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Cyst-motile stage relationship and molecular phylogeny of a new freshwater dinoflagellate *Gymnodinium plasticum* from Plastic Lake, Canada

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Abstract :

The dinophyceaen genus *Gymnodinium* was established with the freshwater species *G. fuscum* as type. According to Thessen *et al*. (2012), there are 268 species, with the majority marine species. In recently published molecular phylogenies based on ribosomal DNA sequences, *Gymnodinium* is polyphyletic. Here, a new freshwater *Gymnodinium* species, *G. plasticum*, is described from Plastic Lake, Ontario, Canada. Two strains were established by incubating single cysts, and their morphology was examined with light microscopy and scanning electron microscopy. The cyst had a rounded epicyst and hypocyst with a wide cingulum and smooth surface. Vegetative cells were characterized by an elongated nucleus running vertically and a deep sulcal intrusion. The apical structure complex was horseshoe-shaped and consisted of two pronounced ridges with a deep internal groove, encircling 80% of the apex. Small subunit ribosomal DNA (SSU rDNA), large subunit ribosomal DNA (LSU rDNA) and internal transcribed spacer (ITS) sequences were obtained from cultured strains. Molecular phylogeny based on concatenated SSU, LSU and ITS sequences supports the monophyly of the Gymnodiniales sensu stricto clade but our results suggest that many *Gymnodinium* species might need reclassification. *Gymnodinium plasticum* is closest to *Dissodinium pseudolunula* in our phylogeny but distant from the type species *G. fuscum*, as are the other gymnodiniacean taxa.

Keywords : apical structure complex, cyst, Gymnodiniales sensu stricto, Gymnodinium fuscum

INTRODUCTION

About 2,300 dinoflagellate species have been described in the world (Gómez 2012), only around 350 of them inhabiting freshwater (Mertens *et al.* 2012), suggesting that only a small fraction of marine dinoflagellate lineages has succeeded in crossing the marine-freshwater boundary (Logares *et al.* 2007b). Some genera (e.g. *Peridinium* Ehrenberg, *Peridiniopsis* Lemmermann) are exclusively freshwater and do not have marine representatives. Most transitions to freshwater appear to have occurred long ago based on molecular evidence (Logares *et al.* 2009), possibly because it would have been easier for marine immigrants to transition into freshwater environments before locally adapted communities with high species richness were established (Levine & D'Antonio 1999). Recent transitions have been reported in the phototrophic brackish *Apocalathium malmogiense* (G. Sjöstedt) Craveiro, Daugbjerg, Moestrup & Calado (=*Scrippsiella hangoei* (J. Schiller) J. Larsen) / freshwater *Apocalathium aciculiferum* (Lemmermann) Craveiro, Daugbjerg, Moestrup & Calado (=*Peridinium aciculiferum* Lemmermann), which share identical small subunit (SSU), large subunit (LSU) ribosomal DNA (rDNA) and internal transcribed spacer (ITS) region sequences (Logares *et al.* 2007a; Craveiro et al. 2016).

A few dinoflagellates are known to inhabit both marine and freshwater, e.g. *Huia caspica* (Ostenfeld) H. Gu, K.N. Mertens, T. Liu, *Durinskia baltica* (Levander) Carty & Cox, *Kolkwitziella acuta* Lindemann, probably due to recent transitions (Mertens *et al.* 2015; Gu *et al.* 2016).

The genus *Gymnodinium* F. Stein was erected by F. Stein (1878) with the

freshwater species *Gymnodinium fuscum* F. Stein as type (see Hansen *et al.* 2000 for more details). *Gymnodinium* was broadly defined and encompassed athecate dinoflagellates from both marine and freshwater whose cingulum displacement was less than 20% of the cell length (Kofoid & Swezy 1921). This initial classification based on cingulum displacement is now considered arbitrary and later more emphasis was placed on the systematic significance of the apical groove (Takayama 1985). *Gymnodinium* was then revised as possessing a horseshoe-shaped apical groove, a nuclear envelope with vesicular chambers and a nuclear or dorsal fibrous connector (NFC) (Daugbjerg *et al.* 2000). These revisions were based on detailed examinations of the freshwater species *G. fuscum* (the type species of *Gymnodinium*). Hansen *et al.* (2000) point out that *G. fuscum* lacks a transverse striated flagellar root and striated collars around the flagellar canals, which are rarely found in dinoflagellates; therefore, many species of *Gymnodinium* might need reclassification.

Moestrup *et al.* (2014) renamed the apical groove as apical structure complex (ASC) and revealed that the ASC comprises three elongated vesicles in *Levanderina fissa* (Levander) Ø. Moestrup, P. Hakanen, G. Hansen, N. Daugbjerg & M. Ellegaard. Such vesicles have also been reported in other gymnodinioid species, such as *Gymnodinium impudicum* (S. Fraga & I. Bravo) G. Hansen & Moestrup, *Barrufeta bravensis* N. Sampedro & S. Fraga and *B. resplendens* (Hulburt) H. Gu, Z. Luo & K.N. Mertens (Sampedro *et al.* 2011; Gu *et al.* 2015). The morphological details of the ASC in *G. fuscum*, however, were not investigated in detail (Hansen *et al.* 2000).

A review of the literature led Thessen *et al.* (2012) to propose that there are 268

extant *Gymnodinium* species, with only 66 from freshwater habitats. Most of these species, however, were described long ago without much detail. Therefore, once detailed examinations were conducted using modern techniques, some were transferred to other genera. The freshwater *Gymnodinium limneticum* Wołoszyńska and *G. palustre* A.J. Schiller were transferred into *Spiniferodinium* Horiguchi & Chihara because they exhibit capsoid cells as predominant life-history stage (Kretschmann *et al.* 2015), and the freshwater *G. acidotum* Nygaard and *G. aeruginosum* F. Stein were transferred into *Nusuttodinium* Takano & Horiguchi as they steal a temporary chloroplast by preying on cryptophytes (Takano *et al.* 2014).

