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A GENETICS-BASED DESCRIPTION OF *SYMBIODINIUM MINUTUM* SP. NOV. AND *S. PSYGMOPHILUM* SP. NOV. (DINOPHYCEAE), TWO DINOFLAGELLATES SYMBIOTIC WITH CNIDARIA[1]

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Traditional approaches for describing species of morphologically cryptic and often unculturable forms of endosymbiotic dinoflagellates are problematic. Two new species in the genus *Symbiodinium* Freudenthal 1962 are described using an integrative evolutionary genetics approach: *Symbiodinium minutum* sp. nov. are harbored by widespread tropical anemones in the genus *Aiptasia*; and *Symbiodinium psygmophilum* sp. nov. are harbored by subtropical and temperate stony corals (e.g., *Astrangia*, *Cladocora*, and *Oculina*) from the Atlantic Ocean and Mediterranean Sea. Both new species are readily distinguished from each other by phylogenetic disparity and reciprocal monophyly of several nucleic acid sequences including nuclear ribosomal internal transcribed spacers 1 and 2, single copy microsatellite flanker Sym15, mitochondrial cytochrome *b*, and the chloroplast 23S rRNA gene. Such molecular evidence, combined with well-defined differences in cell size, physiology (thermal tolerance), and ecology (host compatibility) establishes these organisms as distinct species. Future descriptions of *Symbiodinium* spp. will need to emphasize genetics-based descriptions because significant morphological overlap in this group obscures large differences in ecology and evolutionary divergence. By using molecular evidence based on conserved and rapidly evolving genes analyzed from a variety of samples, species boundaries are defined under the precepts of Evolutionary and Biological Species Concepts without reliance on an arbitrary genetic distance metric. Because ecological specialization arises through genetic adaptations, the Ecological Species Concept can also serve to delimit many host-specific *Symbiodinium* spp.

Key index words: **dinoflagellate; species recognition; *Symbiodinium*; symbiont; taxonomy; zooxanthellae**

Abbreviations: ***AA*, amino acid; *cob*, cytochrome *b*; *cp23S*, chloroplast 23S rRNA gene; DGGE, denaturing-gradient gel electrophoresis; *ITS*, internal transcribed spacer**

Studies on the diversity, physiology, and ecology of the genus *Symbiodinium* have spanned nearly four decades. Yet it was not until widespread and frequent coral bleaching began that research into cnidarian-dinoflagellate symbioses expanded almost exponentially, with many studies focused on how the identity of the symbiont relates to thermal tolerance of reef-building corals (e.g., Berkelmans and van Oppen 2006, Jones et al. 2008, LaJeunesse et al. 2009a, 2010b, Fisher et al. 2012). In addition to this ecological significance, the intracellular nature of these symbiotic associations has also attracted interest in developing a cnidarian model system in the study of animal–microbe interactions and their cellular biology (Weis et al. 2008). Most research currently combines DNA sequencing with phylogenetic analyses to assign identity to those symbionts under investigation (for review, see Sampayo et al. 2009). However, progress connecting genetic diversity to a formal nomenclatural framework is hampered by disagreement in the interpretation of observed genetic diversity and its taxonomic and ecological significance (e.g., Correa and Baker 2009, LaJeunesse and Thornhill 2011, Stat et al. 2011).

Investigations into the species diversity of *Symbiodinium* began in the 1970s using various morphological, biochemical, physiological, behavioral, and genetic approaches (Schoenberg and Trench 1980a,b,c). These analyses found significant differences among cultured isolates, suggesting that "zooxanthellae" comprised much more than a single species as was previously assumed (Fitt et al. 1981, Blank and Trench 1985, Trench and Blank 1987, Banaszak et al. 1993, Trench 1993). Numerous DNA base substitutions in conserved ribosomal and mitochondrial

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genes from cultured and natural samples provide confirmatory evidence that the genus *Symbiodinium* likely originated in the Mesozoic Era and comprises distantly related monophyletic groups, or clades (Rowan and Powers 1991, 1992, McNally et al. 1994, LaJeunesse 2001, Tchernov et al. 2004, Stern et al. 2010).

The basic subdivision of the genus *Symbiodinium* into clades is well established, but there is dispute over how to interpret the genetic and ecological diversity observed within them. Complementary genetic and ecological data indicate that hundreds of genetically distinct lineages (i.e., species) of *Symbiodinium* may exist (LaJeunesse 2001, Sampayo et al. 2009, Finney et al. 2010, LaJeunesse and Thornhill 2011). Most have no formal species description and are often named according to letter (signifying clade) and number combinations, and referred to by various authors as "subclades," "types," "species," or "strains." These alphanumeric taxonomic schemes differ among members of the research community and create additional taxonomic confusion (e.g., LaJeunesse 2001, 2002, van Oppen et al. 2001, Santos et al. 2003, Fabricius et al. 2004, Stat et al. 2011). Clearly, nomenclatural clarity and taxonomic stability are greatly needed. Without valid scientific names, the accurate communication of *Symbiodinium* diversity, physiology, ecology, and evolution will remain problematic (Blank and Trench 1986, LaJeunesse et al. 2009b).

The life cycle of *Symbiodinium* alternates between coccoid and motile phases (Fitt and Trench 1983). The motile cells possess morphological variations utilized for traditional species description, but the proper imaging of this stage often requires culturing (currently problematic for most *Symbiodinium*) and the meticulous characterization of slight differences in external and internal morphology (Blank 1986, Trench and Blank 1987, Blank and Huss 1989, Trench and Thinh 1995, Hansen and Daugbjerg 2009). Of the nine phylogenetic clades of *Symbiodinium* (designated clades A-I; *sensu* Rowan and Powers 1991), formal species descriptions using conventional morphological features are published for clade A (*S. microadriaticum* Freudenthal 1962, *S. pilosum* Trench and Blank 1987, *S. natans* Hansen and Daugbjerg 2009, *S. linucheae* Trench and Thinh 1995), clade C (*S. goreaui* Trench and Blank 1987), and clade F (*S. kawagutii* Trench and Blank 1987, Trench 2000). Nine additional binomials (*S. bermudense*, *S. californium*, *S. cariborum*, *S. corculorum*, *S. fitti*, *S. glynni*, *S. meandrinae*, *S. muscatinei*, *S. pulchrorum*, *S. trenchi*) appear in the literature as provisional names for taxa that have not yet been formally described (i.e., *nomina nuda*, Table 1), and yet collectively these represent only a small fraction of the probable global species diversity (LaJeunesse 2005). The formal recognition of species comprising this large undescribed diversity will serve to standardize *Symbiodinium* nomenclature and promote knowledge by allowing researchers the ability to directly compare and build off one another's cumulative findings.

