Phycologia
2020, Volume 59, Issue 1, Pages 6-23
https://doi.org/10.1080/00318884.2019.1663693
https://archimer.ifremer.fr/doc/00589/70143/

Attributing *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* to Protoceratiaceae (Dinophyceae), with descriptions of *Ceratocorys malayensis sp. nov*. and *Pentaplacodinium usupianum sp. nov*

Luo Zhaohe [1] , Lim Zhen Fei [2], Mertens Kenneth [3], Krock Bernd [4], Teng Sing Tung [5], Tan Toh Hii [2], Leaw Chui Pin [2], Lim Po Teen [2], Gu Haifeng [1, *]

[1] Third Institute of Oceanography, Ministry of Natural Resources, Xiamen 361005, China
[2] Bachok Marine Research Station, Institute of Ocean and Earth Sciences, University of Malaya, Bachok 16310, Malaysia
[3] Ifremer, LER BO, Station de Biologie Marine, Place de la Croix, BP40537, Concarneau CEDEX F-29185, France
[4] Alfred Wegener Institute for Polar and Marine Research, Ecological Chemistry, Am Handelshafen 12, Bremerhaven D-27570, Germany
[5] Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan 94300, Malaysia

* Corresponding author : Haifeng Gu, email address : guhaifeng@tio.org.cn

Abstract :

The gonyaulacean family Protoceratiaceae is characterised by five precingular plates. It currently encompasses the type genus *Ceratocorys* and the fossil genus *Atopodinium*. Fourteen strains of *Ceratocorys*, *Pentaplacodinium*, and *Protoceratium* were established from Malaysian and Hawaiian waters, and their morphologies were examined using light and scanning electron microscopy. Two new species, *Ceratocorys malayensis sp. nov*. and *Pentaplacodinium usupianum sp. nov*., were described from Malaysian waters. They share a Kofoidean plate formula of Po, Pt, 3', 1a, 6", 6C, 6S, 5"', 1p, 1''''. *Ceratocorys malayensis* has a short first apical plate (1') with no direct contact with the anterior sulcal plate (Sa) whereas *Pentaplacodinium usupianum* had a parallelogram-shaped 1' plate which often contacted the Sa plate. The genera *Ceratocorys* and *Pentaplacodinium* were emended accordingly to incorporate species bearing five or six precingular plates. The *Protoceratium* strain from Hawaii was morphologically similar to *P. reticulatum*, but differed in the lack of a ventral pore in plate 1′ and slight or lack of contact between plates 1′ and Sa, and is here designated as *P*. cf. *reticulatum*. The maximum-likelihood and Bayesian inference analyses based on SSU, LSU and ITS ribosomal DNA sequences revealed that these three genera are monophyletic and form a well-resolved group. Our results support *Protoceratium* and *Pentaplacodinium* as members of the family Protoceratiaceae, characterised by the presence of one anterior intercalary plate. Seven strains of *Protoceratium* cf. *reticulatum*, *Ceratocorys malayensis* and *Pentaplacodinium usupianum* were examined for yessotoxin production by LC-MS/MS but none produced a detectable amount of toxin.

Keywords : Ceratocoryaceae, Gonyaulacaceae, Molecular phylogeny, Protoceratium reticulatum, Yessotoxin

INTRODUCTION

The Gonyaulacales is a major order of dinophytes that is subdivided into five suborders based only on morpho-anatomy (Fensome *et al.* 1993). One of these, Gonyaulacineae, encompasses two extant families Gonyaulacaceae and Ceratocorythaceae and one fossil family Areoligeraceae. Ceratocorythaceae have five precingular plates, L-type ventral organization and strong dextral torsion, whereas Gonyaulacaeae have six precingular plates, L- to S-type ventral organization and sinistral to dextral torsion (Fensome *et al.* 1993). The key difference between Gonyaulacaceae and Ceratocorythaceae is thus the number of precingular plates (six versus five). The Gonyaulacaceae was subdivided further into three subfamilies by Fensome *et al.* (1993): Cribroperidinioideae (with L-type ventral organization and dextral torsion), Leptodinioideae (with L-type ventral organization and sinistral or neutral torsion), and Gonyaulacoideae (with S-type ventral organization and neutral torsion). The criteria used to distinguish subfamilies of Gonyaulacaceae, however, were sometimes inconsistent (Helenes 2000) or gradational in nature, and in some instances tentative (Fensome *et al.* 1993).

Currently, the Ceratocorythaceae includes only the extant genus *Ceratocorys* F.Stein and the fossil genus *Atopodinium* Drugg (Fensome *et al.* 1993). *Ceratocorys* is characterised by three apical plates, one small anterior intercalary plate and five precingular plates (3′, 1a, 5″). The third Kofoidean precingular plate in *Ceratocorys* is considered homologous to both the third and fourth precingular plates in other gonyaulacoid dinoflagellates genera (Mertens *et al.* 2018b). Additionally, cells of *Ceratocorys* are often characterised by an angular body, shorter epitheca relative to

hypotheca, small to large spines on the hypotheca and heavily ornamented theca (Carbonell-Moore 1996). Twelve *Ceratocorys* species have been described, e.g. *Ceratocorys anacantha* M.C.Carbonell-Moore, *C. armata* (Schütt) Kofoid, *C. bipes* (Cleve) Kofoid, *C. horrida* Stein; all are exclusively marine and found only in tropical and subtropical waters (Graham 1942; Carbonell-Moore 1996). However, sequences of *Ceratocorys* are available only for three species: *Ceratocorys armata, C. horrida* and *C. gourretii*, which share high nucleotide similarity for the SSU (100%) and LSU ribosomal gene markers (>99%; Mertens *et al.* 2018b).

Pentaplacodinium Mertens, Carbonell-Moore, Pospelova & Head was established for strains formerly identified as *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli (Mertens *et al.* 2018b). Salgado *et al.* (2018) considered this species to belong to *Ceratocorys*. Mertens *et al.* (2018a, b) disagreed because of the insert type epithecal configuration in these strains, unlike the episert type in *Ceratocorys horrida*, the type species of *Ceratocorys*. However, episert (type I) was also reported in *Pentaplacodinium saltonense* (Salgado *et al.* 2018, as *Ceratocorys mariaovidiorum*). *Pentaplacodinium* is a sister clade of *Ceratocorys* in the molecular phylogeny based on ribosomal DNA sequence, but has not been assigned to a family.

Protoceratium reticulatum is a common dinoflagellate originally described from Bergen Fjord, Norway by Claparède & Lachmann (1858) as *Peridinium reticulatum* Claparède & Lachmann. *Protoceratium* Bergh was erected by Bergh (1881, p. 242) with *P. aceros* as the type species (fig. 36) collected at Strib, Denmark. The plate formula for *P. reticulatum* was first provided by Wołoszyńska (1929) as 4′, 0a, 6″, 6‴, 1p, 1⁗ based

on the Baltic Sea specimens. Reinecke (1967) described a similar species as *Gonyaulax grindleyi* Reinecke based on specimens from Elands Bay in Cape Town, South Africa, and provided its tabulation as 3′, 1a, 6″, 6‴, 1p, 1⁗. The difference between *Protoceratium reticulatum* and *Gonyaulax grindleyi* was considered to be the number of apical plates and anterior intercalary plates. Hansen *et al.* (1997) restudied specimens close to the type locality of *P. aceros*, and confirmed that the epithecal tabulations can be 3′, 1a, 6″ or 4′, 0a, 6″. Therefore, Hansen *et al.* (1997) concluded that *P. reticulatum*, *P. aceros* and *G. grindleyi* were conspecific (Hansen *et al.* 1997). Eleven other *Protoceratium* species have been described, e.g., *Protoceratium splendens* Meunier, *Protoceratium aculeatum* (von Stein) Schiller, *Protoceratium areolatum* Kofoid and *Protoceratium spinulosum* (Murray & Whitting) Schiller (e.g. Schiller 1937, p. 322–326). None of these species have sequences available except *P. reticulatum*.

Protoceratium reticulatum was questionably assigned to subfamily Cribroperidinioideae of Gonyaulacaceae by Fensome *et al.* (1993); however, molecular phylogenetic inference showed that *P. reticulatum* was closely related to *Ceratocorys* (Saldarriaga *et al.* 2004; Orr *et al.* 2012). Kawai & Nakayama (2015) suggested using Protoceratiaceae instead of Ceratocorythaceae. Sequences of several unidentified strains from Malaysia and Hawaii were previously reported by Mertens *et al.* (2018b). The morphology of these and other related strains of *Ceratocorys* and *Pentaplacodinium* are investigated in this study in detail. Furthermore, to address the phylogenetic relationships among *Protoceratium*, *Ceratocorys* and *Pentaplacodinium*, two SSU, five partial LSU and eleven ITS rDNA sequences were determined for the cultured strains

and the molecular phylogeny was inferred.

MATERIAL AND METHODS

Sample collection and treatment

Surface sand samples (upper 5 cm) were collected by SCUBA divers using plastic bottles from Semariang and Talang-Talang Island (Sarawak), Rawa Island (Terengganu), Malaysia from 2010 to 2016 (Table 1). The samples were rinsed with filtered seawater and transferred into a polycarbonate bottle. Single motile ceratocorioid cells were immediately isolated by means of drawn-out Pasteur pipettes and using an Eclipse TS100 inverted microscope (Nikon, Tokyo, Japan) to establish clonal cultures. Thirteen strains of *Ceratocorys* and *Pentaplacodinium* were initially established in ES-DK medium at 25 ºC (Kokinos & Anderson 1995) in Malaysia. These clonal cultures were transferred to Xiamen and maintained with f/2-Si medium (Guillard & Ryther 1962) at 20 ºC, 90 µmol quanta·m^{-2}·s^{-1} under a 12:12 h light: dark cycle (hereafter called 'standard culture conditions'). Plankton samples were also collected using a 20 µm mesh-size plankton net by vertical and horizontal hauls at sub-surface water of Rawa Island in 2016. The samples were fixed with 2% Lugol's solution, and later for SEM examination.

In Hawaii, sediment sampling was done using an Ekman grab in nearby Haleiwa Harbor on 04 March 2014 (water depth 3.0 m; Table 1). The top 2 cm of sediment were sliced off and stored in the dark at 4 °C until further treatment. Approximately 5 g of wet sediment was mixed with 20 ml of filtered seawater and stirred vigorously to

dislodge detrital particles. The settled material was subsequently sieved through 120 μm and 10 μm filters. The 10–120 μm fractions were rinsed with f/2-Si medium (Guillard & Ryther 1962) and transferred into a 96-well culture plate. The culture plate was incubated under standard culture conditions. Single motile cells of *Protoceratium* were isolated with a micropipette with the above microscope and incubated in 96-well plate with f/2-Si medium under standard culture conditions. The strain HWYD1 was established in clonal cultures (Table 1).

Morphological study of motile cells with microscopy

Live cells were examined and photographed using a Zeiss Axio Imager light microscope (Carl Zeiss, Göttingen, Germany) equipped with a Zeiss Axiocam HRc digital camera and fluorescence. The cell size of 30 cells was measured using Axiovision v4.8.2 software at ×1000 magnification. To observe shape and location of nuclei, cells were stained with 1:100,000 SYBR Green (Sigma Aldrich, St. Louis, USA) for 1 min, and photographed with the same Zeiss microscope with a Zeiss-38 filter set (excitation BP 470/40, beam splitter FT 495, emission BP 525/50). Autofluorescence of the chloroplast in live cells was observed using the above microscope equipped with a Chroma filter cube (emission filter ET480/20x, dichromatic mirror AT505dc, suppression filter AT515lp), and digitally photographed using a Zeiss Axiocam HRc digital camera.

For scanning electron microscopy (SEM), mid-exponential batch cultures were concentrated by centrifugation (Universal 320 R centrifuge, Hettich-Zentrifugen,

Tuttlingen, Germany) at 850 *g* for 10 min at room temperature. Cells were fixed with 2.5% glutaraldehyde for 3 h at 8 ºC, rinsed with Milli-Q water twice and post-fixed with 1% OsO_4 overnight at 8 ºC. The supernatant was removed and settled cells were transferred to a coverslip coated with poly-L-lysine (molecular weight 70,000–150,000). The cells attached to the cover slip were rinsed in Milli-Q water twice. The samples were then dehydrated in a graded ethanol series (10, 30, 50, 70, 90 and 3 × in 100%, 10 min at each step), critical point dried (K850 Critical Point Dryer, Quorum/Emitech, West Sussex, UK), sputter-coated with gold, and examined with a Zeiss Sigma FE (Carl Zeiss Inc., Oberkochen, Germany) scanning electron microscope. Labeling of tabulation follows a modified Kofoid system that recognizes homologs (e.g. Fensome *et al.* 1993); sulcal plate labeling follows Balech (1980).

