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# DIVERSITY OF CRYPTHECODINIUM SPP. (DINOPHYCEAE) FROM OKINAWA PREFECTURE, JAPAN

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# DIVERSITY OF *Crypthecodinium* spp. (DINOPHYCEAE) FROM OKINAWA PREFECTURE, JAPAN

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Key words: *Crypthechodinium chonii*, taxonomy, phylogeny, diversity, heterotrophic dinoflagellates.

## ABSTRACT

The genus *Crypthecodinium* (Dinophyceae) currently consists of only one species: *C. cohnii*, a heterotrophic marine dinoflagellate widely known able to produce prolific amount of DHA. However, previous studies revealed that there are morphological and genetic differences among *Crypthedodinium cohnii*-like strains, indicating the potential of undiscovered diversity of this dinoflagellate. Attempts of isolating heterotrophic marine dinoflagellate strains were made from submerged mangrove leaves and seaweed. A total of nine strains were established, cultured and maintained using seawater-based GTY medium for taxonomic identification and phylogenetic analyses based on SSU, ITS1, 5.8S, ITS2 and LSU rDNA. Morphological observation revealed all strains shared similar morphology, e.g.: motile cells were delicately thin armored with single or double flagella surrounding the cingulum and protruding along the sulcus posteriorly, while non-motile cell stage (cyst) may retain more than two daughter cells inside the transparent and thick cell wall. Both cell stages varied in sizes and shapes even within the same clonal culture. Numerous lipid granules can be observed in the cytoplasm. Two types of cingulum encirclement were observed: partially or completely surrounding the cell body. All strains indicated close relatedness (>95% sequence similarity) with previously reported *Cryphtecodinium cohnii* strains, except for *C. cohnii* CAAE-CL2 (87.2-87.4% similarity) in SSU and *C. cohnii* CCMP316 (79.9-80.4% similarity) in ITS. Moreover, molecular phylogenetic tree grouped the Okinawan isolates into two genetically distinct clades with high similarity (>98%) among members of each clade in SSU and LSU but only shared 80.5% similarity in ITS rDNA. The results of this study showed evidences of undiscovered diversity in the heterotrophic dinoflagellates currently regarded as *Crypthecodinium*.

## I. INTRODUCTION

Traditionally, early taxonomic identification of dinoflagellates species were performed only based on morphological observation using light microscopy. Several morphological criteria were developed to define major genera of dinoflagellate, i.e.: armored or unarmored cells, thecae plate pattern, the shape and size ratio of the epicone and hypocone, cingulum location and displacement, apical groove, etc. [11, 15]. Despite implementation of electron microscopy (EM) helped in obtaining detailed ultra structural characters of dinoflagellate species and resolved some of the taxonomic challenges, many species remained unidentified, especially unarmored dinoflagellate species [27, 28]. Molecular analyses had revealed that many dinoflagellates previously considered monophyletic were in fact paraphyletic or polyphyletic and highly diverse [8, 26]. Since then, classification of dinoflagellates has combined both detailed morphological identification and molecular phylogenetic analyses [11, 19, 31].

The important aspect of studying dinoflagellate classification and distribution are based on the fact that many members of this organism are able to produce toxins causing massive fish mortality during blooming event or accumulated by shellfish and caused shellfish poisoning if consumed by human [15]. However, several dinoflagellate species have been reported also capable of producing beneficial chemical substances such as carotenoids and fatty acids [16, 17, 21]. *Crypthecodinium cohnii*, in particular, produces prolific amount of docosahexaenoic acid (DHA), an omega-3 fatty acid essential for the neurological development of infants, health and growth enhancer in aquaculture fish [17].

*Crypthecodinium cohnii* has undergone a series of taxonomical revision. It was originally described as *Glenodinium cohnii* by Seligo [28] and later transferred to the genus *Gyrodinium* (Kofoid and Swezy [14]) as *Gyrodinium cohnii* (Seligo) Schiller [27]. In separate study, Biecheler [5]

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**Table 1. Strains isolated from different sample types and locations.**

| Strains | Isolated from | Isolation Date | Locality |
|---|---|---|---|
| SZ7-1 | Submerged mangrove leaf | 2011.09.29 | Suzaki River |
| SZ13-1 | Submerged mangrove leaf | 2011.09.29 | Suzaki River |
| SZ13-2 | Submerged mangrove leaf | 2011.09.29 | Suzaki River |
| SZ13-3 | Submerged mangrove leaf | 2011.09.29 | Suzaki River |
| SS2-2 | Submerged mangrove leaf | 2011.11.21 | Nanjo |
| ISK1-2 | Submerged mangrove leaf | 2012.01.31 | Ishikawa River |
| ISK1-2 | Submerged mangrove leaf | 2012.01.31 | Ishikawa River |
| ISG40-1 | Submerged mangrove leaf | 2012.07.05 | Miyara River |
| OKI5-1 | Seaweed | 2012.11.12 | Cape Chinen |

*Note: All strains were isolated from samples taken in Okinawajima Island, except for ISG40-1, which was isolated from Ishigaki Island.

established the new genus *Crypthecodinium* with the single species *C. setense* Biecheler as the type species characterized by thin thecal plates. *C. setense* shared similar morphology to that of *G. cohnii* and Biecheler [5] suspected the possibility of synonymy between them. To accommodate this taxonomical confusion, Chatton [6] considered the two as conspecific and introduced the combination *Crypthecodinium cohnii* (Seligo) Chatton with *C. setense* retained as the type species of the genus. The genus *Crypthecodinium* Biecheler currently consists only one species: *Cryptecodinium cohnii* (Seligo) Javornicky [12], in which the name is presently accepted taxonomically [18].

Early taxonomic studies of this species were based on morphological observation, reproduction compatibility experiments and early molecular analyses. Further study revealed that *C. cohnii* has diversified into a complex of sibling species that are indistinguishable using light microscopy [3]. Parrow *et al*. [22] provided the first clear plates tabulation of a *Crypthecodinium* sp. isolated from brackish-water fish aquarium. Despite the general morphology of this strain fit best with the description of *C. cohnii* [5], the strain was suggested not identical compared to a *C. cohnii* strain reported by Gajadhar *et al*. [10] as it only shared 87% similarity in SSU rDNA. Perret *et al*. [23] also reported *Crypthedodinium cohnii* having cingulum characters that differed from the original description by Seligo [28]. Hence, the taxonomy and diversity of this genus might need to be reinvestigated.

Nine heterotrophic marine dinoflagellate strains were isolated from submerged mangrove leaves and seaweed taken in Okinawa Prefecture, Japan. Based on light microscopy, the isolates appeared to be *Crythecodinium cohnii-like* species. However, some distinctions based on morphology and molecular analyses were found between our isolates and previously published strains of *Crypthecodinium cohnii*. The aim of this study is to investigate the diversity of this dinoflagellate by examining the morphology and molecular phylogeny based on the SSU, ITS and LSU rDNA sequences.