TABLE 1. List of all binomials (valid and invalid) used in connection with *Symbiodinium*. Names in bold are formally described species, while names in quotation marks are *nomina nuda* (published specific epithets without formal diagnosis). Clade assignments follow LaJeunesse (2001).

Species	Clade	Author(s)
Symbiodinium "bermudense"	B	Banaszak et al. (1993)
Symbiodinium "californium"	E	Banaszak et al. (1993)
Symbiodinium "cariborum"	A	Banaszak et al. (1993)
Symbiodinium "corculorum"	A	Banaszak et al. (1993)
Symbiodinium "fitti"	A	Pinzón et al. (2011)
Symbiodinium "glynni"	D	LaJeunesse et al. (2010b)
Symbiodinium goreaui	C	Trench and Blank (1987)
Symbiodinium kawagutii	F	Trench and Blank (1987)
Symbiodinium linucheae	A	(Trench and Thinh 1995)* LaJeunesse (2001)
Symbiodinium "meandrinae"	A	Banaszak et al. (1993)
Symbiodinium microadriaticum	A	Freudenthal (1962), Trench and Blank (1987)
Symbiodinium microadriaticum* var. *condylactis	A	Blank and Huss (1989)
Symbiodinium "muscatinei"	B	LaJeunesse and Trench (2000)
Symbiodinium natans	A	Hansen and Daugbjerg (2009)
Symbiodinium pilosum	A	Trench and Blank (1987)
Symbiodinium "pulchrorum"	B	Banaszak et al. (1993)
Symbiodinium "trenchi"	D	LaJeunesse et al. 2005

*First described as belonging to *Gymnodinium*.

Cnidarian hosts usually harbor monospecific populations of *Symbiodinium* comprising a single dominant genotype (Goulet and Coffroth 2003a,b, Pettay and LaJeunesse 2007, 2009, Thornhill et al. 2009, Andras et al. 2011, Pettay et al. 2011, Pinzón et al. 2011, Wham et al. 2011). Thus, most host individuals and/or colonies act as culture vessels, and sampling from them provides access to relatively purified genotypes or strains (i.e., individual clones) of a particular *Symbiodinium* sp. Multiple genetic analyses may be employed on each sample with the understanding that there is little contamination from other genomes (e.g., Sampayo et al. 2009).

Multi-locus data are increasingly used to test for reproductive isolation and genetic divergence among morphologically cryptic and closely related organisms (e.g., Hausdorf and Hennig 2010, Gazis et al. 2011). It was recently demonstrated that a lineage-based approach (*sensu* Avise and Wollenberg 1997, de Queiroz 2007, Gazis et al. 2011) combining sequences of mitochondrial (*cob*), chloroplast (*cp23S*), and ribosomal genes (*LSU*), as well as spacer regions (*ITS*), could identify and classify *Symbiodinium* into biologically meaningful units (i.e., species; Sampayo et al. 2009). We proceed with this methodology by using a combination of slow- and fast-evolving nuclear, mitochondrial, and plastid DNA sequences. These genetic data are combined with available

morphological (cell size), physiological (thermal tolerance), and ecological traits (host association) to describe two new species of *Symbiodinium* in clade B, a group dominant among symbiotic cnidarians in the Western Atlantic. By introducing an integrative genetics-based approach for testing the reproductive/genetic isolation of these lineages, we seek to unify the nomenclature of a well-studied, but taxonomically problematic (i.e., morphologically cryptic), eukaryotic microbial group.

MATERIALS AND METHODS

Specimen collection. Cultured *Symbiodinium* characterized as *ITS2* types *B1* and *B2* were originally isolated from host tissues by Schoenberg and Trench (1980a) using modified methods of McLaughlin and Zahl (1959). Initial crude cultures were established by inoculating several drops of a heavy suspension of symbiont cells into nutrient-enriched filtered seawater (Provasoli 1968) and then spread onto semi-solid agar (0.8%) containing this medium. Vegetative cells from viable colonies on agar were transferred to liquid medium. In generating isoclonal lines, only motile cells were transferred to fresh medium. Final cultures were maintained in liquid medium ASP-8A (Ahles 1967) and grown under Philips fluorescent tubes (Koninklijke Philips Electronics, Amsterdam, the Netherlands) delivering 80–120 μmol quanta $\cdot$ m^{-2} $\cdot$ s^{-1} photosynthetically active radiation (PAR) on a 14:10 (light:dark) photoperiod.

Wild-collected, noncultured *Symbiodinium* populations were included in this study to augment the phylogenetic analyses and to demonstrate reciprocal monophyly between cultured and natural genotypes. These additional representatives were taken from various cnidarian host samples collected from the Gulf of Mexico (Florida Panhandle, USA), Western Atlantic (Rhode Island, USA and Bermuda), Mediterranean (Israel), Pacific (Gulf of California and Hawaii, USA) and Indian Ocean (Zanzibar) and preserved in high-salt, 20% DMSO buffer (Seutin et al. 1991) and stored at −20°C until extraction.