Molecular phylogeny supports the transfer of these freshwater *Gymnodinium* species and supports a well resolved Gymnodiniales sensu stricto clade, consisting of *Gymnodinium* and a variety of genera, e.g. *Barrufeta* N. Sampedro & S. Fraga, *Dissodinium* Klebs, *Gyrodiniellum* N.S.Kang, H.J. Jeong & Moestrup, *Lepidodinium* Watanabe, Suda, Inouye, Sawaguchi & Chihara, *Nematodinium* C.A. Kofoid & O. Swezy, *Paragymnodinium* N.S. Kang, H.J. Jeong, Moestrup & W. Shin, *Polykrikos* Bütschli, S*piniferodinium*, *Warnowia* Lindemann (Hoppenrath & Leander 2007; Hoppenrath *et al.* 2009; Kang *et al.* 2010; Hansen & Daugbjerg 2011; Kang *et al.* 2011; Kretschmann *et al.* 2015). To fully understand the genus *Gymnodinium*, it is essential to incorporate more sequences from freshwater species. Sequences are currently available for only three freshwater *Gymnodinium* species, i.e. *G. fuscum*, *G. baicalense* N.L. Antipova and *G. impatiens* Skuja, and the closest relative of *G. fuscum* is not clear (Kretschmann *et al.* 2015). Moreover, the SSU sequence of *G.*

impatiens is close to *Gyrodinium* and falls outside the Gymnodiniales sensu stricto clade (Kang *et al.* 2014).

Approximately 84 freshwater dinoflagellates are known to produce cysts, including 16 *Gymnodinium* species (Mertens *et al.* 2012). The cyst morphology of *Gymnodinium* species is extremely variable, ranging from round to ellipsoidal, with or without ornamentations. Some (e.g. *Gymnodinium dodgei* Sarma & Shyam) have been transferred based on cyst morphology (Luo *et al.* 2016), but cyst-motile cell relationship of additional *Gymnodinium* species is needed. Here we isolate two cysts from surface sediments collected from Plastic Lake, Canada and establish the cyst-motile cell relationship of a new *Gymnodinium* species. We also sequence the SSU, LSU rDNA and ITS sequences to infer its phylogenetic position by concatenating the sequences.

MATERIAL AND METHODS

Surface sediment samples were collected from ca. 3 m water depth on the lakebed of Plastic Lake, Canada (45°18.34' N, 78°83.29' W) using a grab sampler. The sediment samples were stored in the dark at 4°C until further treatment. Approximately 5 g of wet sediment were mixed with 20 mL of sterilized MilliQ water and sonicated for 2 min (100 watts) to dislodge detrital particles. Single cysts were isolated by means of drawn-out Pasteur pipettes under an AE30 inverse microscope (Motic, Xiamen, China) and incubated in 96 well culture plate filled with 300 μL carefoot medium (Carefoot 1968) at 20ºC, 90 μE m^{-2} s^{-1} under a 12:12 h light: dark cycle (hereafter called

"standard culture conditions"). Germinated cells were maintained under standard culture conditions and two strains (TIO826 and TIO827) were established.

Vegetative cells of both strains were examined under a Zeiss Axio Imager microscope (Carl Zeiss, Göttingen, Germany) equipped with both differential interference illumination and epifluorescence. Light micrographs were obtained using a Zeiss Axiocam HRc digital camera. Approximately 1 mL of live, healthy culture in mid exponential growth phase was transferred to a 1.5 mL microcentrifuge tube, and DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) stain (Sigma-Aldrich, St. Louis, Missouri, USA) was added at a final concentration of 10 μg mL^{-1}. The cells were viewed and photographed through a Zeiss Filterset (emission: BP 365-445; beamsplitter: FT 395). Cells in mid exponential growth phase were fixed with 5% Lugol's solution, and cell size was measured at 400× magnification. Forty cells were measured for strains TIO826 and TIO827.

Mid-exponential batch cultures (600 μL) were fixed for 1 h at 4°C with 4% OsO_4 (200 μL) prepared with filtered MilliQ water. The material was then dehydrated in an ethanol series (once in 10, 30, 50, 70 and 90%, followed by three times in 100%; 10 min at each step), critical point dried (K850 Critical Point Dryer, Quorum/Emitech, West Sussex, UK), sputter-coated with gold, and examined using a Zeiss Sigma FE (Carl Zeiss Inc., Oberkochen, Germany) scanning electron microscope.

Single cells were isolated and washed three times with sterilized bi-distillate water and were used as the template to amplify about 1,430 bp of the LSU rRNA gene (D1-D6 domains), using the primers D1R (forward,

5′-ACCCGCTGAATTTAAGCATA-3′) (Scholin *et al.* 1994), 28-1483R (reverse, 5′-GCTACTACCACCAAGATCTGC-3′) (Daugbjerg *et al.* 2000), 1,740 bp of the SSU rRNA gene, using the primers SR1 (forward, 5′-TACCTGGTTGATCCTGCCAG-3′) and SR12b (reverse, 5′-CGGAAACCTTGTTACGACTTCTCC-3′) (Takano & Horiguchi 2006), and 600 bp of the total ITS1–5.8S–ITS2, using the primers ITSA (forward, 5′-CCTCGTAAC AAGGHTCCGTAGGT-3′), ITSB (reverse, 5′-CAGATGCTTAARTTCAGCRGG) (Adachi *et al.*, 1996). A 50 µL PCR cocktail containing 0.2 µM forward and reverse primer, PCR buffer, 50 µM dNTP, 1U of Taq DNA polymerase (Takara, Dalian, China) was subjected to 35 cycles using a Mastercycler PCR (Eppendorf, Hamburg, Germany). The PCR protocol was identical to that of (Gu *et al.* 2015). PCR products were sequenced directly in both directions using the ABI Big-Dye dye-terminator technique (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. New sequences were deposited in GenBank with accession numbers from KY688184 to KY688189.

New sequences obtained in this study were incorporated into those of closely related species available in the GenBank and that of outgroup taxa were first aligned using 'BioEdit' v7.0.0 (Hall 1999), and then using Mafft (Katoh *et al.* 2005) (http://mafft.cbrc.jp/alignment/server/). The program JModelTest (Posada 2008) was used to select the most appropriate model of molecular evolution with Akaike information criterion (AIC). This test chose the general time-reversible (GTR) model of substitution (Rodriguez *et al.* 1990) following a gamma distribution shape

parameter (0.4080) (GTR+ G). Maximum likelihood-based analyses were conducted using with RAxML v7.2.6 (Stamatakis *et al.* 2008) on the T-REX web server (Boc *et al.* 2012). The Gamma model was selected and 1,000 bootstraps were carried out.

