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**NOTE**

# MITOCHONDRIAL DNA PHYLOGENY OF THE SYMBIOTIC DINOFLAGELLATES (*SYMBIODINIUM*, DINOPHYTA)[1]

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**Symbiotic dinoflagellates belonging to the genus *Symbiodinium* (Freudenthal) are found worldwide in association with shallow-water tropical and subtropical marine invertebrates. Most phylogenetic studies of *Symbiodinium* have used nuclear rRNA (nrDNA) genes to infer relationships among members of the genus. In this report, we present the first phylogeny of *Symbiodinium* based on DNA sequences from a mitochondrial protein-coding gene (cytochrome oxidase subunit I [*cox*1]). Two principal groups, one comprised of *Symbiodinium* clade A and the second encompassing *Symbiodinium* clades B/C/D/E/F, are strongly supported in the *cox*1 phylogeny. Relationships within *Symbiodinium* clades B/C/D/E/F, however, are less well resolved compared with phylogenies inferred from nrDNA and chloroplast large subunit (cp23S)-rDNA genes. Statistical tests between alternative tree topologies verified, with an exception being the position of one controversial member of *Symbiodinium* clade D, that relationships inferred from *cox*1 are congruent with those inferred from nrDNA and cp23S-rDNA. Taken together, the relationships between the major *Symbiodinium* clades are robust, and there appears to be no evidence of hybridization or differential introgression of nuclear and plastid genomes between clades.**

***Key index words:*** ***cox*1; dinoflagellate; mitochondrial; phylogeny; *Symbiodinium*; symbiosis; zooxanthella**

***Abbreviations:*** ***cox*1, cytochrome oxidase subunit I; cp23S-rDNA, chloroplast large subunit rDNA; ITS, internal transcribed space; nrDNA, nuclear rDNA; SH-test, Shimodaira and Hasegawa test**

Dinoflagellates in the genus *Symbiodinium* (Freudenthal 1962) form mutualistic symbioses with a wide array of marine invertebrates and protists and are an integral part of tropical and subtropical marine ecosystems (Hallock 2001). Historically, species descriptions of these dinoflagellates have relied on characteristics such as morphology and biochemistry (Freudenthal 1962, Trench and Blank 1987, Banaszak et al. 1993, Trench 1993). With the application of DNA sequence analyses, our understanding of the diversity and phylogenetic relationships within and between the symbiotic dinoflagellates has been greatly advanced (Rowan and Powers 1991, LaJeunesse 2001, Santos et al. 2002). However, a further understanding of *Symbiodinium* taxonomy and phylogenetics is essential for interpreting inter- and intraspecific physiological/ecological differences and exploring questions of host–symbiont coevolution and specificity within an evolutionary context.

Molecular taxonomic studies of *Symbiodinium* have not used many loci, with most studies over the last decade confined to genes of the nuclear rDNA (nrDNA) operon. Analyses of ribosomal small subunit (18S; Rowan and Powers 1991, McNally et al. 1994) and large subunit (28S; Wilcox 1998, Baker 1999, Pochon et al. 2001) RNA genes as well as the internal transcribed spacer regions (ITS1, ITS2, and 5.8S; Hunter et al. 1997, Baillie et al. 2000, LaJeunesse 2001) have shown that *Symbiodinium* comprises several large groups, commonly referred to as clades. The exact taxonomic significance of these clades, designated A–F, and those associated with the soritid foraminiferans (Pawlowski et al. 2001, Pochon et al. 2001) are currently unclear because few descriptions encompass morphology, physiology, and molecular data (but see LaJeunesse 2001). The first independent genetic evidence to support the established cladal divisions of *Symbiodinium* came from sequence analyses of chloroplast large subunit (cp23S)-rRNA genes (Santos et al. 2002). Phylogenies inferred from the chloroplast-encoded protein *psbA* also appear

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to be congruent with nrDNAs and cp23S-rRNA (Takishita et al. 2003). These findings of congruence are in contrast to discrepancies between nuclear and chloroplast phylogenies among free-living dinoflagellates (Zhang et al. 2000), with the discrepancies probably attributable to significant differences in evolutionary rates between genomes and taxa.