PCR amplifications and sequencing

The total algal DNA of *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* was extracted from 10 ml of exponentially growing cultures using a MiniBEST Universal DNA Extraction Kit (Takara, Tokyo, Japan) according to manufacturer's protocol. PCR amplifications were carried out using 1×PCR buffer, 50 μM dNTP mixture, 0.2 μM of each primer, 10 ng of template genomic DNA, and 1 U of ExTaq DNA Polymerase (Takara, Tokyo, Japan) in 50 μl reactions. The SSU rDNA was amplified using the primer pair PRIMER A/PRIMER B (Medlin *et al.* 1988). The LSU rDNA was amplified using the primer pair D1R/28-1483R (Scholin *et al.* 1994; Daugbjerg *et al.* 2000). The total ITS1–5.8S–ITS2 was amplified using the primer pair ITSA/ITSB (Adachi *et al.*

1996). The thermal cycle procedure was 4 min at 94 ºC, followed by 30 cycles of 1 min at 94 ºC, 1 min at 45 ºC, 1 min at 72 ºC, and final extension of 7 min at 72 ºC with a Mastercycler (Eppendorf, Hamburg, Germany). The PCR product was purified using a DNA purification kit (Shengong, Shanghai, China) and sequenced directly in both directions on an ABI PRISM 3730XL (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Newly obtained sequences were deposited in GenBank with accession numbers MN137888 to MN137906.

Sequence alignment and phylogenetic analysis

The newly obtained sequences of *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* (SSU, partial LSU and ITS rDNA) were incorporated into independent datasets of closely related dinoflagellates sequences via taxon sampling in the NCBI GenBank nucleotide database. All sequences were aligned using MAFFT v7.110 (Katoh & Standley 2013) online program (http://mafft.cbrc.jp/alignment/server/) using the default settings. Alignments were manually checked with BioEdit v7.0.5 (Hall 1999). For Bayesian inference (BI), jModelTest (Posada 2008) was used to select the most appropriate model of molecular evolution with Akaike Information Criterion (AIC). Bayesian reconstruction of the data matrix was performed using MrBayes 3.2 (Ronquist & Huelsenbeck 2003) with the best-fitting substitution model (GTR+G). Four Markov chain Monte Carlo (MCMC) chains ran for 1,000,000 generations, sampling every 100 generations. The first 10% of burn-in trees were discarded. A majority rule consensus tree was created to examine the posterior probabilities of each clade. Maximum-

likelihood (ML) analyses were conducted with RaxML v7.2.6 (Stamatakis 2006) on the T-REX web server (Boc *et al.* 2012) using the model GTR+G. Node support was assessed with 1000 bootstrap replications.

The ITS1-5.8S-ITS2 sequences of *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* species were aligned using MAFFT v7.110 (Katoh & Standley 2013) online program with the default settings. Completed alignments were saved as NEXUS files and imported into PAUP*4b10 software (Swofford 2002) so that divergence rates could be estimated using simple uncorrected pairwise (p) distance matrices.

Yessotoxin analysis

Cultures of two strains of *Ceratocorys malayensis*, four strains of *Pentaplacodinium usupianum* and one strain of *Protoceratium* cf. *reticulatum* were grown in 200-ml Erlenmeyer flasks under standard culture conditions. A total of $10^5 – 10^6$ cells at exponential phase (determined using sequential cell counts) were collected by centrifugation. Exponential phase was determined via linear regression of log-transformed cell count time series.

Algal pellets were ultrasonicated (70 s, 40% power, 70 cycles) in 100 µl methanol with a sonotrode (model HP2070, Bandelin, Berlin, Germany). After homogenization, samples were centrifuged (16,000 *g*, 15 min, 4 °C, Centrifuge 5415R, Eppendorf, Hamburg, Germany) and supernatants were transferred to spin-filters (pore-size 0.45 mm, Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 3,220 *g*. Filtrates were transferred into HPLC vials (Agilent Technologies, Waldbronn, Germany)

and stored at -20 °C until analysis.

Yessotoxin measurements were carried out on a triple quadruple mass spectrometer (API 4000 QTrap, Sciex, Darmstadt, Germany) as detailed in Sala-Pérez *et al.* (2016). In brief, separation was performed on a reversed phase C8 column (50 × 2 mm, 3 µm) at a flow rate of 0.3 ml min^{-1} using an elution gradient with two eluents (water and acetonitrile/methanol (1:2 v/v)). Yessotoxins were detected in the selected reaction monitoring (SRM) mode and 25 transitions were used (Sala-Pérez *et al.* 2016).

RESULTS

Family Protoceratiaceae Lindemann 1928

Ceratocorys malayensis Z.Luo, P.T.Lim & H.Gu *sp. nov.*

Figs 1–22

DESCRIPTION: Cells heavily reticulated, 40.2–58.0 µm long and 40.9–54.6 µm wide, with rounded epitheca and hypotheca with several short antapical spines; ratio of epitheca/hypotheca around 0.84. Cells with numerous radiating chloroplasts and a U-shaped posterior nucleus. Cells with plate formula of Po, Pt, 3′, 1a, 6″, 6C, 6S, 5‴, 1p, 1⁗ and with L-type ventral organization and sinistral torsion. Pore plate oval with a λ-shaped cover plate (Pt). The first apical plate short and narrow, not contacting plate Sa (episert type I) with a ventral pore at the border with plate 3′.

HOLOTYPE: SEM stub from strain A10-49-A55 designated as TIO201901, deposited at Third Institute of Oceanography, Ministry of Natural Resources, Xiamen, 361005,

China.

TYPE LOCALITY: Rawa Island, Terengganu, Malaysia (South China Sea, 5°57.70′ N; 102°40.88′ E). Collection date: 17 May 2016.

HABITAT: Marine, sand.

ETYMOLOGY: The epithet '*malayensis*' derived from Malay Archipelago, where the species was recovered.

GENBANK ACCESSIONS: MN137904, MN137899 and MN137896, the nuclear-encoded SSU, LSU and ITS rDNA sequences of strain A10-49-A55.

DISTRIBUTION: Terengganu and Sarawak of Malaysia.

Morphology

Ceratocorys malayensis strains were morphologically indistinguishable from each other. Cells of strain A10-49-A55 were 40.2–58.0 µm long (49.2 ± 4.9 µm, n = 30) and 40.9–54.6 µm wide (46.6 ± 3.9 µm, n = 30), with brownish cell contents due to the presence of chloroplasts (Fig. 1). The cells had a rounded epitheca and hypotheca with several short spines 3.0–4.1 µm long at the antapical end (Fig. 2). The epitheca was smaller than the hypotheca with the ratio of epitheca/hypotheca ranging from 0.72 to 0.98 (0.84

± 0.08, n = 23) (Figs 1, 2, 7 and 8). Nmerous chloroplasts radiated from the central part of the cell (Fig. 3). The nucleus was U-shaped and located in cell posterior ('N' in Fig. 4).

The thecae displayed a plate formula of Po, Pt, 3′, 1a, 6″, 6C, 6S, 5‴, 1p, 1⁗ (Figs 1–18), and had a sexiform gonyaulacoid tabulation (cf. Fensome *et al.* 1993, their text-fig. 64B; Figs. 10, 13) with a L-type ventral organization (cf. Fensome *et al.* 1993, Text-figs. 82A, C) (Figs 9, 17) and sinistral torsion (cf. Fensome *et al.* 1993, text-fig. 83C) (Fig. 8). The plates were heavily reticulated with one pore inside each reticulation, although two or more pores might occur in reticulations adjacent to a suture. The reticulations were weakly expressed on the sulcus and cingulum (Figs 11, 12). The pore plate (Po) was oval with a λ-shaped cover plate (Pt) and perforated by approximately 10 pores (Figs 15, 16). The first apical plate (1′) was asymmetric and narrowed posteriorly with a ventral pore on its right side (Fig. 18). Plate 1′ was short and narrow and did not contact anterior sulcal plate (Sa) (Figs 6, 17), i.e. episert type I (Paez-Reyes & Head, 2013). The second and third apical plates (2′, 3′) were much larger and irregularly shaped (Figs 6, 14, 17). The anterior intercalary plate was small and pentagonal without contacting the pore plate (Figs 6, 9, 14). The precingular series consisted of six plates, with 3″ and 4″ much smaller than the rest (Figs 5, 9, 14, 17). The cingulum was descending, lined with narrow lists, and comprised six cingular plates with two rows of pores along their anterior and posterior margins (Figs 8, 11, 12). The ends of the cingulum did not overhang, and were displaced by twice the cingulum width (Fig. 12).

The sulcus was narrow anteriorly, slightly widened posteriorly, and consiseds of six

plates. The Sa plate was relatively small and intruded between plates 1″ and 6″ without contacting plate 1′. The anterior left sulcal plate (Ssa) was similar in size to the anterior right sulcal plate (Sda). The left posterior sulcal (Ssp) was larger than the posterior right sulcal (Sdp). The large posterior sulcal (Sp) was at the bottom of the sulcus, with pores lined up along the left suture with plate 1′″′ (Fig. 12).

The hypotheca comprised five postcingular plates and one antapical plate. Plate *2‴ was triangular and the smallest in the series (Fig. 13). All other postcingular plates were large, although *6‴ was relatively smaller (Fig. 10). The posterior intercalary plate (1p) was small and elongated, located adjacent to plate Sp with a conspicuous flange on its right margin (Fig. 13). The antapical plate (1′″′) was six-sided and large, located in the middle of the hypotheca with several spines emerging from the margins (Figs 10, 13). Cells of *C. malayensis* from the field had identical morphology with those in culture (Figs S1, S2). Schematic drawings of *C. malayensis* are provided (Figs 19–22). Cysts were not observed in cultures.

Pentaplacodinium usupianum **Z.Luo, Leaw & H.Gu sp. nov.**

Figs 23–39

DESCRIPTION: Thecae reticulated more heavily on hypotheca than on epitheca, 26.6–31.3 µm long and 22.7–27.7 µm wide. Cells with conical epitheca and rounded hypotheca with similar size. Cells with numerous radiating chloroplasts and U-shaped posterior nucleus. Thecae with a plate formula of Po, Pt, 3′, 1a, 6″, 6C, 6S, 5‴, 1p, 1′″′ and a L-type ventral organization and neutral torsion; pore plate oval. The first apical

plate parallelogram and slightly contacts plate Sa (insert type).

HOLOTYPE: SEM stub from strain DBS02 designated as TIO201902, deposited at Third Institute of Oceanography, Ministry of Natural Resources, Xiamen, 361005, China.

TYPE LOCALITY: Semariang, Sarawak, Malaysia (South China Sea, 1°36.00″ N; 110°19.00′ E). Collection date: 21 December 2016.

HABITAT: Marine, sand.

ETYMOLOGY: The epithet '*usupianum*' is in honor of Gires Usup, who did pioneering work on harmful algal blooms in Malaysia.

GENBANK ACCESSIONS: MN137906, MN137903 and MN137898, the nuclear-encoded SSU, LSU and ITS rDNA sequences of strain DBS02.

DISTRIBUTION: Sarawak, Malaysia.

Morphology

Strains of *Pentaplacodinium usupianum* were morphologically indistinguishable from each other. Cells of strain DBS02 were 26.6–31.3 μm long (28.8 ± 1.3 μm, n = 30) and

22.7–27.7 μm wide (24.7 ± 1.4 μm, n = 30). Cells were brownish due to presence of chloroplasts (Fig. 23). Cells had a conical epitheca and rounded hypotheca with several spines 1.0–1.2 μm long at antapical end (Figs 23–26, 29). These antapical spines were not always present (Fig. 31). The cingulum was situated in the equatorial part of the cell (Fig. 24). The cells showed numerous chloroplasts radiating from the central part of the cell (Fig. 27). The nucleus was U-shaped and posterior ('N' in Figs 26, 28).