Culture and cell size analyses. Isolates were photographed during log phase growth under 80–120 μmol quanta $\cdot$ m^{-2} $\cdot$ s^{-1} PAR on a 14:10 (light:dark) photoperiod. To avoid the effect of age on appearance and size, cells were photographed at approximately the same stage in culture (between day 10 and 15 after re-inoculation into fresh medium), during the middle of logarithmic growth. Cells were photographed under bright-field illumination at a magnification of 400–1000× using an Olympus BX51 compound microscope (Olympus Corp., Tokyo, Japan) with a Jenoptik ProgRes CF Scan digital camera (Jenoptik, Jena, Germany). An autoexposure setting within ProgRes Capture Pro 2.8 software (Jenoptik) was used to expose and capture the cell images. Cell sizes for at least 40 individuals per culture were calculated with the program ImageJ (Abramoff et al. 2004). Size differences between ITS types *B1* and *B2* were assessed via Student's *t*-test on a combined dataset of all measurements of cultured and natural material belonging to each group.

DNA extraction, PCR amplification, sequencing, and phylogenetic analyses. Nucleic acid extractions were conducted as described by LaJeunesse (2001). Table 2 lists the nuclear, mitochondrial, and plastid DNA marker sequences targeted, and the primers and thermal cycler conditions used for amplification. Amplifications were performed in 25 μL reaction volumes containing 2.5 μL of 2.5 mM dNTPs, 2.5 μL of 25 mM $MgCl_2$, 2.5 μL standard Taq Buffer (New England Biolabs, Ipswich, MA, USA), 0.13 μL of 5 U $\cdot$ μL^{-1} Taq DNA Polymerase (New England Biolabs), 1 μL of each forward and reverse primer at 10 μM, and 1 μL of 5–50 ng DNA template. Products were directly sequenced on an Applied Biosciences sequencer (Applied Biosciences, Foster City, CA, USA) at the Pennsylvania State University Genomics Core Facility. Chromatograms were checked and sequences aligned using CodonCode Aligner software (CodonCode, Dedham, MD, USA). Paup 4.0b10 (Swofford 2000) was used to perform phylogenetic analyses on aligned data sets under maximum parsimony (with indels included as a 5th character state), maximum likelihood, and distance to test for reciprocal monophyly. Additionally, Bayesian posterior probabilities were calculated with the software Mr. Bayes (Huelsenbeck and Ronquist 2001), using the HKY85+G substitution model (Hasegawa et al. 1985) with a chain length of 1,100,000 and a burn-in of 100,000.

RESULTS

Currently, there are over 25 genetically distinct types in clade B that are characterized from host samples obtained from field surveys and/or as cultured isolates. Types *B1* and *B2*, analyzed here, were first recognized among various cultured isolates by their distinct *ITS* sequences (LaJeunesse 2001), and later identified in certain samples from collections of symbiotic cnidarians acquired from the western Atlantic (e.g., LaJeunesse 2002, Finney et al. 2010). Therefore, *B1* and *B2* are unusual in that they both occur in their hosts as normal dominant symbionts and can grow in artificial culture medium (LaJeunesse 2002).

Taxonomic assignment of species. Complementary genetic evidence unequivocally supported the designation of the first two *Symbiodinium* spp. in clade B (Fig. 1, A–D). The concordant reciprocal monophyly observed from analysis of various genetic markers originating from chloroplast (*cp23S*, 670 bp), mitochondrial (*cob*, 921 bp), and nuclear genomes (*ITS1/5.8S/ITS2*, 667 bp), and a single copy microsatellite flanker (Sym15, 191 bp) indicates the maintenance of long-standing genetic isolation between these lineages. Maximum parsimony as well as likelihood and distance analyses (data not shown) all produced the same phylogenetic reconstructions. Cultured isolates and field-collected specimens of both species appeared similar using light microscopy at maximum resolution (1000×; Fig. 2, A and B), but differed significantly in cell dimension between species (t [596] = 23.28, $P < 0.001$ for maximum diameter; Fig. 2C). The measured sizes of freshly isolated cells corresponded to their in vitro counterparts, indicating that the culture process had a limited effect on cell size or volume (Fig. 2C). The photophysiologies of these symbionts exposed to low temperatures in vitro also demonstrated functional differences that appear to influence their ecological distributions (Thornhill et al. 2008; Fig. 2D). Based on these genetic data and supporting morphological, physiological, and ecological data, we therefore assign the following formal binomials.

TABLE 2. Gene regions targeted for analyses, gene types, primer pairs used for PCR, primer sequences, approximate sizes of amplified DNA fragments, and annealing temperatures used to delineate species in clade B of the genus *Symbiodinium*. For analysis of ITS regions using denaturing gradient gel electrophoresis, a GC-rich area (clamp) is attached to the primer (underlined).

Region	Type	Primer	Primer sequence (5′–3′)	Size (bp)	T_m (°C)
ITS1 rDNA	Nuclear	ITS1CLAMP[1]	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCC	~420	62–53 (TD)
			GGGATCCGTTTCCGTAGGTGAACCTGC		
		ITS1intrev2[1]	TTCACGGAGTTCTGCAAT		
ITS2 rDNA	Nuclear	ITS2CLAMP[2]	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCC	~360	62–52 (TD)
			GGGATCCATATGCTTAAGTTCAGCGGGT		
		ITSintfor2[2]	GAATTGCAGAACTCCGTG		
Sym15	Nuclear	B7Sym15 (forward)[3]	CTCACCTTGAAATCAGTAGCCA	~200–250	59
		B7Sym15 (reverse)[3]	CGTAGCTTCTGAAGGTACGACAC		
Cytochrome *b*	Mitochondrial	cob1 (forward)[4]	TCTCTTGAGGKAATTGWKMACCTATCCA	~950	55
		dinocob1 (reverse)[4]	CACGACGTTGTAAAACGACGGCTGTAACTATAACGGTCC		
cp23S domain V	Plastid	23S1M13[5]	GGATAACAATTTCACACAGGCCATCGTATTGAACCCAGC	~670	55
		23S2M13[5]	GCAGCTCATGGTTATTTTGGTAGAC		

[1]LaJeunesse et al. (2008).
[2]LaJeunesse and Trench (2000).
[3]Pettay and LaJeunesse (2007).
[4]Zhang et al. (2005).
[5]Santos et al. (2002).
TD, touchdown PCR protocol.

