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Pyramidodinium spinulosum sp. nov. (Dinophyceae), a sand-dwelling non-motile dinoflagellate from the seafloor (36 m deep) off Mageshima Island, Kagoshima, Japan

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Running title: *Pyramidodinium spinulosum* sp. nov.

SUMMARY

A new species of benthic marine dinoflagellate, *Pyramidodinium spinulosum* Horiguchi, Moriya, Pinto & Terada is described from the deep (36 m) seafloor off Mageshima Island, Kagoshima Prefecture, Japan in the subtropical region of the northwest Pacific. The life cycle of the dinoflagellate consists of a dominant, attached, dome-shaped, vegetative form and short-lasting, motile cell. Asexual reproduction takes place by the formation of two motile cells within each non-motile cell. The released motile cells swim only for a short period and transform directly into the dome-shaped vegetative form. The duration of the cell cycle varies and can be extremely long, ranging 5 to 38 days under culture conditions. The non-motile cell is enclosed by a cell wall and its surface is covered with many (80–130) spines of various length. The dinoflagellate is photosynthetic and contains many (more than 50) discoidal chloroplasts. Phylogenetic analysis reveals that the dinoflagellate is closely related to the type species of the genus *Pyramidodinium*, *P. atrofuscum* which also possesses a dominant, attached, non-motile

form. However, *P. spinulosum* can be clearly distinguished from *P. atrofuscum* by the cell shape (dome-shaped vs. pyramid-shaped) and surface ornamentation (spines vs. wart-like processes) of the non-motile form. Based on these morphological differences together with molecular evidences, it was concluded that this organism from a deep water sand sample should be described as a second species of the genus *Pyramidodinium*, *P. spinulosum*.

Key words: benthic dinoflagellate, phylogeny, SSU rRNA gene, taxonomy, ultrastructure.

INTRODUCTION

Some of the free-living dinoflagellates have the unique habit of a dominant, attached, non-motile cell cycle stage. Such dinoflagellates have been recorded in both freshwater and marine habitats (see Popovský & Pfiester 1990; Fensome *et al.* 1993; Hoppenrath *et al.* 2014). They were tentatively grouped in the order Phytodiniales for convenience, because their true phylogenetic affinities were mostly unknown (Fensome *et al.* 1993). However, recent molecular work has shown that these coccoid dinoflagellates are mostly polyphyletic (Hoppenrath *et al.* 2014). Dinoflagellates with this character set (marine, benthic, attached, non-motile) include *Stylodinium littorale* Horiguchi & Chihara (Horiguchi & Chihara 1983), *Halostylodinium arenarium* Horiguchi & Yoshizawa-Ebata (Horiguchi *et al.* 2000), *Spiniferodinium galeiforme* Horiguchi & Chihara (Horiguchi & Chihara 1987; Houpt & Hoppenrath 2006), *Spiniferodinium palauense* Horiguchi, Hayashi, Kudo & Hara (Horiguchi *et al.* 2011), *Gymnodinium quadrilobatum* Horiguchi & Pienaar (Horiguchi & Pienaar 1994), *Pyramidodinium atrofuscum* Horiguchi & Sukigara (Horiguchi & Sukigara 2005) and *Galeidinium rugatum* Tamura & Horiguchi (Tamura *et al*. 2005).

Recently Yamada *et al.* (2013) described a new genus and a species of sand-dwelling dinoflagellate, *Bispinodinium angelaceum* N. Yamada & Horiguchi from a sandy seafloor at a depth of 36 m near Mageshima Island, Kagoshima Prefecture, Japan in the subtropical region of the Pacific Ocean. More recently, Pinto *et al.* (2017) described a new sand-dwelling species, *Testudodinium magnum* Pinto, Terada & Horiguchi from a similarly deep (35 m) environment in the same area of the sea, but in the different date (30 May 2012). The dinoflagellate described in this study was from the same sample,

which we obtained *B. angelaceum* (Yamada *et al*. 2013). This same sample also yielded an attached, coccoid dinoflagellate, covered with a dome-shaped, spiny cell wall. The molecular phylogenetic analysis using the small subunit ribosomal RNA gene (SSU rDNA) sequences revealed that this third species formed a robust clade with *Pyramidodinium atrofuscum*, from Jellyfish Lake, Republic of Palau, a dinoflagellate with a pyramid-shaped, non-motile attached form (Horiguchi & Sukigara 2005). Based on light and electron microscopical observations using cultured material together with molecular data, we conclude that the species described here belongs to the genus *Pyramidodinium*. This paper describes the morphology, ultrastructure and cell cycle of a second species of the genus *Pyramidodinium, P. spinulosum* Horiguchi, Moriya, Pinto et Terada sp. nov.

MATERIALS AND METHODS

The sand samples were collected from the seafloor at a depth of 36 m, off Mageshima Island, Kagoshima Prefecture, Japan on 3 July 2011 using a Smith-McIntyre bottom sampler (Rigosha, Tokyo). The light conditions and seawater temperature of this region have been described in Yamada *et al.* (2013). A spoonful of the sand sample was placed in a disposable transparent plastic dish with a lid and enriched with Daigo's IMK medium (Nihon Pharmaceutical Co., Ltd., Tokyo) and cultured at 25ºC with an illumination of 50 µmol photons m^{-2} s^{-1} with 16:8 hour light:dark regime. The non-motile cells attached to the wall of the plastic cup and were isolated using capillary pipettes while viewing with an inverted microscope (Olympus CKX-41, Tokyo) and subsequently a clonal culture was established. The culture strain (number HG289) was maintained in IMK medium under the same conditions described above and is available from the author upon request.

Light and scanning electron microscopy

Living cells were observed using a Carl Zeiss Axioskop 2 microscope equipped with Nomarski interference optics (Carl Zeiss Japan, Tokyo). Photographs were taken using a Leica MC120HD camera (Leica-microsystems, Tokyo). To recover intact attached cells for direct observation, pieces of broken cover slips were submerged in a culture of HG289 strain grown in a petri dish and the cells allowed to attach to them over several days. Cover slip fragments with attached cells were mounted onto a glass slide and

covered by a further cover slip, and observed as usual. Lateral views of the attached cells were obtained by observing the edges of the broken cover slips. To observe the cell cycle, the actively growing culture in the petri dish was used. Individual motile cells were tracked at low power using an inverted microscope (Olympus CKX-41, Tokyo) until they settled on the bottom of the petri dish. The transformation from the motile to the non-motile state and all subsequent changes (cell division, etc.) in the non-motile cell were followed. The change of cell morphology was recorded directly from cultures using a ScopePad-500 camera (Gellex International, Tokyo) fitted to the inverted microscope. The time from settling (0 h) of any morphological change was recorded. All such recording was achieved by keeping the petri dish on the microscope stage at room temperature (25±3°C) and with conventional ceiling fluorescent light illumination and without precise control of the light:dark period. The morphological change was observed continuously for the first 3 hours until cell wall was formed and after this period, the cells were monitored three times a day (morning/noon/night). The cells were exposed to additional illumination from the microscope during periods of observation. The shape and number of chloroplasts were examined under the fluorescent microscope (Carl Zeiss Axioskop 2 with a filter set No. 15).

