

INVESTIGATING THE BIODIVERSITY, ECOLOGY, AND PHYLOGENY OF ENDOSYMBIOTIC DINOFLAGELLATES IN THE GENUS *SYMBIODINIUM* USING THE ITS REGION: IN SEARCH OF A “SPECIES” LEVEL MARKER¹

Todd C. LaJeunesse²

Department of Ecology, Evolution and Marine Biology, University of California at Santa Barbara, Santa Barbara, California 93106

The internal transcribed spacer (ITS) regions from 47 *Symbiodinium* (Freudenthal) isolates cultured from 34 different host species and two populations sampled from nature were sequenced and compared. Of these, 17 distinct ITS types were identified. The described species *Symbiodinium goreauii*, *S. kawagutii*, *S. pilosum* (Trench and Blank), *S. microadriaticum* (Freudenthal), and *S.* (= *Gymnodinium*) *linucheae* (Trench and Thinh) had ITS sequences distinct from each other. Four of these species share identical ITS sequences with uncharacterized isolates. Sequence differences among other isolates indicate that at least seven other cultured types await formal species descriptions, whereas numerous others most likely exist in nature. The *Symbiodinium* phylogeny is positively correlated with cell size, mycosporine-like amino acid production (UV protection), and host infectivity, whereas the production of water-soluble peridinin-chl *a*-protein homodimer and monomer apoproteins and isoenzyme similarity do not correlate. There is evidence, based on the lack of phylogenetic congruency with allelic variability, that sexual recombination occurs at some frequency among *Symbiodinium* populations. *Symbiodinium* isolates from the Caribbean possess identical ITS sequences to isolates originating from the Red Sea or the western Pacific. These findings indicate that some *Symbiodinium* species may have global biogeographic distributions.

Key index words: biogeography; dinoflagellate; internal transcribed spacer (ITS); isoenzyme; phylogeny; specificity; *Symbiodinium*; symbiosis; zooxanthellae

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit; sPCP, water-soluble peridinin-chl *a*-protein; SSU, small subunit; MAA, mycosporine-like amino acid

There are many kinds of symbiotic algae (Trench 1993). Of the eight dinoflagellate genera reported to have symbiotic representatives (Trench 1997), species of the genus *Symbiodinium* are the most common endosymbionts among coral reef-dwelling cnidarians, platyhelminths, mollusks, and protists (Trench 1993, Rowan 1998). Growing concerns over environmental change and worldwide reef ecosystem degradation

has heightened the need to characterize the diversity of these dinoflagellates and to use this information to learn more about their associations with reef-dwelling hosts (Rowan and Knowlton 1995). There is wide speculation as to how symbiotic organisms in coral reef communities will adjust to changes in climactic conditions (Baker 2001). If a component of host susceptibility to environmental change depends on the algal taxon it harbors (Iglesias-Prieto and Trench 1994, Warner et al. 1996, Iglesias-Prieto and Trench 1997a,b), then characterizing symbiont populations with different physiologies, observing how hosts associate with them, and determining how competition and environmental factors affect the distribution of these algal populations can provide insight into how these symbioses as a community will react to climactic change (Rowan et al. 1997).

Reviews by Blank and Trench (1986) and Trench (1997) provide an historical perspective of the initial morphological and taxonomic characterizations made on endosymbiotic dinoflagellates. The primary difficulty in distinguishing *Symbiodinium* species is due to the lack of readily discernible morphological features, especially in the nonmotile coccoid (spherical) stage. Initial estimates of diversity, based on light microscopy, were very low (Freudenthal 1962, Taylor 1974). *Symbiodinium microadriaticum* (Freudenthal), isolated from *Cassiopeia xamachana*, for many years represented the only known symbiont species. However, detailed investigations on cultures originating from different host species and geographic locations have revealed the existence of significant morphological, biochemical, and genetic differences within this genus (Schoenberg and Trench 1980a,b,c, Chang et al. 1983, McNally et al. 1994).

The early concept of low symbiont diversity (Taylor 1974, Smith and Douglas 1987) has given way to the recognition that diverse species assemblages occur within coral reef ecosystems (Trench 1993, 1997, Rowan 1998). Marked differences in cell morphology, ultrastructure, circadian rhythms, growth rates, host infectability, and photoacclimation have been identified between cultured isolates (Schoenberg and Trench 1980a,b,c, Fitt et al. 1981, Chang et al. 1983, Colley and Trench 1983, Fitt 1985, Trench and Blank 1987, Iglesias-Prieto and Trench 1994, 1997b). It is now accepted that the genus *Symbiodinium* contains many species, but only a few are formally described (Trench 1993, Rowan 1998, Baker 1999).

The use of molecular analyses to distinguish organisms at all taxonomic ranks has gathered wide acceptance (Avice 1994). DNA sequence diversity of the small

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²Author for correspondence and present address: Department of Botany, Plant Sciences Building, University of Georgia, Athens, GA 30602. E-mail: lajeunes@dogwood.botany.uga.edu.

ribosomal subunit (SSU rDNA) in *Symbiodinium* was first assessed by Rowan and Powers (1991). Their phylogeny revealed divergent lineages within the genus (presently referred to in the literature as clades, types, phylotypes, or groups—"A," "B," "C," etc.). Analyses of additional SSU rDNA sequences have confirmed and expanded the systematics of this group (McNally et al. 1994, Langer and Lipps 1994, Carlos et al. 1999). This gene was later used as a marker in examining and documenting some important ecological host-symbiont interactions (Rowan and Knowlton 1995, Rowan et al. 1997). Still, it is recognized that the conserved SSU is not suitable for species level distinctions (Rowan and Powers 1991, McNally et al. 1994, Baker and Rowan 1997). Use of variable domains from the large subunit (LSU) rRNA gene was subsequently added to the phylogenetic analysis (Lenaers et al. 1991, Wilcox 1998, Baker 1999), but Baker (1999) observed that the LSU rDNA did not adequately resolve the phylogenetic relatedness between genetic types within each of the major phylogenetic lineages.

The present study improves the phylogenetic resolution within these lineages and provides a marker that more closely approximates physiologically distinct populations for use in ecological investigations. The internal transcribed spacer (ITS) region, consisting of the ITS1, 5.8S, and ITS2 sequenced from protists, animals, plants, fungi, and macrophyte algae, typically provide phylogenetic resolution at or below the species level in each of these groups (Lee and Taylor 1992, Gonzalez et al. 1990, Goff et al. 1994, Coleman et al. 1994, Baldwin et al. 1995, Hunter et al. 1997). The ITS sequence is examined from 46 isolates of *Symbiodinium* cultured from 34 host species originating from the Red Sea, western Pacific, central Pacific region, eastern Pacific, Caribbean Sea, and western Atlantic. Two distinct noncultured *Symbiodinium* populations were also analyzed. Many of these isolates have been used previously in morphological, physiological, and biochemical studies (Colley and Trench 1983, Fitt 1985, Trench and Blank 1987, Iglesias-Prieto and Trench 1994, 1997b, Banaszak et al. 2000), and now their phylogenetic identity is determined for the first time. Certain attributes related to cell morphology and size, photophysiology, biochemistry, and host infectivity, determined in these earlier studies, are compared with phylogenies based on ITS sequences. This combination of molecular and traditional approaches provides a more complete picture of *Symbiodinium* diversity and systematics.

MATERIALS AND METHODS

Culturing, sample collection, and DNA extractions. Forty isolates of *Symbiodinium* were obtained from diverse host taxa collected at locations in the Red Sea (Gulf of Aqaba), west Pacific (Australia, Enewetak, Palau), central Pacific (Hawaii), east Pacific (North America), the Caribbean Sea (Bahamas, Florida, Jamaica, Puerto Rico), and Atlantic Ocean (Bermuda) (see Table 1 for a complete list of isolates and corresponding host origin and geographic location). They were cultured in 1 L of ASP-8A (Blank 1987) for 2 months at 23° C illuminated by banks of Vi-

taLite (Full Spectrum Solutions, Jackson, MI) fluorescent tubes delivering 80–120 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation on a 14:10 (light:dark) photoperiod. Algae were then harvested by centrifugation at 800–1000g in a Sorvall RC-5B centrifuge. DNA extractions followed a procedure described by Sadler et al. (1992). Between 1 and 2 g of algal material were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The material was thawed in 20 mL of extraction buffer (5 M guanidine thiocyanate, 10 mM Na2EDTA, 50 mM HEPES pH 7.6, and 5% by volume β -mercaptoethanol), and the extraction mixture was rendered to 4% sarcosyl (by volume) and stirred for 1 h. The extraction slurry was centrifuged at 8000g in a Sorvall RC-5B centrifuge for 5 min at 4° C to remove cellular debris. Cesium chloride was added to the supernatant (for a final concentration of 1.5 M) and combined with ethidium bromide; 9 mL of this solution was added slowly to a 12-mL ultracrimp (Sorvall) tube containing a 3-mL cushion of 5.7 M CsCl. Each tube was loaded into a TV-865B Sorvall fixed angle rotor and centrifuged for 16 h at 130,000 RCF in a RC80 Sorvall ultracentrifuge. Several milliliters containing the DNA band from the resulting gradient were obtained, the EtBr removed with water-saturated butanol, and then dialyzed overnight in 2 L of 1 \times TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) at 4° C. DNA was precipitated by the addition of one-tenth volume 3 M sodium acetate and 2 vol 100% ETOH. After centrifugation, the DNA pellet was lyophilized and resuspended in 500 μL 0.1 \times TE.