A Bayesian reconstruction of the data matrix was performed with MrBayes 3.0b4 (Ronquist & Huelsenbeck 2003) using the best-fitting substitution model. Four Markov chain Monte Carlo (MCMC) chains ran for five million generations, sampling every 1,000 generations, with an appropriate burnin (10%). A majority rule consensus tree was created in order to examine the posterior probabilities of each clade.

RESULTS

Gymnodinium plasticum N. Wang, Z. Luo, K. N. Mertens, F.M.G. McCarthy, H. Gu sp. nov.

Diagnosis

Vegetative cells with rounded epicone and hypocone, 25.5–36.1 µm long, and 21.2–27.6 µm wide, inhabiting fresh water. Chloroplasts in the periphery forming a network and nucleus elongated longitudinally. There are 9–10, 5 and 8–10 rows of vesicles on the epicone, cingulum and hypocone, respectively. The cingulum is deeply incised with a descending displacement of approximately one cingular width. The sulcus intrudes deeply into the epicone. There is a circular orange stigma in the sulcal area. The apical structure complex is horseshoe shaped encircling the apex around 80%. The ASC consists of two pronounced ridges and a deep groove inside. The cyst has a rounded epi- and hypocyst, 30.8–40.6 µm long, 28.0–35.4 µm wide and

23.8–29.5 µm deep. The cyst is rectangular in lateral view and trapezoidal in polar view. The cyst has a wide cingulum and covered by a thin wall with smooth surface.

Holotype

SEM stub of strain TIO826 deposited at Third Institute of Oceanography, SOA, Xiamen 361005, China.

Type locality

Plastic Lake (45°18.34' N, 78°83.29' W), a small oligo-mesotrophic holomictic lake dating back to retreat of the Laurentide Ice Sheet from south-central Ontario, Canada, ca. 11,000 years ago.

Etymology

'plasticum' derives from Plastic Lake, refers to the type locality.

GenBank accession number sequences

KY688188 (SSU), KY688184 (LSU) and KY688186 (ITS) of strain TIO826.

Description

The vegetative cells of *Gymnodinium plasticum* (strain TIO826) are 25.5–36.1 µm long (mean = 30.2±2.5 µm, n = 42) and 21.2–27.6 µm wide (mean = 24.7±2.5 µm, n =42). Vegetative cells of strain TIO827 are 25.0–34.1 µm long (mean =29.7±2.4 µm, n = 40) and 19.8–26.4 µm wide (mean = 23.5±2.6 µm, n = 40). They share the same cell morphology with TIO826. They display a characteristic golden colour (Fig. 1), and only a small fraction can form paired chains in optimal conditions (Fig. 2). Many band-like chloroplasts are interconnected to form a network in the periphery of the cell (Figs 3, 4). A few round starch rings, presumably stalked pyrenoids are located

near the cingulum and a circular orange stigma is present in the sulcal area (Fig. 5). The nucleus is elongated running nearly vertically, and occupies most of the cell spanning from the epicone to the hypocone (Figs 3, 6). The epicone is nearly equal in size to the hypocone. Both the epicone and the hypocone are rounded (Figs 3–5). The cingulum is deeply incised and has sharp edges, with a descending displacement of approximately one cingular width. The sulcus is narrow and deeply excavated, especially in the intercingular area due to the projection of right epicone (Fig. 1).

Under SEM, the cells show amphiesmal vesicles which are either pentagonal or hexagonal (Figs 7–10). As many as seven amphiesmal pores (around 0.5 μm in diameter) are present in the epicone and hypocone (Fig. 9). There are around 9–10, 5 and 8–10 rows of vesicles on the epicone, cingulum and hypocone, respectively. The sulcus intrudes around 60% of the epicone where it connects to the onset of the apical structure complex (ASC) (Figs 7, 8). The ASC is horseshoe-shaped and joins the sulcal intrusion, forming the shape of a question mark. The ASC runs anticlockwise, and encircles the apex around 80%. The ASC consists of two pronounced ridges and a deep groove inside (Figs 10, 12). The outer ridge is ornamented with numerous small knobs (Figs 13, 14).

The cyst from the field has a rounded epicyst and a rounded to conical hypocyst (Figs 15, 16), around 30.8–40.6 μm long, 28.0–35.4 μm wide and 23.8–29.5 μm deep (n = 6). The cyst wall is thin, and is underlain by a thicker endospore layer. The cyst is rectangular in lateral view and trapezoidal in apical and antapical view and is excavated on the ventral side (Figs 17, 18). The cyst is filled with brown granules and

there is a conspicuous red accumulation body inside and a spherical nucleus (Figs 15–17). The cyst has a wide cingulum, accounting for approximately 40% of the cyst length, and has a smooth surface (Figs 19, 20). We could not observe the archeopyle.

Molecular phylogenetics

Two strains of *Gymnodinium plasticum* share identical SSU, LSU and ITS sequences, and they differ from *G. fuscum* at 40, 193 and 233 positions (97.64%, 74.20% and 61.42% similarity), respectively. *Gymnodinium plasticum* differs from *Dissodinium pseudolunula* Swift ex Elbrächter & Drebes at 40, 133 positions (96.58% and 86.66% similarity) of partial SSU and LSU sequences, and differs from *G. baicalense* at 7 and 69 positions (99.0% and 80.5% similarity) of partial SSU (709 bp) and ITS sequences.