For scanning electron microscopy, the same methods employed for the preparation of both non-motile and motile cells of *Spiniferodinium palauense* (Horiguchi *et al.* 2011) were used. The samples were dried with CO_2 using a critical point drier (Hitachi HCP-2, Tokyo) and coated with gold in a sputter coater (Hitachi E-1045, Tokyo). The observations were made using a S-3000N scanning electron microscope (Hitachi, Tokyo).

Transmission electron microscopy

Non-motile cells attached on the surface of petri dish were harvested by sweeping gently by a sterilized paint brush and were concentrated by centrifugation. These cells were fixed in 5% glutaraldehyde made up in IMK medium for 3 h at room temperature. They were rinsed in the same medium and subsequently post fixed in 2% OsO_4 made up in the same medium for 2 h at room temperature. The sample was dehydrated through an acetone series and finally embedded in Agar LV resin (AGAR Scientific, Essex). Sections were cut with a diamond knife on an ME-Ultracut S ultramicrotome (Leica, Wetzlar) and viewed with a Hitachi H-7650 transmission electron microscope

(Hitachi, Tokyo) without staining.

Molecular analysis

Total genomic DNA was extracted from the clonal culture strain (HG289) using the benzyl chloride method of Zhu *et al*. (1993). To amplify almost the entire region of the small subunit of the ribosomal RNA gene (SSU rDNA), the primer sets reported by Takano and Horiguchi (2006) were used. Both forward and reverse strands were sequenced.

The sequences were aligned manually, based on the published secondary structure of the SSU rRNA molecule, using alveolate taxa available at the rRNA server. A total of 3,041 aligned sites (1,751 bases augmented by the gaps from the rRNA secondary structure) were used for the analyses. *Perkinsus marinus* (Mackin, Owen & Collier) Levine (Perkinsozoa) was used as an outgroup for the SSU rDNA analyses. The original description of *Pyramidodinium atrofuscum* (Horiguchi & Sukigara 2005) did not include molecular characterization, but the current phylogenetic analysis includes its newly-determined SSU rDNA sequence, that is now linked with the strain number, HG226. The aligned sequences were analysed by the ML method using PAUP* version 4.0a150 (Swofford 2002) and the Bayesian method using MrBayes 3.2.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). The programme in the PAUP* was used to calculate the evolutionary model that was the best fit for ML analysis of the dataset (GTR + I + G). The heuristic search for the ML analysis was performed with the following options: a TBR branch-swapping algorithm and the Kimura 2-parameter NJ tree as the starting tree. The parameters used for the analysis were as follows: assumed nucleotide frequencies A = 0.2427, C = 0.1889, G = 0.2529, and T = 0.3155; substitution rate matrix with A<->C = 1.4897, A<->G = 4.1955, A<->T = 1.3850, C<->G = 0.8244, C<->T = 7.5500, G<->T = 1.0000; proportion of sites assumed to be invariable = 0.34152; rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.56597, and number of rate categories = 4. For the Bayesian analysis, the GTR + I + G evolutionary model was selected by MrModeltest 2.3 (Nylander *et al.* 2004). Markov chain Monte Carlo iterations up to 1,500,000 generations were undertaken, when the average standard deviations of split frequencies fell below 0.01, indicating the convergence of the iterations.

RESULTS

Cell cycle

Pyramidodinium spinulosum spends most of the time in its cell cycle as a dome-shaped non-motile form (Fig. 1a–d). This form is covered with a thick cell wall, within which the cytoplasm divides equally into two daughter cells (Fig. 1c). These daughters are subsequently released as motile cells, leaving behind an empty cell wall (arrowhead in Fig. 1d). The motile cells have the typical gymnodinioid morphology (Fig. 1e). They swim for only a few hours, very slowly with rotatory movement.

The motile cells settle and directly transform into the non-motile forms (Fig. 2a–c). The motile cells are extremely sensitive to changes in the environment and even the change in light brought about by light microscope observation can induce them to settle into the non-motile form. The quiescent cells become spherical and both flagella are shed from the cell body (Fig. 2b, c). Within a minute, the rounded cells expand in all dimensions and become more or less spherical to somewhat square in surface view (Fig. 2c, d). During this process, no cell wall is formed and it only becomes visible after 40 min to 2 hours of settling (Fig. 2e, f). Interestingly, non-motile cells attract other motile cells and as a result groups of non-motile cells are common (Figs 1d, 2g, Fig. S1 in Supporting Information). In such instances, the edges of non-motile cell often overlap those of their neighbours (Figs 1d, 2g, Fig. S1 in Supporting Information). After the transformation into the non-motile cell, a further three to four days are required before the commencement of cell division and the subsequent formation of two motile cells within the parent cell wall (Fig. 2h, i). Two to three more days were required for the release of the motile cells; thus the completion of a cell cycle requires a minimum of 5 to 7 days in total (Fig. 2j). However, it can be very protracted too and the longest cell cycle recorded under the same culture conditions (within the same petri dish) was an astonishing 38 days (Fig. S1 in Supporting Information).

Light and scanning electron microscopy

The non-motile vegetative cells are dome-shaped (Fig. 1a), being more or less spherical in surface view (Fig. 1b–d). The cell wall is covered with 80 to 130 spines/cell (n = 10) (Figs 1a, 3a–c). The spines are almost even in distribution (Fig. 3b), but their lengths are quite variable (Fig. 3a–c). The spines near the cell periphery tend to be much shorter than the ones in the central area (Fig. 3b) and the longest spines are 4.5 µm in length.

Each spine is an elongated cone with an acute tip (Fig. 3a, c). Non-motile cells (almost round in face view) are 30.0 – 42.5 µm (avg. = 35.8 µm ± 4.2 µm, n = 30) in diameter and 12.5 – 22.5 µm (avg. = 16.9 µm ± 2.6 µm, n = 30) in height. The numerous chloroplasts (mostly more than 50 (40–78, n=10)) are small, elliptical to strap-shaped, yellowish brown, and are peripherally arranged (Fig. 1f, g). Starch grains are scattered throughout the cytoplasm. The nucleus is spherical to oblong (Fig. 1b, d) and is located in the middle of the cell (Fig. 1b). The red spot thought to be an accumulation body has been occasionally observed (Fig. 1b). No eyespot is present. Numerous dark small granules are scattered throughout the cytoplasm (Fig. 1c).

