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T.C. LaJeunesse

Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs

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Abstract A community ecology approach to the study of the most common group of zooxanthellae, dinoflagellates in the genus *Symbiodinium*, was applied to symbiotic invertebrate assemblages on coral reefs in the western Caribbean, off the Yucatan peninsula (Puerto Morelos, Mexico) and over 1000 km away in the northeastern Caribbean, at Lee Stocking Island, Bahamas. Sequence differences and intragenomic variation, as determined by denaturing gradient gel electrophoresis and sequencing of the internal transcribed spacer 2 (ITS 2) region, were used to classify these symbionts. Twenty-eight genetically distinct *Symbiodinium* types were identified, eleven of which were found in hosts from both Caribbean locations. A single symbiont population was detected in 72% of hosts from the Yucatan and 92% of hosts from the Bahamas. The reef-wide community distribution of these symbionts is dominated by a few types found in many different host taxa, while numerous rare types appear to have high specificity for a particular host species or genus. Clade or lineage A *Symbiodinium* spp. was restricted to compatible hosts located within 3–4 m of the surface, while *Symbiodinium* spp. types from other lineages displayed differences in vertical zonation correlated with ITS type but were independent of clade designation. A comparison of the symbiont types found in field-collected hosts with types previously cultured from these hosts indicates the existence of low density or "background"-symbiont populations and cryptic, potentially non-mutualistic types in some hosts.

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T.C. LaJeunesse
Department of Ecology, Evolution and Marine Biology,
University of California at Santa Barbara,
Santa Barbara, CA 93106, USA

Present address: T.C. LaJeunesse
Department of Botany,
Plant Sciences Building, University of Georgia,
Athens, GA 30602, USA,
e-mail: lajeunes@dogwood.botany.uga.edu,
Tel.: +1-706-5420279, Fax: +1-706-5421805

Introduction

Tropical marine ecosystems possess the highest diversity and abundance of epibenthic invertebrates that contain zooxanthellae. Dinoflagellates in the genus *Symbiodinium* represent the most common group of these photosynthetic endosymbionts, and their presence contributes substantially to the productivity, survival, and success of their hosts (Muscatine and Porter 1977). These symbiont populations may reach densities of several million or more per square centimeter of host tissue, levels that would not otherwise exist freely in the nutrient-poor, external environment. As symbionts, they are one of the most abundant and important photosynthetic protist groups in tropical reef ecosystems.

Over the past several decades, analysis of various cultured *Symbiodinium* spp. isolates have repeatedly shown that high morphological, biochemical, and physiological diversity exists within the genus (Schoenberg and Trench 1980a,b,c; Chang et al. 1983; Tytler and Trench 1986; Trench and Blank 1987; Govind et al. 1990; Markell and Trench 1993; Trench 1993; Iglesias-Prieto and Trench 1994, 1997b). Though there is no consensus regarding what defines a species among the *Symbiodinium* spp., a consensus is emerging that the genus comprises a yet to be defined number of ecologically distinct types or "species" (Trench 1993; Rowan 1998; Carlos et al. 1999; LaJeunesse 2001).

Molecular methods incorporating the small and large ribosomal subunits (SSUrDNA and LSUrDNA) and, more recently, the internal transcribed spacer (ITS) region have rapidly advanced our understanding of the diversity, ecology, and evolution within the genus *Symbiodinium* (Rowan and Powers 1991; McNally et al. 1994; Rowan and Knowlton 1995; Baker and Rowan 1997; Wilcox 1998; Carlos et al. 1999; LaJeunesse and

Trench 2000; LaJeunesse 2001; Pawlowski 2001; Pochon et al. 2001; Van Oppen et al. 2001). Most of these molecular studies assessed the diversity and distribution patterns of *Symbiodinium* spp. populations in symbiosis with particular host groups (e.g. scleractinians), or species (e.g. *Montastraea* spp., *Anthopleura elegantissima*) (Baker and Rowan 1997; Rowan et al. 1997; Baker 1999; LaJeunesse and Trench 2000). However, the ecosystem-wide diversity and community ecology of these symbionts is poorly understood (Baker and Rowan 1997).

The present study addresses the diversity, prevalence, and distribution of *Symbiodinium* spp. in two coral reef ecosystems separated by over 1000 km: Puerto Morelos on the Yucatan Peninsula, western Caribbean, and Lee Stocking Island, Bahamas, northeastern Caribbean. Past observations have indicated that one symbiont type usually occurs in hosts of the same species from similar environments (Rowan and Powers 1991; Baker and Rowan 1997; Baker 1999; LaJeunesse and Trench 2000), but distinct symbiont types can be found in different host species and/or from individuals sampled from different environments (Rowan et al. 1997; Baker 1999). Therefore, sampling from a wide diversity of hosts and from different environments is expected to uncover still greater *Symbiodinium* spp. diversity. For these reasons, symbiotic invertebrates including scleractinians, hydrozoans (e.g. the firecoral genus *Millepora*), scyphozoans (e.g. jellyfish, *Cassiopeia xamachana*), actiniarians (anemones), zooanthids, corallimorphs, gorgonians, and a mollusc (queen conch) were sampled from the lagoon, back reef, reef crest, and fore reef zones to define the symbiont diversity within entire reef invertebrate communities. The unusual *Symbiodinium* spp. assemblages found in soritid foraminiferans were not analyzed in this study (cf. Pochon et al. 2001).

The variable ITS region is presently the most useful genetic marker for distinguishing symbiont types that differ in their ecological distribution, physiological fitness, and/or host infectability (cf. LaJeunesse 2001). The high level of molecular resolution provided by this region is deemed necessary because the sub-generic clades of the genus *Symbiodinium* (e.g. A, B, C, etc.), as assessed by the low resolution of ribosomal DNA RFLP (restriction fragment length polymorphism) methodology, correlate poorly with their ecological distribution; each "clade" contains representatives with markedly different physiological attributes and host specificities, and therefore different ecologies (Iglesias-Prieto and Trench 1997a,b; LaJeunesse 2001). Thus the ITS region is used to provide a more definitive assessment of the biodiversity of *Symbiodinium* spp.

Here, PCR (polymerase chain reaction) amplification of DNA extracts for ITS 2 from symbiont populations obtained from host tissues were coupled with denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla 1998; Abrams and Stanton 1992). This technique produced high-resolution banding profiles, from which diagnostic bands were further characterized by sequencing and phylogenetic analyses. By assigning specific profiles to particular symbiont types, the DGGE method is shown to provide precise identifications of symbionts from large numbers of field samples, minimizing the need for DNA sequencing.

Materials and methods

Collection of invertebrate hosts

In August 1998 and September 1999 symbiotic invertebrates were collected by SCUBA or snorkel at several locations on the barrier reef off Puerto Morelos, northeast coast of the Yucatan Peninsula in the province of Quintana Roo, Mexico (20°50′N; 086°52′W). Collections were made from the shallow lagoon (1–3 m), back reef (3–5 m), and reef crest (1.5 m) at Bocana reef, and the fore reef at Petem Pitch (10–15 m). Host species were collected from those reef zones where they were most common. Sixty-nine host species representing seven cnidarian orders (Scleractinia, Actinaria, Gorgonacea, Zoanthidea, Corallimorpharia, Rhizostomae, Milleporina) and one molluscan species (the queen conch, *Strombus gigas*) were sampled (see Table 1). Some species were collected from additional environments to determine how physical conditions modulates the identity of symbiont populations. The specimens were processed immediately after collection or maintained in a covered flow-through seawater tank and processed usually within 36 h after collection.