Two noncultured *Symbiodinium* populations that possess divergent ITS sequences were included in this study to augment the phylogenetic analyses. "*Symbiodinium muscatinei*" was collected from the sea anemone *Anthopleura elegantissima*, taken from Puget Sound, Washington State. The algae were isolated from host tissues and their nucleic acids extracted following the procedures described by LaJeunesse and Trench (2000). The phylogenetically divergent "D1" type *Symbiodinium* (cf. Baker 1999) was collected from the scleractinian, *Montastraea faveolata*, from a reef near Puerto Morelos, Mexico. Animal tissue, containing algae, was removed from the skeleton using a Water Pik and filtered (1.2 μm) seawater and EDTA (5 or 10 mM). Algal cells were separated from host tissue by centrifuging for 5 min at 1000g in a swinging bucket rotor using a Beckman model TJ-6. A tissue grinder (Pyrex no. 7727-07) was used to resuspend the algal pellet in 30 mL of fresh filtered seawater-EDTA (5 mM) and 0.025% Triton X-100. This process further purified the algal component by breaking up most of the remaining host cells and mucus as determined by inspection under 200 \times magnification. The slurry was centrifuged again and the pellet was resuspended in filtered seawater-EDTA (5 mM), this time by gentle mixing. This step was repeated and finally the resulting pellet stored in a high salt, DMSO, preservation buffer (Seutin et al. 1991). DNA extractions were conducted using the DNeasy plant mini prep kit (Qiagen Corporation, Santa Clarita, CA).

ITS amplification and DNA sequencing. ITS flanking primers "a" and "b" (Coleman et al. 1994), which target the entire ITS region, were used for PCR amplification. Reactions were carried out on a Perkin-Elmer Thermal Cycler 2400 under the following conditions: an initial denaturing step of 3 min at 92° C followed by 35 to 40 cycles of 30 s at 92° C, 40 s at 48° C and 30 s at 72° C, followed by a single cycle of 10 min at 72° C. Overlapping forward and reverse internal primers, ITSintfor1 (RGC ACC WWT GAA GGG CGC) and ITSintrev2 (5' TTC ACG GAG TTC TCG AAT), were designed to conserved sites within the 5.8S gene and used to cycle sequence the entire ITS. Reagents and reaction conditions for sequencing were supplied and specified by the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). Reaction products were analyzed on an Applied Biosystems 310 Genetic Analyzer (Division of Perkin Elmer, Foster City, CA). The resulting chromatograms were checked and edited using Sequence Navigator, version 1.0 (ABI, a Division of Perkin Elmer).

Denaturing gradient gel electrophoresis (DGGE) (Abrams and Stanton 1992) was incorporated in this study to rapidly compare cultured isolates and screen for ITS sequence varia-

TABLE 1. List of cultured isolates, their ITS sequence type, host and geographic origin, and Genbank accession numbers.

Isolate number	Symbiont species	ITS type	Geographic origin		Host origin	Accession numbers
61	<i>Symbiodinium microadriaticum</i> subsp. <i>microadriaticum</i>	A1	Caribbean	Florida	<i>Cassiopeia xamachana</i> (Rhizostomeae)	AF333505
362		A1	Red Sea	G. of Aqaba	<i>C. andromeda</i> (Rhizostomeae)	
370		A1	"	"	<i>Stylophora pistillata</i> (Scleractinaria)	
48		A1.1	Caribbean	Jamaica	<i>Cassiopeia frondosa</i> (Rhizostomeae)	
80	<i>S. microadriaticum</i> subsp. <i>condylactis</i> ^a	A1.1	"	Jamaica	<i>Condylactis gigantea</i> (Actiniaria)	AF333504
89		A2	Western Atlantic	Bermuda	<i>Gorgonia ventallina</i> (Gorgonacea)	
97		A2	Caribbean	Puerto Rico	<i>Gorgonia ventallina</i> (Gorgonacea)	
102		A2	"	Jamaica	<i>Gorgonia ventallina</i> (Gorgonacea)	
185	<i>S. pilosum</i>	A2	"	Jamaica	<i>Zoanthus sociatus</i> (Zoantharia)	AF333506
130	" <i>S. meandrinae</i> "	A2	"	"	<i>Meandrina meandrites</i> (Scleractinaria)	
23/24		A2	"	Barbados	<i>Bartholomea annulata</i> (Actiniaria)	
169		A2	West Pacific	Australia	<i>Tridacna gigas</i> (Bivalvia)	
104		A2	"	Enewetak	<i>Heliopora</i> sp. (Helioporacea)	
350	" <i>S. corculorum</i> "	A2	"	Palau	<i>Corculum cardissa</i> (Bivalvia)	
77		A3	Central Pacific	Hawaii	<i>Cassiopeia mertensii</i> (Rhizostomeae)	
168		A3	West Pacific	Australia	<i>Tridacna crocea</i> (Bivalvia)	
174		A3	"	Enewetak	<i>T. maxima</i> (Bivalvia)	
292		A3	"	Palau	<i>T. maxima</i> (Bivalvia)	
220		A3	"	"	<i>T. crocea</i> (Bivalvia)	
265		A3	"	"	<i>T. derasa</i> (Bivalvia)	
272		A3	"	"	<i>T. gigas</i> (Bivalvia)	
338		A3	"	"	<i>Hippopus hippopus</i> (Bivalvia)	
328		A3	"	"	<i>Mastigias</i> sp. (Rhizostomeae)	AF333507
368	<i>S. (=Gymnodinium) linucheae</i>	A4	Western Atlantic	Bermuda	<i>Linuche unguiculata</i> (Coronatae)	AF333509
379		A4	Caribbean	Bahamas	<i>Plexaura homamallia</i> (Gorgonacea)	
315		A5	West Pacific	Palau	<i>T. squamosa</i> (Bivalvia)	AF333508
8	" <i>S. pulchrorum</i> "	B1	Central Pacific	Hawaii	<i>Aiptasia pulchella</i> (Actiniaria)	AF333511
351		B1	"	"	<i>Pocillopora damicornis</i> (Scleractinaria)	
83		B1	West Pacific	Palau	<i>Corbulifera</i> sp. (Hydrozoa)	
13	" <i>S. bermudense</i> "	B1	Western Atlantic	Bermuda	<i>Aiptasia tagetes</i> (Actiniaria)	
146		B1	"	"	<i>Oculina diffusa</i> (Scleractinaria)	
12		B1	Caribbean	Puerto Rico	<i>A. tagetes</i> (Actiniaria)	
64		B1	"	Jamaica	<i>C. xamachana</i> (Rhizostomeae)	
74		B1	"	"	<i>C. xamachana</i> (Rhizostomeae)	
2		B1	"	Florida	<i>Aiptasia pallida</i> (Actiniaria)	
125		B1	"	Jamaica	<i>Lebrunia danae</i> (Actiniaria)	
147		B1	"	Jamaica	<i>Pseudoterogorgia bipinnata</i> (Gorgonacea)	
141		B2.1	Western Atlantic	Bermuda	<i>Oculina diffusa</i> (Scleractinaria)	AF333513
5		B2	Caribbean	Florida	<i>Aiptasia pallida</i> (Actiniaria)	AF333512
384		B3	Caribbean	Bahamas	<i>Dichotomia</i> sp. (Coronatae)	AF333514
113	<i>S. goreauii</i>	C1	Caribbean	Jamaica	<i>Rhodactis (Heteractis) lucida</i> (Corallimorph.)	AF333515
152		C1	"	"	<i>Discosoma sancti-thomae</i> (Corallimorph.)	
203		C2	West Pacific	Palau	<i>Hippopus hippopus</i> (Bivalvia)	AF333518
383	" <i>S. californium</i> "	E1	East Pacific	California	<i>Anthopleura elegantissima</i> (Actiniaria)	AF334659
135	<i>S. kawagutii</i>	F1	Central Pacific	Hawaii	<i>Montipora verrucosa</i> (Scleractinaria)	AF333517
133		F2	Caribbean	Jamaica	<i>Meandrina meandrites</i> (Scleractinaria)	AF333516

^a Later referred to as *Symbiodinium cariborum* (Banaszak et al. 1993).

tion. DGGE was also used to identify intragenomic variation at paralogous loci (Buckler et al. 1997) and to detect the presence of pseudogenes (Scholin et al. 1993). The protocol for DGGE analysis of the ITS2 delineated by LaJeunesse and Trench (2000) was done with all cultured and noncultured algae investigated in this study.