The thecae displayed a plate formula of Po, Pt, 3′, 1a, 6″, 6C, 6S, 5‴, 1p, 1⁗ (Figs 29–35), and had a sexiform gonyaulacoid tabulation (Fig. 31) with a L-type ventral organization (Fig. 29) and neutral torsion (Figs S3, S4). The plates were more heavily reticulated on the hypotheca than the epitheca with one pore inside each reticulation (Figs 29–31). The pore plate (Po) was oval to elliptical with a λ-shaped cover plate (Pt) and perforated by around seven pores (Figs 33, 34). The first apical plate (1′) was parallelogram-shaped, lacked a ventral pore, and slightly contacted the anterior sulcal plate (Sa) (Figs 25, 29), i.e. the insert type (Fensome *et al.* 1996, text-fig. 34). The second and third apical plates (2′, 3′) were slightly larger and irregularly shaped (Figs 30, 33). The anterior intercalary plate was five-sided and contacted plates 2′, 3′, 3″, 4″ and 5″ (Figs 30, 33). The precingular series consisted of two four-sided plates (2″, 4″) and four five-sided plates (1″, 3″, 5″ and 6″; Figs 29, 30). Occasionally, five precingular plates were observed (Fig. S4). The cingulum was descending, lined with narrow lists, and comprised six cingular plates (Fig. 32). The ends of the cingulum did not overhang, and were displaced by one cingulum width (Fig. 29).

The sulcus was narrow anteriorly and widened slightly posteriorly. It consisted of at

least six plates. The Sa plate was large and hook-shaped, and intruded the epitheca to contact plate 1′. Occasionally, the Sa plate was small and did not contact plate 1′ (Figs S5, S6). The anterior left sulcal plate (Ssa) was slightly larger than the anterior right sulcal plate (Sda). The left posterior sulcal (Ssp) was similar in size to the posterior right sulcal (Sdp) (Fig. 35). The large posterior sulcal (Sp) was at the bottom of the sulcus, which bore lines of pores along its sutures with plates *6‴ and 1‴′ (Figs 29, 31).

The hypotheca comprised five postcingular plates and one antapical plate. Plate *2‴ was triangular and the smallest in the series (Figs 29, 31). All other postcingular plates were trapezoid and large, although *6‴ was relatively smaller (Figs 29, 31). The posterior intercalary plate (1p) was small and elongated, located adjacent to plate Sp (Fig. 29). The antapical plate (1‴′) was six-sided and located in the middle of the hypotheca (Fig. 31). Schematic drawings of *Pentaplacodinium usupianum* are provided (Figs 36–39). Cysts were not observed in cultures.

Protoceratium cf. *reticulatum*

Figs 40–50

Morphology

Cells of the strain HWYD1 were 23.6–29.1 μm long (26.2 ± 1.1 μm, n = 30) and 20.2–24.0 μm wide (21.9 ± 0.9 μm, n = 30). The cell was brownish due to chloroplasts (Figs 40, 41). The thecae had a rounded epitheca and hypotheca (Fig. 40). The cingulum was situated in the pre-equatorial part of the cell. Many chloroplasts radiated from the central part of the cell to form a network (Fig. 42). The nucleus was curved and located

posteriorly ('N' in Fig. 43).

The thecae showed a plate formula of Po, Pt, 3′, 1a, 6″, 6C, 6S, 5‴, 1p, 1⁗ (Figs 44–50), and had a sexiform gonyaulacoid tabulation (Fig. 48) with a L-type ventral organization (Fig. 45) and dextral torsion (Figs 46, 47). The plates were reticulated with one pore inside each reticulation; the reticulation was weakly expressed on sulcus and cingulum (Figs 44–50). The pore plate was sigmoidal with a λ-shaped cover plate and perforated by around five pores (Fig. 49). The first apical plate (1′) was rhombic and lacked a ventral pore and contacted the anterior sulcal plate (Sa) (Fig. 45), thus belonged to insert type. Sometimes, plate 1′ did not contact plate Sa, thus showing a episert type I (Fig. 44). The second and third apical plates (2′, 3′) were much larger and irregularly shaped (Fig. 45). The anterior intercalary plate was five-sided which was either separated from or contacted the pore plate (Fig. 45, 46). The precingular series consisted of four four-sided plates (2″ and 4″) and four five-sided plates (1″, 3″, 5″ and 6″) (Fig. 45). Occasionally, five precingular plates were observed (Fig. 46). The cingulum was descending, lined with narrow lists, and comprised six cingular plates (Figs 44, 47). The ends of the cingulum did not overhang, and were displaced by one cingulum width (Fig. 44).

The sulcus was narrow anteriorly and slightly widened posteriorly. It consisted of six plates. The Sa plate was relatively large and hook-shaped, and intruded the epitheca either to contact plate 1′ slightly (Fig. 45) or did not contact plate 1′ (Fig. 44). The anterior left sulcal plate (Ssa) was similar in size with the anterior right sulcal plate (Sda). The left posterior sulcal (Ssp) was larger than the posterior right sulcal (Sdp) (Fig.

50). The large posterior sulcal (Sp) was at the bottom of the sulcus (Fig. 50).

The hypotheca comprised five postcingular plates and one antapical plate. All postcingular plates were trapezoidal and large except that *3‴ was five-sided, although *2‴ and *6‴ were much smaller (Figs 44, 48). The posterior intercalary plate (1p) was small and elongated, located adjacent to plate Sp (Fig. 44). The antapical plate (1⁗) was six-sided and located in the middle of the hypotheca (Fig. 48). Cysts were not observed in cultures.

Molecular analysis and phylogeny

When SSU rDNA sequences were compared, *Pentaplacodinium usupianum* strains DSB01, GgSm01, GgSm07, GgSm10 and GgSm11 showed 0–1 base pair divergence (99.94% similarity) but differed from *Pentaplacodinium saltonense* (MG646323) in 6 positions (99.65% similarity). *Protoceratium* cf. *reticulatum* HWYD1 differed from *Protoceratium reticulatum* (AY421790) at 12 positions (99.30% similarity). *Ceratocorys malayensis* strain A10-49-A55 differed from *Ceratocorys horrida* (DQ388456) and *Ceratocorys* sp. (LC054924) in 1 and 4 positions (99.77% and 99.94% similarity), respectively.

Pairwise comparison of LSU rDNA sequences revealed that *Pentaplacodinium usupianum* strains DSB01, DSB02, GgSm01, GgSm03, GgSm07, GgSm10, and GgSm11 differed in 0–2 positions (99.64% similarity), but up to 25 divergent positions were observed when compared to *Pentaplacodinium saltonense* (FJ155820) (95.57% similarity). *Protoceratium* cf. *reticulatum* HWYD1 differed from *Protoceratium*

reticulatum (FJ155821) in 13 positions (97.70% similarity). *Ceratocorys malayensis* strains A10-49-A55, A10-49-A56, A10-49-A61, PrTT01, PrTT02 and PrTT03 differed from each other in 0–2 positions (99.64% similarity) and differed from *Ceratocorys horrida* in 2–3 positions (99.47–99.64% similarity).

When ITS rDNA sequences were compared, *Pentaplacodinium usupianum* strains DSB01, DSB02 GgSm01, GgSm03, GgSm07, GgSm10, and GgSm11 differed from each other in 0–4 positions (99.28% similarity), and from *Pentaplacodinium saltonense* (EU532485) in 123 positions (77.83% similarity). *Protoceratium* cf. *reticulatum* HWYD1 differed from *Protoceratium reticulatum* (AB727654) in 105 positions (79.65% similarity). *Ceratocorys malayensis* strains A10-49-A55, A10-49-A56, A10-49-A61, PrTT01, PrTT02 and PrTT03 shared identical sequences and differed from *Ceratocorys horrida* in 33 positions (94.10% similarity). Genetic distances among *Pentaplacodinium*, *Ceratocorys* and *Protoceratium* species were greater than 0.18, but between *Ceratocorys malayensis* and *C. horrida* was only 0.06 (Table 2).

The maximum likelihood (ML) and Bayesian inference (BI) topologies based on the SSU rDNA sequences yielded similar phylogenetic trees. The ML tree is illustrated in Fig. 51. Protoceratiaceae formed a monophyletic clade with maximal support (100 BS/1.0 BPP) and comprised *Ceratocorys, Protoceratium* and *Pentaplacodinium* with an early divergence of *Ceratocorys*. Gonyaulaceceae was monophyletic with strong ML support (100) but low BI posterior probability (< 0.7) comprising *Gonyaulax* and *Lingulodinium*. *Ceratocorys malayensis* grouped together with *C. horrida* and *Ceratocorys* sp. with maximal support. *Protoceratium* cf. *reticulatum* was closest to

Protoceratium reticulatum with strong support (100 BS/0.94 BPP). *Pentaplacodinium usupianum* and *P. saltonense* formed a clade with strong support (100 BS/0.88 BPP) which was a sister clade of *Protoceratium reticulatum* with strong support (100 BS/0.99 BPP).

ML and BI analysis based on partial LSU rDNA sequences yielded identical phylogenetic trees. The ML tree is illustrated in Fig. 52. *Ceratocorys malayensis* was well-resolved (100 BS/0.72 BPP) and grouped with *C. horrida*, *C. gourretii* and *C. armata* with maximal support. *Pentaplacodinium usupianum* was well-resolved (100 BS/0.96 BPP) and formed a sister clade to *Pentaplacodinium saltonense* with high ML bootstrap support (100) but low BI posterior probability (< 0.7). *Protoceratium* cf. *reticulatum* was closest to *Protoceratium reticulatum* with strong support (100 BS/0.97 BPP).

ML and BI analysis based on ITS rDNA sequences yielded identical phylogenetic trees too. The ML tree is illustrated in Fig. 53. *Ceratocorys malayensis* grouped with *C. horrida* with maximal support, which diverged early in the tree. *Pentaplacodinium usupianum* was well-resolved (100 BS/0.98 BPP) and formed a sister clade to *P. saltonense* with maximal support. They formed a sister clade to *Protoceratium* cf. *reticulatum* and *P. reticulatum* with strong support (100 BS/0.85 BPP).

Analysis of yessotoxin:

None of the examined strains of *Ceratocorys malayensis*, *Pentaplacodinium usupianum* and *Protoceratium* cf. *reticulatum* produced detectable yessotoxins (Table 1). Limits of

detection for YTX ranged between 0.09 – 2.8 fg cell^{-1} depending on available biomass.

DISCUSSION

Morphology

A characteristic feature of the genus *Ceratocorys* is the broad contact of the sixth precingular homolog (*6″) with the first precingular plate (1″), thus leading to an episert type I topology where plate 1′ does not contact Sa (Paez-Reyes & Head 2013). *Ceratocorys* is also characterised by an angular body and a much larger hypotheca compared to epitheca (Graham 1942; Carbonell-Moore 1996). The strain of A10-49-A55 (*Ceratocorys malayensis*) matches the description of *Ceratocorys* except that it possesses six precingular plates instead of five. It would appear that a new genus is needed to incorporate our strains; however, the fourth precingular plate in *Ceratocorys* has been considered homologous to the fourth and fifth precingular plates in other gonyaulacoids (Carbonell-Moore 1996); therefore, our strains justify classification in *Ceratocorys* if this variability is allowed through emendation of the genus.

Our strain of A10-49-A55 is morphologically similar to *Ceratocorys anacantha* in terms of a ventral pore of plate 1′, the dorsal position of plate 3″, but it differs in the absence of spines in the hypotheca of *C. anacantha* and the number of precingular plates (six versus five; Carbonell-Moore 1996). The antapical spine length can be variable in *Ceratocorys*, e.g. *C. horrida* can reduce spine size, or even lose them completely, due to hydrodynamic forces (Zirbel *et al.* 2000). Strain A10-49-A55 can be differentiated from other *Ceratocorys* species based on the number of precingular plates,

as well as the relative size and configuration of plate 3″. Plate 3″ of strain A10-49-A55 is symmetrical and its central median line nearly coincides with the epitheca. In contrast, plate 3″ of *C. anacantha, C. grahamii, C. armata, C. aultii, C. bipes, C. gourretii, C. reticulata, C. skogsbergii* are either not symmetrical, or not dorsal (Graham 1942; Carbonell-Moore 1996). Therefore, strain A10-49-A55 was described as a new species.

Pentaplacodinium differs from *Ceratocorys* in that it has a first apical plate of an insert type which touches the Sa plate. As a consequence, there is no contact between plates 1″ and 6″ in *Pentaplacodinium*, whereas in *Ceratocorys* the contact is broad (Mertens *et al.* 2018a). Our strain DBS02 (*Pentaplacodinium usupianum*) displays an insert type of plate 1′, but occasionally plate 1′ does not contact plate Sa, i.e. showing episert type I (Figs S5, S6), as also reported in *P. saltonense* (Salgado *et al*. 2018) and *Protoceratium* cf. *reticulatum* strain HWYD1 (Figs 44, 45). Presence of both insert type and episert type within a single strain is possibly due to culture artefacts, thus field samples are needed for verification. Our strain DBS02 thus fits the definition of *Pentaplacodinium* except that it had six precingular plates instead of five, and the torsion was more neutral. Regardless, a new genus is unnecessary since the third precingular plates in *Pentaplacodinium* is homologous to the third and fourth precingular plates in other gonyaulacoids (Mertens *et al.* 2018b). Thus, our strains justify classification in *Pentaplacodinium* pending its emendation. To date, only *Pentaplacodinium saltonense* has been described for this genus. The strain studied here differed from *P. saltonense* in the absence of a ventral pore on plate 1′ and the number of precingular plates (Mertens *et al.* 2018b). Therefore, it was described as a new

species.

