

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

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

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### ABSTRACT

The gonyaulaclean family Protoceratiaceae is characterised by five precingular plates. It currently encompasses the type genus *Ceratocorys* and the fossil genus *Atopodinium*. Fourteen strains of *Ceratocorys*, *Pentaplagodinium*, 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 *Pentaplagodinium usupianum* sp. nov., were described from Malaysian waters. They share a Kofoidian 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 *Pentaplagodinium usupianum* had a parallelogram-shaped 1' plate which often contacted the Sa plate. The genera *Ceratocorys* and *Pentaplagodinium* 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 *Pentaplagodinium* as members of the family Protoceratiaceae, characterised by the presence of one anterior intercalary plate. Seven strains of *Protoceratium cf. reticulatum*, *Ceratocorys malayensis* and *Pentaplagodinium usupianum* were examined for yessotoxin production by LC-MS/MS but none produced a detectable amount of toxin.

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### 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 organisation and strong dextral torsion, whereas Gonyaulacaceae have six precingular plates, L- to S-type ventral organisation and sinistral to dextral torsion (Fensome *et al.* 1993). Thus, the key difference between Gonyaulacaceae and Ceratocorythaceae is 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 organisation and dextral torsion), Leptodinioideae (with L-type ventral organisation and sinistral or neutral torsion), and Gonyaulacoideae (with S-type ventral organisation 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 include 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 Kofoidian precingular plate in *Ceratocorys* is considered homologous to both the third and fourth precingular plates in other gonyaulacoid dinoflagellate 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 (Carbonell-

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Moore 1996; Graham 1942). However, sequences of *Ceratocorys* are available for only 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).

*Pentaplagodinium* 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 epithelial configuration in these strains, unlike the episert type in *Ceratocorys horrida*, the type species of *Ceratocorys*. However, episert (type I) was also reported in *Pentaplagodinium saltonense* (Salgado et al. 2018, as *Ceratocorys mariaovidiorum*). *Pentaplagodinium* 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 epithelial 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). Of these species, only *P. reticulatum* has an available sequence.

*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* (Orr et al. 2012; Saldarriaga et al. 2004). 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 *Pentaplagodinium* are investigated in this study in detail. Furthermore, to address the phylogenetic relationships among *Protoceratium*, *Ceratocorys* and *Pentaplagodinium*, 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 *Pentaplagodinium* were initially established in ES-DK medium at 25 °C (Kokinos & Anderson 1995) in Malaysia. These clonal cultures were transferred to Xiamen and maintained with f/2-Si medium (Guillard & Ryther 1962) at 20 °C, 90 µmol quanta·m<sup>-2</sup>·s<sup>-1</sup> 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 subsurface 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 Harbour on 4 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, Missouri, 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). Chloroplast autofluorescence in live cells was observed using the above microscope equipped using 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

**Table 1.** Information on isolates used in this study. Species designations, strain identification, origin, isolator, isolation date and yessotoxin. NA: not available.

Species	Strains	Latitude (N)	Longitude (E)	Location	Isolator	GenBank no. (SSU/ITS/LSU)	Isolation date	Yessotoxin
<i>Pentaplagodinium usupianum</i>	DBS01	1°36.00'	110°19.00'	Semariang, Sarawak, Malaysia	Teng Sing Tung	MN137905/ MN137897/MN137902	21 Dec. 2016	NA
<i>Pentaplagodinium usupianum</i>	DBS02	1°36.00'	110°19.00'	Semariang, Sarawak, Malaysia	Teng Sing Tung	MN137906/ MN137898/MN137903	21 Dec. 2016	NA
<i>Pentaplagodinium usupianum</i>	GgSM01	1°36.00'	110°19.00'	Semariang, Sarawak, Malaysia	Tan Toh Hii	MG646302/ MN137888/MG646324	08 Aug. 2010	None
<i>Pentaplagodinium usupianum</i>	GgSM03	1°36.00'	110°19.00'	Semariang, Sarawak, Malaysia	Tan Toh Hii	MG646303/ MN137889/-	22 Sep. 2010	NA
<i>Pentaplagodinium usupianum</i>	GgSM07	1°36.00'	110°19.00'	Semariang, Sarawak, Malaysia	Tan Toh Hii	MG646304/ MN137890/MG646325	17 Jan. 2013	None
<i>Pentaplagodinium usupianum</i>	GgSM10	1°36.00'	110°19.00'	Semariang, Sarawak, Malaysia	Kam Bao Juan	MG646305/ MN137897/MG646326	17 Jan. 2013	None
<i>Pentaplagodinium usupianum</i>	GgSM11	1°36.00'	110°19.00'	Semariang, Sarawak, Malaysia	Kam Bao Juan	MG646306/ MN137897/MG646327	17 Jan. 2013	None
<i>Ceratocorys malayensis</i>	A10-49-A55	5°57.70'	102°40.88'	Rawa Island, Terrenganu, Malaysia	Lim Zhen Fei	MN137904/ MN137896/MN137899	17 May 2016	None
<i>Ceratocorys malayensis</i>	A10-49-A56	5°57.70'	102°40.88'	Rawa Island, Terrenganu, Malaysia	Lim Zhen Fei	-/-/MN137901	17 May 2016	NA
<i>Ceratocorys malayensis</i>	A10-49-A61	5°57.70'	102°40.88'	Rawa Island, Terrenganu, Malaysia	Lim Zhen Fei	-/-/MN137900	17 May 2016	None
<i>Ceratocorys malayensis</i>	PrTT01	1°55.02'	109°46.47'	Talang-Talang Island, Sarawak, Malaysia	Teng Sing Tung	MG646307/ MN137893/-	28 Mar. 2013	NA
<i>Ceratocorys malayensis</i>	PrTT02	1°55.02'	109°46.47'	Talang-Talang Island, Sarawak, Malaysia	Teng Sing Tung	MG646308/ MN137895/-	28 Mar. 2013	NA
<i>Ceratocorys malayensis</i>	PrTT03	1°55.02'	109°46.47'	Talang-Talang Island, Sarawak, Malaysia	Teng Sing Tung	MG646309/ MN137894/-	28 Mar. 2013	NA
<i>Protoceratium cf. reticulatum</i>	HWYD1	21°35.65'	-158°6.00'	Haleiwa Harbour, north coast of O'ahu, USA	Anne de Vernal and Geneviève Vautour	MG646320/MG646295/ MG646298	04 Mar. 2014	None

2.5% glutaraldehyde for 3 h at 8 °C, rinsed with Milli-Q water twice and post-fixed with 1% OsO<sub>4</sub> overnight at 8 °C. The supernatant was removed and settled cells were transferred to a coverslip coated with poly-L-lysine (molecular weight 70,000–150,000). Cells attached to the coverslip were rinsed in Milli-Q water twice. 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 using a Zeiss Sigma FE (Carl Zeiss Inc., Oberkochen, Germany) scanning electron microscope. Labelling of tabulation follows a modified Kofoid system that recognises homologs (e.g., Fensome *et al.* 1993); sulcal plate labelling follows Balech (1980).

