Symbiodinium tridacnidorum sp. nov., a dinoflagellate common to Indo-Pacific giant clams, and a revised morphological description of Symbiodinium microadriaticum Freudenthal, emended Trench & Blank

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Symbiodinium tridacnidorum sp. nov., a dinoflagellate common to Indo-Pacific giant clams, and a revised morphological description of Symbiodinium microadriaticum Freudenthal, emended Trench & Blank

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The dinoflagellate genus Symbiodinium contains numerous genetically distinct lineages that appear ‘morphologically cryptic’. However, detailed morphological assessments of plate formulae visible in the motile phase (mastigote) of two distantly related Symbiodinium spp., representing Clades ‘A’ and ‘E’, were recently shown to be different. While there are several formally described species in Clade A, genetic evidence indicates that many more remain uncharacterized. We focused on closely related phylogenetic lineages within this group to examine whether differences in morphology can be used together with genetic and ecological evidence to describe new species. We found fixed differences in nuclear (internal transcribed spacer (ITS) and large subunit rDNA), chloroplast (cp23S) and mitochondrial (cob) gene sequences from cultured and field-collected samples of Symbiodinium microadriaticum (sensu Trench & Blank, 1987) and Symbiodinium sp. associated predominantly with giant clams and Pacific Cassiopea jellyfish (comprising members of the ITS2 A3 lineage, sensu LaJeunesse 2001). Amphimesial plate tabulations were formulated for the strain of S. microadriaticum (CCMP2464) used by Trench & Blank (1987) in their emended description, and two strains of type A3 (CCMP832 and rt-272) cultured from Indo-Pacific giant clams in the subfamily Tridacninae. The Kofoidian plate formula for type A3 consists of the small plate (x), the elongated amphiesmal vesicle (EAV), 5', 6a, 8", 9–11s, a 2-row cingulum, 7"" and 3"" (and occasionally 2""), and is different from S. microadriaticum, which has a plate formula of x, EAV, 4', 5a, 8", 9–13s, a 2-row cingulum, 6"" and 2"".". Based on morphological and genetic comparisons, we recognized Symbiodinium tridacnidorum sp. nov., a new Indo-Pacific species. The plate arrangement exhibited by S. microadriaticum appears to be more similar to the distantly related S. natans (also in Clade A). When the tabulations for all three Clade A species are compared with S. voratum (Clade E), the amount of morphological differentiation between species does not correspond to their degree of genetic divergence.

Key words: Cassiopea, Dinoflagellates, Kofoidian plate tabulation, Pacific Ocean, Symbiodinium Clade A, taxonomy, Tridacninae

INTRODUCTION

Dinoflagellates in the genus Symbiodinium, commonly known as ‘zooxanthellae’, are symbiotic with various invertebrates such as corals, sponges, sea anemones, jellyfish, nudibranchs, clams and single-cell hosts including ciliates and foraminifera (Trench, 1993; LaJeunesse, 2002; Lobban et al., 2002; Pochon et al., 2007), although some occur free-living on various substrata and in the water column (Hirose et al., 2008; Porto et al., 2008; Jeong et al., 2012, 2014; Yamashita & Koike, 2013). The symbioses that many form with a broad diversity of stony corals, order Scleractinia, are crucial for building tropical reef ecosystems. However, despite their ecological and economic importance in marine ecosystems, the taxonomy of Symbiodinium remains limited because few biologically discrete entities have formal binomials.

After Symbiodinium microadriaticum was initially described by Freudenthal (1962), the description was emended by Kevin et al. (1969). Owing to the prevailing dogma which assumed that Symbiodinium was a monotypic genus (McLaughlin & Zahl, 1966; Kevin et al., 1969; Taylor, 1974), there was little interest in investigating species diversity further until Loeblich & Sherley (1979) attempted to rename the species Zooxanthella microadriatica on the basis that no previous effort had been made to differentiate it from the symbiont cells that Brandt (1881) had examined from...
the radiolarian, *Collozoum inerme*. Loeblich & Sherley (1979) were the first to provide Kofoidian plate tabulations, based on scanning electron microscopy (SEM), for an isolate obtained from the cnidarian *Cassiopea xamachana*, in the Florida Keys, USA (isolate 406). They included in their study an environmental isolate from the temperate northwestern Atlantic Ocean (isolate 395) whose tabulation was distinct, yet too similar to justify describing it as a separate species.

The revelation that *Symbiodinium* was speciose began with Trench & Blank (1987), who introduced *S. goreauii*, *S. pilosum* and *S. kawagutii*, as well as providing another emendation to *S. microadriaticum* (Trench & Blank 1987; Trench, 2000). The criteria used in the justification of these additional species relied on differences in cell size and ultrastructure including the number of chromosomes, pyrenoids and pyrenoid stalks, as well as in the relative volumes of chromosomes, nuclei, chloroplasts and mitochondria. This work was also the first to utilize molecular evidence on photosystem proteins to substantiate these claims that the lineages were genetically distinct (Trench & Blank, 1987). Molecular sequence data eventually revealed large genetic differences among members of this genus, clades of which were designated as Clades A, B, C, etc. (Rowan & Powers, 1991). The subsequent application of various genetic methods and markers to identify ecologically distinct lineages facilitated a surge in discovery about processes underlying the ecology and evolution of these dinoflagellates (Sampayo et al., 2009; Baums et al., 2014; Thornhill et al., 2014).

Since the early 1990s, genetic analyses have dominated many aspects of *Symbiodinium* research (Coffroth & Santos, 2005; Sampayo et al., 2009), and are the de facto diagnostic to examine ecological and geographic patterns of diversity (LaJeunesse et al., 2010). While there is some contention regarding how rDNA data are interpreted (Stat et al., 2011; LaJeunesse & Thornhill 2011), the convergence of evidence from a combination of several genetic markers can delimit reproductively isolated lineages (LaJeunesse & Thornhill, 2011; Pochon et al., 2012; Thornhill et al., 2014) and has led to the collective use of these data in formally describing species including *Symbiodinium* spp. that are not in culture (LaJeunesse et al., 2012, 2014). However, it is important to relate, when possible, these new approaches with more traditional methods for describing dinoflagellate species.