***Symbiodinium minutum*, sp. nov**.

Diagnosis. Coccoid cells range in average size from 6.5 to 8.5 μm in diameter during log phase growth and in hospite (Fig. 2, A). The combined nucleotide sequences of the *cp23S* (JX213587–JX213588), microsatellite flanker Sym15 (JN602464, JX263427), nuclear ribosomal *ITS1/5.8S/ITS2* (AF333511), and mitochondrial *cob* (JX213579–JX213581) define this species.

Holotype designation. Cryopreserved culture strain CCMP 2460 housed at the Provasoli-Guillard National Center for Marine Algae and Microbiota (East Boothbay, Maine USA).

Type locality. Collected from the brown sea anemone, *Aiptasia* sp., Florida Keys, USA.

Etymology. The Latin *minutum* (small) refers to the small size of this organism relative to other *Symbiodinium*.

Other notes. The authentic strain (CCMP2460) was originally isolated in mid-1970s by David A. Schoenberg; it is also known as culture rt-002 from the Robert K. Trench collection. This species is equal to type *B1*, which was based on *ITS2* sequence data (LaJeunesse 2001); it is also equal to B184 based on *cp23S* (Santos et al. 2003). Ecologically distinct *B1* lineages exist that possess identical *cob* and *ITS* sequences, yet sequences from *cp23S*, Sym15, other microsatellite flankers (Santos et al. 2004, Finney et al. 2010), and the *psbA* noncoding region (*sensu* LaJeunesse and Thornhill 2011) unequivocally separate these from *S. minutum* (T. C. LaJeunesse unpubl. data).

***Symbiodinium psygmophilum*, sp. nov.**

Diagnosis. Coccoid cells range in average size from 8.5 to 10.5 μm in diameter during log phase growth and in hospite (Fig. 2, B). The combined nucleotide sequences of the *cp23S* (JX213589-JX213593), microsatellite flanker Sym15 (JN602465, JN602461, JX263228-JX263230), nuclear ribosomal *ITS1/5.8S/ITS2* (AF333512), and mitochondrial *cob* (JX213582-JX213586) define this species.

Holotype designation. Cryopreserved culture strain CCMP 3320 housed at the Provasoli-Guillard National Center for Marine Algae and Microbiota (East Boothbay, Maine USA).

Type locality. Collected from the ivory bush coral *Oculina diffusa*, Bermuda.

Etymology. From the Greek "*psygmophilia*" and latinized to *psygmophilum* to mean "cold-loving;" refers to its common association with coral hosts from cold-water, temperate, and sub-tropical Atlantic and Mediterranean environments.

Other notes. The authentic strain CCMP3320 was originally isolated in mid-1970s by David A. Schoenberg; it is also known as culture rt-141 from the Robert K. Trench collection. This species is equal to type *B2* derived from *ITS* sequence data (LaJeunesse 2001); it is also equal to B224 based on *cp23S* (Santos et al. 2003). Populations of *S. psygmophilum* from

