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 discordant 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 foraminifersans, 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, Ambaryianto 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, Darius 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,
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 mollusk hosts 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, zooxanthellae 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 zooxanthellae 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 1/2 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 1/2 medium by centrifugation at 700g for 5 min. Zooxanthellae were brought into bulk culture by inoculating a cell concentrator with 10% of the approximately 750-base pair (bp) PCR products using 5 μg/mL Actinomycin D for 20 h, irradiance level of 80 μmol photons·m−2·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 1/2 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, after purification, DNA samples were digested with Taq I restriction enzyme to generate RFLPs (Rowan and Powers 1991b). RFLP analysis of 18S rDNA PCR products was determined by comparison with the reverse sequence or coded using the IUPAC ambiguity codes. Consensus sequences of 657 bp were aligned with PAUP*4.0 (Swofford 2000). The ITS-rDNA sequences and analyses. The relatedness between cultured zooxanthellae and in hospite populations of the host species from which they originate was determined by establishing cultures from the Caribbean gorgonians Plexaur a 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 identity and PCR-template quality. The ribosomal ITS-rDNA region was PCR amplified using the primers ZITSUPM13 (5′-CAGGCG TTGTAAGAACGCCGGGTTAGATTTGCGACTGACCGAGT GCT-3′) and ZITSDNM13 (5′-GGATACAAATTCACACAGC GCTTAGTTCCTTTTCCTGC-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 μM Tris-SC (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 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., Watertown, MA). PCR products generated with the primer set ZITSUPM13/ZITSDNM13 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 Symbiodi num 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 originate was determined by establishing cultures from the Caribbean gorgonians Plexaura kun a, 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 identity and PCR-template quality. The ribosomal ITS-rDNA region was PCR amplified using the primers ZITSUPM13 (5′-CAGGCG TTGTAAGAACGCCGGGTTAGATTTGCGACTGACCGAGT GCT-3′) and ZITSDNM13 (5′-GGATACAAATTCACACAGC GCTTAGTTCCTTTTCCTGC-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 μM Tris-SC (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 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 (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 Sequencer™ 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 scel-
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. treebase.org/treebase/). Microsatellite amplifications were performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 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 30 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 zoanthellae 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 zooxanthellae 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'-TGCACCTGCGGCGCAAATG-3' | 155 |
| GA2.8DN | 5'-GGGATGAAACACTGGGATAATCCAG-3' | 170 |
| GA4.84UP | 5'-GACCAACCTTTGATGAC-3' | 119 |
| GA4.84DN | 5'-GTCAGATTTGTCATCAAGACTGC-3' | 119 |
| CA6.10UP | 5'-GGTCCAGCTGGAACGAC-3' | 119 |
| CA6.10DN | 5'-CCCTAGAGAGACACCATAGAGAC-3' | 119 |

“CA” or “GA” in primer name designates type of dinucleotide repeat.
amplification with primers ss5 and ss3z (Rowan and Powers 1991b) of RFLP from amplification with Antithesis pallida zooxanthella cultures initially and 1 year after isolation.

This sampling revealed that the sides of the flask at the air–medium interface. These areas included three spots 120 degrees apart. Samples of cells from various areas of nonhostive culture via a founder effect. Given these observations, antibiotic treatment may act as a selective force during culturing. This possibility deserves further investigation.

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 zooxanthellae 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 zooxanthellae 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 zooxanthellae 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.