## II. MATERIAL AND METHODS

### 1. Sampling, Isolation and Cultures

Samples of seawater, sand, mud, seaweed, seagrass, submerged leaves and plant debris were collected from several mangrove and estuary areas in Okinawa Prefecture, Japan, in 2012-2013. Upon collection, samples were enriched using modified seawater-based 1/10 glucose-triptone-yeast extract (GTY) culture medium (0.1 gr/l glucose, 0.2 gr/l triptone, and 0.05 gr/l yeast extract in 40% GF/F-filtered seawater) added with 125 mg/l of antibiotics (ampicillin and streptomycin) for 2-3 days. Single motile dinoflagellate cells were isolated, serially washed with the aid of micropipette and transferred into a sterile glass tube containing 5 ml of the same medium used for enrichment of samples. A total of nine heterotrophic dinoflagellate strains were established (Table 1) and maintained under 22 ± 1°C for 20-25 days before transferred into new medium.

### 2. Morphological Observation

Motile and non-motile cells (cysts) were observed using a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan) equipped with Differential Interference Contrast (DIC) optics. Micrographs were obtained using SPOT Idea 5 MP CCD digital camera (Diagnostic Instruments, Miami, USA). SEM (scanning electron microscope) preparations followed a modified protocol by Yamaguchi *et al.* [35]: 1 ml of actively growing cell cultures were mixed with equal volume of fixatives containing a 2.5% glutaraldehyde, 0.2% $OsO_4$ and 0.25 M sucrose in 0.2 M sodium cacodylate buffer (pH 7.2). After 1 hour of fixation, the cells were then slowly and gently filtered using a 0.2 μm Millipore filters, rinsed with 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated by ethanol series (30%, 50%, 70%, 90%, 95%, 100%) and transferred into t-butyl alcohol. Subsequently, the filtered specimens were freeze-dried using VFD-21S t-BuOH freeze dryer (Shinku Device, Ibaraki, Japan), mounted on metal stubs and coated with Au/Pd using Eiko IB-3 ion sputter-coater (Eiko, Tokyo, Japan). Finally, the specimens were observed using a JEOL JSM 6060 LV (JEOL, Tokyo, Japan) scanning electron microscope at 15 Kv.

### 3. DNA Extraction, Amplification and Sequencing

Prior DNA extraction, 20 mL cultures were harvested for pellets by centrifugation at 91 × 100 G (10,000 rpm) using

**Table 2. GenBank accession number of organism/strain included in the phylogenetic.**

| Organism/Strain | GenBank Accession Number | | |
|---|---|---|---|
| | SSU-rDNA | ITS-rDNA | LSU rDNA |
| *Adenoides eludens* | AF274249 | - | - |
| *Akashiwo sanguinea* | AF276818 | - | AF260397 |
| *Alexandium catenella* | AJ535392 | - | AF200667 |
| *Alexandrium tamarense* | AF022191 | - | AF200668 |
| *Amphidinium asymmetricum* | AF274250 | - | - |
| *Amphidinium carterae* | AF009217 | - | AY460578 |
| *Amphidinium corpulentum* | AF274252 | - | - |
| *Amphidinium gibbosum* | L13719 | - | - |
| *Amphidinium herdmanii* | AF274253 | - | AY455675 |
| *Amphidinium semilunatum* | AF274256 | - | AY460591 |
| *Amphidinium steinii* | - | - | AY455673 |
| *Amyloodinium ocellatum* | AF080096 | - | - |
| *Cachonina hallii* | AF033865 | - | - |
| *Ceratium fusus* | AF022153 | - | - |
| *Ceratium lineatum* | - | - | AF260391 |
| *Ceratium tripos* | - | - | AF260389 |
| *Ceratocorys horrida* | AF022154 | - | - |
| *Crypthecodinium cohnii* | M64245 | - | - |
| *Crypthecodinium cohnii* | FJ821501 | - | - |
| *Crypthecodinium cohni* | DQ241737 | - | - |
| *Crypthecodinium cohnii* CCMP316 | - | FJ823534 | FJ939575 |
| *Crypthecodinium* sp. CAAE-CL2 | DQ322643 | - | - |
| *Crypthecodinium* sp. D31 | AB811790 | - | - |
| ***Crypthecodinium cohnii* ISG40-1** | AB871546 | AB871528 | AB871537 |
| ***Crypthecodinium cohnii* ISK1-1** | AB871544 | AB871526 | AB871535 |
| ***Crypthecodinium cohnii* ISK1-2** | AB871545 | AB871527 | AB871536 |
| ***Crypthecodinium cohnii* OKI5-1** | AB871551 | AB871533 | AB871542 |
| ***Crypthecodinium cohnii* SS2-2** | AB871552 | AB871534 | AB871543 |
| ***Crypthecodinium cohnii* SZ7-1** | AB871550 | AB871532 | AB871541 |
| ***Crypthecodinium cohnii* SZ13-1** | AB871547 | AB871529 | AB871538 |
| ***Crypthecodinium cohnii* SZ13-2** | AB871548 | AB871530 | AB871539 |
| ***Crypthecodinium cohnii* SZ13-3** | AB871549 | AB871531 | AB871540 |
| *Dinophysis acuminata* | AJ506972 | - | - |
| *Dinophysis norvegica* | AF239261 | - | AY571375 |
| *Eimeria tenella* | U67121 | - | AF026388 |
| *Fragilidium subglobosum* | AF033869 | - | - |
| *Glenodiniopsis steinii* | AF274257 | - | - |
| *Gloeodinium viscum* | L13716 | - | - |
| *Gonyaulax baltica* | - | | AF260388 |
| *Gonyaulax spinifera* | AF022155 | - | - |
| *Gymnodinium breve* | | | AF200677 |
| *Gymnodinium catenatum* | AF022193 | - | AF200672 |
| *Gymnodinium chlorophorum* | - | | AF200669 |
| *Gymnodinium fuscum* | AF022194 | - | AF200676 |
| *Gymnodinium galatheanum* | AF172712 | - | AF200675 |
| *Gymnodinium mikimotoi* | AF022195 | - | AF200682 |
| *Gymnodinium venator* | - | - | AY455681 |
| *Halostylodinium arenarium* | AB036837 | - | - |
| *Heterocapsa rotundata* | - | - | AF260400 |
| *Heterocapsa triquetra* | AF022198 | - | AF260401 |
| *Karenia brevis* | AF274259 | - | - |
| *Kryptoperidinium foliaceum* | AF274268 | - | - |

