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*Plagiodinium ballux* sp. nov. (Dinophyceae), a deep (36 m) sand dwelling dinoflagellate from subtropical Japan.

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Short running title: *Plagiodinium ballux* sp. nov.

## SUMMARY

A new species of marine sand-dwelling dinoflagellate, *Plagiodinium ballux* N. Yamada, Dawut, Terada & Horiguchi is described from a deep (36 m) seafloor off Takeshima Island, Kagoshima Prefecture, Japan in the subtropical region of the northwest Pacific. The species is thecate and superficially resembles species of *Prorocentrum*, but possesses an extremely small epitheca. The cell varies from ovoid to a rounded square, and is small (15.0 – 22.5 µm in length) and laterally compressed. The thecal plates are smooth and the thecal plate arrangement (Po, 1', 0a, 5'', 5C, 2S, 5'''', 0p, 1''''') is similar to that of *Plagiodinium belizeanum*, the type species of the genus. Molecular phylogenetic analyses based on SSU rDNA and partial LSU rDNA reveal that the dinoflagellate is closely related to *P. belizeanum*, but it can be clearly distinguished by its size and cell shape. This suite of morphological and molecular differences leads to the conclusion that this deep benthic dinoflagellate represents a new species of the genus *Plagiodinium*.

Key words: benthic, LSU rDNA, morphology, phylogeny, SSU rDNA, taxonomy,

ultrastructure

## INTRODUCTION

Marine benthic dinoflagellates inhabit various habitats, such as sandy beaches, tidal flats and the surfaces of corals, rocks and seaweeds/seagrasses (Hoppenrath *et al.* 2014). Recently, a novel type of habitat for benthic dinoflagellates, i.e. sandy seafloor at a depth of 30 – 40 m, has been explored in the subtropical region of the northwest Pacific (Yamada *et al.* 2013; Horiguchi *et al.* 2017; Pito *et al.* 2017). Although the habitat is deeper compared with the habitats reported for most of other benthic dinoflagellates, the survival of microalgae remains feasible because the water temperature remains relatively warm (around 20°C) throughout the year, and the water column has a high transparency (30 – 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at 30 m depth) due to influence of the Kuroshio warm current (Yamada *et al.* 2013). Until now, several new taxa, including *Bispinodinium angelaceum* N. Yamada & Horiguchi (Yamada *et al.* 2013), *Testudodinium magnum* Pinto, Terada & Horiguchi (Pinto *et al.* 2017) and *Pyramidodinium spinulosum* Horiguchi, Moriya, Pinto & Terada (Horiguchi *et al.* 2017) were found from this deep seafloor habitat. A series of these discoveries indicates that the dinoflagellate community in the habitat might contain diverse species.

Here, we found a small dinoflagellate that superficially resembles species of *Prorocentrum*, but which actually was revealed by molecular phylogenetic analysis to be related to the genus *Plagiodinium*. The type species of the genus *Plagiodinium*, *P. belizeanum* Faust & Balech from Twin Cays, Belize (Faust & Balech 1993), is sand-dwelling, armoured, laterally-flattened dinoflagellates with a small epitheca (Faust & Balech 1993; Wakeman *et al.* 2018). The novel species has a much smaller epitheca than *P. belizeanum*. Its cingulum is inconspicuous making the recognition of an epitheca difficult and resulting in morphology more similar to the genus *Prorocentrum*. We investigated the morphology, ultrastructure and phylogenetic affinities of this new species, and described it as a second species of the genus *Plagiodinium*, *Plagiodinium ballux*.

## MATERIALS AND METHODS

Collection and culturing

Sand samples were collected from the seafloor, at a depth of 36 m, off Takeshima Island, Kagoshima Prefecture, Japan (30°49'1" N, 130°24'0" E) on 10th May 2011 using a Smith-McIntyre bottom sampler (Rigosha, Tokyo). A spoonful of each sand sample was placed in a plastic dish and it was supplemented with Daigo's IMK medium (salinity of the seawater = 34‰) (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan). One milliliter of germanium dioxide (1 mg/mL) was added to the culture dish to prevent the growth of diatoms. This was cultured under 50  $\mu\text{mol photon s}^{-1} \text{m}^{-2}$  with a 16:8 h light:dark regime at 25°C. Single cells of the targeted alga that appeared in this crude culture were picked up with a micropipette while observing them with an inverted light microscope (CKX41, Olympus, Tokyo, Japan) and inoculated into sterile IMK medium and resulted in the establishment of a culture strain (HG177). The culture strain was maintained under the same conditions described above.

#### Light and scanning electron microscopy

Light microscopic observations were made using a Carl Zeiss Axioskop 2 microscope equipped with Nomarski interference optics (Carl Zeiss Japan, Tokyo, Japan). Photographs were taken using a Leica MC-120HD digital camera (Leica Microsystems, Wezlar, Germany). The shape and number of plastids were characterised using chlorophyll *a* autofluorescence (Carl Zeiss Axioskop 2 with a filter set No. 15) and the nucleus was visualized using 1  $\mu\text{g/mL}$  of 4',6-diamidino-2-phenylindole (DAPI) made up in distilled water (with filter set No. 49).

For scanning electron microscopy, we used cells from cultures fixed in Lugol's solution four years previously and stored in a refrigerator. The cells were harvested by centrifugation and the resultant pellet was rinsed in distilled water. This was dehydrated through an ethanol series (30, 50, 80, 90, 95 and 100%). Dehydrated cells were placed in a gelatinous capsule with holes in a small metal basket and dried in a Hitachi HPC-2 (Hitachi, Tokyo, Japan) critical point drier. Although some of the cells leaked through the holes of the capsule, most were retained. The dried cells were sprinkled over double sided tape on a stub and were coated with gold for 180 s at 12 mA using an E-1045 Hitachi sputter coater. Observations were made using a S-3000N Hitachi scanning electron microscope.

#### Transmission electron microscopy

Live cells were harvested by centrifugation at 300 g. After removing the supernatant, the sample was fixed in a mixture of 1% (w/v) OsO<sub>4</sub> and 2.5% (v/v) glutaraldehyde and 0.5 M Sucrose in 0.5 M Phosphate buffer (pH 7.1) for 30 min at room temperature. Cells were rinsed once with 0.5 M sucrose in 0.5 M phosphate buffer and twice with phosphate buffer, each rinse lasting 10 min, and post-fixed in 2% aqueous OsO<sub>4</sub> for 1 hour at room temperature. The sample was dehydrated for 10 min in each of the following concentrations of an acetone series, 30%, 50%, 70%, 80%, 90%, and 95% and then completely dehydrated in two rinses of 100% acetone, each lasting 30 min. The cells were then infiltrated with a 1:1 acetone-resin mixture and finally with full resin (Low Viscosity Resin, Agar Scientific Limited, Essex, UK) which was then polymerized at 65°C for 24 hour and sectioned using a diamond knife on a Leitz Ultra-cut (LEICA EM UC6) microtome. Sections were picked up with Formvar-coated one-slot grids. The sections were viewed without staining with a Hitachi H-7650 transmission electron microscope.

