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Molecular phylogeny of the benthic dinoflagellate *Cabra matta* (Dinophyceae) from Okinawa, Japan

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ABSTRACT: *Cabra* is a genus of marine heterotrophic thecate dinoflagellates with a peculiar cell shape and thecal plate tabulation. Previous studies suggested it was related to the benthic genus *Roscoffia* based on morphological features. A previous molecular phylogenetic study using 18S rDNA showed that *Roscoffia capitata* and members of the family Podolampadaceae formed a well-supported monophyletic group; however, the classification of *Cabra* remains uncertain. In this study, the first molecular phylogenetic analysis based on 18S rDNA sequence for the genus was performed using specimens of *Cabra matta* collected in Okinawa, Japan. Molecular phylogeny showed that *C. matta* formed a clade with *R. capitata* and the planktonic oceanic members of Podolampadaceae including *Podolampas* spp. and *Blepharocysta* sp. This result supported previous studies that inferred the close affinity between *Cabra*, podolampadaceans and *R. capitata* based on a very narrow and long 1′ plate, and the diminutive 2′ and 3′ plates, the larger precingular plates, smaller apical plates, the presence of three cingular plates, the hypothecal plate pattern of five postcingular plates and one antapical plate. In contrast, there are clear morphological differences between the planktonic podolampadaceans and the benthic genera *Roscoffia* and *Cabra*: the planktonic members have a cryptic cingulum and lack an apparent sulcus, while the benthic members have an apparent cingulum and sulcus. Molecular phylogeny in the present study did not resolve the evolutionary branching order for *Cabra*, *Roscoffia*, *Podolampas* and *Blepharocysta*.

KEY WORDS: Epiphytic, Morphology, Podolampadaceae, *Rhinodinium*, *Roscoffia*, Systematics

INTRODUCTION

Cabra is a marine, heterotrophic, thecate dinoflagellate established from samples collected from Australia (Murray & Patterson 2004). The genus was monotypic, having only one representative species, *Cabra matta* S.Murray & D.J.Patterson, until Chomérat & Nézan (2009) described *Cabra reticulata* Chomérat & Nézan from Groix Island, South Brittany, France. Since that time, two more species, *Cabra aremorica* Chomérat, Couté & Nézan and *Cabra levis* Selina, Chomérat & Hoppenrath, have been described from France and Russia (Chomérat *et al.* 2010; Selina *et al.* 2015). Although research has examined the detailed morphology of these species using light microscopy (LM) and scanning electron microscopy (SEM), there have been no molecular phylogenetic studies on the taxa.

Cabra can be sand-dwelling (*C. matta*, *C. reticulata*, *C. aremorica* and *C. levis*) and has been reported as epiphytic on macroalgae (*C. matta*, *C. reticulata* and *C. levis*) (Selina *et al.* 2015). The genus has a peculiar cell shape and thecal plate tabulation. The original description of *C. matta* described the plate formula as P_o 4′ 4″ ‘x’ 3c ?s 5‴ 1⁗ (Murray & Patterson 2004). The plate pattern and other cellular features of *C. matta* differed from any described dinoflagellate genera at that time but was most similar to *Roscoffia* in that they shared similar hypothecal plate patterns and had three cingular plates, a narrow first apical plate and the ascending or slightly ascending cingulum (Murray & Patterson 2004). *Roscoffia* contains two described species, *Roscoffia capitata* Balech and *Roscoffia minor* Horiguchi & Kubo (Balech 1956; Horiguchi & Kubo 1997; Hoppenrath & Elbrächter 1998). Because this genus also has a peculiar morphology, the phylogenetic position among dinoflagellates is difficult to infer. However, it was suggested that the thecal plate pattern of the genus is related to the planktonic oceanic family Podolampadaceae in the order Peridiniales (Horiguchi & Kubo 1997; Hoppenrath & Elbrächter 1998). A previous molecular phylogenetic study using 18S rDNA showed that members of Podolampadaceae (four species of *Podolampas* and a species of *Blepharocysta*) and *R. capitata* formed a well-supported monophyletic group (Gómez *et al.* 2010).

Cabra also shares morphological similarities to species with the family Podolampadaceae. They share similar features in hypothecal plate arrangement, apical pore structure and presence of a long narrow first apical plate (Murray & Patterson 2004). Carbonell-Moore (1994) described the thecal plate formula of the family Podolampadaceae as P_o P_i canal plate (CP) 3′ 1a 5″ 3c 4–5s 4–5‴ 1⁗. When Chomérat *et al.* (2010) described the third species of *Cabra*, *Cabra aremorica*, they also observed the type species, *Cabra matta*, and emended *Cabra*. They gave a new interpretation of the plate pattern as P_o P_t 3′ 1a 5″ (4″ ‘x’) 3c ?s 5‴ 1⁗. This new plate pattern is generally similar with that of Podolampadaceae.

Cabra is currently assigned to the phylum Dinophyta, order Peridiniales, and its familial assignment is still

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uncertain (Murray & Patterson 2004; Chomérat & Nézan 2009; Chomérat *et al*. 2010; Gómez 2012). Shah *et al*. (2013) included it in Podolampadaceae, Peridiniales. The unique morphology of *Cabra* makes positioning of the genus among the other dinoflagellates uncertain. Because morphology alone is not sufficient to infer the phylogenetic position of the genus, molecular DNA sequences of *Cabra* should help infer relationship both within the genus and to other dinoflagellates. In this study, the first molecular phylogenetic analysis based on 18S rDNA sequence of the genus was performed using *Cabra matta* collected in Okinawa, Japan, in the subtropical region of the north-western Pacific Ocean.

