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Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae)

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ABSTRACT **Zooxanthellae are unicellular algae that occur as endosymbionts in many hundreds of common marine invertebrates. The issue of zooxanthella diversity has been difficult to address. Most zooxanthellae have been placed in the dinoflagellate genus *Symbiodinium* as one or several species that are not easily distinguished. We compared *Symbiodinium* and nonsymbiotic dinoflagellates using small ribosomal subunit RNA sequences. Surprisingly, small ribosomal subunit RNA diversity within the genus *Symbiodinium* is comparable to that observed among different orders of nonsymbiotic dinoflagellates. These data reinforce the conclusion that *Symbiodinium*-like zooxanthellae represent a collection of distinct species and provide a precedent for a molecular genetic taxonomy of the genus *Symbiodinium*.**

Animal–algal symbioses are ubiquitous and typically dominant features of shallow tropical seas. Of the several kinds of unicellular algal participants (1), the most abundant is a collection of coccoid yellow-brown dinoflagellates that are colloquially known as zooxanthellae (2, 3). Taxonomic studies of these algae have been hindered by their problematic biology: a paucity of informative morphology, especially in the vegetative (symbiotic) state (4, 5); the possible complications of host-associated phenotypic plasticity, restricting definitive studies to those zooxanthellae that can be cultured *in vitro* (6–8); the absence of sexual reproduction, a lack that precludes genetic investigations (3). The poor status of zooxanthella taxonomy in turn limits the study of the ecology and evolution of their symbioses. Whether distinct species of algae are mutually specific for particular species of hosts and how such symbiont–host specificity might change in response to environmental factors or over evolutionary time scales are examples of fundamental issues that have been difficult to address.

Morphological features associate zooxanthellae with two distinct forms of free-living dinoflagellates. A minority of isolates produce the amphidinioid form during at least some stage in their life cycle and have been placed in the genus *Amphidinium* (2, 3). Most zooxanthellae, including the symbionts from reef-building corals, soft corals, anemones, and giant clams, appear to have gymnodinioid affinities. Many or all of these were originally (2, 9) believed to be the single pandemic species *Symbiodinium microadriaticum* Freudenthal. Subsequent morphological, cytological, biochemical, physiological, and behavioral analyses of *Symbiodinium* (3, 10–12) refuted this conclusion and lead to the description of three additional *Symbiodinium* species from *in vitro* cultures (13). Because *Symbiodinium*-like algae undoubtedly represent a collection of many cryptic taxa, these algae in nature can only be referred to *Symbiodinium* sp. (14).

The present study was undertaken as a step toward developing a molecular genetic taxonomy for the genus *Symbiodinium*. Many of the problematic aspects of zooxanthella systematics can be obviated by applying molecular methods. DNA sequences are excellent phylogenetic data (for reviews, see refs. 15 and 16) that are especially useful for identifying and classifying morphologically depauperate organisms like zooxanthellae. Furthermore, *Symbiodinium* genes can be obtained from intact symbioses using the polymerase chain reaction (PCR; ref. 17), removing the obstacle of culturing zooxanthellae for the purpose of taxonomy (18).

Various DNA sequences evolve at very different rates; which sequences are informative for a group depends upon how closely related its members are. Having no *a priori* information for *Symbiodinium*, we examined nuclear genes that encode small ribosomal subunit RNA (ssRNA; 16S-like RNA). ssRNA is a mosaic of domains with different evolutionary rates (19–21). This feature and a large base of ssRNA sequence data (22) make this molecule the logical choice for investigations of "unknown" organisms (23). We present nearly complete ssRNA sequences from two cultured isolates of *Symbiodinium* and partial ssRNA sequences from two other cultures of *Symbiodinium* and from nine cultured nonsymbiotic dinoflagellates.† These data allow us to evaluate *Symbiodinium* diversity in the larger context of dinoflagellate taxonomy. *Symbiodinium* ssRNA sequences are surprisingly diverse and will be useful in *Symbiodinium* taxonomy.