The motile cells have a typical gymnodinioid form (Figs 1e, 3d). The cell is oblong and slightly compressed in a dorsoventral plane, with the epicone and hypocone being of near-equal length. In some cells, the right side of the hypocone is shorter than that of left side (Fig. 1e). The cells are 25.0 – 32.5 µm in length (mean = 27.1 µm ± 3.0 µm, n = 7) and 15.0 – 20.0 µm in width (mean = 17.9 ± 2.2 µm, n = 7). The descending cingulum is displaced by a distance about its own width (Fig. 3d). The sulcus is short and extends only half the way down the hypocone (Fig. 3d). The transverse flagellum is thin and delicate compared with those of ordinary planktonic dinoflagellates (Fig. 3d). No thecal plates are present and the surface of the cells is smooth. No apical grooves or longitudinal striae have been observed (Fig. 3d).

Transmission electron microscopy

Figure 4a shows a vertical section of a non-motile cell, which had completed cell division, i.e. two daughter cells are included within a parental cell wall. The cytoplasm of the non-motile stage is covered by a continuous cell wall (Fig. 4a). The cell wall is layered and is considered a pellicle layer complex *sensu* Sekida *et al.* (2001). This complex is composed of two layers (Fig. 4b, c), i.e. the first (PI) and the second (PII) pellicle layer. The PI, which is about 18 nm thick, is composed of a trilaminar (dark-light-dark) structure (Fig. 4c). The PII layer comprises the main part of the cell wall (Fig. 4b, c). The spines represent an extension of the main part of cell wall, made up of the same material (Fig. 4b), and, as seen using the SEM, each possesses an acute apex (Fig. 3b). The surface of cell wall is covered by amorphous material (Fig. 4b). Below the pellicle layer complex (cell wall), the amphiesma of the newly formed motile cell is present (Fig. 4d). The plasma membrane is underlain by a single layer of flattened

vesicles (= amphiesmal vesicles). Each vesicle contains an interrupted membrane-like layer (Fig. 4d).

The nucleus is a typical dinokaryon (Fig. 4a). The chloroplasts are typical for dinoflagellates (Fig. 4e), i.e. the chloroplast envelope consists of three membranes (Fig. 4e, inset) and each lamella consists of three stacked thylakoids (Fig. 4e). A stack of grana-like thylakoids has been observed in some chloroplasts (not shown). Other organelles, such as mitochondria with tubular cristae (not shown) and trichocysts (Fig. 4f), are typical for dinoflagellates. No pusule has been detected. The cytoplasm is filled with numerous starch granules of various size (Fig. 4a) and often substantial part of cytoplasm is filled with dark-stained lipid grains of various size (Fig. 4a). The dark-coloured granules seen with the light microscope are due to the presence of many rhomboidal crystalline structures (Fig. 4g) which are distributed mainly in the peripheral areas of the cell (Fig. 4a, g).

Molecular phylogeny

We have analysed the dataset by both maximum likelihood (ML) and Bayesian methods. The topology resulting from these analyses are almost congruent and we present only the topology of the ML tree (Fig. 5). The accession numbers of species included in the alignments are presented in the tree (Fig. 5). In this tree, *Pyramidodinium spinulosum* formed a strongly supported clade with the type species of the genus *Pyramidodinium*, *P. atrofuscum* (1.0 posterior probability (PP), 100% ML bootstrap). The sister to this *Pyramidodinium* clade was the *Togula* clade, but with very weak statistical support. The exact phylogenetic position of the genus *Pyramidodinium* within the Dinophyceae was not clearly demonstrated. Other dinoflagellates with dome-shaped non-motile cells, such as *Galeidinium rugatum* in 'Dinotom-clade' and *Spiniferodinium* spp. in '*Gymnodinium s.s.*-clade' are well-separated from the *Pyramidodinium* clade (Fig. 5).

DISCUSSION

Non-motile dinoflagellates with dome-shaped, helmet-shaped or pyramid-shaped vegetative cells have been described, but are rather rare. These include *Pyramidodinium atrofuscum, Galeidinium rugatum, Spiniferodinium galeiforme* and *S. palauense.* Two species of *Spiniferodinium* possess a helmet-shaped 'shell' with surface spines, but the shell consists of fibrous materials and its nature is very different from the cell wall of

Pyramidodinium allowing for ease in distinguishing them (Horiguchi & Chihara 1987; Horiguchi & Sukigara 2005, Horiguchi *et al.* 2013). The genus *Galeidinium* also possesses a helmet-shaped, attached cell with double transverse ridges. However, *Galeidinium* is characterised by having a diatom endosymbiont (= dinotom, *sensu* Imanian *et al.* 2010) and thus very distinct from *Pyramidodinium* spp. Our molecular tree shows that *Spiniferodinium* spp. and *Galeidinium rugatum* are phylogenetically distinct from *Pyramidodinium* spp. The genus *Spiniferodinium* is included in the *Gymnodinium s.s.* clade, while *Galeidinium* positioned in the dinotom clade together with other dinotoms.

Our molecular analysis indicated that the new species formed a robust clade with *Pyramidodinium atrofuscum*, the type species of the genus. *P. atrofuscum* was originally described from a bottom sand sample taken from Jellyfish Lake in the Republic of Palau (Horiguchi & Sukigara 2005). The species is characterised by the possession of a pyramidal sessile form with a single longitudinal and double transverse ridges on its surface. In addition, the surface of non-motile cell is covered with many wart-like processes. The dinoflagellate alternates a long-lasting, non-motile, vegetative stage (up to 10 days) with a very short (few hours) motile gymnodinioid stage in its cell cycle. The motile cells are produced within the non-motile cells and upon release they swim for only a short time before directly returning to the non-motile form. Therefore, these two species share very similar habits and mode of cell cycle. In addition they share some morphological features. Both possess numerous rhomboidal crystalline structures throughout the cytoplasm (Horiguchi & Sukigara 2005) and the ultrastructure of the cell covering (the cell wall plus the amphiesma) of the two is similar, including the presence of an interrupted membrane-like structure within each amphiesmal vesicle (Horiguchi & Sukigara 2005). In addition, both species also share the characteristic in motile stage, i.e. lack of apical groove. Most of the unarmoured dinoflagellates are known to possess apical grove (e.g. Daugbjerg *et al*. 2000) and the unarmoured dinoflagellates without apical groove is rather rare, although *Togula* is one such example (Flø Jørgensen *et al*. 2004). Therefore, as the molecular tree suggests, it makes sense to accommodate *Pyramidodinium atrofuscum* and *P. spinulosum* in the same genus. However, these two species are distinctly different from each other. The overall cell morphology of the sessile form is different. The new species possesses no longitudinal or transverse ridges, but instead has an even covering of spines of various

size. Therefore, the two species can be easily distinguished from each other making it clear that the organism described here is a new species. Because in the original description (Horiguchi & Sukigara 2005), no separate genus description was given and now the dinoflagellate with slightly different morphology is included in the genus, it is appropriate to define the genus here.