**Table 2. (Continued)**

| Organism/Strain | GenBank Accession Number | | |
|---|---|---|---|
| | SSU-rDNA | ITS-rDNA | LSU rDNA |
| *Lepidodinium viride* | AF022199 | - | - |
| *Lessardia elongata* | AF521100 | - | - |
| *Lingulodinium polyedrum* | AF274269 | - | - |
| *Pentapharsodinium tyrrenicum* | AF022201 | - | - |
| *Peridiniella catenata* | - | - | AF260398 |
| *Peridinium bipes* | - | - | AF260385 |
| *Peridinium willei* | AF274272 | - | AF260384 |
| *Peridinium pseudolaeve* | - | - | AF260395 |
| *Pfiesteria piscicida* | - | - | AY112746 |
| *Polarella glacialis* | - | - | AY036081 |
| *Prorocentrum cordatum* | - | - | AF260379 |
| *Prorocentrum lima* | Y16235 | - | - |
| *Prorocentrum mexicanum* | Y16232 | - | AF260379 |
| *Prorocentrum micans* | M14649 | - | AF260377 |
| *Prorocentrum minimum* | Y16238 | - | - |
| *Protoceratium reticulatum* | AF274273 | - | AF260393 |
| *Pseudopfiesteria shumwayae* | AF080098 | - | - |
| *Pyrocystis noctiluca* | AF022156 | - | - |
| *Roscoffia capitata* | AF521101 | - | - |
| *Scrippsiella nutricula* | U52357 | - | - |
| *Scrippsiella trochoidea* | - | - | AF260393 |
| *Symbiodinium microadriaticum* | M88521 | - | - |
| *Thoracosphaera elongata* | AF274278 | - | - |
| *Togulla jolla* | - | - | AY568559 |
| *Togulla britanica* | - | - | AY455679 |
| *Toxoplasma gondii* | U00458 | - | X75429 |
| *Woloszynskia pesudopalustris* | - | - | AF260402 |

Tomy MX-201 highspeed refrigerator micro centrifuge (Tomy Tech Inc., Fremont, CA, USA). Pellets were then immersed in liquid nitrogen for 5-10 seconds and immediately ground thoroughly using homogenizer followed by total DNA extraction using Qiagen Plant Mini Kit (Qiagen, Tokyo, Japan). PCR protocols and amplification primers used in this study followed those of Takano and Horiguchi [32] for partial SSU (SR1 and SR12b primers), ITS1-5.8S-ITS2 (SR12cF and 25F1R primers) and partial LSU D1-D3 (D1R and R2 primers) rDNA. Amplifications were performed by means of an Eppendorf Mastercycle EP (Harlow Scientific, Arlington, Massachusetts, USA) with the following PCR conditions: one initial cycle of denaturation at 93°C for 1 min, 35 cycles of denaturation at 93°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min and final extension cycle at 72°C for 4 min. PCR products were sent to Macrogen Japan (http://www.macrogen-japan.co.jp/) for sequencing. Both forward and reverse strands were sequenced.

## 4. Sequence Alignment and Phylogenetic Analyses

The SSU (1650 bp), LSU (1046 bp) and additional sequences from GenBank were aligned using multiple alignment tool of ClustalW in MEGA 5.22 [33], examined and corrected manually by eye. Phylogenetic tree based on ITS dataset (610 bp) was also generated as comparison. All sequences of *Crypthecodinium cohnii*-like strains reported in this study were deposited to DNA Data Bank of Japan (DDBJ). Organisms included in the phylogenetic analyses and their GenBank accession numbers are presented in Table 2.

Model tests and phylogenetic analyses based on maximum likelihood (ML) for each analysis were performed using MEGA 5.22 [33]. Bayesian inference of posterior probability (PP) was calculated using MrBayes version 3.2.2 [25]. The best fit model for SSU and LSU were TN93+G+I, while HKY+G was chosen for ITS analysis, based on the lowest Bayesian information criterion (BIC). Bootstrap values [9] were obtained from analyses of 1000 replicates using Nearest-Neighbor-Interchange (NNI) of ML heuristic method to infer the tree. Gaps or missing data were treated with complete deletion. For Bayesian analysis, four chains of Markov chain Monte Carlo (MCMC) iterations were carried out for 1,000,000 generations with burn in fraction of 0.25 and sample frequency of 1000. Bayesian inferred tree was visualized using FigTree version 1.4.0 (http://tree.bio.ed. ac.uk/software/

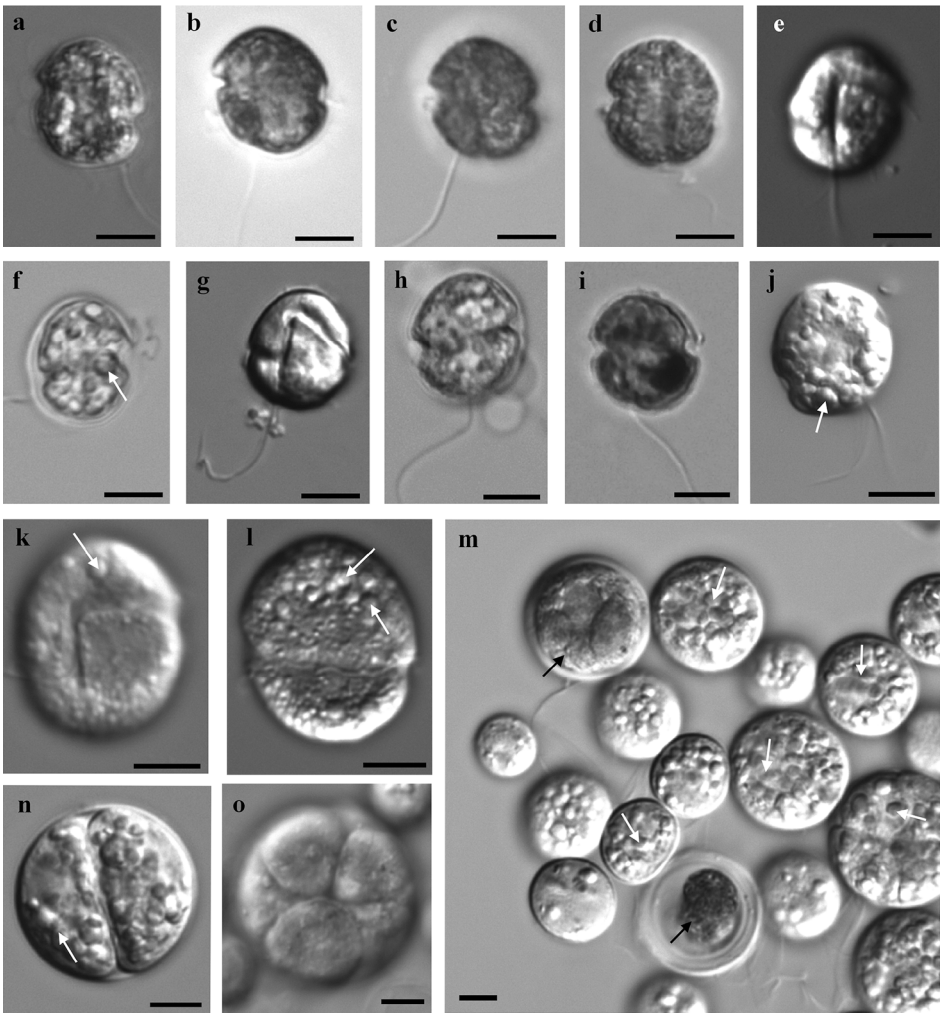


**Fig. 1. Morphology of *Crypthecodinium*-like strains based on light microscopy (a-o). Motile cell of each strains are presented in the following order: ISG40-1 (a), ISK1-1 (b), ISK1-2 (c), OKI5-1 (d), SS2-2 (e), SZ7-1 (f), SZ13-1 (g), SZ13-2 (h), and SZ13-3 (i). Sexual reproduction of two cells produces a planozygote with double longitudinal flagella (j). Ventral and dorsal view of large motile cells with numerous starch-like granules (white arrow) (k, l). Vegetative cell cycle of single cell formed cyst with colored food vacuole (black arrow) and cyst with starch-like granules (white arrow) (m). Cyst producing two or multiple daughter cells inside cell wall (n, o). Scale bar = 5 μm.**

figtree/). Sequence identity of the Okinawan isolates and *Crypthecodinium* sequences retrieved from GenBank were estimated based on *p*-distance calculation for each rDNA genes using MEGA 5.22 [33].