MATERIAL AND METHODS

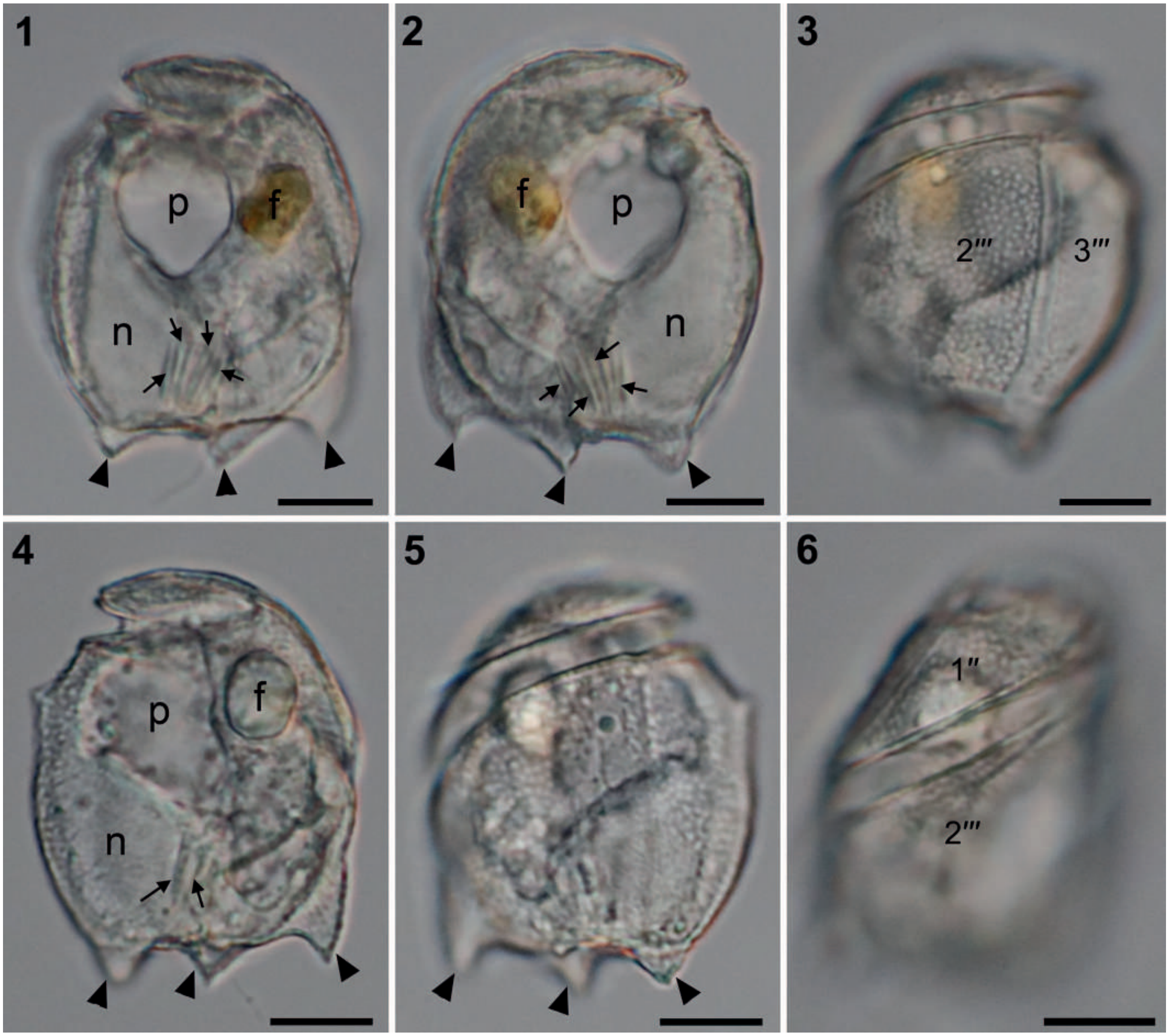
Sand samples were collected from a tidal flat near Onna Village, Okinawa (26°28′57″N, 127°50′44″E), in April 2017. Crude cultures were established using Daigo's IMK culture medium (Nihon Pharmaceutical Co., Tokyo Japan) prepared using 0.126 g of powder per litre of seawater. A 50 ml volume of culture medium was put in a plastic cup, sealed with a plastic lid and maintained at 20°C in a 16:8 h light:dark cycle for 3 wk. Since the lid was sealed on the container, evaporation of the medium was considered negligible and no additional medium was added to the crude culture.

Single living cells of *Cabra matta* were isolated from crude cultures and washed several times in serial drops of 0.22 μm filtered seawater by micropipetting. Cells were transferred to a glass slide with a vinyl tape frame (Horiguchi *et al*. 2000) and sealed with a cover glass. Each cell was observed using a BX-50 compound microscope with Nomarski optics (Olympus, Tokyo Japan) equipped with an EOS Kiss X8i digital camera (Canon, Tokyo Japan). Photographed cells were transferred to a polymerase chain reaction (PCR) tube, each containing 10 μl of Quick Extract formalin-fixed, paraffin-embedded (FFPE) DNA extraction solution (Epicentre, Madison, Wisconsin USA). Each PCR tube containing a single cell was then incubated for 1 h at 56°C, then 2 min at 98°C. The resulting extract was used as a DNA template for subsequent PCR amplifications.

The initial PCR was performed using a total volume of 25 μl with EconoTaq 2X Master Mix (error rate 1 per 20,000–40,000) (Lucigen, Middleton, Wisconsin USA) following manufacturer's protocols. Nearly the entire 18S rRNA gene was amplified using the sets of universal eukaryote primers: SR1 (5′-TACCTGGTTGATCCTGCCAG-3′) and SR12 (5′-CCTTCCGCAGGTTCACCTAC-3′) (Nakayama *et al*. 1996). The PCR protocol had an initial denaturation stage of 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min and final extension at 72°C for 7 min. The first PCR product was used as a DNA template for the second PCR. The second PCR was performed using EconoTaq 2X Master Mix where the following combinations of primer pairs were used separately: (1) SR1b (5′-GATCCTGCCAGTAGTCATATGCTT-3′) and SR5TAK (5′-ACTACGAGCTTTTTAACYGC-3′); (2) SR4 (5′-AGGGCAAGTCTGGTGCCAG-3′) and SR9p (5′-AACTAAGAACRGCCATGCAC-3′); and (3) SR8P (5′-GGATTGACARDTTGATARCT-3′) and SR12b (5′-CGGAAACCTTGTTACGACTTCTCC-3′) (Nakayama *et al*. 1996; Takano & Horiguchi 2004; Yamaguchi & Horiguchi 2005). The PCR protocol had an initial denaturation stage at 94°C for 2 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 45 s and final extension at 72°C for 7 min. Amplified DNA fragments corresponding to the expected size were purified by QIAquick PCR Purification Kit (Qiagen, Hilden Germany). The cleaned PCR products were sequenced directly by Fasmac sequencing service (Fasmac, Kanagawa Japan). In the course of the experiment, eight cells of *C. matta* were isolated, photographed and used in PCR reactions. Of the eight isolates, only two cells were sequenced successfully. These new sequences have been deposited in DDBJ/EMBL/GenBank under the accession numbers (LC333936, LC333937).

The 18S rDNA sequences were aligned using MUSCLE (Edgar 2004; http://www.ebi.ac.uk/Tools/msa/muscle/) with the default settings and then manually refined in Mesquite v3.11 (Maddison & Maddison 2015). The final alignment of the 18S rDNA dataset consisted of 64 taxa and 1283 sites. The apicomplexan *Neospora caninum* Dubey, Carpenter, Speer, Topper & Uggla was used as the outgroup. The alignment is available from the corresponding author upon request. Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian analysis. For ML, the alignment was analysed by GARLI v0.951 (Zwickl 2006). The general time reversible (GTR) + proportion of invariable sites (I) + gamma distribution (G) model of nucleotide substitution was chosen by the Akaike information criterion as implemented in jModelTest 2.1.10 (Darriba *et al*. 2012; Guindon & Gascuel 2003). The parameters were as follows: assumed nucleotide frequencies were A = 0.2321, C = 0.2145, G = 0.2594, T = 0.2939; substitution rate matrix with A–C substitutions = 1.1713, A–G = 4.8250, A–T = 1.2586, C–G = 0.6366, C–T = 8.5686, G–T = 1.0000; proportion of sites assumed to be invariable = 0.2940. In addition, rates for variable sites were assumed to follow a gamma distribution with shape parameter = 0.5370. Bootstrap analysis was carried out for ML with 500 replicates to evaluate statistical reliability. MrBayes v3.2.5 was used to perform Bayesian analyses (Ronquist & Huelsenbeck 2003) with the GTR + I + gamma evolutionary model. The program was set to operate four Monte-Carlo–Markov chains starting from a random tree. A total of 1,500,000 generations were calculated, with trees sampled every 100 generations. The first 3750 trees in each run were discarded as burn-in. Posterior probabilities (PP) correspond to the frequency at which a given node was found in the post–burn-in trees.