### PCR amplifications and sequencing

Total algal DNA of *Ceratocorys*, *Pentaplagodinium* 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). LSU rDNA was amplified using the primer pair DIR/28-1483R (Daugbjerg *et al.* 2000; Scholin *et al.* 1994). 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 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C, and final extension of 7 min at 72 °C with a Mastercycler (Eppendorf, Hamburg, Germany). 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, California, 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*, *Pentaplagodinium* and *Protoceratium* (SSU, partial LSU and ITS rDNA) were incorporated into independent datasets of closely related dinoflagellate sequences via taxon sampling in the NCBI GenBank nucleotide database. All sequences were aligned using MAFFT v7.110 (Katoh & Standley 2013) online programme (<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 using 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

were run 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*, *Pentaplecodinium* 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 *Pentaplecodinium 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  $\mu$ l methanol with a sonotrode (model HP2070, Bandelin, Berlin, Germany). After homogenisation, 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  $\mu$ m, Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 3220 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  $\times$  2 mm, 3  $\mu$ m) at a flow rate of 0.3 ml min<sup>–1</sup> 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  $\mu$ m long and 40.9–54.6  $\mu$ m 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 Po, Pt, 3', 1a, 6'', 6C, 6S, 5'', 1p, 1'''' and with L-type ventral organisation and sinistral torsion. Pore plate oval with a  $\lambda$ -shaped cover plate (Pt). 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.

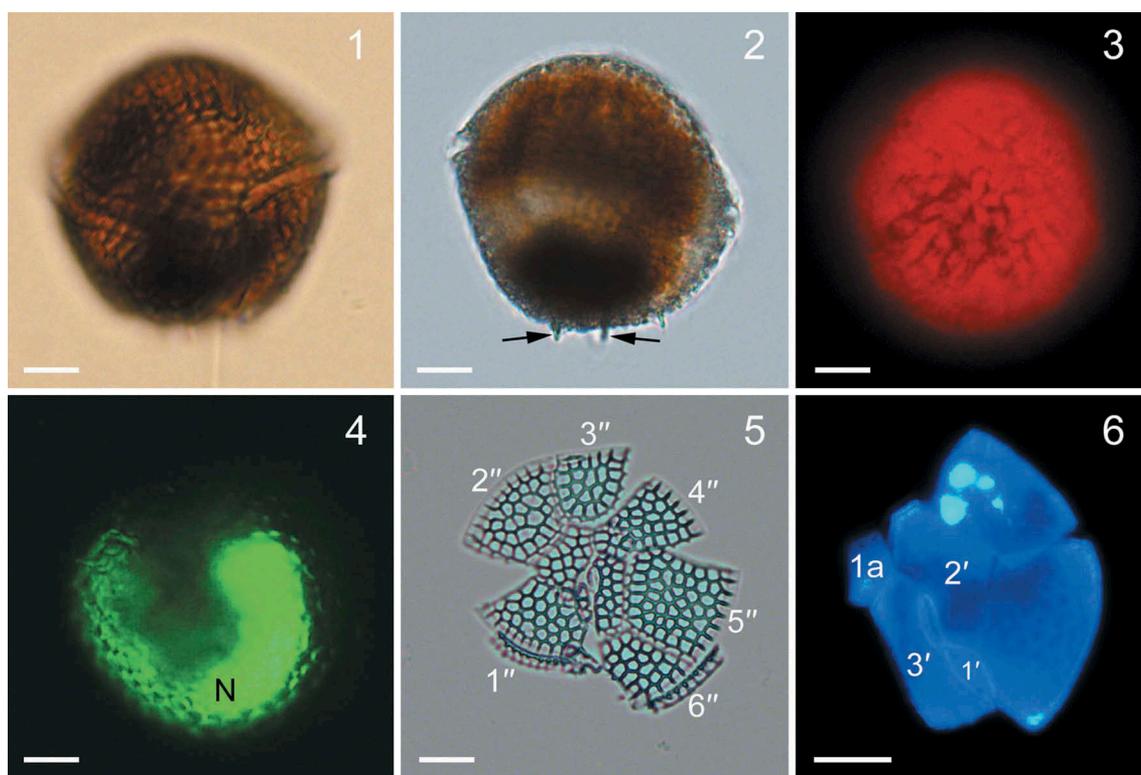
**DISTRIBUTION:** Terengganu and Sarawak of Malaysia.

### Morphology

Strains of *Ceratocorys malayensis* were morphologically indistinguishable from each other. Cells of strain A10-49-A55 were 40.2–58.0  $\mu$ m long ( $49.2 \pm 4.9$   $\mu$ m,  $n = 30$ ), 40.9–54.6  $\mu$ m wide ( $46.6 \pm 3.9$   $\mu$ m,  $n = 30$ ), and brownish cell contents due to the presence of chloroplasts (Fig. 1). Cells had a rounded epitheca and hypotheca with several short spines 3.0–4.1  $\mu$ m 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 ( $0.84 \pm 0.08$ ,  $n = 23$ ) (Figs 1, 2, 7 and 8). Numerous 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).

Thecae displayed plate formula Po, Pt, 3', 1a, 6'', 6C, 6S, 5'', 1p, 1'''' (Figs 1–18), and had a sexiform gonyaulocoid tabulation (cf. Fensome *et al.* 1993, fig. 64B; Figs 10, 13) with a L-type ventral organisation (cf. Fensome *et al.* 1993, 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  $\lambda$ -shaped cover plate (Pt) and perforated with 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 consisted 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



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

**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.** Epithelial plates stained with calcofluor showing three apical plates (1'–3') and one anterior intercalary plate (1a).

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 morphology identical to those in culture (Figs S1, S2). Schematic drawings of *C. malayensis* are provided (Figs 19–22). Cysts were not observed in cultures.

### *Pentaplaconium 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  $\mu$ m long and 22.7–27.7  $\mu$ m wide. Cells with conical epitheca and rounded hypotheca with similar size. Cells with numerous radiating chloroplasts and U-shaped posterior nucleus. Thecae with plate formula Po, Pt, 3', 1a, 6'', 6C, 6S, 5'', 1p, 1'''' and an L-type ventral

organisation and neutral torsion; pore plate oval. The first apical plate a parallelogram slightly contacting 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.

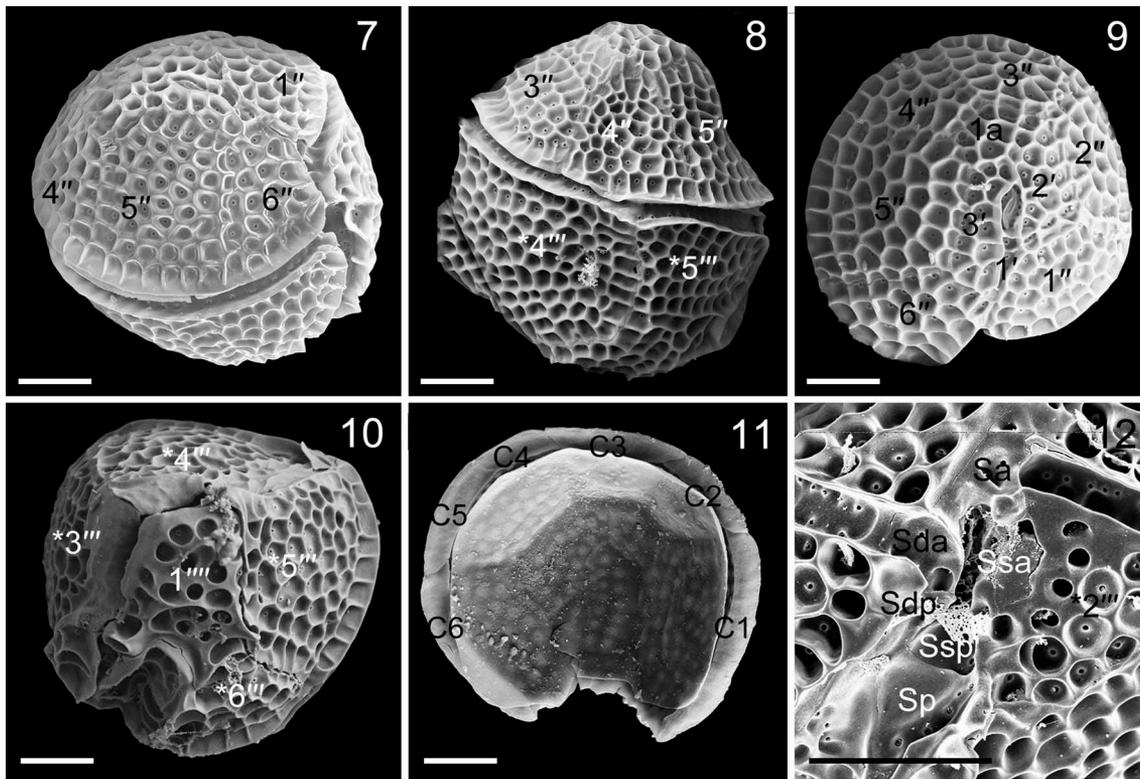
**ETYMOLOGY:** The epithet '*usupianum*' is in honour of Gires Usup, who did pioneering work on harmful algal blooms in Malaysia.