Maximum likelihood and Bayesian inference generate identical trees (Fig. 21) by using concatenated data from SSU, ITS and LSU sequences. *Gymnodinium plasticum* and *Dissodinium pseudolunula* form a clade with maximum bootstrap support (100), but low Bayesian posterior probability (0.73), which is a sister clade comprising *G. baicalense*, *G. aureolum* (Hulburt) G. Hansen, *G. corollarium* A. M. Sundström, Kremp & Daugbjerg, *Chytriodinium* sp., and *Pheopolykrikos beauchampii* Chatton with maximum bootstrap support (100), but low Bayesian posterior probability (0.67). They are nested within the well resolved Gymnodiniales sensu stricto clade (1.0/100) comprising amongst others *Barrufeta*, *Polykrikos*, *Nematodinium*, *Nusuttodinium*, *Spiniferodinium*, *Lepidodinium*, *Paragymnodinium*, *Warnowia*, *Nematodinium*, *Gyrodiniellum* and *Gymnodinium*. *Gymnodinium fuscum* diverges earliest in the Gymnodiniales clade but is not well supported.

DISCUSSION

The equatorial cingulum with slight displacement and the horseshoe-shaped ASC justifies classifying our strains in the genus *Gymnodinium* (Kofoid & Swezy 1921; Daugbjerg *et al.* 2000). Under light microscope, *G. plasticum* was characterized by a deep sulcal intrusion and an elongated nucleus running nearly vertically. Few freshwater species reported are similar to *G. plasticum* (Table 1). Both *Gymnodinium limitatum* Skuja and *G. mirabile* Penard have deep sulcal intrusions, but *G. mirabile* is much larger (48–68 µm long, 46–62 µm wide) and has a small nucleus located in the hypocone (Pénard 1891, plate V, fig. 1). *Gymnodinium limitatum* has a spherical nucleus and produce spherical cysts (Skuja 1956, Tafel LXI, figs 29–31). *Gymnodinium impatiens* has no sulcal intrusion and is much smaller and produces ellipsoidal cysts (Skuja 1964, Tafel LXVI, figs 31–34). *Gymnodinium uberrimum* (Allman) Kofoid & Swezy is of similar size and can form paired cell chains too but its nucleus is rounded and small and it has slight sulcal intrusion (Allman 1855, pl. 3, figs. 9–17). *Gymnodinium plasticum* is also similar to *G. excavatum* Nygaard but the latter has a pronounced eyespot (Nygaard 1945), thus was considered as a synonym of *Biecheleria pseudopalustris* (J. Schiller) Moestrup, Lindberg & Daugbjerg (Moestrup *et al.* 2009). *Gymnodinium plasticum* differs from *G. fuscum* in the shape of hypocone and nucleus, the configuration of chloroplasts and the cyst morphology (Hansen *et al.* 2000). *Gymnodinium plasticum* is also similar to some marine athecate species in general shape and sulcal intrusion, including *Gymnodinium mundulum* Campbell, *Gymnodinium aureolum* , *Gymnodinium polycomma* Larsen, *Gymnodinium*

corollarium and *G. litoralis* A. Reñé (Campbell 1973; Larsen 1994; Sundström *et al.* 2009; Hansen et al. 2000; Reñé *et al.* 2011), but differ in the cell size, elongated nucleus running vertically, and banded chloroplasts forming a network (Table S1).

The cyst of *G. plasticum* resembles some previously described cysts. It superficially resembles the cyst of the freshwater species *Woloszynskia tylota* (H. Mapletoft, M. Montgomery, J. Waters & P. Wells) B.T. Bibby & J.D. Dodge in the general shape and a wide cingulum, but differs in the lack of a pronounced apical horn and eight additional protuberances in the four corners (Mapletoft *et al.* 1966). It also somewhat resembles the cyst of the freshwater species *G. chiastosporum* (Harris) Cridland, but differs in the absence of projections at each edge (Harris 1940). The cyst is also different from that of the freshwater species *Glenodinium segriense* Dangeard, which has a pronounced apical and antapical horn (Dangeard 1939). The cyst of *G. plasticum* also somewhat resembles that of the marine species *G. trapeziforme* Attaran-Fariman & Bolch (Attaran-Fariman *et al.* 2007), but differs in the absence of microreticulation on the surface and in its transparent color (as opposed to brown). Based on the differences from previously reported motile cells and cysts, we consider our strains to belong to a new *Gymnodinium* species, *Gymnodinium plasticum*.

The ASC of *Gymnodinium plasticum* consists of a deep groove surrounded by two pronounced ridges. Such a type of ASC was also observed in the marine species *G. trapeziforme* (Attaran-Fariman *et al.* 2007), *G. aureolum* (Tang *et al.* 2008), *G. corollarium* (Sundström *et al.* 2009), *G. catenatum* Graham, and *G. microreticulatum* Bolch & Hallegraeff (Gu *et al.* 2013), as well as in the freshwater species *G. fuscum*, *G.*

mirabile, and *G. obesum* Schiller (Hansen & Flaim 2007). Species from closely related genera, e.g. *Polykrikos lebourae* Herdman (Hoppenrath & Leander 2007), *Nematodinium armatum* (Dogiel) Kofoid & Swezy (Takayama 1985), *Spiniferodinium limneticum* (Wołoszyńska) Kretschmann & Gottschling (Kretschmann *et al.* 2015), *Nusuttodinium acidotum* (Nygaard) Takano & Horiguchi (Takano *et al.* 2014) also have a similar ASC, which was tentatively named "Type I". Another kind of ASC consists of three elongated vesicles, which might correspond to the two prominent ridges and a deep groove inside observed in *Gymnodinium plasticum*. This ASC has been reported for *Barrufeta bravensis, B. resplendens, G. litoralis,* and *Gymnodinium impudicum* (Reñé *et al.* 2011; Sampedro *et al.* 2011; Gu *et al.* 2015). These vesicles can be ornamented with numerous small knobs (Sampedro *et al.* 2011; Moestrup *et al.* 2014; Gu *et al.* 2015) or without small knobs (Reñé *et al.* 2011). The last kind of ASC ("Type II") consists of one row of amphiesmal vesicles with numerous small knobs, reported in *Gymnodinium smaydae* Kang, Jeong & Moestrup (Kang *et al.* 2014) and *Gyrodiniellum shiwhaense* Kang, Jeong & Moestrup (Kang *et al.* 2011).