Pyramidodinium spinulosum has been isolated from a sample collected from the seafloor at a depth of 36 m in the subtropics. Recently, other dinoflagellates were described from similar depths in the same locality. *Bispinodinium angelaceum* (Yamada *et al.* 2013) was obtained from the same sample from which *P. spinulosum* was isolated (July 2011) and *Testudodinium magnum* (Pinto *et al.* 2017) was collected from the same locality at a depth of 35 m but on a different occasion (May 2012). Interestingly, both species are known to possess unique internal structures for dinoflagellates. *Bispinodinium angelaceum* possesses a pair of spine-like structures, termed the spinoid apparatus (Yamada *et al.* 2013), while *Testudodinium magnum* is characterised by possessing many (about 100/cell) internal props, which span the breadth of the cell. These two species only have been encountered from deep environments and thus, these special structures might be interpreted as adaptations to the high pressure at the bottom of the sea. However, it should be noted that we have isolated many other unarmoured dinoflagellates, for example, *Amphidinium operculatum* Claparède & Lachmann and *A. gibbosum* (Maranda & Shimizu) Flø Jørgensen & Murray, from the same deep environment, which do not have any specialised internal structures (Horiguchi personal observation, 2012). The latter species also inhabit shallow waters. Therefore, the presence of such internal structures is not essential for the survival of dinoflagellates in deep (around 30 m) water (Pinto *et al.* 2017). Cells of *Pyramidodinium spinulosum* do not have any such special internal structures, but the cell is protected by a thick cell wall. This continuous wall might contribute to the tolerance of high pressure, but again, there is no clear evidence that this structure represents an adaptation to high pressure, because the closely-related *P. atrofuscum* from shallow water also possesses such a type of cell wall.

Pyramidodinium Horiguchi et Sukigara emend. Horiguchi, Moriya, Pinto et Terada

Non-motile cell sessile, pyramidal or dome-shaped, covered with a continuous wall; cell wall covered with wart-like or spine-like projections; cytoplasm filled with dark brown

crystals; chloroplasts many; no eyespot present. Motile cell, *Gymnodinium*-like, without apical groove.

Type species: *Pyramidodinium atrofuscum* Horiguchi et Sukigara

Pyramidodinium spinulosum Horiguchi, Moriya, Pinto et Terada sp. nov.

Non-motile cell dome-shaped; 30.0 – 42.5 µm in diameter in face view and 12.5 – 22.5 µm in height; enclosed by a continuous cell wall, the surface of which is covered with regularly distributed spines. Cytoplasm contains many, small, dark brown crystals; chloroplasts small, many (more than 50), ellipsoidal to strap-shaped, yellowish-brown; no eyespot present. Motile cell, *Gymnodinium*-like, without apical groove, 25.0 – 32.5 µm in length and 15.0 – 20.0 µm in width. Dinoflagellate marine, sand-dwelling.

Holotype: An embedded specimen in epoxy resin (permanent slide) has been deposited in the herbarium of the Graduate School of Science, Hokkaido University as SAP 97816 (Isotype as SAP 97817).

Type locality: Seafloor off Mageshima Island (36 m deep), Kagoshima Prefecture, Japan (30°38’28.68” N 130°39’2.95”E).

Etymology: Latin *spinulosum* (spine-bearing) indicating possession of spines on the upper surface of non-motile cell, which are longer than the wart-like processes of *P. atrofuscum* (type species).

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REFERENCES

Daugbjerg, N., Hansen, G., Larsen, J. and Moestrup, Ø. 2000. 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 unarmoured dinoflagellates. *Phycologia* **39**: 302–17.

Fensome, R. A., Taylor, F. J. R., Norris, G., Sarjeant, W. A. S., Wharton, D. I. and

Williams, G. L. 1993. A classification of living and fossil dinoflagellates. *Micropaleontology, Special Publication Number* **7**: 1–351.

Flø Jørgensen, M., Myrray, S. A. and Daugbjerg, N. 2004. A new genus of athecate interstitial dinoflagellates, *Togula* gen. nov., previously encompassed within *Amphidinium sensu* lato: Inferred from light and electron microscopy and phylogenetic analyses of partial large subunit ribosomal DNA sequences. *Phycol. Res*. **52**: 284–99.

Hoppenrath, M., Murray, S. A., Chomérat, N. and Horiguchi, T. 2014. *Marine Benthic Dinoflagellates. – Unveiling their worldwide biodiversity.* Kleine Senckenberg-Reihe 54, Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obermiller), Stuttgart, Germany.

Horiguchi, T. and Chihara, M. 1983. *Stylodinium littorale*, a new marine dinococcalean alga (Pyrrophyta). *Phycologia* **22**: 23–8.

Horiguchi, T. and Chihara, M. 1987. *Spiniferodinium galeiforme*, a new genus and species of benthic dinoflagellates (Phytodiniales, Pyrrophyta) from Japan. *Phycologia* **26**: 478–87.

Horiguchi, T., Hayashi, Y., Kudo, H. and Hara, Y. 2011. A new benthic dinoflagellate *Spiniferodinium palauense* sp. nov. (Dinophyceae) from Palau. *Phycologia* **50**: 616–23.

Horiguchi, T. and Pienaar, R. N. 1994. Ultrastructure of a new marine sand-dwelling dinoflagellate *Gymnodinium quadrilobatum* sp. nov. (Dinophyceae) with special reference to its endosymbiotic algae. *Eur. J. Phycol*. **29**: 237–45.

Horiguchi, T. and Sukigara, C. 2005. *Pyramidodinium atrofuscum* gen. et sp. nov. (Dinophyceae), a new marine sand-dwelling coccoid dinoflagellate from tropical waters. *Phycol. Res.* **53**: 247–54.

Horiguchi, T., Yoshizawa-Ebata, J. and Nakayama, T. 2000. *Halostylodinium arenarium*, gen. et sp. nov. (Dinophyceae), a coccoid sand-dwelling dinoflagellate from subtropical Japan. *J. Phycol.* **36**: 960–71.

Houpt, P. and Hoppenrath, M. 2006. First record of the marine, benthic dinoflagellate *Spiniferodinium galeiforme* (Dinophyceae) from a temperate region. *Phycologia* **45**: 10–2.

Huelsenbeck, J. P. and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–5.

Imanian, B., Pombert, J. and Keeling, P. 2010. The complete plastid genomes of the two 'dinotoms' *Durinskia baltica* and *Kryptoperidinium foliaceum*. *PlosOne* **5**:e10711.

Nylander, J. A. A., Ronquist, F., Huelsenbeck, J. P. and Nieves-Aldrey, J. 2004. Bayesian phylogenetic analysis of combined data. *Syst. Biol.* **53**: 47–67.

Pinto, S. K., Terada, R. and Horiguchi, T. 2017. *Testudodinium magnum* sp. nov. (Dinophyceae), a novel marine sand-dwelling dinoflagellate from subtropical Japan. *Phycologia* **56**: 136–46.

Popovský, J. and Pfiester, L. A. 1990. *Süsswasserflora von Mitteleuropa. Band 6. Dinophyceae.* Gustav Fischer Verlag, Jena, Stuttgart.

Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–4.