MATERIALS AND METHODS

Dinoflagellate Cultures and DNA Isolation. *Symbiodinium microadriaticum* (from the jellyfish *Cassiopeia xamachana*; Jamaica), *Symbiodinium pilosum* (from the zoanthid *Zoanthus sociatus*; Jamaica), and *Symbiodinium #8* (from the anemone *Aiptasia pulchella*; Hawaii) were provided by R. K. Trench (Department of Biological Sciences, University of California at Santa Barbara); *Symbiodinium* sp. (from *Aiptasia pallida*; Bermuda) was provided by G. Muller-Parker (Shannon Point Marine Center of Western Washington University); and *Prorocentrum mariae-labouriae* was provided by L. W. Harding (Chesapeake Bay Institute, Johns Hopkins University). These cells were harvested by centrifuging 1 ml of culture (5×10^4 to 1×10^6 cells) at 12,000 rpm for ≈15 sec at room temperature (Sorvall Microspin centrifuge). *Gymnodinium simplex* (clone WT582), *Gymnodinium varians* (clone CHANG4), *Gymnodinium galatheanum* (clone 76D), *Ceratium fusus* (clone NEPCC655), *Heterocapsa ildefina* (clone CILL), *Heterocapsa niei* (clone CNIEI), *Peridinium foliaceum* (clone FOLI), and *Thoracosphaera heimii* (clone L603) were from unialgal "starter cultures" (containing 1×10^4 to 1×10^5 cells) obtained from the Provasoli–Guillard Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME). These cultures were harvested at room temperature by

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Abbreviation: ssRNA, small ribosomal subunit RNA.
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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M88509–M88521).

filtration using disposable filter units [Nalgene type A, 0.45-μm (pore-size) filters], and the cells were rinsed from the filters with buffer (0.4 M NaCl/40 mM $MgSO_4$/10 mM EDTA/20 mM Tris·HCl, pH 7.6) and concentrated by centrifugation as above.

Dinoflagellate cell pellets (2 μl–10 μl) were resuspended in 0.4 ml of 0.4 M NaCl/0.05 M EDTA, pH 8.0/1% SDS and heated to 65°C for 30 min, followed by incubation at 45°C in the presence of proteinase K (Boehringer Mannheim; 0.5 mg/ml, final concentration) for 6 h. NaCl was then increased to 0.8 M, 20 μg of *Escherichia coli* tRNA (Boehringer Mannheim) was added as carrier nucleic acid, cetyltrimethylammonium bromide was added to a final concentration of 1%, and the lysates were heated to 65°C for 30 min. The lysates were extracted with equal volumes of chloroform (once) and then phenol (twice), and the nucleic acids were precipitated with ethanol. Nucleic acid precipitates were resuspended in 100 μl of 0.3 M sodium acetate, precipitated again with ethanol, then resuspended in 50 μl of water, and stored at −20°C.

Gene Amplification, Cloning, and Sequencing. ssRNA sequences were obtained from PCR-amplified ssRNA nuclear genes, as described by Medlin *et al.* (24). "Universal eukaryotic" PCR primers for amplifying ssRNA-encoding DNA were designed from a published ssRNA sequence comparison (22). Primers ss5 (5′-GGTTGATCCTGCCAGTAGT-CATATGCTTG-3′) and ss3 (5′-GATCCTTCCGCAGGT-TCACCTACGGAAACC-3′) are located 4 nucleotides from the 5′ and 3′ ends, respectively, of the ssRNA coding sequence in the dinoflagellate *Prorocentrum micans* (25).

DNA amplifications were performed using the GeneAmp PCR kit and the DNA thermal cycler (Perkin–Elmer/Cetus) according to the manufacturer's instructions. Amplification mixtures (total volume = 100 μl) contained 4 μl of dinoflagellate nucleic acid (corresponding to the DNA recovered from 1×10^3 to 1×10^4 cells) and consisted of 30 cycles of the following profile: 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Completed amplifications were extracted once with phenol/chloroform, 50:50 (vol/vol), and the amplified DNA was precipitated with ethanol and then resuspended in water.