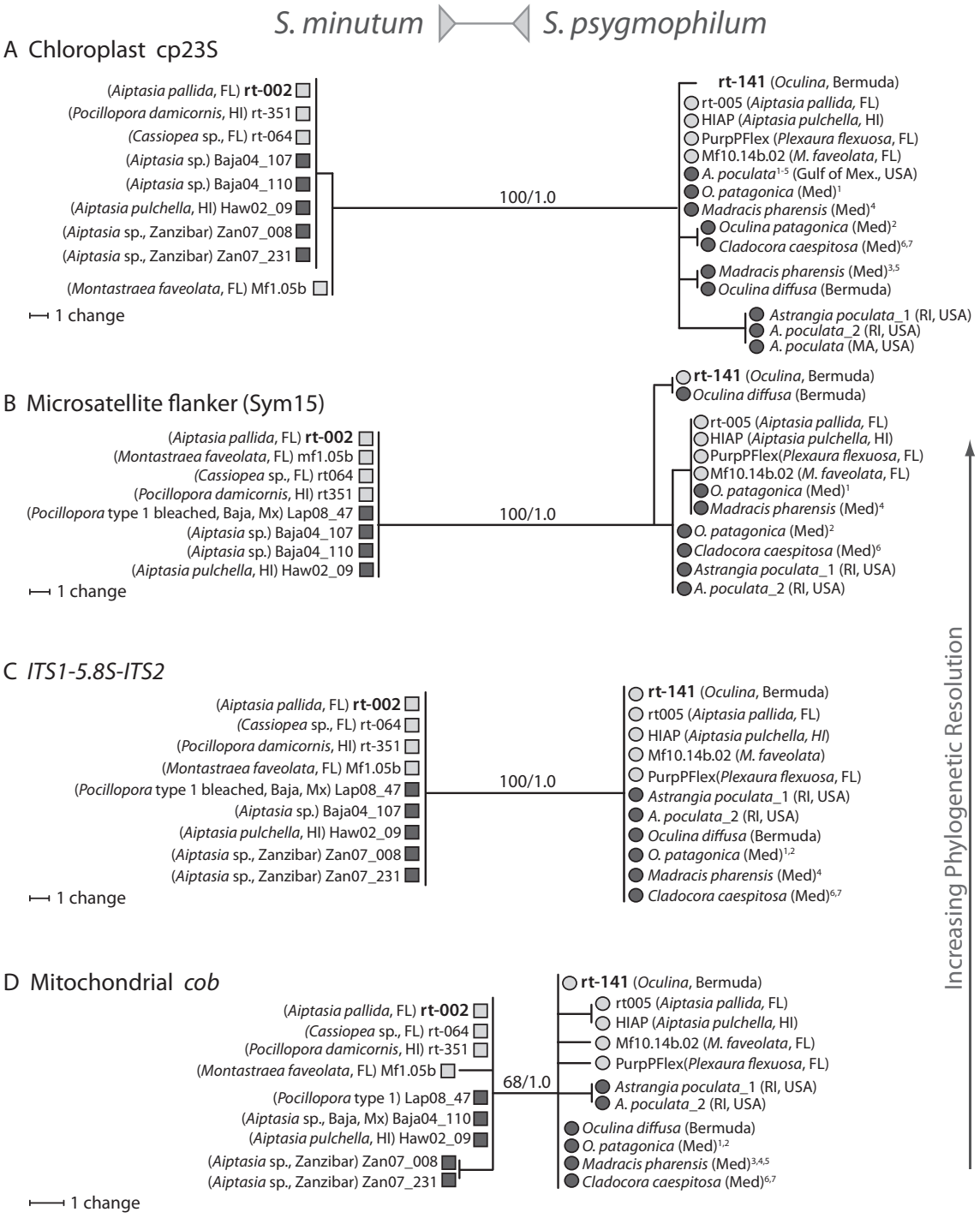

FIG. 1. Multiple phylogenetic analyses resolve cultured isolates and field-collected samples into separate lineages designated here as *S. minutum* (left, square symbols) and *S. psygmophilum* (right, circle symbols). From these independent comparisons, the *cp23S* (A) was the most rapidly evolving due to large differences in the hyper-variable region. (B) The flanker sequences of the microsatellite locus, Sym15, differentiate members of each species, and provided twice the resolving power as the *ITS1–5.8S-ITS2* region (C). The *cob*, the most conserved and only protein-encoding gene analyzed, contained fixed nonsynonymous differences at base positions 586 and 879, producing changes at amino acids 195 and 293, respectively (D). Small amounts of sequence variation observed within each lineage may represent genetic variation characteristic of different individuals (i.e., clone genotypes) and/or isolated populations. Solid symbols refer to samples collected naturally from host specimens. The numbers above branches separating each species are bootstrap values based on 1,000 iterations, followed by Bayesian posterior probabilities. Superscripts were used to refer to independent samples acquired from a particular location.