Sekida, S., Horiguchi, T. and Okuda, K. 2001. Development of the cell covering in the dinoflagellate *Scrippsiella hexapraecingula* (Peridiniales, Dinophyceae). *Phycol. Res*. **49**: 163–76.

Swofford, D. L. 2002. PAUP* Phylogenetic analysis using parsimony [*and other methods]. Version 4. Sinauer Associates, Sunderland, Massachusetts.

Takano, Y. and Horiguchi, T. 2006. Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell. *J. Phycol.* **42**: 251–6.

Yamada, N., Terada, R., Tanaka, A. and Horiguchi, T. 2013. *Bispinodinium angelaceum* gen. et sp. nov. (Dinophyceae), a new sand-dwelling dinoflagellate from the seafloor off Mageshima Island, Japan. *J. Phycol.* **49**: 555–69.

Zhu, H., Qu, F. and Zhu, L. H. 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benizyl chloride. *Nucl. Acids Res.* **21**: 5279–80.

Figure legends

Fig. 1. Light micrographs (LM) of *Pyramidodinium spinulosum* cells. (a) Side view of attached, non-motile cell, showing surface spines. (b) Face view of attached, non-motile cell. The nucleus (N) is located in the centre of cell. The cytoplasm is surrounded by a hyaline cell wall, the surface of which is covered by spines (arrowhead). The spherical red body thought to be an accumulation body can be seen (double arrowhead). (c) Face view of dividing cell. Almost-equal daughter cells are produced. Many small dark granules can be seen throughout the cytoplasm (arrowheads). (d) Aggregation of non-

motile, attached cells. An empty cell wall, representing a non-motile cell that has released its daughter cells, can be seen (arrowhead). Most of cells have already divided and contain two daughter cells – two nuclei (pale areas) can be seen. (e) Motile cell. (f, g) Nomarski optics and fluorescent micrographs, respectively, of the same non-motile, attached cell, showing numerous elliptical to strap-shaped chloroplasts (g). Scale bars, (a) – (g) = 10 µm.

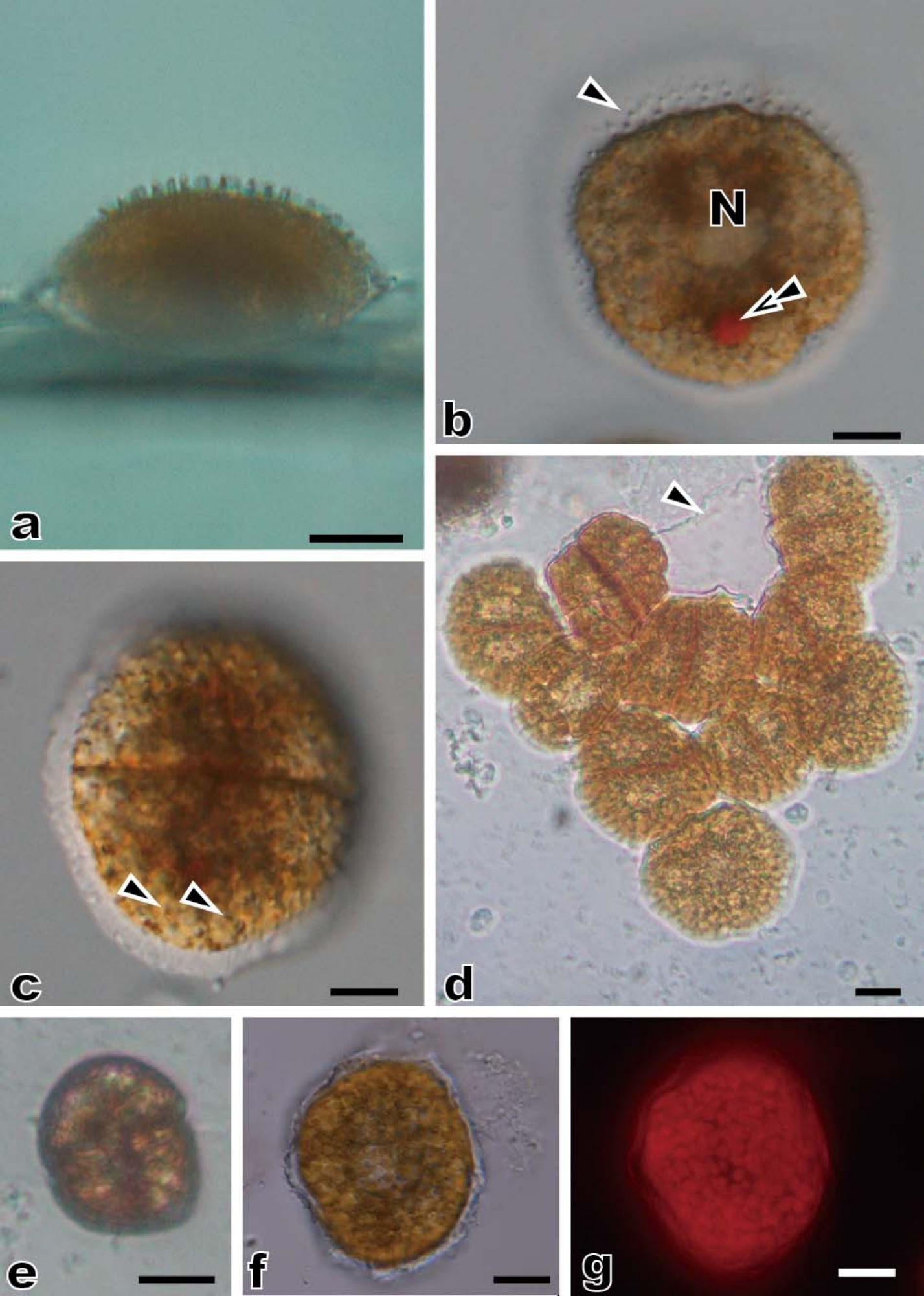
Fig. 2. Time lapse observation of cell cycle stages of *Pyramidodinium spinulosum* (a–j), following the transformation of a motile cell (0 min) (a) to a fully-formed non-motile phase (2 h) (f). By 40 minutes (e), the cell wall was visibly developing (arrowhead) and by 2 hours was fully-formed (arrowhead) (f). Eighteen hours after settling (g), another non-motile cell settled, partially overlapping the first cell. After 3 days and 18 hours, cell division took place in the first cell (h) and the second cell followed suit after 6 days and 18 hours (i). Arrows indicate planes of cell division. By 7 days and 18 hours (j), motile cells have been released from both parental cells, leaving the empty parental cell walls (arrowheads). N: nucleus. Scale bar in (a) = 10 µm and that can be applicable to (b) – (j).