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

**DISTRIBUTION:** Sarawak, Malaysia.

### Morphology

Strains of *Pentaplaconium usupianum* were morphologically indistinguishable from each other. Cells of strain DBS02 were 26.6–31.3  $\mu$ m long ( $28.8 \pm 1.3 \mu$ m,  $n = 30$ ) and 22.7–27.7  $\mu$ m wide ( $24.7 \pm 1.4 \mu$ m,  $n = 30$ ). 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  $\mu$ m long at antapical end (Figs 23–26, 29). These



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

**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 one antapical plate (1''').

**Fig. 11.** Cingulum showing six cingular plates (C1–C6).

**Fig. 12.** 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).

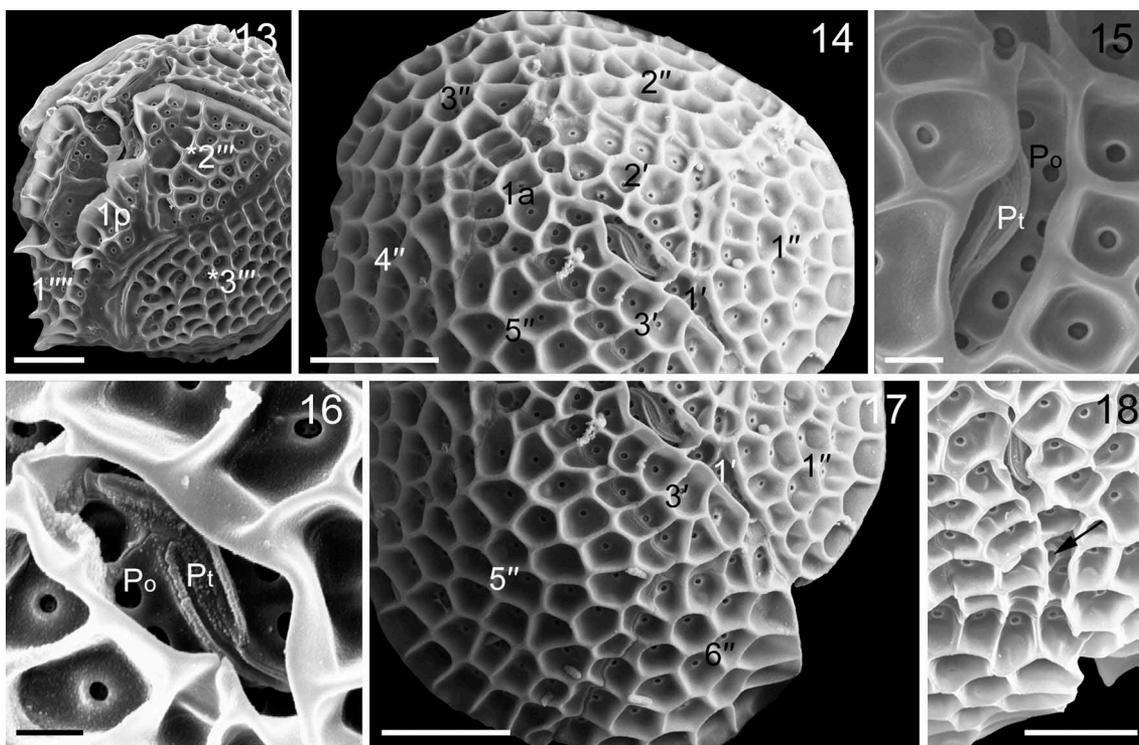
antapical spines were not always present (Fig. 31). The cingulum was situated in the equatorial part of the cell (Fig. 24). The cells showed numerous chloroplasts radiating from the central part of the cell (Fig. 27). The nucleus was U-shaped and posterior ('N' in Figs 26, 28).

Thecae displayed plate formula Po, Pt, 3', 1a, 6'', 6C, 6S, 5''', 1p, 1'''' (Figs 29–35), and had a sexiform gonyaulacoid tabulation (Fig. 31) with an L-type ventral organisation (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  $\lambda$ -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 slightly widened posteriorly consisting 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.



**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  $\mu$ m.

**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  $\mu$ m.

**Figs 15, 16.** Detail of oval pore plate (Po) with a  $\lambda$ -shaped cover plate (Pt) perforated by pores. Scale bar = 1  $\mu$ m.

**Fig. 17.** Apical view showing first apical plates (1'), and wide contact between first and sixth precingular plates (1'', 6''). Scale bar = 10  $\mu$ m.

**Fig. 18.** Apical view showing first apical plate with ventral pore at its right margin (arrow). Scale bar = 10  $\mu$ m.

### *Protoceratium cf. reticulatum*

Figs 40–50

#### Morphology

Cells of strain HWYD1 were 23.6–29.1  $\mu$ m long ( $26.2 \pm 1.1$   $\mu$ m,  $n = 30$ ) and 20.2–24.0  $\mu$ m wide ( $21.9 \pm 0.9$   $\mu$ m,  $n = 30$ ). Cells were 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 an L-type ventral organisation (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  $\lambda$ -shaped cover plate and perforated by around five pores (Fig. 49). The first apical plate (1') was rhombic, lacked a ventral pore, and contacted the anterior sulcal plate (Sa; Fig. 45), thus belonging to insert type. Sometimes, plate 1' did not contact plate Sa, thus showing an 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 (Figs 45, 46). The precingular series consisted of 4

