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Brandtodinium gen. nov. and B. *nutricula* comb. Nov. (Dinophyceae), a dinoflagellate commonly found in symbiosis with polycystine radiolarians

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Abstract :

Symbiotic interactions between pelagic hosts and microalgae have received little attention, although they are widespread in the photic layer of the world ocean, where they play a fundamental role in the ecology of the planktonic ecosystem. Polycystine radiolarians (including the orders Spumellaria, Collodaria and Nassellaria) are planktonic heterotrophic protists that are widely distributed and often abundant in the ocean. Many polycystines host symbiotic microalgae within their cytoplasm, mostly thought to be the dinoflagellate Scrippsiella nutricula, a species originally described by Karl Brandt in the late nineteenth century as Zooxanthella nutricula. The free-living stage of this dinoflagellate has never been characterized in terms of morphology and thecal plate tabulation. We examined morphological characters and sequenced conservative ribosomal markers of clonal cultures of the free-living stage of symbiotic dinoflagellates isolated from radiolarian hosts from the three polycystine orders. In addition, we sequenced symbiont genes directly from several polycystine-symbiont holobiont specimens from different oceanic regions. Thecal plate arrangement of the free-living stage does not match that of Scrippsiella or related genera, and LSU and SSU rDNA-based molecular phylogenies place these symbionts in a distinct clade within the Peridiniales. Both phylogenetic analyses and the comparison of morphological features of culture strains with those reported for other closely related species support the erection of a new genus that we name Brandtodinium gen. nov. and the recombination of S. nutricula as B. nutricula comb. nov.

Keywords : symbiosis, taxonomy, Scrippsiella, Peridiniales, Radiolaria, polycystines, dinoflagellate, Zooxanthella

Abbreviations

ICBN	International Code for Botanical Nomenclature
ITS	internal transcribed spacer
LM	light microscopy
LSU	large subunit (ribosomal DNA)
ML	maximum likelihood
PCR	polymerase chain reaction
RCC	roscoff culture collection
SEM	scanning electron microscopy
SSU	small subunit (ribosomal DNA)

Introduction

Mutualistic associations involving photosynthetic microalgae are common in both benthic and pelagic ecosystems and are essential for establishing and maintaining the structure of marine communities (Caron 2000). Symbiosis between corals and the dinoflagellate genus *Symbiodinium* Freudenthal is fundamental for the survival and ecological success of coral reef ecosystems. Members of the genus *Symbiodinium* have been intensively studied with respect to their morphology and life cycle (Freudenthal 1962; Fitt and Trench 1983; Trench and Blank 1987), and genetic diversity (Coffroth and Santos 2005; Sampayo et al. 2009; LaJeunesse and Thornhill 2011; Stat et al. 2011). Studies on this coastal benthic symbiotic relationship significantly increased when the coral-bleaching phenomenon was brought to global attention and associated to increases in sea surface temperature, enhanced light intensity, and ocean acidification (Hoegh-Guldberg et al. 2007).

Symbiotic interactions between pelagic hosts and microalgae have received less attention, despite the fact that they are widespread in the photic layer of the world ocean where they play a fundamental role in the ecology of the planktonic ecosystem (Stoecker et al. 2009; Decelle et al. 2012). Recent studies have demonstrated that dinoflagellate symbionts of Foraminifera belong to *Pelagodinium* Siano, Montresor, Probert et de Vargas, a genus that is related to *Symbiodinium* within the order Suessiales (Siano et al. 2010), and that Acantharia typically associate with members of the prymnesiophyte genus *Phaeocyst~~s~~is* Lagerheim (Decelle et al. 2012), although one taxon, *Acanthochiasma* sp., can contain multiple symbiotic partners, including distantly related dinoflagellates (from the genera *Pelagodinium*, *Heterocapsa* Stein, *Azadinium* Elbrächter et Tillmann and *Scrippsiella* Balech ex Loeblich III) as well as a haptophyte (Decelle et al. 2012b).

Polycystine radiolarians (including the orders Spumellaria, Collodaria and Nassellaria) are single-celled, heterotrophic, biomineralizing planktonic protists from the Rhizaria lineage that are widely distributed in the ocean and are found throughout the entire water column (Boltovskoy et al. 2010). Many polycystines host microalgae within their cytoplasm (Anderson 1983). Cells containing photosynthetic microalgae have been shown to survive for longer periods in nutrient-poor water than those that do not have microalgal partners and the microalgae are therefore assumed to be symbionts that play a nutritive role for the hosts (Anderson 1983).

Polycystines form associations with various dinoflagellate, prymnesiophyte and prasinophyte partners (usually not at the same time), with dinoflagellates being the most common symbiotic partners (Anderson 1976, 1983; Anderson et al. 1983). In the late nineteenth century, Karl Brandt was the first to recognize that the "yellow cells" within polycystines, actinian corals and hydrozoans were microalgae, which he collectively described in the new genus *Zooxanthella* Brandt (Brandt 1881), although they were not immediately recognized as dinoflagellates. Soon afterwards, the species *Z. nutricula* Brandt was proposed for the symbiont of the collodarian polycystine *Collozoum inerme* collected from the western Mediterranean Sea and it was stated in the description that this species was presumably identical to the yellow cells of other polycystines (Brandt 1882). The subsequent taxonomic history of this genus and species have been very confused (see review by Blank ~~&~~ and Trench 1986), and the plural noun 'zooxanthellae' has persisted as a colloquialism used to describe marine microalgal endosymbionts in general.

The symbionts of the 'by-the-wind sailor' hydrozoan jellyfish *Velella velella* were reported to be similar to those of polycystines ~~initially~~ by Hovasse (1922), who initially described the *in hospite* symbionts of Mediterranean *V. velella* as *Endodinium* *chattoni* Hovasse (*E. chattonii* under ICBN Art. 73). Taylor (1971) and Hollande and Carré (1974) further characterized the *in hospite* stage of *E. chattonii* and the latter authors proposed the reclassification of the polycystine symbionts (*Z. nutricula*) as *E. nutricola* (Brandt) Hollande et Carré (*E. nutricula* under ICBN Art. 73), despite the fact that Hovasse (1924) had in fact previously recombined *E. chattonii* as *Z. chattonii* (Hovasse) Hovasse. Banaszak et al. (1993) isolated a culture of the symbiont of *V. velella* from the Pacific, which they considered slightly different from *E. chattonii* (larger cell size and presence of trichocysts *in hospite* and in culture). Based on SEM observations of the morphology and arrangement of thecal plates in the motile stage, Banaszak et al. (1993) classified their organism in the genus *Scrippsiella* as a new species, *S. velellae* Banaszak, Iglesias-Prieto et Trench (a name later validated by Trench 2000). These authors also transferred *E. chattonii* and *E. nutricula* to *Scrippsiella* as *S. chattonii* (Hovasse) Banaszak, Iglesias-Prieto et Trench and *S. nutricula* (Brandt) Banaszak, Iglesias-Prieto et Trench, respectively (Banaszak et al. 1993), but these names remain technically invalid because reference was not made to the exact page of the basionym.

Using molecular methods, Gast and Caron (1996) found that the dinoflagellate symbionts in six different polycystine species from the Sargasso Sea (the collodarians *Collozoum caudatum* and *Thalassicolla nucleata*, three unidentified collodarian species and the spumellarian *Spongostaurus* sp.) had identical SSU rDNA sequences that they assigned to *Scrippsiella nutricula*. These molecular analyses indicate that taxonomically divergent radiolarians can contain the same symbiotic dinoflagellate. Since these analyses were conducted directly on symbionts extracted from the hosts (i.e., not cultured), the morphology of the motile stage of the symbiotic algae assigned to *S. nutricula* was not investigated, and has still never been reported. Gast and Caron

(1996) also sequenced the SSU rDNA of the symbiont of *V. velella* from the Sargasso Sea and found that the sequence was very similar to those of the radiolarian symbionts (4 differences out of 1802 base pairs). They therefore also assigned this *V. velella* symbiont to *S. nutricula*.

Here we examined the morphology and molecular phylogenetic position of clonal cultures of the free-living stage of dinoflagellates isolated from several different polycystine radiolarian hosts, including *Collozoum*, the taxon from which *Zooxanthella nutricula* was originally described. In addition, we sequenced symbiont genes directly from several polycystine-symbiont holobiont specimens (including collodarian, spumellarian and nassellarian hosts) from different oceanic regions.

Accurate morpho-molecular characterization and taxonomic designation of symbionts from the genus *Symbiodinium* has been key for studies of the ecology and functioning of coral reef systems and ~~is~~ it is likewise likely to prove important for future studies on the widespread pelagic symbiosis involving polycystine radiolarian hosts.

Material and Methods

Samples and culture isolation

The radiolarian specimens from which the holobiont sequences or cultures originated were isolated from samples collected in 2010-2012 by net tows (20 to 150 micron mesh size) in the bay of Villefranche-sur-Mer (France), off Sesoko Island, Okinawa (Japan) and in the South Pacific Ocean during the Tara Oceans expedition (Table 1, Supplementary Figs 1 and 2). The polycystines were first sorted from fresh net samples under a binocular microscope, cleaned by successive transfers in sterile seawater in Petri dishes, then left in an illuminated and temperature-regulated incubator for several hours to self-clean. Individual clean specimens were then identified based on their morphology and imaged under an inverted microscope. Some specimens were then transferred to guanidinium isothiocyanate (GITC) buffer for direct DNA extraction from holobionts. The dinoflagellate cultures were obtained by micropipette isolation of single symbiont cells released from live radiolarian specimens that were microdissected under an inverted microscope. The resulting monoclonal cultures were maintained in filter-sterilized seawater with K/2(-Tris, -Si) medium supplements (Keller et al. 1987) at 22°C with an irradiance of 70–80 µmol photons $m^{-2}s^{-1}$ in a 12:12 light:dark regime. The cultures have been deposited in the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/RCC). LM images of radiolarian holobionts from which sequences / cultures were obtained are shown in Supplementary Figures 1 and 2. Detailed information related to each of the samples used in this study can be found in the RENKAN database at http://abims.sb-roscoff.fr/renkan/.