Our strain HWYD1 is morphologically similar to *Protoceratium reticulatum*, except that it lacks a ventral pore on the plate 1′ and is much smaller (26 μm long versus 40 μm long) (Hansen *et al.* 1997). Another morphological feature that differentiates both is that in *P. reticulatum* the contact between plates 1′ and Sa is always wide (plates 6″ and 1″ are well separated), whereas in *P.* cf. *reticulatum* strain HWYD1 the contact is slight (Fig. 45) or absent (Fig. 44). *Gonyaulax grindleyi* also lacks a ventral pore, but it was regarded as a junior synonym of *Protoceratium reticulatum* (Hansen *et al.* 1997). From the type locality of *G. grindleyi*, cells of *P. reticulatum* were confirmed through ITS rDNA sequences comparison (Mertens *et al.* 2018b). It is possible that *G. grindleyi* is not a synonym of *P. reticulatum* but that both are present at Elands Bay, Cape Province of South Africa. However, this will not be clarified until more specimens from Cape Province are examined. *Protoceratium reticulatum* from the Mexican Pacific also lacks a ventral pore and toxin production was not detected either (Hernandez-Becerril *et al.* 2010). However, strain PRPV-1 of *P. reticulatum* from the Mexican Pacific (Gulf of California) was grouped with other strains of the species from variable geographic locations in the molecular phylogenetic tree of Salgado *et al.* (2018). In the same area of the Mexican Pacific, Morquecho *et al*. (2009) reported *Protoceratium globosum* characterised by a large ventral pore, but it was considered to be a synonym of *Pentaplacodinium saltonense* (Mertens *et al.* 2018b). *Protoceratium splendens* from the Kara Sea is possibly a junior synonym of *P. reticulatum* as suggested by Gómez (2012). Whether or not *P. splendens* has a ventral pore was not clarified (Meunier 1910).

Protoceratium encompass many other species, e.g. *Protoceratium aculeatum*, *Protoceratium areolatum* and *Protoceratium spinulosum*, but some of them even do not have illustrations, e.g. *P. cancellorum, P. pellucidissimum, P. pepo, P. globosum* and *P. promissum* (Kofoid & Michener 1911). More sequences of *Protoceratium* species are necessary to confirm the validity of these species. An unidentified *Protoceratium* species was reported from the southwestern Atlantic Ocean characterised by spines throughout the cell body (Balech 1988), and its correct identity remains to be determined.

Molecular phylogeny and genetic differentiation

Our results support the classification of strains HWYD1, A10-49-A55, and DBS02 within *Protoceratium*, *Ceratocorys* and *Pentaplacodinium* respectively, with the latter two new to science. Genetic distance based on ITS rDNA sequences is greater than 0.3 between species of *Pentaplacodinium*, much higher than the threshold value (0.04) to differentiate dinoflagellates at inter-specific level (Litaker *et al.* 2007). In contrast, the ITS rDNA sequences based genetic distances between *Ceratocorys malayensis* and *C. horrida* is only 0.06, and *C. horrida* even shares identical LSU rDNA sequences with *C. gourretii*, suggesting that speciation in *Ceratocorys* might have occurred quite recently. The ITS rDNA sequence based genetic distances between strain HWYD1 and *Protoceratium reticulatum* was 0.18, suggesting that this strain might represent a different species. However, strain HWYD1 shares similar morphology with that of *P. reticulatum* except that it lacks a ventral pore, and the contact between plates 1″ and Sa

is much slighter. A ventral pore has been regarded as the key feature to differentiate *Azadinium* species (Luo *et al.* 2017), but it can be present or absent in some *Alexandrium* species (e.g. John *et al.* 2014). Strain HWYD1 must have a cyst stage as it originated from direct incubation of sediments. The cyst-theca relationship, however, is not available at the moment although we did observe cysts of *P. reticulatum* from the same sample of Hawaii (Gu personal observations). Therefore, strain HWYD1 is not described as a new species at the moment, especially because only one *Protoceratium* species has sequences available.

Our molecular phylogeny based on SSU rDNA sequences supports the close relationship between the gonyaulacoid families Protoceratiaceae and Gonyaulaceceae, as previously reported with concatenated data from ribosomal DNA, mitochondrial, and nuclear protein genes (Orr *et al.* 2012). These molecular results are consistent with the fact that both families have been included within the suborder Gonyaulacineae based on morphological evidence (Fensome *et al.* 1993). Previously, the key difference between Gonyaulacaceae and Ceratocorythaceae (here considered a junior synonym of Protoceratiaceae) was the number of precingular plates (six versus five) (Fensome *et al.* 1993), but we show that *Ceratocorys* incorporates species with both five and six precingular plates. The number of anterior intercalary plates appears promising to separate Protoceratiaceae from Gonyaulacaceae. Protoceratiaceae are characterised by always having one anterior intercalary plate, whereas in Gonyaulacaceae, the anterior intercalary plate may be absent, but when present, two or more anterior intercalary plates occur (Dodge, 1989). The pore plate can vary from oval to elliptic in

Pentaplacodinium usupianum (Figs 33, 34) and *Protoceratium reticulatum* (Sala-Pérez *et al.* 2016), but a λ-shaped pore is always present in *Pentaplacodinium*, *Ceratocorys* and *Protoceratium* (Sala-Pérez *et al.* 2016, present study). In contrast, species of *Gonyaulax* show a lanceolate shaped pore (Dodge 1989; Escalera *et al.* 2018). As a consequence, *Protoceratium* and *Pentaplacodinium* are here classified within the Protoceratiaceae.

Ventral organization and dextral torsion were also used to define the family Protoceratiaceae as with a L-type sulcus and strong dextral torsion. However, our new species of *Ceratocorys* has sinistral torsion. Torsion can even be variable within one genus or species. *Protoceratium* strain HWYD1 had sexiform gonyaulacoid tabulation with a L-type ventral organization and dextral torsion. Pronounced dextral torsion was also reported for specimens from the North Sea (the type area for *Protoceratium reticulatum*) (Röder *et al.* 2012) and from the Pacific Ocean off Chile (Álvarez *et al.* 2011). However, neutral torsion occurred in samples from Plymouth, UK (Lebour 1925, pl. 12, fig.7), and specimens from the Mexican Pacific appear slightly sinistral to neutrally contorted (Hernandez-Becerril *et al.* 2010, figs 23 and 24, respectively).

The close morphological similarity between *Pentaplacodinium* and *Protoceratium* is also reflected in our SSU, ITS rDNA sequence-based phylogeny (Figs 51, 53) and V4 region of LSU rDNA sequences based phylogeny (Mertens *et al.* 2018b). Both share a first apical plate of an insert type, and the number of precingular plates can be the same too. Unlike these two genera, *Ceratocorys* shows an episert type in the first apical plate. The early divergence of *Ceratocorys* suggests that the insert type is a derived character.

Protoceratiaceae Lindemann 1928 emend. H.Gu & Mertens

Gonyaulacineans with five or six precingular plates and a midventral, L-type sulcus; only one anterior intercalary plate present.

Gonyaulacaceae Lindemann 1928 emend. H.Gu & Mertens

Gonyaulacineans with the sulcus more or less midventral and straight, oblique from upper right to lower left, or sigmoidal. Antapical outline more or less symmetrical and strong dorsoventral compression lacking. Six precingular plates and, when present, two or more anterior intercalary plates.

***Ceratocorys* F.Stein emend. H.Gu & Mertens**

A gonyaulacinean genus with polygonal theca bearing reticulated plates, with tabulation Po, Pt, 3′, 1a, 5–6″, 6C, 6–7S, 5‴, 1p, 1′‴′ and oval pore plate; plate 1′ of episert type I. Resting cysts unknown.

***Pentaplacodinium* Mertens, Carbonell-Moore, Pospelova & Head emend. H.Gu & Mertens**

A gonyaulacinean genus with roundish theca bearing reticulated plates and tabulation Po, Pt, 3′, 1a, 5–6″, 6C, 6–7S, 5‴, 1p, 1′‴′, and an oval to elliptical pore plate. Plate 1′ of insert type with minimal contact between plates 1″ and 6″. *Operculodinium* type cysts described.

***Protoceratium* Bergh emend. H. Gu & Mertens**

Small, oval to broadly biconical cell bearing reticulated plates, with plate formula Po, Pt, 3′, 1a, 6″, 6C, 6S, 5‴, 1p, 1⁗; pore plate round with a λ-shaped cover plate; plate 1′ of insert type. *Operculodinium* type cysts described.

The classification of *Pentacalodinium* and *Protoceratium* within Protoceratiaceae is supported by the morphological similarity with *Ceratocorys* in possessing only one anterior intercalary plate and possibly a λ-shaped pore too. The latter feature has not been confirmed in other *Ceratocorys* species. The configuration of anterior sulcal plate appears to be conservative in *Ceratocorys* but not in *Pentacalodinium* and *Protoceratium* since both insert type and episert type can be present in the latter two genera. Future research on the cyst-theca relationships of *Protoceratium* and related species and more sequences of gonyaulacoid species will provide futher inight into the relationship between Protoceratiaceae and Gonyaulacaceae.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for constructive suggestions that improved the manuscript greatly.

FUNDING

This work was supported by the National Key Research and Development Program of

China (2016YFE0202100), the National Natural Science Foundation of China (41676117) and China-ASEAN Maritime Cooperation Fund and partially funded by Helmholtz-Gemeinschaft Deutscher Forschungszentren through the research program "Polar regions And Coasts in the changing Earth System" (PACES) of the Alfred Wegener Institut-Helmholtz Zentrum für Polar- und Meeresforschung, and International Collaborative Fund (CF001-2018) from Malaysia Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC), and Ministry of Education HICoE and RU fund (IOES-2014C; TU001A-2018).

REFERENCES

Adachi M., Sako Y. & Ishida Y. 1996. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *Journal of Phycology* 32: 424–432.

Álvarez G., Uribe E., Díaz R., Braun M., Mariño C. & Blanco J. 2011. Bloom of the yessotoxin producing dinoflagellate *Protoceratium reticulatum* (Dinophyceae) in northern Chile. *Journal of Sea Research* 65: 427–434.

Balech E. 1980. On the thecal morphology of dinoflagellates with special emphasis on circular and sulcal plates. *Anales del Centro de Ciencias del Mar y Limnologia, Universidad Nacional Autonomia de Mexico* 7: 57–68.

Balech E. 1988. Los dinoflagelados del Atlántico sudoccidental. *Publicaciones Especiales. Instituto Español de Oceanografía* 1: 1–310.

Bergh R.S. 1881. Der Organismus der Cilioflagellaten. Eine phylogenetische Studie.

Morphologisches Jahrbuch 7: 177–288.

Boc A., Diallo A.B. & Makarenkov V. 2012. T-REX: a web server for inferring, validating and visualizing phylogenetic trees and networks. *Nucleic acids research* 40: W573–W579.

Carbonell-Moore M. 1996. *Ceratocorys anacantha*, sp. nov., a new member of the family Ceratocoryaceae Lindemann (Dinophyceae). *Botanica Marina* 39: 1–10.

Daugbjerg N., Hansen G., Larsen J. & 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: 302–317.

Dodge J. 1989. Some revisions of the family Gonyaulacaceae (Dinophyceae) based on a scanning electron microscope study. *Botanica Marina* 32: 275–298.

Escalera L., Italiano A., Pistocchi R., Montresor M. & Zingone A. 2018. *Gonyaulax hyalina* and *Gonyaulax fragilis* (Dinoflagellata), two names associated with ‘mare sporco', indicate the same species. *Phycologia* 57: 453–464.

Fensome R.A., Taylor F.J.R., Norris G., Sarjeant W.A.S., Wharton D.I. & Williams G.L. 1993. A classification of fossil and living dinoflagellates. *Micropaleontology Special Publication* 7: 1–245.