## II. RESULT AND DISCUSSION

### 1. Habitat and Morphological Characteristics

This study was a part of research project aimed to find potential microalgae strains to be used for various applications (e.g. alternative source for food, feed, fine chemicals, biofuel, etc.) from local sources in Okinawa, Japan. In order to be selected as a potential strain, the isolated microalgae need to exhibit the following characters: fast growing rate, easy to maintain and able to produce high amount of commercial and important substances (e.g. oil, essential fatty acids, carotenoids, etc.). We successfully isolated several *Crypthecodinium cohnii*-like strains that showed fast growing characters during culture. *C. cohnii* has been regarded highly as a prolific producer of DHA. This heterotrophic dinoflagellate has been proposed to replace fish oil as the common source of DHA [24].

Among many sample types collected from various mangrove areas and estuaries in Okinawa Prefecture, Japan, isolates were found mostly from organic materials such as submerged fallen mangrove leaves and seaweed. Whereas, similar isolates were not found in seawater, mud and sand samples. This is consistent with previous reported studies that strains of *C. cohnii* were recovered from several organic sources, e.g.: living or decaying seaweed samples [17, 34], submerged mangrove leaves in mangrove areas and estuaries [1, 20] and brackish water [22]. In laboratory culture, the strains grew relatively fast and abundant in matter of several days after transferred to a fresh GTY medium. It has been also

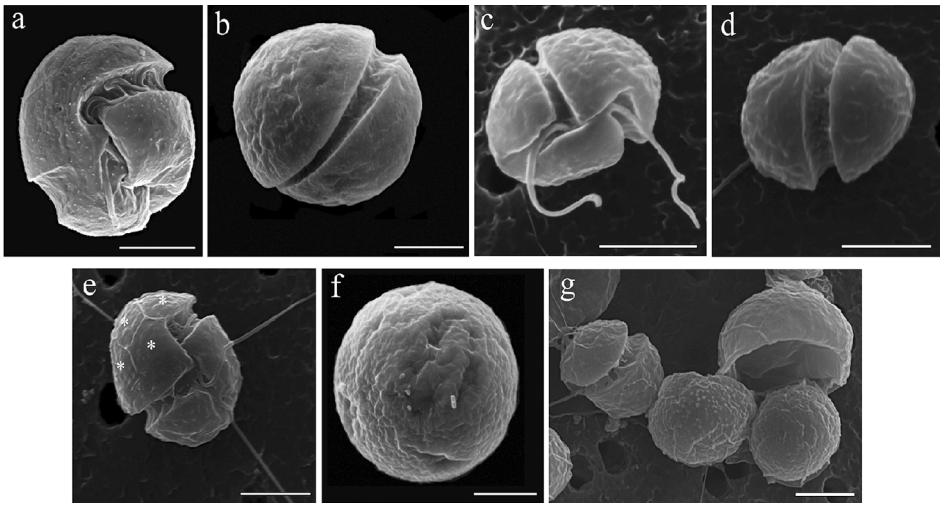


**Fig. 2. Scanning electron micrographs (SEM) showing two different cell morphologies viewed ventral and dorsal: round cell with cingulum not completely encircled the body (a, b) and ovoid cell with descended cingulum completely encircle the body with a deep groove and converged with the sulcus (c, d). Reproductive cells as seen as: a planozygote with two longitudinal flagella and incomplete thecal plates (asterisk) (e), cyst (f) and daughters cells hatches from a cyst capsule (g). Scale bar = 5 µm**

reported that some strains of *C. cohnii* were able to prey on other algal cell [34] or fed on fish cell line offered as food source [22]. However, the later prey preference was not observed in our cultured strains since they were not fed with other food source other than GTY medium.

All isolated strains shared similar morphological characters, thus making them difficult to distinguish based on light microscopy, or in some cases, even using scanning electron microscopy. Cells varied in sizes, both small cells (5-20 µm) (Figs. 1a-i) and large cells (8-30 µm) (Figs. 2k, l) were observed in the same clonal culture. The cell size variation might indicate different stages of the cell life cycle, in which small cell represents young offspring and the latter represent adult cell. Two types of cell stages were observed: motile and non-motile cells (cysts) with various dimensions. Motile cells were colorless and appeared generally oval in shape and occasionally round in dorsal and ventral view under LM or SEM (Fig. 1; Fig. 2). Numerous lipid-like granules were also observed in the cytoplasm (Figs. 1f, j, l, m, n). Cingulum were displaced and descended leftward, dividing the cell body into three equal parts: epicone, middle part and the hypocone (Figs. 1e, g; Figs. 2a-e) or only slightly displaced and divided the cell into epicone and hypocone that were almost equal in size (Figs. 1a, b, c, d, f, h, i, j). The cingulum also appeared partially (Fig. 2a) or completely encircling the cell body, in which the latter converged in the longitudinal sulcus (Fig. 2c). Strains having partial cingulum encirclement are ISK1-1, ISK1-2, SZ7-1, and SZ13-3 while strains of ISG40-1, SZ13-1, SZ13-2, SS2-2, and OKI5-1 having a complete cingulum encircling the cell body. Both types of cingulum encirclement were even present in the strains that are genetically closely related (discussed in the molecular phylogeny). These morphological characters remained unclear whether it was only present in certain cell stages or permanent novel feature of the cell.

Two unequal types of flagella were present: transverse and longitudinal flagella, which provide fast cell locomotion. Double transverse and longitudinal flagella were observed in putative planozygote; cells resulted from sexual reproduction in which two vegetative and motile cells merged into one active motile cell (Fig. 1j; Fig. 2e). Non-motile cell (cyst) were generally round in shape and appeared larger than the motile cells (Fig. 1m). The non-motile cells are able to produce more than two daughter cells inside the encapsulated cyst (Figs. 1n, o; Fig. 2g). Observation by SEM revealed that motile cells had thin and structured thecal plates (Fig. 2e). Unfortunately, we were unable to obtain satisfactory scanning electron micrographs in order to confirm the tabulation patern of these thin and delicate thecal plates. Nonetheless, in comparison with the original description by Seligo [28] our strains shared similar characteristics, except for the type of cells in which the cingulum encircled completely the cell body. Parrow *et al*. [22] showed that the left descended cingulum end of *C. cohnii* CAAE-CL2 was not visible in ventral view as it ended relatively far from the sulcus. In our strains, the end of the cingulum was notably visible even though it did not fully encircle the cell body (Fig. 2a). Despite the SEM images showed the presence of thecal plates partially covering the cell surface, the tabulation pattern remained unresolved in this study. Staining the thin armor plates using calcofluor-white in this study also did not successfully visualize the thecal plates.