Sequence alignment and molecular phylogenetics. 5.8S sequences for *Gymnodinium sanguineum* (AF131075), *Gyrodinium impudicum* (AF13074), *Proocentrum micans* (M14649), and *Cryptocodinium cohnii* (M25116) were acquired from Genbank, and the sequence for *G. catenatum* was obtained from Adachi et al. (1997). These sequences were used to delineate the 5.8S gene for *Symbiodinium* and its boundaries within the ITS region. The ITS1 boundary with the SSU rRNA gene corresponds to Adachi et al. (1997) and the ITS2 boundary with the LSU rRNA corresponds to the determination by Lenaers et al. (1989).

Sequences were manually aligned using Sequence Navigator (version 1.0) provided by PE Applied Biosystems. Cladistic anal-

yses of aligned sequences were done using the method of parsimony and maximum likelihood with the PAUP 4.0b4 computer program package using the default settings (Swofford 1993). Both deletions and insertions were omitted from these analyses.

Microscopy. Isolates were photographed during log phase growth under 40 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation on a 14:10 (light:dark) photoperiod. Cell size and cytoplasmic accumulations in the form of starch and lipid storage products, accumulation bodies, or oxalate crystals increase in older cultures (Freudenthal 1962, Domotor and D'Elia 1986). To avoid the effect of age on appearance and size, cells were photographed at approximately the same stage in culture (between days 10 and 15 after reinoculation into fresh media), during the middle of logarithmic growth. Cells were photographed under bright-field illumination at a magnification of 1000 \times using an Olympus BX60 compound microscope with an Optronics Engineering DEI-750 digital camera. An autoexposure setting of 1/125 (++) using Flashpoint FPG 3.10 software (Integral

Technologies Inc. 1997) was used to expose and capture the cell images. Figure 3 consists of solitary and doublet cells in late mitosis that represent the typical cellular morphology and size of each isolate in culture.

RESULTS

Symbiodinium ITS region. The sizes of various 5.8S, ITS1, and ITS2 regions and their respective combined lengths are presented in Table 2. Sequences from noncultured *Symbiodinium*, B4 (“*S. muscatinei*”) (AF333510) and D1 (AF334660) were incorporated to augment the data set and to demonstrate the existence of distinct noncultured taxa. The 5.8S gene was uniform in size within a lineage. The total length of the ITS region had considerably greater variation (521–584 bp) than the range (518–535 bp) observed for dinoflagellate species within the genus *Alexandrium* (Adachi et al. 1996). For most *Symbiodinium* the ITS1 is 10%–15% longer than the ITS2. This disparity is greatest within lineage A, whereas the spacers 1 and 2 are nearly identical in size in lineage B.

DGGE profiles of the ITS2 from these isolates (data not shown) produced a single prominent band for all, except one. Culture 384, the isolate designated B3, consistently produced four different bands of similar intensity. These bands were most likely the product of secondary structural conformations that migrated and melted differently in the denaturing gel and/or are paralogous loci (Buckler et al. 1997). Although there was no evidence that more than one ribosomal locus exists in these cultured isolates, paralogous loci have been identified from samples of natural populations (unpublished data).

Symbiodinium phylogeny based on the 5.8S. The 5.8S sequences were aligned with selected outgroups and an inferred phylogeny was constructed (Fig. 1). The tree topology based on maximum likelihood and verified by parsimony is consistent with and supported by trees previously constructed using the SSU and LSU rRNA genes (Rowan and Powers 1991, Saunders et al. 1997, Wilcox 1998, Baker 1999). Though much smaller in size, the 5.8S gene appears to provide essentially the

same phylogenetic information as larger LSU rRNA and SSU rRNA genes. It has been useful in delineating the divergence of higher phylogenetic ranks (Maroteaux et al. 1985, Hori and Osawa 1987); however, like the LSU and SSU rRNA genes, in many instances it does not effectively resolve genetic differences between closely related taxa (e.g. lineage A, Fig. 1).

ITS phylogenies and sequence variation. Phylogenies were constructed from full ITS sequences for lineages A, B, and C–F, respectively (Figs. 2, A–C). Considerable sequence divergence precluded accurate alignments between distantly related *Symbiodinium* (i.e. full sequences were not alignable between algae from different lineages). An ITS phylogeny of lineages D and E could not be constructed because only one representative from each was sequenced.

Six ITS types were distinguished among 27 isolates from lineage A. Four of the six A ITS sequences correspond with previously described species. The ITS sequences for *Symbiodinium microadriaticum* subsp. *microadriaticum* (Freudenthal 1962, Blank and Huss 1989) (type A1), *S. microadriaticum* subsp. *condylactis* (Blank and Huss 1989; it is referred to as “*S. cariborum*” in Banaszak et al. 1993) (A1.1), *S. pilosum* (Trench and Blank 1987) (type A2), and *S.* (= *Gymnodinium*) *linucheae* (Trench and Thinh 1995) (type A4) were readily distinguishable and their phylogenetic affinities clearly established (Fig. 2A). The closely related types A3 and A5 were also identified by this analysis. *Symbiodinium pilosum* (Trench and Blank 1987, Trench 2000), “*S. meandrinae*,” and “*S. corculorum*” (Banaszak et al. 1993) are not distinguished by their ITS sequences. Isolates with the same ITS type originated from different hosts collected from distant oceans (e.g. A1 isolates originated from *Stylophora pistillata* in the red sea and *Cassiopeia frondosa* in the Caribbean).

Three ITS types from 14 lineage B isolates in culture, not resolved by the 5.8S gene, were identified (Fig. 2B). Two undescribed but informally named “species” from this lineage, “*S. pulchrorum*” and “*S. bermudense*,” possess identical ITS sequences (type B1). Cultures possessing this same B1 sequence originated from nine different host species, one from the west Pacific, two from the central Pacific, and six from the Caribbean. The B2 type was obtained from the coral *Oculina diffusa* and the anemone *Aiptasia pallida* in the Caribbean. A closely related ITS type B3 is characterized by a single isolate obtained from the Caribbean jellyfish *Dichotomia* sp. “*Symbiodinium muscatinei*” (type B4) is different from other lineage B algae, and its overall phylogenetic divergence from types B1, B2, and B3 supports the species designation proposed by LaJeunesse and Trench (2000).

Analysis of sequences from five isolates whose RFLPs of the SSU rRNA gene are indicative of lineage C (sensu Rowan and Powers 1991) identified four distinct ITS types (Fig. 2C). C1 types (e.g. *S. goreauii*) from the Caribbean are closely related to type C2 isolated from the giant clam *Hippopus hippopus* in the Pacific. *Symbiodinium kawagutii* (no. 135) (F1) from Montipora ver-

TABLE 2. Length of the 5.8S and ITS regions among ITS types from cultured and some noncultured *Symbiodinium*.

ITS Type	ITS1	5.8S rDNA	ITS2	Total length
A1 (A1.1)	203	160	162	525
A2	214	160	164	538
A3	203	160	158	521
A4	202	160	162	524
A5	203	160	159	522
B1	202	158	198	558
B2 (B2.1)	202	158	194	554
B3	203	158	194	555
B4	229	158	197	584
C1	221	159	192	572
C2	224	159	192	575
D1	232	159	190	581
E1	222	160	192	574
F1	222	159	192	573
F2	222	159	192	573