Microscopy preparations and observations

Light micrographs of living cells were taken using a Zeiss Axiophot light microscope equipped with a Zeiss AxioCam digital camera system (Carl Zeiss, Oberkochen, Germany). For scanning electron microscopy (SEM), dinoflagellate cells were fixed in 1% (v:v) formol for 2 hours at room temperature. Samples were then gently filtered onto 3µm pore-size Nucleopore polycarbonate filters (Pleasanton, CA, USA), washed with distilled water, dehydrated in an ethanol series (25%, 50%, 75%, 95%, 100%), and critical point dried. The filters were mounted on stubs, sputter coated with gold, and examined with a FEI Quanta™ 200 SEM (FEI, Hillsboro, Oregon, USA)

DNA extraction, sequencing and phylogenetic analyses

Genomic DNA was extracted from exponentially growing cultures of the strains using a NucleoSpin Plant II DNA extraction kit (Macherey-Nagel), or from holobionts using the method described in De Vargas et al. (2002).

Partial nuclear LSU and SSU rDNA genes were PCR amplified using Phusion high-fidelity DNA polymerase (Finnzymes) in a 25 µl reaction volume and the following thermocycler steps : an initial denaturation step at 98°C for 30 sec, followed by 35 cycles at 98°C for 10 sec, 30 sec at the temperature of semi-hybridization chosen for each set of primers, and 30 sec at 72°C, with a final elongation step of 10 min at 72°C. The eukaryote primer set 63F (ACGCTT GTCTCAAAGATT) / 1818R (ACGGAAACCTTGTTACGA) (Tm 50°C) (Lepere et al. 2011) was used to amplify the SSU rDNA of the dinoflagellate cultures, whereas the dinoflagellate specific primer set DIN464F (TAACAATACAGGGCATCCAT) / S69 (CCGTCADTTCCTTTRAGDTT) (Tm 53°C) was used to target the dinoflagellates in the holobiont samples. The D1-D2 fragment of the LSU rDNA was amplified using the dinoflagellate specific primers Ldino6 (MCC CGCTGAATTTAAGCATA) / Ldino1 (AACGATTTGCAGGTCAGTACCGC) (Tm 55°C) from both cultures and holobionts. PCR products were then sequenced at the GENOSCOPE (CEA, Evry, France).

The sequences generated from the studied strains and holobionts (GenBank accession numbers~~: XXXX~~ KF557491 to KF557545~~ to XXXX~~) were aligned with other LSU and SSU rDNA sequences from GenBank (release 194.0, February 2013) attributed to *Scrippsiella* and related Peridiniales genera, as well as representatives of the Suessiales as an outgroup. Alignments were generated using MUSCLE implemented in Seaview v.4.0 (Gouy et al. 2010) with subsequent manual verification. The LSU rDNA data set contained 48 sequences (675 unambiguously aligned positions) and the SSU rDNA data set contained 57 sequences (652 unambiguously aligned positions). Phylogenetic analyses were conducted with Maximum Likelihood (ML) and Bayesian methods. The ML analysis was carried out using MEGA v. 5.1 (Tamura et al. 2011) with the General Time Reversible (GTR) as the best model of nucleotide substitution and considering a gamma distribution with a proportion of invariable sites (I) set at 5 by default. Bootstrap supports for the tree were obtained after 1000 replicates. ~~The tree was visualized and edited in Fig Tree v 1.3.1 (Rambaut 2010).~~ Bayesian analyses were conducted using Mr Bayes v.3.2.1 (Huelsenbeck and Ronquist 2001) using the same model of evolution. For each gene marker, two Markov Chain Monte Carlo (MCMC) chains were run for 1 million generations, sampling every 500 generations (diagnostic frequency = 5000). The standard deviation of split frequencies between the 2 runs was <0.01 in both LSU and SSU rDNA analyses. ~~T~~For both ML and Bayesian analyses, the trees w~~as~~ere visualized and edited in Fig Tree v 1.3.1 (Rambaut 2010). ~~The~~ In the trees presented herein the posterior probabilities (PP) associated to each node in the Bayesian topologies ~~were~~ are reported on the ML topologies.

Results

Microscopy Observations

In our culture conditions, ~~our~~ the clonal strains of polycystine symbionts tended to contain a mixture of motile thecate cells and larger, irregularly-shaped non-motile cells devoid of the typical features of motile cells (theca, cingulum, sulcus), the latter more closely resembling the *in hospite* symbiotic state. The proportion of motile and non-motile cells varied between strains and through growth cycles for each strain. The overall morphology and thecal plate pattern of motile cells was identical for several different strains observed. The following descriptions and illustrations are based on observations of strain ~~VFR1-1~~RCC3387.

Cells are 10.5 to 15 µm in length (average 13.1 µm, n=30) and 9.1 to 11.2 µm in width (average 10.4 µm, n=30). The epitheca is larger than the hypotheca. Observed under LM, cells have a slightly convex conical epitheca with a well-pronounced apical horn (Fig. 1A, 1B, 1D). The hypotheca is rounded (Fig. 1A, 1D). The nucleus is large and occupies the center of the cells (Fig. 1B, 1D). One or two golden-yellow chloroplasts are present around the cell periphery, sometimes appearing as a single plastid bordering the cell periphery (Fig. 1D). One large circular pyrenoid (sometimes two) is often visible in LM (Fig. 1A-D). No eyespot is visible in light microscopy. Cells swim steadily in a straight line, rotating around the transapical axis. They suddenly stop, change direction at different angles from the original path, often back-tracking.

In SEM, the epitheca appears conical (Fig. 2A) to rounded (Fig. 2C), and the smaller hypotheca is symmetrical and rounded in ventral (Fig. 2A) and dorsal (Fig. 2C) view. The plate tabulation is Po, X, 4', 3a, 7'', 5C, 4S, 5''', 1'''' (Figs 2A-E, 3A-D). The pore plate (Po) is circular and surrounded by a high collar and is connected to the first apical plate by a long well-defined rectangular canal plate (X) (Figs 2A, 3A, 3C). Three intercalary plates are interposed on the dorsal side of the cell between the apical series and the second epithecal (precingular) series (Figs 2C-D, 3B-C). The first intercalary plate (1a) is five-sided and borders only one of the apical plates (2’), whereas the second and third intercalary plates (2a and 3a) are six-sided and both border two apical plates (Figs 2C-D, 3C). The cingulum is located in the median portion of the cell and descends slightly, displaced by approximately one third of its own width (Figs 2A, 2C, 3A-B). It is very wide and shallow and is constituted by a single series of five rectangular plates, the first being much narrower than the others (Fig. 2A-C, 2E, 3A-B). The sulcus is fairly shallow and narrows towards the antapical end (Fig. 2A-B). The sulcal area comprises four plates (Fig. 2B, 3A). One of these (Sd) forms a conspicuous flange extending over the median area of the sulcus, partially covering the sulcal area (Fig. 2B). There appears to be a single plate (Ss) beneath this flange (Fig. 2B). Flagella were not preserved in our SEM preparations. In the hypotheca, a series of 5 trapezoid plates of similar size borders the cingulum. A single six-sided antapical plate completes the hypothecal tabulation (Fig. 2E, 3D). The cell surface is mostly smooth. We have never observed a peduncule in either LM or SEM preparations.

Phylogenetic Analyses

PCR amplifications of DNA extracts from culture strains and uncultured holobionts led to generation of 35 partial SSU rDNA (~650 bp) and 22 partial LSU rDNA (~675 bp) sequences of dinoflagellate symbionts from spumellarian, collodarian and nassellarian hosts collected in the Mediterranean Sea and in the North and South Pacific oceans (Table 1). For each gene the vast majority of these sequences were identical (see below) and hence only a subset of 15 SSU rDNA and 10 LSU rDNA sequences, representing a cross-section of host diversity, were included in datasets for phylogenetic reconstructions. Phylogenetic analyses on the SSU and LSU rDNA datasets demonstrated that all of our sequences grouped together in a distinct and highly supported clade (hereafter called clade B) within the dinoflagellate order Peridiniales (full ML and Bayesian statistical support; Figs. 4 and 5). In both SSU and LSU rDNA phylogenies, this clade included two distinct sub-clades, B1 and B2, each containing sequences that are 100% identical irrespective of host taxon and oceanic region. In our SSU rDNA phylogenetic tree (Fig. 4), sub-clade B1 included the majority of symbiont sequences recovered in this study (including those from five culture strains isolated from *Collozoum* colonies from the Mediterranean Sea and Pacific Ocean), as well as published sequences that correspond to the symbionts of five collodarians and one spumellarian collected in the Atlantic Ocean (Gast and Caron 1996). Sub-clade B2 contained the sequences generated in the present study of the symbionts of two collodarian holobionts as well as one published sequence (U52357) of the symbiont of the jellyfish *Velella velella* (Gast and Caron 1996). In both phylogenetic reconstructions, the monophyletic clade B containing the sequences of polycystine symbionts was phylogenetically distinct from the well-supported clade containing members of the genus *Scrippsiella* (including the holotype species *S. sweeneyae* Loeblich III), but overall the phylogenetic relationships between clades within the Peridiniales were not clearly resolved in our analyses. When sequences of members of the genus *Bysmatrum*, which have a plate tabulation pattern similar to *Scrippsiella*-like peridinaleans (Table 2), were included in phylogenetic analyses, they formed a distinct mono-generic clade which fell on a long branch that altered overall tree topology (Supplementary Figure 3). In the SSU rDNA phylogeny (Figure 4), note that the sequence labeled "uncultured alveolate from Nasselaria" (DQ916409) and the two sequences labeled "Dinophyceae from Collodaria" (DQ116021 and DQ116022) correspond to non-photosynthetic dinoflagellate parasites of Radiolaria (Gast 2006).