Individual cells of *C. matta* were isolated and placed in a small container covered on one side with a 10 μm mesh plankton net containing acidic 1% Lugol's solution in culture seawater. They were then washed three times in distilled water to remove the fixative and salt, and dehydrated through a graded series of ethanol (50%, 70%, 80%, 90% 100%). Samples were critical point dried with CO_2, sputter-coated with 5 nm gold, and observed using a Hitachi 4700 Electron Microscope (Hitachi, Tokyo Japan).



Figs 1–6. Light micrographs of the single cells of *Cabra matta* used for rDNA sequencing. Scale bars = 10 μm.

Fig. 1. *Cabra matta* individual number 1. Right lateral view showing the pusule (p), food vacuole (f), nucleus (n), extrusomes (arrows) and three protrusions (arrowheads).

Fig. 2. *Cabra matta* individual number 1. Left lateral view showing the pusule (p), food vacuole (f), nucleus (n), extrusomes (arrows) and three protrusions (arrowheads).

Fig. 3. *Cabra matta* individual number 1. Left lateral surface view showing the second and third postcingular plates ($2'''$ and $3'''$) and thecal ornamentation.

Figs 4–6. *Cabra matta* individual number 2.

Fig. 4. *Cabra matta* individual number 2. Right lateral view showing the pusule (p), food vacuole (f), nucleus (n), extrusomes (arrows) and three protrusions (arrowheads).

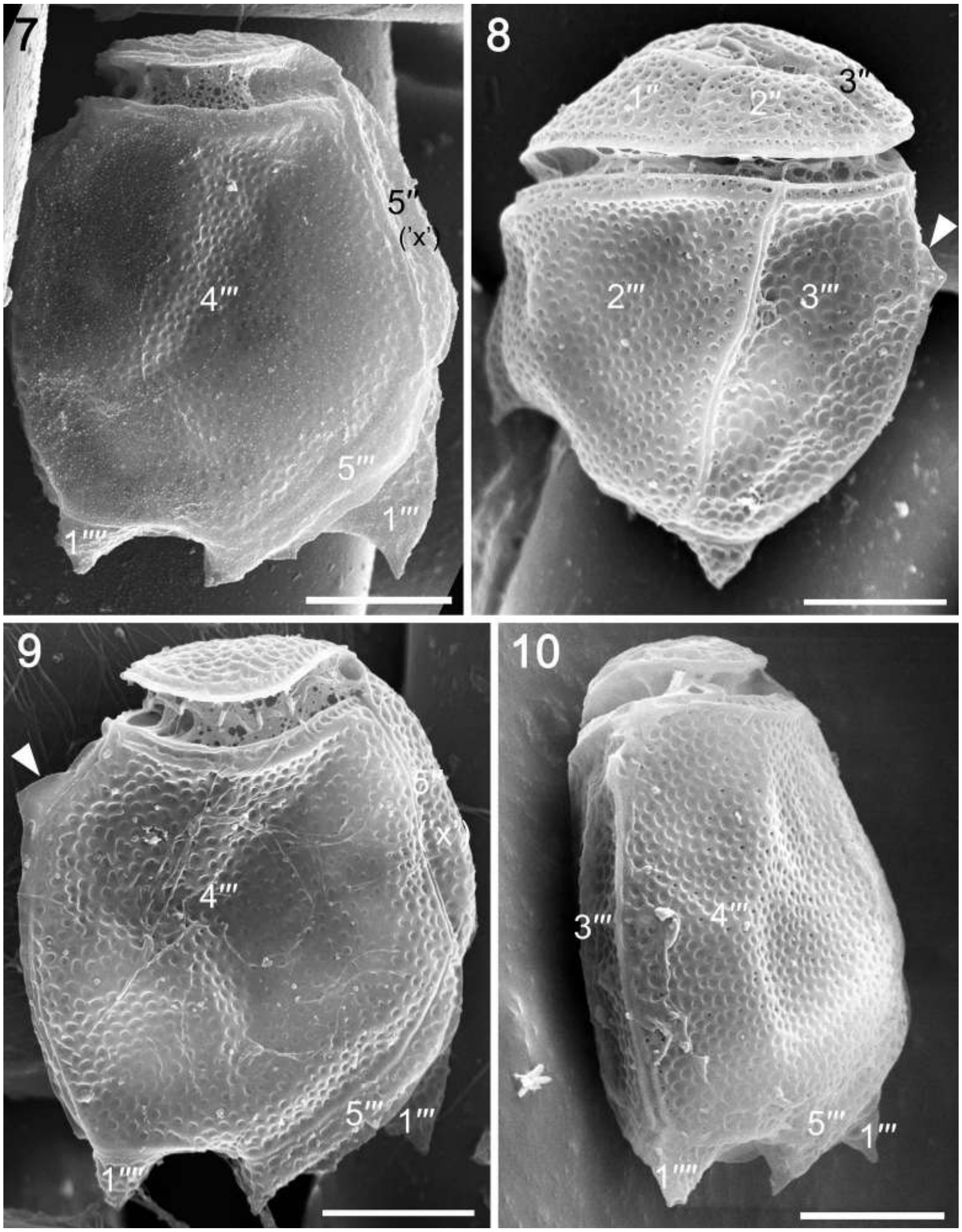
Fig. 5. *Cabra matta* individual number 2. Left lateral view showing the three protrusions (arrowheads).

Fig. 6. *Cabra matta* individual number 2. Left dorsal view showing the first precingular plate ($1'$) and the second postcingular plate ($2'''$).

RESULTS

Morphology of *Cabra matta* from Okinawa, Japan

Cells of *Cabra matta* (Figs 1–14) were laterally compressed (Figs 6, 10–14), 34.4–43.5 μm long (8.0 ± 2.9 μm, mean ± *s*, *n* = 11) and 29.0–34.2 μm deep in lateral view (dorsoventral width; 31.9 ± 1.9 μm, *n* = 11). In lateral view, cells were oval with three short antapical protuberances (Figs 1, 2, 4, 5, 7–9). The pusule was observed in the anterior–dorsal part of the cell (Figs 1, 2, 4). Most cells had a pigmented food vacuole in the anterior–ventral part of the hypotheca (Figs 1, 2, 4). Some cells contained large extrusomes (trichocysts?) in the posterior part of the cells (Figs 1, 2, 4). The nucleus was positioned in



Figs 7–10. SEM micrographs of *Cabra matta* from Okinawa, Japan. Scale bars = 10 μm.