In this study we sequenced a highly conserved mitochondrial gene, *cox*1, which encodes subunit 1 of cytochrome *c* oxidase, and used these sequences to infer relationships among symbiotic dinoflagellates that have been previously characterized as clades A–F by nuclear and/or chloroplast rDNAs. This is the first phylogenetic analysis of symbiotic dinoflagellates based on mitochondrial DNA and only the second to use a protein-coding gene (Takishita et al. 2003). Cytochrome *c* oxidase is the final protein complex in the electron transport chain and is composed of subunits encoded by both nuclear and mitochondrial genes. Thus, cytochrome *c* oxidase activity reflects, to some extent, the coordinated function of the two genomes (Edmands and Burton 1999). We report that the mitochondrial phylogeny of symbiotic dinoflagellates is congruent (with the exception of the placement of one unusual isolate of *Symbiodinium* clade D) with those constructed from nuclear and chloroplast rDNAs, suggesting that the molecular taxonomy of the currently recognized *Symbiodinium* clades is robust and the point of their differentiation is ancient.

Most cultured isolates of *Symbiodinium* sp. used in this study are described in Santos et al. (2002) as part of a phylogenetic study of the genus using cp23S-rDNA. Other isolates included the *Symbiodinium* clade D cultures HpiH-2 and PSP1-05 (generously donated by Drs. M. Ishikura and T. Maruyama of the Marine Biotechnology Institute, Japan) and cultures 12B1, 141B2, and 385B3 (kindly provided by Dr. T. C. LaJeunesse, University of Georgia), which represent *Symbiodinium* ITS types B1, B2, and B3, respectively (LaJeunesse 2001). Other symbionts were freshly isolated from host tissue. These hosts included the sea anemones *Aiptasia pallida* (Key Largo, FL, USA) and *Condylactis gigantea* (Long Key, FL, USA) and the octocorals *Briareum asbestinum*, *Pseudopterogorgia americana*, and *Pterogorgia citrina* (Long Key, FL, USA). DNA was isolated from fresh samples and quantified as described in Takabayashi et al. (1998), whereas DNA from cultured cells was extracted and quantified as described in Santos et al. (2001).

For many of the samples in this study, the 18S-rDNA RFLP profiles (Santos et al. 2002), cp23S-rDNA genotypes (Santos et al. 2003), or ITS types (LaJeunesse 2001) have been reported. The phylogenetic identity of previously uncharacterized samples was assessed by established 18S-rDNA RFLP methods (Rowan and Powers 1991). To amplify *cox*1, two primer sets, located within the gene's open reading frame, were used in combination to produce an amplicon of approximately 1000 base pair. The first primer set came from Norman and Gray (1997) [forward: 5′-TTATTTTGRTTTTTTGGTCATCCTGARGT-3′, reverse: 5′-TCTGGGTAGTCTGGTATTCKTCKTGGCA-3′]. Sequences for the second primer set were kindly provided by Dr. S. Lin (University of Connecticut) [forward: 5′-AAAAATTGTAATCATAAACGCTTAGG-3′, reverse: 5′-TGTTGAGCCACCTATAGTAAACATTA-3′]. Both primer sets were found to be dinoflagellate specific by the original authors.

PCRs consisted of $1\,ng \cdot \mu L^{-1}$ DNA, 0.5 mM total dNTP, 0.1 M Tris-HCl, 50 mM KCl, 4 mM $MgCl_2$, $0.4\,mg \cdot mL^{-1}$ DNAase-free BSA (Promega, Madison, WI, USA), 0.1 μM of each primer, and $25\,U \cdot mL^{-1}$ Taq polymerase (Promega). Thermocycling conditions for both primer sets were as follows: initial denaturation of 4 min at 94° C, 30 cycles of 94° C for 30 s, 55° C for 1 min, and 72° C for 1 min, followed by a final extension of 7 min at 72° C. Amplicons were cloned using the TOPO-TA Cloning Kit® (Invitrogen, Carlsbad, CA, USA) and sequenced in the forward and reverse directions by the Interdisciplinary Center for Biotechnology Research, University of Florida. To ensure that host DNA was not amplified with the primer sets, we isolated template DNA from tissue of aposymbiotic (symbiont-free) *A. pallida* and ran control PCRs. No amplification was found (data not shown).