## 2. Molecular Phylogeny

In order to evaluate the relationship among the strains, early molecular studies of *C. cohnii* were based on different approaches, such as DNA buoyant density values, restriction endonuclease cleavage, DNA-DNA hybridization and soluble enzyme studies [3, 29]. Current molecular phylogenetic

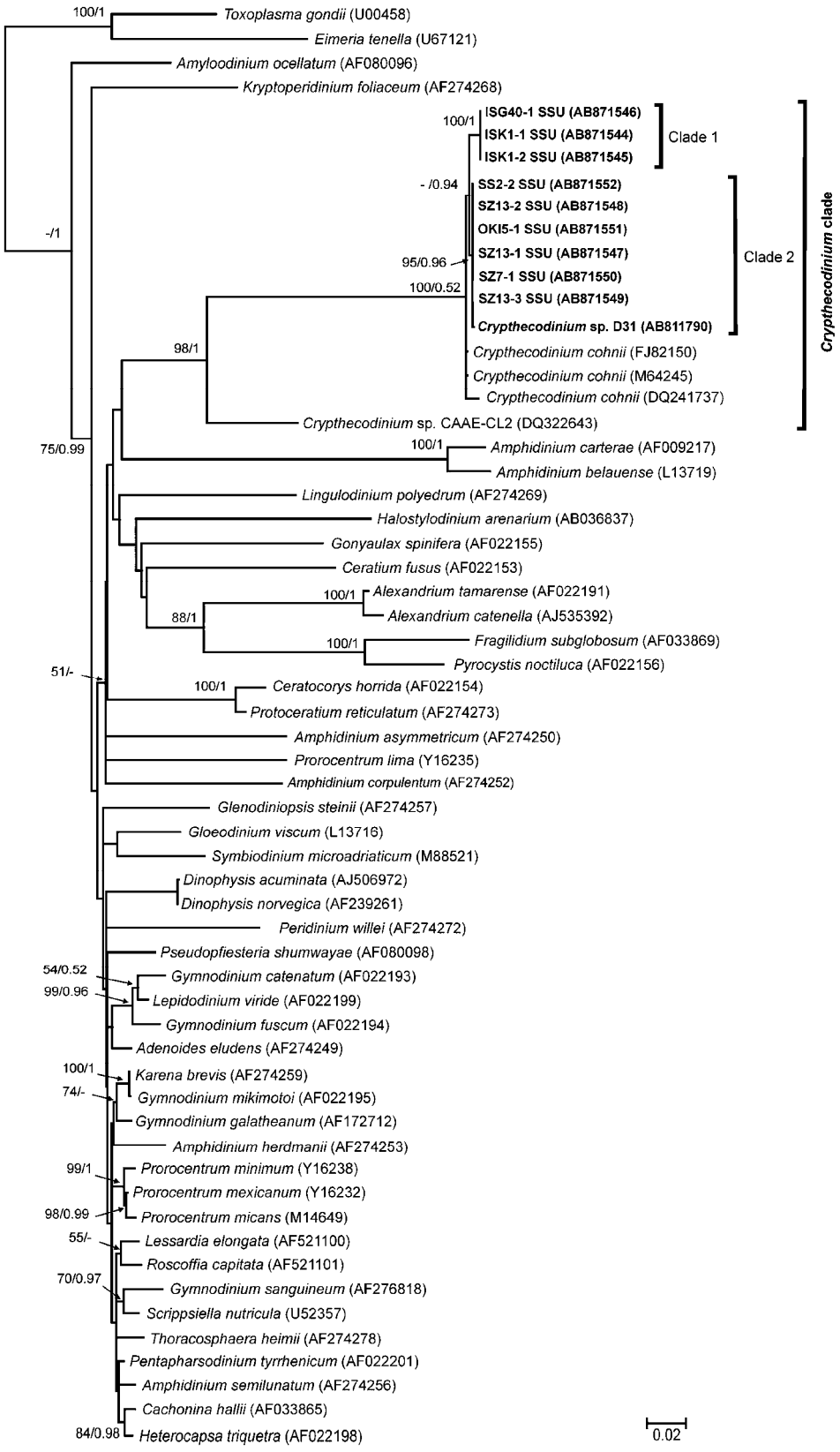


**Fig. 3. Maximum likelihood (ML) phylogenetic tree of major dinoflagellate genera derived from partial SSU rDNA sequences. The ML analysis was calculated using TN93 + G +I evolutionary model resulted the highest log likelihood of -12859.0046. ML bootstrap values (>50%) and Bayesian inference posterior probabilities (>0.5) are indicated at branches. *Crypthecodinium* strains isolated in this study are indicated in bold letters and the tree is rooted with two apicomplexans, *Toxoplasma gondii* (U00458) and *Eimeria tenella* (U067121).**