Gómez F. 2012. A checklist and classification of living dinoflagellates (Dinoflagellata, Alveolata). *CICIMAR Oceánides* 27: 65–140.

Graham H.W. 1942. Studies in the morphology, taxonomy, and ecology of the Peridiniales. *Scientific Results Cruise VII Carnegie Biology Series* 3: 1–129.

Guillard R.R.L. & Ryther J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Canadian Journal of Microbiology* 8: 229–239.

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Series* 41: 95–98.

Hansen G., Moestrup Ø. & Roberts K.R. 1997. Light and electron microscopical observations on *Protoceratium reticulatum* (Dinophyceae). *Archiv für Protistenkunde* 147: 381–391.

Helenes J. 2000. *Exochosphaeridium alisitosense* n. sp.; a new gonyaulacoid dinoflagellate from the Albian of Baja California, Mexico. *Micropaleontology* 46: 135–142.

Hernandez-Becerril D.U., Rodriguez-Palacio M.C. & Lozano-Ramirez C. 2010. Morphology of two bloom-forming or potentially toxic marine dinoflagellates from the Mexican Pacific, *Heterocapsa pygmaea* and *Protoceratium reticulatum* (Dinophyceae). *Cryptogamie, Algologie* 31: 245–254.

John U., Litaker R. W., Montresor M., Murray S., Brosnahan M. L. & Anderson D. M. 2014. Formal revision of the *Alexandrium tamarense* species complex (Dinophyceae) taxonomy: the introduction of five species with emphasis on molecular-based (rDNA) classification. *Protist* 165: 779–804.

Katoh K. & Standley D.M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772–780.

Kawai H. & Nakayama T. 2015. Division Dinophyta. In: *Syllabus of plant families: photoautotrophic eukaryotic algae* (Ed. by W. Frey), pp. 29–49. Borntraeger Science Publishers, Stuttgart, Germany.

Kokinos J.P. & Anderson D.M. 1995. Morphological development of resting cysts in cultures of the marine dinoflagellate *Lingulodinium polyedrum* (= *L. machaerophorum*). *Palynology* 19: 143–166.

Litaker W.R., Vandersea M.W., Kibler S.R., Reece K.S., Stokes N.A., Lutzoni F.M., Yonish B.A., West M.A., Black M.N.D. & Tester P.A. 2007. Recognizing dinoflagellate species using ITS rDNA sequences. *Journal of Phycology* 43: 344–355.

Luo Z., Krock B., Mertens K.N., Nézan E., Chomérat N., Bilien G., Tillmann U. & Gu H. 2017. Adding new pieces to the *Azadinium* (Dinophyceae) diversity and biogeography puzzle: non-toxigenic *Azadinium zhuanum* sp. nov. from China, toxigenic *A. poporum* from the Mediterranean, and a non-toxigenic *A. dalianense* from the French Atlantic. *Harmful Algae* 66: 65–78.

Medlin L., Elwood H.J., Stickel S. & Sogin M.L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491–499.

Mertens K.N., Pospelova V. & Head M.J. 2018a. *Ceratocorys mariaovidiorum* P.Salgado, S.Fraga, F.Rodríguez, P.Riobó & I.Bravo is a junior synonym of *Pentaplacodinium saltonense* K.N.Mertens, M.C.Carbonell-Moore, V.Pospelova & M.J.Head. *Notulae algarum* 45: 1–2.

Mertens K.N., Carbonell-Moore M.C., Pospelova V., Head M.J., Highfield A., Schroeder D., Gu, H., Andree K. B., Fernandez M., Yamaguchi A. *et al.* 2018b. *Pentaplacodinium saltonense* gen. et sp. nov. (Dinophyceae) and its relationship to the cyst-defined genus *Operculodinium* and yessotoxin-producing *Protoceratium reticulatum*. *Harmful Algae* 71: 57–77.

Morquecho L., Góngora-González D.T. & Okolodkov Y.B. 2009. Cyst-theca relationships on Gonyaulacales and Peridiniales (Dinophyceae) from Bahía Concepción, Gulf of California. *Acta Botánica Mexicana* 88: 9–29.

Orr R.J.S., Murray S.A., Stüken A., Rhodes L. & Jakobsen K.S. 2012. When naked became armored: an eight-gene phylogeny reveals monophyletic origin of theca in dinoflagellates. *PLoS ONE* 7: e50004.

Paez-Reyes M. & Head M.J. 2013. The cenozoic Gonyaulacacean dinoflagellate genera *Operculodinium* Wall, 1967 and *Protoceratium* Bergh, 1881 and their phylogenetic relationships. *Journal of Paleontology* 87: 786–803.

Posada D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* 25: 1253–1256.

Röder K., Hantzsche F.M., Gebühr C., Miene C., Helbig T., Krock B., Hoppenrath M., Luckas B. & Gerdts G. 2012. Effects of salinity, temperature and nutrients on growth, cellular characteristics and yessotoxin production of *Protoceratium reticulatum*. *Harmful Algae* 15: 59–70.

Reinecke P. 1967. *Gonyaulax grindleyi* sp. nov.: a dinoflagellate causing a red tide at Elands Bay, Cape Province, in December 1966. *South African Journal of Botany*

33: 157–160.

Ronquist F. & Huelsenbeck J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Sala-Pérez M., Alpermann T.J., Krock B. & Tillmann U. 2016. Growth and bioactive secondary metabolites of arctic *Protoceratium reticulatum* (Dinophyceae). *Harmful Algae* 55: 85–96.

Saldarriaga J.F., Taylor F.J.R.M., Cavalier-Smith T., Menden-Deuer S. & Keeling P.J. 2004. Molecular data and the evolutionary history of dinoflagellates. *European Journal of Protistology* 40: 85–111.

Salgado P., Fraga S., Rodríguez F., Riobó P. & Bravo I. 2018. *Ceratocorys mariaovidiorum* sp. nov. (Gonyaulacales), a new dinoflagellate species previously reported as *Protoceratium reticulatum*. *Journal of Phycology* 54: 126–137.

Schiller J. 1937. Dinoflagellatae (Peridineae). Teil 2. In: Kryptogamen Flora (Ed. by L. Rabenhorst), pp. 1-589. Akademische Verlagsgesellschaft, Leipzig.

Scholin C.A., Herzog M., Sogin M. & Anderson D.M. 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *Journal of Phycology* 30: 999–1011.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690.

Swofford D.L. 2002. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Wołoszyńska J. 1929. Dinoflagellatae Polskiego Baltyku i blot nad Piasnica. *Archivum Hydrobiologji i Rybactwa* 3: 153–278. [In Polish]

Zirbel M.J., Veron F. & Latz M.I. 2000. The reversible effect of flow on the morphology of *Ceratocorys horrida* (Peridiniales, Dinophyta). *Journal of Phycology* 36: 46–58.

Figure captions

Figs 1–6. Light micrographs of live cells of *Ceratocorys malayensis* strain A10-49-A55. Scale bar = 10 μm.

Fig. 1. Ventral view showing gross morphology and cingulum displacement.

Fig. 2. Lateral view with several spines in hypotheca (arrows).

Fig. 3. Cell with numerous autofluorescent chloroplasts.

Fig. 4. Sybr Green stained cell, with U-shaped nucleus (N).

Fig. 5. Apical view with six precingular plates.

Fig. 6. Epithecal plates stained with calcofluor showing three apical plates (1′ – 3′) and one anterior intercalary (1a) plate.

Figs 7–12. Scanning electron micrographs of *Ceratocorys malayensis* strain A10-49-A55. Scale bar = 10 μm.

Fig. 7. Oblique apical view showing cingulum displacement.

Fig. 8. Dorsal view showing precingular plates (3″–5″) and postcingular plates (*4‴, *5‴) with evident sinistral torsion.

Fig. 9. Apical view showing three apical plates (1′ – 3′), one anterior intercalary (1a) plate and six precingular plates (1″ – 6″).

Fig. 10. Antapical view showing four postcingular plates (*3‴ – *6‴) and antapical plate (1⁗).

Fig. 11. The cingulum showing six cingular plates (C1–C6).

Fig. 12. The sulcus showing anterior sulcal plate (Sa), anterior left sulcal plate (Ssa),

anterior right sulcal plate (Sda), posterior left sulcal plate (Ssp), posterior right sulcal plate (Sdp) and posterior sulcal plate (Sp).

Figs 13–18. Scanning electron micrographs of *Ceratocorys malayensis* strain A10-49-A55.

Fig. 13. Left lateral view showing two postcingular plates (*2‴, *3‴), posterior intercalary plate (1p) and antapical plate (1⁗). Scale bar = 10 μm.

Fig. 14. Apical view with three apical plates (1′ – 3′), one anterior intercalary (1a) plate and five of the six precingular plates (1″ – 5″). Scale bar = 10 μm.

Figs 15, 16. Detail of oval pore plate (Po) with a λ-shaped cover plate (Pt) perforated by pores. Scale bar = 1 μm.

Fig. 17. Apical view showing first apical plates (1′), and wide contact between first and sixth precingular plates (1″, 6″). Scale bar = 10 μm.

Fig. 18. Apical view showing first apical plate with a ventral pore at its right margin (arrow). Scale bar = 10 μm.

Figs 19–22. Schematic drawings of thecal plate patterns of C*eratocorys malayensis.*

Fig. 19. Ventral view.

Fig. 20. Dorsal view.

Fig. 21. Apical view.

Fig. 22. Antapical view.

Figs 23–28. Light micrographs of live cells of *Pentaplacodinium usupianum* strain DBS02. Scale bar = 10 μm.

Fig. 23. Ventral view showing cingulum displacement and posterior widening of sulcus.

Fig. 24. Dorsal view showing a conical epitheca and rounded hypotheca.

Fig. 25. Ventral view, showing anterior sulcal plate (Sa) intruding the epitheca.

Fig. 26. Dorsal view, with nucleus (N) and several spines at antapex (arrow).

Fig. 27. Dorsal view, with numerous autofluorescent chloroplasts.

Fig. 28. SYBR Green stained cell, with U-shaped nucleus (N).

Figs 29–35. Scanning electron micrographs of *Pentaplacodinium usupianum* strain DBS02.

Fig. 29. Ventral view showing first apical plate (1′), anterior sulcal plate (Sa), first and sixth precingular plates (1″, 6″), second and sixth postcingular plates (*2‴, *6‴), posterior intercalary plate (1p), first antapical plate (1⁗) and posterior sulcal plate (Sp). Scale bar = 10 μm.

Fig. 30. Apical view showing three apical plates (1′ – 3′), one anterior intercalary (1a) plate and six precingular plates (1″ – 6″). Scale bar = 10 μm.

Fig. 31. Antapical view showing five postcingular plates (*2‴ – *6‴), posterior intercalary plate (1p) and antapical plate (1⁗). Scale bar = 10 μm.

Fig. 32. Cingulum with six plates (C1–C6). Scale bar = 10 μm.

Figs 33, 34. Detail of oval to elliptical pore plate with λ-shaped cover plate and perforated by pores. Scale bar = 2 μm.

Fig. 35. Sulcus showing anterior sulcal plate (Sa), anterior left sulcal plate (Ssa), anterior right sulcal plate (Sda), posterior left sulcal plate (Ssp), and posterior right sulcal plate (Sdp). Scale bar = 2 μm.

Figs 36–39. Schematic drawings of thecal plate patterns of *Pentaplacodinium usupianum.*

Fig. 36. Ventral view.

Fig. 37. Dorsal view.

Fig. 38. Apical view.

Fig. 39. Antapical view.

Figs 40–43. Light micrographs of live cells of *Protoceratium* cf. *reticulatum* strain HWYD1. Scale bars: 5 μm.

Fig. 40. Ventral view showing cingulum displacement and posterior widening of sulcus.

Fig. 41. Ventral view showing anterior sulcal plate (Sa) intruding the epitheca.

Fig. 42. Dorsal view, showing autofluorescent chloroplast network.

Fig. 43. A SYBR Green stained cell, showing curved nucleus (N).

Figs 44–50. Scanning electron micrographs of *Protoceratium* cf. *reticulatum* strain

HWYD1.

Fig. 44. Ventral view showing first and sixth precingular plates (1″, 6″), second, third and sixth postcingular plates (*2‴, *3‴ and *6‴), posterior intercalary plate (1p) and three cingular plates (C1, C2 and C6). Scale bar = 5 μm.

Fig. 45. Apical view showing three apical plates (1′ – 3′), one anterior intercalary (1a) plate and six precingular plates (1″ – 6″). Scale bar = 5 μm.