For comparison, 27 species of host invertebrates were collected at several locations off Lee Stocking Island (North Norman's Reef, Rainbow Gardens Reef, and South Perry) in the Bahamas (24°15′N; 076°30′W) in November 1999, March 2000, and August–September 2001 (see Table 2). This sampling was conducted to test whether symbiont diversity from reefs at one location was similar to that on reefs located approximately 1050 km away.

Symbiodinium spp. isolation from host tissues

The isolation of *Symbiodinium* spp. from host tissues followed one of three procedures depending on the type of host tissue. Gorgonian tissues were processed immediately upon collection. Colony branches were cut into 2- to 3-cm fragments, placed in a mortar and ground with a pestle in 100 ml of extraction buffer (1.2 μm filtered seawater and 5 mM EDTA). The resulting slurry was sometimes filtered through a 100 μm mesh to remove large fragments of host tissue and mucus.

In the case of actinarians, corallimorphs, and zoanthids, the soft fleshy bodies of these species were first cleaned by hand of debris. For each individual, the entire oral disk, including tentacles, was removed and diced into small pieces using a razor blade. This material was combined with 15 ml of extraction buffer and processed in a tissue grinder (Pyrex no. 7727-07) to separate algal cells from host tissues.

To extract symbionts from scleractinians, tissue was removed from 5 to 50 cm^2 of coral skeleton surface using a Water Pik and extraction buffer. This process was conducted in a plastic zip-lock bag to collect the effluent. For those species that produced large amounts of mucus, the bag was sealed and shaken vigorously for 1–3 min to disrupt the mucopolysaccarides.

All host extracts were centrifuged in 250-ml screw cap containers for 5 min at 1000 *g* using a swinging bucket rotor in a Beckman model TJ-6 centrifuge. The pellet, often viscous with mucus, was resuspended in 30 ml of extraction buffer containing a non-ionic detergent (0.01%Triton X-100), and homogenized in a tissue grinder (Pyrex no. 7727-07). The slurry was centrifuged at 925 *g* using a fixed angle rotor. This step helped purify the algal component further by lysing and removing most of the remaining host cell debris as determined microscopically at ×200 magnification. The pellet was immediately resuspended in filtered seawater (1.2 μm) with EDTA (5 mM) by gentle mixing. This wash step was repeated once more, and the resulting pellet stored in a high salt

Table 1. Host species, collection depth, number of symbiont types detected by PCR-DGGE, and their genetic identity. Collections were from reefs at Puerto Morelos, northeastern coast of Yucatan peninsula, Mexico. *Numerals in parentheses* indicate the number of colonies independently sampled

Host sampled	Collection depth (m)	No. of symbiont types	Genetic identity
Phylum Cnidaria			
Scyphozoa (jellyfish)			
Cassiopeia xamachana	0.5	3	Al, A3, C1
Hydrozoa (firecorals)			
Millepora alcicornis	2	2	A4a, A3
Millepora complanata	2	1	B1
Anthozoa			
Scleractinia (corals)			
Acropora cervicornis	4	1	A3
Acropora palmata	1.5	1	A3
Agaricia agaricites f. *agaricites* (2)	15	1	C3a
Agaricia agaricites f. *danai*	4.5	1	C3a
Agaricia fragilis	11	1	C3a
Agaricia humilis	2.5	3	C3a, C1, D1a
Agaricia tenuifolia	4.5	1	C3a
Cladocora arbuscula	2	1	B1
Colpophyllia natans	4	2	B6, C1
Dendrogyra cylindrus	4	1	B1
Diploria c1ivosa	2.5	1	B1
Diploria labyrinthiformis	12	1	B1
Diploria strigosa	2.5	2	B1, C1
Dichocoenia stokesii	2.5	1	B1
Eusmilia fastigiata	4	1	B1
Eusmilia fastigiata	12	2	B1, C1
Favia fragum	1.5	2	B1, C1
Isophyllastrea rigida	5	1	C3
Isophyllia sinuosa	5	1	C3c
Leptoseris cucullata	10	1	C3
Madracis decactis	13	1	B7
Manicina aereolata	2.5	2	B1, C1
Meandrina meandrites	14	1	B1
Montastraea faveolata	2	1	D1a
Montastraea faveolata	15	1	C7
Montastraea cavernosa	2.5	1	C3d (C3e)
Mycetophyllia danaana	13	1	C3c
Mycetophyllia lamarckiana (3)	12	1	C3c
Mycetophyllia sp.	11	3	C3c, C7, C6
Porites astereoides	2.5	3	A4a, A3, B1
Porites colonensis (2)	12	1	C1a
Porites divaricata	2.5	1	C9
Porites furcata	1.5	2	A4, B1
Porites furcata (6)	5	1	C4
Siderastrea radians	2.5	1	B5a
Siderastrea siderea	2.5	1	C1
Stephanocoenia intercepta	2.5	1	A3
Actinaria (anemones)			
Actinoporus elegans	2	1	B1
Bartholomea annulata	2	1	C1
Condylactis gigantea	2.5	3	A4a, C1, A3
Lebrunia danae	10	1	C1
Rhodactis (*Heteractis*) *lucida*	3	1	C1
Viatrix antilliensis (= *Bunodeopsis lobulifera*) (5)	2.5	1	C3
Stichodactyla helianthus	2	1	A4a
Gorgonacea (sea fans, sea whips)			
Erythropodium caribaeorum	12	1	C3
Eunicea mammosa	4	1	B1
Eunicea tourneforti	12	2	B1, C1
Eunicea laciniata	2.5	1	B1
Eunicea ciavigera	15	2	B1, C1
Gorgonia flabellum	4.5	1	B1
Gorgonia mariae	4	1	B1
Muricea muricata	15	1	B1
Plexaura homamalla	2.5	2	B1, C1
Plexaura flexuosa	2.5	2	B1b
Plexaurella nutans	13	1	B1a
Pseudoplexaura wagenaari	2.5	1	B1
Pseudoplexaura flagellosa (2)	2.5	1	B1

Table 1. (Continued)

Host sampled	Collection depth (m)	No. of symbiont types	Genetic identity
Pseudopterogorgia rigida	4.5	1	B1
Pseudopterogorgia kallos	4.5	1	B1
Pseudopterogorgia americana	2.5	1	B1
Pseudopterogorgia americana	15	1	B1
Pterogorgia anceps	2.5	1	B1
Zoanthidea (button polyps)			
Palythoa caribaeorum (2)	1.5	1	C1
Palythoa grandis	12	1	C3
Zoanthus sociatus	3	4	A4, A3, B1, C1
Zoanthus sp. (grey)	15	1	C5
Corallimorpharia (mushroom polyps)			
Discosoma carlgreni	4	1	C1
Discosoma sanctithomae (2)	4	1	C1
Ricordea forida	12	1	C3c
Phylum Mollusca (Gastropoda)			
Strombus gigas (digestive gland)	5	1	C4
Strombits gigas (digestive tact)	5	2	C4, B1

Table 2. Host species, collection depth, number of symbiont types detected by PCR-DGGE, and their genetic identity. Collections were from reefs at Lee Stocking island, Bahamas. *Numerals in parentheses* indicate number of colonies independently sampled