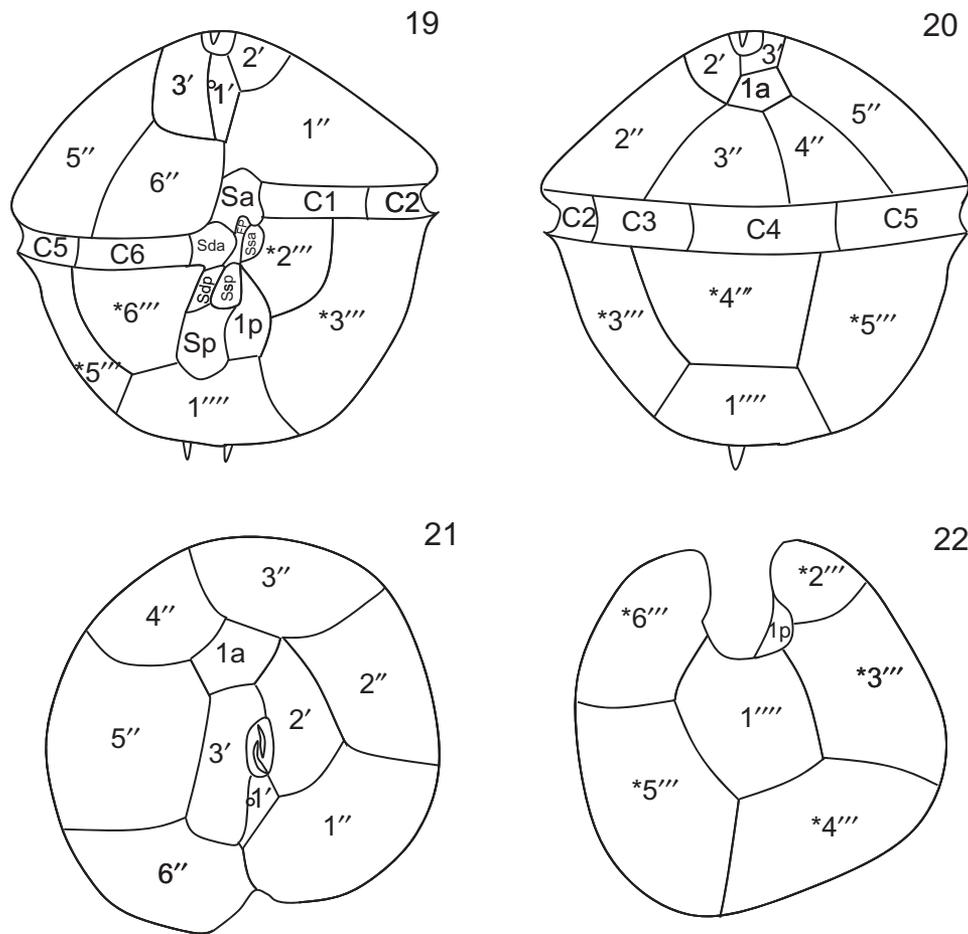
four-sided plates (2'' and 4'') and 4 five-sided plates (1'', 3'', 5'' and 6'') (Fig. 45). Occasionally, five precingular plates were observed (Fig. 46). The cingulum was descending, lined with narrow lists, and comprised six cingular plates (Figs 44, 47). The ends of the cingulum did not overhang and 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 plate (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, and \*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, *Pentaplagodinium usupianum* strains DSB01, GgSm01, GgSm07, GgSm10 and GgSm11 showed 0–1 base pair divergence (99.94% similarity)



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

- Fig. 19.** Ventral view.  
**Fig. 20.** Dorsal view.  
**Fig. 21.** Apical view.  
**Fig. 22.** Antapical view.

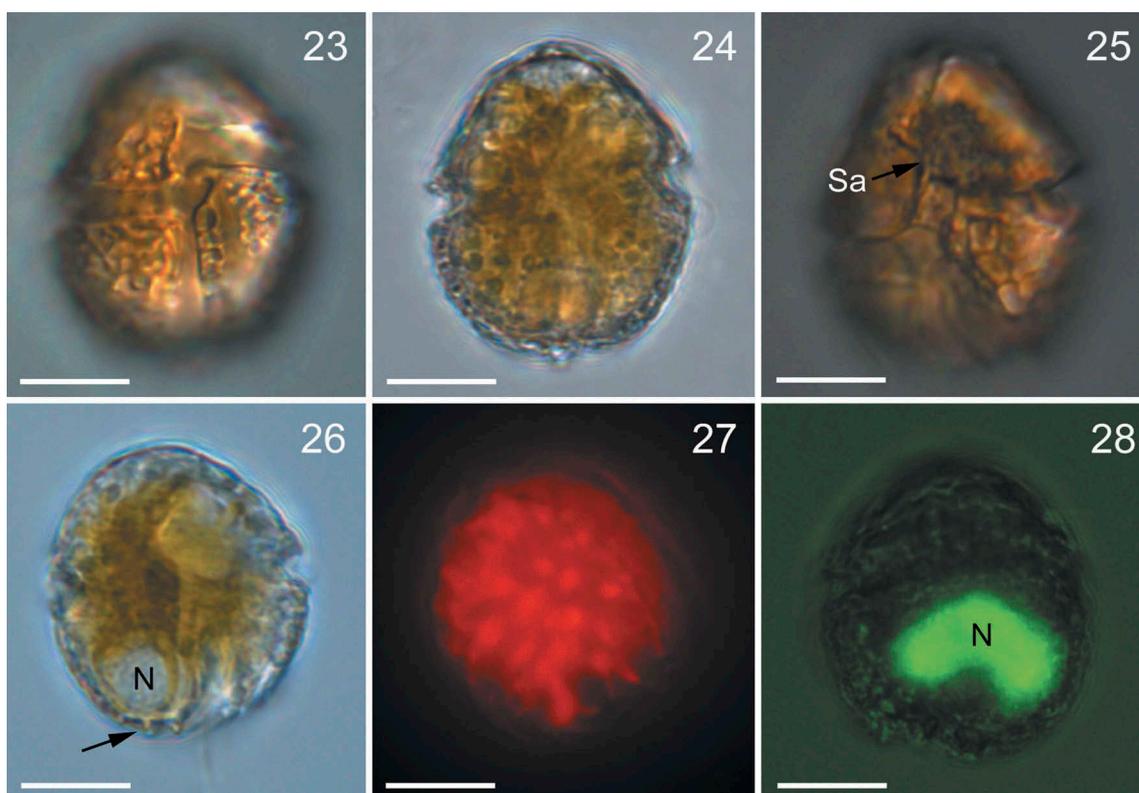
but differed from *Pentapladodinium saltonense* (MG646323) in six positions (99.65% similarity). *Protoceratium* cf. *reticulatum* HWYD1 differed from *Protoceratium reticulatum* (AY421790) at 12 positions (99.30% similarity). *Ceratocorys malayensis* strain A10-49-A55 differed from *Ceratocorys horrida* (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 *Pentapladodinium 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 *Pentapladodinium 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, *Pentapladodinium usupianum* strains DSB01, DSB02 GgSm01, GgSm03, GgSm07, GgSm10, and GgSm11 differed from each other in 0–4 positions (99.28% similarity), and from

*Pentapladodinium 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 *Pentapladodinium*, *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 *Pentapladodinium* with an early divergence of *Ceratocorys*. Gonyaulaceae was monophyletic with strong ML support (100) but low BI posterior probability (< 0.7) comprising *Gonyaulax* and *Lingulodinium*. *Ceratocorys malayensis* grouped 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). *Pentapladodinium usupianum* and *P. saltonense* formed a clade with strong support



**Figs 23–28.** Light micrographs of live cells of *Pentaplecodinium usupianum* strain DBS02. Scale bar = 10  $\mu\text{m}$ .

**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).

(100 BS/0.88 BPP) which was a sister clade of *Protoceratium reticulatum* with strong support (100 BS/0.99 BPP).

ML and BI analyses 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. *Pentaplecodinium usupianum* was well-resolved (100 BS/0.96 BPP) and formed a sister clade to *Pentaplecodinium 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. *Pentaplecodinium 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*, *Pentaplecodinium usupianum* and *Protoceratium* cf.