The shape of ASC can be much more variable, although the term "horseshoe-shaped" was generally used to describe ASC in *Gymnodinium* species. The shape of ASC can be circular (e.g. *Gymnodinium mirabile*) (Hansen & Flaim 2007), fish-hook shaped (e.g. *Spiniferodinium limneticum*) (Kretschmann *et al.* 2015), or Smurfcap-shaped (e.g. *Barrufeta bravensis*) (Sampedro *et al.* 2011). Even for species with an ASC of similar shape, the details can vary. For instance, *G. impudicum* has small knobs on each elongated vesicle, whereas *Barrufeta bravensis* only has

small knobs on the middle row of vesicles (Sampedro *et al.* 2011).

Our molecular phylogeny generally supports previous results based on LSU or SSU sequences regarding the monophyly of Gymnodiniales sensu stricto clade (Reñé *et al.* 2011; Kang *et al.* 2014; Reñé *et al.* 2015). Kretschmann *et al.* (2015) demonstrated that *Gymnodinium fuscum* is distant from other *Gymnodinium* species using concatenated data of SSU, LSU ribosomal DNA, ITS sequences, mitochondrial and actin gene sequences although with low support, which is confirmed by our molecular phylogeny. As a result, nomenclature changes might be expected for many *Gymnodinium* species. This has been suggested previously as *G. fuscum* is unique in the ultrastructure detail, i.e. lack of a transverse striated flagellar root and striated collars around the flagellar canals (Hansen *et al.* 2000).

The close relationship between *Dissodinium pseudolunula* and Gymnodiniales sensu stricto species was reported by Kim *et al.* (2008), and here we further demonstrate that it is closest to G. *plasticum*, which might be explained by the fact that *D. pseudolunula* has *Gymnodinium*-like swarmers (Kofoid & Swezy 1921; Swift 1973). They group together with *G. baicalense*, *G. aureolum*, *G. corollarium*, and *Chytriodinium* sp., encompassing both marine and freshwater species, with a lifestyle varying from autotrophic to parasitic. We follow Reñé *et al.* (2015) in using the Gymnodiniales sensu stricto clade to incorporate those species previously also known as *Gymnodinium* sensu stricto clade (Reñé *et al.* 2011; Kang *et al.* 2014). This clade incorporates three groups of freshwater species, supporting the idea that marine-fresh water transitions are infrequent (Logares *et al.* 2007b). The evolution of species like *G.*

plasticum may have been facilitated during Quaternary deglaciations by the creation of numerous freshwater lakes lacking established ecosystems, as suggested by Levine and D'Antonio (1999).

According to the diagnosis by Daugbjerg *et al.* (2000), *Gymnodinium* is characterized by horseshoe-shaped ASC, nuclear chambers and a NFC (Daugbjerg *et al.* 2000). These features are also shared by *Gymnoxanthella radiolariae* T. Yuasa & T. Horiguchi, and *Lepidodinium viride* M. M. Watanabe, S. Suda, I. Inouye Sawaguchi & Chihara (Hansen *et al.* 2007; Yuasa *et al.* 2016). Therefore, the unequivocal definition of *Gymnodinium* might require more details from ASC and additional ultrastructure. Because our strains of *Gymnodinium plasticum* could not be maintained, it was not possible to obtain ultrastructural information; this will be the focus of future work.

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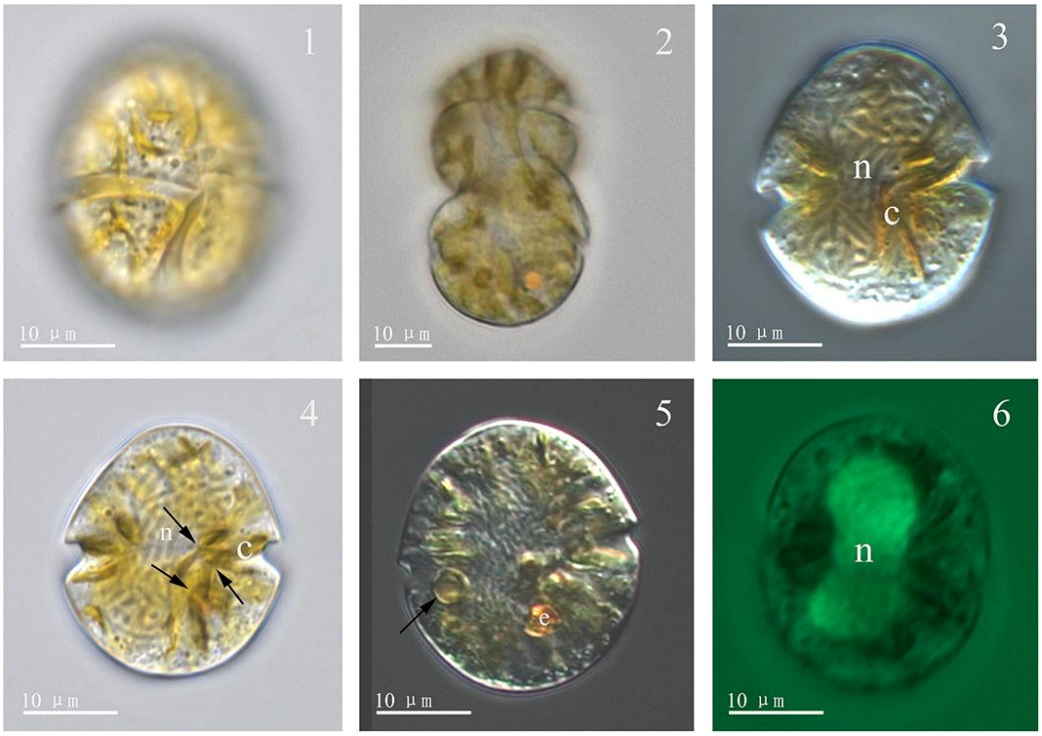
Figs 1–6. Light microscopy (LM) of vegetative cells of *Gymnodinium plasticum* strain TIO826. 1. Ventral view showing the sulcus. 2. A two-cell chain. 3. Ventral view showing the elongated nucleus (n) and several chloroplasts (c). 4. The same cell in fig. 3 with different focus showing the peripheral chloroplasts (c) connections (arrows). 5. Ventral view showing a presumable pyrenoid (arrow) and eyespot (e). 6. A DAPI-stained cell, showing the elongated nucleus (n).