Host sampled	Collection depth (m)	No. of symbiont types	Genetic identity
Phylum Cnidaria			
Scyphozoa (jellyfish)			
Linuche unguiculata (polyp)	7	1	C1
Hydrozoa (firecorals)			
Millepora alcicornis	4	1	B1
Myrionema ambionense	0.2	1	A4
Anthozoa			
Scleractinia (corals)			
Acropora cervicornis (4)	3	1	A3
Acropora cervicornis	12	1	C12
Acropora palmata (2)	3	1	A3
Agaricia agaricites	12	1	C3c
Colpophyllia natans	12	2	B6, B9
Eusmilia fastigiata	4	1	B1
Montastraea annularis (5)	4	1	B1
Montastraea annularis	4	2	B1, C3
Montastraea annularis	14	1	D1a
Montastraea annularis	14	1	B1
Montastraea cavernosa	12	1	C3
Montastraea faveolata	4	1	D1a
Montastraea faveolata (3)	4	1	B1
Montastraea faveolata	14	1	C12
Madracis decactis	12	1	B7
Madracis formosa	4	1	B1
Porites astereoides	4	1	A4a
Porites divarieata	4	1	C1
Porites porites (2)	4	1	C10
Scolymia cubensis	4	1	C11
Scolymia cubensis	12	1	C11
Siderastrea radians	1.5	1	B5a
Stephanocoenia intercepta	4	1	A3
Actinaria (anemones)			
Condylactis gigantea	1	1	A4
Lebrunia danae	4	1	C1
Gorgonacea (sea fans, sea whips)			
Eunicea mammosa	11	1	B9
Eunicea mammosa	4	1	B1
Gorgonia mariae	12	1	B1
Plexaurella nutans	4.5	1	B1
Plexaura homamalla	4	1	B1a
Pseudoplexaura flexuosa	4	1	B8
Zoanthidea (button polyps)			
Zoanthus sociatus	0.5	1	A3

(NaCl-saturated), DMSO (20%), EDTA (0.25 M) preservation buffer (Seutin et al. 1991).

DNA extraction, PCR, restriction digestion, and DGGE

About 15–40 mg of pelleted algae was rinsed with 1 M Tris-EDTA to remove excess preservation buffer. A spin-column DNA purification protocol described in the DNeasy plant mini kit (Qiagen, Santa Clarita, Calif.) was utilized and found to yield higher quantity and quality genomic DNA than the Qiagen animal tissue kit used previously (LaJeunesse and Trench 2000). The final eluate from each sample was diluted 1:10, and 1 µl was used as a template for amplification.

The small subunit ribosomal RNA gene (SSUrDNA) from each sample was amplified and restriction digested. The primers ss5z and ss3z of Rowan and Powers (1991) were used in SSUrDNA amplifications conducted under the following conditions: an initial denaturing step of 3 min at 92°C followed by 35 cycles of 30 s at 92°C, 40 s at 52°C, and 30 s at 72°C, followed by two cycles of 5 min at 72°C. Restriction digests of these amplification products were performed by incubating with 1–5 U of *Taq* I (New England BioLabs, Beverly, Mass.) at 65°C for 3–5 h. Products of the digests were separated by electrophoresis in a 2.5% high-melt agarose gel at a constant 70 V for 3 h, and the resulting pattern was scored as *Symbiodinium* spp. lineages A, B, C, or D based on the designations of Rowan and Powers (1991) (cf. Baker 1999, 2001).

The internal transcribed spacer 2 region (ITS 2) was then amplified for DGGE analysis. Primers for PCR-DGGE analyses were designed previously (LaJeunesse and Trench 2000) to amplify a 330–360 bp product containing the ITS 2. An internal primer "ITSintfor2" (5′GAATTGCAGA ACTCCGTG-3′) anneals to a conserved region of the 5.8S rDNA and is paired with the conserved 3′ flanking primer, ITS-Reverse (Coleman et al. 1994). This end primer, called the "ITS2CLAMP", was modified with a 39 bp GC clamp (underlined) (Sheffield et al. 1989) (5′CGCCCGCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC GGGATC-CATA TGCTTAAGTT CAGCGGGT-3′). A "touchdown" amplification protocol with annealing conditions 10°C above the final annealing temperature of 52°C was designed to ensure specificity (Don et al. 1991). Every two cycles, the annealing temperature was decreased 1°C. After 20 cycles, the annealing temperature was maintained at 52°C for another 15–18 cycles.

Reaction products from samples containing *Symbiodinium* spp. belonging to the same phylogenetic lineage, as previously determined from SSUrDNA RFLPs, were run on a gradient gel along with ITS 2 standards prepared from cultured species (see below). All samples were loaded with a 2% Ficoll loading buffer (2% Ficoll-400, 10 mM Tris-HCL pH 7.8, 1 mM EDTA, 1% bromophenol blue) onto an 8% polyacrylamide denaturing gel containing a gradient of 3.15 M urea/18% deionized formamide to 5.6 M urea/37% deionized formamide and separated by electrophoresis for 9.5 h at 150 V at a constant temperature of 60°C. The gel was either stained in a 1× TAE and 5 µg ml^{-1} ethidium bromide solution for 15 min and washed in deionized water for 15 min, or stained with Syber Green (Molecular Probes, Eugene, Ore.) for 25 min using the manufacturer's specifications, and finally photographed.

Standards for PCR-DGGE analyses

Cultures representing 14 different ITS types were used to test the utility and sensitivity of DGGE (see Fig. 1). The signature profiles of these isolates were characterized and some used as standards for comparison with DGGE profiles from natural symbiont populations. Culturing conditions and DNA extraction procedures were described previously (LaJeunesse 2001).

Isolation of PCR products, DNA elution, re-amplification, and sequencing

Discreet, prominent bands from denaturing gels were excised and placed separately into 1.5 ml Eppendorf tubes containing 200 mg of 1 mm glass beads and 500 µl of 0.1 M Tris-EDTA. The tube was vortexed vigorously for 2 min to disrupt the acrylamide and then stored overnight at 4°C. Re-amplification was later performed with 3–4 µl of eluate using the "ITSintfor2" forward and the conserved 3′ flanking reverse primer lacking the GC clamp (Coleman et al. 1994). A touchdown PCR protocol similar to that described above, but with only 12–14 final cycles at 52°C, re-amplified the ITS 2 for sequencing. After purification using a Promega Wizard PCR-prep DNA kit (Promega, Madison, Wis.), cycle sequencing was accomplished in both directions using 3.2 pmol of the forward and reverse amplification primers separately. Reagents and reaction conditions were as specified in the ABI Prism Big Dye Terminator Cycle Sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif.). Reaction products were analyzed on an Applied Biosystems 310 genetic analyzer (Division of Perkin Elmer, Foster City, Calif.).