DNA extraction, polymerase chain reaction and *molecular* phylogenetic analyses

The small subunit ribosomal RNA gene (SSU rDNA) sequence for the strain HG177 has been deposited in DDBJ/GenBank by Yamada et al. (2015) (Accession No. LC054938 as *Plagiodinium* sp. HG177). However, the length of the sequence determined at that time was short, i.e. 1,679 bp, and thus a new sequence of 1,770 bp was obtained and replaced with same accession number for this study. The method and the primers used are the same as those of Yamada *et al.* (2015).

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 3,146 aligned sites (1,770 bases augmented by gaps from the rRNA secondary structure) were used for the analyses. *Perkinsus marinus* (Mackin, Owen & Collier) Levine (Perkinsozoa) was used as an outgroup. The aligned sequences were analysed by the maximum likelihood (ML) method using PAUP\* version 4.0a154 for Microsoft (Swofford 2002) and the Bayesian inference (BI) using MrBayes 3.2.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). The programme in the PAUP\* version 4.0a150 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 = 0.2471, C = 0.1949, G = 0.2585, and T = 0.2995; substitution rate matrix with A $\leftrightarrow$ C = 1.3485, A $\leftrightarrow$ G = 3.7888, A $\leftrightarrow$ T = 1.3218, C $\leftrightarrow$ G = 0.77183, C $\leftrightarrow$ T = 7.6668, G $\leftrightarrow$ T = 1.0000); proportion of sites assumed to be invariable = 0.31381; rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.49203, 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.

For the partial large subunit ribosomal RNA gene (LSU rDNA) sequence analysis, a total of 1598 aligned sites (1094 bases augmented by gaps from the rRNA secondary structure) were used. The manually aligned sequences were analysed by the same method described for SSU rDNA sequences. The selected model for ML analysis was the GTR+I+G model. The parameters used for the analysis were as follows: assumed nucleotide frequencies (A=0.274425 C=0.189376 G=0.2779 and T=0.258299; substitution rate matrix with A $\leftrightarrow$ C=0.759515, A $\leftrightarrow$ G=2.35024, A $\leftrightarrow$ T=1, C $\leftrightarrow$ G=0.759515, C $\leftrightarrow$ T=5.86343, G $\leftrightarrow$ T=1); proportion of sites assumed to be invariable=0.189565; rates for variable sites assumed to follow a gamma distribution with shape parameter=0.635142; and the number of rate categories=4. For the Bayesian analysis, GTR+I+G was selected as the best evolutionary model by MrModeltest 2.3 (Nylander *et al.* 2004). Markov-chain Monte Carlo iterations were carried out until 3,000,000 generations were attained, when the average standard deviations of split frequencies fell below 0.01, indicating the convergence of the iterations.

## RESULTS

### Light and scanning electron microscopy

The cells were either almost rectangular with rounded corners or ovoid in lateral view (Fig. 1) and laterally compressed. The cell dimensions were 15.0 – 22.5  $\mu\text{m}$  (ave.  $19.4 \pm 2.4 \mu\text{m}$ , n = 30) in length, 10.0 – 16.5  $\mu\text{m}$  (ave.  $12.6 \pm 2.2 \mu\text{m}$ , n = 30) in depth (dorsoventral length) and 6.3 – 9.5  $\mu\text{m}$  (ave.  $7.6 \pm 0.9 \mu\text{m}$ , n = 30) in width. The cingulum was hardly recognizable and thus it is also difficult to recognize an epitheca

clearly (Fig. 1a, b). However, a slightly-raised part of the epitheca could be observed under high magnification (Fig. 1a). A very short sulcus could be seen near the apex of the cell (Fig. 1a, b). The antapical end of the cell was often wider than the apical region (Fig. 1b) and sometimes marked near the base by a small notch formed by the junction between the antapical and postcingular plates (Fig. 1b). The nucleus was small, almost spherical and located in the posterior dorsal part of the cell (Fig. 1a-c). The chloroplast was single, yellow to yellowish brown and showed reticulation near the surface of the cell (Fig. 1d). The pyrenoid was circular, surrounded by conspicuous donut-shaped starch sheaths and located on the ventral side of the middle of the cell (Fig. 1).

The cell surface was smooth (Fig. 2a-c) and small pores were distributed mostly along the sutures (Fig. 2b, c). The thecal plate arrangement interpreted here was: Po, 1', 0a, 5'', 5C, 2S, 5''', 0p, 1'''' (Figs 2, 3). The epitheca consisted of one apical plate and 5 precingular plates (Fig. 2b). No apical pore complex was observed, but a small potential apical pore was observed between 1' and 1'' plates (Fig. 2c). The cingulum consisted of 5 plates and, by our interpretation, the cingulum did not completely encircle the epitheca. The C5 plate made contact with the 4'' plate but did not directly touch the sulcal plate (Fig. 2b). The sulcus was very short and consisted only of two plates, the Sa and Sp plates (Figs 2c, 3b). The hypotheca consisted of 6 plates, i.e. 5 postcingular plates and one antapical plate. The 1'''' plate positioned directly below the sulcus. The 2'''' and 4'''' plates were the largest of all the plates and positioned on the left and right sides of the cell respectively (Figs 2a, 2b-d, 3a). The antapical plate was single and positioned at the bottom of the cell (Fig. 2e).

#### Transmission electron microscopy

The cell possessed the typical ultrastructure for dinoflagellates with a dinokaryotic nucleus with thick chromosomes (Fig. 4a). The chloroplast lobes radiated from the central pyrenoid (Fig. 4a). The pyrenoid was spherical and was surrounded by thick starch sheaths (Fig. 4a, b). The pyrenoid matrix was traversed by many irregular sinuous thylakoid bands (Fig. 4b). Typical trichocysts, although not many, were present (Fig. 2d). The mitochondrial profiles showed typical tubular cristae (not shown). The cytoplasm contained starch granules and lipid granules. The pusule with a branching chamber was observed (Fig. 2c). Vesicles containing virus-like particles were sometimes observed (Fig. 2d).

### Molecular phylogenetic analysis

The phylogenetic affinities of *Plagiodinium ballux* were investigated using both SSU rDNA (Fig. 5) and LSU rDNA (Fig. 6) alignments. Two species (three strains) of *Plagiodinium* formed a clade which was moderately supported (86%) by bootstrap value of ML and strongly supported (0.98) by posterior probability of BI in the SSU rDNA tree. In the LSU rDNA tree, these two species formed a very strongly supported (100%/1.0) clade. The two strains identified as *Plagiodinium belizeanum* (accession numbers LC054937, KX008973) formed a clade (93%/1.0), but the distance between these two strains was unexpectedly large (6%) in SSU rDNA alignment, however the distance in LSU rDNA was 1.9%. *P. ballux* was resolved as a sister to the *P. belizeanum* clade. The phylogenetic affinities of the genus *Plagiodinium* with other taxa of dinoflagellates were not resolved due to low support of deeper branches.