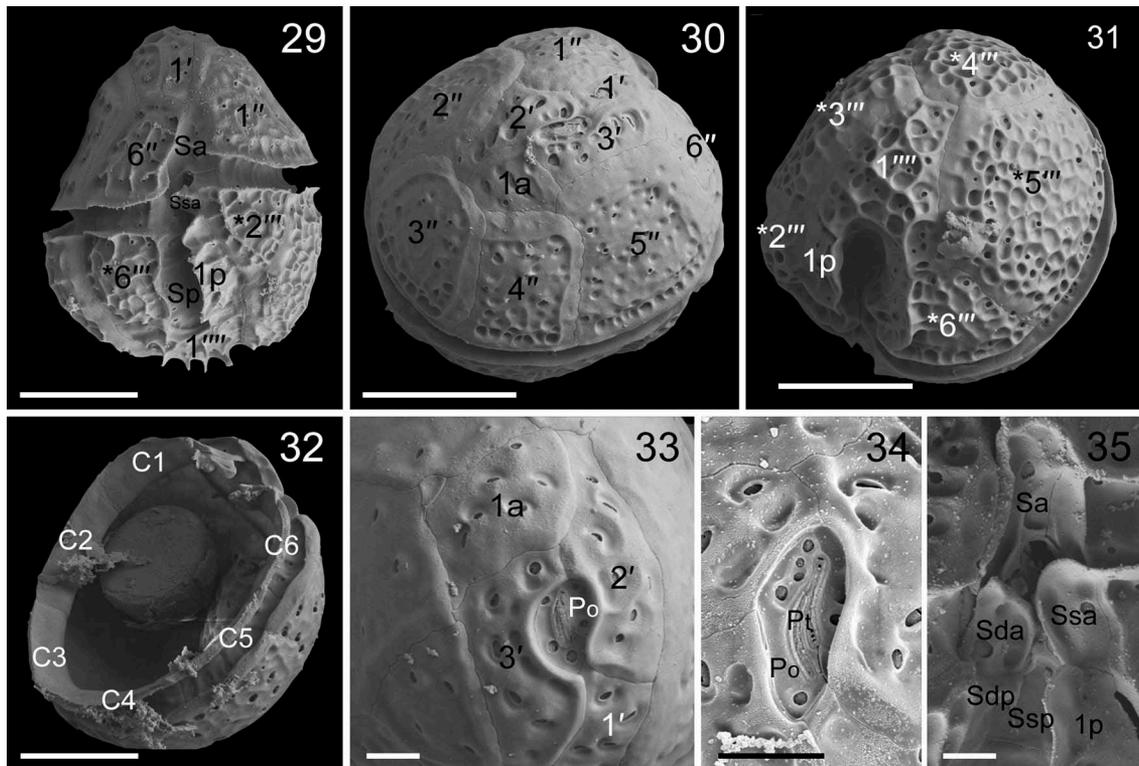
*reticulatum* produced detectable yessotoxins (Table 1). Limits of detection for YTX ranged between 0.09 and 2.8  $\text{fg cell}^{-1}$  depending on available biomass.

## 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 than epitheca (Carbonell-Moore 1996; Graham 1942). The strain 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 A10-49-A55 is morphologically similar to *Ceratocorys anacantha* in terms of a ventral pore of plate 1',



**Figs 29–35.** Scanning electron micrographs of *Pentaplagodinium 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  $\mu$ m.

**Fig. 30.** Apical view showing three apical plates (1' – 3'), one anterior intercalary (1a) plate and six precingular plates (1'' – 6''). Scale bar = 10  $\mu$ m.

**Fig. 31.** Antapical view showing five postcingular plates (\*2'' – \*6'''), posterior intercalary plate (1p) and antapical plate (1'''). Scale bar = 10  $\mu$ m.

**Fig. 32.** Cingulum with six plates (C1–C6). Scale bar = 10  $\mu$ m.

**Figs 33, 34.** Detail of oval to elliptical pore plate with  $\lambda$ -shaped cover plate and perforated by pores. Scale bar = 2  $\mu$ m.

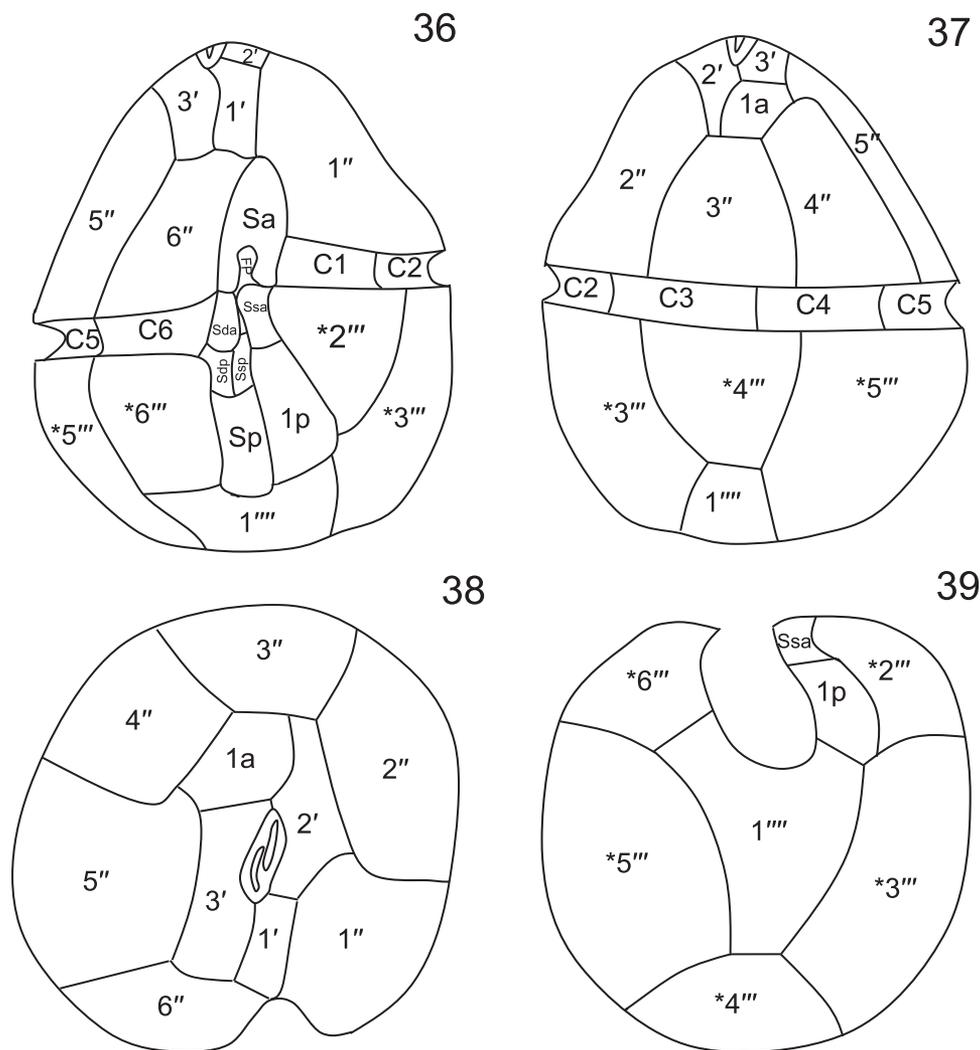
**Fig. 35.** Sulcus showing anterior sulcal plate (Sa), anterior left sulcal plate (Ssa), anterior right sulcal plate (Sda), posterior left sulcal plate (Ssp), and posterior right sulcal plate (Sdp). Scale bar = 2  $\mu$ m.

and 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 vary 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*, and *C. skogsbergii* is either not symmetrical, or not dorsal (Carbonell-Moore 1996; Graham 1942). Therefore, strain A10-49-A55 was described as a new species.