Fig. 3. Scanning electron micrographs (SEM) of *Pyramidodinium spinulosum* cells. (a) Side view of a non-motile, attached cell. Part of the cell bottom has detached from the substratum. (b) Face view of a non-motile, attached cell, showing the arrangement of spines. (c) Detail of the surface of a non-motile, attached cell. (d) Ventral view of a motile cell. Transverse flagellum (tf) and longitudinal flagellum (lf) are visible. White arrows indicate degree of displacement of the cingulum at the sulcus. Black arrow indicates posterior terminus of sulcus. Scale bars, (a, b, d) = 10 µm, (c) = 1 µm.

Fig. 4. Transmission electron micrographs (TEM) of *Pyramidodinium spinulosum* cells. (a) Vertical section through a divided, non-motile, attached cell. The vertical division plane (arrowheads) is visible. The daughter cells are completely surrounded by a continuous parental cell wall (CW). Some spines (arrows) can be seen on the surface of the upper half of the cell wall. The cytoplasm of each daughter cell contains a dinokaryotic nucleus (N), chloroplasts (c) and starch granules (s). Most of cytoplasm is occupied by lipid granules (L). Much of the space between the lipid granules and the

other organelles is occupied by many small crystalline granules (see also (Fig. 4g)). (b) Detail of one of the spines. It is obvious that the spine is an extended portion of the cell wall. The surface of the cell wall (CW) is covered with fine fibrous material. (c) Detail of an unspined portion of the cell wall (CW), which consists of an outermost trilaminar thin layer (PI) and the bulk of the cell wall (PII). (d) Detail of the amphiesmal vesicles. The plasma membrane (double arrowhead) is underlain by flattened amphiesmal vesicles (arrowheads). Each amphiesmal vesicle contains an interrupted membrane like structure (arrow). (e) Detail of portion of a chloroplast (C), showing the lamellae consisting of three-thylakoid bands. Lipid granules (L) and trichocysts (T) border the chloroplast profile. (e inset) The three membranes of the chloroplast envelope that surround the chloroplast (C), are indicated (arrows). (f) Detail of trichocysts (T). (g) Detail of a portion of cytoplasm showing crystalline rhomboidal granules (arrowheads). Scale bars, (a) = 10 μm, (b, c, e) = 500 nm, (d, e inset) = 100 nm, (f, g) = 200 nm.