Fig. 46. Apical view showing three apical plates (1′ – 3′), one anterior intercalary (1a) plate and five precingular plates (1″ – 5″). Scale bar = 5 μm.

Fig. 47. Dorsal view showing three cingular plates (C3, C4 and C5), two precingular plates (3″, 4″) and two postcingular plates (*4‴, *5‴) with evident dextral torsion. Scale bar = 5 μm.

Fig. 48. Antapical view showing four postcingular plates (*3‴ – *6‴) and the antapical plate (1⁗). Scale bar = 5 μm.

Fig. 49. Detail of the sigmoidal pore plate with a λ-shaped cover plate and perforated by pores. Scale bar = 2 μm.

Fig. 50. The sulcus showing the anterior sulcal plate (Sa), anterior left sulcal plate (Ssa), anterior right sulcal plate (Sda), posterior left sulcal plate (Ssp), posterior right sulcal plate (Sdp), and posterior sulcal plate (Sp). Scale bar = 2 μm.

Fig. 51. Phylogeny of *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* inferred from SSU rDNA sequences using maximum likelihood (ML). New sequences indicated in bold. Branch lengths drawn to scale, with scale bar indicating number of nucleotide

substitutions per site. Numbers on branches are statistical support values to clusters on their right (left: ML bootstrap support values; right: Bayesian posterior probabilities). Asterisk indicates maximal support (ML BS = 100% and BPP = 1.00).

Fig. 52. Phylogeny of *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* inferred from partial LSU rDNA sequences using maximum likelihood (ML). New sequences indicated in bold. Branch lengths drawn to scale, with scale bar indicating number of nucleotide substitutions per site. Numbers on branches are statistical support values to clusters on their right (left: ML bootstrap support values; right: Bayesian posterior probabilities). Dashed lines indicate half length.

Fig. 53. Phylogeny of *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* inferred from ITS rDNA sequences using maximum likelihood (ML). New sequences indicated in bold. Branch lengths drawn to scale, with scale bar indicating number of nucleotide substitutions per site. Numbers on branches are statistical support values to clusters on their right (left: ML bootstrap support values; right: Bayesian posterior probabilities). Asterisk indicates maximal support (ML BS = 100% and BPP = 1.00). Dashed lines indicate half length.

Supplementary Figures

Figs S1, S2. Scanning electron micrographs of *Ceratocorys malayensis* cells from the field. Scale bar = 10 μm.

Fig. S1. Oblique ventral view showing two apical plates (1′, 3′), three precingular plates (1″, 5″, 6″) and three postcingular plates (*2‴, *5‴, *6‴).

Fig. S2. Apical view showing three apical plates (1′ – 3′), one anterior intercalary (1a) plate and six precingular plates (1″ – 6″).

Figs S3, S4. Scanning electron micrographs of *Pentaplacodinium usupianum* strain DBS02. Scale bar = 10 μm.

Fig. S3. Lateral view showing three precingular plates (1″ – 3″) and two postcingular plates (*3‴, *4‴).

Fig. S4. Apical view showing three apical plates (1′ – 3′), one anterior intercalary (1a) plate and five precingular plates (1″ – 5″).

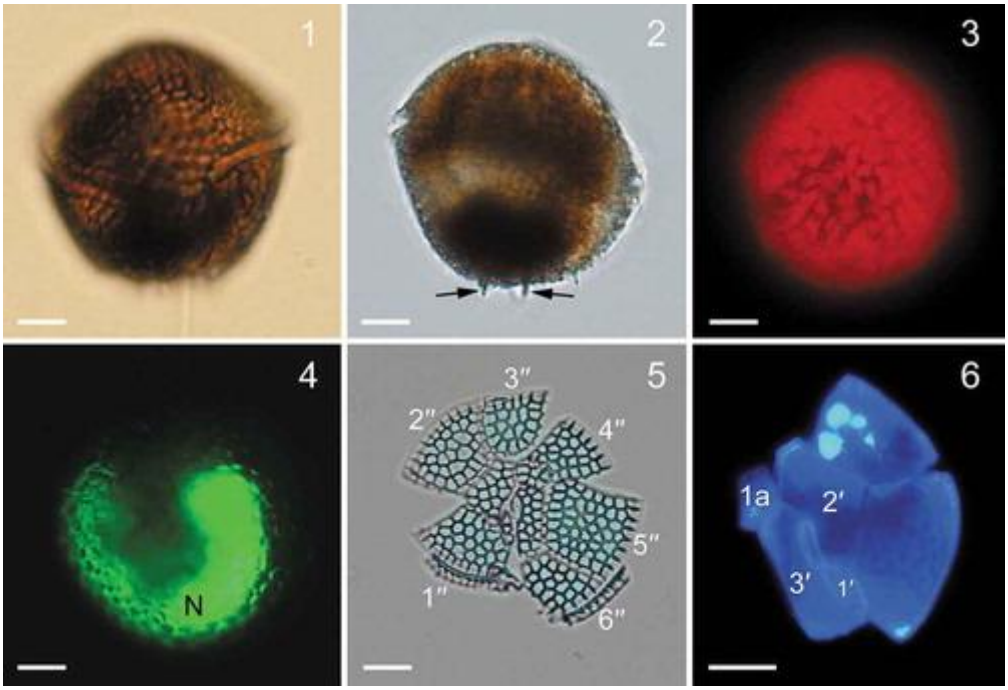
Figs S5, S6. Scanning electron micrographs of *Pentaplacodinium usupianum* strain DBS02. Scale bar = 10 μm.

Fig. S5. Oblique apical view showing three apical plates (1′ – 3′), four precingular plates (1″, 2″, 5″ and 6″), anterior sulcal plate (Sa) with broad contact between plates 1″ and 6″.

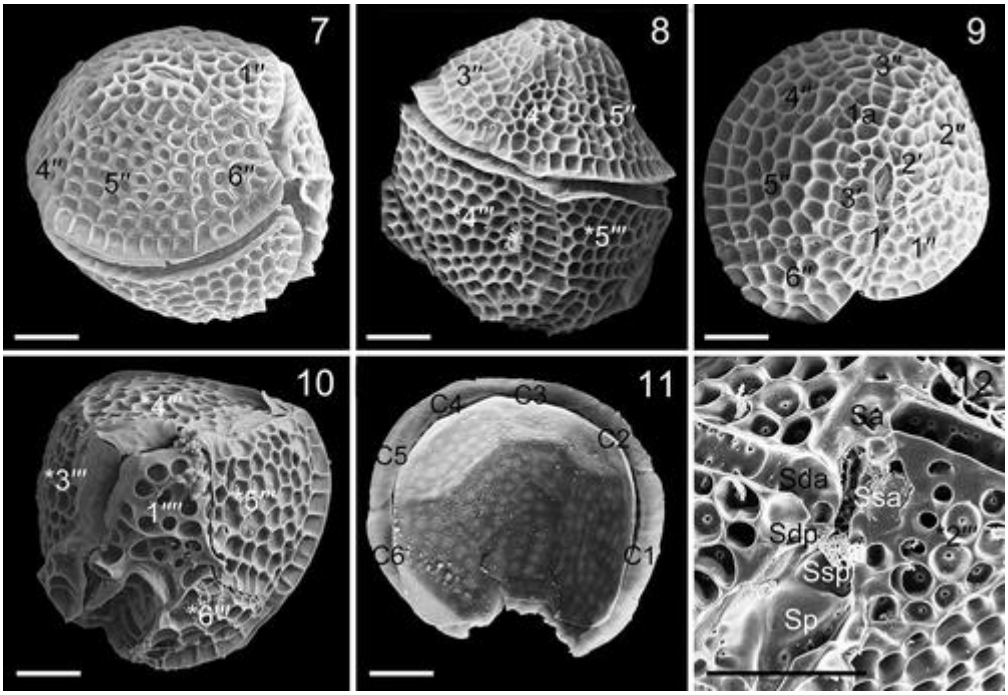
Fig. S6. Apical view showing three apical plates (1′ – 3′), one anterior intercalary plate (1a), six precingular plates (1″ – 6″), anterior sulcal plate (Sa) with narrow

contact between plates 1″ and 6″.