*Pentaplagodinium* 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 *Pentaplagodinium*, whereas in *Ceratocorys* contact is broad (Mertens et al. 2018a). Our strain DBS02 (*Pentaplagodinium 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 *Pentaplagodinium* 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 plate in *Pentaplagodinium* is homologous to the third and fourth precingular plates in other gonyaulacoids (Mertens et al. 2018b). Thus, our strains justify classification in *Pentaplagodinium*, pending its emendation. To date, only *Pentaplagodinium 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 plate 1' and is much smaller (26  $\mu$ m long versus 40  $\mu$ m 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*



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

**Fig. 36.** Ventral view.

**Fig. 37.** Dorsal view.

**Fig. 38.** Apical view.

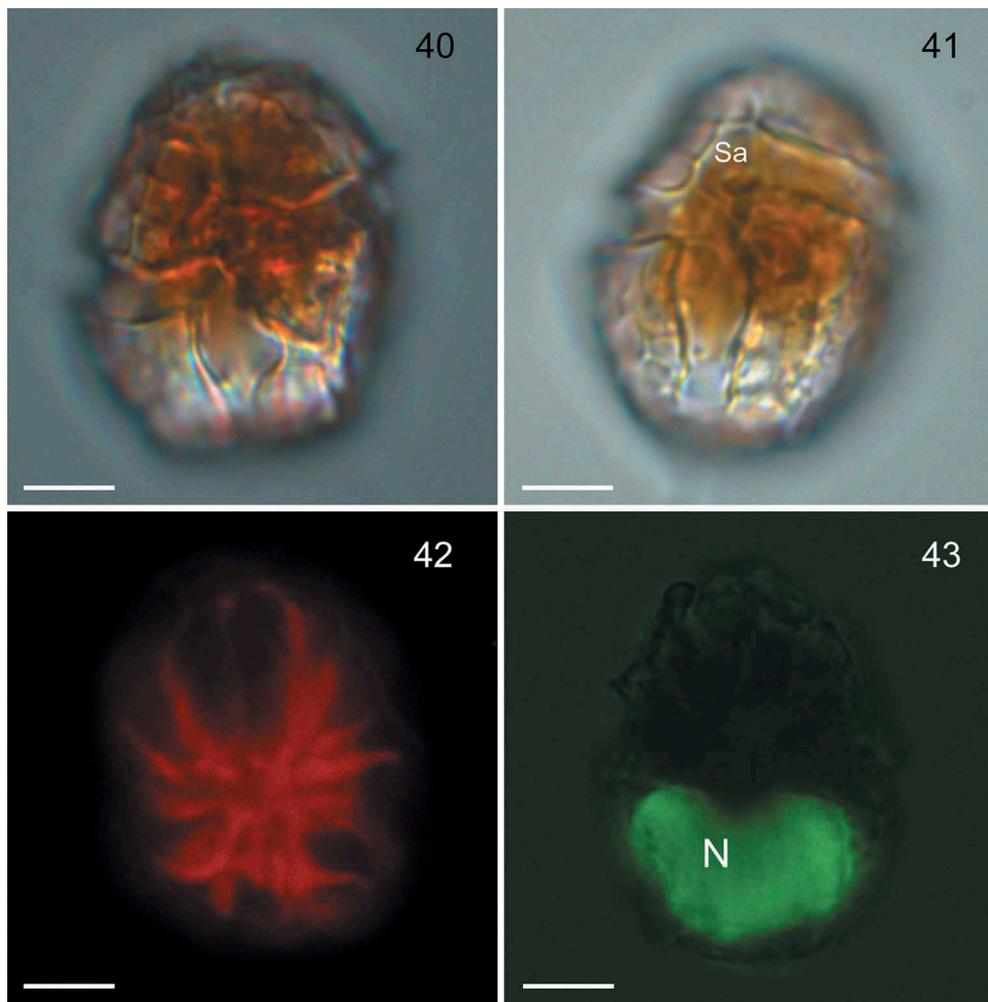
**Fig. 39.** Antapical view.

*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 sequence 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, 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 various 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 *Pentaplecodinium 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 descriptions do not even 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



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

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

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

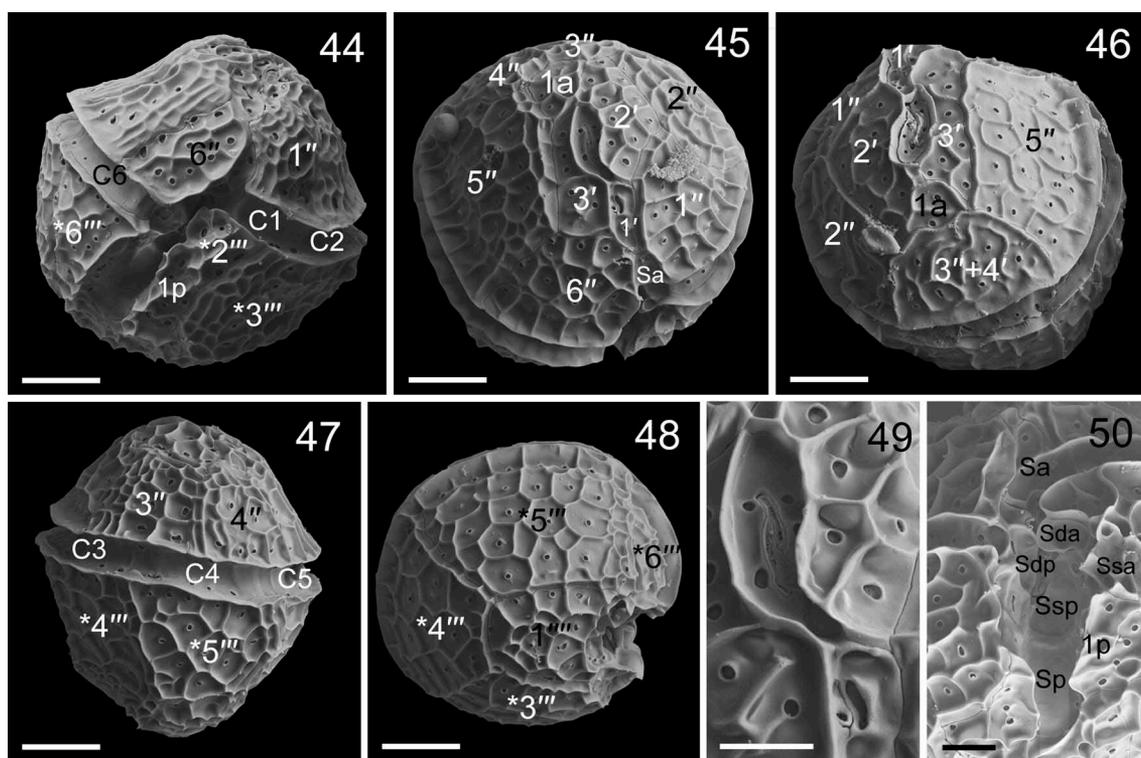
**Fig. 42.** Dorsal view, showing autofluorescent chloroplast network.