Discussion

Dinoflagellates that form symbiotic relationships with metazoan or protistan hosts are characterized by complex life cycles, with an alternation of symbiotic and free-living stages with considerable morphological and physiological differentiation between them. Within the host cells, the symbionts are typically coccoid without flagella, and the cingulum and sulcus are no longer apparent (Trench and Blank 1987). In the free-living stage, cells tend to regain their original morphology (Freudenthal 1962; Spero 1987, Siano et al. 2010). Since the taxonomy of dinoflagellates is largely based on comparison of the number, shape and arrangement of the thecal plates (or amphiesmal vesicles in athecate species) that form the periplast of free-living motile cells, the establishment of clonal cultures from symbionts extracted from their hosts is critical for accurate taxonomic assignation.

The genus *Zooxanthella* was originally created to collectively describe the symbionts of diverse hosts from the Mediterranean Sea, including polycystines, corals and hydrozoans (Brandt 1881) and *Z. nutricula* was created to describe the symbionts of the collodarian polycystine *Collozoum inerme* (Brandt 1882). The taxonomic history of *Zooxanthella* has subsequently been confusing, with *Z. nutricula* being alternately combined within *Endodinium*, *Amphidinium* Claperède et Lachmann (see review of the nomenclatural history of endosymbiotic dinoflagellates by Blank and Trench, 1986) and most recently (albeit technically invalidly) within *Scrippsiella* (Banaszak et al. 1993).

Our observations of the plate tabulation pattern of cultured motile cells of the free-living stage of the dinoflagellate isolated from diverse polycystine hosts clearly show that it is a member of the order Peridiniales (bilateral symmetry, cingulum only slightly displaced, presence of Po and X plates, presence of 3 intercalary plates in the epitheca) and that it should not be classified in the genus *Scrippsiella*, nor in the related genera *Calciodinellum*, *Bysmatrum*, *Pentapharsodinium,* or *Ensiculifera*. All of these latter genera are described as possessing 2 antapical plates, whereas the polycystine symbiont reported here possesses a single antapical plate (Table 2, Figs 2E and 3D). The presence of a single antapical plate is rare in the order Peridiniales, occurring notably in a group of heterotrophic genera (*Podolampas* Stein, *Blepharocysta* Ehrenberg, and *Lissodinium* Matzenauer) characterized by the absence of both a cingulum and a depressed sulcus (Gómez et al. 2010) and a group of heterotrophic taxa (*Diplopsalis* Bergh, *Preperidinium* Mangin, *Boreadinium* Dodge et Hermes) characterized by having large lenticular-shaped cells. The radiolarian symbionts are clearly morphologically and ecologically distinct from these other peridinialeans ~~with~~ that have a single antapical plate.

The polycystine symbionts also differ from *Scrippsiella* and *Bysmatrum* (but not from *Pentapharsodinium* and *Ensiculifera*) in possessing 5 (rather than 6) cingular plates. The wing-like flange that covers the sulcal area has not been described in any of these related genera. This structure resembles the peduncule cover plate (PC) of heterotrophic dinoflagellates in the peridinialean family Pfiestereaceae Steidinger et Burkholder emend. Litaker. Motile forms of members of the Pfiestereaceae feed myzocytotically by means of a peduncule that emerges close to the flagella and that can attach to microalgal prey or epidermal cells of live fish (e.g. Steidinger et al. 2006). We have not observed a peduncle in the taxon described here, but should it be present, the Sd plate should rather be termed PC and the plate formula would become: Po, X, 4’, 3a, 7’’, 5c, ~~,~~ 3s, PC, 5’’’, 1’’’’.

Comparison of morphological characters strongly supports a generic level separation of the polycystine symbiont reported here from other described Peridiniales taxa, a conclusion that is corroborated by phylogenetic analyses. In both SSU and LSU phylogenies (Figs 4 and 5), the analyzed polycystine symbionts (including several cultures isolated from *Collozoum* colonies) formed a well-supported clade within the

Peridiniales, clearly distinct from *Scrippsiella* and related genera and distant from other dinoflagellate taxa known to form symbiotic relationships such as the suessialeans *Symbiodinium* and *Pelagodinium*.

In light of both morphological and genetic differences from existing genera, this taxon should clearly be classified in a distinct genus. Although *S. nutricula* was previously classified within the genus *Endodinium*, this genus was created to describe the symbiont of *Velella velella* from the Mediterranean and there is sufficient doubt as to whether these organisms are actually closely related (see below) to preclude reinstatement of this combination, which in any case should be considered synonymous with *Z. nutricula*. Strict adherence to nomenclatural rules would hence dictate the use of the genus *Zooxanthella* for this species, but we agree with numerous previous authors (e.g. Blank and Trench 1986; Trench and Blank 1987; Banaszak et al. 1993) who have convincingly argued that *Zooxanthella* should be rejected as a confusing name that has been widely applied to divergent taxa. We therefore propose the erection of a new genus, which we name *Brandtodinium* Probert et Siano in reference to Karl Brandt who first described this species (Brandt 1882), and the transfer of *Z. nutricula* to this new genus as *Brandtodinium nutriculum* comb. nov.. In the absence of a holotype, not provided in the original description of the species, we designate Fig. 2, SEM illustrations of plate tabulation of the motile stage of the culture strain ~~VFR1-1~~RCC3387 of this species, as the neotype for the species.

Whereas the generic level distinction of *Brandtodinium* from other peridinialeans is obvious, the relationship of this genus to other genera within the Peridiniales is not clear. In terms of overall morphology of the motile stage (e.g. cell size and shape, plate tabulation), *Brandtodinium* has several features in common with members of the Calciodinellaceae Taylor, a family that includes *Scrippsiella*. The Calciodinellaceae, however, are characterized by the production of calcified resting cysts, a feature that we have not observed in *Brandtodinium*. As discussed above, *Brandtodinium* also has certain morphological similarities with members of other groups such as the Pfiestereaceae. An unexpectedly close genetic relationship between *B. nutriculum* (as *Z. nutricula*) and a small group of taxa in which photosynthesis takes place by a tertiary endosymbiont derived from a diatom (Horiguchi and Pienaar 1994), the 'dinotoms' (Imanian et al. 2011), was recently reported (Gottschling and McLean 2013). These investigators employed a 'maximal taxon sample' approach by inferring relationships based on a concatenated SSU, LSU and ITS rDNA sequence alignment irrespective of whether all of these sequences were available for the taxa included (i.e. an alignment with significant gaps). Our individual SSU and LSU phylogenies do not recover this relationship. The present study provides strong evidence from two highly conserved phylogenetic markers (SSU and LSU rDNA) to support the conclusion from our observations of the morphology of free-living cells that *Brandtodinium* is a taxonomically distinct genus within the Peridiniales. We chose not to employ an approach comparable to that of Gottschling and McLean (2013) because in-depth assessment of evolutionary and phylogenetic relationships between *Brandtodinium* and other members of the order Peridiniales goes beyond the scope of our research. We nevertheless provide evidence that *Brandtodinium* is distinct from the dinotom genera (*Durinskia* Carty et Cox, *Galeidinium* Tamura et Horiguchi, *Kryptoperidinium* Lindemann, and some species currently assigned to *Peridiniopsis* Lemmermann or *Peridinium* Ehrenberg) on the basis of morphological criteria, notably because dinotom genera all have 2 antapical plates whereas *B. nutriculum* possesses a single antapical plate, but also because the characteristic highly visible eyespot of dinotoms is absent in *B. nutriculum*.