### DISCUSSION

*Plagiodinium ballux* sp. nov. possesses a small ovoid cell with lateral compression. Because of the insignificant epitheca and the presence of ring-shaped central pyrenoid, it is possible that this dinoflagellate has been described before as a species of the genus *Prorocentrum*. Although any described benthic *Prorocentrum* species do not correspond to the size and the cell morphology to *P. ballux* (Hoppenrath et al. 2013, 2014), a small planktonic individual, *Prorocentrum cassubicum* (Wołoszyńska) Dodge, has a slightly similar cell shape to that of *P. ballux* in point of smooth thecal plates and a central ring-shaped pyrenoid (Dodge 1975). However, in addition to having a different habitat, *P. cassubicum* has a larger cell body and the periplagellar area can be clearly seen at the apex of the cell (Wołoszyńska 1928). In addition, no other sand-dwelling thecate dinoflagellates of similar morphology have been known, therefore, it is highly likely that this current organism has not been described and represents a new species.

Because the morphological similarity of the thecal plate arrangement in the genera *Pileidinium* and *Plagiodinium* among the benthic dinoflagellates with a single antapical plate (1''') (Table 1), we compared these two genera with *Plagiodinium ballux*. The genus *Pileidinium*, which contains only a single species, *P. ciseropse* Tamura & Horiguchi, possesses thecal plate arrangement; 1', 5'', 4C, 4S, 5''', 1'''' and

its major plate arrangement (other than those of cingulum and sulcus) is similar to that of *P. ballux*. The species possesses an incomplete cingulum and short sulcus as in *P. ballux*. On the other hand, the number of cingular and sulcal plates is different from those of *P. ballux* (Table 1). Furthermore, although the cell surface in *P. ballux* is smooth, *P. ciseropse* possesses highly reticulated cell surface with number of small meshes (Tamura & Horiguchi 2005).

The genus *Plagiodinium* was established as a monotypic genus with a type species, *P. belizeanum* (Faust and Balech 1993). The basic thecal plate arrangement reported for *P. belizeanum* was; Po, 5', 0a, 5'', 5C, 5S, 5''', 0p, 1'''' (Faust & Balech 1993, Hoppenrath et al. 2014) and this arrangement seems quite different from that of *P. ballux*. More recently, however, Wakeman *et al.* (2018) proposed a different interpretation of the thecal plate arrangement for *P. belizeanum*, due to discovery of an additional tiny apical plate, i.e. Po, 1', 0a, 5'', 5(6)C, 4S, 5''', 0p, 1'''''. Our interpretation of the thecal plate arrangement of *P. ballux* (Po, 1', 0a, 5'', 5C, 2S, 5''', 0p, 1''''') basically similar to that of *P. belizeanum* by Wakeman *et al.* (2018). When the cingulum, i.e. the plate series consisting of horizontally elongated plates with depression, is used as a landmark the plate series touching upper side of the cingulum were interpreted as precingular series ("). The plates 4'' and 5'' plates do not have depression and thus regarded as part of the precingular series rather than part of the cingulum. A single plate surrounded by precingular series was assigned as 1st apical plate (1'). In the hypotheca, the 1'''' plate in the hypotheca positioned directly below the sulcus and thus could be interpreted as a sulcal posterior plate (Sp). However, considering its large size, almost equal to that of the 5'''' plate, we interpreted this plate as the 1'''' plate (Fig. 2a). As a result, the sulcus was interpreted as consisting of only two small plates. The main difference between *P. belizeanum* and *P. ballux* lies with the number of sulcal plates and lack of complete cingulum in the latter. Another difference is that in *P. belizeanum* the presence of a distinct apical pore was demonstrated (Wakeman *et al.* 2018), while in *P. ballux*, the pore identified as apical pore is rather obscure. The location of the pore of both species is also slightly different, i.e. in *P. belizeanum*, the pore is located dorsal side of the 1'' plate, while in *P. ballux*, it is situated between 1' and 1'' plates.

The ultrastructure of *Plieidinium ciseropse* and *Plagiodinium belizeanum* have been reported by Tamura and Horiguchi (2005) or by Wakeman *et al.* (2018), respectively.

These species are phototrophic and the fundamental structure of the chloroplast is the same as is the pyrenoid type, i.e. multi-stalked pyrenoid. All three taxa possess a central pyrenoid the matrix of which is traversed by many curved thylakoid bands. However, the ultrastructure of pyrenoid matrix of *Pileidinium*, which is traversed by many regularly arranged loop-shaped thylakoids, is more similar to that of *P. belizeanum* rather than to that of *P. ballux*, which possesses rather irregular sinuous invasion of thylakoids. The pyrenoid matrix in *P. ballux* and *Pileidinium* is surrounded by a conspicuous ring-shaped starch sheath that facilitates the clear recognition of the pyrenoid at the light microscope (LM) level. However, in *P. belizeanum* the starch granules surrounding the pyrenoid matrix do not form a clear ring-like structure making it difficult to localize the pyrenoid at the LM level (Wakeman *et al.* 2018). Other fundamental dinoflagellate organelles, such as the dinokaryon, mitochondria and trichocysts are indistinguishable between three species, but a pusule has been only identified in *P. ballux* and *P. ciseropse*. It is interesting that, in all three species, a vacuole containing virus-like particles was found (described as polyvesicular body in *P. ciseropse* by Tamura and Horiguchi 2005). Overall, although there are differences between each species pair, these three species seem to share basic internal structures.

As discussed previously, it is certain that *P. ballux* is a new species. However, to which genus the species should be accommodated is difficult question to answer. There are three options, i.e. 1) to accommodate *P. ballux* in the genus *Pileidinium* based on morphological resemblance, including thecal plate arrangement, the presence of an incomplete cingulum, and overall similarity of the ultrastructure, 2) to classify in the genus *Plagiodinium*, and 3) to establish a new genus to accommodate this species. As to the option 1), although there are morphological resemblances between *Pileidinium* and *P. ballux* as mentioned above, the phylogenetic closeness between *Pileidinium* and *P. ballux* was not proven by SSU rDNA analysis (unfortunately, the culture of *P. ciseropse* is no longer available to study other gene sequences). Therefore, we believe it is not appropriate to accommodate our species in the genus *Pileidinium*. The difference in thecal plate ornamentation might supports this separation at generic rank. As to the option 2), our molecular study based on SSU and LSU rDNA revealed that the dinoflagellate described here was phylogenetically close to *P. belizeanum*. The two registered sequences of *P. belizeanum* were found to differ more than one might reasonably expect for one species (see Fig. 5). The sequence LC054937, was linked

with only a single colour micrograph of strain HG225 (Yamada *et al.* 2015), the cell of which showed a morphology typical of *P. belizeanum*. The morphology of the strain linked with the registered sequence KX008973 (Wakeman *et al.* 2018) was found to be morphologically identical to the original description of *P. belizeanum*. The base pair differences between these two strains in the LSU rDNA alignment is not so large, but still difference exists. Therefore, it is possible that cryptic species exist within a *P. belizeanum* ‘species complex’. However, this subject is beyond the scope of this paper and a more detailed comparative study and the recovery of a sequence from material collected at the type locality are required to clarify this problem. If these two species are truly closely related as indicated by both SSU and LSU rDNA trees and morphological resemblance, including thecal plate arrangement, although there are a few discrepancies, such as the presence of an incomplete cingulum and number of sulcal plates. It appears that the sulcus became substantially reduced in size and subsequently the number of sulcal plates also decreased in the line leading to *P. ballux* and at the same time, the morphology of ventral region in the epitheca has also been modified.