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

*Pentaplagodinium* respectively, with the latter two new to science. Genetic distance based on ITS rDNA sequences is greater than 0.3 between species of *Pentaplagodinium*, 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 represents a different species. However, strain HWYD1 shares similar morphology with *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 either 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 from 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 Gonyaulaceae, 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 pre-cingular plates (six versus five; Fensome *et al.* 1993), but we show that *Ceratocorys* incorporates species with both five and six pre-cingular plates. The number of anterior intercalary plates appears promising to separate Protoceratiaceae from Gonyaulacaceae.



**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''', \*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 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.** 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). Scale bar = 2 μm.

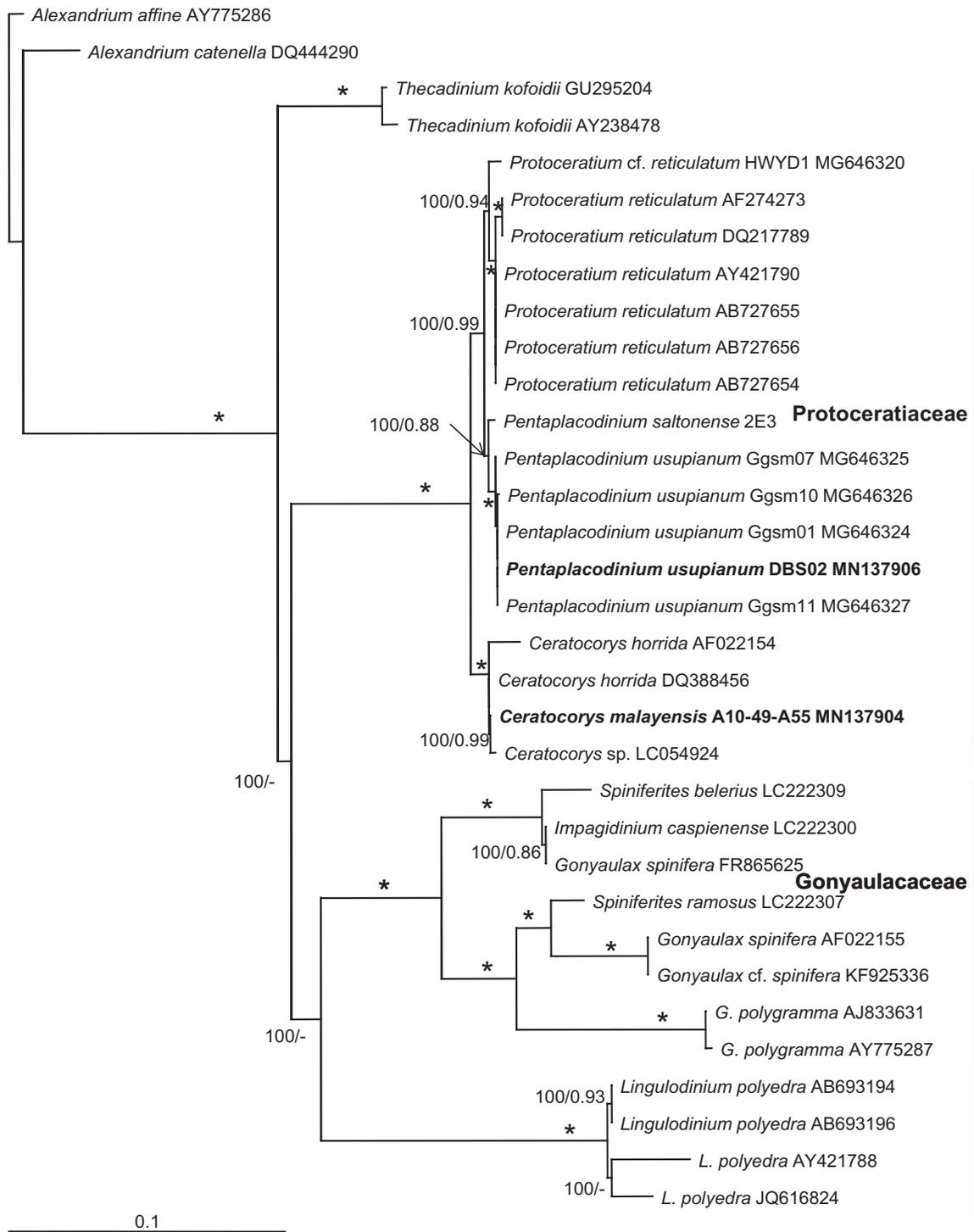
**Table 2.** Pairwise genetic distances based on ITS rDNA sequences for *Protoceratium*, *Pentapladodinium* and *Ceratocorys* species.

Species (GenBank numbers)	AB727654	MG646295	DBS02	EU532485	A10-49-A55	EU927577
<i>Protoceratium reticulatum</i> (AB727654)	-					
<i>Protoceratium cf. reticulatum</i> (MG646295)	0.18	-				
<i>Pentapladodinium usupianum</i> (MN137898)	0.31	0.30	-			
<i>Pentapladodinium saltonense</i> (EU532485)	0.35	0.35	0.19	-		
<i>Ceratocorys malayensis</i> (MN137896)	0.37	0.34	0.36	0.35	-	
<i>Ceratocorys horrida</i> (EU927577)	0.37	0.35	0.36	0.37	0.06	-

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

Ventral organisation and dextral torsion were also used to define the family Protoceratiaceae, e.g. an L-type sulcus and