While most *Symbiodinium* are unable to flourish in artificial growth media, there are some that proliferate in vitro. Preliminary genetic evidence suggests that many of these strains represent undescribed species for which a combination of modern and conventional taxonomic approaches can be applied (e.g. Jeong et al., 2014). When viewed with light microscopy, *Symbiodinium* are morphologically nondescript, except for obvious differences in cell size. Scanning electron microscopy (SEM) allows the resolution of Kofoidian plate patterns on the motile cells (mastigotes) of cultured isolates (Jeong et al., 2014). Amphiansmal plate formulae are different between *S. natans* (Clade A; Hansen & Daugbjerg, 2009), *S. voratum* (Clade E; Jeong et al., 2014), and *S. minutum* and *S. physigiphilum* (Clade B; LaJeunesse et al., 2012; Lee et al., 2014), yet these species are genetically divergent from each other. It is not known whether closely related species (i.e. members of the same clade) differ in the number and arrangement of their amphiansmal plates. An assessment of morphological variation and evolution among distantly and closely related *Symbiodinium* would benefit from expanding our comparative analyses of thecal morphology, using SEM, when new species are described.

In this report, we have characterized the amphiansmal plate morphology and pattern found on *S. microadriaticum* (*Symbiodinium* strain CCMP2464, or rt-061) isolated from the jellyfish *Cassiopea xamachana* (ITS2 type A1, sensu LaJeunesse, 2001) because the plate tabulation of this isolate was not described by Trench & Blank (1987). The genetic identities of the isolates Loeblich & Sherley (1979) characterized morphologically and assigned to the species *Zooxanthella microadriatica* as reported above, were used to resolve their phylogenetic relationships within the genus *Symbiodinium*. We combined these results with the morphological characterization of two cultured strains (CCMP832 and rt-272) isolated from giant clams in the Pacific Ocean and for which genetic evidence indicated that they represented a novel species in Clade A (initially designated ITS2 type A3, sensu LaJeunesse, 2001). The morphological comparison between *S. microadriaticum* and isolates from two giant clam hosts were combined with additional sequence analyses of the large subunit (LSU) rDNA, partial chloroplast large subunit (cp23S) and mitochondrial cytochrome b (cob). The genetic variation in these markers was analysed from multiple cultures as well as field-collected specimens from several ecological and geographic sources. Finally, we examined to what extent morphological differences (i.e. plate tabulations) were concordant with phylogenetic relationships among *Symbiodinium* spp. for which these data were available.

**MATERIALS AND METHODS**

Cultures and growth conditions of *Symbiodinium* spp.

*Symbiodinium microadriaticum* (CCMP2464, formally rt-061 sensu Trench & Blank, 1987) and strain CCMP832 were obtained from the National Center for Marine Algae and Microbiota (NCMA) (Table 1). Strain CCMP 2464 of *S. microadriaticum* was isolated originally from the jellyfish *Cassiopea xamachana* in the Florida Keys, USA.
The culture CCMP832 was originally obtained from the giant clam *Hippopus hippopus* living on the Great Barrier Reef, Australia; and isolate rt-272 was cultured from *Tridacna gigas* from Palau (LaJeunesse, 2001). The cultured isolates 395 and 406 analysed by Loeblich & Sherley (1979) were obtained from the University of Texas at Austin culture collection (UTEX LB 2281 and UTEX LB 2282, respectively). Cultures were grown in L1 seawater medium without silicate (Guillard & Hargraves, 1993), at a temperature of 25°C with continuous illumination of 20 μmol photons m⁻² s⁻¹ cool white fluorescent light under a 14:10 h light-dark cycle. They were transferred approximately every 2–3 weeks to new 250 ml PC bottles containing fresh media. When culture volumes were large enough to analyse, genetic and morphological characterizations were performed.

**Scanning electron microscopy (SEM)**

Cell size and the analyses of the amphiesmal plate shape, number and arrangement of culture CCMP2464 (*S. microadriaticum*), CCMP832 and rt-272 were performed by SEM. Cells from dense cultures were fixed for 10 min in osmium tetroxide at a final concentration of 0.3% (w/v) in seawater. Cell collection, dehydration, drying and mounting were performed as described in Kang et al. (2010).

**Transmission electron microscopy (TEM)**

TEM was used for counting the permanently condensed chromosome bodies typical of dinoflagellates. For sample preparation and sectioning, cells from dense cultures were fixed in 2.5% (w/v) glutaraldehyde (final concentration). After 1.5–2 h, cells were concentrated at 1610 x g for 10 min in a Vision Centrifuge VS-5500 (Vision Scientific Company, Bucheon, Korea). Next, the supernatants were discarded, and the pellets were transferred to 1.5 ml tubes and rinsed several times with 0.2 M pH 7.4 sodium cacodylate buffer. Then, the pellets were post-fixed with 1% (w/v) osmium tetroxide in deionized water for 90 min. After fixation, the pellets were embedded in agar. Dehydration was performed via a graded ethanol series (50%, 60%, 70%, 80%, 90%, and 100% ethanol, followed by two changes in 100% ethanol). The agar-embedded pellet was then re-embedded in Spurr’s low-viscosity resin and dried for 3 days at 70 °C. Samples were then serially sectioned (80–100 nm) using an RMC MT-XL ultramicrotome (Boeckeler Instruments Inc., Tucson, Arizona, USA), and stained with 3% (w/v) aqueous uranyl acetate followed by lead citrate. Finally, samples were observed with a JEOL-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan). The chromosome number (± SE) for the strains was determined using these serial sections.

**Nucleic acid extraction, PCR amplification, sequencing and phylogenetic analyses**

Nucleic acids were extracted from 10–15 ml of a dense culture of CCMP832 using the *AccuPrep*® Genomic DNA Extraction Kit (Bioneer Cooperation, Daejeon, Korea). The DNA extracts from cultures and field-collected samples corresponding to *Symbiodinium microadriaticum* (= type A1), *S. pilosum* (= type A2) and that of type A3 from the Pacific Ocean obtained and used in previously published or
unpublished research were included in subsequent genetic analyses. Details such as culture number, host, depth and geographic origin as well as journal citations where particular samples were initially analysed are listed in supplemental Table S1. A request to acquire DNA from S. natans to complete sequencing of the cp23S and cob genes was denied.