Banaszak et al. (1993) described the dinoflagellate symbiont of the jellyfish *Velella velella* from the Pacific as *Scrippsiella velellae* and also (albeit invalidly) transferred *Endodinium (=Zooxanthella*) *chattonii*, the symbiont of Mediterranean *V. velella*, to *Scrippsiella*, as *S. chattonii*. These authors gave the thecal plate formula for *S. velellae* as pp (=Po, X), 4', 3a, 7'', 5c, 3s, 5''', 2'''', which corresponds neither to that of *Scrippsiella* nor to that of *Brandtodinium* (Table 2). The spine-like protuberance on the first cingular plate illustrated in Figure 11 (page 520) of Banaszak et al. (1993) is a characteristic feature of the genus *Ensiculifera*, to which we believe this species should have been assigned. However, the SEM images illustrated in Banaszak et al. (1993) do not permit verification of whether this organism really has 3 sulcal plates (as stated in the description), rather than 5, as diagnostic for members of the genus *Ensiculifera*. It could also be inferred that *S. chattonii*, the symbiont of Mediterranean *V. velella*, might also be transferred to *Ensiculifera*, but unfortunately no morphological data has ever been provided for the free-living stage of this taxon. It is noteworthy that the only existing sequence (SSU rDNA) of a symbiont of *V. velella* (from the Sargasso Sea, Atlantic Ocean) produced by Gast and Caron (1996) falls within our *Brandtodinium* clade, in the sub-clade B2 composed of three identical sequences, two of which we generated from Pacific polycystine holobionts. This sub-clade is distinct from the sub-clade B1 formed by the group of identical sequences from all of our Pacific (South and North) and Mediterranean culture strains of *B. nutriculum* isolated from polycystines, from several Pacific polycystine holobionts that we sequenced, and from the Sargasso Sea polycystine symbionts sequenced by Gast and Caron (1996). Gast and Caron (1996) did not observe the morphology of the dinoflagellate symbionts of Sargasso Sea *V. velella* that they sequenced, but we predict that they would have plate tabulation consistent with our description of *Brandtodinium*. If this were the case, it would mean that *V. velella* is capable of forming symbiotic associations with different dinoflagellate genera (*Brandtodinium* and *Scrippsiella* (or *Ensiculifera*)), possibly with a biogeographical pattern (*Brandtodinium* in the Atlantic and possibly Mediterranean, *Scrippsiella* (or *Ensiculifera*) in the Pacific). The capacity of hosts to form associations with different symbionts has already been observed for other pelagic organisms (Siano et al. 2010; Decelle et al. 2012b). A comparison of genetic sequences from morphologically characterized cultured *V. velella* symbionts from the Pacific Ocean, Sargasso Sea and Mediterranean Sea could be helpful in establishing the validity of historical descriptions of these symbionts and their relationship to *B. nutriculum*.

Brandtodinium has been found (in this and previous studies) in association with diverse polycystine radiolarian hosts from the North and South Pacific Ocean, Sargasso Sea, and Mediterranean Sea. In light of the abundance of symbiotic polycystines in the world ocean, *Brandtodinium* likely plays a key ecological role in primary and secondary production at a global scale. Putting aside associations with parasitic alveolates (Gast 2006; Bråte et al. 2012~~1~~) that can be considered as a form of symbiosis, all Collodaria investigated so far harbor only *Brandtodinium* species as symbionts. At present, *Brandtodinium* is the only symbiont identified for Nassellaria, but information for this radiolarian group remains extremely scarce. *Brandtodinium* has now been found in association with numerous spumellarian hosts, but unlike the other polycystine lineages, other types of (non-dinoflagellate) microalgal and cyanobacterial symbionts have also been reported for this group (Anderson 1983; Gast and Caron 2001; Yuasa et al. 2005). With *Brandtodinium* also probably found in symbiosis with jellyfish, it is clear that *Brandtodinium*, like the suessialean dinoflagellates *Pelagodinium* and *Symbiodinium*, is a generalist symbiont. In this context it is interesting to note that the known genetic diversity (in terms of SSU and LSU rDNA sequences) of *Brandtodinium* and *Pelagodinium*, both of which form symbiotic relationships with planktonic hosts, is relatively low (2 clades described within each of these genera) compared to that of *Symbiodinium* (9 divergent clades and multiple sub-clades, Stat et al. 2008; Pochon and Gates 2010) that is predominately found in association with benthic host organisms. This apparent trend might be explained by the relatively low number of studies on symbiosis in the pelagic realm, but might also be real and reflect inherent differences between life and symbiotic processes in planktonic and benthic ecosystems (Decelle 2013).

Taxonomic appendix

Brandtodinium Probert et Siano *gen. nov.*

Diagnosis: Photosynthetic dinoflagellate. Motile cells covered by 6 series of thecal plates: 3 in the epitheca, 2 in the hypotheca (including single antapical plate), and 1 in the cingulum. One transverse and one longitudinal flagellum. Large nucleus located in central part of cell. One or two peripheral chloroplasts, golden-yellow in color. One or two large circular pyrenoids.

Type species: *Brandtodinium nutriculum* (Brandt) Probert et Siano *comb. nov.*

Etymology: the genus name for this dinoflagellate (= *dinos*) derives from Karl Brandt who first described *Zooxanthella* in 1882.

Brandtodinium nutriculum (Brandt) Probert et Siano *comb. nov.*

Basionym: Zooxanthella *nutricula* Brandt in Brandt (1882): 140

Synonyms: *Endodinium nutricula* (Brandt) Hollande et Carré in Holland and Carré (1974); *Scrippsiella nutricula* (Brandt) Banaszak, Iglesias-Prieto et Trench in Banaszak et al. (1993).

Neotype : Fig. 2 in this publication.

Diagnosis: Plate tabulation: Po, X, 4’, 3a, 7’’, 5c, 4s, 5’’’, 1’’’’. Epitheca larger than hypotheca. Epitheca convex conical with well-pronounced apical horn. Hypotheca rounded. Wide and shallow cingulum located in the median portion of the cell, displaced by a small fraction of its own width. Sulcal area with 4 plates, one of which forms a wing-like flange over the median part of the sulcus. Single antapical plate. Cells on average 13.1µm in length by 10.4µm in width. Symbiont of polycystine radiolarians.

Type locality: Bay of Villefranche sur Mer (France), Western Mediterranean Sea

Authentic culture strain: RCC3387 in the Roscoff Culture Collection.

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Table 1. List of specimens used to obtain symbiont sequences (images of host cells are shown in Supplementary Figures 1 and 2).

Host ID	**Host taxonomy**	**Sampling site**	**Strain code**	**~~18S~~ SSU rDNA GenBank ~~acc~~ #accession number**	**~~28S~~ LSU rDNA GenBank ~~acc~~ #accession number**
Holobionts					
PAC1	Collodaria (solitary)	South Pacific 21°17.462 S, 105°9.476 W	n.a.	~~xxxxxxx~~KF557503	KF557534~~xxxxxxx~~
PAC2	Collodaria (colony)	South Pacific 21°17.462 S, 105°9.476 W	n.a.	KF557504~~xxxxxxx~~	n.a.
PAC3	Collodaria (solitary)	South Pacific 23°42.949 S, 107°20.141 W	n.a.	KF557505~~xxxxxxx~~	KF557535~~xxxxxxx~~
PAC4	Collodaria (solitary)	South Pacific 23°42.949 S, 107°20.141 W	n.a.	KF557506~~xxxxxxx~~	KF557536~~xxxxxxx~~
PAC6	Collodaria (solitary)	South Pacific 24°48.085 S, 110°33.307 W	n.a.	KF557507~~xxxxxxx~~	n.a.
PAC7	Collodaria (colony)	South Pacific 24°48.085 S, 110°33.307 W	n.a.	KF557508~~xxxxxxx~~	n.a.
PAC8	Collodaria (colony)	South Pacific 24°48.085 S, 110°33.307 W	n.a.	n.a.	KF557537~~xxxxxxx~~
PAC 9	Collodaria (colony)	South Pacific 24°48.085 S, 110°33.307 W	n.a.	KF557509~~xxxxxxx~~	KF557538~~xxxxxxx~~
PAC10	Collodaria (solitary)	South Pacific 24°48.085 S, 110°33.307 W	n.a.	KF557510~~xxxxxxx~~	n.a.
PAC11	Collodaria (solitary)	South Pacific 24°23.025 S, 113°58.068 W	n.a.	KF557511~~xxxxxxx~~	KF557539~~xxxxxxx~~
PAC14	Collodaria (solitary)	South Pacific 24°23.025 S, 113°58.068 W	n.a.	KF557512~~xxxxxxx~~	KF557540~~xxxxxxx~~
PAC15	Collodaria (solitary)	South Pacific 24°23.025 S, 113°58.068 W	n.a.	KF557513~~xxxxxxx~~	~~xxxxxxx~~n.a.
PAC16	Collodaria (colony)	South Pacific 24°23.025 S, 113°58.068 W	n.a.	KF557514~~Xxxxxxx~~	n.a.
PAC17	Collodaria	South Pacific	n.a.	KF557515~~xx~~	KF557541~~xxxx~~

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	(colony)	23°42.289 S, 131°12.744 W		~~xxxxx~~	~~xxx~~
PAC19	Collodaria (colony)	South Pacific 23°42.289 S, 131°12.744 W	n.a.	KF557516~~xx~~ ~~xxxxx~~	KF557542~~xxxx~~ ~~xxx~~
PAC21	Collodaria (colony)	South Pacific 23°42.289 S, 131°12.744 W	n.a.	KF557517~~xx~~ ~~xxxxx~~	KF557543~~xxxx~~ ~~xxx~~
PAC22	Collodaria (colony)	South Pacific 23°42.289 S, 131°12.744 W	n.a.	KF557518~~xx~~ ~~xxxxx~~	KF557544~~xxxx~~ ~~xxx~~
PAC24	Collodaria (colony)	South Pacific 23°42.289 S, 131°12.744 W	n.a.	KF557519~~xx~~ ~~xxxxx~~	n.a.
PAC26	Collodaria (colony)	South Pacific 23°42.289 S, 131°12.744 W	n.a.	KF557520~~xx~~ ~~xxxxx~~	n.a.
PAC27	Collodaria (colony)	South Pacific 23°42.289 S, 131°12.744 W	n.a.	KF557521~~xx~~ ~~xxxxx~~	n.a.
SES47	Collodaria (colony)	Sesoko, Japan 26°37’20 N, 127°52’15 E	n.a.	KF557502~~xx~~ ~~xxxxx~~	KF557546~~xxxx~~ ~~xxx~~
SES19	Spumellaria	Sesoko, Japan 26°37’20 N, 127°52’15 E	n.a.	KF557501~~xx~~ ~~xxxxx~~	n.a.
SES28	Nassellaria	Sesoko, Japan 26°37’20 N, 127°52’15 E	n.a.	n.a.	KF557545~~xxxx~~ ~~xxx~~
Vil 210	Spumellaria?	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	n.a.	KF557522~~xx~~ ~~xxxxx~~	n.a.
Vil 217	Spumellaria	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	n.a.	KF557523~~xx~~ ~~xxxxx~~	n.a.
Vil 219	Spumellaria	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	n.a.	KF557524~~xx~~ ~~xxxxx~~	n.a.
Vil 231	Spumellaria	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	n.a.	KF557525~~Xx~~ ~~xxxxx~~	n.a.
Culture strains					
SES46	Collodaria (*Collozoum* colony)	Sesoko, Japan 26°37’20 N, 127°52’15 E	RCC3378	KF557500~~xx~~ ~~xxxxx~~	KF557526~~xxxx~~ ~~xxx~~
			RCC3379	KF557499	n.a.
~~SES46B~~	~~Collodaria~~ ~~(*Collozoum*~~ ~~colony)~~	~~Sesoko, Japan~~ ~~26°37’20 N, 127°52’15 E~~	~~RCC3379~~	~~xxxxxxx~~	~~n.a.~~
VFPO14~~-8~~	Collodaria (*Collozoum*	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	RCC3380	KF557494~~xx~~ ~~xxxxx~~	KF557530~~xxxx~~ ~~xxx~~