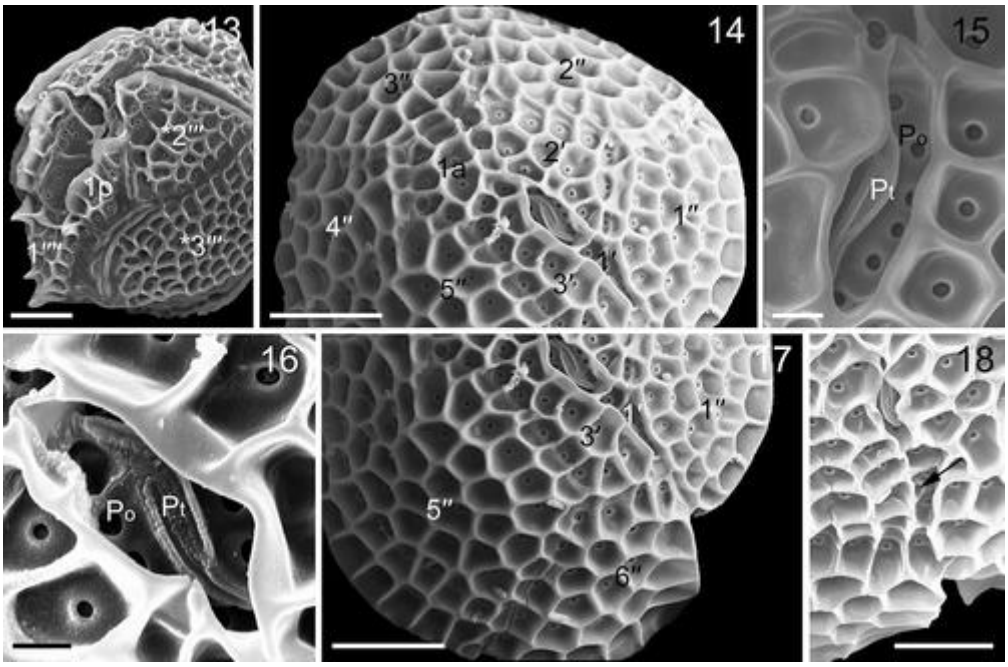
Figures 1-6



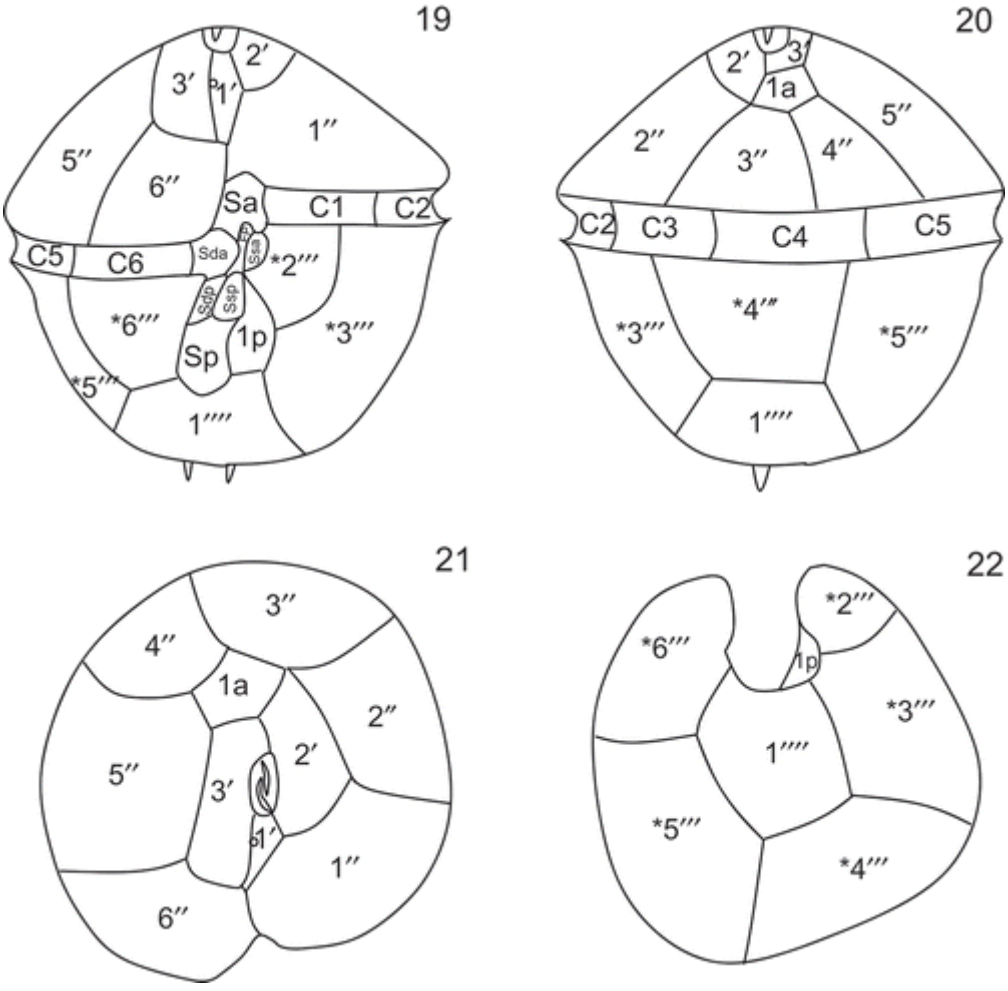
Figures 7-12



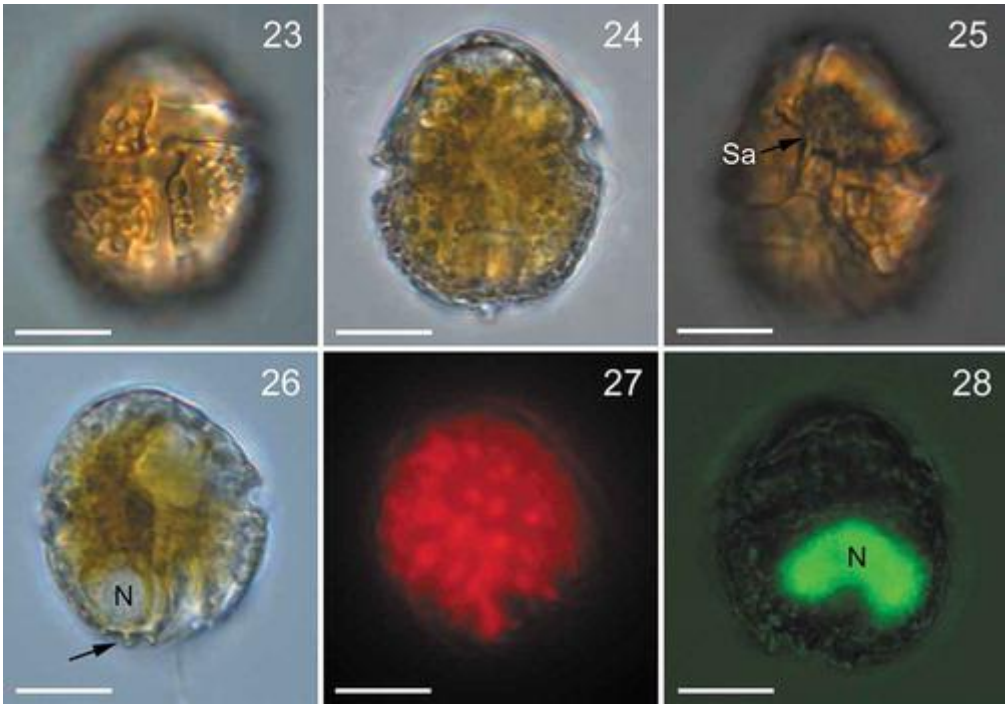
Figures 13-18



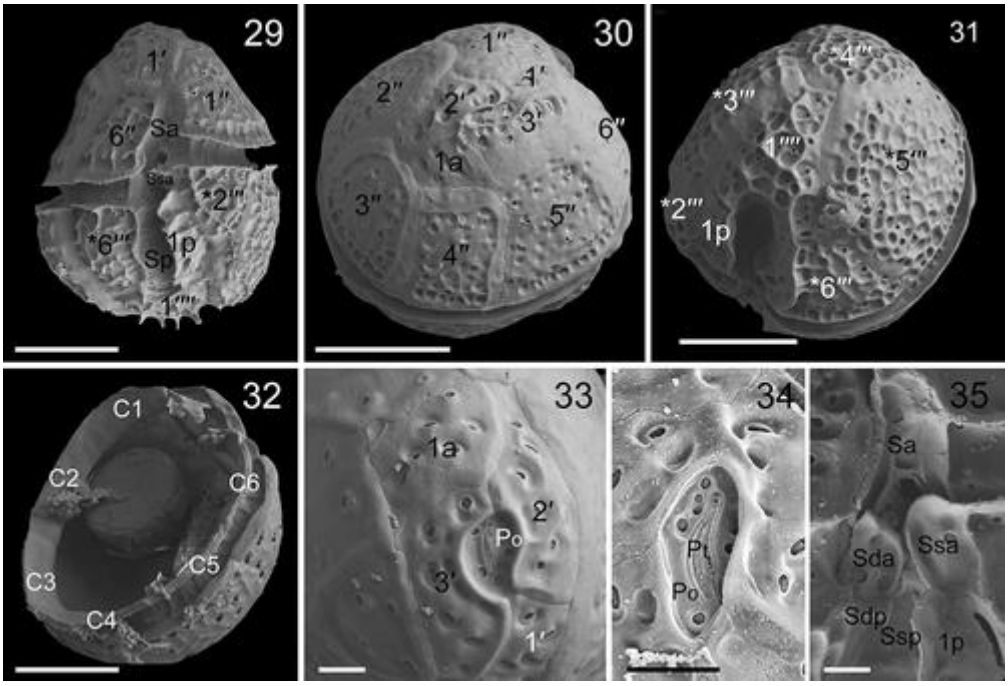
Figures 19-22



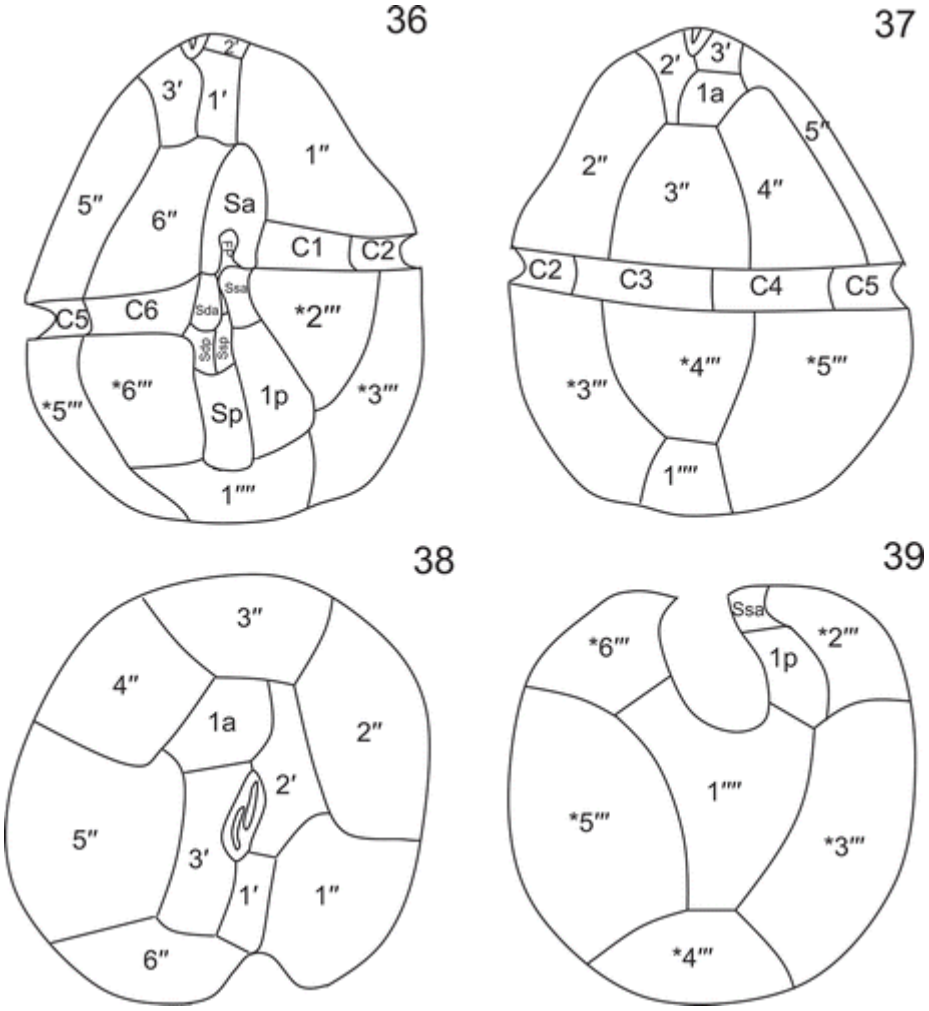
Figures 23-28



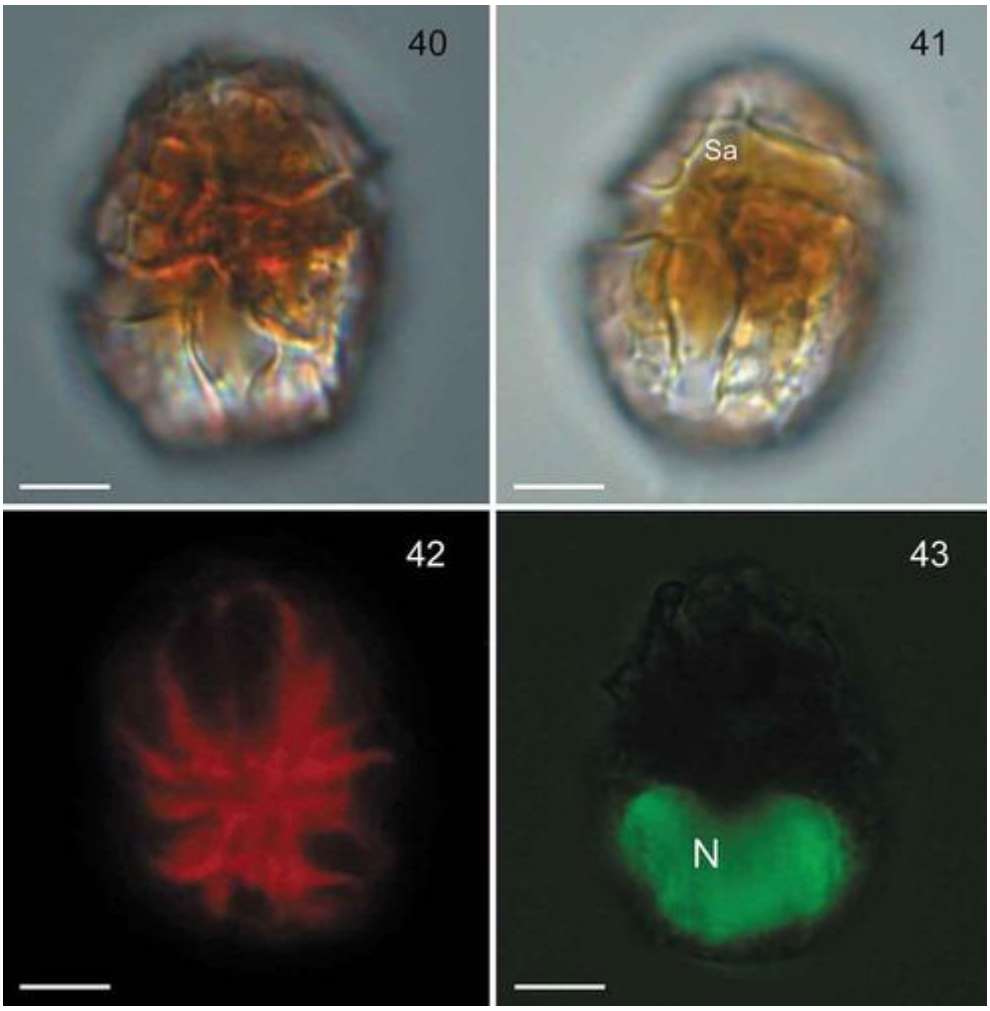
Figures 29-35



Figures 26-39



Figures 40-43



Figures 44-50

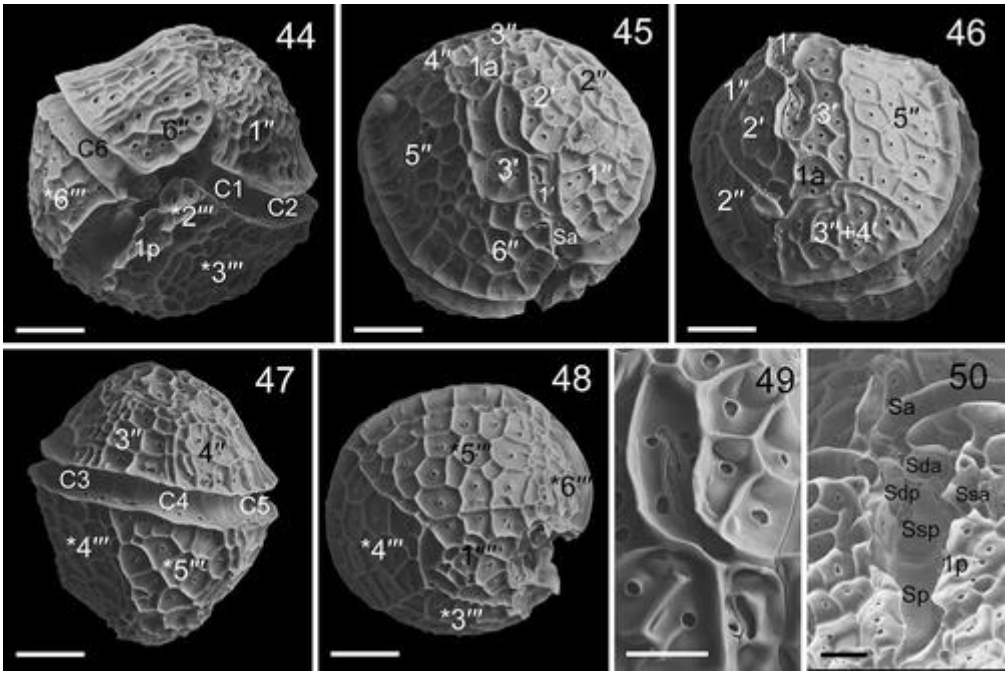


Figure 51

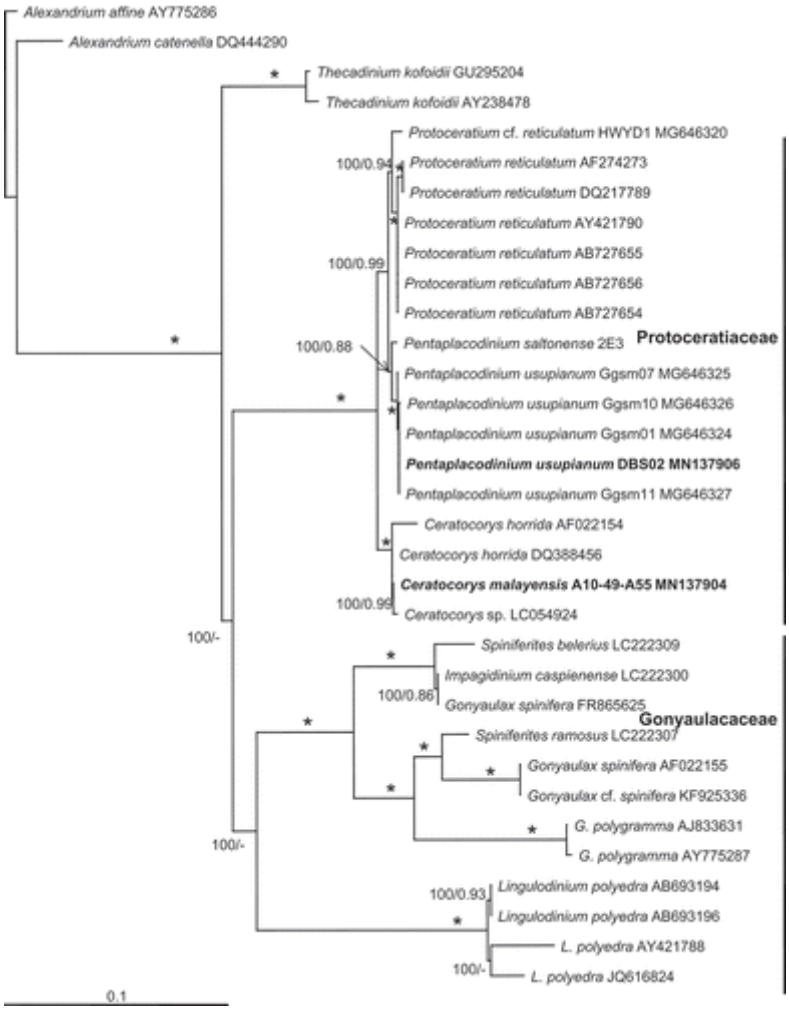


Figure 52

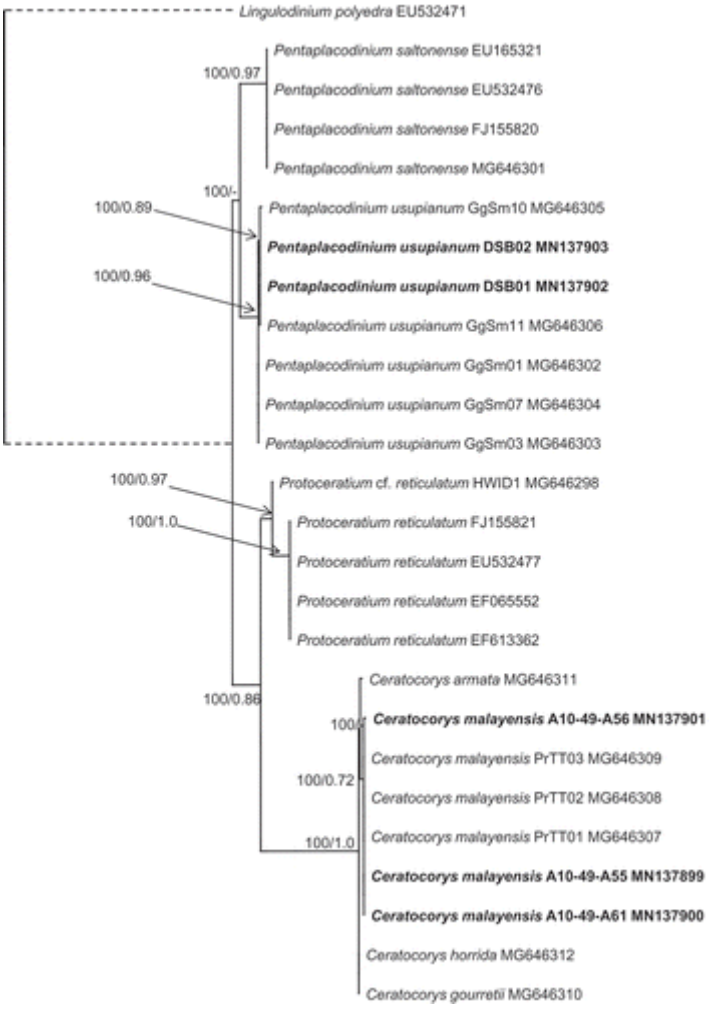
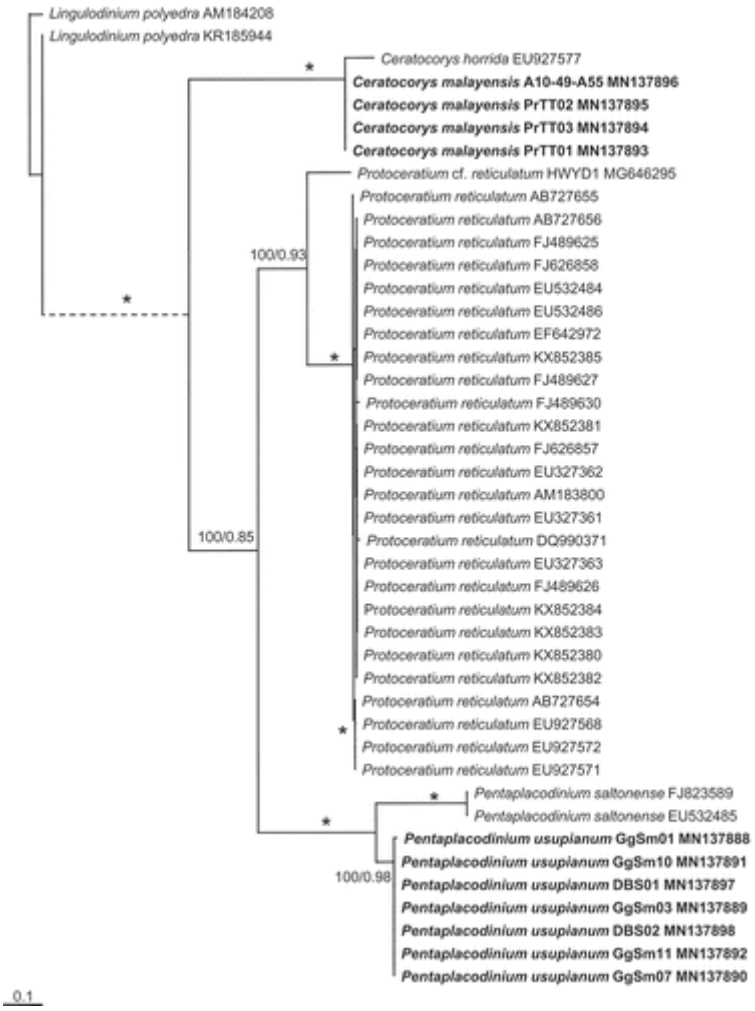

Lingulodinium polyedra EU532471
Pentaplacodinium saltonense EU165321
100/0.97
Pentaplacodinium saltonense EU532476
Pentaplacodinium saltonense FJ155820
Pentaplacodinium saltonense MG646301
100/-
100/0.89
Pentaplacodinium usupianum GgSm10 MG646305
Pentaplacodinium usupianum DSB02 MN137903
100/0.96
Pentaplacodinium usupianum DSB01 MN137902
Pentaplacodinium usupianum GgSm11 MG646306
Pentaplacodinium usupianum GgSm01 MG646302
Pentaplacodinium usupianum GgSm07 MG646304
Pentaplacodinium usupianum GgSm03 MG646303
100/0.97
Protoceratium cf. reticulatum HWID1 MG646298
100/1.0
Protoceratium reticulatum FJ155821
Protoceratium reticulatum EU532477
Protoceratium reticulatum EF065552
Protoceratium reticulatum EF613362
Ceratocorys armata MG646311
100/0.86
100/-
Ceratocorys malayensis A10-49-A56 MN137901
Ceratocorys malayensis PrTT03 MG646309
100/0.72
Ceratocorys malayensis PrTT02 MG646308
100/1.0
Ceratocorys malayensis PrTT01 MG646307
Ceratocorys malayensis A10-49-A55 MN137899
Ceratocorys malayensis A10-49-A61 MN137900
Ceratocorys horrida MG646312