PCR amplifications for the ITS region of CCMP832 (ITS1, 5.8S and ITS2) were performed using the forward primer Euk1209F (Giovannoni et al., 1988) and the reverse primer ITS2R (Litaker et al., 2003). All other ITS2 amplifications used the primer-set and reaction conditions specified in Sampayo et al. (2009). Amplifications of the LSU region D1/D2 was performed using the forward primer D1RF (Scholin et al., 1994) and the reverse primer LSB3 (Litaker et al., 2003), or using the primer set and conditions developed by Zardoya et al. (1995). For PCR amplification, Solq™ f-Taq DNA Polymerase® (SolGent Co., Daejeon, Korea) was used according to the manufacturer’s instructions. The partial chloroplast cp23S and mitochondrial cob genes were amplified and directly sequenced for all cultures and field-collected samples according to Zhang et al. (2000) and Zhang et al. (2005), respectively (Table S1). Sanger sequencing of these plastid markers and the LSU was performed using Big Dye 3.1 reagents (Life Sciences) and the products analysed on an Applied Biosystems 3730XL instrument.

The phylogenetic analysis of the ITS (ITS1, 5.8S, ITS2) and LSU rDNA regions of the Symbiodinium strains was conducted using MEGA v.4 (Tamura et al., 2007) and Clustal X2 (Larkin et al., 2007), and the sequences from outgroup Symbiodinium from Clades D and E were obtained from recently published results (Jeong et al., 2014; LaJeunesse et al., 2014). Maximum likelihood (ML) analyses were conducted using the RAxML 7.0.4 program with a GTRGAMMA model (Stamatakis, 2006). Further, 200 independent tree inferences were used to identify the best tree. ML bootstrap values were determined using 1000 replicates. Bayesian analyses were conducted using MrBayes v.3.1 (Ronquist & Huelsenbeck, 2003) in the default GTR + G + I model to determine the best available model for the data for each region. For all sequence regions, four independent Markov Chain Monte Carlo runs were performed as described in Kang et al. (2010).

RESULTS

Taxonomic descriptions


EMENDATION: This dinoflagellate has a Kofoidian plate formula of x, EAV, 4′, 5a, 8′, 9–13s, 2 row cingulum, 6′′, 2′′′.

Nucleotide sequences of the large ribosomal subunit rDNA (Accession no. KM972549), partial chloroplast large subunit, cp23S (Accession no. KF740693) and mitochondrial cytochrome b, cob (Accession no. KF206025) genetically define this species.

TYPE LOCALITY: Key Largo, Florida, USA (25°00′00″N, 80°50′00″W).

Symbiodinium tridacnidorum Lee, Jeong, Kang, & LaJeunesse sp. nov. (Figs 4–6, 16–23, 28–35)

DIAGNOSIS: Mastigote cells are 9.4–12.9 μm in length and 7.5–10.6 μm in width. The cell epism is slightly larger than the hyposome. An EAV is present on the apex. The cingulum consists of 2 rows of pentagonal plates. The cingulum is displaced by 0.06–0.2 cell lengths and 0.2–0.7 cell widths. Plates are arranged as x, EAV, 5′, 6a, 8′, 9–11s, 17–19e, 7″′ and 2–3″′. The 5a plate is hexagonal. The 6″ plate is pentagonal. The 2′ plate touches the 4′ plate. The size of the 5′ plate is similar to that of the 2′ plate. A single, 2-stalked pyrenoid is present. A type E eyespot is located beneath the sulcus in motile cells. A well-developed PE emerges between the longitudinal and transverse flagella. The nucleus contains a nucleolus and 77–83 condensed chromosomes.

Nucleotide sequences of the large ribosomal subunit rDNA (Accession nos KM816405, KM816406, KM972551), ITS2 rDNA (Accession no. KM816410), partial chloroplast large subunit, cp23S (Accession nos KM816407–KM816409) and mitochondrial cytochrome b, cob (Accession no. KM816411) genetically define this species.

HOLOTYPE: A holotype slide labelled USNM stub 1251931 of a culture fixed with 0.3% (w/v) osmium tetroxide has been deposited in the Protist Type Specimen Slide Collection, US Natural History Museum, Smithsonian Institution, Washington, District of Columbia, USA.

TYPE LOCALITY: Tridacna Reef, Great Barrier Reef, Australia (20°00′00″S, 149°00′00″E).

HABITAT: Isolated from mantle tissue of the giant clam species Hippopus hippopus.

ETYMOLOGY: The specific name ‘tridacnidorum’ is based on the subfamily of bivalves (Family Cardioidae Lamark, 1809), commonly referred to as giant clams.

Morphology of Symbiodinium microadriaticum

Motile cells of S. microadriaticum (strain CCMP2464) were mushroom shaped, with an epism (epitheca) slightly larger than the hemispherical hyposome (hypotheca; Figs 1–3). The cells (n = 30) in log phase culture were 8.0–13.8 μm in length and 6.3–11.9 μm in width, respectively (Table 2). In addition, the ratio of the length to width of the living cells was 1.1–1.9 μm (Table 2). However, when fixed and observed under SEM, the cells were slightly smaller and were 7.6–10.0 μm in length and 5.8–7.7 μm in width (Table 2). The ratio of the length to the width under SEM was 1.2–1.5 μm (Table 2).
The Kofoidian plate formula of *S. microadriaticum* cells was x, EAV, 4′, 5a, 8′′, 9–13s, the 2 cingulum rows 22–24c, 6′′, and 2′′′ (Table 2; Figs 7–15 and 24–27). At the cell’s apex, the elongated amphiesmal vesicle (EAV) possessed 6–8 aligned knobs and measured 1.33–2.65 μm in length and 0.13–0.33 μm in width. This structure was bordered ventrally by the x plate and surrounded by apical plates, 2′, 3′ and 4′ (Figs 12, 14, 26). Apical plates consisted of a rhomboid-shaped and relatively large 1′, a quadrangular 2′ plate and hexagonal 3′ plate. Surrounding these were the pentagonal 4′ apical plate and 1a–5a intercalary plates (Figs 12, 13, 26). The 5 intercalary plates were hexagonal (plates 1a and sometimes 3a) pentagonal (plates 2a, 5a and sometimes 3a), and heptagonal (plate 4a) (Figs 8–10, 12, 13, 24–26). Of the 8 precingular plates, 1′′, 2′′, 4′′′, 5′′′ and 6′′′ plates were quadrangular, but the 3′′ plate was pentagonal (Figs 15, 27). Both antapical plates, 1′′′ and 2′′′, were pentagonal or hexagonal (Table 2; Figs 15, 27). A total of 13 sulcal plates, including 2 S (?) plates and 1 S.p. plate were located in the sulcus. A peduncle (PE) was present in the middle of the sulcal plates (Table 2; Figs 7, 11, 24).