Fig. 5. Phylogenetic position of *Pyramidodinium spinulosum* and *P. atrofuscum* (type species) based on maximum likelihood analysis inferred from SSU rDNA sequences. Each species name is followed by an accession number. The posterior probability (PP) (>0.8) and bootstrap values (>50) are indicated at each node. The black dots at the nodes represent maximal statistical support, i.e. 1.0 PP and 100% bootstrap value. The organisms marked with a star are dinoflagellates with dome-shaped non-motile cells.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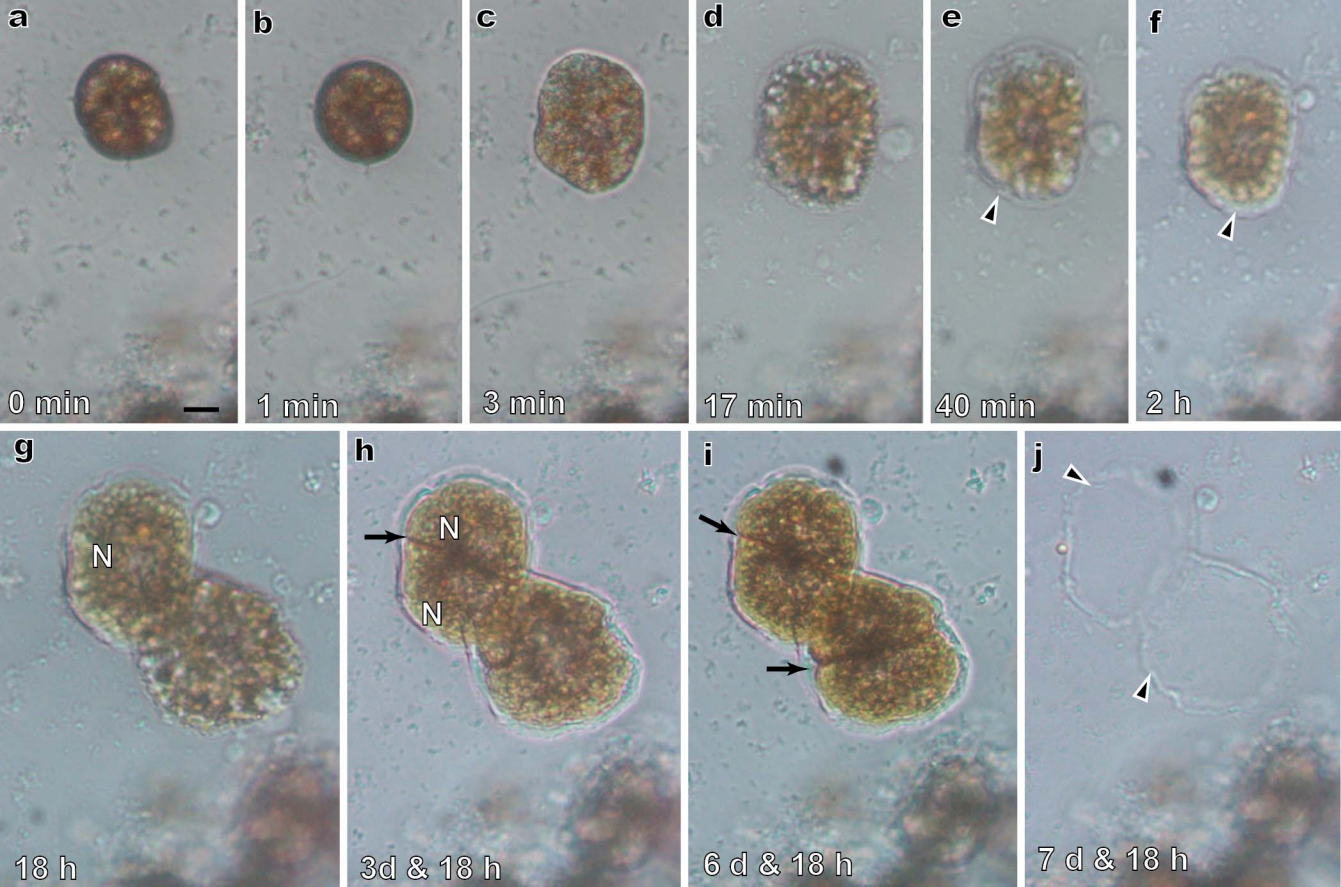
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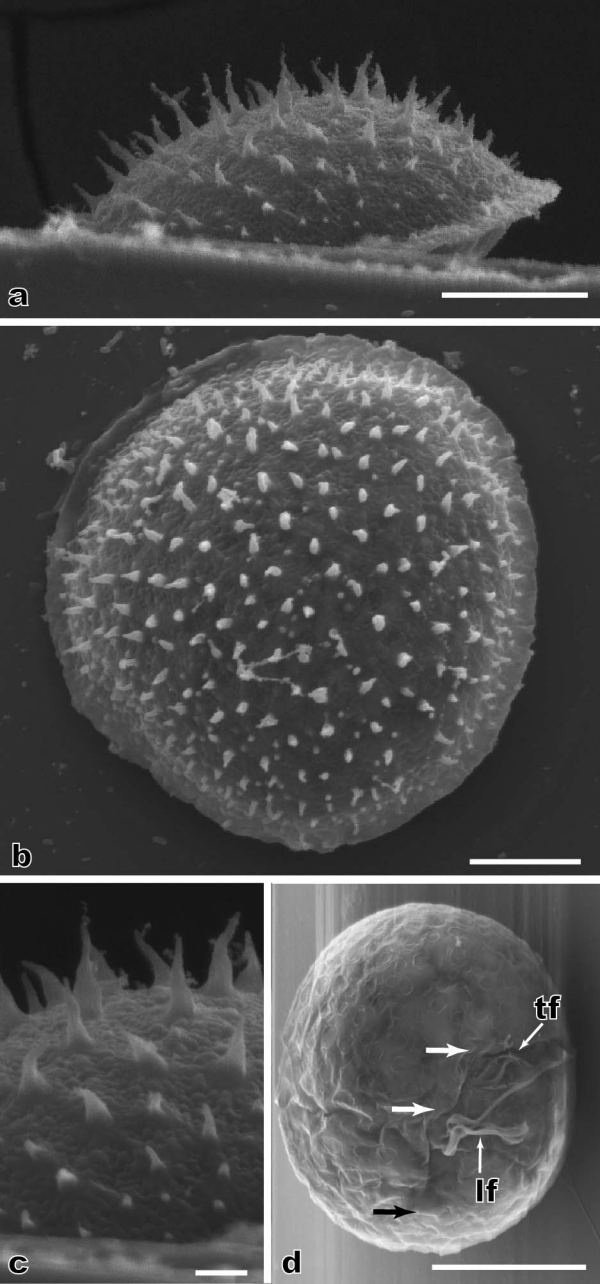
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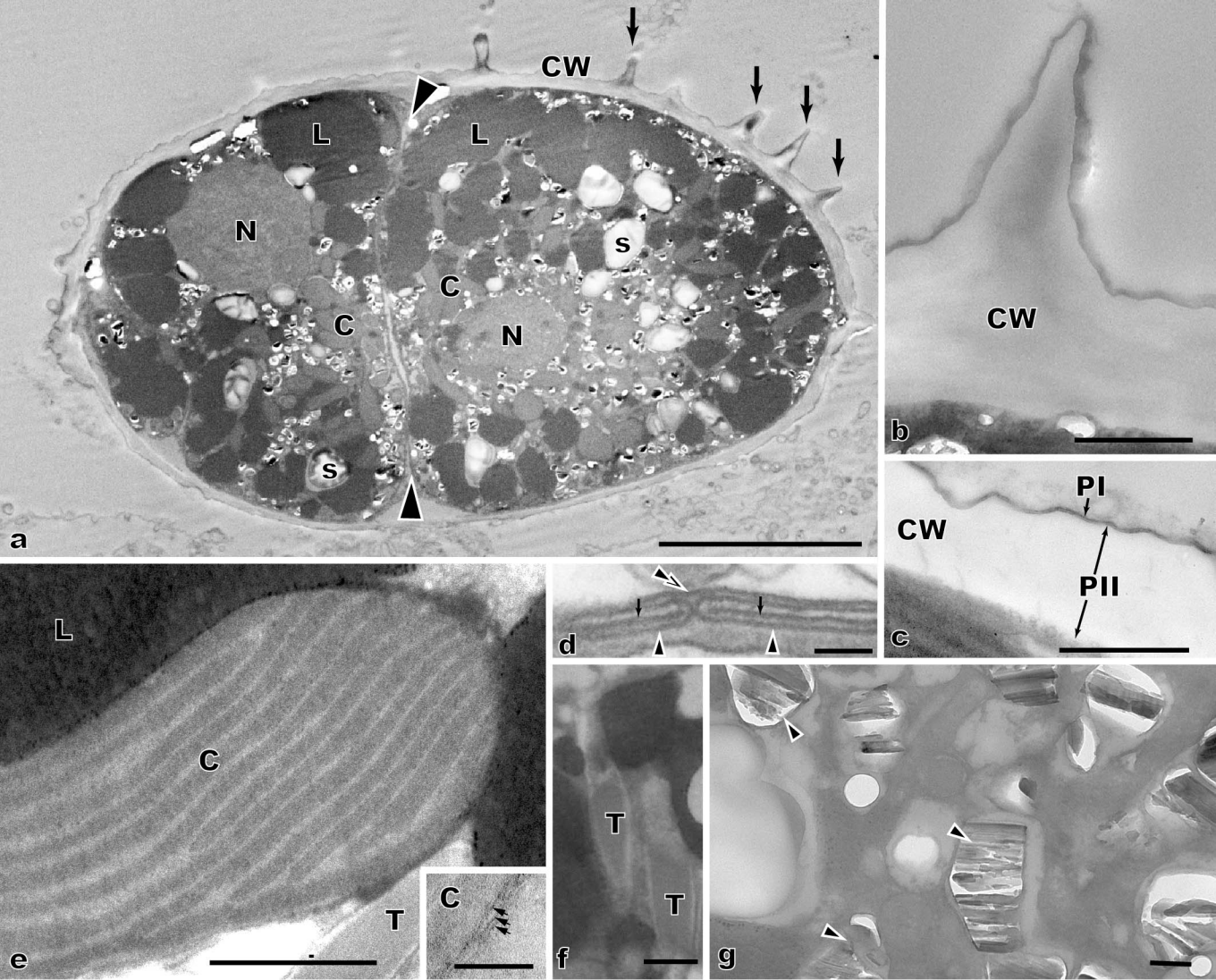
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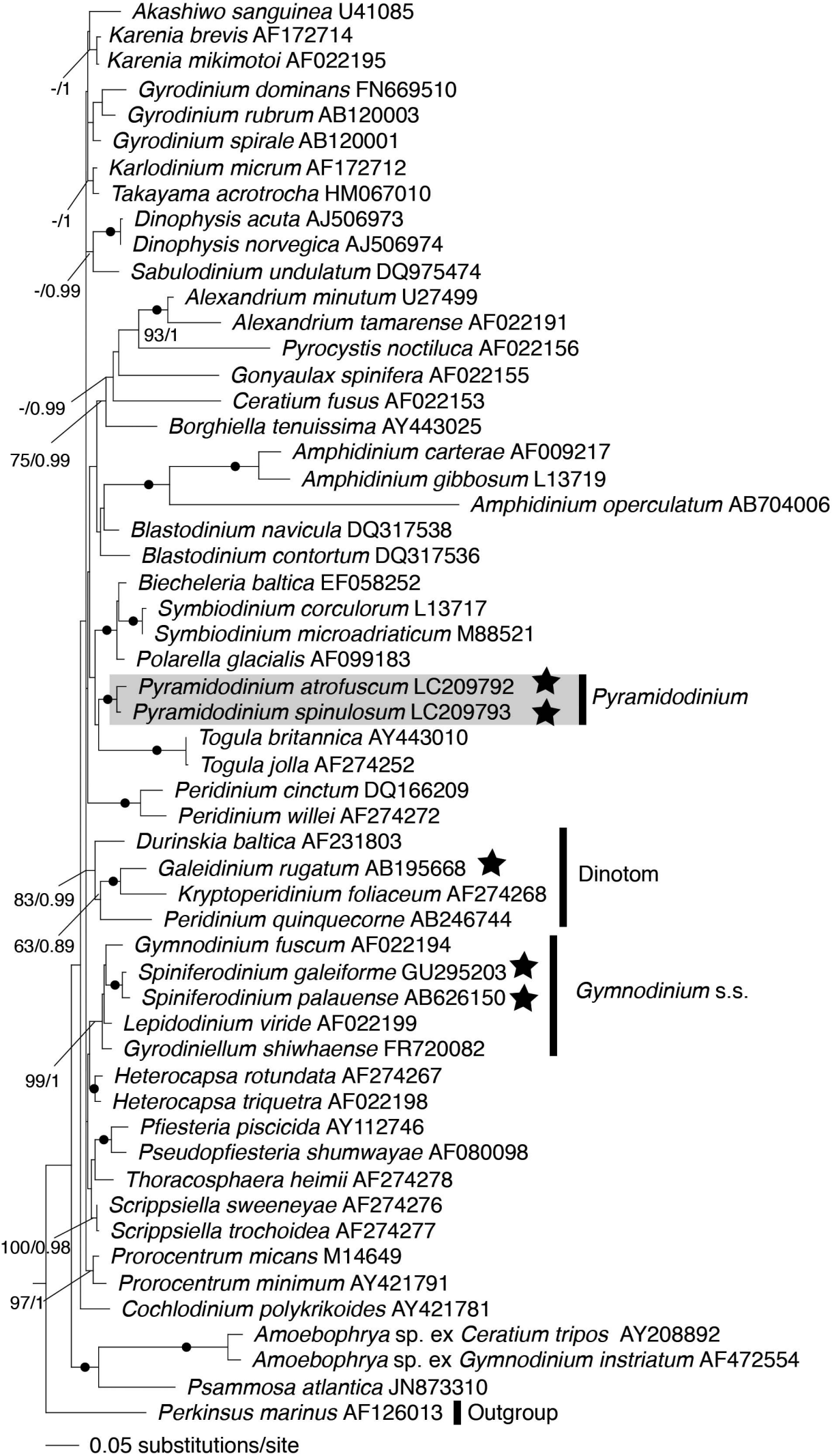
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Pyramidodinium
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SUPPORTING INFORMATION