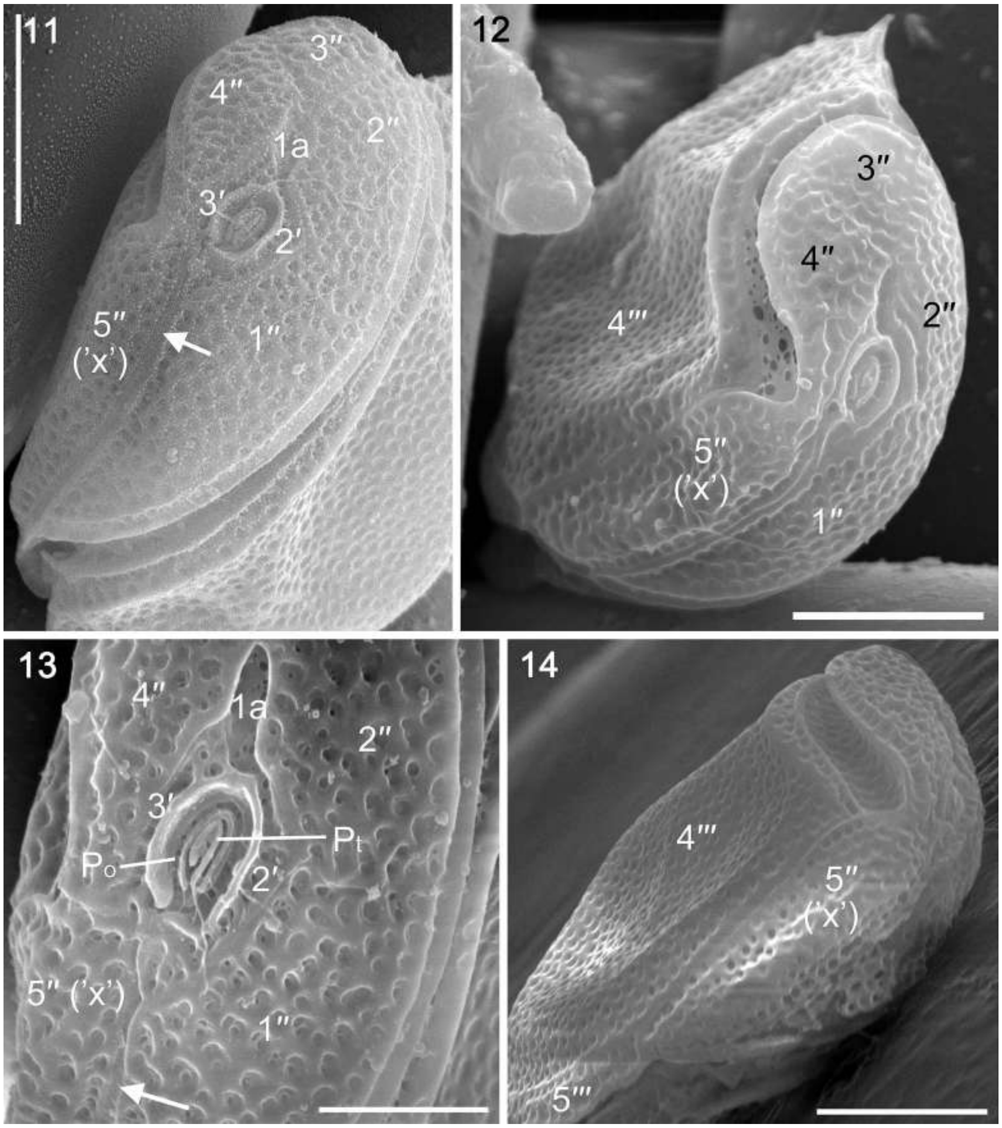
Fig. 7. Right lateral view showing the fifth precingular plate ($5''$), which can also be interpreted as 'x' plate; the first, fourth and fifth postcingular plates ($1'''$, $4'''$ and $5'''$) and the antapical plate ($1''''$).

Fig. 8. Left lateral view showing the first to the third precingular plates ($1''$, $2''$ and $3''$), the second and third postcingular plates ($2'''$ and $3'''$) and a small spine on the upper side of the $3'''$ plate (white arrowhead).

Fig. 9. Right lateral view showing the fourth and fifth postcingular plates ($4'''$ and $5'''$) and a small spine on the upper side of the $3'''$ plate (white arrowhead).

Fig. 10. Dorsal view showing the first, third, fourth and fifth postcingular ($1'''$, $3'''$, $4'''$ and $5'''$) and the first antapical ($1''''$) plates.

the dorsal part of the hypotheca (Figs 1, 2, 4). Thecal plates had pores, which were present mostly in circular depressions (Figs 3, 7–14). The epitheca was smaller than the hypotheca. The cingulum was wide and strongly ascending (Figs 6, 12, 14). The apical pore complex (APC) was composed of a cover plate (P_t) and a pore plate (P_o) surrounding the P_t (Fig. 13, Carbonell-Moore 1994). The P_o plate was oval and the P_t plate was positioned in the centre of the P_o plate. There were small pores on the P_o plate (Fig. 13). The first apical plate ($1'$) was long and narrow (Fig. 11) and was positioned next to the second ($2'$) and third ($3'$) apical plates and the first ($1''$) and fifth ($5''$) precingular plates (Fig. 11). The $2'$ and $3'$ plates surrounded the APC (Figs 11, 13). There was a narrow and small first anterior intercalary plate (1a), which was deeply indented and positioned at the dorsal part of the cell, surrounded by the $2'$–$3'$ plates and the second ($2''$) to the



Figs 11–14. SEM micrographs of *Cabra matta* from Okinawa, Japan. Scale bars = 10 μm for Figs 11–12, 14. Scale bars = 5 μm for Fig. 13.

Fig. 11. Dorso-apical view showing the first apical plate (arrow), second and third apical plates (2′ and 3′), the first anterior intercalary plate (1a) and the first to fifth precingular plates (1″–5″). The fifth precingular plate can also be interpreted as 'x' plate.

Fig. 12. Apical view showing the first to fifth precingular plates (1″–5″) and the fourth postcingular plate (4‴).

Fig. 13. Apical view focused on the apical pore complex showing the pore plate (P_o), the cover plate (P_t), the 1′ plate (arrow), the 2′ and 3′ plates, the first anterior intercalary plate (1a) and the 1″, 2″, 4″ and 5″ plates.

Fig. 14. Ventral view showing the fifth precingular plate (5″) and the fourth and the fifth postcingular plates (4‴ and 5‴).

fourth (4″) precingular plates. The 5″ plate ('x' plate) touched the 1′ plate on the left side (Fig. 11) and the fourth postcingular plate (4‴) on the right side (Figs 12, 14). The hypotheca was formed by five postcingular plates (1‴–5‴) and one antapical plate (1⁗) (Figs 7, 9, 12, 14). There was a small spine on the upper side of the third postcingular plate (3‴), which was positioned at the dorsal side of the hypotheca (Figs 8, 9). Three antapical protuberances were formed by the 1‴ and 5‴ plates and the 1⁗ plate (Figs 7, 9, 10).