Figs 7–13. Scanning electron microscopy (SEM) of vegetative cells of *Gymnodinium plasticum* strain TIO826. 7. Left lateral view showing the projection of right epicone. 8. Apical view showing the sulcal intrusion and the apical structure complex. 9. Several amphiesmal pores in the dorsal epicone. 10. Lateral view showing the sulcal intrusion and polygonal vesicles on the epicone. 11. Lateral view showing polygonal vesicles on the cingulum and hypocone. 12. Detail of the apical structure complex

showing two prominent ridges and a deep groove inside. 13. Detail of the apical structure complex showing small knobs in the outer ridge (arrows).

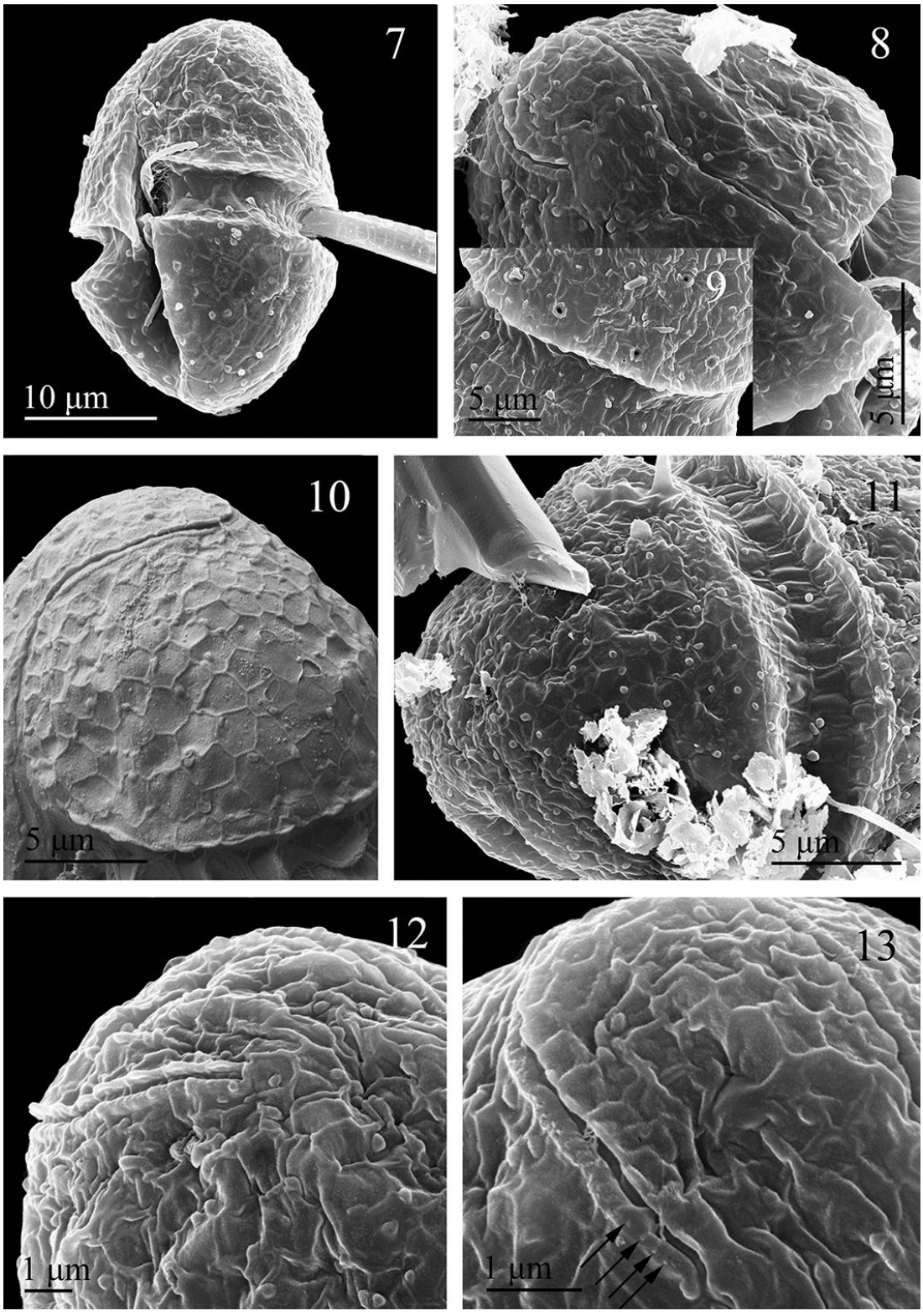
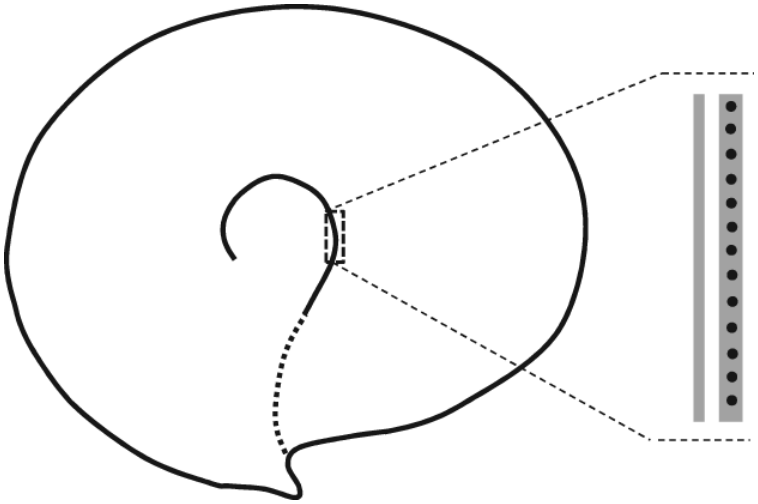


Fig. 14. Schematic illustration of the apical structure complex of *Gymnodinium plasticum*.



Figs 15–20. Light microscopy (LM) and scanning electron microscopy (SEM) micrographs of live cysts of *Gymnodinium plasticum*. 15. Ventral view of the cyst yielding strain TIO826, showing a wide cingulum and a pronounced red accumulation body (ab) in the hypocyst (LM). 16. Ventral view of a cyst from the field, showing a round nucleus (n) (LM). 17. Lateral view of a cyst from the field, showing the rectangular shape (LM). 18. Apical view of a cyst from the field, showing the trapezoidal shape. 19, 20. Cysts formed in culture of strain TIO826, showing the wide cingulum (C) (SEM).