**Fig. 1–6.** Light micrographs of the mastigote (motile), coccoid (spherical) and doublet (dividing) cells from *Symbiodinium microadriaticum* (Figs 1–3) and *S. tridacnidorum* sp. nov. (Figs 4–6). **Fig. 1.** *S. microadriaticum* mastigote; pyrenoid (PY, white arrowhead), nucleus (N). **Fig. 2.** *S. microadriaticum* coccoid. **Fig. 3.** *S. microadriaticum* doublet. A single PY is generally located in the centre of the cell. **Fig. 4.** *S. tridacnidorum* mastigote; pyrenoid (PY, white arrowhead), nucleus (N). **Fig. 5.** *S. tridacnidorum* coccoid. **Fig. 6.** *S. tridacnidorum* doublet. All cells contained a reticulated chloroplast located at the periphery of the cell. All scale bars = 2 μm.

**Morphology of *Symbiodinium tridacnidorum***

The motile cells of this culture were mushroom-shaped, with an episeome that was slightly larger than the hemispherical hyposome (Figs 4–6; Figs S1–S3). The cells (n = 30) in log phase growth were found to be 9.4–12.9 μm in length and 7.5–10.6 μm in width (Table 2). In addition, the ratio of the length of the living cells to the width was 1.1–1.4 μm (Table 2). When fixed and observed under SEM, the cells were found to be 8.1–10.1 μm in length and 5.6–7.1 μm in width, and the ratio of the length to width was 1.3–1.5 μm (Table 2).
The Kofoidian plate formula of *S. tridacnidorum* cells is x, EAV, 5’, 6a, 8”, 9–11s, 2 cingulum rows 17–19c, 7”” and 3””” (rarely 2”””); Table 2; Figs 16–23; Figs S4–S11). At the cell’s apex, the EAV possessed 7–9 aligned knobs and measured 0.76–1.84 μm in length and 0.09–0.37 μm in width (Table 2). This
Figs 16–23. Scanning electron micrographs of *Symbiodinium tridacnidorum* motile cells. **Fig. 16.** Ventral view showing the episome, cingulum (C), sulcal plates (s), peduncle (PE) and hyposome. **Fig. 17.** Ventral-left lateral view showing the episome, cingulum (C) and hyposome. **Fig. 18.** Dorsal view showing the episome, cingulum (C) and hyposome. **Fig. 19.** Ventral-right lateral view showing the episome, cingulum (C), sulcus (s) and hyposome. **Fig. 20.** Apical view showing the episome and elongated amphiesmal vesicle (EAV) plate. **Fig. 21.** Apical view showing the EAV plate with small knobs (arrowhead). **Fig. 22.** Antapical view showing the hyposome. **Fig. 23.** Antapical-ventral view showing the sulcal plates (s) and peduncle (PE). All scale bars = 1 μm.
structure was bordered ventrally by the x plate and surrounded by 4 apical amphiesmal plates (2’, 3’, 4’ and 5’ plates; Figs 20, 30; Fig. S8). There are 6 intercalary plates. Of these 4 are hexagonal (plates 1a, 3a, 4a and 5a), one was pentagonal (2a), and one is either pentagonal-, hexagonal- or heptagonal-shaped depending on the cell imaged (6a) (Figs 16–20, 28–30; Figs S4–S8). For the 8 precingular plates, the 1” plate is invariably quadrangular, the 2”, 5”, 6” and 8” plates are pentagonal, however the 3”, 4” and 7” plates are variable and either quadrangular or pentagonal (Table 2; Figs 16–20, 28, 29; Figs S4–S8). Two wide cingulum rows consisting of 17–19 pentagonal plates create the groove around the middle of the cell where the transverse flagellum lies. The cingulum is displaced in the region of the sulcus groove by ~0.1–0.2 times the cell length and ~0.2–0.7 times the cell width (Table 2). Of the 7 postcingular plates, 1’”, 2’”, 5’”, 6’” and 7’” plates are quadrangular, while the 3’” and 4’” plates are pentagonal (Table 2; Figs 22, 31). There are usually 3, but sometimes 2, antapical plates (Table 2; Figs S10, S12, S13). The 1’” and 2’” plates are either pentagonal or hexagonal, while the 3’” is hexagonal when present (Fig. 22; Figs S4–S13). A total of between 9–11 sulcal plates including 2 S (?) plates and one posterior sulcal plate (S.p.) was observed. An accessory sulcal plate positioned between where the transverse and longitudinal flagella emerge from the cell (Table 2; Figs 23, 28; Fig. S11).

Through TEM analysis, the periphery of the cell interior of *S. tridacnidorum* (CCMP832) is often occupied by chloroplast lobes. A single pyrenoid located in the central part of each cell is connected by 2 stalks to the adjacent chloroplast and surrounded by a distinct starch cap (Figs 32, 35). No chloroplast thylakoid lamellae penetrated the pyrenoid. A large number of lipid globules and starch were observed (Fig. 32). Furthermore, an eyespot (type E), consisting of multiple layers of rectangular electron-translucent vesicles or crystalline deposits, was observed in
sectioned mastigote cells (Fig. 33). A mean of $80 \pm 3$ (SE) condensed chromosomes were estimated from serial sections of cell nuclei ($n=4$ cells; Table 2; Fig. 34).