The amplification products from *S. microadriaticum*, *S. pilosum*, *Symbiodinium #8*, and *Prorocentrum mariae-labouriae* were purified by electrophoresis in 1% SeaPlaque agarose (FMC) and by chromatography on Elutip-d columns (Schleicher & Schuell) according to the manufacturers' directions and then were cloned as blunt-ended fragments into bacteriophage M13mp18 (26). The DNA amplified from *Symbiodinium* sp. *G. simplex*, *G. varians*, *G. galatheanum*, *Ceratium fusus*, *H. ildefina*, *H. niei*, *Peridinium foliaceum*, and *T. heimii* was digested with the restriction endonuclease *Xba* I, and a fragment (positions 150–841, see Fig. 2) was cloned into the *Xba* I site of M13mp18. Single-stranded DNA from recombinant bacteriophage was sequenced using a Sequenase kit (United States Biochemical) and, as additional sequencing primers, synthetic oligonucleotides that correspond to conserved ssRNA sequences were used.

RESULTS

The complete sequence of the nuclear ssRNA gene from the dinoflagellate *Prorocentrum micans* (25) provided a reference for our investigation. A single PCR amplification product of ≈1800 base pairs was obtained from each dinoflagellate (data not shown), in agreement with a prediction of 1793 base pairs according to the *Prorocentrum micans* ssRNA sequence. Complete sequences of the *S. microadriaticum* and *Symbiodinium #8* amplification products are presented in Fig. 1. The accuracy of these data and their usefulness for phylogenetic analyses will be limited by errors in DNA synthesis during the PCR and by sequence heterogeneity of the multicopy ssRNA genes that were amplified (17, 28). To estimate this limitation, two independent amplification products (clones of opposite polarity were obtained from separate amplification reactions) were sequenced and compared to each other.

Two clones from *S. microadriaticum* differed by a single nucleotide (Fig. 1), and one clone contained a perfect tandem duplication of 30 base pairs. This duplication was not present in two other *S. microadriaticum* clones nor in any of the >30 cloned (PCR-amplified) ssRNA genes from other dinoflagellates that have been partially sequenced (ref. 27 and unpublished data) and, therefore, remains an unexplained (but identifiable) artifact. Two clones from *Symbiodinium #8* differed from each other by two single-nucleotide substitutions (Fig. 1). We conclude that the sequences of *Symbiodinium* ssRNA genes can be obtained from cloned PCR-amplified DNA with a high degree of certainty (1726 of 1733 total positions were determined unambiguously on both clones; 1724 of 1726 positions correspond to 99.9% for *Symbiodinium #8*). Medlin *et al.* (24), in an identical analysis of ssRNA genes from a diatom, reported this same high level of precision.

Overall, the two *Symbiodinium* ssRNA sequences differ by 3.3% (58 of 1733 sequence positions; an insertion/deletion of 2 nucleotides at position 235 is scored as one event). The significance of this difference was evaluated using nonsymbiotic dinoflagellates that, unlike isolates of *Symbiodinium*, are obviously different species. Data from two variable regions of the ssRNA gene (see Fig. 2) were easily obtained and included enough nucleotide substitutions for this comparison. Together, these regions contain 478 nucleotide positions that account for 52% (30 of 58 positions) of the difference between *S. microadriaticum* and *Symbiodinium #8*. Two additional *Symbiodinium* sequences were also determined. Phylogenetic analyses of the aligned ssRNA sequences (Fig. 2) were conducted using programs in J. Felsenstein's PHYLIP collection (29). The unrooted tree that is consistently defined by genetic distances is presented in Fig. 3. In this tree, branch lengths are proportional to the estimated amounts of nucleotide substitution in the partial ssRNA sequences (31). The program DNAPARS (29) identifies Fig. 3 as the single most parsimonious tree topology for the data, whereas compatibility criteria (DNACOMP, ref. 29) identify this tree plus 11 other trees that differ only in the relative positioning of *T. heimii*, *H. ildefina*, and *H. niei* with respect to one another, as equally parsimonious. Bootstrapped parsimony analysis using DNABOOT (29) gives Fig. 3 as the unrooted majority rule consensus tree topology (32) and shows that the data strongly support the groups of taxa labeled A and B as distinct from each other (in 100 of 100 instances) and the two taxa labeled C as distinct from both groups A and B (in 98 of 100 instances).