Establishing a new genus for this species is another option (option 3) based on morphological differences from all other dinoflagellates so far described as mentioned above (Table 1). However, we believe that these difference are rather minor and based on phylogenetic affinities, the possession of similar thecal plate arrangement, smooth surface thecal plates and many similarities in the internal structures with *Plagiodinium belizeanum*, we believe it is appropriate that this deep water benthic, organism should belong to the genus *Plagiodinium*, rather than to a new genus.

*Plagiodinium ballux* N. Yamada, Dawut, Terada et Horiguchi sp. nov. Figs 1 – 4.

Cells almost rectangular with rounded corners or ovoid in lateral view, laterally compressed, measuring 15.0 – 22.5  $\mu\text{m}$  in length, 10.0 – 16.5  $\mu\text{m}$  in depth and 6.3 – 9.5  $\mu\text{m}$  in width; epitheca extremely small, cingulum incomplete and hardly recognizable; sulcus very short and located near the apex of the cell; nucleus almost spherical and located in the dorsal posterior side of the cell; plastid single, yellow to yellowish brown, reticulated; pyrenoid circular, surrounded by conspicuous starch sheaths and located in the ventral side of the middle of cell; cell surface smooth with pores

distributed mostly along the sutures; thecal plate arrangement, Po, 1', 0a, 5'', 5c, 2s, 5''', Op, 1''''.

*Holotype*: A SEM stub used for this study has been deposited in the herbarium of the Faculty of Science, Hokkaido University as SAP 115372 (Isotype as SAP 115373).

*Type locality*: Seafloor off Takeshima Island (36 m deep), Kagoshima Prefecture, Japan (30°49'1'' N, 130°24'0'' E)

*Etymology*: *ballux* (noun in apposition = 'gold dust') refers to the low magnification view of culture of this species – scattered yellowish small particles (cells) look like gold dust.

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#### REFERENCES

- Chomérat, N., Couté, A. and Nézan, E. 2010. Further investigations on the sand-dwelling genus *Cabra* (Dinophyceae, Peridinales) in South Brittany (northwestern France), including the description of *C. aremorica* sp. nov. *Mar. Biodiv.* **40**: 131–42.
- Chomérat, N. and Bilien, G. 2014. *Madanidinium loirii* gen. et sp. nov. (Dinophyceae), a new marine benthic dinoflagellate from Martinique Island, Eastern Caribbean. *Eur. J. Phycol.* **49**: 165–78.
- Dodge, J. D. 1975. The Prorocentrales (Dinophyceae). II. Revision of the taxonomy within the genus *Prorocentrum*. *J. Linn. Soc. London, Bot.* **71**: 103–25.

- Faust, M. A. and Balech, E. 1993. A further SEM study of marine benthic dinoflagellates from a mangrove island, Twin Cays, Belize, including *Plagiodinium belizeanum* gen. et sp. nov. *J. Phycol.* **29**: 826–32.
- Hoppenrath, M. and Elbrächter, M. 1998. *Roscoffia capitata* (Dinophyceae) refound: notes on morphology and biology. *Phycologia* **37**: 450–7.
- Hoppenrath, M. 2000. Morphology and taxonomy of the marine sand-dwelling genus *Thecadinium* (Dinophyceae), with the description of two new species from the North German Wadden Sea. *Phycologia* **39**: 96–108.
- Hoppenrath, M., Horiguchi, T., Miyoshi, Y., Selina, M., Taylor, 'Max' F. J. R., and Leander, B. 2007. Taxonomy, phylogeny, biogeography, and ecology of *Sabulodinium undulatum* (Dinophyceae), including an emended description of the species. *Phycol. Res.* **55**: 159–75.
- Hoppenrath, M., Chomérat, N., Horiguchi, T., Schweikert, M., Nagahama, Y. and Murray, S. 2013. Taxonomy and phylogeny of the benthic *Prorocentrum* species (Dinophyceae) – A proposal and review. *Harmful Algae* **27**: 1–28.
- 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., Moriya, R., Pinto, S. K. and Terada, R. 2017. *Pyramidodinium spinulosum* sp. nov. (Dinophyceae), a sand-dwelling non-motile dinoflagellate from the seafloor (36 m deep) off Mageshima Island, Kagoshima, Japan. *Phycol. Res.* **65**: 272–9.
- Huelsenbeck, J. P. and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–5.
- 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.
- Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–4.
- Saunders, R. D. and Dodge, J. D. 1984. An SEM study and taxonomic revision of some

- armoured sand-dwelling marine dinoflagellates. *Protistologica* **10**: 271–83.
- Swofford, D. L. 2002. PAUP\* Phylogenetic analysis using parsimony [\*and other methods]. Version 4. Sinauer Associates, Sunderland, Massachusetts.
- Tamura, M. and Horiguchi, T. 2005. *Pileidinium ciceropse* gen. et sp. nov. (Dinophyceae), a sand-dwelling dinoflagellate from Palau. *Eur. J. Phycol.* **40**: 281–91.
- Wakeman, K. C., Hoppenrath M., Yamaguchi, A., Gavelis, G. S., Leander, B. S. and Nozaki, H. 2018. Ultrastructure of the marine benthic dinoflagellate *Plagiodinium belizeanum* (Dinophyceae) from the southeast Pacific island of Okinawa, Japan. *Phycologia* **57**: 209–22.
- Wołoszyńska, J. 1928. Dinoflagellates der polnischen Ostsee sowie der an Piasnica gelegenen Sumpfe. *Arch. Hydrobiol. Rybact.* **3**: 153-278.
- 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.
- Yamada, N., Tanaka, A., Horiguchi, T. 2015. Pigment compositions are linked to the habitat types in dinoflagellates. *J. Plant Res.* **128**: 923–32.

#### Figure legends

Fig. 1. Light micrographs of *Plagiodinium ballux* sp. nov. (a) Lateral view of the cell, showing nucleus (N) and pyrenoid (Py) which is surrounded by a circular starch sheath. The slightly raised part at upper left corner in this photograph represents the epitheca (arrow). (b) Lateral view, showing the emergence point (arrow) of the transverse flagellum (TF) and the longitudinal flagellum (LF). The pusule is visible near this point (double arrowhead). N: nucleus. The junction between the postcingular plates and the antapical plate is notched (arrowhead). (c) Fluorescent micrograph, showing the circular nucleus (N). (d) Fluorescent micrograph showing reticulated chloroplast.

Fig. 2. Scanning electron micrographs of *Plagiodinium ballux* sp. nov. (a) Left lateral view. (b) Apical view of the cell, showing the epithecal arrangement. (c) Detail of the sulcal area. Arrowhead indicates a small apical pore (Po). (d) Right lateral view of the cell. (e) Antapical view of the cell.