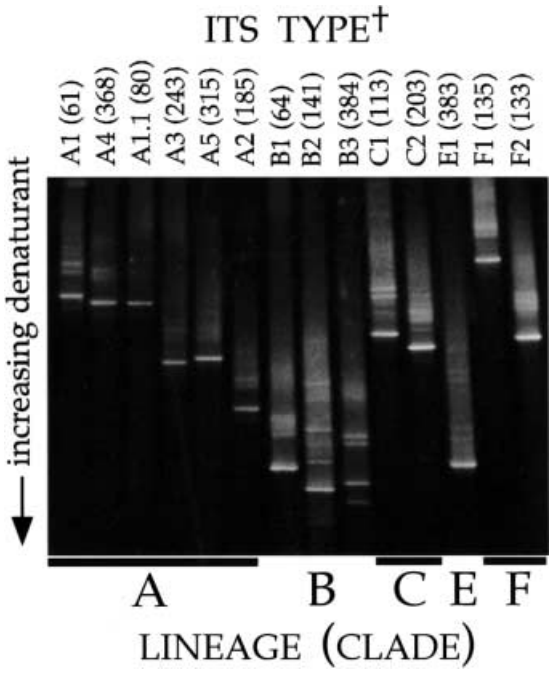

Fig. 1. *Symbiodinium* spp. PCR-DGGE (polymerase chain reaction–denaturing gradient gel electrophoresis) analysis of the ITS 2 (internal transcribed spacer 2) (300–350 bp) from cultured *Symbiodinium* spp. the ITS region of which was previously sequenced (LaJeunesse 2001). Among these profiles, several can be attributed to described species [A1, *Symbiodinium microadriaticum*; A2, *S. pilosum* (="*S. meandrinae*", ="*S. corculorum*"); A4, *Symbiodinium* (=*Gymnodinium*) *linucheae*; B1, "*S. pulchrorum*" (="*S. burmudense*"); C1, *S. goreaui*; F1, *S. kawagutii*]. Profiles are typically characterized by one distinctive band, however, the occurrence of more than one prominent band or lesser, fainter, bands is diagnostic and thus considered the "signature" of a distinct *Symbiodinium* spp. type (*cross* cf. LaJeunesse 2001)

Sequence analysis

Chromatograms were checked, edited, and sequences aligned using Sequence Navigator version 1.0 software (ABI, Division of Perkin Elmer, Foster City, Calif.). Cladistic analyses, using maximum parsimony and maximum likelihood were conducted on aligned data sets using PAUP 4.0b8 software under default settings (Swofford 1993). Under maximum parsimony, sequence gaps were designated as a fifth character state. A bootstrap re-sampling was conducted for 500 replicates to assess relative branch support (Felsenstein 1985).

Results

Analysis and interpretation of DGGE profiles

The DGGE profiles from cultured isolates were typically characterized by a single distinctive band sometimes accompanied by faint "background" bands (Fig. 1). Single base substitutions in the ITS 2 sequence were readily resolved by this method (e.g. Fig. 1, A1 vs. A1.1). Profiles from the cultured isolate B3 (culture 384) consistently produced four distinctive bands with similar intensities, yet only one ITS sequence was detected from direct cycle-sequencing of the amplification product. DGGE signatures containing four distinctive bands were commonly observed in analyses of natural populations (Fig. 2A). The presence of intragenomic variation or paralogous loci were inferred where migration differences between co-occurring bands could be attributed to minor sequence variation. These are believed to result when differences in ribosomal repeats have not been homogenized by concerted evolution (Arnheim 1983; Buckler et al. 1997). The "quartet" band profiles (Fig. 2A) result from two dominant paralogous loci present in the rDNA repeat region and are represented by the two lower bands most resistant to denaturation. The upper pair of bands are heteroduplexes formed by the denaturing and re-annealing of heterologous DNA strands during the PCR amplification process (Myers et al. 1989). In examples where paralogous loci were observed, the "combined" signature was scored as a distinct symbiont type. For example, the profile designated A4a represents a distinct *Symbiodinium* sp. type possessing two paralogous loci A4 and A4a. Regardless of the number and intensity of the bands produced, banding profiles from a genetically distinct *Symbiodinium* sp. type were highly repeatable and therefore diagnostic.

Complex profiles from field samples could be attributed to the presence of more than one *Symbiodinium* spp. type (Fig. 2B). A band or set of bands from these mixed profiles were indicative of a different symbiont type if they were observed independently or associated with different signature profiles in other samples. In samples with more than one symbiont type, heteroduplexes were not observed, presumably because substantial differences in ITS sequence prevented their formation.

In several cases, the host ITS 2 region was amplified together with that of the symbiont. Sequencing and NCBI GenBank BLAST searches identified those bands attributable to host DNA contamination. Thus, DGGE profiles with multiple bands were carefully assessed and attributed to one or more of the possibilities described above.

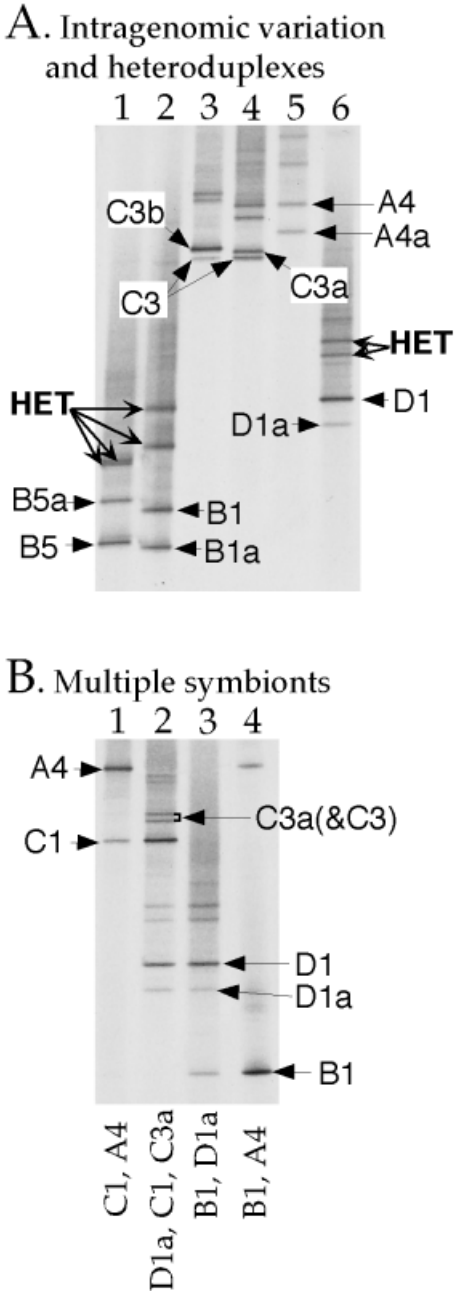

Fig. 2A, B. *Symbiodinium* spp. **A** A negative image of PCR-DGGE on *Symbiodinium* spp. types possessing two diagnostic paralogous rRNA genes. ITS sequence identity of each paralogue (lower homoduplex bands) is indicated. Paralogues are usually distinguished by a single base substitution or deletion. Heteroduplexes (*HET*) are formed during re-annealing stage of PCR that allows re-assortment creating two mismatched strands. The heteroduplexes are destabilized by these differences and melt before the homoduplexes. **B** Negative image of PCR-DGGE profiles indicating presence of more than one symbiont type. Designations beneath the image indicate the symbiont types detected

Diagnostic DGGE profiles and ITS 2 phylogenies

DGGE profiles diagnostic of different symbiont types in lineages (clades) A, B, and C are presented in Figs. 3, 4, and 5, respectively. Bands excised from DGGE gels and sequenced are indicated by arrows and labeled with their corresponding ITS designation. Distinct sequences, even if distinguished by a single base substitution, were assigned a "type" designation corresponding to their phylogenetic affiliation to previously characterized types