Phylogenetic analysis of 18S rDNA

We analysed the phylogenetic position of *Cabra matta* from Okinawa, Japan, based on the dataset including various dinoflagellate 18S rDNA sequences (Fig. 15). We tried to get the molecular data through isolating, photographing using LM and then isolating for DNA extraction eight single cells of *C. matta*. As a result, we obtained the 18S rDNA sequences from two cells of *C. matta* (accession numbers LC333936, LC333937) that were identical. *Cabra matta* formed a moderately supported clade with members of Podolampadaceae (*Blepharocysta* sp., *Podolampas spinifera* Okamura, *Podolampas palmipes* F.Stein, *Podolampas bipes* F.Stein, *Podolampas elegans* Schütt and *Roscoffia capitata*). In this clade, four species of *Podolampas* formed a clade, and also *Blepharocysta* sp. and *R. capitata* formed a clade. The photosynthetic and benthic *Plagiodinium belizeanum* M.A.Faust & Balech was positioned at the base of the clade

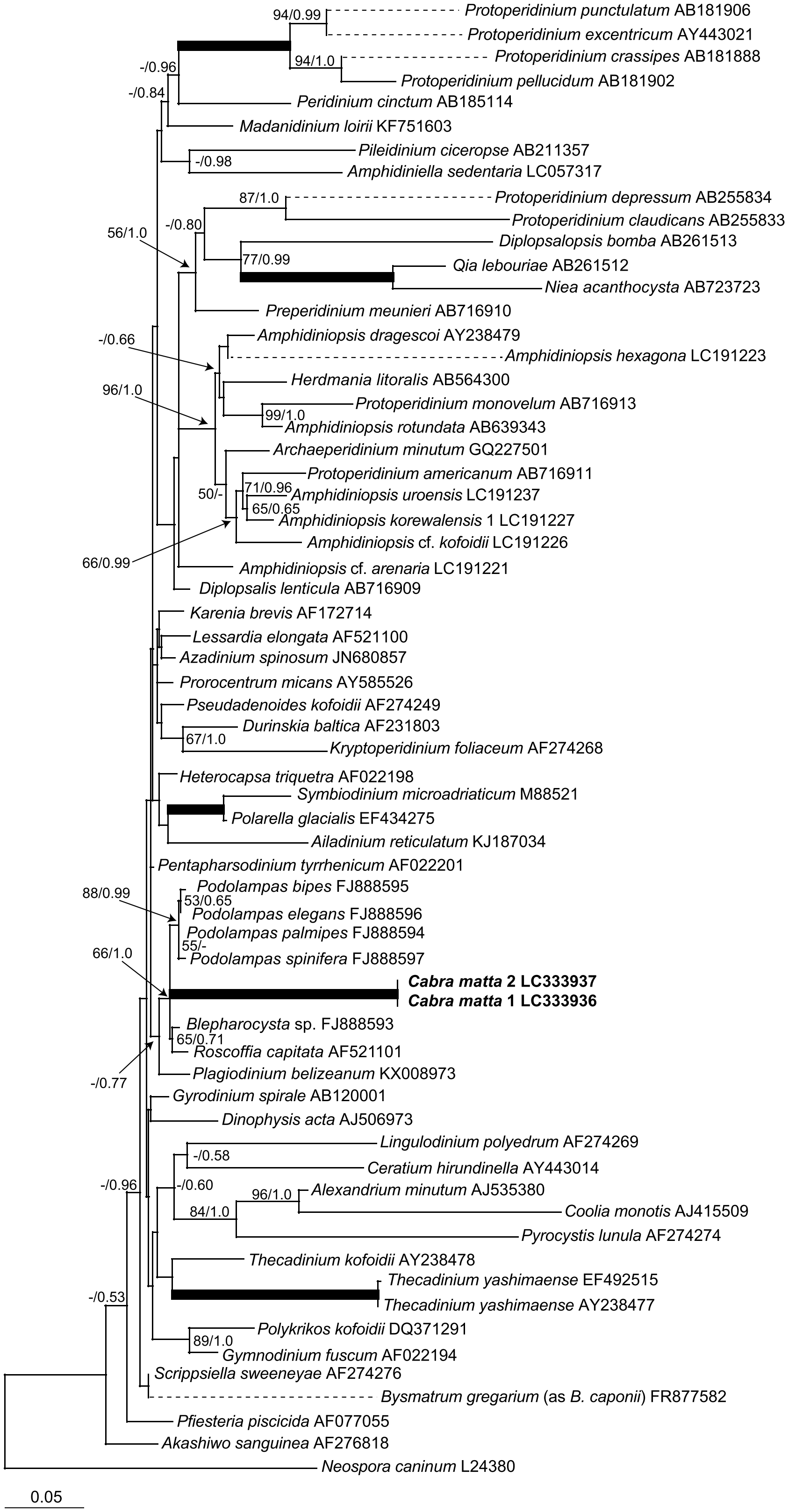


Fig. 15. Maximum-likelihood (ML) tree inferred from 18S rDNA sequences. ML bootstrap values over 50% and Bayesian posterior probabilities (PP) over 0.50 are shown at the nodes (ML/PP). Thick branches indicate maximal statistical support (100/1.0). The branches leading to the fast-evolving taxa are indicated by dashed and shortened by one half. *Cabra matta* from this study are indicated in bold. The scale bar represents inferred evolutionary distance in changes/site.

comprised of *Podolampas* spp., *Blepharocysta* sp., *R. capitata* and *C. matta* with weak statistical support. Three diplopsalids, *Diplopsalopsis bomba* (F.Stein) J.D.Dodge & Toriumi, *Qia lebouriae* T.Liu, K.N.Mertens & H.Gu, and *Niea acanthocysta* (Kawami, Iwataki & Matsuoka) T.Liu, K.N.Mertens & H.Gu, formed a clade with moderate statistical support. However, the other diplopsalids, *Preperidinium meunieri* (Pavillard) Elbrächter and *Diplopsalis lenticula* Bergh, did not show affinity with the other dinoflagellates. None of the benthic species of *Amphidiniopsis* and *Thecadinium* included in the present analysis showed a close relationship with *Podolampas* spp., *Blepharocysta* sp., *R. capitata* or *C. matta*. Other included taxa with unknown affinities to other dinoflagellates – such as *Ailadinium reticulatum* Saburova & Chromérat, *Pileidinium ciceropse* Tamura & Horiguchi, *Amphidiniella sedentaria* Horiguchi, *Pseudadenoides kofoidii* (Herdman) Gómez, Onuma, Artigas & Horiguchi, *Bysmatrum gregarium* (Lombard & Capon) Horiguchi & Hoppenrath, *Lessardia elongata* Saldarriaga & F.J.R.Taylor and *Madanidinium loirii* Chomérat – showed no definitive phylogenetic positions among other dinoflagellates.