Corresponding *cox*1 DNA sequences were acquired from GenBank for *Crypthecodinium cohnii* (AF186994) and *Heterocapsa circularisquama* (AB049708) and served as outgroups. The partial sequences of *Symbiodinium* and outgroup *cox*1, homologous to positions 2187–3124, were aligned using Sequencher 4.1 (Gene Codes, Ann Arbor, MI, USA) or Clustal W (Thompson et al. 1994) and adjusted manually. To assess the phylogenetic signal of the alignment, the skewness of tree length distributions ($g_1$) was calculated on the basis of $10^6$ randomly sampled parsimony trees (Hillis and Huelsenbeck 1992). In addition, to examine the level of noise (i.e. homoplasy) in the data set, a permutation tail probability analysis (Fu and Murphy 1999) of 10,000 replicates was used. The $g_1$-test and permutation tail probability analysis were conducted with PAUP* v4.0b10 (Swofford 2001). Phylogenetic relationships from the *cox*1 sequences were inferred using maximum parsimony (MP) and maximum likelihood (ML) methods in PAUP*. The MP analysis was performed using 10 random sequence additions with tree-bisection-reconnection and branch support assessed by bootstrap analysis of 1000 replicates. The ML phylogenetic tree was constructed under the best-fit model of DNA evolution (F81 + G; $-\ln L = 3845.97$) determined by hierarchical likelihood ratio tests in Modeltest v3.06 (Posada and Crandall 1998) and used stepwise addition and branch swapping by tree-bisection-reconnection. Branch support in the ML tree was tested by bootstrap analysis of 200 replicates. Branch stability was further analyzed by calculation of decay indices. Serial consensus MP trees of tree length < S + 1, S = 2, S = 3, etc. (S = length of most parsimonious tree) were analyzed using PAUP* to determine how many steps it takes for a given node to collapse (Bremer 1994). Statistical testing of alternative tree topologies

were conducted with a Shimodaira-Hasegawa test (SH-test; Shimodaira and Hasegawa 1999), using a RELL bootstrap of 10,000 replicates, as implemented in PAUP*. For the SH-tests, ML trees, with ($L_1$) and without ($L_2$) constraints, were constructed using the heuristic search option under the same ML parameters as described above.

According to their 18S-rDNA RFLP profiles, algal samples used in this study belonged to *Symbiodinium* clades A, B, C, D, or E. The *cox*1 sequences generated from these isolates have been assigned GenBank accession numbers AY289689–AY289712. Some clades (e.g. clade E) are represented by only a single strain because of difficulties with sampling and/or PCR