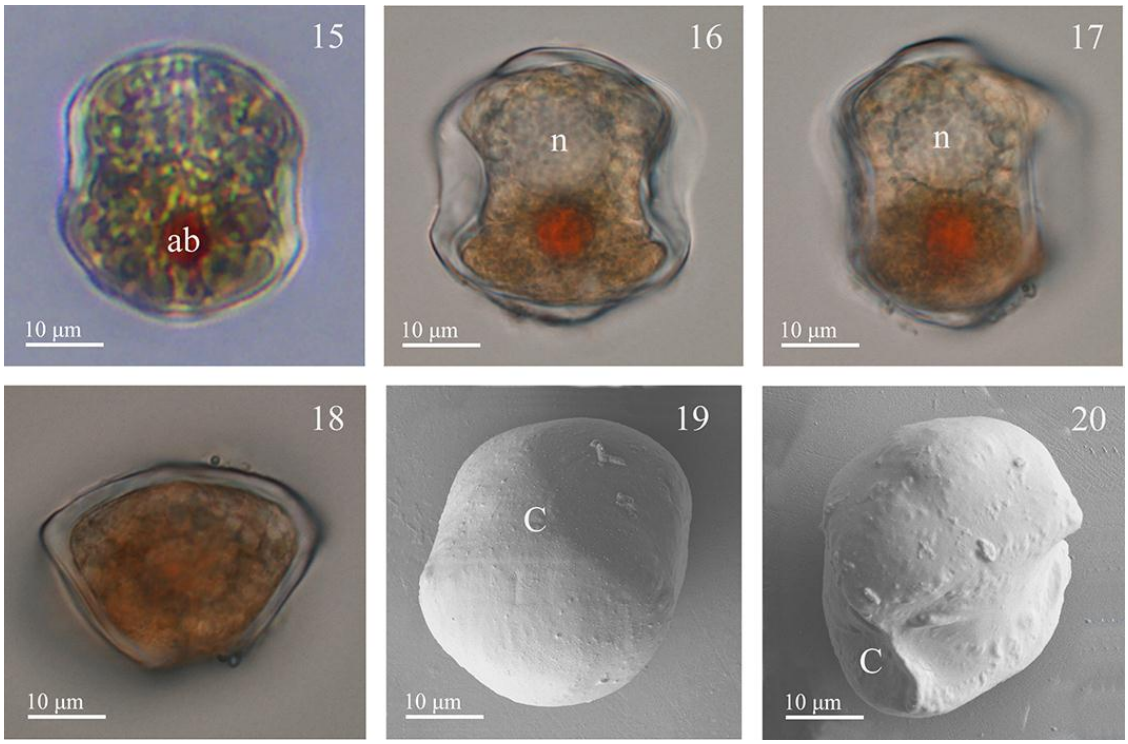
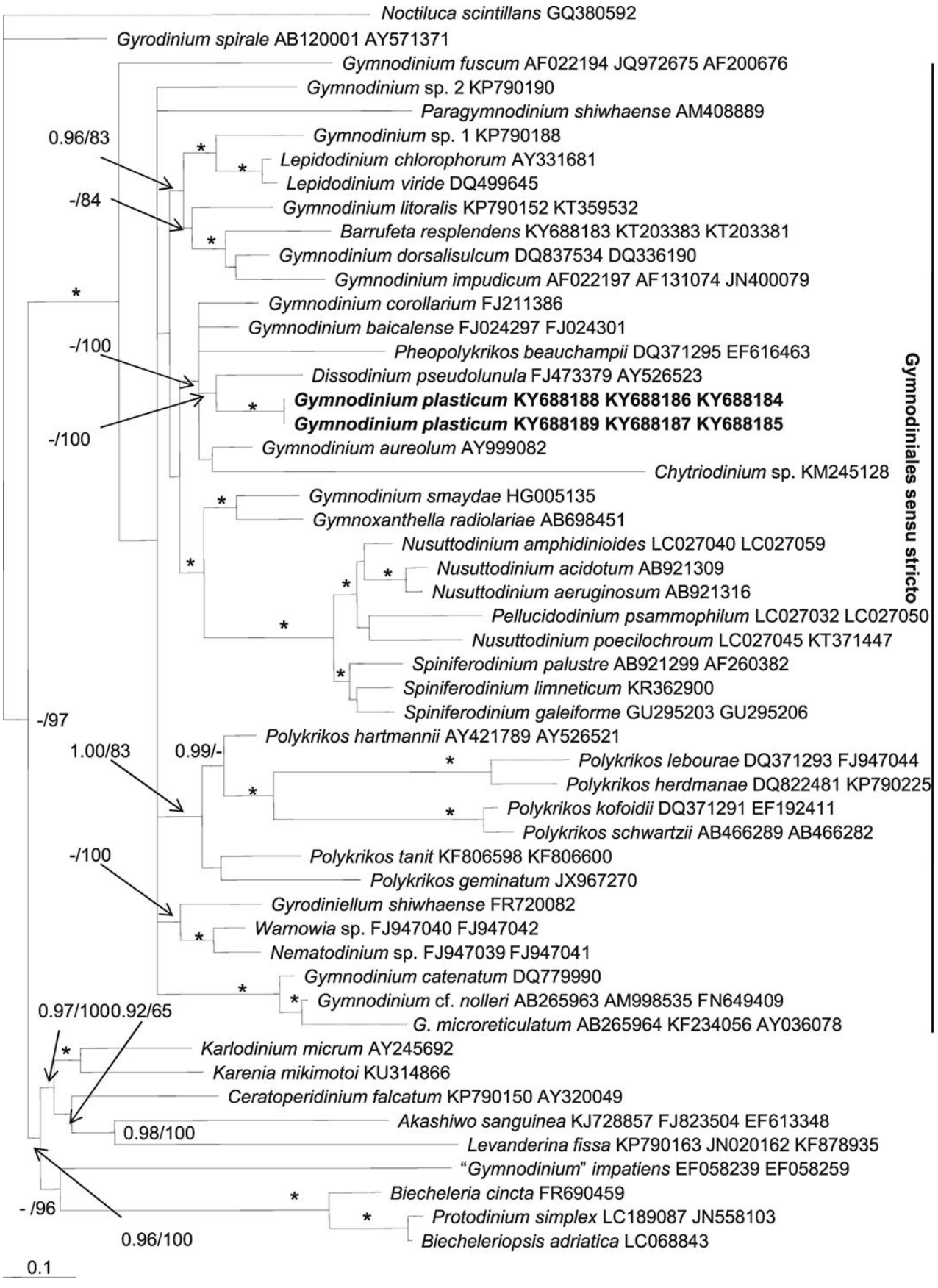