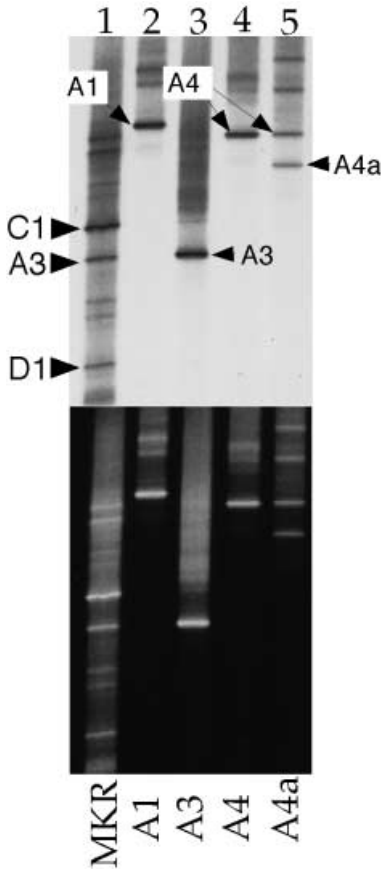

Fig. 3. *Symbiodinium* spp. PCR-DGGE profiles diagnostic of lineage A types observed in nature. Upper image is a negative of the lower and shows the identity of bands excised from the gel and sequenced. Designated symbiont types are indicated at bottom of gel by their phylogenetic designation; the *capital letter* indicates lineage (clade), the *number* represents ITS type, and *lower case letter* denotes a characteristic rDNA paralogue, where one exists. Standards (*MKR*) in lane 1 were pooled PCR amplifications from cultured C1 and A3 isolates and the non-cultured D1a type

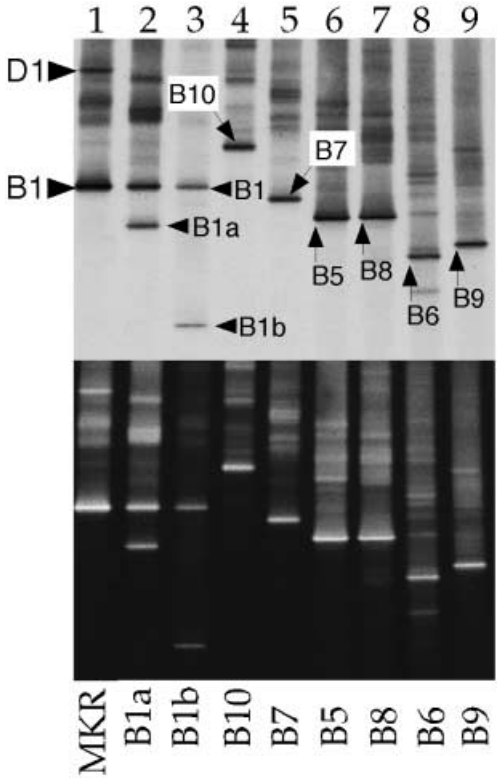

Fig. 4. *Symbiodinium* spp. Diagnostic PCR-DGGE profiles of lineage B types observed in nature. Upper image is a negative of the lower and shows the identity of bands excised from the gel and sequenced. Designated symbiont types are indicated at bottom of gel by their phylogenetic designation; the *capital letter* indicates lineage (clade), the *number* represents ITS type, and *lower case letter* denotes a characteristic rDNA paralogue, where one exists. Type B10 (lane 4) was identified in some *Montastraea annularis* from the Florida Keys. Standards (*MKR*) in lane 1 are pooled PCR amplifications from a cultured B1 isolate and the non-cultured D1a type

(cf. LaJeunesse 2001). Tables 1 and 2 summarize the distribution of these symbiont types in different host species collected at various depths and from different habitats.

ITS 2 phylogenies inferred from maximum parsimony are presented independently for each of the three major *Symbiodinium* spp. lineages (Figs. 6, 7, and 8). Sequences from types in different clades were not alignable and prohibited a genus-wide phylogenetic analysis. Only one distinct type, containing two ITS paralogues (D1 and D1a), was found from lineage D.

Symbiodinium spp. diversity at the ITS level

Twenty-eight distinctive *Symbiodinium* spp., distinguished by DGGE profiles and sequencing as described above, were identified from the hosts sampled in this study. Of these, 4 lineage A types, 9 lineage B types (type B10 originates from *Montastraea annularis* from Florida) 14 lineage C types, and 1 lineage D type were recognized.

At both Caribbean locations, the majority of host species sampled contained populations with only one diagnostic ITS 2 profile (50 of 69 from Yucatan reefs and 24 of 26 in the Bahamas). However, the sensitivity of DGGE for identifying types at very low concentrations was not determined. Scleractinians, actiniarians, zoanthids, and scyphozoans all had representatives that possessed mixed or polymorphic symbiont populations, and as many as four different symbiont types were identified from a single host. The greatest proportion of those hosts found to have polymorphic symbiont populations on Yucatan reefs were collected within 3 m of the surface (60%).

Community structure and the distribution of specific symbiont types

Figure 9 shows the prevalence of *Symbiodinium* spp. types in hosts collected at three different depths on reefs off Puerto Morelos, Yucatan, Mexico: 0–3 m corresponds with the lagoon and reef crest zones, 3–5 m with the back reef (rear zone), and 10–15 m with

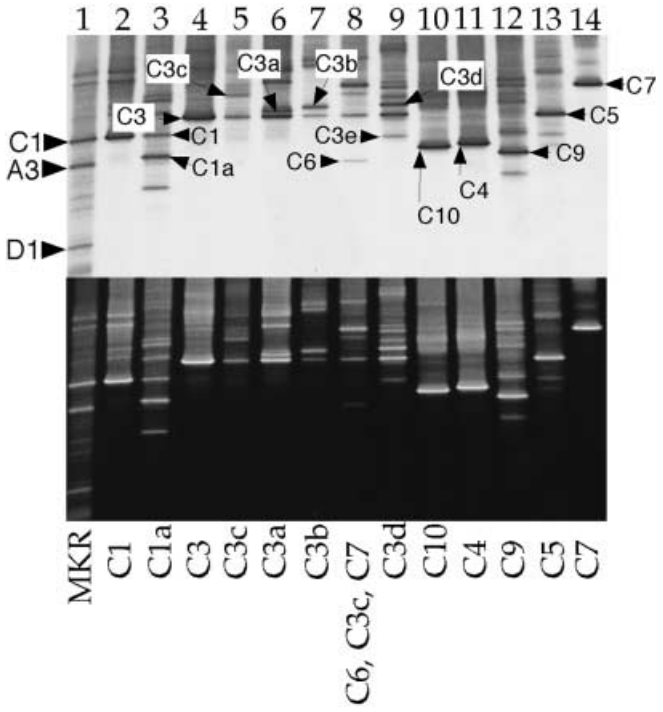

Fig. 5. *Symbiodinium* spp. Diagnostic PCR-DGGE profiles of lineage C types observed in nature. Upper image is a negative of the lower and shows the identity of bands excised from the gel and sequenced. Lanes 8 and 9 contain profiles from more than one symbiont type. Designated symbiont types are indicated at bottom of gel by their phylogenetic designation; the *capital letter* indicates lineage (clade), the *number* represents ITS type, and *lower case letter* denotes a characteristic rDNA paralogue, where one exists. Profiles for types C11 and C12 are not shown. Standards (*MKR*) in lane 1 are pooled PCR amplifications from cultured C1 and A3 isolates and the non-cultured D1a type

