

Schoenberg, D. A. & Trench, R. K. 1980b. Genetic variation in *Symbiodinium* (=*Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. II. Morphological variation in *Symbiodinium microadriaticum*. *Proc. R. Soc. Lond. B* 207:428–44.

Schoenberg, D. A. & Trench, R. K. 1980c. Genetic variation in *Symbiodinium* (=*Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. III. Specificity and infectivity of *Symbiodinium microadriaticum*. *Proc. R. Soc. Lond. B* 207:445–60.

Steen, R. G. 1987. Evidence for facultative heterotrophy in cultured zooxanthellae. *Mar. Biol.* 95:15–23.

Sterrer, W. 1986. *Marine Fauna and Flora of Bermuda*. Wiley-Interscience, New York. 774 pp.

Stochaj, W. R. & Grossman, A. R. 1997. Differences in protein profiles of cultured and endosymbiotic *Symbiodinium* sp. (Pyrrophyta) from the anemone *Aiptasia pallida* (Anthozoa). *J. Phycol.* 33:44–53.

Swofford, D. L. 2000. *PAUP**. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sinauer Associates, Sunderland, MA.

Taylor, D. L. 1974. Symbiotic marine algae: taxonomy and biological fitness. *In* Vernberg, W. B. [Ed.] *Symbiosis in the Sea*. University of South Carolina Press, South Carolina, pp 245–62.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tools. *Nucleic Acids Res.* 24:4876–82.

Trench, R. K. & Fisher, C. R. 1983. Carbon dioxide fixation in *Symbiodinium microadriaticum*: problems with mechanisms and pathways. *Endocytobiol.* 2:659–73.

Trench, R. K. 1987. Dinoflagellates in non-parasitic symbioses. *In* Taylor, F. J. R. [Ed.] *The Biology of Dinoflagellates*. Blackwell, Oxford, pp. 530–70.

Trench, R. K. & Blank, R. J. 1987. *Symbiodinium microadriaticum* Freudenthal, *S. goreauii* sp. nov., *S. kawagutti* sp. nov., and *S. pilosum* sp. nov.: gymnodinioid dinoflagellate symbionts of marine invertebrates. *J. Phycol.* 23:469–81.

Trench, R. K. 1993. Microalgal-invertebrate symbioses: a review. *Endocyto. Cell. Res.* 9:135–75.

Trench, R. K. & Thinh, L. V. 1995. *Gymnodinium linucheae* sp. nov.: the dinoflagellate symbiont of the jellyfish *Linuche unguiculata*. *Eur. J. Phycol.* 30:149–54.

Warner, M. E., Fitt, W. K. & Schmidt, G. W. 1999. Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching. *Proc. Natl. Acad. Sci. USA* 96:8007–12.

Watanabe, M. M., Kasai, F. & Sudo, R. 1988. NIES collection list of strains. Microalgae and protozoa. *In* Watanabe, M. M., Kasai, F. & Sudo, R. [Eds.] *The Microbial Culture Collection*, 2nd ed. The Natural Institute for Environmental Studies, Tsukuba, Japan, p. 148.

Wilcox, T. P. 1998. Large-subunit ribosomal RNA systematics of symbiotic dinoflagellates: morphology does not recapitulate phylogeny. *Mol. Phylo. Evol.* 10:436–48.

Wintzingerode, F. V., Gobel, U. B. & Stackebrandt, E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* 21:213–29.

Zahl, P. A. & McLaughlin J. J. A. 1957. Isolation and cultivation of zooxanthellae. *Nature* 180:199–200.

Zahl, P. A. & McLaughlin J. J. A. 1959. Studies in marine biology. IV. On the role of algal cells in the tissues of marine invertebrates. *J. Protozool.* 6:344–52.