Phylogenetic delineation of Clade A Symbiodinium
Concordant data from ribosomal DNA (ITS2 and LSU), mitochondrial cob and chloroplast cp23S sequences show that *S. tridacnidorum* is a reproductively isolated, evolutionarily divergent lineage and therefore distinct from other described Clade A species (Figs 36, 37, 38). Some sequence variation in rDNA was found among cultured and field-collected specimens originating from giant clams in the subfamily Tradacnaeae (formerly the family Tridacnidae) and the upside-down jellyfish (*Cassiopea*; Figs 36, 38; Table S1). In addition to the ITS2 ‘A3’ sequence, several additional sequence variants, *A3a*, *A6* and *A3* *A3x’ sensu Weber 2009) were found to be diagnostic of this *Symbiodinium* lineage (Fig. 36). These sequences were recovered from direct sequencing of PCR products or from screening using denaturing gradient electrophoresis (DGGE; see Sampayo et al., 2009 for explanation of the method for identifying those ITS sequences of diagnostic value in organisms with high intragenomic rDNA variation). The culture CCMP832 is characterized by the *A3* ITS2 sequence, which differentiated from *Symbiodinium A3* by a single base substitution (Fig. 36). The LSU sequence of *A3* (e.g. CCMP832) also contained two base substitutions (Fig. 38). The rDNA variant of CCMP832 matched with a field-collected sample of *Tridacna maxima* originating from 12–15 m depth in Palau.

Figs 28–31. Drawings of *Symbiodinium tridacnidorum* motile cells showing the external morphology. Fig. 28. Ventral view. Fig. 29. Dorsal view. Fig. 30. Apical view. Fig. 31. Antapical view. All scale bars = 1 μm.
Fig. 32–35. Transmission electron micrographs of *Symbiodinium tridacnidorum* cells. **Fig. 32.** A transverse section of a mastigote cell from *S. tridacnidorum* showing the position of the pyrenoid in the middle of cell, the chloroplasts and type E eyespot (stigma) near the cell’s surface, lipid globules and starch. **Fig. 33.** A magnified view of the type E eyespot consisting of multiple layers of rectangular electron-translucent vesicles, or crystalline deposits. **Fig. 34.** Serial sectioning through the nucleus showing large number of condensed chromosomes (approximately 80 ± 3). **Fig. 35.** A magnified view of a single pyrenoid with 2 stalks, located in the central part of each cell and surrounded by a distinct polysaccharide cap. Scale bar = 1 μm for Figs 32–34 and 0.5 μm for **Fig. 35.**

(Fig. 38) and from samples obtained from the Indo-West Pacific, West Pacific and Central South Pacific (Figs 36, 37; Weber, 2009).

The LSU (and cp23S; data not shown) sequences from the two isolates examined by Loeblich & Sherley (1979) placed them in the genus *Symbiodinium*. Isolate 406 is a strain of *S. microadriaticum* (Fig. 38), while isolate 395 matched sequences of *S. voratum* obtained from populations in the northwestern Pacific (Fig. 38).

Mitochondrial cob and partial chloroplast cp23S sequences were identical for most samples of *S. tridacnidorum*. The only exceptions found were from samples of *Cassiopea andromeda* collected in Palau. These were differentiated from the others by a single base substitution in domain V of the cp23S marker (Fig. 39).

The rDNA (ITS2 and LSU) and plastid (cp23S and cob) sequences used to diagnose cultures and field-collected samples of *S. microadriaticum* were invariant despite originating from several locations in the north central and western Caribbean (Florida Keys, the Mexican Yucatan and Belize), Red Sea (Gulf of Aqaba) and one culture (KB8) from O’ahu, Hawaii.
Table 2. Morphological differences among Clade A Symbiodinium in comparison to S. voratum (Clade E) based on electron microscopy.

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<th>S. microadriaticum</th>
<th>S. natans</th>
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</thead>
<tbody>
<tr>
<td>AP length (μm; living cells)</td>
<td>9.4–12.9 (10.9)</td>
<td>8.0–13.8 (9.8)</td>
<td>9.5–11.5 (10)</td>
<td>10.8–16.2 (13.1)</td>
</tr>
<tr>
<td>Cell width (μm; living cells)</td>
<td>7.5–10.6 (8.8)</td>
<td>6.3–11.9 (8.1)</td>
<td>7.4–9.0 (8)</td>
<td>7.8–11.5 (9.5)</td>
</tr>
<tr>
<td>Ratio of length to width (living cells)</td>
<td>1.1–1.4 (1.3)</td>
<td>1.1–1.9 (1.2)</td>
<td>Unk.</td>
<td>1.1–1.8 (1.4)</td>
</tr>
<tr>
<td>AP length (μm; SEM)</td>
<td>8.1–10.1 (9.2)</td>
<td>7.6–10.0 (9.2)</td>
<td>10.38*</td>
<td>8.5–12.4 (10.5)</td>
</tr>
<tr>
<td>Cell width (μm; SEM)</td>
<td>5.6–7.1 (6.5)</td>
<td>6.3–11.9 (8.1)</td>
<td>8.25*</td>
<td>6.4–9.8 (8.2)</td>
</tr>
<tr>
<td>Ratio of length to width (SEM)</td>
<td>1.3–1.5 (1.4)</td>
<td>1.2–1.5 (1.3)</td>
<td>1.26*</td>
<td>1.2–1.4 (1.3)</td>
</tr>
<tr>
<td>EAV length (μm)</td>
<td>0.76–1.84 (1.42)</td>
<td>1.33–2.65 (1.94)</td>
<td>2</td>
<td>1.75–3.09 (2.45)</td>
</tr>
<tr>
<td>EAV width (μm)</td>
<td>0.09–0.37 (0.18)</td>
<td>0.13–0.33 (0.21)</td>
<td>0.2</td>
<td>0.15–0.27 (0.2)</td>
</tr>
<tr>
<td>Numbers of aligned knobs on EAV</td>
<td>7–9</td>
<td>6–8</td>
<td>12</td>
<td>9–13</td>
</tr>
<tr>
<td>Cingulum displaced by cell length</td>
<td>0.06–0.20 (0.12)</td>
<td>0.06–0.24 (0.14)</td>
<td>0.23*</td>
<td>0.13–0.21</td>
</tr>
<tr>
<td>Cingulum displaced by cell width</td>
<td>0.23–0.70 (0.40)</td>
<td>0.38–0.71 (0.61)</td>
<td>1.0</td>
<td>0.48–0.85 (0.65)</td>
</tr>
<tr>
<td>Numbers of cingular plates</td>
<td>17–19</td>
<td>22–24</td>
<td>20</td>
<td>17–20</td>
</tr>
<tr>
<td>Numbers of sulcal plates</td>
<td>9–11</td>
<td>9–13</td>
<td>Unk.</td>
<td>9</td>
</tr>
<tr>
<td>Numbers of apical plates</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Numbers of intercalary plates</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Numbers of precingular plates</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Numbers of postcingular plates</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Numbers of antapical plates</td>
<td>3 (rarely 2)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>No. of chromosomes</td>
<td>80 ± 3 (n=4)</td>
<td>97 ± 2 (n=6)*</td>
<td>Unk.</td>
<td>74 ± 1.5 (n=5)</td>
</tr>
<tr>
<td>Plate formula</td>
<td>x, EAV, 5′, 6a, 8′, 9–11s, 17–19c, 7″′, 2′–3′′, PE</td>
<td>x, EAV, 4′, 5a, 8′, 9–13s, 22–24c, 6′′, 2″′, PE</td>
<td>x, EAV, 4′, 5a, 8′, 9s, 6, 17–20c, 6′′, 2″′, PE</td>
<td>x, EAV, 4′, 5a, 8′, 9s, 6, 17–20c, 6′′, 2″′, PE</td>
</tr>
</tbody>
</table>