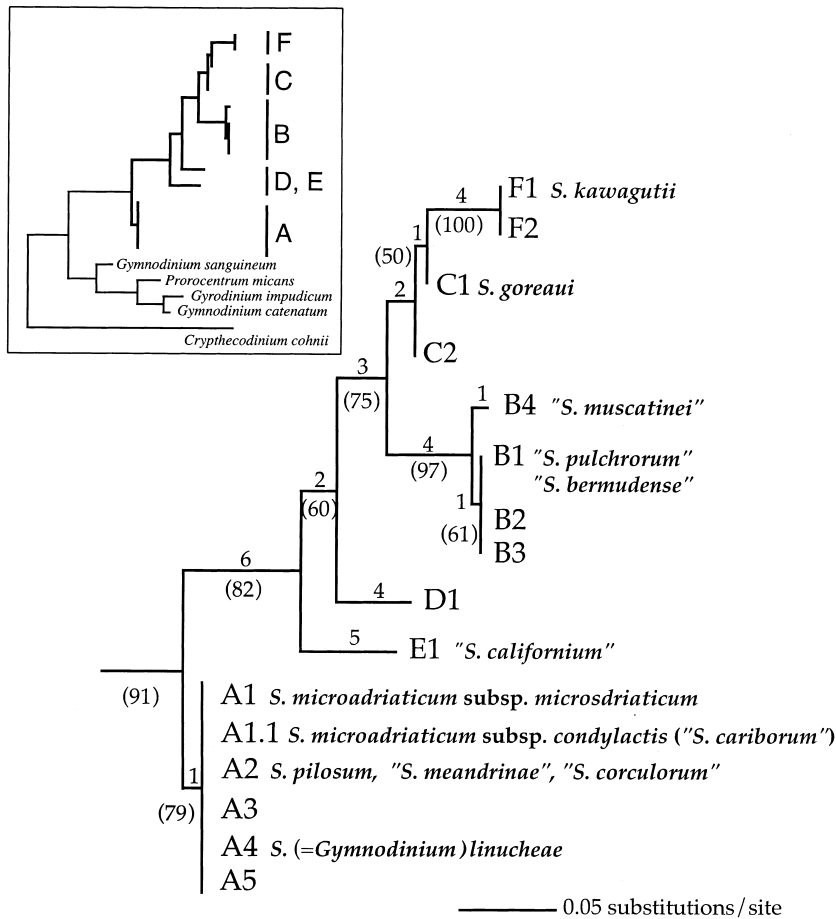


FIG. 1. Phylogenetic reconstruction of the genus *Symbiodinium* inferred from 5.8S rDNA based on the method of maximum likelihood. *Gymnodinium sanguineum*, *G. catenatum*, *Gyrodinium impudicum*, and *Prorocentrum micans* are included here as sister taxa. *Cryptothecodinium cohnii* was designated the out group (inset). *Symbiodinium* lineages A through F are distinguished from each other, but differences between taxa within each lineage are either poorly resolved or unresolved. The designations A1, A2, and so on are distinct ITS designations as determined from the full ITS sequence. Species with formal descriptions and informal names given to taxa without formal descriptions are included. See Table 1 for geographic location and host origin. The numerals above each branch indicate the number of informative base pair differences based on parsimony analysis. Numerals in parentheses below the branches indicate the bootstrap value for 100 replicates.

rucosa in the Pacific is related to the F2 isolate (no. 133) from *Meandrina meandrites* from the Caribbean. Isolates 133 and 135 are referred to lineage F because analysis of available LSU rDNA and 5.8S sequence data indicates that they belong to one of several lesser lineages whose members most commonly associate with foraminiferans (Pawlowski et al. 2001, group Fr5). Though still alignable, the divergence between lineages C and F approaches the upper limit of the ITS for phylogenetic reconstruction.

ITS versus the 5.8S, SSU rRNA, and LSU rRNA genes. A comparison of genes within the ribosomal array shows marked differences in phylogenetic resolution. The noncoding ITS1 and 2 regions clearly exhibit greater phylogenetic resolution than the 5.8S rRNA, SSU rRNA, and LSU rRNA genes (Table 3).

Morphology of cultured Symbiodinium. The photomicrographs in Figure 3 illustrate morphological diversity on a qualitative level. Under light microscopy, the lack of distinguishing morphological characters limits a comprehensive interpretation of the qualitative differences observed between these isolates and demonstrates the difficulty early microscopists had in assessing diversity. Pigmentation and cell size are noticeably different for the various isolates presented. Because cultures were grown

under similar irradiance conditions, differences in pigmentation are possibly due to differences in photoacclimation (Iglesias-Prieto and Trench 1994). More compelling are differences in cell size, which appear to be phylogenetically informative. Types A2 and E1 are visually larger than cells from the other ITS types. Other representatives of lineage A and members of lineage C and F range in size, whereas lineage B types are typically small (cf. Fig. 3). Figure 4 is reproduced from the findings of Fitt (1985) and is a quantitative observation

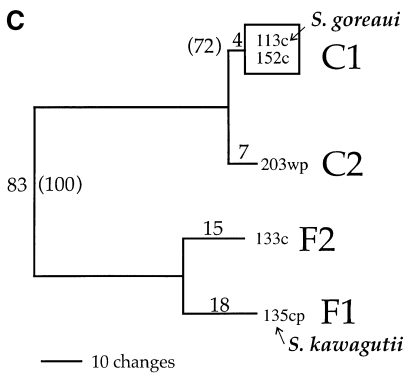
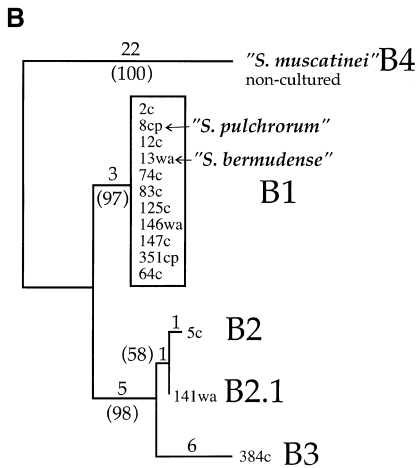
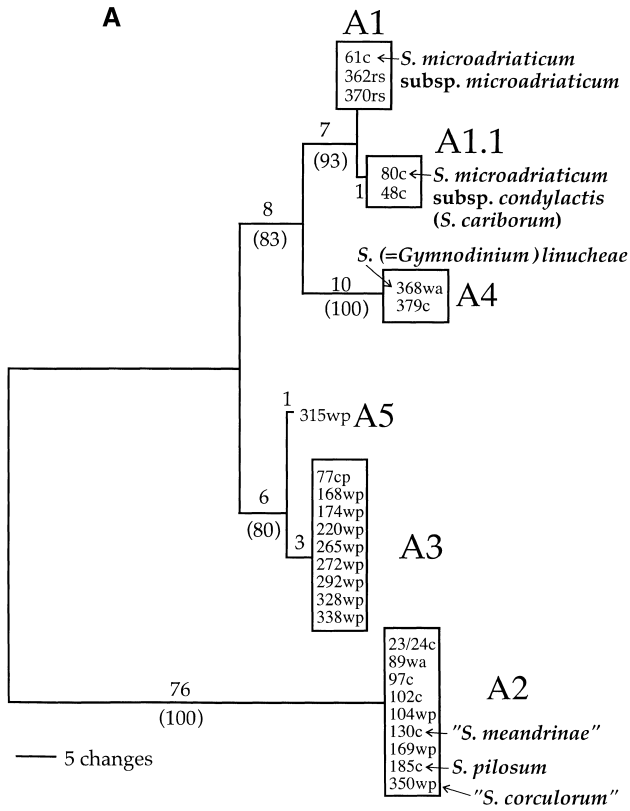
TABLE 3. Pairwise comparisons of percent ribosomal sequence difference between *Symbiodinium microadriaticum* and other genetic types of lineage A: *S. pilosum*, *Symbiodinium* sp. type A3, and *S. (=Gymnodinium) linucheae*, respectively.

	<i>Symbiodinium microadriaticum</i> (A1)			
	5.8S	SSU1 ^a	LSU ^b	ITS 1 and 2
<i>S. pilosum</i> (A2)	0.0	0.3	4.1	20.0
<i>Symbiodinium</i> sp. (A3)	0.0	0.0	1.5	5.2
<i>S. (=G.) linucheae</i> (A4)	0.0	n.d.	0.8	4.0

There is a marked increase in sequence variation from the most conserved 5.8S to the unconserved ITS spacers.

^a McNally et al. 1994, unpublished data.

^b Wilcox 1988, Baker 1999.



that cell size can distinguish some ITS types from each other. Finally, it is possible to observe marked differences in size and morphology of the motile form, especially in the relative lengths and widths of the hypocone and epicone (Trench and Blank 1987, personal observation). These are not shown here because of the current difficulty in photographing this rapidly moving life stage.

Isoenzyme similarity versus gene phylogeny. A dendrogram based on the distance matrix of mean similarity coefficients (Jaccard) (Sneath and Sokal 1973) obtained from 11 isoenzyme profiles is shown in Figure 5 (Colley 1984). The ITS identity and region of origin for each culture is included for comparison. There is little or no congruence between a phylogeny based on rDNA sequences and the dendrogram created from the similarity of enzyme loci.

DISCUSSION

Symbiodinium species with formal descriptions are strongly supported by differences in ITS sequences. The inferred phylogenies of Figure 2 show that some of these species form distinct sequence clusters with uncharacterized isolates. A preliminary interpretation is that such clusters containing no sequence variability may represent or closely approximate, species populations (cf. Baillie et al. 2000b). These findings also show that there is a need for formal taxonomic descriptions on many uncharacterized isolates whose sequences are separated by equivalent or greater differences (Fig. 2, A–C).