DISCUSSION

Morphological, biochemical, physiological, and behavioral characters have been used to assess variation among superficially similar zooxanthellae (3, 10–13). The present study examined the utility of ssRNA sequence data in *Symbiodinium* taxonomy. Because ssRNA has been widely studied and since homologous ssRNAs occur in all complete organisms, this molecule is an especially useful metric of diversity.

Differences between nearly complete ssRNA sequences from *S. microadriaticum* and *Symbiodinium #8* define two distinct "types" of zooxanthellae (Fig. 1). Two partial ssRNA sequences refer one additional zooxanthella isolate to each type (Fig. 3). Because two independent sequences from one isolate (*Symbiodinium #8*) differed by two nucleotides within the region covered by these partial sequences, the two members of each zooxanthella pair are not distinguishable by

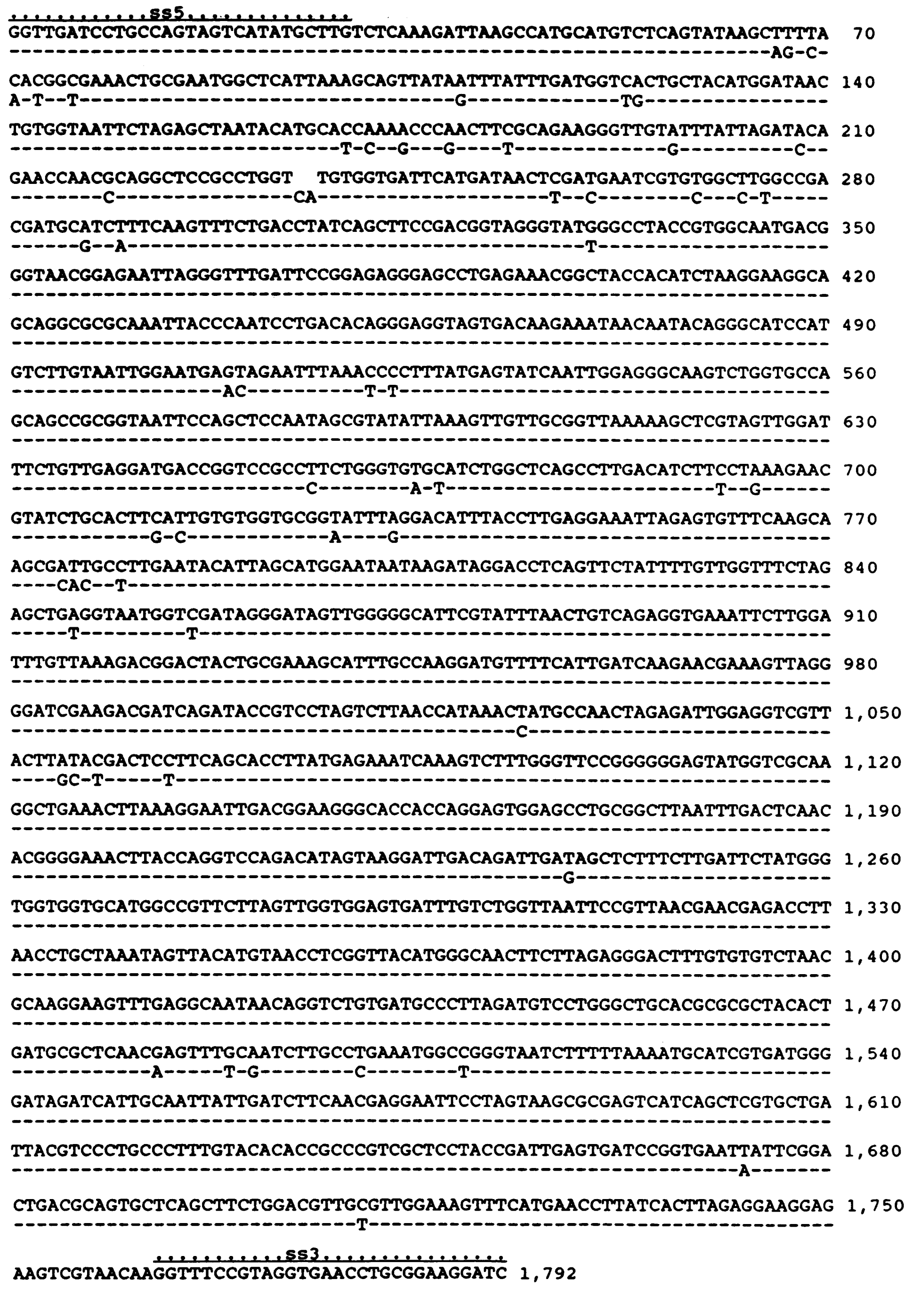

FIG. 1. Sequences of nuclear-encoded ssRNA from *S. microadriaticum* (upper line) and *Symbiodinium #8* (lower line) obtained from PCR-amplified ssRNA genes. Positions are numbered from 5′ to 3′. ss5 and ss3 identify PCR primers. Two independent *S. microadriaticum* ssRNA gene clones differed at position 639 (G vs. A), and two independent *Symbiodinium #8* clones differed at positions 195 (G vs. A) and 267 (T vs. C). These positions were assigned according to consensus with 20 other *Symbiodinium* ssRNA sequences (27).