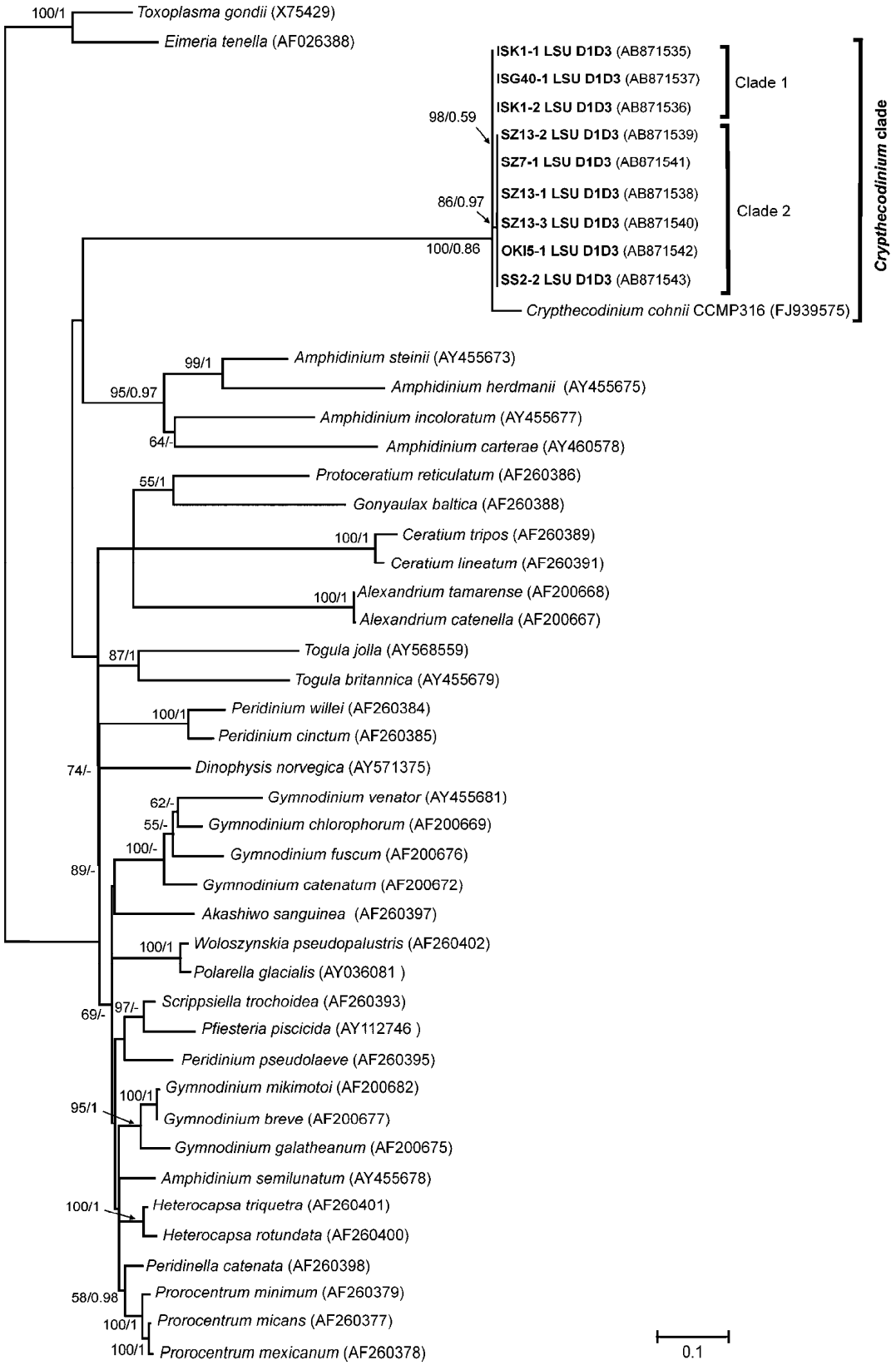


**Fig. 4. Maximum likelihood (ML) phylogenetic tree of major dinoflagellate genera derived from partial LSU rDNA domain D1-D3 sequences. The ML analysis was calculated using TN93 + G +I evolutionary model resulted the highest log likelihood of -9236.9767. ML bootstrap values (>50%) and Bayesian inference posterior probabilities (>0.5) are indicated at branches. *Crypthecodinium* strains isolated in this study are indicated in bold letters and the tree is rooted with two apicomplexans, *Toxoplasma gondii* (X75429) and *Eimeria tenella* (AF026388).**

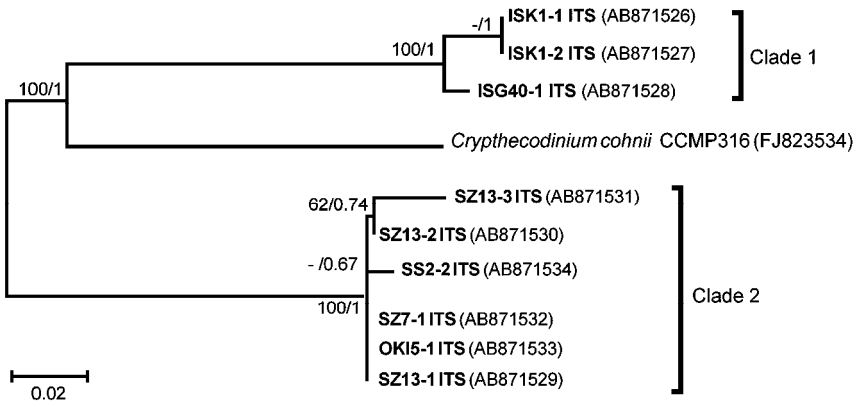


**Fig. 5. Maximum likelihood (ML) of unrooted phylogenetic tree of *Crypthecodinium* strains derived from partial ITS1, 5.8S, and partial ITS2 region. The ML analysis was calculated using HKY evolutionary model and resulted the highest log likelihood of -1702.1285. ML bootstrap values (>50%) and Bayesian inference posterior probabilities (>0.5) are indicated at branches. *Crypthecodinium* strains isolated in this study are indicated in bold.**

analyses of microalgae have implemented the use of single or multiple DNA genes/markers to determine the taxonomical delineation of genera and species. Among those genes, the SSU and LSU rDNA were vastly used, especially in dinoflagellates [13], while the ITS rDNA gene were subsequently suggested as a potential barcoding marker [31]. Concatenation or combining SSU, ITS and LSU rDNA data sets in the analyses have been also suggested as this method might provide better understanding of genetic diversity in dinoflagellates [19, 26]. However, to the best of our knowledge, previous studies of *C. cohnii* mostly used SSU rDNA sequences as the single marker in the phylogenetic analyses [10, 20, 22]. Moreover, there was only one sequence of. *C. cohnii* available for the ITS and LSU rDNA genes from the GenBank (Table 2). Therefore, it was not possible to compare and perform phylogenetic analyses by combining SSU, ITS and LSU rDNA data sets of the Okinawan isolates with other reported *C. cohnii* strains due to the lack of available sequences. Nevertheless, in order to accommodate this issue, we performed separate phylogenetic analyses for each rDNA gene based on the availability of *C. cohnii* sequences for each respective marker (Figs. 3, 4 and 5).

Phylogenetic tree of SSU (Fig. 3), LSU (Fig. 4) and ITS (Fig. 5) sequences indicated close relatedness between the Okinawan strains and previously published strains of *Crypthecodinium cohnii*. Despite overall branching orders in the phylogenetic trees were not well resolved, the isolates formed a monophyletic group in SSU and LSU with other *Crypthecodinium* species, although *Crypthecodinium* sp. CAAE-CL2 [22] was positioned separately from other *C. cohnii* strains in SSU (Fig. 3). The *Crypthecodinium* clade was supported with high bootstrap values (>98%) and robust Bayesian PP in the base branch of the tree in SSU and LSY (Figs. 3 and 4). Subsequently the strains were grouped into two genetically distinct clades, the clade 1 and clade 2, in all analyses. Sequence similarity based on uncorrected *p*-distance estimation of multiple sequences alignment showed that within each clade, the strains shared very high sequence similarity (>97%) in SSU, ITS and LSU analyses (Table 3).