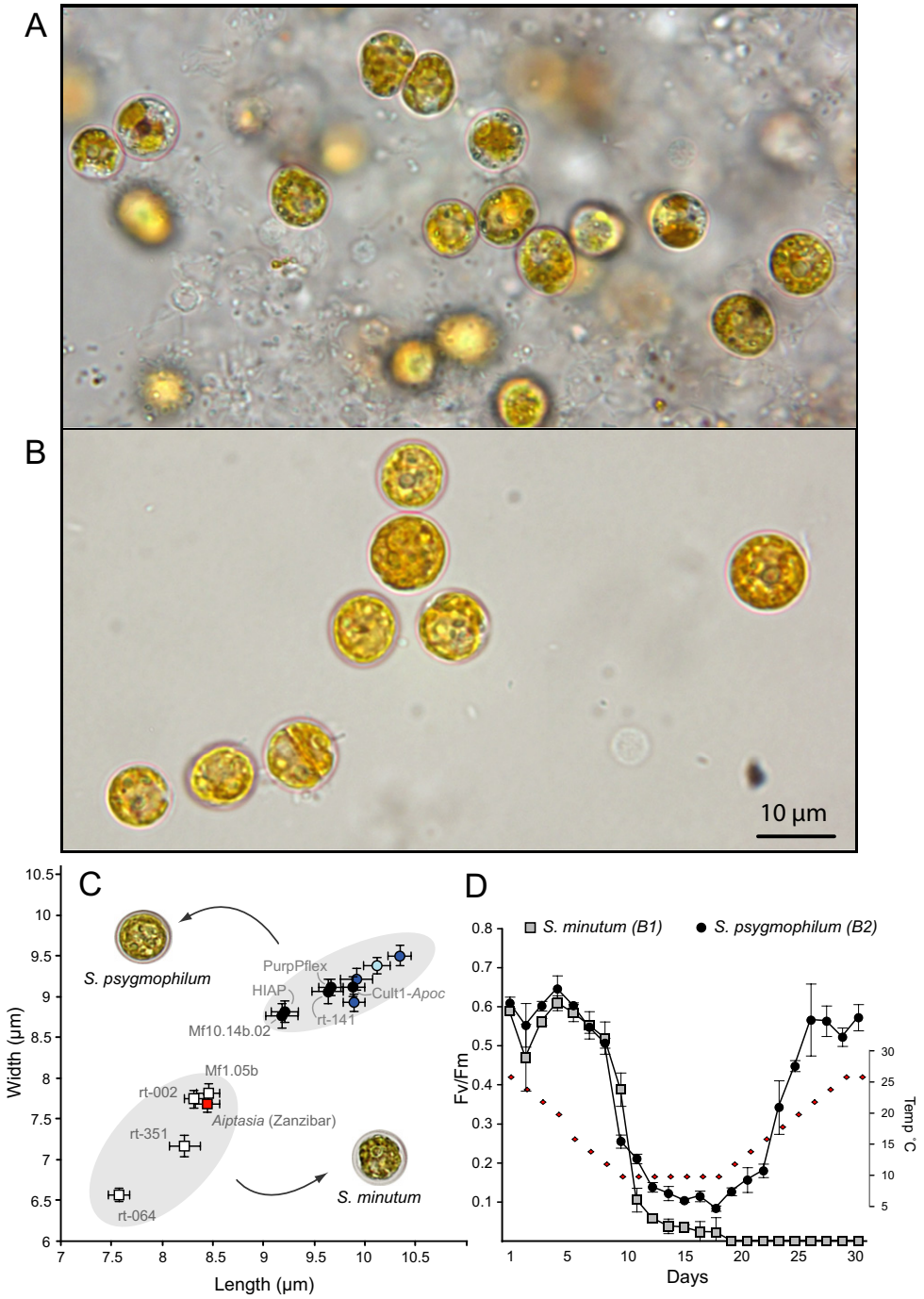

FIG. 2. Light micrographs of cultures rt-002 and rt-141 representing (A) *S. minutum* (*ITS2* type *B1*), and (B) *S. psygmophilum* (*ITS2* type *B2*), respectively. (C) Each species differed significantly in cell size and measurements were consistent across cultured isolates obtained from different host taxa and geographic locations, as well as across cells freshly isolated from host tissues. Square symbols correspond to *S. minutum*, while circles refer to *S. psygmophilum*. Colored symbols identify samples measured from freshly isolated cells, dark blue circles correspond to samples of *Astrangia poculata* collected from the Gulf of Mexico, and the light blue circle was from a sample collected in Woods Hole, Massachusetts, USA. (D) The photophysiologies of example cultures of *S. minutum* and *S. psygmophilum* show marked differences in thermal (cold-water) tolerance that relate to their tropical and temperate distributions, respectively (modified from Thornhill et al. 2008 with permission).

Rhode Island, USA, may be genetically isolated, and subsequent taxonomic treatment of this northern population may identify these as a separate variety or species. A second numerically common *ITS* sequence (AF333513) that differs by a single base substitution occurs in the genome of the cultured *S. psygmophilum*, rt-141, and is also observed in denaturing gradient gel electrophoresis (DGGE) profiles of field collected samples.

Discussion. On the use of genetics to describe S. minutum *and*
S. pysgmophilum The lack of a standardized taxonomy for delimiting within-clade diversity among *Symbiodinium* hampers progress in a rapidly expanding field by obfuscating direct comparisons between studies employing different terminologies and genetic markers. This paper's contribution represents a critical and long-anticipated step toward developing a common nomenclature by streamlining the process of defining species diversity based mostly on molecular evidence—the most practical and frequently used criteria for discerning morphologically indistinct taxa (Ald et al. 2007). Our approach puts into practice the recommendations of a large and growing body of protistologists who advocate the use of genetic evidence above morphology in describing species (e.g., Ald et al. 2007, Lilly et al. 2007, Moniz and Kaczmarska 2010).

The selection of genetic markers appropriate to resolve species admittedly requires some subjectivity (as does the analysis of morphological traits). Different markers evolve at different rates among different lineages, and genes useful in some groups do not provide adequate resolution in others (for e.g., *cp23S* poorly resolves closely related clade C *Symbiodinium*, Sampayo et al. 2009). The selection of markers also requires some background knowledge. Our use of the microsatellite flanker Sym15 was inspired by the previous broad analysis of clade B *Symbiodinium* from eastern and western regions in the Caribbean (Finney et al. 2010, who referred to this marker as Si15). However, this marker works only on members of clade B and therefore has limited utility. To prevent reliance on a single marker, we targeted genes from various parts of the cell's genome that presumably sort independently during meiosis. Violation of reciprocal monophyly at any level of genetic resolution would bring into question the reproductive isolation of the two lineages under comparison.

It is tempting to provide a nucleotide sequence divergence cut-off value at which the lines between species can be drawn consistently, but such arbitrary distinctions have been problematic for other taxa, and the same issues would apply to *Symbiodinium*. The number of fixed sequence differences between *S. minutum* and *S. psygmophilum* differs depending on the degree of conservation inherent to each gene or noncoding region. Cytochrome *b* is a proposed barcode marker for dinoflagellates because it provides slightly better resolution than cytochrome oxidase 1 in the analysis of diversity present in plankton assemblages (Lin et al. 2009). Only two fixed base changes in the *cob* gene (921 bases) distinguished *S. minutum* from *S. psygmophilum* (Fig. 1D). While this may not seem significant, both nucleotide substitutions encode amino acid substitutions. However, because these changes involve similar classes of amino acids, the structure and function of this enzyme are probably unaffected (the *cob* of *S. minutum* has a leucine at AA 195 and serine at AA 293, which are replaced by phenylalanine and threonine, respectively, in *S. psygmophilum*; Fig. S1). As a species marker, *cob* is relatively conserved and will not discern among ecologically distinct *Symbiodinium* resolved by more rapidly evolving markers, and therefore, it has limited taxonomic utility (Sampayo et al. 2009).

Nuclear ribosomal internal transcribed spacers are commonly relied on to resolve species boundaries among plants, fungi, and micro-algae (Seifert 2009, Moniz and Kaczmarska 2010, Yao et al. 2010). *S. minutum* and *S. psygmophilum* were well differentiated by this marker (~2% *ITS1–5.8S-ITS2* sequence divergence). The lack of interindividual variation among cultured isolates and naturally collected samples of each species is likely due to the concerted manner in which this tandem multi-copy marker evolves among individuals in a species population (Dover 1982). In their review of dinoflagellate ITS data, Litaker et al. (2007) suggest that 1–4% divergence represents intraspecific variation. These calculations are likely confounded by (i) the misidentification and lumping of cryptic species (e.g., Lilly et al. 2007); and (ii) the use of bacterial cloning for sequencing rDNA, a process that recovers low copy number functional and nonfunctional intragenomic variants and artifacts from gene amplification (PCR) reactions (Thornhill et al. 2007). The presence of substantial intragenomic variation can obscure the correct recognition of distinct genotypes (LaJeunesse and Thornhill 2011, Miranda et al. 2012). We highly recommend direct sequencing and/or the use of acrylamide gels such as DGGE to screen PCR products and target the numerically dominant sequence variant representative of the organism's genome (Thornhill et al. 2007, Sampayo et al. 2009, LaJeunesse and Thornhill 2011).

Nucleotide differences were greatest for the Sym15 microsatellite flanker and *cp23S* gene compared between *S. minutum* and *S. psygmophilum*. While interindividual variation among *S. psygmophilum* was greater than *S. minutum*, these differences were minimal relative to the genetic distance separating each species lineage. Among the undescribed species diversity in clade B (Finney et al. 2010), *S. minutum* and *S. psygmophilum* are relatively divergent and present no ambiguity in separating them as distinct species. Future studies involving the characterization of species that are more closely related may require

the incorporation of additional genetic markers (e.g., microsatellites, *psbA* noncoding region, etc.).