	colony)		RCC3381	KF557495	KF557531
			RCC3382	KF557496	KF557532
~~VFPO14-13~~	~~Collodaria (*Collozoum* colony)~~	~~Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E~~	~~RCC3381~~	~~xxxxxxx~~	~~xxxxxxx~~
~~VFPO14-14~~	~~Collodaria (*Collozoum* colony)~~	~~Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E~~	~~RCC3382~~	~~xxxxxxx~~	~~xxxxxxx~~
VFPO2~~-1~~	Spumellaria	Villefranche-sur-Mer, France 43°41’10N, 7°18’50E	RCC3383	KF557491~~xxxxxxx~~	KF557527~~xxxxxxx~~
			RCC3384	n.a.	KF557528
~~VFPO2-2~~	~~Spumellaria~~	~~Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E~~	~~RCC3384~~	~~n.a.~~	~~xxxxxxx~~
VFPO5	Spumellaria	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	RCC3385	KF557492~~xxxxxxx~~	n.a.
VFPO22~~-2~~	Spumellaria	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	RCC3386	KF557497~~xxxxxxx~~	n.a.
VFR1~~-1~~	Spumellaria	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	RCC3387	KF557498~~xxxxxxx~~	KF557533~~xxxxxxx~~
VFPO10	Nassellaria	Villefranche-sur-Mer, France 43°41’10 N, 7°18’50 E	RCC3388	KF557493~~xxxxxxx~~	KF557529~~Xxxxxxx~~

Table 2. Kofoidian plate tabulation of *Brandtodinium* and related genera

Scrippsiella	Po, X, 4’, 3a, 6-7’’, 6c, 4-7s, 5’’’, 2’’’’
Calciodinellum	Po, X, 4’, 3a, 7’’, 6c, 5s, 5’’’, 2’’’’
Bysmatrum	Po, X, 4’, 3a, 7’’, 6c, 4-5s, 5’’’, 2’’’’
Pentapharsodinium	Po, X, 4’, 3a, 7’’, 5c, 4s, 5’’’, 2’’’’
Ensiculifera	Po, X, 4’, 3a, 7’’, 5c, 5s, 5’’’, 2’’’’
Brandtodinium	Po, X, 4’, 3a, 7’’, 5c, 4s, 5’’’, 1’’’’

Figure captions

Figure 1. Light micrographs of *Brandtodinium nutriculum* gen. nov., comb. nov.. (arrow indicates large pyrenoid). A, B. Ventral view of the cell showing the large nucleus in the central portion of the cell. C. Lateral (slightly antapical) view of the cell. D. Dorsal view of the cell. Scale bars = 5µm.

Figure 2. SEM micrographs of *Brandtodinium nutriculum* gen. nov., comb. nov.. (enumeration of plates follows the Kofoidian tabulation system). A. Ventral view of a cell (flagella lost during fixation). B. Detail of the sulcal region. C. Dorsal view. D. Apical view. E. Antapical view. Scale bars = 2µm.

Figure 3. Schematic representation of plate patterns of *Brandtodinium nutriculum* gen. nov., comb. nov. (enumeration of plates follows the Kofoidian tabulation system). A. Ventral view (generalized). B. Dorsal view (generalized). C. Apical view (generalized). D. Antapical view (generalized).

Figure 4. SSU rDNA phylogenetic tree inferred by Maximum Likelihood (ML) analysis. ~~650~~ 652 unambiguously aligned positions were considered from an alignment of 57 sequences, including *Brandtodinium* gen. nov.. Sequences obtained in this study are indicated in bold (followed by the type of host from which the sequence was obtained and the number of holobiont specimens or culture strains in parentheses). The tree was rooted with Suessiales (*Symbiodinium* spp. and *Pelagodinium béii*) as the outgroup. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches

are statistical support values for the clusters to the right of them (first: ML bootstrap support values, values under 0.5 are not shown; second: Bayesian posterior probabilities, values under 0.5 are not shown; black dots at nodes represent a statistical support of 1 for both methods).

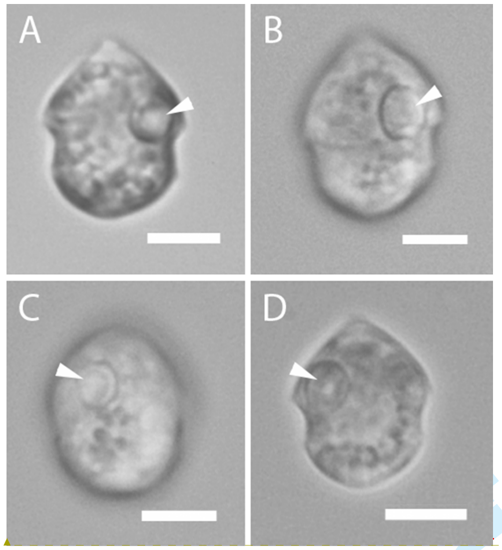
Figure 5. LSU rDNA phylogenetic tree inferred by Maximum Likelihood (ML) analysis. 675 unambiguously aligned positions were considered from an alignment of 48 sequences, including *Brandtodinium* gen. nov.. Sequences obtained in this study are indicated in bold (followed by the type of host from which the sequence was obtained and the number of holobiont specimens or culture strains in parentheses). The tree was rooted with Suessiales (*Symbiodinium* spp. and *Pelagodinium beii*) as the outgroup. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values for the clusters to the right of them (first: ML bootstrap support values, values under 0.5 are not shown; second: Bayesian posterior probabilities, values under 0.5 are not shown; black dots at nodes represent a statistical support of 1 for both methods).

Supplementary Figure 1. LM images of host cells from which uncultured symbiont (holobiont) sequences were retrieved.

Supplementary Figure 2. LM images of host cells from which cultures were isolated.

Supplementary Figure 3. SSU rDNA phylogenetic tree inferred by Maximum Likelihood (ML) analysis. 652 unambiguously aligned positions were considered from an alignment of 59 sequences, including *Bysmatrum*. The tree was rooted with Suessiales (*Symbiodinium* spp. and *Pelagodinium béii*) as the outgroup. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values for the clusters to the right of them (first: ML bootstrap support values, values under 0.5 are not shown; second: Bayesian posterior probabilities, values under 0.5 are not shown; black dots at nodes represent a statistical support of 1 for both methods).

~~Figure 1.~~



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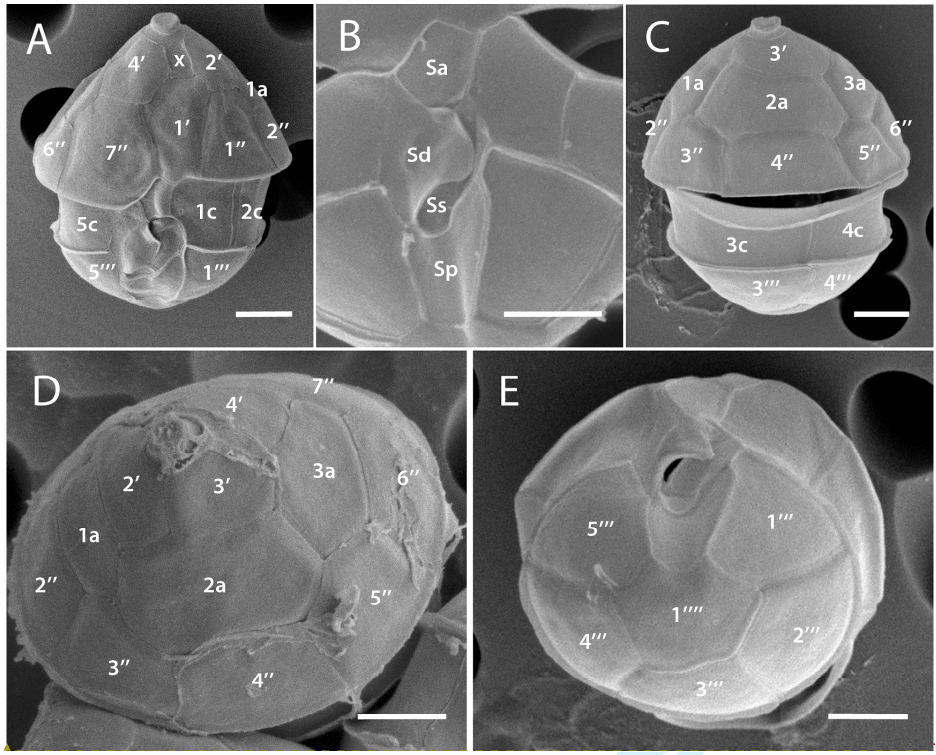
~~Figure 2.~~