**Table 3. Sequence similarity (%) of *Crypthecodinium* strains within each clade.**

| | SSU | ITS | LSU |
|---|---|---|---|
| Clade 1 | 100% | 98.9% | 99.9% |
| Clade 2 | 100% | 97.8% | 99.9% |

Overall sequence identities between members of the two clades showed high similarity (>98%) in SSU and LSU analyses but only shared 80.5% similarity in ITS (Table. 4). Moreover, in comparison with other *Crypthecodinium* species, strains in clade 1 and clade 2 shared more than 98% of base pairs in SSU and LSU, except for *Crypthecodinium* sp. CAAE-CL2 in which the clades shared only 87.2% and 87.4% sequence similarity respectively in SSU rDNA (Table 4; Fig. 3). However, in ITS rDNA, members of both clades shared only 80.4% and 79.9% sequence similarity with the solely published ITS sequence of *C. cohnii* CCMP316 available from GenBank (Table 4, ITS rDNA). Based on this, the highly diverse ITS rDNA region of *C. cohnii*-like strains might potentially be used as barcoding marker for strains identification in future studies of this dinoflagellate. Based on the similarity found in the morphology and the high sequence similarity in SSU and LSU rDNA, all Okinawan isolates in this study were identified as related species of *Crypthecodinium cohnii* (Seligo) Javornicky [12] in this study. However, we refrain from suggesting further taxonomical delineation until more comprehensive data are available for this heterotrophic dinoflagellate.

The Okinawan isolates of *Crypthecodinium cohnii*-like species showed two genetically distinct clades in which members in each clade shared very high sequence similarity (>97%) in all analyses (Table 3). Interestingly, clade 1 consisted of strains that were isolated from two geographically distant locations: Ishigaki Island (ISG40-1) and Okinawajima Island

**Table 4. Sequence similarity (%) between clades and *Crypthedodinium cohnii* strains**

| Clade/strains | SSU rDNA | | ITS rDNA | | LSU rDNA | |
|---|---|---|---|---|---|---|
| | Clade 1 | Clade 2 | Clade 1 | Clade 2 | Clade 1 | Clade 2 |
| Clade 1 | - | 99.3 | - | 80.5 | - | 98.1 |
| Clade 2 | 99.3 | - | 80.5 | - | 98.1 | - |
| *C. cohnii* (M64245) | 99.2 | 99.4 | - | - | - | - |
| *C. cohnii* (FJ821501) | 99.2 | 99.4 | - | - | - | - |
| *C. cohnii* (DQ241737) | 98.7 | 98.9 | - | - | - | - |
| *Crypthecodinium* sp. CAAE-CL2 (DQ322643) | 87.2 | 87.4 | - | - | - | - |
| *C. cohnii* D31 (AB811790) | 99.2 | 99.9 | - | - | - | - |
| *C. cohnii* CCMP316 (FJ823534) | - | - | 80.4 | 79.9 | - | - |
| *C. cohnii* CCMP316 (FJ939575) | - | - | - | - | 95.1 | 95.0 |

(ISK1-1 and ISK1-2). Beam and Himes [1] suggested that global distribution of *C. cohnii* species were aided by tides and currents on which they attached on the surface fragments of seaweeds or other mediator. This might also explain why the strains presented in this study showed very high sequence similarity among them, although they were distantly dispersed in two different localities.

The current study showed that SSU and LSU rDNA genes were sufficient to be used for assigning the Okinawan isolates into two genetically distinct clades. However, we also suggest for future study, incorporating the molecular analyses of this dinoflagellate with ITS rDNA data set may provide better understanding in the diversity of *Crypthecodinium* species. For instance, *Symbiodinium* was once considered to consist only a single species based on morphology, but further study using ITS rDNA analysis revealed that *Symbiodinium* could be grouped into several genetically distinct clades containing many undescribed species [7]. The use of ITS rDNA gene for dinoflagellate barcoding has been also proposed by Stern *et al*. [31] and showed successful attempts in identifying and revealing hidden diversity of dinofalgellate species in culture collection.

### 3. Diversity of *Crypthecodinium* species from Okinawa

Previous studies have been performed to assess the diversity of the *Crypthecodinium cohnii* because isolated heterotrophic marine dinoflagellates having roughly similar morphology were often regarded only as strains or related species of this dinoflagellate. Most of the taxonomical studies of *C. cohnii* strains involved morphological investigations [1, 12, 23, 34], cell cycles study [4], reproductive compatibility assessment [2] and genetic analyses [3, 10, 29]. Unfortunately, the rDNA sequences of *C. cohnii* from these studies were not available for comparison in the current study. Despite variation in morphological characters were observed in our isolates (e.g. cingulum encirclement), the molecular phylogenetic grouping did not correspond well with the grouping based on morphological characteristic. Moreover, there has been only one study that combined detailed morphological characterization and phylogenetic analyses based on the SSU rDNA sequences of *Crypthecodinium* sp. [22]. Hence, it has not been possible to understand how the molecular properties corresponded with the morphological features of this heterotrophic marine dinoflagellate. Nevertheless, it was clear that the molecular phylogenetic analyses grouped the Okinawan isolates into therefore might well indicate that the taxonomy of *Crypthecodinium* is much more complex than it is currently considered. Geographic barrier might not be the cause of diversification of *C. cohnii* in this study as explained earlier.

In the present studies, the diversity of the *C. cohnii*-like species isolated from Okinawa Prefecture, Japan, is only partially resolved. There are at least two genetically distinct clades and two morphotypes based on the cingulum encirclement. This showed some evidence of diversification within the strains of *C. cohnii*, which potentially could be be assigned for taxonomical characterization in the future. However, further study involving more *C.cohnii* strains from other localities together with comprehensive morphological investigation and molecular analyses are needed to clarify the current taxonomy of this heterotrophic marine dinoflagellate.

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## REFERENCES

1. Beam, C. A. and Himes, M., "Distribution of members of the *Crypthecodinium cohnii* (Dinophyceae) species complex," *Journal of Protozoology*, Vol. 29, pp. 8-15 (1982).
2. Beam, C. A. and Himes, M., "Sexual isolation and genetic diversification among some strains of *Crypthecodinium cohnii* dinoflagellates: evidence of speciation," *Journal of Protozoology*, Vol. 24, pp. 532-539 (1977).
3. Beam, C. A., Preparata, R. M., Himes, M., and Nanney, D. L., "Ribosomal RNA sequencing of members of the *Crypthecodinium cohnii* (Dinophyceae) species complex; comparison with soluble enzyme studies," *Journal of Eukaryotic Microbiology*, Vol. 40, No. 5, pp. 660-667 (1993).
4. Bhaud, Y., Barbier, M., and Soyer-Gobillard, M. O., "A detailed study of the complex cell cycle of the dinofagellate *Crypthecodinium cohnii* B, and evidence for variation in histone H1 kinase activity," *Journal of Eukaryotic. Microbiology*, Vol. 41, pp. 519-526 (1994).