The ITS of *Symbiodinium*, as determined for other organismal groups, provides better phylogenetic resolution than the SSU and LSU rRNA genes (Fig. 3). Considerable differences in ITS sequence between lineages A, B, C, D, E, and F support the notion that this genus has a long evolutionary history (Rowan and Powers 1992). Furthermore, the finding that identical ITS sequences come from cultures originating from different host species collected from distant geographic locations raises the possibility that some *Symbiodinium* are capable of dispersing great distances and may form symbioses with a wide range of host types.

FIG. 2. Phylogenies inferred from ITS1, ITS2, and 5.8S rDNA sequences. Trees were constructed using PAUP software and the method of parsimony. Culture numbers correspond with those in Table 1. The lowercase letters ep (east Pacific), cp (central Pacific), wp (west Pacific), rs (Red Sea), c (Caribbean), and wa (western Atlantic) next to each culture number refer to geographic origin. Differences resulting from insertions and deletions were omitted from each analysis. Numerals above or adjacent to branch lengths indicate number of informative differences. Numbers in parentheses below the branches indicate the bootstrap value for 100 replicates. (A) Phylogeny of lineage A species. Type A2 was designated the outgroup. (B) Phylogeny of lineage B species. Type B4 was designated as the outgroup based on SSU sequence data (LaJeunesse and Trench 2000). (C) Phylogeny of lineages C and F. No outgroup was used.

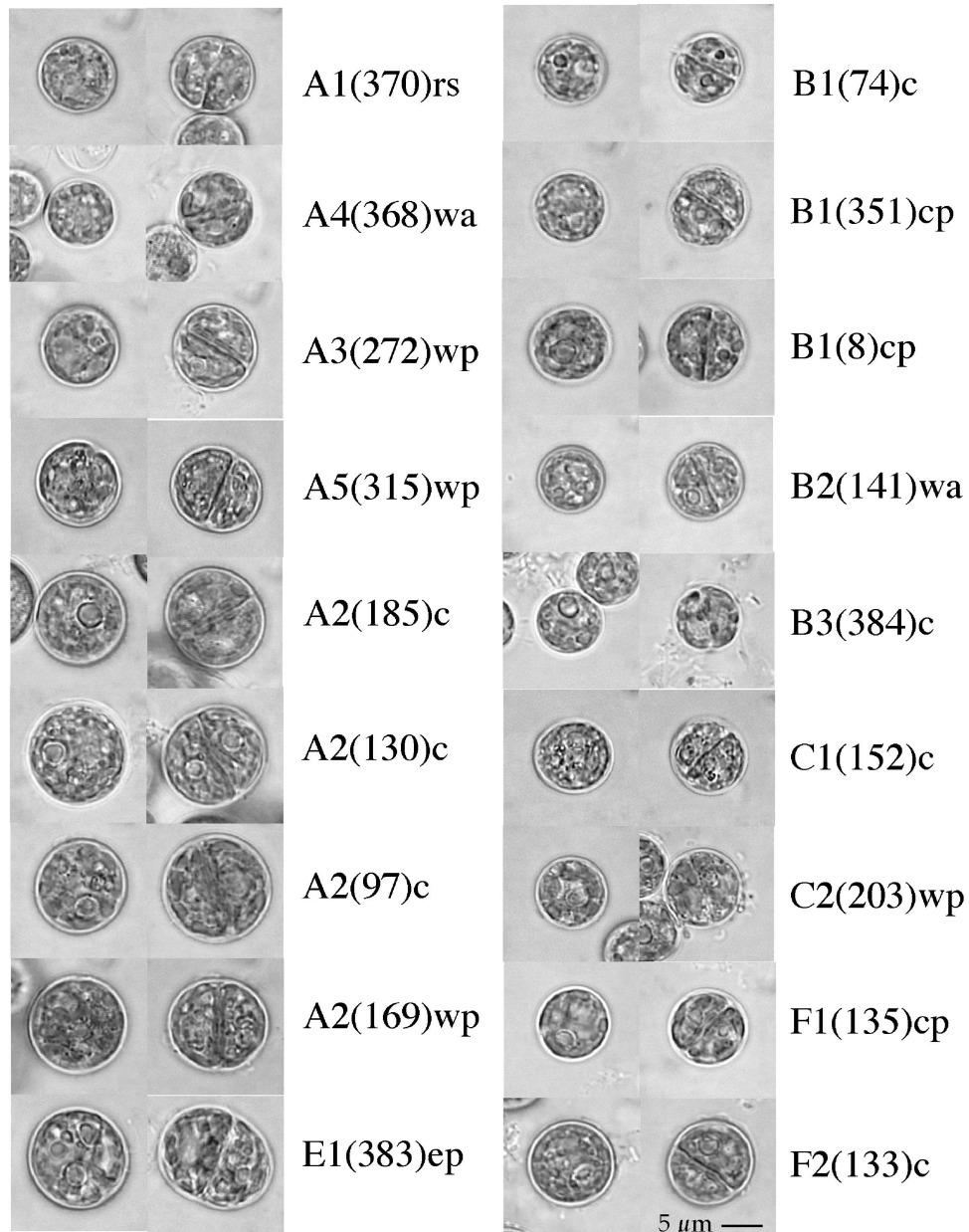


FIG. 3. Cellular morphology of *Symbiodinium* isolates. Photographs from bright-field light microscopy of cultured isolates growing in log phase under identical irradiance. Solitary cells (left image of each column) and doublet cells in the mitotic phase of cytokinesis (right image) are depicted from selected cultures. The ITS type (e.g. A1, B2, etc.) and isolate number with geographic origin (in parentheses) are provided to the right of each pair of images. Isolates show notable differences in size and subtle differences in pigmentation, cytoplasmic refractiveness, and chloroplast morphology. See text for further discussion. Magnification, $\times 1000$; Scale bar, $5.0 \mu\text{m}$.

How do ITS classifications of Symbiodinium correlate with morphology, biochemistry, physiology, and host selectivity? Most research on *Symbiodinium* biology has involved investigations on morphology (Trench and Blank 1987), photo-physiology (Iglesias-Prieto and Trench 1994, 1997b) or genetic diversity (Rowan and Powers 1991, Wilcox 1998, Carlos et al. 1999). However, there have been few attempts to bring together these research directions (cf. Schoenberg and Trench, 1980a,b,c, Banaszak et al. 2000). Because these approaches to studying *Symbiodinium* are

rarely combined, it has not been possible to learn how phylogenetic classification corresponds with morphological, biochemical, and physiological attributes. Studies were conducted earlier on many of the same cultures analyzed in this study, including cell size (Fitt 1985), ultrastructure (Trench and Blank 1987, Trench and Thinh 1995), synthesis of mycosporine-like amino acids (MAAs) (Banaszak et al. 2000), differential expression of monomer and/or dimer apoprotein subunits of the water-soluble peridinin-chl a -protein (sPCP)

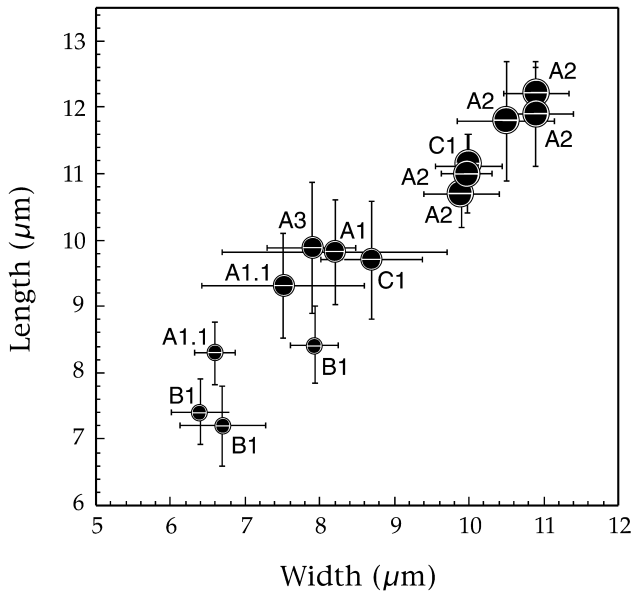


FIG. 4. Size (length and width \pm SD) of dividing cells ($n = 10$) and corresponding ITS identity of 14 cultured isolates (cf. Table 4). (From Fitt 1985.) Though variation exists between isolates with the same ITS sequence, relative size appears to correspond with ITS phylogeny.

complex (Govind et al. 1990), and infectivity with aposymbiotic *Cassiopeia xamachana* (Colley and Trench 1983, Fitt and Trench 1983). The relevant cultures and their ITS types along with the corresponding attributes, when available, are listed in Table 4.