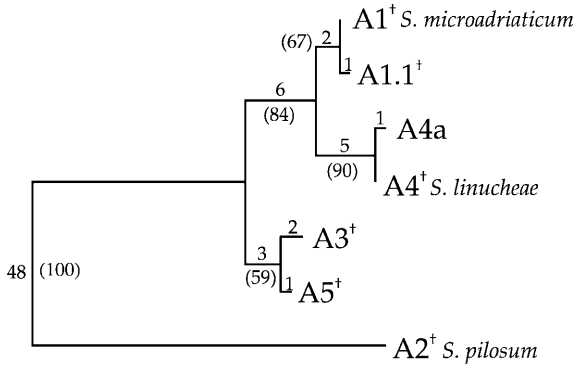

Fig. 6. *Symbiodinium* spp. Phylogenetic reconstructions of ITS 2 genes from lineage A types based on an alignment of 267 characters using maximum parsimony. Distinctive ITS 2 bands identified from the PCR-DGGE analyses of natural symbiont populations were sequenced and aligned. ITS sequences A1 (*Symbiodinium microadriaticum*) and A3 and A4 (*S. linucheae*) were observed in natural populations. Cultured A2 (*S. pilosum*) was never detected in field-collected hosts. The phylogram was rooted with *S. pilosum* (A2) as the outgroup. Gaps were included as a fifth character state. *Numbers above branch segments* indicate informative base pair differences. *Numbers in parentheses* are bootstrap values for 500 replicates (*cross* cf. LaJeunesse 2001)

the fore reef zone. The community of symbiotic invertebrates was dominated by several very common, relatively non-host-specific symbiont types (Fig. 9). Types B1, C1, C3 (C3c), and A3 were associated with a majority (about 70%) of the host species sampled. Prevalence is defined by the number of different hosts associated with a particular symbiont. Type B1 represents the most prevalent *Symbiodinium* sp. in the Caribbean. It was found associated with a wide diversity of host taxa, and nearly all species of gorgonian sampled at each collection depth possessed this type. However, the majority of symbionts characterized occurred rarely (Tables 1 and 2; Fig. 9). These uncommon *Symbiodinium* sp. types appear to associate specifically with certain host genera or species. Examples include, A1 with *Cassiopeia xamachana*, B5a with *Siderastrea radians*, B6 with *Colpophyllia natans*, B7 with *Madracis decactis*, C1a with *Porites colonensis* (*Porites* sp.), C5a and C5b with agaricids, and C11 with *Scolemia cubensis*.

The symbiont assemblage, while still characterized by several prevalent types, changes in host communities sampled from different environments. Symbionts from lineage A are major components of the shallowest host communities (0–3 m) but absent in hosts collected below 5 m. Differences in depth zonation were also observed between types within a lineage (e.g. C1 vs. C3 types). C3 and C3c symbiont types dominated the coral community at greater depths especially below 5 m in reefs off the Yucatan. Their hosts included mussid corals (*Mycetophyllia* spp., *Isophyllia sinuosa*, *Isophyllastrea rigida*), corallimorphs (*Ricordea florida*), zoanthids (*Palythoa grandis*), and encrusting soft corals (*Erythropodium caribaeorum*).

Shared diversity between distant Caribbean coral reef ecosystems

Eleven of 28 types (A4, A4a, A3, B1, B1a, B5a, B6, B7, C1, C3, and D1a) were documented from reefs in both the northeastern and western Caribbean. The limited sampling (less than half the number of host species) from the Bahamas may account for the lower diversity (17 types vs. 22 at Puerto Morelos) currently discovered at this location.

Discussion

This is the first study to examine the diversity and community structure of *Symbiodinium* spp. populations over an entire coral reef invertebrate assemblage. These data indicate that symbiont diversity is high, and although these dinoflagellates are mutual endosymbionts, they show patterns of dominance or prevalence similar to communities of free-living organisms.

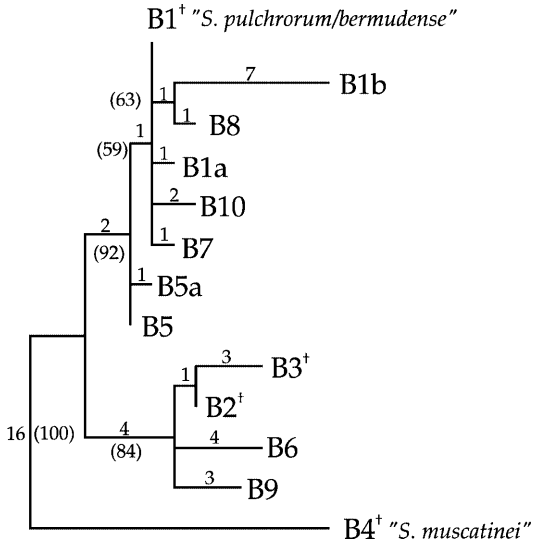

Fig. 7. *Symbiodinium* spp. Phylogenetic reconstructions of ITS 2 genes from lineage B types based on an alignment of 317 characters using maximum parsimony. Type B1 ("*Symbiodinium pulchrorum*"/"*S. burmudense*") was the only cultured isolate identified in natural populations. It is also the most prevalent symbiont found among different host species. The phylogram was rooted with "*S. muscatinei*" (B4) as the outgroup. Gaps were included as a fifth character state. *Numbers above branch segments* indicate informative base pair differences. *Numbers in parentheses* are bootstrap values for 500 replicates (*cross* cf. LaJeunesse 2001)

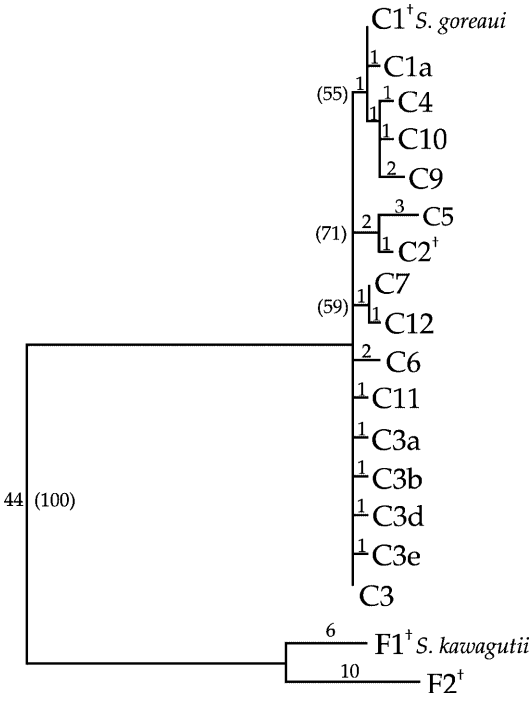

Fig. 8. *Symbiodinium* spp. Phylogenetic reconstructions of ITS 2 genes from lineage C types based on an alignment of 296 characters using maximum parsimony. Type C1 (*Symbiodinium goreaui*) and type C3 are distinguished by a single informative base substitution but occupy ecologically distinct niches based on their depth and host distributions. Type F1 (*S. kawagutii*) was used as the outgroup. Gaps were included as a fifth character state. *Numbers above branch segments* indicate informative base pair differences. *Numbers in parentheses* are bootstrap values for 500 replicates (*cross* cf. LaJeunesse 2001)

Prevalence

There are several explanations why the reef community of symbiotic invertebrates was dominated by certain *Symbiodinium* spp. Size, high rates of growth, nutrient uptake, and photosynthetic efficiency, along with a wide host range and photoacclimation ability, are likely attributes that may affect the distribution and competition and, hence, the prevalence and abundance of a particular symbiont type. On the Yucatan and Bahaman reefs, *Symbiodinium* sp. B1 types were observed in almost half the host taxa. What factors are most important in determining why B1 types are so prevalent awaits further physiological study. However, preliminary work on cultured isolates showed that type B1 possessed the greatest ability to photo-acclimate to high and low irradiances (Iglesias-Prieto and Trench 1997a). Lineage B types are morphologically the smallest group of *Symbiodinium* spp. in culture (LaJeunesse 2001). If these attributes, small size and photosynthetic plasticity, are applicable to natural populations, they may largely account for the prevalence of B1 in numerous Caribbean host species from different reef habitats.