Sequence data from conserved rDNA (small subunit 18S) and the mitochondrial cytochrome oxidase 1 indicate that the genus *Symbiodinium* comprises lineages (i.e., "clades" A, B, C, etc.) whose genetic divergence is similar to differences observed among dinoflagellates from different genera, families, and even orders (Rowan and Powers 1992, Stern et al. 2010). Future taxonomic revision of this genus is required and many of these "clades" will probably be reclassified into distinct genera. As members of clade B, *S. minutum* and *S. psygmophilum* are well differentiated from all other described species of *Symbiodinium* (Fig. 3). Large portions of the ITS region are unalignable between species from different clades, further supporting revision in the systematics of these dinoflagellates. Clearly, *Symbiodinium* "clades" comprise many reproductively isolated, genetically distinct lineages, exhibiting different ecological, physiological, and biogeographic distributions (see below), which is why the use of only clade-level taxonomic designations oversimplifies our understanding of complex host–symbiont interactions.

Supporting morphological, physiological, and ecological evidence in the recognition of species Cell size can substantially influence cellular function, DNA content, relative growth rate, and photosynthesis among unicellular algae (Banse 1976, LaJeunesse et al. 2005). The way in which size differences affect cell physiology, growth, and ecology among *Symbiodinium* spp. requires further study. The average cell sizes of cultured isolates and natural samples of *S. minutum* are smallest among described *Symbiodinium* species. The obvious difference in size between species is clearly under genetic control, but, as with many morphological characters, it is not possible to assess the underlying genetic changes necessary to affect this trait.

Preliminary evidence indicates that *S. minutum* and *S. psygmophilum* differ in their physiology. Tolerance of cold temperatures (Fig. 2D) is consistent with the idea that *S. psygmophilum* is adapted to subtropical and temperate environments (Thornhill et al. 2008). When representative cultures of each species were subjected to a gradual reduction in temperature, and then maintained under cold-stress ($\sim$10°C) for 10 days before returning to normal culture conditions ($\sim$26°C), only *S. psygmophilum* (culture rt-141) was capable of returning to prestress levels of photosynthetic efficiency, while *S. minutum* (culture rt-064) and other cultured *Symbiodinium* with tropical distributions failed to regain function (Thornhill et al. 2008). Additionally, when subjected to high-temperature stress (~32°C), turnover of the D1 protein in photosystem II was sensitive in culture rt-141 (Warner et al. 1999), further indicating that *S. psygmophilum* is "cold-water" adapted and may simply tolerate low temperatures better than many other *Symbiodinium*.

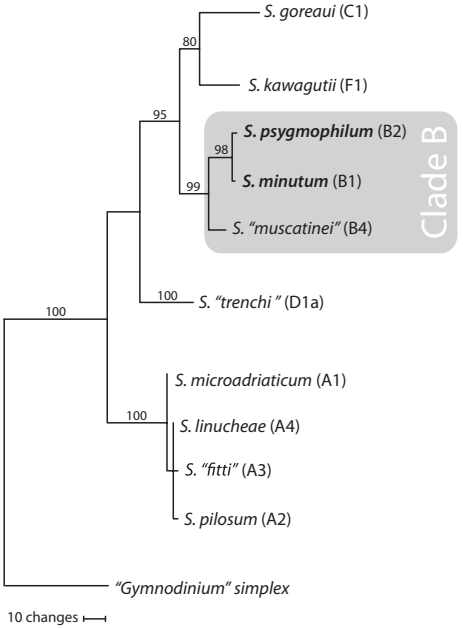

FIG. 3. The phylogenetic comparison of the partial cp23S gene from described species of *Symbiodinium* including several taxa provisionally named in the literature. The hyper-variable domain V was removed for improving alignments between members of each "clade." Next to each species name is its designated *ITS2* type in parentheses. This phylogeny was constructed using maximum parsimony on 647 aligned nucleotides including insertions and deletions as 5th character states. Bootstrap values listed above a corresponding branch were based on 1,000 replicates.

These observations support the growing acknowledgment of significant physiological differences among closely related *Symbiodinium* spp. (Rodriguez-Lanetty et al. 2004, Frade et al. 2008, Hennige et al. 2009).

It is challenging to discern differences in ecology among "free-living" planktonic dinoflagellates and other micro-algae (Hutchinson 1961). The host specificity exhibited by many *Symbiodinium* provides a strong ecological characteristic. When identified in wild-collected samples, *S. minutum* and *S. psygmophilum* associate with different hosts and have different (yet overlapping) geographic distributions, therefore representing separate ecological niches. *S. minutum* is part of a group of *Symbiodinium* (*B1*/B184) initially designated as a single ecological entity defined by conventional markers (e.g., *ITS* and *cp23S*) and associated with a wide diversity of host species found in various reef habitats (LaJeunesse 2002). However, several additional lineages were resolved upon the analysis of genetic markers that improved phylogenetic resolution (Santos et al. 2004, Finney et al. 2010). *S. minutum* corresponds to the lineage designated *B1*[1] by Finney et al. (2010) that associates worldwide with the common brown sea anemone *Aiptasia* sp. (Fig. 1, A–D). It may also associate with Caribbean fire corals, gorgonian sea fans, and sea whips, pending further analysis. In

contrast, *S. psygmophilum* is predominant in temperate waters of the Atlantic, where it occurs commonly with *Oculina* and *Astrangia* (Fig. S2 Thornhill et al. 2008). It also occurs in the species *Oculina patagonica, Cladocora caespitosa,* and *Madracis pharensis* from the eastern Mediterranean Sea, and is known from one sample of *Parazoanthus* sp. collected from depth off the coast of Barbados (Finney et al. 2010), indicating that *S. psygmophilum* is not excluded from tropical environments.