5. Biecheler, B., "Recherches sur les péridiniens," *Bulletin Biologique de France et de Belgique Supplément*, Vol. 36, pp. 1-149 (1952).
6. Chatton, E., "Classe des dinoflagellés ou péridiniens," in: Grass, P. P. (Ed.), *Traité de Zoologie*, Masson (1952).
7. Coffroth, M. A. and Santos, S. R., "Genetic diversity of symbiotic dinoflagellates in the genus *Symbiodinium*," *Protist*, Vol. 156, pp. 19-34 (2005).
8. Daugbjerg, N., Hansen, G., Larsen, J., and Moestrup, Ø, "Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmored dinoflagellates," *Phycologia*, Vol. 39, No. 4, pp. 302-317 (2000).
9. Felsenstein, J., "Confidence limits on phylogenies: an approach using the bootstrap," *Evolution*, Vol. 39, pp. 783-791 (1985).
10. Gajadhar, A. A., Marquardt, W. C., Hall, R., Gunderson, J., Ariztia-Carmona, E. V., and Sogin, M. L., "Ribosomal RNA sequences of *Sacrocystis muris*, *Theileria annulata* and *Crypthecodinium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates," *Molecular and Biochemical Parasitology*, Vol. 45, pp. 147-154 (1991).
11. Hansen, G. and Daugbjerg, N., "*Moestrupia oblonga* gen. & comb. nov. (syn.: *Gyrodinium oblongum*), a new marine dinoflagellate genus characterized by light and electron microscopy, photosynthetic pigments and LSU rDNA sequence," *Phycologia*, Vol. 50, No. 6, pp. 583-599 (2011).
12. Javornicky, P., "Two scarcely known genera of the class Dinophyceae: *Bernardinium* Chodat and *Crypthecodinium* Biecheler," *Preslia*, No. 34, pp. 98-113 (1962).
13. Ki, J. S., "Hypervariable regions (V1-V9) of the dinoflagellate 18S rRNA using a large dataset for marker considerations," *Journal of Applied Phycology*, Vol. 24, pp. 1035-1043 (2012).
14. Kofoid, C. and Swezy, O., "The free-living unarmored dinoflagellata," *Memoirs of the University of California*, Vol. 5 (1921).
15. Lewitus, A. J., Horner, R. A, Caron, D. A., Garcia-Mendoza, E., Hickey, B. M., Hunter, M., Huppert, D. D., Kudela, R. M., Langlois, G. W., Largier, J. L., Lessard, E. J., RaLonde, R., Jack Rensel, J. E., Strutton, P. G., Trainer, V. L., and Tweddle, J. F., "Harmful algal blooms along the North American west coast region: History, trends, causes, and impacts," *Harmful Algae*, Vol. 19, pp. 133-159 (2012).
16. Mansour, M. P., Volkman, J. K., Jackson, A. E., and Blackburn, S. I., "The fatty acid and sterol composition of five marine dinoflagellates," *Journal of Phycology*, Vol. 35, No. 4, pp. 710-720 (1999).
17. Mendes, A., Reis, A., Vasconcelos, R., Pedro Guerra, P., and da Silva, T. L., "*Crypthecodinium cohnii* with emphasis on DHA production: a review," *Journal of Applied Phycology*, Vol. 21, pp. 199-214 (2008).
18. Michael Guiry in Guiry, M. D. & Guiry, G. M., *AlgaeBase*, Retrieved December 10, 2013 from http://www.algaebase.org/search/species/ detail/?species_id=52213.
19. Murray, S., JØrgensen, M. F., Ho, S. Y. W., Patterson, D. J., and Jermiin, L. S., "Improving the analysis of dinoflagellate phylogeny based on rDNA," *Protist*, Vol. 156, pp. 269-286 (2005).
20. Okuda, T., Ando, A., Sakuradani, E., and Ogawa, J., "Isolation and characterization of a docosahexaenoic acid-phospholipids producing microorganism *Crypthecodinium* sp. D31," *Journal of the American Oil Chemists' Society*, Vol. 90, pp. 1837-1844 (2013).
21. Oren, A., "A hundred years of *Dunaliella* research: 1905-2005," *Saline Systems*, Vol. 1, No. 2 (2005).
22. Parrow, M. W., Elbrächter, M., Krause, M. K., Burkholder, J. M., Deamer, N. J., Htyte, N., and Allen, E. H., "The taxonomy and growth of a *Crypthecodinium* species (Dinophyceae) isolated from a brackish-water fish aquarium," *African Journal of Marine Science*, Vol. 28, No. 2, pp. 185-191 (2006).
23. Perret, E., Davout, J., Albert, M., Besseau, L., and Soyer-Gobillard, M.-O., "Microtubule organization during the cell cycle of the primitive eukaryote dinoflagellate *Crypthecodinium cohnii*," *Journal of Cell Science*, Vol. 104, pp. 639-651 (1993).
24. Ratledge, C., "Single cell oils for the 21st century," in: Cohen, Z., Ratledge, C. (Eds.), *Single Cell Oils*, AOCS Press, pp. 1-20 (2005).
25. Ronquist, F. and Huelsenbeck, J. P., "MRBAYES 3: Bayesian phylogenetic inference under mixed models," *Bioinformatics*, Vol. 19, pp. 1572-1574 (2003).
26. Saldarriaga, J. F., Taylor, F. J. R., Cavalier-Smith, T., Menden-Deuer, S., and Keeling, P. J., "Molecular data and the evolutionary history of dinoflagellates," *European Journal of Protistology*, Vol. 40, pp. 85-111 (2004).
27. Schiller, J., "Dinoflagellatae (Peridineae) in monographischer behandlung," in: Kolkwitz, R. (Ed.), *Rabenhorst's Kryptogamenflora*, Leipzig (1933).
28. Seligo, A., "Untersuchungen uber flagellaten," *Beiträge zur Biologie der Pflanzen*, Vol. 4, pp. 45-180 (1885).
29. Steele, R. and Rae, P. M. M., "Comparison of DNAs of *Crypthecodinium cohnii*-like dinoflagellates from widespread geographic locations," *Journal of Protozoology*, Vol. 27, pp. 479-493 (1980).
30. Steidinger, K. A., Landsberg, J. H., Truby, E. W., and Blakesley, B. A., "The use of scanning electron microscopy in identifying small "gymnodinioid" dinoflagellates," *Nova Hedwigia*, Vol. 112, pp. 415-422 (1996).
31. Stern, R. F., Andersen, R. A., Jameson, I., Küpper, F. C., Coffroth, M.-A., Vaulot, D., Le Gall, F., Véron, B., Brand, J. J., Skelton, H., Kasai, F., Lilly, E. L., and Keeling, P. J., "Evaluating the ribosomal internal transcribed spacer (ITS) as a candidate dinoflagellate barcode marker," *PLOS ONE*, Vol. 7, No. 8 (2012).
32. Takano, Y. and Horiguchi, T., "Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell," *Journal of Phycology*, Vol. 42, No. 1, pp. 251-256 (2005).
33. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., "MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods," *Molecular Biology and Evolution*, Vol. 28, pp. 2731-2739 (2011).
34. Ucko, M., Elbrächter, M., and Schnepf, E. A., "*Crypthecodinium cohnii* like dinoflagellate feeding myzocytotically on the unicellular red alga *Porphyridium* sp.," *European Journal of Phycology*, Vol. 32, pp. 133-140 (1997).
35. Yamaguchi, H., Nakayama, T., Kai, A., and Inouye, I., "Taxonomy and phylogeny of a new kleptoplastidal dinoflagellate, *Gymnodinium myriopyrenoides* sp. nov. (Gymnodiniales, Dinophyceae), and its cryptophyte symbiont," *Protist*, Vol. 162, No. 4, pp. 650-667 (2011).