our methods: *S. microadriaticum* and *S. pilosum* differ by three nucleotides, and *Symbiodinium #8* and *Symbiodinium* sp. differ by two nucleotides (Fig. 2). The similarity of *Symbiodinium #8* and *Symbiodinium* sp. correlates with the similarity of their natural hosts, the anemones *A. pulchella* (from Hawaii) and *A. pallida* (from Bermuda), respectively. The similarity of the *S. microadriaticum* and *S. pilosum* ssRNA sequences is surprising. These Caribbean isolates (from the jellyfish *Cassiopeia xamachana* and the zoanthid *Zoanthus sociatus*, respectively) are very different from each other by morphological, biochemical, physiological, and behavioral criteria (3, 13). Indeed, *S. pilosum* is phenotypically distinct from all other cultured zooxanthellae that have been studied in detail, and the characteristic appearance of *S. pilosum* cultures (13) assures that the material used in the present study was correctly identified. Thus these observations suggest that phenotypes evolve at different rates in different lineages of *Symbiodinium*. Since biochemical, physiological, and behavioral phenotypes must be significant aspects of symbiosis biology (3), this phenomenon deserves further study.

There is no convention for relating ssRNA dissimilarity to taxonomic rank or to time of divergence, but these data do provide estimates of similarity that can be compared across traditional taxonomic boundaries. *S. microadriaticum* and *Symbiodinium #8* appear about as distinct from each other as from the nonsymbiotic dinoflagellate *G. varians* (Fig. 3). In pair-wise comparisons by the number of observed nucleotide substitutions, *G. varians*, *G. simplex*, *S. microadriaticum*, and *Symbiodinium #8* are all about equally similar/dissimilar