Cell size and morphology. The taxonomic classification of dinoflagellates, as with most protists, has relied on distinctions made from morphological comparisons. Taxonomic studies on *Symbiodinium* have remained scarce, primarily due to the difficulty in making formal descriptions based on morphology, especially on the nonmotile coccoid form (Taylor 1974, Trench and Blank 1987). The species *S. microadriaticum*, *S. pilosum*, *S. kawagutii*, *S. goreauii*, and *Gymnodinium linucheae* are classified based on a "morphological species" concept (Freudenthal 1962, Trench and Blank 1987, Trench and Thinh 1995). Cell size, chromosome number ("condensed DNA regions": Udy et al. 1993) and volume, nuclear volume, surface morphology, chloroplast and pyrenoid ultrastructure, and cingulum orientation were among the attributes used to characterize these species. *Symbiodinium microadriaticum* was subsequently divided into two subspecies, subsp. *microadriaticum* and subsp. *condylactis*, by Blank and Huss (1989). Their reclassification was based primarily on the characteristics of DNA/DNA hybridization and phenotypes. *Symbiodinium microadriaticum* subsp. *condylactis* was later referred to as *S. cariborum* by Banaszak et al. (1993).

For purposes of naming *Symbiodinium* species, formal descriptions were based on cultured specimens grown under controlled environmental conditions to avoid the confounding effects of phenotypic plasticity

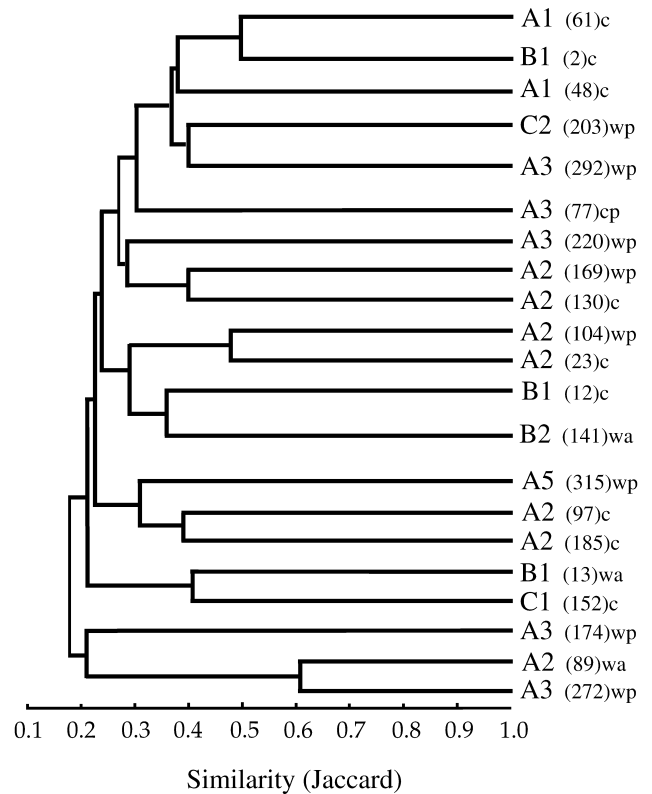


FIG. 5. Distance matrix based on the mean similarity coefficients obtained from 11 isoenzyme profiles from 21 cultured isolates (Colley 1984). ITS identity and geographic origin (see Fig. 2A) are assigned to each isolate. The overall incongruence of isoenzyme similarity and phylogenetic relatedness is an indicator of sexual recombination (Tibayrenc et al. 1991).

(Trench and Blank 1987). Morphological or phenotypic plasticity is evident, especially in natural populations, and may confound attempts to distinguish types based on morphometric analyses. Culture phase (log vs. stationary), nutrient exposure while *in hospite*, and irradiance are all factors that cause variation in cell size, chloroplast size, and the relative concentrations of lipid and starch bodies in the cytoplasm (Doyle and Doyle 1940, Muller-Parker et al. 1996, personal observation).

Most ultrastructural examinations were done on species from lineage A, perhaps because they arise regularly under culturing conditions. Therefore, the species diversity of this lineage is well characterized. *Symbiodinium pilosum* (no. 185) and other A2 types are ancestral to the rest of lineage A (Fig. 2A). *Symbiodinium pilosum* is noticeably distinct from other A taxa by possessing a peripheral thylakoid lamella within its chloroplast. It is not known whether "*S. meandrinae*" (no. 130), "*S. corculorum*" (no. 350), and other A2 types possess this same feature. *Symbiodinium pilosum* (A2), "*S. meandrinae*" (A2), and "*S. corculorum*" (A2) each possesses ejectile organelles, termed mucocysts, and each has an external pilose or tufted surface. They are similar in

TABLE 4. Cultures from lineages A, B, C, and F *Symbiodinium* with corresponding ITS sequence type and available published data on size (Fitt 1985), MAA synthesis (Banaszak et al. 2000), results from infection experiments with *Cassiopeia xamachana* (C.x.) (Colley and Trench 1983, Fitt and Trench 1983), PCP apoprotein subunit size (Govind et al. 1990), condensed DNA regions and chloroplast number (Trench and Blank 1987, Blank and Huss 1989, Trench and Thinh 1995).

Isolate number	Symbiont species	ITS type	Size (μm) (width \times length)	MAA synthesis in culture ¹	Symbiosis with C.x. ²	PCP apoprotein ³	Condensed DNA regions	Chloroplast number
61	<i>S. microadriaticum</i> subsp.	A1	9.6 \times 9.9	Yes	+	15 & 35	97 \pm 2	1
362		A1		Yes	+			
370		A1		Yes	n.d.			
48	<i>S. microadriaticum</i> subsp. <i>condylactis</i> ^a	A1.1	6.6 \times 8.3		n.d.	15 & 35	98 \pm 2	1
80		A1.1	7.5 \times 9.3		n.d.	15		
89		A2	10.9 \times 11.9		–	n.d.		
97		A2		Yes	–	n.d.		
185	<i>S. pilosum</i>	A2	13.0 \times 13.6	Yes	–	15	78 \pm 2	1
130		" <i>S. meandrinae</i> "	A2	9.7 \times 11.0	Yes	–		
23/24		A2	10.9 \times 12.2	Yes	–	35		
169		A2	10.5 \times 11.8	Yes	–	n.d.		
104		A2		Yes	–	n.d.		
350	" <i>S. corculorum</i> "	A2		Yes	n.d.	15		
77		A3			+	n.d.		
168		A3		Yes	n.d.	n.d.		
174		A3	8.2 \times 9.8		+	15 & 35		
292		A3	7.9 \times 9.9		+	n.d.		
220		A3		Yes	+	n.d.		
272		A3			+	n.d.		
328		A3		Yes	+	n.d.		
368	<i>S. (=Gymnodinium)</i> <i>linucheae</i>	A4	10.58 \times ?	Yes	n.d.	n.d.		2
315		A5			+	n.d.		
8	" <i>S. pulchrorum</i> "	B1	7.9 \times 8.4	No	+	35		
351		B1		No	+	n.d.		
83		B1		No	n.d.	n.d.		
13	" <i>S. bermudense</i> "	B1	6.4 \times 7.4		+	n.d.		
146		B1			n.d.	n.d.		
12		B1		No	+	15		
2		B1			+	n.d.		
125		B1	6.7 \times 7.2	No	+	n.d.		
147		B1		No	n.d.	n.d.		
141		B2.1		No	+/-	35		
113	<i>S. goreau</i>	C1	10.0 \times 11.1		+	35	74 \pm 2	5
152		C1	8.7 \times 9.7	No	+	35		
203		C2		No	+	n.d.		
383	" <i>S. californium</i> "	E1		No	–	15 & 35		
135	<i>S. kawagutii</i>	F1		No	+	35	26	1
133		F2		No	n.d.	n.d.		

The taxonomic names, both valid and invalid, are given next to their respective isolates. (n.d. = no data)

^a Later referred to as *Symbiodinium cariborum* (Banaszak et al. 1993).

overall size and relatively larger than most other *Symbiodinium* (Figs. 3 and 4). All three appear to have different mucocyst morphologies and are distinguishable by the thecal plate patterns of their mastigote stage (Trench 1993, McNally et al. 1994, R. K. Trench, University of California, Santa Barbara, personal communication). The SSU rDNA differences between *S. pilosum* and "*S. corculorum*" reported by McNally et al. (1994) are likely the result of incorrect sequence reads because their base substitutions are located in highly conserved domains. In an attempt to distinguish them on the basis of sequence variation, the 3' end of the hyper-variable nontranscribed spacer region was analyzed, and no differences were detected (unpublished data).

