Pentaplacodinium saltonense gen. et sp. nov. (Dinophyceae), and its relationship to the cyst-defined genus *Operculodinium* and the yessotoxin-producing *Protoceratium reticulatum*

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Abstract

Strains of a dinoflagellate from the Salton Sea, previously identified as *Protoceratium reticulatum* and yessotoxin producing, have been reexamined morphologically and genetically and *Pentaylacodinium saltonense* n. gen. et sp. wasis erected to accommodate this species. *Pentaylacodinium saltonense* differs from *Protoceratium reticulatum* (Claparede et *Lachmann 1859) Butschli 1885* in the number of precingular plates (-5-five vs. six-6), cingular displacement (2two widths vs. lone), and distinct cyst morphology. Incubation experiments (excystment and encystment) show that the resting cyst of *Pentaylacodinium saltonense* is morphologically most similar to the cyst-defined species *Oyerculodinium israelianum* (Rossignol 1962) Wall 1967 and *O. ysilatum* Wall 1967.

Collections of comparative material from around the globe (including *Protoceratium reticulatum* and the genus *Ceratocorys*) and single cell PCR wasere used to clarify molecular

phytogenies. Variable regions in the LSU (3three new sequences), SSU (12 new sequences) and intergenic ITS 1-2 (14 new sequences) were obtaineded. These show that *Pentaylacodinium saltonense* and *Protoceratium reticulatum* form two distinct clades. *Pentaylacodinium saltonense* formeds a monophyletic clade with several unidentified strains from Malaysia. LSU and SSU rDNA sequences of three species of Ceratocorys (*C. armata, C. gourreti, C. horrida*) from the Mediterranean and several other unidentified strains from Malaysia form a well-supported sister clade. The unique phylogenetic position of an unidentified strain from Hawaii is also documented thatand requires further examination. In addition, based on the V9 SSU topology (bootstrap values >80%), specimens from Elands Bay (South Africa), originally described as *Gonyaulax grindleyi* by Reinecke (1967), cluster with *Protoceratium eticulatum.* The known range of *Pentaplacodinium saltonense* is tropicalto subtropical, *and its cyst is recorded as a fossil in lateupper Cenozoic* sediments.

Proloceralium reliculatum and *Penlaplacodinium sallonense* seem to inhabit different niches: motile stages of these dinoflagellates have not been found in the same plankton sample.

Keywords

Penlaplacodinium, *Proloceralium,* precingular plates, Salton Sea, *Ceralocorys, Operculodinium,* Cribroperidinioideae

1. Introduction

The dinoflagellate genus *Protoceratium* was erected by Bergh (1881, p. 242) with *Protoceratium aceros* as the type species (fig. 36), which was recovered from Strib, Denmark. Butschli (1885, p. 1007, plate 52, fig. 2) considered *Peridinium reticulatum* as described earlier by Claparede and Lachmann (1858) from Bergen Fjord, Norway, as a senior synonym, and he proposed the combination *Protoceratium reticulatum.* He also considered *Clathrocysta reticulata* as described by Stein (1883) a junior synonym. The plate formula for *P. reticulatum*, 4', 0a, 6", 6"', 1p, 1"", was first provided by Wdoszynska (1929) through the study of Baltic Sea specimens. Reinecke (1967) erected the name *Gonyaulax grindleyi* for specimens from Elands Bay in Cape Town, South Africa, with the tabulation 3', 1a, 6", 6"',
1p, 1"". Based on a detailed study of the theca of *Protoceratium reticulatum* from the North Sea, Stosch (1969) considered *G. grindleyi* to be a junior synonym of *P. reticulatum*, although he considered it assignable to the genus *Gonyaulax.* Dodge (1989) agreed with the tabulation of Reinecke (1979), but retained the genus *Protoceratium* because he considered it different from his emendation of the genus *Gonyaulax,* by having only one intercalary plate. Hansen et al. (1997) restudied specimens close to the type locality of *P. aceros*, and based on the plate analysis concluded that *P. reticulatum, P. aceros* and *G. grindleyi* were conspecific, and agreed with the tabulation of Wdoszynska (1929). Paez-Reyes and Head (2013) reviewed the morphological variability reported for *P. reticulatum* and concurred with Dodge (1989) in maintaining *Protoceratium* as a distinct genus from *Gonyaulax.*

Seven other *Protoceratium* species have been described since the early 1900s, and the latest review of these taxa was having been performed by Schiller (1937 p. 322-326). Kofoid (1907) described *P. areolatum* from the tropical Pacific and emended the genus for the first time. Meunier (1910) described a very similar species from the Kara Sea that he named