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TCTAGAGCTAATACATGCACCAAAACCCAACTTCGCAGAAGGGTTGTATTTATTAGATACAGAACCAACGCAGGCTCCGCCTGGT  TGTGG TGATTCATGATAACTC GATGAATCGTGTGGCTTG GCCGACGATGC 140
-------------------------------------------------------------------------------------  ----- ---------------- ------------------ -----------
-------------------------------------------------------------------T---------T-------  ----- ---------------- ------------------ -----------
-------------------T-C--G---G----T-------------G----------C----------C---------------CA----- ---------------T --C--------C---C-T -----------
-------------------T-C--G---G----T-------------G----------C----------C---------------CA----- ---------------T --C--------C---C-A -----------
------------------G--C------G------TG----------G---------------------C----T--GC------AT----- --------AG----CG A-C------CA-A----CC--T-G----AA
------------------G-A-------G----T----G--------G---------------------C-------T------ACA----- ---------------T ----------------CC -----------
----------------------------G------  ----------G-------------------T-C--A-------T----CT----- ---C------------ ----------A------C --T--------
------------------G--C------G---C--TG----------G--------T------------C---------------TT-T--- --------A-----CG A-C------CA----A-CA----G-----A
-G----------------G--C------G----ATAG----------G--------T----------G-C-----C-GC- ----CC-T--- --------A-----CG A-C------CA-A----C --T-G-----A
-G----------------G--C------G------AG----------G--------T------------C-------T-- --TCTT----- --------A-----CAGA-C------CA----A-CC--T-G-----A
------------------G---------G-----TTG----------G--------T----A-------C---------------CC-C--- --------A-----CG A-C------CA----A-CA--T-G-----A
------------------G--C------G-----TTG----------G--------T----A-------C-------T-------TT-C--- --------A-----CG AGC------CA----A-CA--T-G-----A
---------------C---TA-CCG-----T---T-G------C-A-G--------T--------------------T-----A-GC-T--- ---G-G--AG---AA- A------T-CACA--A-TA--T-G-----A
------------G-------AC-----TG--C-TCA--T-T------G------GAC-T--A-------T--A----T--T--A-GT-T--TA---C-G-------A-T A------T-C-----GCAT--T-G-A---A

ATCTTTCAAGTTTCTGACCTATCAGCTTCCGACGGTAGGGTATGGGCCTACCGTGGCAATGACGGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCGGTTAAAAAGCTCGTAGTTGGATTTCTGTTGGGGATG 280
--------------------------------------------------------------------------------------------------------------------------------------------
G--A---------------------------------------T------------------------------------------------------------------------------------------A-----
G--A---------------------------------------T------------------------------------------------------------------------------------------A-----
---A---------------------------------------T---------------------------------------------------------------------------------------CC-A---C-
---A---------------------------------------T---------------------------------------------------------------------------------------------G--
---A---------------------------------------T------------------------------------------------T--------------------------------------C--A-----
---A---------------------------------------T------------------------------------------------T--------------------------------C-----CC-A---C-
---A---------------------------------------T---------------------------------------------------------------------------------------CC-A---C-
---A---------------------------------------T---------------------------------------------------------------------------------------CC-A---C-
---A---------------------------------------T-----------------------------------------------------------------------------------C---CC-----C-
---A---------------------------------------T---------------------------------------------------------------------------------------CC-----C-
---A------------------------T----------A---A---T---------G-------------------------------------------------------------------------CC-G---C-
--TG--TT-----------------------------AA--------T----------G------------------------------------------------------------------------C---A-CAA