Ceratocorys gourretii MG646310

Figure 53


Lingulodinium polyedra AM184208
Lingulodinium polyedra KR185944
Ceratocorys horrida EU927577
Ceratocorys malayensis A10-49-A55 MN137896
Ceratocorys malayensis PrTT02 MN137895
Ceratocorys malayensis PrTT03 MN137894
Ceratocorys malayensis PrTT01 MN137893
Protoceratium cf. reticulatum HWYD1 MG646295
Protoceratium reticulatum AB727655
Protoceratium reticulatum AB727656
Protoceratium reticulatum FJ489625
Protoceratium reticulatum FJ626858
Protoceratium reticulatum EU532484
Protoceratium reticulatum EU532486
Protoceratium reticulatum EF642972
Protoceratium reticulatum KX852385
Protoceratium reticulatum FJ489627
Protoceratium reticulatum FJ489630
Protoceratium reticulatum KX852381
Protoceratium reticulatum FJ626857
Protoceratium reticulatum EU327362
Protoceratium reticulatum AM183800
Protoceratium reticulatum EU327361
Protoceratium reticulatum DQ990371
Protoceratium reticulatum EU327363
Protoceratium reticulatum FJ489626
Protoceratium reticulatum KX852384
Protoceratium reticulatum KX852383
Protoceratium reticulatum KX852380
Protoceratium reticulatum KX852382
Protoceratium reticulatum AB727654
Protoceratium reticulatum EU927568
Protoceratium reticulatum EU927572
Protoceratium reticulatum EU927571
Pentaplacodinium saltonense FJ823589
Pentaplacodinium saltonense EU532485
Pentaplacodinium usuplanum GgSm01 MN137888
Pentaplacodinium usuplanum GgSm10 MN137891
Pentaplacodinium usuplanum DBS01 MN137897
Pentaplacodinium usuplanum GgSm03 MN137889
Pentaplacodinium usuplanum DBS02 MN137898
Pentaplacodinium usuplanum GgSm11 MN137892
Pentaplacodinium usuplanum GgSm07 MN137890
100/0.93
100/0.85
100/0.98
0.1