Fig. 3. Schematic illustrations of thecal plate arrangement of *Plagiodinium ballux* sp. nov. (not to scale) (a) Apical view. (b) Ventral view.

Fig. 4. Transmission electron micrographs of *Plagiodinium ballux* sp. nov. (a) Longitudinal section of the cell, showing the chloroplast lobes (C), lipid granules (L), nucleus (N), pyrenoid (Py) and starch grains (S). (b) Detail of the pyrenoid (Py). The pyrenoid is surrounded by a sheath of starch grains (S) and the pyrenoid matrix is traversed by many sinuous thylakoid bands. (c) Detail of the pusule. (d) A vesicle containing virus-like particles and a trichocyst (T) in cross section.

Fig. 5. The phylogenetic position of *Plagiodinium ballux* sp. nov. and *P. belizeanum* (type species) based on maximum likelihood analysis inferred from SSU rDNA sequences. Each species name is followed by an accession number. The bootstrap values (>50) of ML and posterior probability (>0.8) of BI of are indicated at each node. Node with full support (100% Bootstrap value and 1.0 of Posterior Probability) is denoted by black circle. The clade consisting of *Plagiodinium* spp. is indicated by grey box and the sequence of *Plagiodinium ballux* is indicated in bold face.

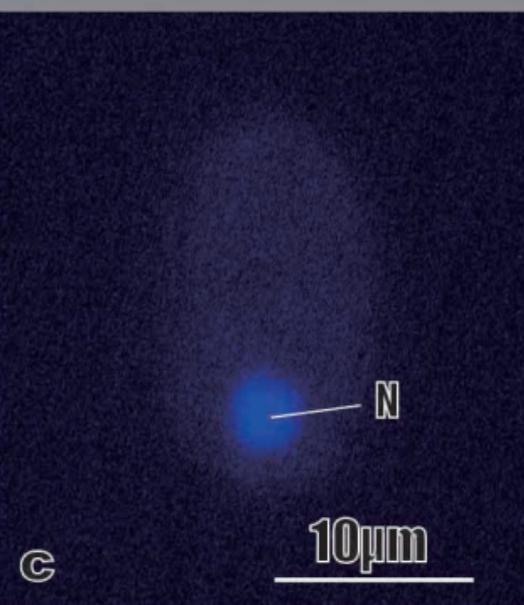
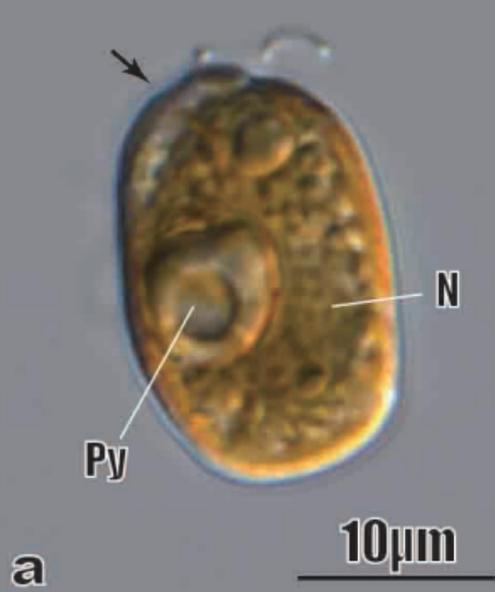
Fig. 6. Phylogenetic position of *Plagiodinium ballux* sp. nov. and *P. belizeanum* based on maximum likelihood analysis inferred from partial LSU rDNA sequences. Each species name is followed by an accession number. The bootstrap values (>50) of ML and posterior probability (>0.8) of BI of each node are indicated at each node. Node with full support (100% Bootstrap value and 1.0 of Posterior Probability) is denoted by black circle. The clade consisting of *Plagiodinium* spp. is indicated by grey box and the sequence of *Plagiodinium ballux* is indicated in bold face.