Symbiodinium microadriaticum subsp. *microadriaticum* and *S. microadriaticum* subsp. *condylactis* ("*S. cariborum*") (A1 and A1.1), *Gymnodinium linucheae* (A4), and A3 types do not appear to possess mucocysts nor do they

have a pilose or tufted exterior (Trench and Blank 1987, Trench and Thinh 1995). They are also smaller than their A2 relatives (Figs. 3 and 4).

The lineage B algae are typically the smallest cells of the genus (7–8 μm) (Figs. 3 and 4). "*Symbiodinium pulchrorum*" (no. 8) and "*S. bermudense*" (no. 12), under traditional classification schemes, possess differences in thecal plate patterns that have been used to distinguish them as distinct species (R. K. Trench, personal communication), yet both possess identical B1 ITS sequences. These two organisms do share several morphological similarities, such as comparatively small size, smooth outer surfaces, and no mucocysts (Trench 1993). The epicone of their mastigote (zoospore) stage contains an accumulation body that does not appear to be present in species with larger mastigotes (Schoenberg and Trench 1980b).

Whether morphological differences within isolates of the same ITS type constitute interspecific or intra-

specific variation or divergence from the effect of progressive subculturing over many years cannot be determined without continued investigation. Further electron microscopy work is required to determine whether the morphological attributes mentioned above are shared by all isolates from the same ITS type and to identify other traits these isolates may have in common. Of particular interest will be to determine if similar traits are shared by field-collected specimens of the same genetic type.

It is important to remember that some morphological traits, historically used in systematics, do not always support molecular phylogenies nor do they always correlate with the level of divergence indicated by sequence differences. Evidence from molecular phylogenies are challenging conventional beliefs concerning what attributes are taxonomically relevant for some dinoflagellate groups (Lenaers et al. 1991, Saunders et al. 1997, Wilcox 1998). For instance, Wilcox (1998) observed that *Gymnodinium linucheae* (Trench and Thinh) (no. 368) and *Symbiodinium microadriaticum* (Freudenthal) (no. 61) possessed closely related LSU rDNA genes (0.8% different). Given the relatively small difference (4%) between their ITS sequences, their morphologies are surprisingly distinct (cf. Trench and Thinh 1995, Trench and Blank 1987). *Gymnodinium linucheae* possesses two pyrenoids and two chloroplasts, whereas *S. microadriaticum* possesses only one of each. The displaced cingulum in *G. linucheae*, a feature absent in all described *Symbiodinium* species, was considered distinctive of the genus *Gymnodinium*. However, the genus *Gymnodinium* is a problematic group whose members, by their morphology, do not conform to phylogenies based on molecular analysis (Saunders et al. 1997). Convergence in morphology is most likely responsible for the misclassification of these taxa. Based on molecular evidence, *Gymnodinium linucheae* should be referred to *Symbiodinium linucheae* comb. nov. Furthermore, a reconsideration of the morphological evidence shows that *Symbiodinium* (= *Gymnodinium*) *linucheae* possesses a nonmotile coccoid morphology that alternates with a gymnodinoid-like motile stage. This alternation in morphology is indicative of all *Symbiodinium* taxa and is perhaps a more important trait than previously estimated. *Gymnodinium beii* (Spero) also possesses a nonmotile oblate spheroid stage while in symbiosis, and from genetic comparison is the closest known sister taxa to *Symbiodinium* (Spero 1987, Gast and Caron 1996).

Physiology and Biochemistry. Though rarely included in formal descriptions, organisms can be characterized by their physiology, faculties that depend on the biochemistry and ultimately the genetic composition of that organism. For *Symbiodinium*, photophysiology is an important component of their existence. The photobiology for perhaps only four or five species of *Symbiodinium* has been examined in any detail (Chang et al. 1983, Iglesias-Prieto and Trench 1994, 1997b). *Symbiodinium microadriaticum*, *S. kawagutii*, *S. pilosum*, and "*S. pulchrorum*" each have their own characteristic photoacclimatory responses to changes in irradiance. The limited data

set on these few species prohibits making any definitive conclusions of phylogenetic significance. However, there are two studies that contain enough information where this may be possible.

MAAs are a class of compound that absorbs UV radiation and provides a form of "sunscreen protection" to organisms that produce them (Neale et al. 1998). Banaszak et al. (2000) detected the production of MAA molecules in cultured *Symbiodinium* all corresponding with lineage A types (Table 4). However, there appears to be little correspondence between the production of any particular MAA molecule and ITS type. No MAAs were detected in the representative isolates from lineages B, C, E, and F. The synthesis of these UV sunscreens may influence the ecology, or niche, of lineage A species. In a survey of *Symbiodinium* populations from a wide diversity of reef animals and habitats, all hosts that possessed populations of A *Symbiodinium* were collected within 3 to 4 m of the surface (unpublished data). In a similar observation, coral colonies of the species *Montastraea faveolata* sampled within the first 3 m of the surface commonly harbored lineage A *Symbiodinium*, whereas in the deepest colonies (below 9 m), these algae were never observed (Rowan and Knowlton 1995). These observations are consistent with the hypothesis that the ecological distribution of *Symbiodinium* is limited, in part, by their physiological attributes (Rowan et al. 1997, Iglesias-Prieto and Trench 1997a).

sPCP are important light-harvesting components of the photosynthetic apparatus of dinoflagellates (Prezelin 1987). The sPCP apoproteins occur as a monomer (31–35 kDa) or as a homodimer (14–15.5 kDa). For some taxa with the capability to express both forms, the monomeric (35 kDa) sPCP was expressed in greater concentrations under high light (250 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions, whereas the 15-kb dimeric sPCP achieved greater expression under low light (40 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions (Iglesias-Prieto and Trench 1997b). Among the cultures analyzed by Govind et al. (1990), there is no clear indication that the expression of the monomer and/or the homodimer follows a phylogenetic pattern (Table 4). Grown under similar irradiances (40 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), the 35-kb monomer was expressed by at least one culture from lineages A, B, C, E, and F. Some cultures, affiliated with lineages A and B, expressed only of the 15-kDa homodimer. The expression of the 15-kDa homodimer was never detected for lineage C and F types; however, this could be the result of under sampling. The expression or nonexpression of a particular apoprotein may be fixed in some lineages (e.g. C and F), whereas for others (e.g. lineages A and B), the presence or absence may depend on allelic differences, similar to the variability found in allozyme profiles (Colley 1984, see Fig. 5).

Host-symbiont specificity. The phenomenon of specificity, where symbiont species partner with species of host with some degree of selectivity, is important to the biology of any symbiotic relationship. The characterization of this specificity ultimately depends on discerning

the specific identity of both partners. Past infection experiments under controlled conditions, conducted without knowledge of symbiont identity (other than their original host origin), showed that most cultured or freshly isolated *Symbiodinium* populations established symbioses with the experimental hosts (Kinzie 1974, Kinzie and Chee 1979, Davy et al. 1997). Several studies identified cultured isolates that did not form associations with polyps (scyphistomae) of the mangrove upside-down jellyfish, *Cassiopeia xamachana*, and aposymbiotic *Aiptasia tagetes* (Schoenberg and Trench 1980c, Colley and Trench 1983, Fitt and Trench 1983). It appeared that *C. xamachana* and *A. tagetes* demonstrated high specificity for certain *Symbiodinium* taxa and rejected others. However, a reexamination of infection experiments by Colley and Trench (1983), Fitt and Trench (1983), and Fitt (1985) shows that many of the “noninfective” isolates correspond to type A2 taxa (Table 4). Except for this group and “*S. californium*” of lineage E, *C. xamachana*, under laboratory conditions, forms symbioses with a wide variety of *Symbiodinium* species from divergent lineages. An individual of *C. xamachana* collected from a lagoon near Cancun, Mexico was found simultaneously harboring *Symbiodinium* types A1, A3, and C1 (unpublished data). Though it is generally believed that specificity is greater under natural conditions (Trench 1993), these findings, both experimental and natural, indicate that *C. xamachana* demonstrates low selectivity for different *Symbiodinium*.