Mean values are shown in parentheses. AP, anteroposterior; EAV, elongated amphiesmal vesicle; PE, Peduncle; Unk, Unknown; *Obtained from Trench & Blank (1987). †Obtained from Jeong et al. (2014) (1) This study. (2) Trench & Blank (1987). (3) Hansen & Daugbjerg (2009). (4) Jeong et al. (2014)

(Figs 36–38). Similarly, the sequences of these markers were identical for each of three cultures of S. pilosum (type A2; sensu LaJeunesse, 2001) obtained from three host genera and two Caribbean locations (Figs 36, 38, 39; Table S1). This species is only known from opportunistic contaminants during the culturing process, as there are no known field-collected samples of S. pilosum (LaJeunesse pers. obs.).

The phylogenetic reconstructions of Clade A Symbiodinium based on sequences from chloroplast and mitochondrial genes corresponded with rDNA phylogenies (cp23S and cob trees not shown). Fixed sequence differences in both the cob and cp23S resolved S. tridacnidorum and S. microadriaticum as evolutionary divergent entities. The phylogeny based on the concatenated sequence alignments of chloroplast and mitochondrial markers, along with the D1/D2 region of LSU, is shown in Fig. 39.

**DISCUSSION**

Clade A Symbiodinium contains phylogenetically and ecologically distinct entities that exhibit unique geographic distributions (LaJeunesse et al., 2009). This group also comprises many species that are both ecologically common and are capable of growth in culture media, which allows for detailed morphological analysis of the motile phase and experimental manipulation (Hennige et al., 2009).

Morphological comparisons among Clade A Symbiodinium

The new description of S. tridacnidorum sp. nov. and emended description of S. microadriaticum show that the mastigotes of genetically similar species are morphologically distinct (Fig. 40). Our analyses of two genetically distinct strains of S. tridacnidorum obtained from different regions of the Pacific Ocean (GBR and Palau) indicate that morphology is stable among individuals (i.e. strains) of this species (Figs 36, 38; Figs S4–S11).

The size, shape, number and arrangement of amphiesmal plates can also be used to discern Clade A species that were initially recognized by genetic and ecological differences (Table 2). Each species possesses different numbers of sulcal and cingular plates (Table 2). The number of knobs arranged on the EAV are similar for S. microadriaticum and S. tridacnidorum (6–8 and 7–9, respectively), but differ significantly from S. natans, which has at least 12 knobs. However, similarities and differences in plate formulae do not necessarily correspond to phylogenetic relationships.

Symbiodinium microadriaticum (CCMP2464) and S. natans contain the same number of apical, intercalary, postcingular and antapical plates whereas S. tridacnidorum had different totals for these plate types (Fig. 40; Table 2), making the overall plate arrangement for S. tridacnidorum more distinctive than the plate tabulation established for S. voratum (Clade E), a distant related species (Fig. 40). Furthermore, the shapes of specific
Figs 36 & 37. Fig. 36. Maximum parsimony (MP) tree created from the alignment of ITS2 data from Clade A *Symbiodinium* identified as *S. microadriaticum* (types A1), *S. pilosum* (A2), *S. tridacnidorum* sp. nov. (comprising mostly Indo-Pacific samples from tridacnid clams and *Cassiopea* sp.) and type A4 found in the Western tropical Atlantic. The numbers above the branches correspond to Bayesian posterior probabilities (left) and MP bootstrap values (right). Fig. 37. A summary of the known geographic distributions for *S. tridacnidorum* associated with *Tridacna maxima* across the Indo-Pacific. The known ranges of ITS2 variants A3a and A3* [A3x sensu Weber (2009)] suggest the possibility that genetic structure among geographically distant populations may exist. Numbers correspond to the publications where these distributions were reported.
plates when compared between species corresponded only partially with phylogenetic data. For example the 5a and 6′′ plates were distinct for S. tridacnidorum being hexagonal and pentagonal, respectively; whereas the shapes of these plates for S. microadriaticum and S. natans matched with S. voratum, pentagonal and quadrangular, respectively. Therefore, while the comprehensive analyses of morphological evidence can define these ‘morphologically cryptic’ species, the overlap in trait values creates some ambiguity when reconciled against gene-based phylogenies.