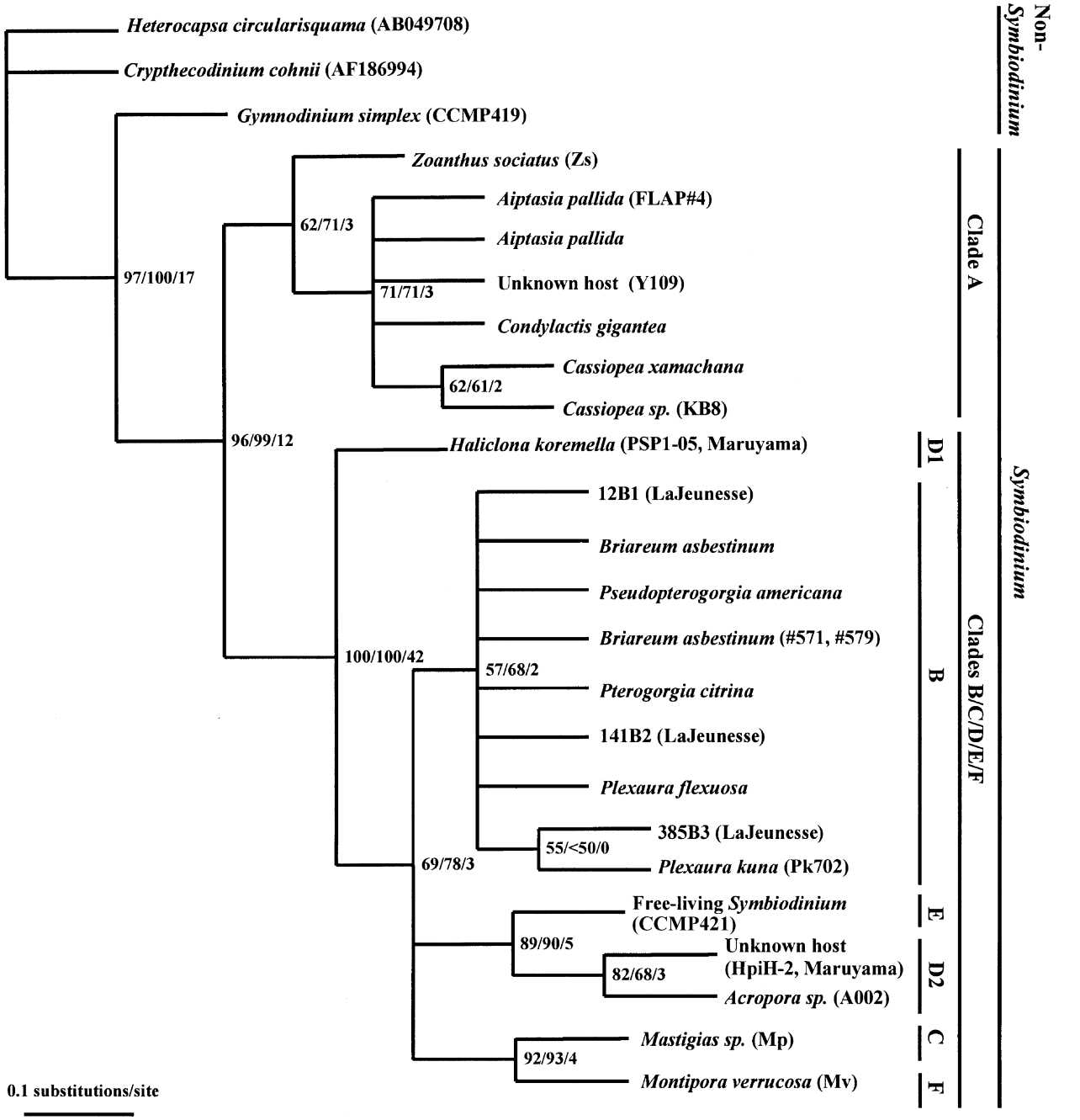


FIG. 1. Maximum likelihood (ML) phylogenetic tree based on mitochondrial cytochrome oxidase 1 (*cox*1) sequences from *Symbiodinium* and other dinoflagellates. For each node, three branch support values are shown in the following order: ML bootstrap value based on 200 replicates/maximum parsimony bootstrap value based on 1000 replicates/decay index. Corresponding sequences of the free-living dinophyceans *Heterocapsa circularisquama* and *Crypthecodinium cohnii* were used as outgroups. With the exceptions of the outgroups, *Gymnodinium simplex* and the free-living *Symbiodinium* isolate, names of the invertebrate hosts are used for *Symbiodinium* samples and follow the culture numbers and codes used in Santos et al. (2002). The nuclear 18S-rDNA cladal designations of the samples are shown to the right.

amplifications. Although including more representative samples would provide better support for our conclusions regarding these clades, significant phylogenetic signal (mean = 931.85, SD = 36.29, $g_1 = -0.577$, $P < 0.01$) relative to noise and homoplasy (permutation tail probability: $P < 10^{-4}$) was present in the alignment, implying that the *cox*1 sequences were appropriate for inferring relationships among the *Symbiodinium* isolates. The average *cox*1 pairwise genetic distance across all *Symbiodinium* strains was 4.5%. The MP and ML analyses revealed a well-supported deep division between *Symbiodinium* clade A and clades B/C/D/E/F (Fig. 1), which is congruent with phylogenies based on nuclear 5.8S-rDNA (LaJeunesse 2001) and cp23S-rDNA (Santos et al. 2002). Within the B/C/D/E/F group, the *cox*1 topology was compared with those seen in nuclear and chloroplast rDNAs (Fig. 2). Although *Symbiodinium* clade B formed a monophyletic group based on *cox*1 (Fig. 1), resolution within the clade was lower than observed in phylogenies generated from ITS sequences (LaJeunesse 2001). This is evident in the inability of *cox*1 to resolve the relationship between ITS types B1 and B2 and the weakly supported separation of type B3 (bootstrap support of 55%). The *cox*1 analysis also splits *Symbiodinium* 18S-rDNA RFLP clade C into sister clades C and F, which is consistent with previous results (LaJeunesse 2001, Pochon et al. 2001, Santos et al. 2002, Takishita et al. 2003). Thus, though *cox*1 is one of the more conserved genes in the mitochondrial genome, the phylogenetic resolution it provides is nearly equal to that seen for nuclear and chloroplast rDNAs.