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 zooxanthellae 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 fulchella (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 zooxanthellae 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 Gracilariaopsis, 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. mutisieris. 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
Pseudopterogorgia americana in hospite (12 ft. Mound Reef, Florida)
Plexaura kuna in hospite (North Bocas del Toro, Panama)
Plexaura kuna in hospite (Nan tu po, Panama)
Plexaura kuna in hospite (South Bocas del Toro, Panama)
Plexaura kuna in hospite (Bache Reef, Florida)
Plexaura kuna in hospite (12 ft. Mound Reef, Florida)
Plexaura kuna in hospite (St. Croix, US Virgin Islands)
Plexaura kuna culture #13 (Bache Reef, Florida)
Plexaura kuna in hospite (Sail Rock, San Blas, Panama)
Plexaura horomallia in hospite (Sail Rock, San Blas, Panama)
Plexaura kuna culture #706 (Sail Rock, San Blas, Panama)
Pseudopterogorgia elisabethae in hospite (San Salvador, Bahamas)
Plexaura flexuosa in hospite (Tennessee Reef, Florida)
Gorgonia sp. in hospite (12 ft. Mound Reef, Florida)
Pseudopterogorgia elisabethae culture (San Salvador, Bahamas)
Plexaura kuna in hospite (Bahamas)
Plexaura kuna culture #704 (San Blas, Panama)
Eunicea sp. in hospite (12 ft. Mound Reef, Florida)
Briareum asbestinum in hospite (Craig Key, Florida)
Plexaura kuna culture #702 (Sail Rock, San Blas, Panama)
Plexaura flexuosa culture (Tennessee Reef, Florida)

5 changes

Montipora verrucosa culture (Symbiodinium kawaguti) (Kaneohe Bay, Hawaii)

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 Symbiodinium 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-
CULTURE VS. **IN HOSPITE** ZOOXANTHALLAE

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**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) 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** population.

![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.](image-url)
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 (Dowell 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 become apparent after culturing with certain growth media. These possibilities deserve further investigation.

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, Rowan et al. 1996, Belda-Baillie et al. 1999, Carlos et al. 1999, Baillie et al. 2000a,b, Carlos et al. 2000).

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.

<table>
<thead>
<tr>
<th>Host species</th>
<th>Banaszak et al. (2000) 18S-rDNA RFLP</th>
<th>This study 18S-rDNA RFLP</th>
<th>In hospite 18S-rDNA RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastigias sp.</td>
<td>A (Belau)</td>
<td>C (Palau)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Caribbean Aiptasia anemones</td>
<td>B (Puerto Rico)</td>
<td>A and B; B (Middle Florida Keys)</td>
<td>A&lt;sup&gt;a&lt;/sup&gt;, A and B&lt;sup&gt;b&lt;/sup&gt;, B&lt;sup&gt;c&lt;/sup&gt;, B&lt;sup&gt;d&lt;/sup&gt;, B&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pocillopora damicornis</td>
<td>B (Hawaii)</td>
<td>B (Hawaii)</td>
<td>C&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Class Alcyonacea (gorgonians)</td>
<td>A (Caribbean) (2 out of 3 cultures)</td>
<td>B (Caribbean)</td>
<td>B&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridacna gigas</td>
<td>A (GBR, Australia)</td>
<td>A (Indo-Pacific)</td>
<td>A and C&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

n.d., no data available. Geographic origins of individual hosts are in parentheses.

* Coffroth et al. (2001).
* This study.
* Rowan et al. (1996), Belda-Baillie et al. (1999), Carlos et al. (1999), Baillie et al. (2000a,b), Carlos et al. (2000).
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 harvested 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

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

<table>
<thead>
<tr>
<th>Name of medium</th>
<th>Formulation by</th>
<th>Used by</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP-8A</td>
<td>Ahles (1967)</td>
<td>Schoenberger and Trench (1980a)</td>
</tr>
<tr>
<td>M series</td>
<td>McLaughlin and Zahl (1959)</td>
<td>McLaughlin and Zahl (1959)</td>
</tr>
<tr>
<td>CSI</td>
<td>McLaughlin and Zahl (1959)</td>
<td>McLaughlin and Zahl (1959)</td>
</tr>
<tr>
<td>f/2</td>
<td>Guillard and Ryther (1962)</td>
<td>Kinzie et al. (1984)</td>
</tr>
<tr>
<td>ES</td>
<td>Provasoli (1968)</td>
<td>Kinzie and Chee (1979)</td>
</tr>
<tr>
<td>ISM</td>
<td>Lee et al. (1980)</td>
<td>Lee et al. (1995)</td>
</tr>
</tbody>
</table>

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...
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.

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