Note in Fig. 2 that cultures of *S. minutum* and *S. psygmophilum* have originated from each other's respective hosts, yet field-sampled specimens exhibit considerable fidelity to specific host taxa. It is well documented that culturing often recovers contaminants that grow successfully in artificial media. Rarely is the symbiont observed in hospite successfully grown in culture (Santos et al. 2001, LaJeunesse 2002, Goulet and Coffroth 2003a). We interpret these results to indicate that *S. minutum* and *S. psygmophilum* are common in the environment and may exist as background or cryptic cells in the tissues of many cnidarians (*sensu* LaJeunesse 2002), yet because they are among the few *Symbiodinium* apparently viable in culture media, they are common contaminants in the culturing process.

Both species can grow opportunistically in other host taxa, yet they may not achieve high population densities nor persist for long periods. *S. minutum* was observed in bleached colonies of *Pocillopora* from the Gulf of California during recovery from a severe cold water event in 2008 (LaJeunesse et al. 2010b). Its presence was initially recognized as light yellow-brown patches on the branch tips of certain colonies; these patterns of host tissue colonization indicate infections by cells from external sources that likely originated from populations found in nearby *Aiptasia* sp., an alien species introduced some decades ago that is now abundant in the region (T. C. LaJeunesse personal observation). Upon full recovery, *S. minutum* was no longer detected by the genetic methods employed, yet given the density of its host anemone, this symbiont may be present at low background levels in some *Pocillopora* colonies (LaJeunesse et al. 2010b). By comparison, newly settled juveniles of the octocoral *Briareum asbestinum* were occasionally infected by *S. psygmophilum* (a.k.a. B224) at several experimental explant sites in the Florida Keys (Poland 2010). Over time, after 6 months of colony growth, *S. psygmophilum* was no longer detected upon the reexamination of each host. These observations further indicate that the *Symbiodinium* described here occur free-living in the environment, and while they may infect a variety of hosts, they associate stably only with a small number of cnidarian species.

Future directions and concluding remarks Although both new species in this study were successfully cultured, the large majority of *Symbiodinium* spp. are nonculturable despite numerous attempts using current methods (e.g., LaJeunesse 2002, Goulet and Coffroth 2003a), therefore preventing the use of traditional morphological and ultrastructural descriptions of the mastigote (motile) phase (e.g., amphiesmal plate number and patterning; Dodge and Greuet 1987, Taylor 1987). We therefore suggest that in the future a voucher specimen of host tissue containing the newly described *Symbiodinium* and/or extracted total DNA be archived in cases where a living culture is not available (nor possible to acquire).

Rules governing the International Code of Nomenclature for algae, fungi, and plants (ICN) are purposely vague with regard to morphological differences, and there are no explicit mandates that morphology is required to describe a species. While slight differences in morphology exist for some species of *Symbiodinium*, genetic data are reliable and have been pervasively used to describe the ecological, biogeographic, and evolutionary patterns of these dinoflagellates since 1991 (e.g., Rowan and Powers 1991, LaJeunesse 2002, Fabricius et al. 2004, Chen et al. 2005, Pochon et al. 2007, Sampayo et al. 2008, Abrego et al. 2009, Stat et al. 2009, LaJeunesse et al. 2010a,b, LaJeunesse and Thornhill 2011, Silverstein et al. 2011). The combination of DNA markers used here range in resolution, but collectively indicate that the two lineages under analysis do not exchange genetic information and have not done so for some time. Therefore, this approach to recognizing species of *Symbiodinium* relies on the principles of evolutionary theory by satisfying Evolutionary (fixed differences in sequence divergence) and Biological (reciprocal monophyly across four independent genetic markers) Species Concepts. Furthermore, unlike "free-living" dinoflagellates, *S. minutum* and *S. psygmophilum* exhibit clear ecological differences in host specificity—an attribute fundamental to the biology of each species. Indeed, many *Symbiodinium* lineages appear to have evolved (i.e., speciated) through ecological specialization (LaJeunesse 2005), a selective process that underlies the speciation of most organisms (Schluter 2009).

The identification of *Symbiodinium* spp. from different clades will likely require a subset of different markers (on a case-by-case basis) depending on the "clade" group to which the proposed species belongs (Ald et al. 2007). Ultimately, genetic evidence based on several markers combined with available ecological, morphological, and physiological data (as demonstrated here) provides a robust framework for making formal descriptions through the direct testing of various species hypotheses (Sites and Marshall 2004). We acknowledge that *S. minutum* and *S. psygmophilum* represent ideal examples to demonstrate the formal species recognition of endosymbiotic dinoflagellates because they (i) were already well differentiated by ITS data, (ii) exhibited clear differences in host specificity (i.e., ecology) and (iii) existed in culture. However, future species delineations in the absence of a complete ecological

understanding or the lack of cultured specimens should be possible, provided a robust genetic analysis is established as described above. An essential criterion—the demonstration of genetic isolation by assessing several independent loci—will always remain the proper test no matter what amount of sequence divergence exists among conserved and rapidly evolving markers. The emphasis on genetic data will allow for the description of cryptic species that are selected in culture, but rarely represent the dominant symbiont in hospite, and for which ecological data are limited or nonexistent. This integrative approach should also be applied to describe commonly occurring *Symbiodinium* of known ecological distribution despite the inability to acquire a cultured voucher specimen.

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Supporting Information

The following supporting information is available for this article:

Figure S1. There are two fixed nucleotide differences between *S. minutum* and *S. psygmophilum* that represent nonsynonymous substitutions, which encode changes in the amino acid sequence of cytochrome *b*. The amino acid replacements characteristic of the *cob* in each species belong to different functional groups and therefore may affect the enzyme's structure and function. The *cob* is aligned according to the published sequence for *S. microadriaticum* (AY456110.1) by Zhang et al. (2005).

Figure S2. Genetic analyses detected only the presence of *S. psygmophilum* in colonies of *A. poculata* and *Oculina sp.* from locations in the Gulf of Mexico, Florida Keys, and northwestern Atlantic.