The SH-test revealed a significant difference ($\delta = L_1 - L_2 = 46.17$; $P = 0.0048$) between a *cox*1 topology constrained to reflect the relationships inferred by 5.8S- and cp23S-rDNA ($L_1$: $-\ln L = 3993.98$) and that of the best *cox*1 tree ($L_2$: $-\ln L = 3947.81$). However, removal of the *Symbiodinium* clade D culture PSP1-05 from the analysis results in no significant difference ($\delta = 17.60$; $P = 0.129$) between the constrained 5.8S/cp23S-rDNA topology ($L_1$: $-\ln L = 3870.20$) and that of *cox*1 ($L_2$: $-\ln L = 3852.60$). Thus, with the exception of the controversial PSP1-05 isolate (see below), the relationships inferred between and among *Symbiodinium* clades by *cox*1 are congruent with those established from nrDNAs and cp23S-rDNA.

The status of *Symbiodinium* clade D deserves special consideration. The *cox*1 phylogeny (Fig. 1) divides clade D into the subclades D1 (i.e. culture PSP1-05) and D2 (i.e. cultures A002 and HpiH-2), as proposed by Pochon et al. (2001) and corroborated by Santos et al. (2002), with strong bootstrap supports for D1 taking an ancestral position to clades B/C/D2/E/F (approximately 100%) and the clustering of D2 with clade E (approximately 89%). Thus, *Symbiodinium* clade D is polyphyletic (Fig. 1). This conclusion is supported by a significant difference (SH-test: $\delta = 48.27$; $P = 0.0024$) between a *cox*1 topology that groups *Symbiodinium* subclades D1 and D2 together (e.g. a monophyletic group; $L_1$: $-\ln L = 3996.08$) and that of the best *cox*1 topology (Fig. 1), in which clade D is polyphyletic ($L_2$: $-\ln L = 3947.81$). Currently, members of subclade D2 have been recovered from cultured isolates as well as *in hospite* (Santos et al. 2002). On the other hand, subclade D1, exemplified by the *Haliclona koremella* isolate PSP1-05, has, to the best of our knowledge, only been recovered from culture (Carlos et al. 1999). Thus, although our *cox*1 tree places PSP1-05 within the genus *Symbiodinium*, comparable with nrDNA (Carlos et al. 1999, Pochon et al. 2001), cp23S-rDNA (Santos et al. 2002), and *psbA* (Takishita et al. 2003) phylogenies, its inclusion within clade D is debatable (Takishita et al. 2003) because this isolate may represent a free-living dinoflagellate (Carlos et al. 1999) derived from a member of *Symbiodinium* clade D (Santos et al. 2002). To clarify this situation, symbionts should be freshly isolated from *H. koremella* and their relationship to PSP1-05 established via infection experiments to determine whether PSP1-05 is capable of forming stable symbiotic associations with invertebrate hosts.

Phylogenies based on nuclear and organellar DNA sequences for a group of taxa are likely to be incongruent if cross-hybridization events have occurred, with differential introgression of nuclear and organellar genomes (Arnold 1992). The agreement in phylogenetic relationships among *Symbiodinium* clades inferred


FIG. 2. Schematic showing the phylogenetic relationships among *Symbionidium* clades inferred by nuclear 5.8S rDNA (A, LaJeunesse 2001), chloroplast large subunit (cp23S)-rDNA (B, Santos et al 2002), and mitochondrial *cox*1 (C, this study).

from nuclear, chloroplast, and mitochondrial genes suggests no significant hybridization events since the clades diverged. Taken together, this high level of congruency between the three genomes of *Symbiodinium* strengthens the robustness and confidence of phylogenetic inferences between the clades and provides a strong foundation on which future ecological and evolutionary studies of symbiotic dinoflagellates can be conducted.

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