ACCGGTCCGCCTTCTGGGTGTGCATCTGGCTCAGCCTTGACATCTTCCTAAAGAACGTATCT GCACTTCATTGTGTGGTGCGGTATTTAGGACATTTACCTTGAGGAAATTAGAGTGTTTCAAGCAAGCGATTGCCTTG 420
----------------------T--------------------------------------- -----------------------------------------------------------------------------
-----------C--------A-T-----------------------T--G------------ ------G-C------------A----G-----------------------------------------CAC--T---
-----------C--------A-T-----------------------T--G------------ ------G-C------------A----G-----------------------------------------CAC--T---
-----------C--------C-T---------G----G-G-------T-GG----------- ------G-C---------------CC-----T-----T--------------------------G--ACAC------
-------------------------------------------------G--------G--- -------GC-------C----C---------T--------------------------------C---CAC------
-----------C--------A-T----------------G---------G------------ ------G-C----------------------------T------------------------------CAC------
-----------C--------A-T---------G----G-G-------T-GG----------- ------G-C---------------CC-A---T-----T--------------------------G--ACAC------
-----------C--------A-T---------G----G-G-------T-GG--------G-- ------G-C---------------CC-----T-----T--------------------------G--TTAC------
----------- --------A-T----- --- ---- -G-------T-GG--------G--G------G-C---------------CC-----T-----T-------------------------C-- TTAC------
-----------C--C-----A-----------G----G-G-------T-GG----------- ------GGC----------------C-----T-----T--------------------------G--ACAC------
-----------C--------A-T---------G----G-G-------T-GG--------A-- ------GGC----------------C-----T-----T--------------------------G--ACAC------
-------------TC-----A--------T--G------G---A--TT-GGCA----CT--- ------G-C----C--------GG-C-A--GC-----T-------------------CT-----G--ATC-------
-----------C--------A----------TTATT---G---A-G-T--G-CTTT-C-G-- ------G-C---------T-AAG C--A-C-------T----------C---------------G -A---------