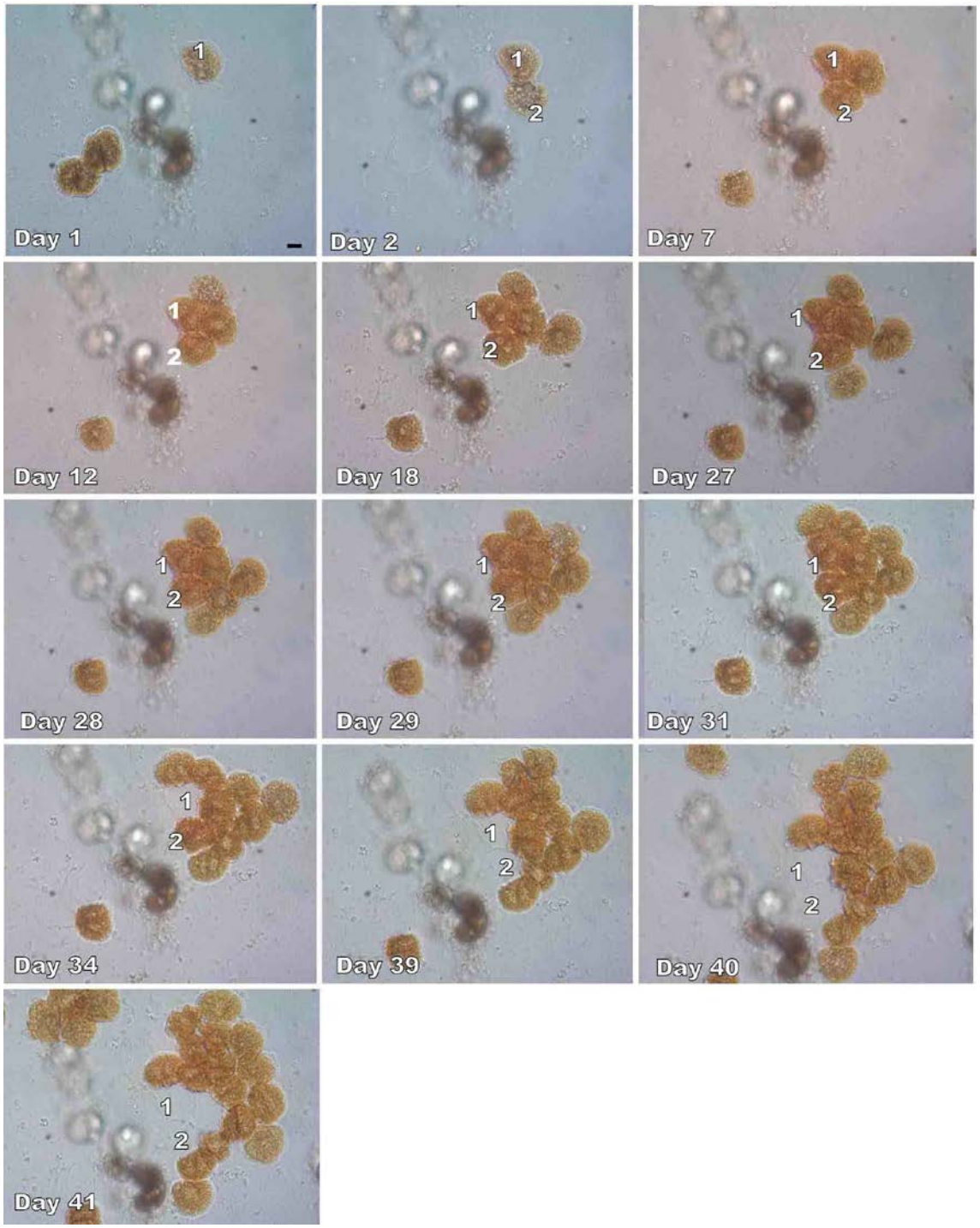

Fig. S1. Time lapse photographs over 41 days of observation. Only the first two cells to attach (1 on day 1 and 2 on day 2) are numbered. By day 34, the daughter cells of the first cell (1) were released and by day 39, the daughters of the second cell (2) were liberated.