Diversity and distribution

The extensive diversity of *Symbiodinium* spp. uncovered by this study is probably a fraction of what exists worldwide. Nonetheless, the finding that the same types occur in different Caribbean locations suggests a limited number of *Symbiodinium* types (or species) exist in this region. These data also confirm findings from cultured isolates that some ITS types are widely distributed (Baillie et al. 2000; LaJeunesse and Trench 2000; LaJeunesse 2001). Reefs in other locations will need extensive sampling similar to that conducted in Puerto Morelos before a more accurate estimation of *Symbiodinium* spp. diversity can be made, but, based on findings from these sites (and unpublished data), it is estimated that as many as 40 or more distinctive types populate invertebrate hosts in the Caribbean.

Several ecological processes may explain how diversity is maintained among so many, potentially competing, *Symbiodinium* spp. types. These mechanisms include niche diversification, environmental disequilibrium, and fugitive dispersal (cf. Venrick 1982). The variability of physical factors (e.g. light, flow, nutrients) on a coral reef coupled with the biochemical and histological differences among hosts may offer the environmental heterogeneity necessary for niche or ecological diversification. Differences in photoacclimation ability (Iglesias-Prieto and Trench 1997a,b) and host specificity (Schoenberg and Trench 1980c; Colley and Trench 1983) are evidence for such diversification among *Symbiodi-*

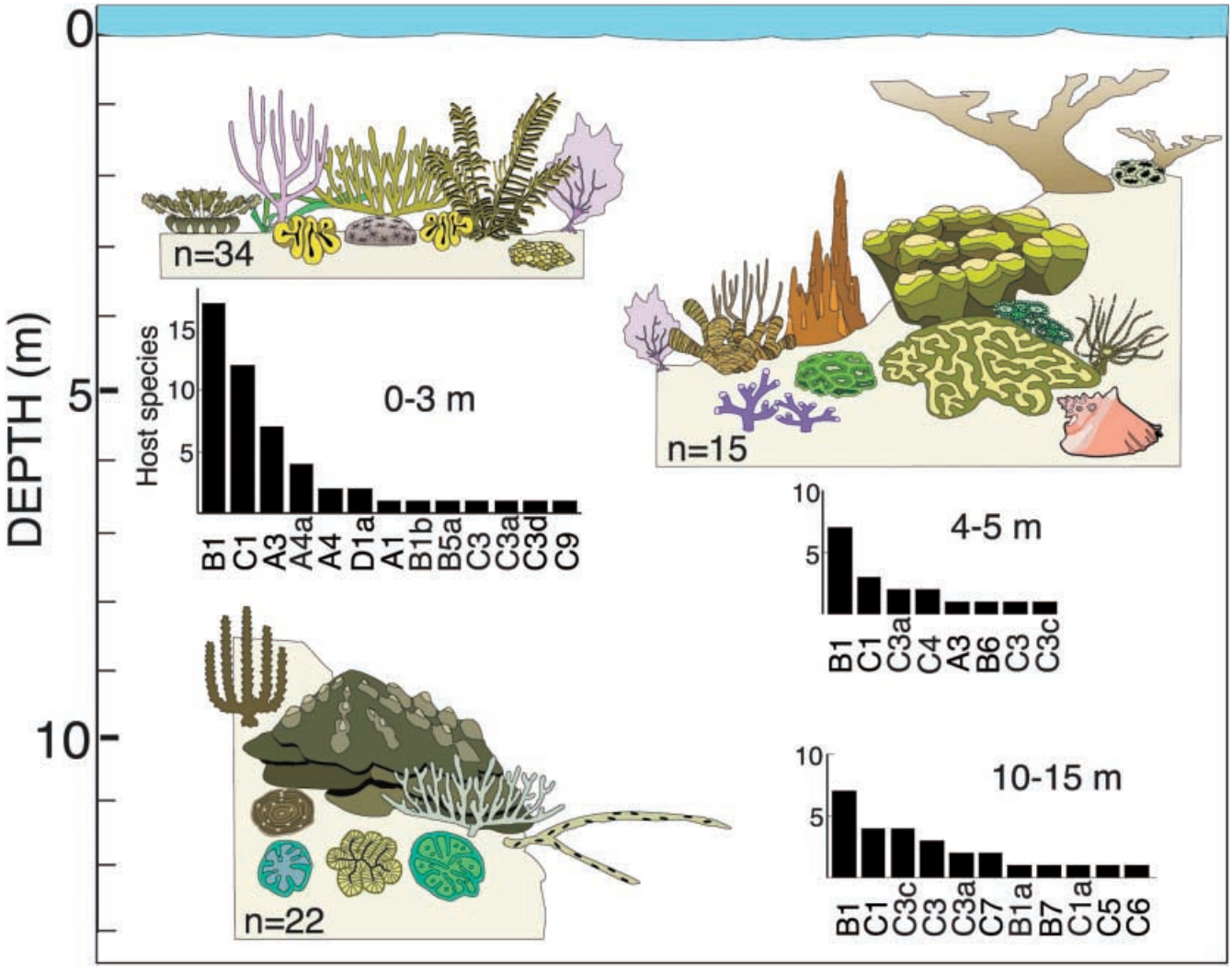

Fig. 9. *Symbiodinium* spp. Community structure and prevalence of *Symbiodinium* spp. types identified by PCR-DGGE from 69 different host species in relation to collection depth and reef zones at Puerto Morelos, Yucatan Peninsula, Mexico. Symbiont designations by ITS type are compared to the number of host species with which each associates. Depth of 0–3 m corresponds with the lagoon and reef crest zones; 3–5 m with the back reef (rear zone), and 10–15 m with the fore reef (*n* number of host species collected from each reef zone)

nium spp. Fugitive species may persist by occupying marginal habitats, such as shallow lagoons, or by rapidly colonizing newly settled aposymbiotic larvae (Coffroth et al. 2001), or by multiplying preferentially in the tissues of bleached adults (Baker 2001). Environments outside the host must also be considered as habitat refuges and an additional source of environmental heterogeneity.

Diversity and specificity

For all symbiotic associations, specialization is potentially important in contributing to species diversity. Until now, the use of rather highly conserved molecular markers has hindered interpretations of partner pairing between hosts and symbionts (Trench 1997). Previous studies have shown that hosts maintain symbioses with *Symbiodinium* spp. from one or more lineages within this genus (e.g. clade A, B, C, etc.) (Rowan and Knowlton 1995; Baker and Rowan 1997). However, it was uncertain whether their specificity extended to a particular type or species within each of the subgeneric clades. At the level of ITS resolution, there is provocative evidence of high specificity between some partners, and that these specific host–symbiont associations occur over wide geographic ranges.

Niche diversification through the formation of specific partnerships may create and maintain symbiont diversity. While there is no evidence of long-term coevolution between partners (Rowan and Powers 1991), there is the possibility that short-term coevolutionary processes during periods of relative stability may drive speciation, as data from the ITS region indicate. Determining how long they may persist over evolutionary or geological time scales would benefit from the calibration of a molecular clock.

The study of host–symbiont specificity is also central to the debate surrounding the effects of climate change on coral reef ecosystems. It is postulated that the long-term adaptive ability of symbiotic reef invertebrates may depend largely on the degree of specificity a host species has for a particular symbiont type (cf. Buddemeier and Fautin 1993). The observations made here and elsewhere (Baker 1999) indicate that certain hosts may be more susceptible than others due to their specificity for a particular symbiont type.