DISCUSSION

The genus *Cabra* has been reported from Australia (Murray & Patterson 2004), the Gulf of Mexico (Okolodkov *et al.* 2007), north-western France (Chomérat & Nézan 2009; Chomérat *et al.* 2010), Russia (Selina & Levchenko 2011; Selina *et al.* 2015), Korea (Shah *et al.* 2013), the Mediterranean Sea, the South Atlantic Ocean, the Caribbean Sea and the eastern Asian coast of China (Gómez & Lopes 2015). To our knowledge, this study is the first published report of *Cabra* from Japan. The cells from Okinawa agreed morphologically with *Cabra matta* in the ornamentation of thecal plates (containing small depressions, some with a thecal pore), the microarchitecture of the apical pore complex and the deeply indented 1a plate. Although the size range of our specimen was larger than that of the original description from Australia (Murray & Patterson 2004), other reports of the species showed a larger size than the original description (Chomérat *et al.* 2010; Shah *et al.* 2013; Selina *et al.* 2015). The majority of the cells in this study had prominent extrusomes, likely trichocysts, that were visible by light microscopy in the posterior part of the cell. These prominent extrusomes have not been previously reported for *Cabra* spp. (Murray & Patterson 2004; Okolodkov *et al.* 2007; Chomérat & Nézan 2009; Chomérat *et al.* 2010; Selina & Levchenko 2011; Shah *et al.* 2013; Gómez & Lopes 2015; Selina *et al.* 2015).

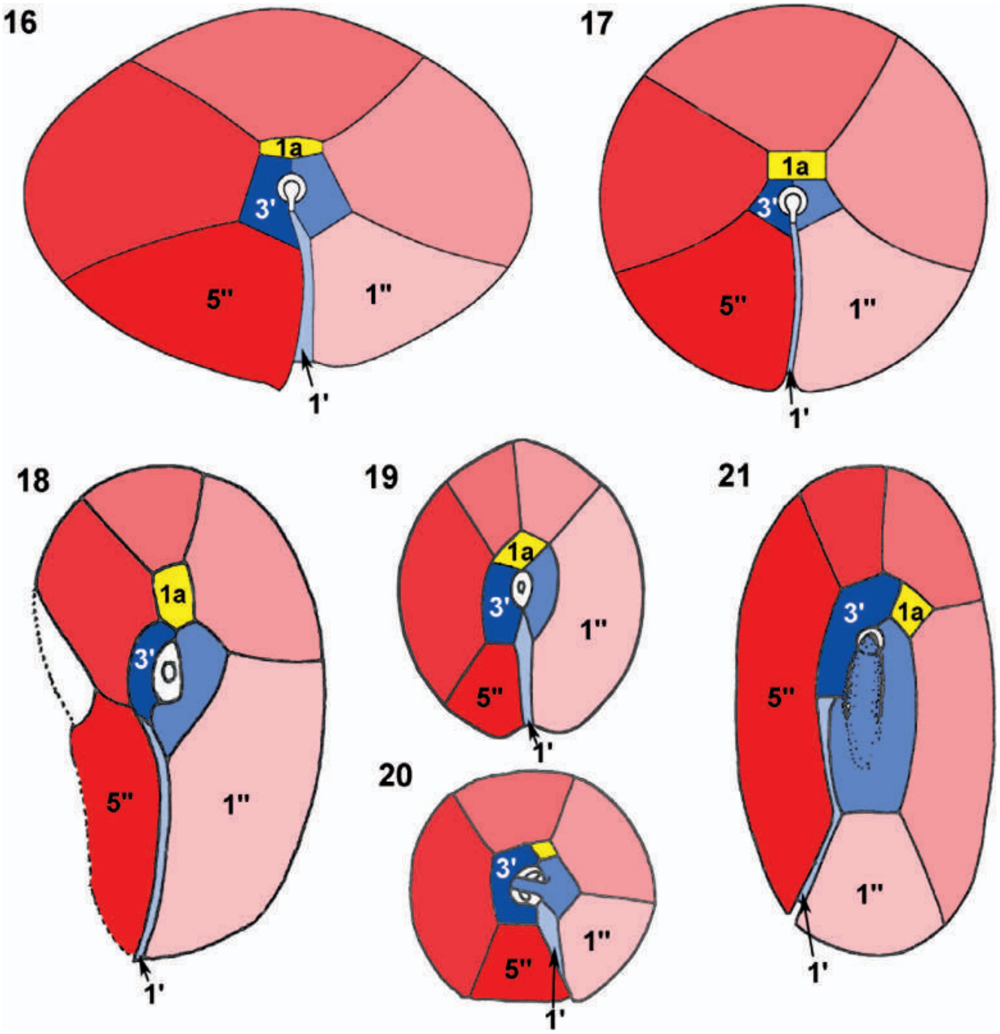
The present study performed the first molecular phylogenetic analyses of the genus *Cabra* from single cells using 18S rDNA sequences. The result showed that *Cabra matta* formed a clade with the benthic species *Roscoffia capitata* and the members of the Podolampadaceae including *Podolampas* spp. and *Blepharocysta* sp. The Podolampadaceae have been traditionally characterised by having a cryptic cingulum and the absence of an apparent sulcus, and includes the planktonic, oceanic genera *Blapharocysta*, *Podolampas* and *Lissodinium* (Carbonell-Moore 1994; Gómez *et al.* 2010). However, *Roscoffia* has an apparent cingulum and sulcus (Horiguchi & Kubo 1997; Hoppenrath & Elbrächter 1998). Our results showed that benthic *Cabra*, which also have a cingulum and sulcus, is closely related to podolampadaceans and *R. capitata*.

There are several morphological similarities among planktonic podolampadaceans (*Podolampas* spp. and *Blepharocysta*), *Roscoffia* and *Cabra* (Figs 16–21), namely, the very narrow, long 1′ plate, the diminutive 2′ and 3′ plates, the larger precingular plates in comparison to the smaller apical plates, possessing three cingular plates and the hypothecal plate pattern of five postcingular plates and one antapical plate (Figs 22–27) (Horiguchi & Kubo 1997; Hoppenrath & Elbrächter 1998; Murray & Patterson 2004; Chomérat & Nézan 2009; Chomérat *et al.* 2010; Gómez & Lopes 2015); the majority of the species within Peridiniales have five postcingular plates and two antapical plates (Fensome *et al.* 1993). Carbonell-Moore (1994) reported that some planktonic Podolampadacea have an elaborate zone of honeycombed pores on plate 1⁗. *Blepharocysta* also has very large pores on 1⁗, and *Podolampas* has two rows of round pores following a unique pattern along the postcingular plates (Carbonell-Moore 1991, 1994). Similar honeycombed pores were reported in *Cabra* spp. as the area of dense pores on 1⁗ (Chomérat & Nézan 2009; Chomérat *et al.* 2010; Selina *et al.* 2015). *Roscoffia capitata* was also reported to have a small area with densely arranged areoles or pores, which Balech (1956) called ‘petit pentagone’ or ‘tache’ (Hoppenrath & Elbrächter 1998).