Evidence for cryptic populations. As studies on *Symbiodinium* ecology expand, it is likely that species with different ecological “strategies” will be characterized. For example, A2 type isolates originated from numerous host species collected from distant geographic locations. These hosts include a colonial zoanthid, *Zoanthus sociatus*, a hard coral, *Meandrina meandrites*, a sea anemone, *Bartholomea annulata*, and a sea fan, *Gorgonia ventalina*, all from the Caribbean, as well as isolates from a blue coral, *Heliopora* sp., tridacnid clam, *Tridacna gigas*, and heart cockle, *Corculum cardissa*, from the west Pacific. Though they do not form symbioses with *C. xamachana*, they do establish symbioses with the giant clams *Tridacna squamosa* and *Hippopus hippopus* (Fitt 1985). Given the high frequency with which it has arisen in culture, surprisingly the genetic signature of A2 algae was never identified in a survey of natural *Symbiodinium* populations from a diverse range of Caribbean host species (unpublished data) nor has been identified from the sampling of others (Carlos et al. 1999, Baker 1999). If continued surveys fail to identify this A2 type in natural populations, one explanation would be that these algae represent a cryptic population that associates with various hosts at low concentrations but, under the culturing process, is opportunistic, grows rapidly, and out competes cells of the dominant natural population.

Biogeography. Studies concerning the biogeography of marine microalgae are often confounded by inadequate sampling and the difficulties of species identifi-

cation (Round 1981). Though there are some discussions (Baker and Rowan 1997, Baker 1999), few studies have attempted to follow the distribution of *Symbiodinium* species over wide geographic ranges (LaJeunesse and Trench 2000, Baillie et al. 2000b). This study reports isolates with identical ITS sequences, originating from hosts collected from extremely distant geographic locations. The nearly global distribution of certain *Symbiodinium* ITS types is similar to that observed for the cosmopolitan planktonic dinoflagellate *Gymnodinium catenatum* (Adachi et al. 1997). Sexually compatible *G. catenatum* isolates from Pacific and Atlantic populations contain low (<1.0%) ITS sequence variation (Adachi et al. 1997). By comparison, the lack of sequence variability observed among *Symbiodinium* originating from the Pacific and Atlantic oceans does indicate that this genus contains cosmopolitan species.

For example, type A3 was acquired from collections in Palau, Enewetak, and the Great Barrier Reef and appears to be widely distributed across the west Pacific. Baillie et al. (2000b) also cultured A3 types from Tridacnid bivalves originating from the Philippines and Palau. Field work in the Atlantic shows that type A3 is also highly prevalent on Caribbean reefs (unpublished data). The barrier to dispersal between the Caribbean Sea and Pacific Ocean is relatively recent (3.0–3.5 mya) (Coates et al. 1992) and perhaps has not allowed enough time for recorded change at the ITS level. The alternative is that these populations remain connected via some other route of dispersal and exchange (e.g. bilge water).

Nothing is known about the dispersal capabilities and rate of gene flow possibly occurring within these algae to provide a plausible explanation for why ITS types are widely distributed. However, there is partial evidence that *Symbiodinium* species are quite capable of living and perhaps remaining viable outside of the host. Free-living *Symbiodinium* have been cultured and identified from environmental samples (Loeblich and Sherley 1979, Carlos et al. 1999). *Gymnodinium varians*, isolated from a water sample taken from Cooks Strait off the coast of New Zealand (Chang 1983), is by molecular standards a *Symbiodinium* and closely related to “*S. californium*” (LaJeunesse and Trench 2000). This discovery indicates that some *Symbiodinium* may have a planktonic existence, which may explain their dispersal capability. Furthermore, many isolates have been grown and maintained in artificial media for over 25 years, indicating that these algae can persist and reproduce for an indefinite period of time outside the host as long as their nutrient requirements are met. Finally, the long-distance dispersal of host planula larvae may also contribute to symbiont dispersal. Symbiotic larvae from the coral *Pocillopora damicornis* can spend up to 100 days in the plankton before settling (Richmond 1987).

The properties of dispersal and the possibility of gene flow among *Symbiodinium* species deserve further investigation. The most basic information concerning

the natural history of free-living *Symbiodinium* is lacking. Future discoveries pertaining to their ecology in the external environment should provide clues to explain the distribution of various *Symbiodinium*. Certain species may have pandemic distributions, whereas others may possess limited regional or local distributions. The occurrence of different *Symbiodinium* species assemblages among regional reef ecosystems may ultimately factor into each system's ability to deal with environmental change (cf. Baker and Rowan, 1997).

Sexual recombination. Sexual recombination among dinoflagellates is rarely described (Pfiester and Anderson 1987). Reports of recombination in *Symbiodinium* are conflicting, and definitive evidence, namely the process of meiosis, has never been observed (Freudenthal 1962, Taylor 1974, Blank 1987, Trench 1993). A long-standing and important question concerning the biology of *Symbiodinium* is whether a sexual stage occurs in their life cycle. Recombination would result in greater variation within and among populations and the outcome would presumably produce populations more resilient to climactic change.

Evidence emerging from genetic analyses strongly suggests that recombination occurs within *Symbiodinium* populations. Isoenzyme (allozyme), RAPD, and DNA fingerprint analyses have revealed unusually high genetic variability among cultured isolates and field sampled populations (Schoenberg and Trench 1980a, Goulet and Coffroth 1997, Baillie et al. 1998, 2000a). Such methods measure differences in genotypes and can distinguish individuals or clonal lineages (Avice 1994, Coffroth 1997). Current speculation proposes that the high variability among sampled *Symbiodinium* populations is attributed to the occurrence of sexual recombination (Baillie et al. 1998, 2000a).

The analyses of Baillie et al. (1998, 2000a) were originally conducted on isolates whose phylogenetic identities were unknown. The high allelic variability they found in isoenzymes and RAPDs could be the result of comparing distantly related clones. It was then shown that all their isolates had virtually identical ITS sequences (type A3) (Baillie et al. 2000b). In support of these findings, isoenzyme profiles generated by the work of Colley (1984) show a similar situation. Figure 5 compares ITS sequence type with a dendrogram derived from a distance matrix based on the mean Jaccard's similarity coefficients (Sneath and Sokal 1973) from 11 enzyme systems (Colley 1984). This comparison shows that phylogenetic similarity is not related to isoenzyme similarity. The level of isoenzyme dissimilarity is in fact just as great for two cultures with identical ITS sequences as it is between isolates from different phylogenetic lineages. The lack of correlation between independent sets of genetic markers (isoenzyme, RAPD, and DNA sequences) indicates that shuffling, or randomization, of alleles by sexual recombination most likely accounts for this high level of genotypic variability (Tibayrenc et al. 1991). Furthermore, the findings here also indicate that *Symbiodin-*

ium species from other lineages, not only from lineage A, appear to demonstrate a high level of allelic variation (cf. Baillie et al. 1998, 2000a).

The lack of congruence between a molecular phylogeny and population genetic structure among *Symbiodinium* isolates is in contrast to the genetic structures of certain clonal protozoan populations. For example, the genetic analyses of *Trypanosoma cruzi* populations demonstrate strong clonality, even though the species is known to have a sexual component in their life histories. Populations of *T. cruzi* are overrepresented by identical genotypes that are widespread, high linkage disequilibrium, and a positive correlation between independent data sets of genetic markers. These data strongly indicate that *T. cruzi* genotypes are replicated units (Tibayrenc et al. 1991, Laurent et al. 1997). For *Symbiodinium*, there are no known examples of widespread identical genotypes. Rather, the allelic variability observed in these dinoflagellates are similar to the allelic variability of organisms displaying sexual recombination (Baillie et al. 1998, 2000a). The data presented here support earlier suppositions of sexual recombination occurring within the genus *Symbiodinium* (Baillie et al. 1998). In the context of these findings, it is hypothesized that a species of *Symbiodinium* consist of numerous clonal populations that are generated by sexual recombination.

Concluding remarks. The history or systematics of an organismal lineage can be viewed as a continuum down to the individual (O'Hara 1993). The use of DNA sequences for phylogenetic reconstruction can be thought of as a discrete sampling of this continuum. The more conserved the gene, the older or more generalized is the phylogenetic picture. By using more variable gene regions, more recent historical phylogenetic divisions are documented. The ITS region provides greater systematic resolution for *Symbiodinium* than the LSU and SSU and is helpful in relating differences and similarities in morphology, physiology, biochemistry, and host-symbiont interactions. The finding that certain functional and morphological attributes correspond with phylogenetic groupings based on ITS sequence data suggests that a phylogenetic or molecular species concept may be useful for classifying *Symbiodinium* (Baum 1992, Manhart and McCourt 1992).

The development of diagnostic or "species specific" molecular markers for the identification and study of different ecological populations should help with studies examining physiology, ecological and biogeographic distribution, and host-symbiont specificity. The level of genetic resolution offered by the ITS will not explain all morphological and physiological differences. The challenge of identifying "species" or ecologically distinct populations may still remain for some types. Ultimately, proper characterization of these populations will depend on a combination of genetic, morphological, and physiological analyses. Finally, it is clear that future investigations require a

greater emphasis on comparative physiology, both on cultures and natural populations, as the profusion of sequence data (this work included) has outpaced the progress of these other disciplinary approaches.

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