AATACATTAGCATGGAATAATAAGATAGGACCTCAGTTCTATTTTGTTGGTTTCTAGA 478
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------G-----------------------
--------------------------------C-------------------------
----------------------------------G-----------------------
---------------------------------TG-----------------------
----------------------------------G-----------------------
----------------------------------G-----------------------
----------------------------------G-----------------------
--------------------------G-------G-----------------------
---------------------G-T-------T--G-----------------------
------C---------------T----T---TGTG---T-----------C-------
```

FIG. 2. Partial ssRNA sequences from various dinoflagellates. The sequence is a composite from two regions: positions 1–206 correspond to positions 150–352 in Fig. 1 and positions 207–478 (end) correspond to positions 571–841 in Fig. 1. The sequences are from top to bottom as follows: *S. microadriaticum*, *S. pilosum*, *Symbiodinium #8*, *Symbiodinium* sp. (from *A. pallida*), *G. galatheanum*, *G. varians*, *G. simplex*, *T. heimii*, *Prorocentrum mariae-labouriae*, *Prorocentrum micans* (from ref. 25), *H. ildefina*, *H. niei*, *Peridinium foliaceum*, and *Ceratium fusus*.

[values in the matrix range (not shown) from 28 to 34 substitutions]. The grouping of these four taxa was anticipated: The gymnodinioid morphology of motile forms that arise in zooxanthella cultures indicates that the genus *Gymnodinium* represents nonsymbiotic dinoflagellates that are closely related to *Symbiodinium* (refs. 33 and 34; see ref. 14 for a discussion of the nomenclature of zooxanthellae and the distinction between *Symbiodinium* and *Gymnodinium*).

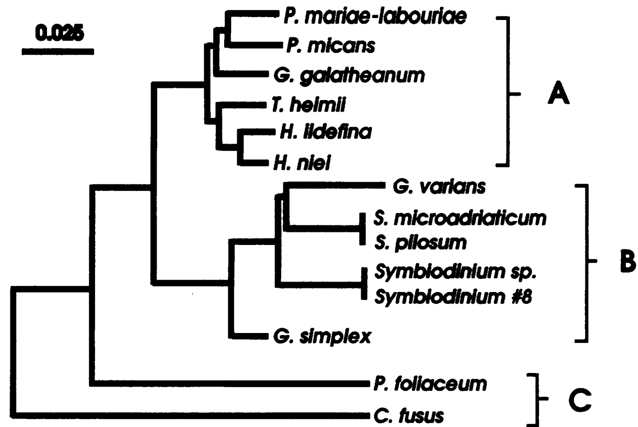

FIG. 3. Inferred phylogenetic relationships of dinoflagellate ssRNAs. The unrooted tree was produced using genetic distances (29, 30) by the method of Fitch and Margoliash (31). Scale indicates the branch length corresponding to an estimated genetic distance of 2.5% nucleotide substitution. The position of the branch tip at *Ceratium fusus* is arbitrary. *P. mariae-labouriae*, *Prorocentrum mariae-labouriae*; *P. micans*, *Prorocentrum micans*; *P. foliaceum*, *Peridinium foliaceum*.

Fig. 3 conflicts with dinoflagellate taxonomy in other instances. One well-supported group of ssRNA sequences (Fig. 3, group A) contains members of the orders Prorocentrales (*Prorocentrum mariae-labouriae* and *Prorocentrum micans*), Gymnodiniales (*G. galatheanum*), and Peridiniales (*T. heimii*, *H. ildefina*, and *H. niei*) to the exclusion of other members of the Gymnodiniales (*G. varians* and *G. simplex*, *Symbiodinium*) and Peridiniales (*Peridinium foliaceum* and *Ceratium fusus*). Our sequence data are in agreement with another study (35) where large ribosomal subunit RNA sequences grouped together a *Heterocapsa* sp., a different isolate of *H.* (*Cachonina*) *niei*, a *Gymnodinium* sp., and *Prorocentrum micans*, to the exclusion of other members of the Peridiniales. These molecular classifications imply that the morphological characters that are traditionally used in dinoflagellate taxonomy might possibly misrepresent phylogenetic groups. Our limited data do not warrant a lengthy discussion of dinoflagellate systematics (36–38). An obvious course for further study would be to assemble a more complete (more taxa and larger sequences) dinoflagellate ssRNA tree and to examine algae for morphological features that are concordant with molecular data.

The present study was designed to assess diversity within the genus *Symbiodinium*. Given that *Symbiodinium*-like

zooxanthellae have often been regarded as a collection of strains or closely related species, it is somewhat surprising to observe (Fig. 3) greater genetic distances between two *Symbiodinium* taxa (*S. microadriaticum/S. pilosum* and *Symbiodinium* sp./*Symbiodinium #8*) than between representatives of different orders of nonsymbiotic dinoflagellates (taxa in group A). Clearly, some *Symbiodinium* spp. are separated by amounts of molecular evolution that are typically accompanied by considerable morphological change in other dinoflagellates. Different isolates of *Symbiodinium* have been distinguished by morphology only through careful analyses of cell architecture (3, 13), if at all. Morphological conservatism among such genetically distinct algae may be a consequence of their adaptation to, and their evolution within, a relatively constant environment—the endozoic milieu (39).

There is no time scale in the evolutionary hypothesis presented in Fig. 3. The possibility that nucleotide substitutions occur at different rates in different lineages (40) cautions against equating genetic distance with time since divergence (the "molecular clock"). There is some suggestion in Fig. 3 of unequal substitution rates: By using *Peridinium foliaceum/Ceratium fusus* to root the tree that contains all other taxa (Fig. 3), the estimated amount of evolution (average root-to-tip branch length) is greater in the *Symbiodinium* group (group B; 6.6% nucleotide substitution, averaging the four distinct taxa) than in the nonsymbiotic group (group A; 3.9% nucleotide substitution). Because of the ad hoc method of tree rooting (outgroups are not obvious given the disparities between dinoflagellate taxonomy and ssRNA sequence similarity) and also the large variance of genetic distances as measured from short DNA sequences (15), this relative rate comparison is only approximate at best. One evolutionary model postulates that genetic change due to recombination is suppressed in endosymbionts (39). Our data do not address this issue, but they do suggest that the rate of genetic change due to nucleotide substitution is not suppressed in symbiotic dinoflagellates.

The important conclusion to be drawn from the present study is that ssRNA genes can be used to identify and classify *Symbiodinium* spp. Immediate applications include further studies of zooxanthella diversity (18) and analyses of host–symbiont specificity (18, 27) and of the evolution (27) of symbioses.

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