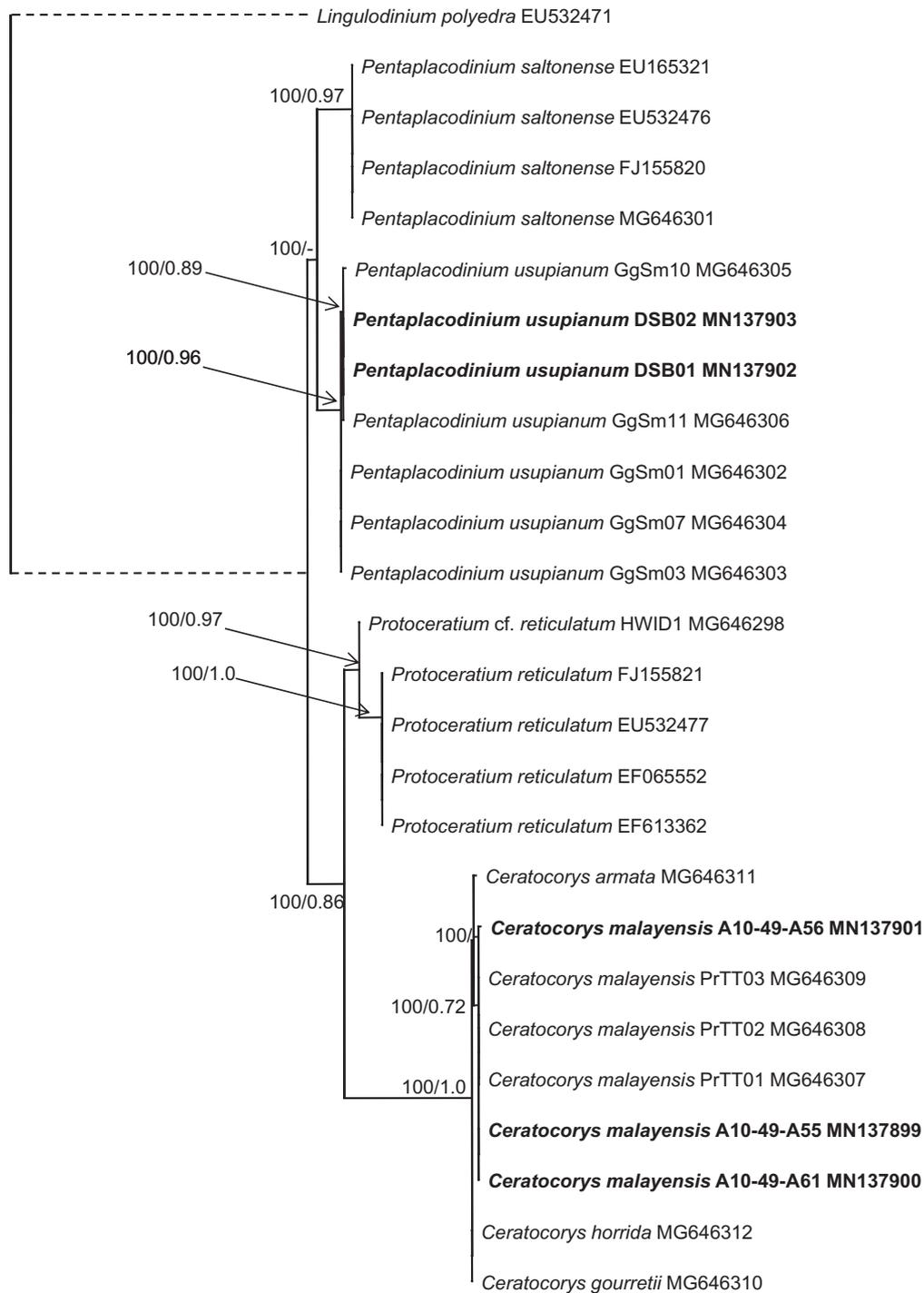
strong dextral torsion. However, our new species of *Ceratocorys* has sinistral torsion. Torsion can even be variable within one genus or species. *Protoceratium* strain HWYD1 had sexiform gonyaulacoid tabulation with an L-type ventral organisation and dextral torsion. Pronounced dextral torsion was 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).



**Fig. 51.** Phylogeny of *Ceratocorys*, *Pentaplagodinium* 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).

The close morphological similarity between *Pentaplagodinium* 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.



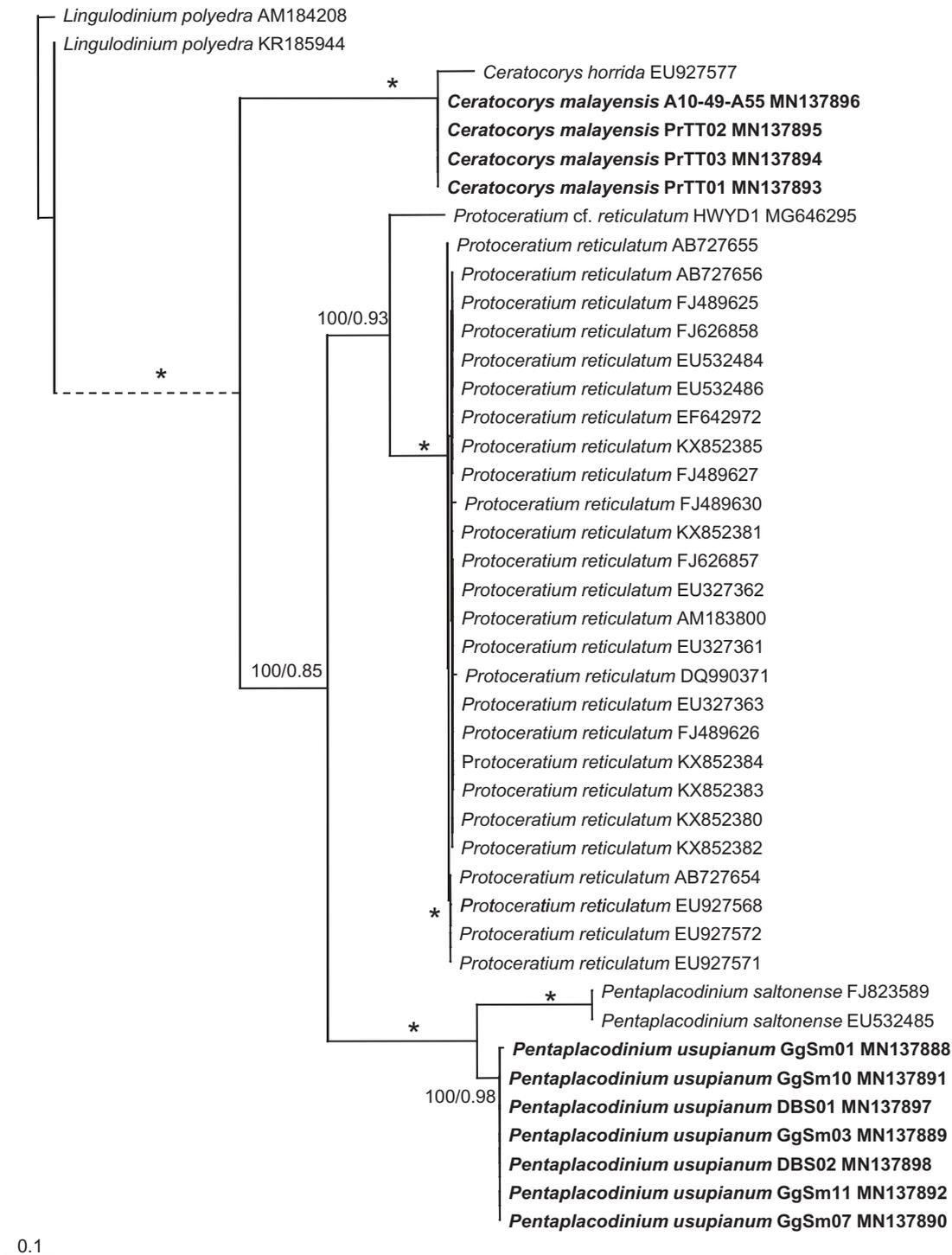
**Fig. 52.** Phylogeny of *Ceratocorys*, *Pentaplacodinium* and *Protoceratium* inferred from partial LSU rDNA sequences using maximum likelihood (ML). New sequences indicated in bold. Branch lengths drawn to scale, with scale bar indicating number of nucleotide substitutions per site. Numbers on branches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

#### 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 sulcus more or less midventral and straight, oblique from upper right to lower left, or sigmoidal. Antapical outline more or less symmetrical and strong.



**Fig. 53.** Phylogeny of *Ceratocorys*, *Pentaplagodinium* 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 line indicates half length.

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.

#### ***Pentaplagodinium* 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  $\lambda$ -shaped cover plate; plate 1' of insert type. *Operculodinium*-type cysts described.

The classification of *Pentaplagodinium* and *Protoceratium* within Protoceratiaceae is supported by the morphological similarity to *Ceratocorys* in possessing only one anterior intercalary plate and possibly a  $\lambda$ -shaped pore too. The latter feature has not been confirmed in other *Ceratocorys* species. The configuration of the anterior sulcal plate appears to be conservative in *Ceratocorys* but not in *Pentaplagodinium* 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 further insight into the relationship between Protoceratiaceae and Gonyaulacaceae.

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