Fig. 21. Phylogeny of *Gymnodinium plasticum* inferred from concatenated data of small subunit, partial large subunit rDNA and internal transcribed spacer sequences using Bayesian inference (BI). Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Dashed lines indicate half of the length. Numbers on branches are statistical support values to clusters on the right of them (left: Bayesian posterior probabilities; right: maximum likelihood bootstrap support values). Posterior probability (>0.9) for BI support/Bootstrap value (>50%) for maximum likelihood (ML) are shown. * indicates maximal support (BI posterior probability = 1.00/ML bootstrap support = 100). New sequences are indicated in bold with the order of SSU, ITS and LSU.


Noctiluca scintillans GQ380592
Gyrodinium spirale AB120001 AY571371
Gymnodinium fuscum AF022194 JQ972675 AF200676
Gymnodinium sp. 2 KP790190
Paragymnodinium shiwhaense AM408889
0.96/83
Gymnodinium sp. 1 KP790188
Lepidodinium chlorophorum AY331681
Lepidodinium viride DQ499645
-/84
Gymnodinium litoralis KP790152 KT359532
Barrufeta resplendens KY688183 KT203383 KT203381
Gymnodinium dorsalisulcum DQ837534 DQ336190
Gymnodinium impudicum AF022197 AF131074 JN400079
Gymnodinium corollarium FJ211386
Gymnodinium baicalense FJ024297 FJ024301
-/100
Pheopolykrikos beauchampii DQ371295 EF616463
Dissodinium pseudolunula FJ473379 AY526523
Gymnodinium plasticum KY688188 KY688186 KY688184
Gymnodinium plasticum KY688189 KY688187 KY688185
-/100
Gymnodinium aureolum AY999082
Chytriodinium sp. KM245128
Gymnodinium smaydae HG005135
Gymnoxanthella radiolariae AB698451
Nusuttodinium amphidinioides LC027040 LC027059
Nusuttodinium acidotum AB921309
Nusuttodinium aeruginosum AB921316
Pellucidodinium psammophilum LC027032 LC027050
Nusuttodinium poecilochroum LC027045 KT371447
Spiniferodinium palustre AB921299 AF260382
Spiniferodinium limneticum KR362900
Spiniferodinium galeiforme GU295203 GU295206
-/97
Polykrikos hartmannii AY421789 AY526521
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0.99/-
Polykrikos lebourae DQ371293 FJ947044
Polykrikos herdmanae DQ822481 KP790225
Polykrikos kofoidii DQ371291 EF192411
Polykrikos schwartzii AB466289 AB466282
-/100
Polykrikos tanit KF806598 KF806600
Polykrikos geminatum JX967270
Gyrodiniellum shiwhaense FR720082
Warnowia sp. FJ947040 FJ947042
Nematodinium sp. FJ947039 FJ947041
Gymnodinium catenatum DQ779990
Gymnodinium cf. nolleri AB265963 AM998535 FN649409
G. microreticulatum AB265964 KF234056 AY036078
0.97/1000.92/65
Karlodinium micrum AY245692
Karenia mikimotoi KU314866
Ceratoperidinium falcatum KP790150 AY320049
Akashiwo sanguinea KJ728857 FJ823504 EF613348
0.98/100
Levanderina fissa KP790163 JN020162 KF878935
"Gymnodinium" impatiens EF058239 EF058259
Biecheleria cincta FR690459
- /96
Protodinium simplex LC189087 JN558103
0.96/100
Biecheleriopsis adriatica LC068843
0.1
Gymnodiniales sensu stricto

Table 1. Morphological comparisons of *Gymnodinium plasticum* with related species (N.A. = not available)

	G. plasticum	*G. fuscum*	*G. impatiens*	*G. limitatum*	*G. mirabile*	*G. uberrimum*
Cell size	26–36 µm long, 21–28 µm wide	50–80 µm long	18–21 µm long, 11–14 µm wide	24–38 µm long, 20–34 µm wide	48–68 µm long, 46–62 µm wide	40–51 µm long, 38–42 µm wide
Cell shape	Rounded epicone and hypocone	Rounded epicone, conical hypocone	Trapezoidal epicone, rounded hypocone	Rounded epicone and hypocone	Rounded epicone and hypocone	Subconical epicone and hypocone
Apical structure complex	Type I, encircles 80% of the apex	Type I, encircles 80% of the apex	N.A.	N.A	Type I, encircles 95% of the apex	N.A.
Cingulum	Descending	Descending	Descending	Descending	Descending	Descending
Sulcal intrusion	Deep sulcal intrusion	A tiny extension	None	Deep sulcal intrusion	Deep sulcal intrusion	Slight sulcal intrusion
Chloroplasts	Banded, forming a	Numerous small	Radially arranged	Radially	Radially arranged	Radially arranged

	network	elongate		arranged		
Nucleus	Elongated running nearly vertically	Spherical	Spherical	Spherical	Spherical	Spherical or ellipsoidal
Cyst size	31–41 µm long, 28–35 µm wide	40 µm in diameter	18–20 µm long, 13–14 µm wide	N.A.	N.A.	N.A.
Cyst shape	Rounded epicyst and hypocyst	Spherical	Ellipsoidal	Spherical	N.A.	N.A.
Reference	Present study	Hansen *et al.* 2000	Skuja 1964	Skuja 1956	Pénard 1891; Hansen 2007	Allman 1855; Kofoid & Swezy 1921