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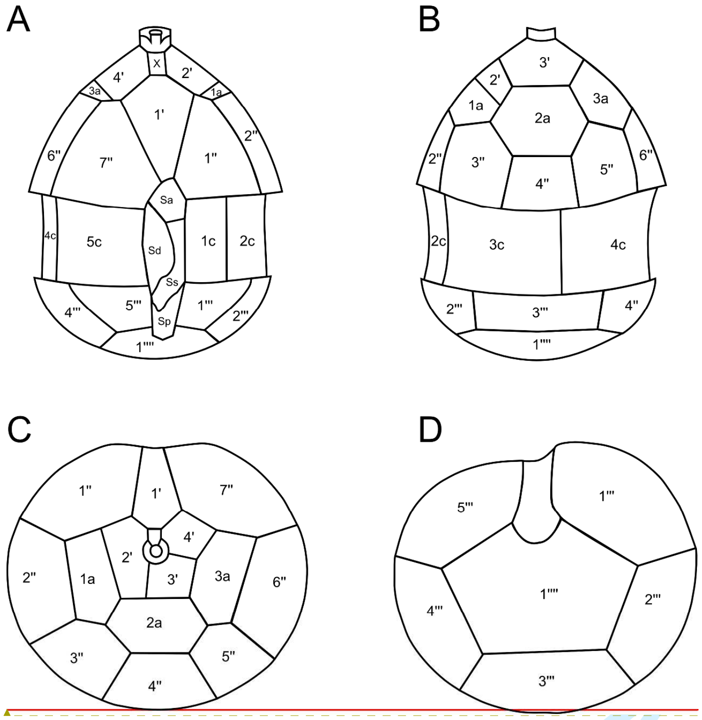
~~Figure 3.~~



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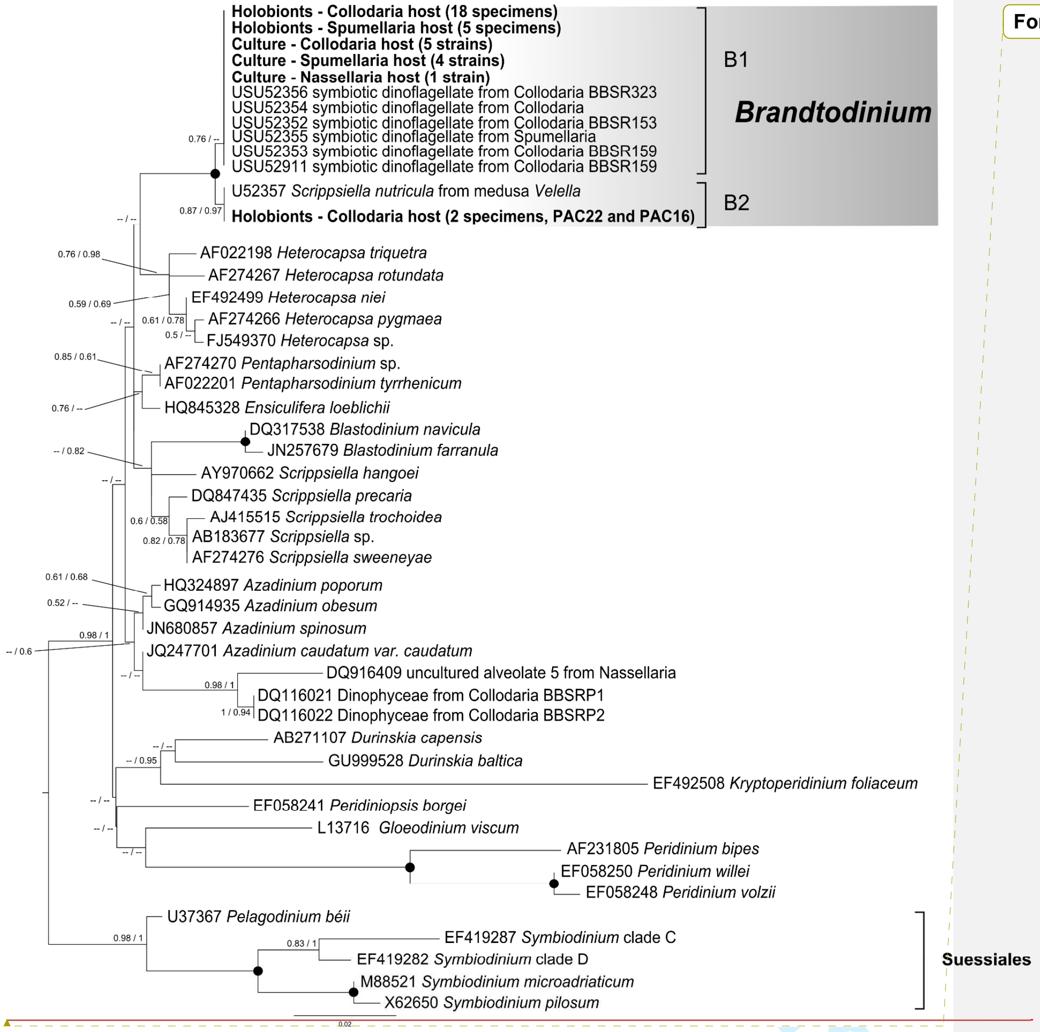
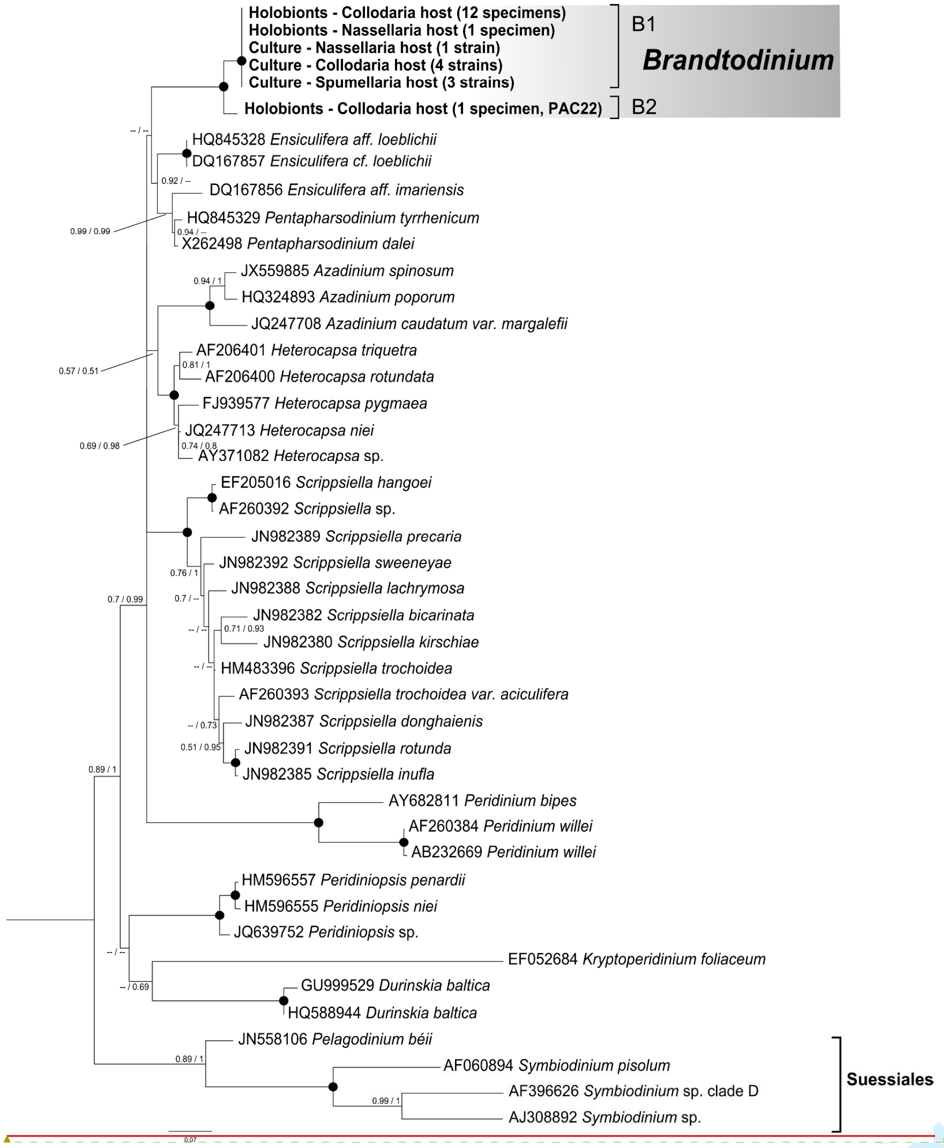