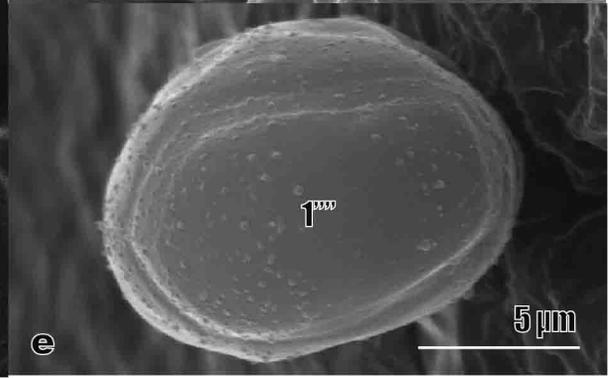
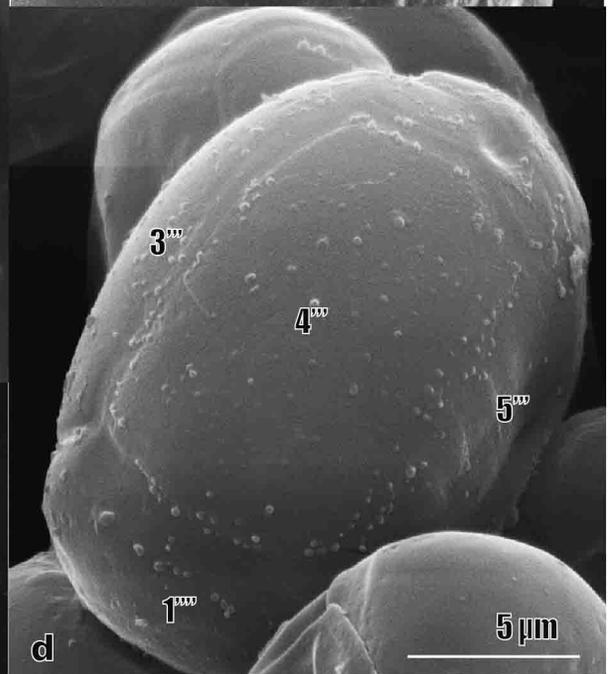
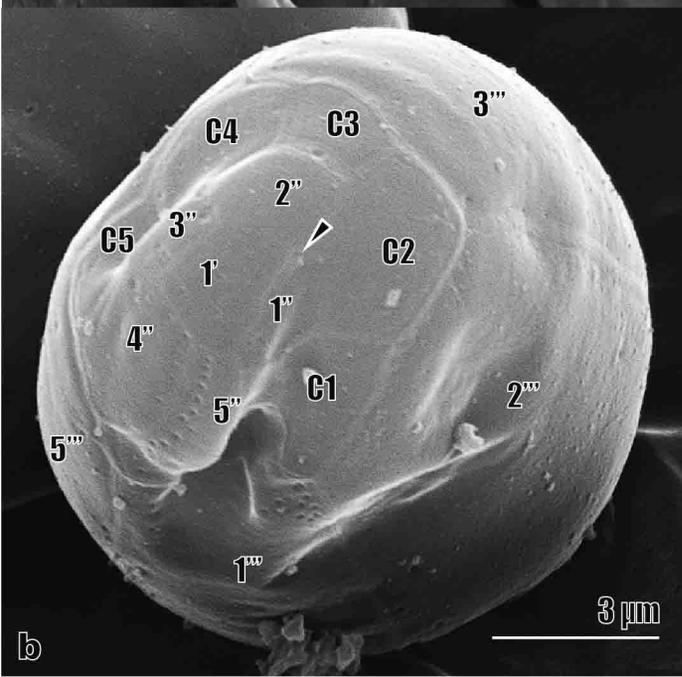
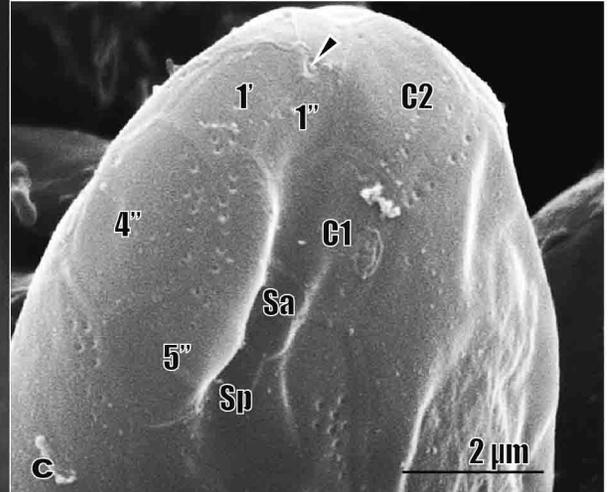
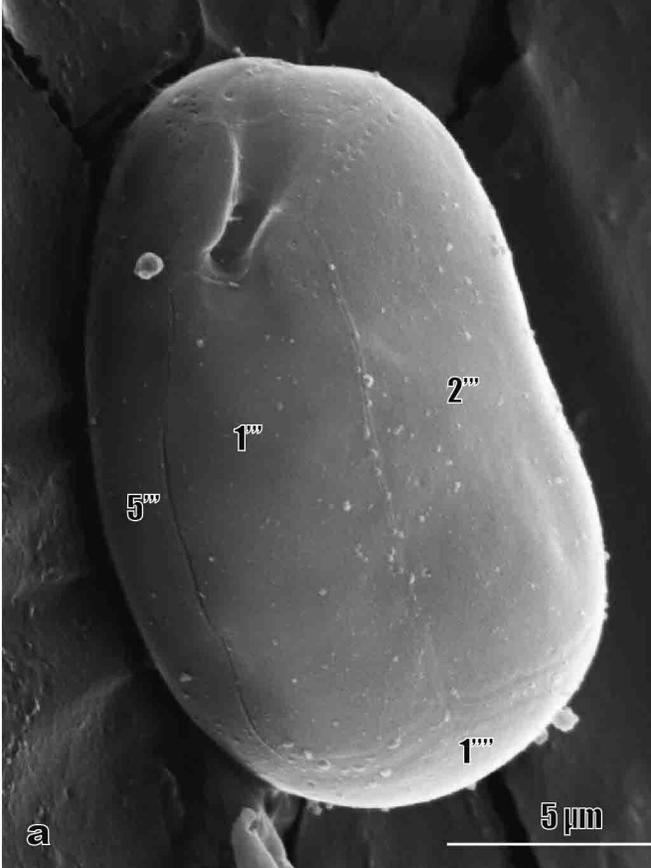
Table 1. Comparative morphological characteristics of *Plagiodinium ballux* and other benthic dinoflagellate genera (based on the characteristics of type species) with a single antapical plate.

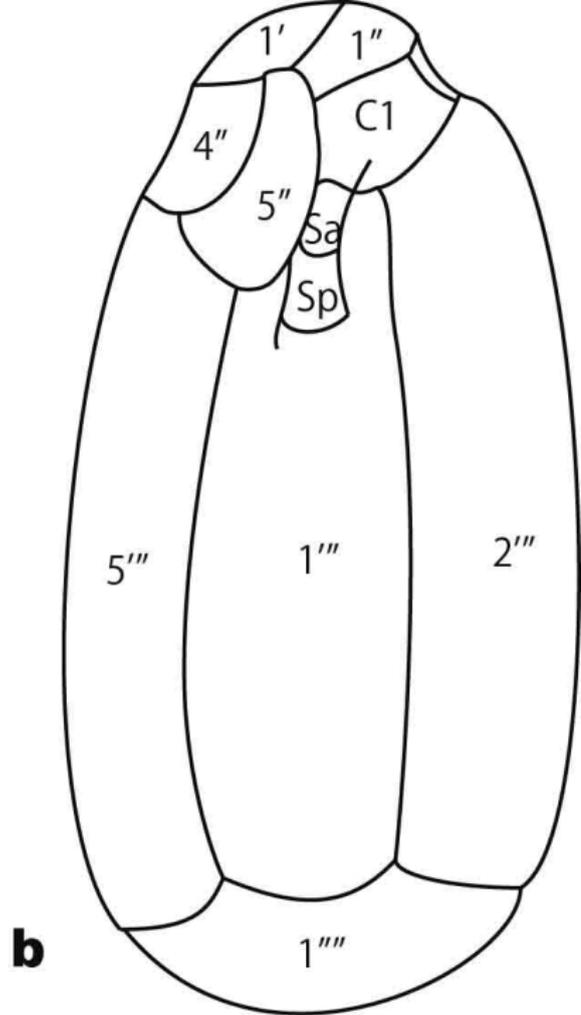
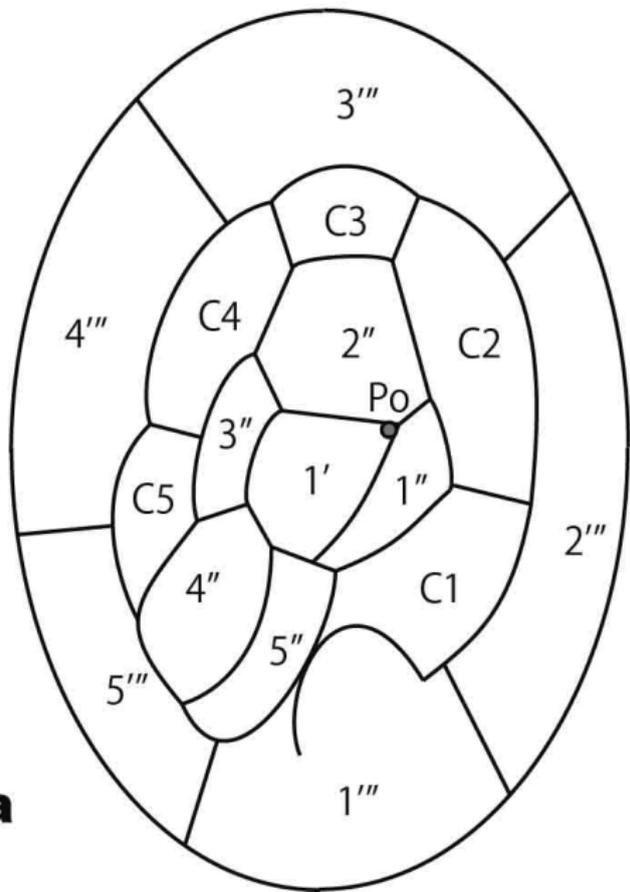
|                       | <i>Plagiodinium ballux</i> | <i>Plagiodinium belizeanum</i> | <i>Cabra</i>                     | <i>Madanidinium</i>      | <i>Pileidinium</i>        |
|-----------------------|----------------------------|--------------------------------|----------------------------------|--------------------------|---------------------------|
| Cingulum              | incomplete                 | complete,                      | complete,                        | complete,                | incomplete                |
| Apical pore           | simple pore                | simple pore                    | APC                              | absent                   | simple pore               |
| Apical plates         | 1'                         | 1'                             | 3'                               | 2'                       | 1'                        |
| Anterior              | 0a                         | 0a                             | 1a                               | 1a                       | 0a                        |
| intercalary plates    |                            |                                |                                  |                          |                           |
| Precingular plates    | 5''                        | 5''                            | 5''                              | 7''                      | 5''                       |
| Cingular plates       | 5C                         | 5C                             | 3C                               | 5C                       | 4C                        |
| Sulcal plates         | 2S                         | 4S                             | ?S                               | 3S                       | 4S                        |
| Postcingular plates   | 5'''                       | 5'''                           | 5'''                             | 5'''                     | 5'''                      |
| Antapical plates      | 1''''                      | 1''''                          | 1''''                            | 1''''                    | 1''''                     |
| Surface ornamentation | smooth                     | smooth                         | reticulate, foveate, or areolate | smooth                   | reticulate                |
| Nutritional mode      | phototrophic               | phototrophic                   | heterotrophic                    | phototrophic             | phototrophic              |
| References            | Present study              | Wakeman <i>et al.</i> (2018)   | Chomérat <i>et al.</i> (2010)    | Chomérat & Bilien (2014) | Tamura & Horiguchi (2005) |