The planktonic Podolampadaceae and *Cabra* have a common tabulation: 3′, 1a, 5″, 3c, ?s (4–5s for the Podolampadaceae by Carbonell-Moore 1994), 5‴, 1⁗ (Carbonell-Moore 1994; Chomérat *et al.* 2010; Figs 16–18, 22–24). Although *R. capitata* has a different tabulation: 3′ (4′), 0a, 5″, 3c, 4s, 5‴, 1‴ (Hoppenrath & Elbrächter 1998), another member of *Roscoffia*, *Roscoffia minor*, has generally the same tabulation as planktonic podolampadaceans (Figs 20, 26) and *Cabra*: 3′, 1a, 5″, 3c, 3s, 5‴, 1⁗ (Horiguchi & Kubo 1997).

There are clear morphological differences between planktonic podolampadaceans and the benthic *Roscoffia* and *Cabra*. The planktonic members have a cryptic cingulum and lack an apparent sulcus, the traditional diagnostic features of Podolampadaceae, while the benthic members have a cingulum and sulcus. Gómez *et al.* (2010) suggested that the cingulum was independently lost in *Podolampas* and *Blepharocysta*, and it is most likely a convergent character. The present study did not show the clear branching order among *Podolampas* spp., *Blepharocysta* sp., *R. capitata* and *C. matta*, and these nodes had low statistical supports. Therefore, it still cannot be clearly concluded that the planktonic and benthic groups are monophyletic.

More dinoflagellate species may be related to *Cabra*, *Roscoffia* and Podolampadaceae. Having three cingular plates is restricted to Podolampadaceae, a few benthic genera including *Cabra* and *Roscoffia* and the planktonic heterotrophic diplopsalids (Table 1, as *Diplopsalis* group); most thecate dinoflagellates have five to six cingular plates (Steidinger & Tangen 1997). None of the diplopsalids in the present phylogenetic analyses show a close relationship with *Cabra*, *Roscoffia* or Podolampadaceae. The benthic hetero-

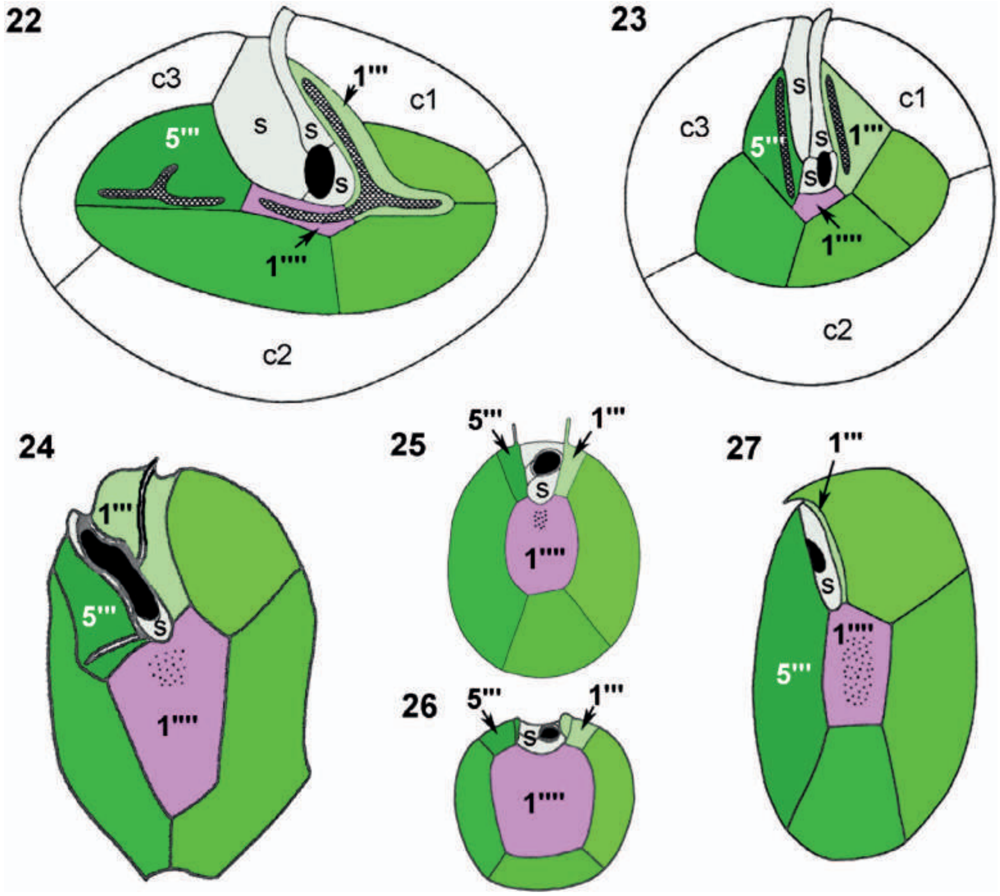


Figs 16–21. Line drawings showing the epithecal tabulation of selected taxa. Blue colours = apical plates, red colours = precingular plates, yellow = anterior intercalary plate.
Fig. 16. *Podolampas* (modified after Fensome *et al.* 1993).
Fig. 17. *Blepharocysta* (modified after Fensome *et al.* 1993).
Fig. 18. *Cabra* (modified after Selina *et al.* 2015).
Fig. 19. *Roscoffia capitata* (modified after Hoppenrath & Elbrächter 1998).
Fig. 20. *Roscoffia minor* (modified after Horiguchi & Kubo 1997).
Fig. 21. *Rhinodinium* (modified after Murray *et al.* 2006).

trophic species of *Amphidiniopsis* – *Amphidiniopsis aculeata* Hoppenrath, Koeman & Leander; *Amphidiniopsis hexagona* Yoshimatsu, Toriumi & J.D.Dodge; *Amphidiniopsis pectinaria* Toriumi, Yoshimatsu & Dodge; *Amphidiniopsis uroensis* Toriumi, Yoshimatsu & J.D.Dodge; and *Amphidiniopsis yoshimatsui* Hoppenrath – have three cingular plates. However, they did not have a close relationship with *Cabra*, *Roscoffia* and Podolampadaceae and formed a clade with other Peridiniales that included other *Amphidiniopsis* spp., *Herdmania litoralis* J.D.Dodge, *Protoperidinium* spp. and *Archaeperidinium minutum* (Kofoid) Jørgensen (i.e. Clade X in Yamaguchi *et al.* 2016).