Depth zonation revisited

It is evident that some *Symbiodinium* spp. show marked zonation with respect to environmental gradients (Rowan and Knowlton 1995; Baker 1999; LaJeunesse and Trench 2000) and that these distribution patterns seem to be due to differences in physiology. The findings of this study confirm that *Symbiodinium* spp. of clade A are shallow-water specialists. Rowan and Knowlton (1995) first observed the distribution of these symbiont types in shallow-dwelling species of boulder coral (*Montastraea faveolata* and *M. annularis*) from Panama. In reefs from the Yucatan and Bahamas, lineage A types were constrained to compatible hosts living within 3–4 m of the surface. It was recently discovered that species in lineage A, albeit cultured, are the only *Symbiodinium* spp. known to produce measurable quantities of mycosporine-like amino acids (MAAs) (Banaszak et al. 2000), compounds providing protection from damaging UV irradiance (Neale et al. 1998). Protection from UV light through MAA synthesis may confer a competitive advantage under conditions of high irradiance. Interestingly, lineage A symbionts are rarely, if ever, observed in hosts from low photic environments. It is possible that the synthesis of MAAs (assuming that production is continuous) places them at a competitive disadvantage in low light environments.

While a portion of shallow-dwelling hosts contain lineage A *Symbiodinium* spp. there are numerous others that associate with *Symbiodinium* spp. in lineages B, C, and D. This is most evident in the Indo-Pacific, where corals at all depths standardly host symbionts from lineage C (Baker and Rowan 1997; Van Oppen et al. 2001). High symbiont diversity maintained in high photic environments (0–3 m) suggests that types from lineages other than clade A also possess adequate photoprotective mechanisms. Another possibility is that anatomical or biochemical differences in host tissues shift or dampen selection pressures attributed to high irradiance. Host tissues with fluorescent pigments or MAAs acquired though diet may provide protective screening (Banaszak and Trench 1995; Salih et al. 2000). The zoanthid *Palythoa caribaeorum* from the reef crest contains type C1 *Symbiodinium* spp. The thick calcified epidermis of this host may physically screen the internal symbiont populations in high PAR and UV environments. Therefore differences in host tissue opaqueness may also affect competition between different symbiont types and ultimately influence specificity.

Background versus cryptic populations

Though a majority of the hosts sampled in this study contained only one detectable symbiont type, evidence from cell cultures suggests that other symbiont types are present at low and presently undetectable concentrations in the host and that, when freshly isolated and placed into artificial growth media, they are potential competitors (Santos et al. 2001). In support of these conclusions, a list was generated of host species, the symbiont types that were cultured from them in the past, and types that were detected in the field (Table 3). In some examples, the symbiont type isolated in culture corresponds with what was observed as the "natural" population of that host. However, in most instances the cultured type is not the symbiont that populates the host in nature. These data indicate that hosts may contain low abundances of *Symbiodinium* spp. not detected by current molecular methods.

There appear to be two categories of symbionts that occur at low densities in host tissues. The first group may exist at low numbers in some host species, but are numerically dominant in others where they contribute significantly to the host's growth and survival. The anemone *Lebrunia danae* sampled at 10 m in the western Caribbean and 4 m from the northeastern Caribbean possessed C1 symbionts. However, the type in culture

Table 3. Comparison of host species, natural *Symbiodinium* spp. types detected (present study), and *Symbiodinium* spp. types previously cultured. *Numerals in parentheses* indicate culture numbers (LaJeunesse 2001)

Host	Natural types	Cultured isolates
Cassiopeia xamachana (Rhizostomae)	Al, A3, C1	Al, B1 (61, 74)
Linuche unguiculata (Rhizostomae)	C1	A4 (368)
Meandrina meandrites (Scleractinia)	B1	A2, F2 (130, 133)
Bartholomea annulata (Actiniaria)	C1	A2 (23, 24)
Condylactis gigantea (Actiniaria)	A3, A4, A4a, B1, C1	Al.1 (80)
Lebrunia danae (Actiniaria)	C1	B1 (125)
Rhodactis (*Heteractis*) *lucida* (Actinaria)	C1	C1 (113)
Plexaura homamalla (Gorgonacea)	B1, C1	A4 (379)
Zoanthus sociatus (Zoanthidea)	A3, Ma, B1, C1	A2 (185)
Discosoma sanctithomae (Corallimorpharia)	C1	C1 (152)

from this host is B1, a dominant type in other host species.

In contrast, a second group has defied detection in natural populations. One example is *Symbiodinium pilosum* (ITS type A2). Cultured from various hosts originating from the Pacific and Atlantic, it has never been detected in host tissues directly analyzed by current molecular methods (LaJeunesse 2001). The possession of an outer pilose surface, ejectile mucocysts (trichocysts) (Trench and Blank 1987), and the inability to infect cnidarian hosts (LaJeunesse 2001) suggest that *S. pilosum* is primarily a free-living species. Other species, like *S. pilosum*, may exist cryptically and occupy different ecological niches in the community. A clarification should be made between *Symbiodinium* spp. that are truly "cryptic" and symbionts that dominate the mutualistic symbiont populations in some hosts but persist in other hosts at very low densities.

A species concept for *Symbiodinium* spp.?

Applying the biological species concept to *Symbiodinium* spp. and protists in general, is impractical at this time. All current formal species in the genus *Symbiodinium* were described using the morphological species concept (cf. Trench and Blank 1987). This labor-intensive approach relies on cultures and electron microscopy to identify genetically fixed ultrastructural differences. Such methods may be utilized in future classifications, especially as more types are brought into culture. However, the lack of readily distinctive morphological traits among this group limits the utility of this concept in ecological investigations. Phylogenetic or "molecular" species concepts are more appropriate for making taxonomic distinctions that have ecological relevance.

The ITS sequences of some symbiont types were distinguished by a single base substitution and/or a unique paralogous locus. These slight differences could be attributed to intraspecific variation, yet types like C1 and C3 showed marked differences in host specificity and depth zonation. In fact, a majority of the symbiont types defined in this study displayed distinctive habitat distributions characterized by different host ranges or occupied hosts from different environments. These distinct patterns of distribution indicate that most types occupy different ecological niches and are therefore unique species by one classical definition (Hutchinson 1957). Regardless of whether an ITS "type" equates to species, minor differences in ITS sequences identify distinctive symbiont populations and are therefore ecologically informative markers.

Knowledge concerning the biology and biodiversity of *Symbiodinium* spp. remains limited. Further host sampling from different regions, characterizing new symbiont types, determining the extent of their host specificity and biogeography, and characterizing their physiology and photobiology are important goals for future investigations.

Coral reef symbioses and environmental change

Recent changes in climate, most notably the increased frequency of El Niño events, combined with increases in human development and our dependence on reef resources have led to the degradation of reefs worldwide (Hoegh-Guldberg 1999). Current research on the acclimatory and adaptive capabilities of dinoflagellate–invertebrate symbioses has shown that certain coral species are more tolerant of environmental stresses than others (Warner et al. 1996). Experiments on cultured isolates indicate that different *Symbiodinium* spp. are adapted to different environments and therefore photosynthesize optimally under different conditions (Chang et al. 1983; Iglesias-Prieto and Trench 1997a,b; Kinzie et al. 2001). These findings indicate that some partner combinations may be more resistant and maintain greater fitness than others with respect to climate change. A central question concerning coral reef ecosystem health is how these important symbiotic associations might adjust to environmental change. A greater understanding of *Symbiodinium* spp. community ecology and diversity, and the mechanisms governing host–symbiont specificity, can help in predicting how these systems will change with the climate.

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