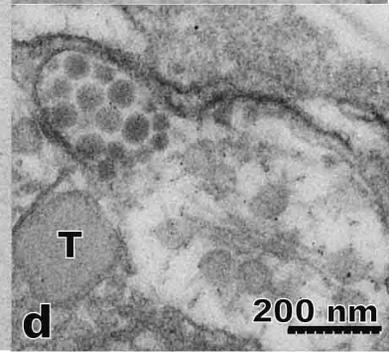
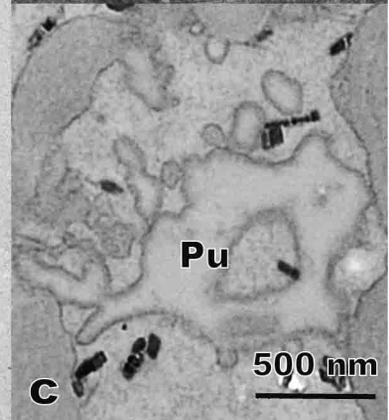
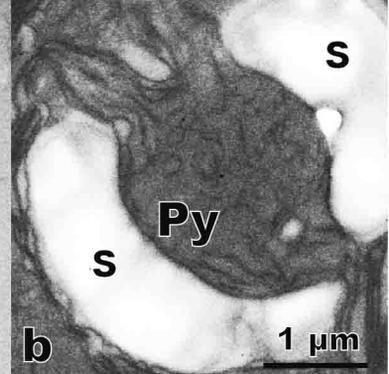
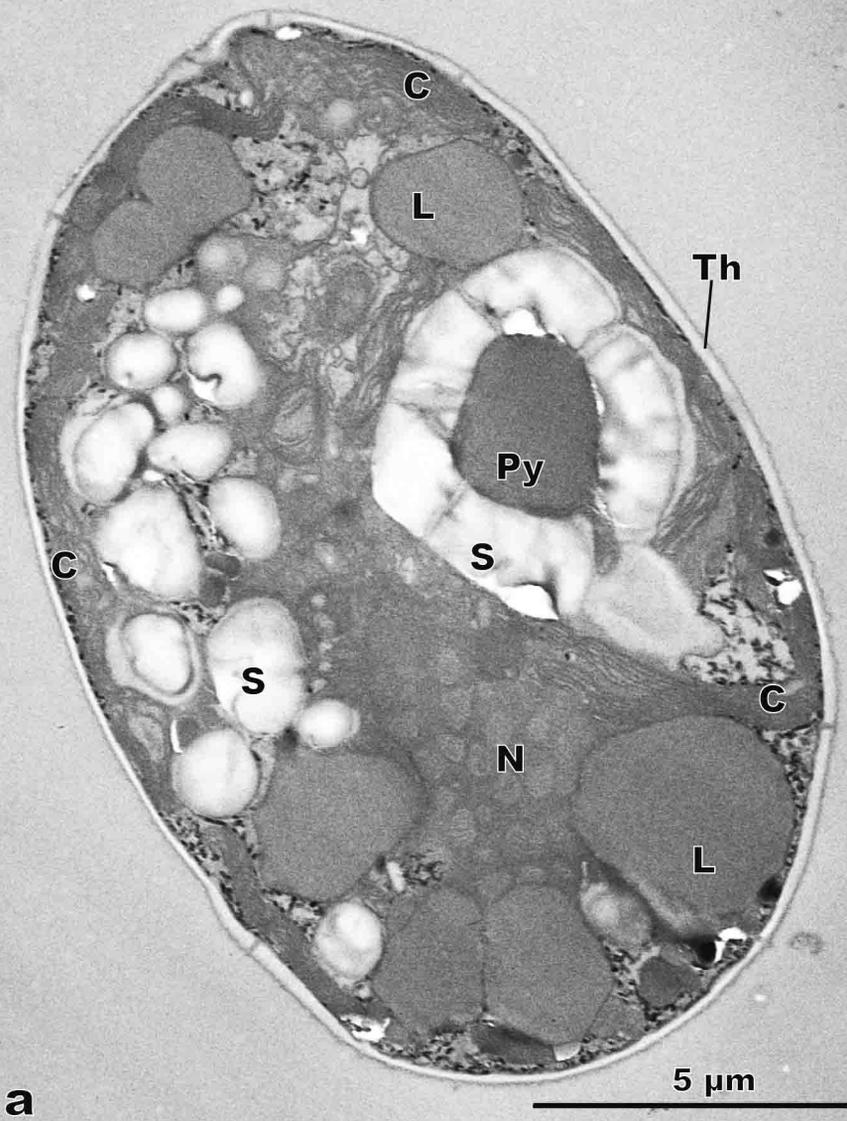
Table 1. (continued)