This work provides morphological analysis of the S. microadriaticum strain (CCMP2464, or rt-061) used by Trench & Blank (1987) to further emend the description of S. microadriaticum, the type species in the genus Symbiodinium (Freudenthal). LaJeunesse (2001) genetically analysed CCMP2464 and designated it as type A1. From the incomplete description provided by Freudenthal (1962), for which the original type material is not available, it is not possible to know whether the particular culture he based his diagnosis on was morphologically and genetically the same as the specimens analysed by Loeblich & Sherley (1979) and by Trench & Blank (1987). However, the entity isolated as CCMP2464 by Trench & Blank (1987) accurately represents the identity of Symbiodinium found in field-collected samples of C. xamachana from the Greater Caribbean (LaJeunesse, 2002; Fig. 38), and not an opportunistic species isolated during the culture process (Santos et al., 2001).
**Fig. 39.** Maximum parsimony (MP) tree created from concatenated alignments of LSU, cp23S and cob sequences. The differently shaded dot symbols along each branch indicate the relative contribution of fixed differences in each genetic marker that differentiate three species of Clade A. The numbers above the branches indicate the Bayesian posterior probability (left) and MP bootstrap values (right). Grey arrowheads signify the cultured isolates used for morphological analysis.

**Fig. 40.** A comparison of apical and antapical plate morphologies from *S. microadriaticum*, *S. tridacnidorum* and *S. natans* (Clade A), and *S. voratum* (Clade E). The occurrence of additional plates and plate connections that differ from *S. microadriaticum* are highlighted in grey. Genetic relationships based on LSU rDNA are drawn to the left.
The two isolates described as *Zooxanthella microadriatica* by Loeblich & Sherley (1979) are morphologically similar to *S. microadriaticum* (CCMP2464). These included strain 406 isolated from *Cassiopeia xamachana* from the Florida Keys, which had a thecal plate formula of 5′, 5–6a, 9–10′, 8–9a, 20c, 7–8′′ and 3′′; and strain 395 isolated from a decaying red alga, *Chondrus crispus*, collected off the coast of Massachusetts, USA, which had a plate formula of 5′, 4–6a, 10–11′, 9–8a, 20c, 7–8′′′ and 3′′′. Our genetic characterization of these isolates unambiguously resolved their identity. Isolate 406 (given to Loeblich by Trench’s student, Schoenber) is likely to be the same isolate later used by Trench & Blank (1987) in their emendation of *S. microadriaticum (rt-061 synonymous with CCMP2464; Fig. 38). Our plate reconstruction of the isolate from *Cassiopeia xamachana* collected from the Florida Keys, USA, differs from their formulation in the number of precingular and antapical plates. The genus *Zooxanthella* was created by Brandt (1881) during his studies on the algae living *S. et al.* replaced with the new genus *Symbiodinium* (Probert et al., 2014).

The free-living strain that Loeblich & Sherley (1979) obtained from cold temperate waters of the Northwest Atlantic, isolate 395, corresponds to *S. voratum* (Fig. 38). Their morphological characterization was similar to that of Jeong et al. (2014), but there appears to be a difference in how antapical vs. post-circular plates were designated by each set of researchers. Also, the number of precingular plates estimated by Loeblich & Sherley (1979) was higher than that reported by Jeong et al. (2014). However, these morphological discrepancies might be explained by the occasional variation in morphology observed among cells of *S. voratum*, which were characterized in the supplementary figure published by Jeong et al. (2014). Further, despite these apparent morphological differences, which may stem from differences in technique, the large cell size, high latitudinal origin and free-living habitat of isolate 395 are consistent with genetic analysis identifying this isolate as *S. voratum*, and not *Zooxanthella microadriatica*.

The genetic, ecological and geographic attributes of described species in *Symbiodinium* Clade A

*Symbiodinium tridacnidorum* is probably the most prevalent Clade A species from the Indo-Pacific. Over its geographic range of distribution, it is harbored by several kinds of animal. Independent researchers, working in the Indo-West Pacific, have commonly identified (genetically) and cultured *S. tridacnidorum* from giant clams in the moluscan subfamily Tradacninae (Rowan et al., 1996; Carlos et al., 1999; Baillie et al., 2000; LaJeunesse, 2001; LaJeunesse et al., 2004a, 2004b; Weber, 2009; DeBoer et al., 2012). In the Indian Ocean, it occurs in the large stinging hydroid (*Aglaophenia*; Figs 36, 38). This symbiont has also been identified and cultured from species of the mangrove upside-down jellyfish in the genus *Cassiopeia*, a rhizostome group in the Class Scyphozoa (Figs 36, 37; LaJeunesse et al., 2004a, 2004b). *Cassiopeia andromeda* from Palau, a likely native of the Pacific Ocean region (host genetic data not shown), contained *S. tridacnidorum* populations, diagnosed by a slightly distinct cp23S sequence variant (Fig. 39). Experiments with *S. tridacnidorum* have shown that isolates from *Tridacna* spp. (rt-272) can infect and induce strobilation in the scyphistome of *Cassiopeia xamachana* (Fitt, 1985). The disparate host specificities exhibited by this symbiont cannot be explained by host phylogenetic relationships, nor the nature of their associations (e.g. extracellular vs. intracellular), yet suggests that unknown biotic or abiotic factors have initiated host shifts during its evolutionary history (Secord & Kareiva, 1996).

Three ITS2 sequence variants, A3a, A3* (listed as ‘A3x’ in Weber, 2009) and A6 diagnose *Symbiodinium* populations found only in the tridacnids (Figs 36, 37). Types A3 and A6 are relatively widespread, but type A3a occurs predominantly in tridacnids from the Indo-West Pacific and Central Indian Ocean, whereas A3* is common in clams from the Southwest and Southcentral tropical Pacific (Fig. 37; Weber, 2009). These different geographic distributions indicate regional differentiation exists between some populations of *S. tridacnidorum* (Weber, 2009). The analysis of microsatellite allelic data can be used in the future to test the possibility of regional endemism/isolation across this widely distributed species (Pinzón et al., 2011; Baums et al., 2014). The broad geographic range and genetic variation among populations of *S. tridacnidorum* associated with the tridacnids indicate that this particular relationship has probably lasted millions of years (Thornhill et al., 2014).