Rhinodinium broomeense S.Murray, Hoppenrath, Yoshimatsu, Toriumi & J.Larsen is also difficult to assign to a specific family within Peridiniales (Murray *et al.* 2006). It has a plate formula similar to *Cabra*, *Roscoffia* and the Podolampadaceae (Table 1; Figs 16–27). *Rhinodinium broomeense* was also reported to have a special pore field with about 30 pores on plate 1'''' (Fig. 27 and Murray *et al.* 2006). Large extrusomes at the posterior cell end, like those shown here for *C. matta*, were demonstrated above this pore field (Murray *et al.* 2006, fig. 1C; Hoppenrath *et al.* 2014, figs 70B, C). Murray *et al.* (2006) performed molecular phylogenetic analyses for *R. broomeense* based on partial 28S rDNA sequences; however, the 28S rDNA data from *Cabra matta* were not sequenced in this study. Although it is likely that *R. broomeense* has a close affinity with these members, it was not included into our analyses because of the lack of a published 18S rDNA sequence.

Madanidinium loirii is a photosynthetic, laterally compressed sand-dwelling species with a reduced epitheca (Chomérat & Bilien 2014). This species has an area of



Figs 22–27. Line drawings showing the hypothecal tabulation of selected taxa. Purple colour = antapical plate, green colours = postcingular plates, s in darker grey = sulcal plates, c in lighter grey = cingular plates.

Fig. 22. *Podolampas* (modified after Fensome *et al.* 1993).

Fig. 23. *Blepharocysta* (modified after Fensome *et al.* 1993).

Fig. 24. *Cabra* (modified after Chomérat & Nézan 2009).

Fig. 25. *Roscoffia capitata* (modified after Hoppenrath & Elbrächter 1998).

Fig. 26. *Roscoffia minor* (modified after Horiguchi & Kubo 1997).

Fig. 27. *Rhinodinium* (modified after Murray *et al.* 2006).

densely arranged pores near the centre of the $2''''$ plate. Our 18S rDNA analyses did not show a close relationship between *Cabra*, *Roscoffia*, Podolampadaceae and this species.

In our analysis, *Plagiodinium belizeanum* was positioned at the base of the clade comprised of *Podolampas* spp., *Blepharocysta* sp., *R. capitata* and *C. matta* with weak statistical support. *P. belizeanum* is a benthic photosynthetic species that has a laterally compressed cell shape. Although it has a different epithecal tabulation, the hypothecal pattern $5'''$ $1''''$ is the same as *Cabra*, *Roscoffia* and Podolampadaceae (Table 1; Faust & Balech 1993; Wakeman *et al.* 2018).

Our phylogenetic results showed *Cabra* with a close affinity to benthic *Roscoffia* and planktonic members of the Podolampadaceae. However, the statistical support for each node was low, and the evolutionary branching order for neither *Cabra* and *Roscoffia* nor the planktonic *Podolampas* and *Blepharocysta* could be resolved. It would be useful to perform further phylogenetic analyses based on 28S rDNA data that included *Rhinodinium*. There is also the possibility that there will be more species assigned to the *Cabra*–*Roscoffia*–Podolampadaceae clade. While the use of single-gene phylogenies such as 18S rDNA can be informative for specific- and generic-level classifications, there are inherent limitations to this approach, especially when considering deeper relationships among dinoflagellates as a whole. The recent multiple studies of dinoflagellate phylogenetics using large multi-gene datasets tend to recover the major orders as monophyletic well-supported groups (Orr *et al.* 2012; Janouškovec *et al.* 2017). Recent work has shown that material from a few or even single cells can be isolated and used to generate large datasets looking at the evolution of organelles, especially those related to photoreception and photosynthesis (Gavelis *et al.* 2015a, b, 2017). In an effort to better understand the evolution of dinoflagellates, these methods can be applied to lineages like *Cabra matta* that are unique yet difficult to culture or rarely encountered in nature.

Table 1. Possible plate interpretations of selected dinoflagellates.

Dinoflagellate	APC	′	a	″	x	c	s	‴	p	⁗	References
Cabra	$P_o + P_t$	3	1	5		3	?	5		1	Chomérat *et al.* (2010)
Cabra (alternative pattern)	$P_o + P_t$	3	1	4	x	3	?	5		1	Chomérat *et al.* (2010)
Cabra	APC	3	1	5		3	?	5		1	Hoppenrath *et al.* (2014)
Cabra matta	P_o	4	0	4	x	3	?	5		1	Murray & Patterson (2004)
Cabra matta	$P_o + P_t$	3	1	5		3	?	5		1	Chomérat *et al.* (2010)
Cabra matta	P_o	4		4	x	3	?	5		1	Selina & Levchenko (2011)
Cabra reticulata	$P_o + P_t$	3	1	4	x	3	?	5		1	Chomérat & Nézan (2009)
Cabra reticulata (alternative pattern)	$P_o + P_t$	4	0	4	x	3	?	5		1	Chomérat & Nézan (2009)
Cabra aremorica	$P_o + P_t$	3	1	5		3	?	5		1	Chomérat *et al.* (2010)
Cabra aremorica (alternative pattern)	$P_o + P_t$	3	1	4	x	3	?	5		1	Chomérat *et al.* (2010)
Cabra levis	APC	3	1	5		3	6	5		1	Selina *et al.* (2015)
Roscoffia minor	P_o	3	1	5		3	3	5		1	Horiguchi & Kubo (1997)
Roscoffia capitata	P	3(4)		5		3	4	5		1	Hoppenrath & Elbrächter (1998)
Rhinodinium broomeense	P_o	3	1	5		4	?	5		1	Murray *et al.* (2006)
Podolampadaceae	$P_o + P_t + X$	3	1	5		3	4–5	4–5		1	Carbonell-Moore (1994)
Lessardia elongata	P_o, P_i, CP	3	1–2	5		3	6	4–5		1	Saldarriaga *et al.* (2003)
Pileidinium ciceropse		1		5		4	4	5		1	Tamura & Horiguchi (2005)
Ailadinium reticulatum	P_o, cover plate	4	2	6		6	4	6	1	1	Saburova & Chomérat (2014)
Amphidiniella sedentaria	P_o	4	1	7		5	4	6		2	Horiguchi (1995)
Plagiodinium belizeanum	P_o	5		0		5	5	5		1	Faust & Balech (1993)
Plagiodinium belizeanum	P_o	1	0	5		5(6)	4	5		1	Wakeman *et al.* (in press)
Diplopsalis group		4	1–2 (1a,2a,4a)	7		3		5		1–2	Gómez *et al.* (2010)
Amphidiniopsis hexagona	APC	4	2	7		3	4+2 acc.	5		2	Yoshimatsu *et al.* (2000)
Amphidiniopsis hexagona	APC	4	2	7		3	4	5		2	Hoppenrath *et al.* (2009)
Amphidiniopsis swedmarkii	APC	4	2	7		3	4+2 acc.	5		2	Yoshimatsu *et al.* (2000)
Amphidiniopsis uroensis	APC	3	3	6		3	4+1 acc.	5		2	Toriumi *et al.* (2002)
Amphidiniopsis uroensis	APC	3	3	6		3	5	5		2	Hoppenrath *et al.* (2012), Selina & Hoppenrath (2013)
Amphidiniopsis kofoidii	APC	4	3	7		3	3?	5		2	Dodge & Lewis (1986)

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