~~Figure 4.~~



~~Figure 5.~~

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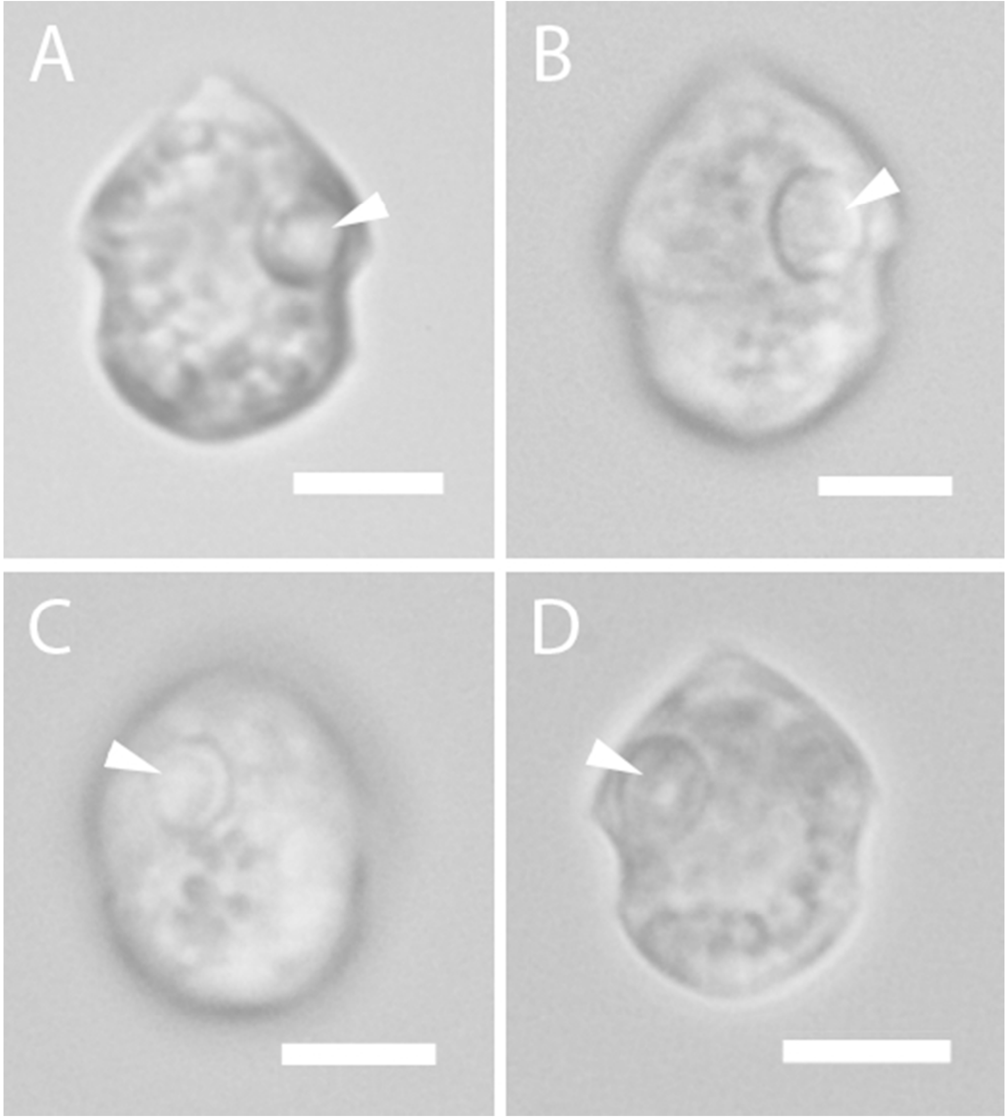

Figure 1. Light micrographs of Brandtodinium nutriculum gen. nov., comb. nov.. (arrow indicates large pyrenoid). A, B. Ventral view of the cell showing the large nucleus in the central portion of the cell. C. Lateral (slightly antapical) view of the cell. D. Dorsal view of the cell. Scale bars = 5µm.
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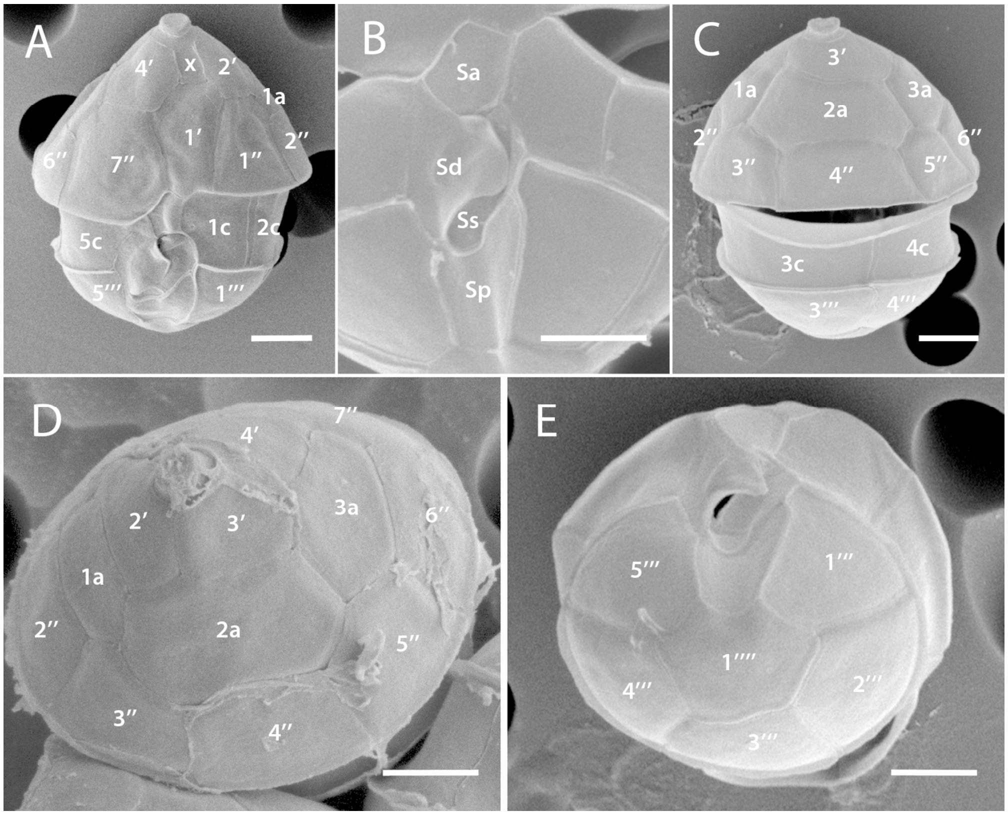

Figure 2. SEM micrographs of Brandtodinium nutriculum gen. nov., comb. nov.. (enumeration of plates follows the Kofoidian tabulation system). A. Ventral view of a cell (flagella lost during fixation). B. Detail of the sulcal region. C. Dorsal view. D. Apical view. E. Antapical view. Scale bars = 2μm.
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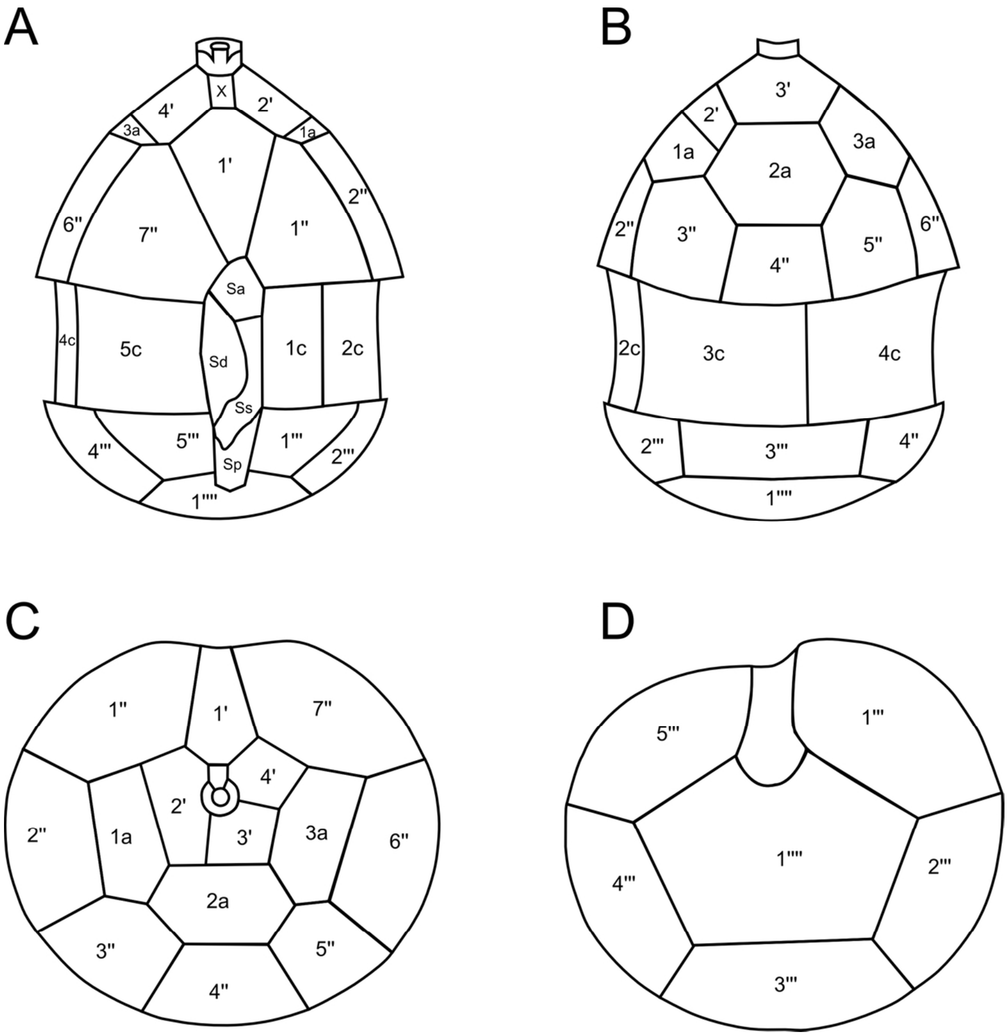