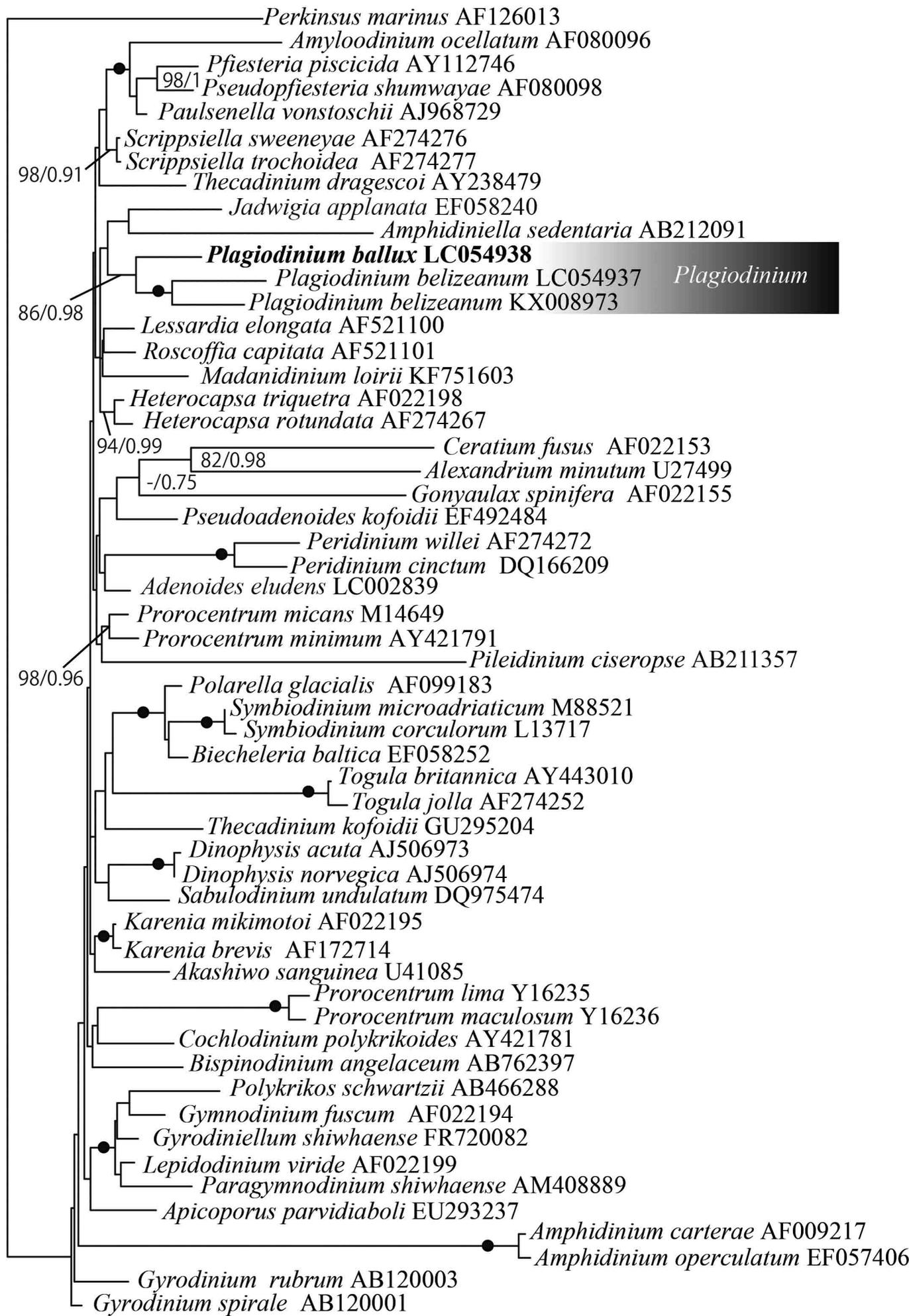
|                     | <i>Planodinium</i>         | <i>Roscoffia</i>                      | <i>Sabulodinium</i>         | <i>Thecadinium</i>          |
|---------------------|----------------------------|---------------------------------------|-----------------------------|-----------------------------|
| Cingulum            | complete                   | complete                              | complete                    | complete                    |
|                     | not displaced              | ascending                             | not displaced               | not displaced               |
| Apical pore         | absent                     | simple pore                           | APC                         | hook-shaped pore            |
| Apical plates       | 3'                         | 5'                                    | 5'                          | 3'                          |
| Anterior            | 0a                         | 0a                                    | 1a                          | 1a                          |
| intercalary plates  |                            |                                       |                             |                             |
| Precingular plates  | 7''                        | 5''                                   | 6''                         | 4''                         |
| Cingular plates     | 6C                         | 3C                                    | 5C                          | 5(6?)                       |
| Sulcal plates       | ?                          | 4S                                    | 4S                          | 5S                          |
| Postcingular plates | 3'''                       | 5'''                                  | 5'''                        | 4'''                        |
| Antapical plate     | 1''''                      | 1''''                                 | 1''''                       | 1''''                       |
| Surfacem            | longitudinal               | foveate                               | smooth                      | smooth (partly reticulated) |
| ornamentation       | striations                 |                                       |                             |                             |
| Nutritional mode    | heterotrophic              | heterotrophic                         | heterotrophic               | phototrophic                |
| References          | Saunders & Dodge<br>(1984) | Hoppenrath &<br>Elbrächeter<br>(1998) | Hoppenrath et al.<br>(2007) | Hoppenrath (2000)           |











*Eimeria tenella* AF026388

*Eimeria tenella* AF026388

*Prorocentrum micans* AF260377

*Prorocentrum minimum* AF260379

*Karlodinium micrum* AF260393

*Takayama acrotrocha* DQ656115

70/-  
*Karenia brevis* AF200675

*Karenia mikimotoi* AF200682

*Alexandrium tamarense* FJ411143

*Alexandrium minutum* EU707466

83/0.95  
*Gonyaulax baltica* AF260388

*Protoceratium reticulatum* AF260386

*Ceratium fusus* AF260390

*Dinophysis norvegica* AY571375

***Plagiodinium ballux* LC375159**

*Plagiodinium*

*Plagiodinium belizeanum* LC375160

*Plagiodinium belizeanum* KX008972

*Peridinium willei* AF260384

*Peridinium cinctum* AF260385

*Scrippsiella trochoidea* AF260393

*Pfiesteria piscicida* AY112746

*Thoracosphaera heimii* EF205018

97/0.99  
*Amphidinium carterae* AY455669

*Amphidinium operculatum* AY455674

*Amphidinium herdmanii* AY455675

*Gyrodinium rubrum* AY571369

*Gyrodinium dominans* AY571370

*Gyrodinium spirale* AY5713717

*Togula britannica* AY455679

*Cochlodinium polykrikoides* AB295050

96/0.98  
*Tovellia coronata* AY950446

*Esoptrodinium gemma* DQ289020

*Gymnodinium fuscum* AF200676

*Spiniferodinium galeiforme* GU295206

*Gymnodinium impudicum* AF200674

*Lepidodinium chlorophorum* AB367942

*Warnowia* sp. FJ947042

*Nematodinium* sp. FJ947041

*Gymnodinium impudicum* AF200674

*Polykrikos schwartzii* EF205013

*Biecheleria baltica* EF205019

*Polarella glacialis* AY571373

*Borghiella dodgei* EU1268011

*Bispinodinium angelaceum* AB762398

*Heterocapsa rotundata* AF260400

*Heterocapsa triquetra* EF613355

*Akashiwo sanguinea* AF260396