*Symbiodinium* species designated type A3 (sensu lato) are associated with a wide range of cnidarians that occur in the Greater Caribbean region, including species of the elk- and stag-horn coral (*Acropora*), shallow colonies of the blushing star coral (*Stephanocenia*), finger corals from seagrass beds (*Porites porites*), the top sides of shallow colonies of boulder star corals (*Orbicella* sp.), and colonies of mat zoanthids (*Zoanthus* sp.; LaJeunesse, 2002; Finney et al., 2010; Baums et al., 2014). Preliminary population genetic and phylogenetic comparison between Pacific and Atlantic *Symbiodinium A3* indicate that they are different species (Pinzón et al., 2011; LaJeunesse unpubl. data). The A3 associated with Caribbean *Acropora* spp. was given the provisional name *Symbiodinium ‘fittii’* (Pinzón et al., 2011; Baums et al., 2014), pending formal description (in...
progress). These genetic data also indicate that type $A_3$ from the Greater Caribbean probably comprises several species (Pinzón, 2011); and the product of a minor adaptive radiation of this lineage in the Atlantic Ocean following its separation from the Pacific 3-4 MYA (Thornhill et al., 2014).

All the genetic variants attributed to $S.$ *tridacnidorum* (i.e. $A_3, A_{3a}, A_{3*}$ and $A_6$) have been isolated and maintained in stable culture at one time or another (Fig. 36). However, the lineages diagnosed as $A_3$ from the Greater Caribbean have yet to be successfully cultured despite numerous attempts (M. A. Coffroth, SUNY Buffalo, pers. comm.). This hints at the fundamental differences in physiology between Pacific and Atlantic members of the ‘$A_3$’ lineage. *Symbiodinium tridacnidorum* lives at high densities in the tubular system of digestive diverticula that ramify the mantle tissues of giant clams (Norton et al., 1992). Perhaps this capacity for extracellular existence explains why it is readily cultured in artificial seawater media (Carlos et al., 1999; Baillie et al., 2000; LaJeunesse, 2001).

On several occasions, strains of *S. microadriaticum* have proliferated opportunistically during failed attempts to culture the normal symbiont found in a particular host coral (e.g. *Orbicella faveolata* from the Florida Keys and *Stylophora* sp. from the Gulf of Aqaba in the Red Sea; Figs 36, 37). Therefore *S. microadriaticum* must exist at low background concentrations in the environment, and yet are only known to occur in high densities from field-collected samples of *Cassiopea xamachana* in the Greater Caribbean (Figs 36, 37, 38; LaJeunesse, 2002). This particular species of upside-down jelly appears to have been introduced to shallow tropical marine environments around the world, but there are no data to indicate from where it originated (Holland et al., 2004). Future genetic analyses of *C. xamachana* specimens collected outside the Atlantic may show that *S. microadriaticum* is found in *C. xamachana* everywhere this host occurs.

Finally, the chromosome numbers counted for *S. tridacnidorum* ($n = 80 \pm 3$) and *S. microadriaticum* ($97 \pm 2$; Table 2; Trench & Blank, 1987) differ to an extent that would make them reproductively incompatible as eukaryotes. The chromosome values for *S. pilosum* ($78 \pm 2$) are similar to *S. tridacnidorum*, but significant sequence divergence at several DNA loci indicates that they are distantly related (Figs 36–39). Additional analysis of the chromosome number from other yet undescribed species in Clade A may offer insights into genome evolution among the closely and distantly related species that comprise this group.

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**SUPPLEMENTARY INFORMATION**

The following supplementary material is accessible via the Supplementary Content tab on the article’s online page at http://dx.doi.org/10.1080/09670262.2015.1018336

**Supplementary table S1.** List of sample ID, type, host, geographic origin, and minimum (Min) and maximum (Max) depths from which isolates were collected.

**Supplementary figs S1–S3.** Light micrographs of the mastigote (motile), coccoïd (spherical) and doublet (dividing) cells from several cultured isolates of *Symbiodinium tridacnidorum* (rt-272) sp. nov. strain. **S1.** *S. tridacnidorum* mastigote. **S2.** *S. tridacnidorum* coccoïd. **S3.** *S. tridacnidorum* doublet. All scale bars = 2 μm.

**Supplementary figs S4–S11.** Scanning electron micrographs of motile cells of *Symbiodinium tridacnidorum* (strain rt-272). **S4.** Ventral view showing the episphere, cingulum (C), sulcus (s), peduncle (PE) and hyposome. **S5.** Ventral-left lateral view showing the episphere, cingulum (C), and hyposome. **S6.** Dorsal view showing the episphere, cingulum (C) and hyposome. **S7.** Ventral-right lateral view showing the episphere, cingulum (C), sulcus (s) and hyposome. **S8.** Apical view showing the episphere and elongated amphiasomal vesicle (EAV) plate. **S9.** Apical view showing the EAV plate with small knobs (arrowhead). **S10.** Antapical view showing the hyposome. **S11.** Antapical-ventral view showing the sulcus (s) and peduncle (PE). All scale bars = 1 μm.

**Supplementary figs S12–S13.** Drawings and micrographs of the antapical plate of *Symbiodinium tridacnidorum* taken using scanning electron microscopy. **S12.** Drawings of the antapical view showing 2 antapical plates. **S13.** Micrographs of the antapical view showing 2 antapical plates. All scale bars = 1 μm.

**AUTHOR CONTRIBUTIONS**

SY Lee, HJ Jeong, TC LaJeunesse: original concept, drafting and editing manuscript; SY Lee, HJ Jeong, TY Jang: culture experiments; SY Lee, NS Kang, SH Jang:
A Species of Giant Clam Symbiont

electron microscopy; SY Lee, SH Jang, TC LaJeunesse: analysis of molecular data.

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