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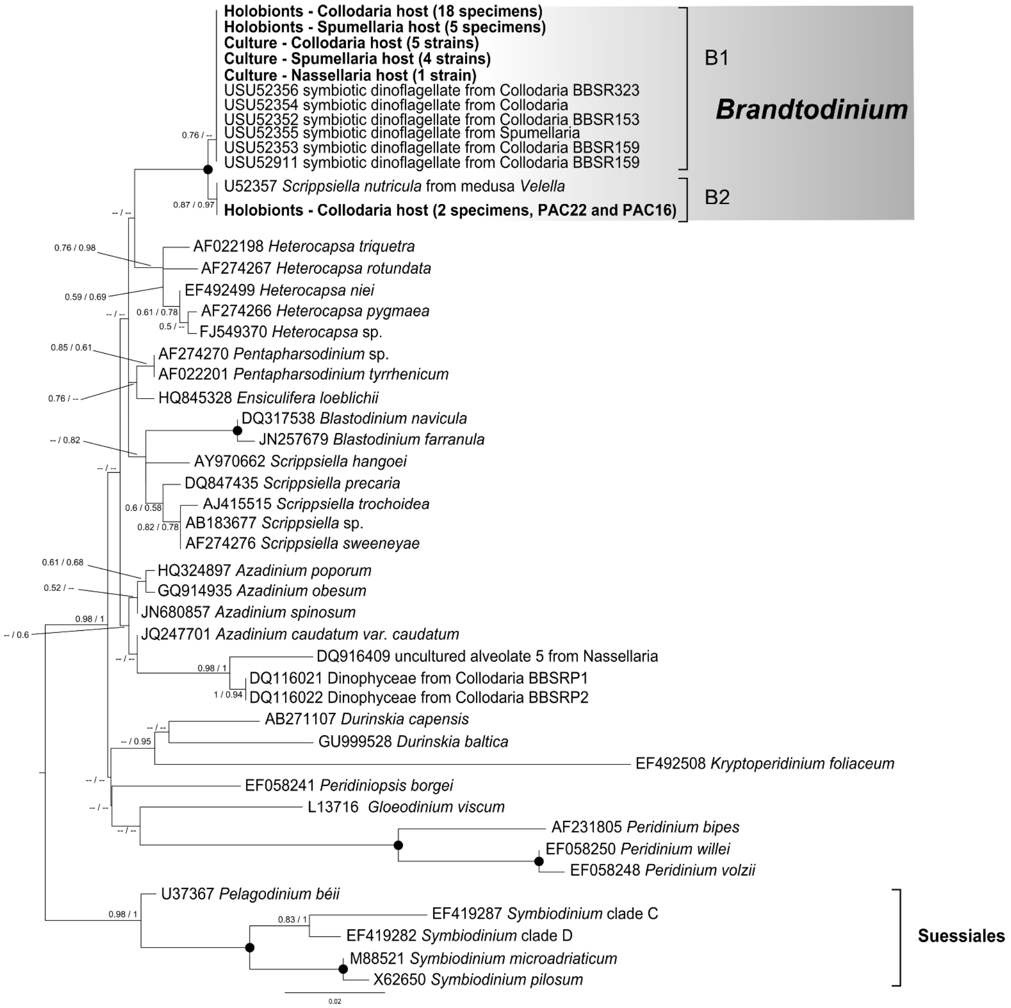

Figure 4. SSU rDNA phylogenetic tree inferred by Maximum Likelihood (ML) analysis. 652 unambiguously aligned positions were considered from an alignment of 57 sequences, including Brandtodinium gen. nov.. Sequences obtained in this study are indicated in bold (followed by the type of host from which the sequence was obtained and the number of holobiont specimens or culture strains in parentheses). The tree was rooted with Suessiales (Symbiodinium spp. and Pelagodinium béii) as the outgroup. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values for the clusters to the right of them (first: ML bootstrap support values, values under 0.5 are not shown; second: Bayesian posterior probabilities, values under 0.5 are not shown; black dots at nodes represent a statistical support of 1 for both methods).
324x323mm (299 x 299 DPI)

Figure 5. LSU rDNA phylogenetic tree inferred by Maximum Likelihood (ML) analysis. 675 unambiguously aligned positions were considered from an alignment of 48 sequences, including Brandtodinium gen. nov.. Sequences obtained in this study are indicated in bold (followed by the type of host from which the sequence was obtained and the number of holobiont specimens or culture strains in parentheses). The tree was rooted with Suessiales (Symbiodinium spp. and Pelagodinium beii) as the outgroup. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values for the clusters to the right of them (first: ML bootstrap support values, values under 0.5 are not shown; second: Bayesian posterior probabilities, values under 0.5 are not shown; black dots at nodes represent a statistical support of 1 for both methods).
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Supplementary Figure 1. LM images of host cells from which uncultured symbiont (holobiont) sequences were retrieved.
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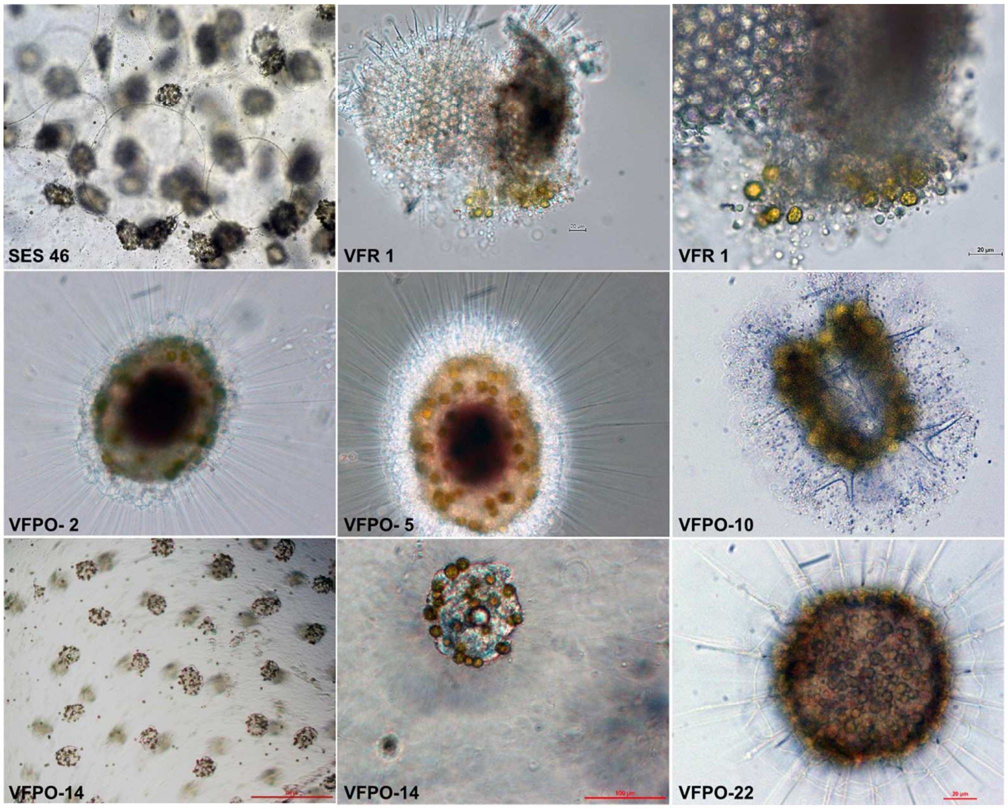

Supplementary Figure 2. LM images of host cells from which cultures were isolated.
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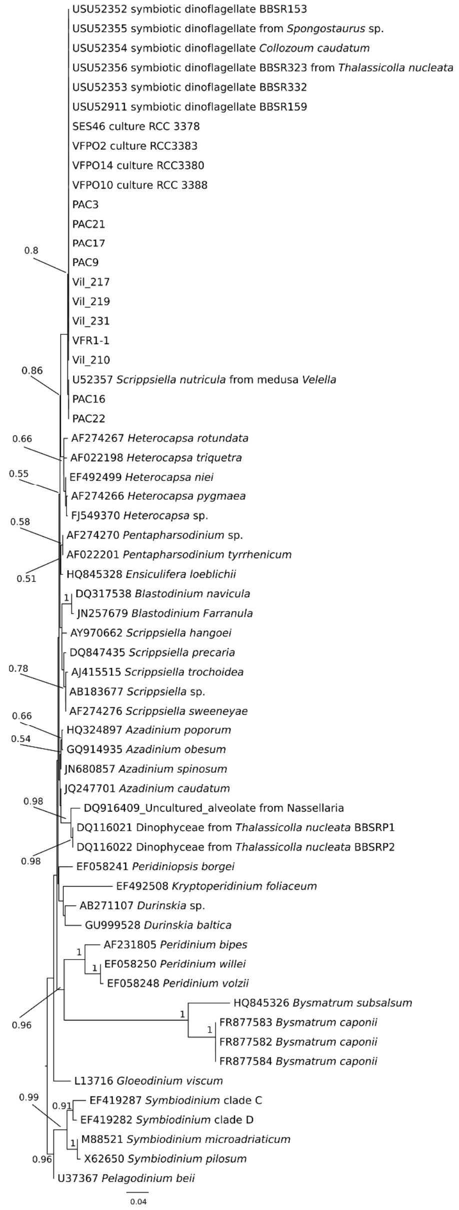

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163x440mm (299 x 299 DPI)