Protoceratium splendens, which is possibly a junior synonym, as suggested by Gomez (2012). Later, Kofoid in Kofoid and Michener (1911) emended *Protoceratium* once more to include several new species from the eastern tropical Pacific that were described without illustration *(P. cancellorum, P. globosum, P. pellucidum, P. pepo, P. promissum),* and he suggested a tabulation formula for the genus: 2', 0a, 6" (?c), 6"', 0p, 3"". Schiller (1937) transferred *Clathrocysta aculeata* as described by Stein (1883) to *Protoceratium aculeatum,* presumably based on the fact that Butschli (1885) had considered the genus *Clathrocysta* described by Stein 1883 as a junior synonym of *Protoceratium.* Schiller (1937) transferred *Peridinium spinulosum* as described by Murray and Whitting (1899) to the genus *Protoceratium.* Later, Balech (1988) rediscovered this species in the South-West Atlantic and suggested yet another variation on the tabulation for *Protoceratium,* 3', 0a, 6", 6"', 2"", based on his observations of *Protoceratium spinulosum.*

Protoceratium reticulatum (Claparede et Lachmann) Butschli 1885 is a very common dinoflagellate found in cold and warm waters, as well as in oceanic and neritic environments (e.g., as *Operculodinium centrocarpum* in Zonneveld et al., 2013). Its resting cyst distribution today reveals a strong link with the North Atlantic Current, an association traceable through the upper Cenozoic fossil record (Hennissen et al., 2017 and references therein).

Protoceratium reticulatum is considered potentially toxic because of its production of yessotoxins (e.g. Paz et al., 2008; Sala-Perez et al., 2016). It has been successfully isolated and cultured from many parts of the world, and grown into cultures. Cysts of *P. reticulatum* were first observed in cultures established from motile cells from the inner Oslofjord (Norway) by Braarud (1945). This cyst was related by Wall and Dale (1966, 1967, 1968) to the cyst-defined species described from the Miocene of Australia, *Operculodinium centrocarpum* (Deflandre et Cookson 1955) Wall 1967. That assignation was challenged by Head and Wrenn (1992) and

Head (1996a) on the grounds that *Operculodinium centrocarpum* was larger and more robust than the cysts recorded by Wall and Dale (1966) from modern sediments. A restudy of the holotype of *Operculodinium centrocarpum* confirmed this, and the name "cyst of *Protoceratium reticulatum*" was recommended (Matsuoka et al., 1997). Wall and Dale (1968) proposed that *P. reticulatum* was also related to the cyst-defined *Operculodinium psilatum* Wall 1967 and furthermore possibly to *Operculodinium israelianum* (Rossignol 1962) Wall 1967. The cyst-defined *Pyxidinopsispsilata* (Wall et Dale in Wall et al., 1973) Head 1994 was subsequently also linked to *Protoceratium reticulatum* (Dale, 1996, as *Tectatodinium psilatum)* although this connection was later questioned (Mertens et al., 2011). Because of uncertainty regarding the links between the cysts produced by *P. reticulatum* and cyst- defined species from the fossil records, Head (1996a, 1996b) and subsequent authors used the term *"Operculodinium centrocarpum* sensu Wall and Dale, 1966" was to describe the cysts that had first been observed by Braarud (1945) and Wall and Dale (1966). With the removal of *Pyxidinopsis psilata* as a potential cyst of *Protoceratium reticulatum,* Paez-Reyes and Head (2013) argued on the basis of non-overlapping geographic distribution that the "cyst of *Protoceratium reticulatum*'" was now unambiguous and should replace the term *"Operculodinium centrocarpum* sensu Wall and Dale, 1966". That approach is followed here. Recent studies of variation in the process length of cysts of *Protoceratium reticulatum* have been related to variations in sea surface salinity and other parameters (e.g., Mertens et al. 2011; Jansson et al., 2014), and the cyst wall appears to be composed of cellulose glucan (Bogus et al. 2014). Resting cyst production through sexualreproduction has recently been demonstrated by Salgado et al. (2017).

Protoceratium reticulatum was assigned questionably to the subfamily Cribroperidinioideae by Fensome et al. (1993) based on the presence of six precingular plates, L-type ventral organization and possible dextral torsion, which at the time had not been

documented. This assignation was confirmed by Paez-Reyes and Head (2013). The description of the similar cyst-defined *Operculodinium bahamense,* with neutral torsion and modified L-type ventral organization, allowing placement in the subfamily Leptodinioideae, either challenges sueh the present subfamilial classification of the Gonyaulacaceae, or implies that *Operculodinium* is polyphyletic, with both outcomes being possible (Paez-Reyes and Head, 2013). Furthermore, molecular phylogenetics show that *Protoceratium reticulatum* is closely related to the family Ceratocoryaceae but not to the other extant cribroperidinean, *Lingulodinium polyedra* (Saldarriaga et al., 2004). It should also be noted that morphological variation and sequencing of cysts has suggested pseudocryptic speciation in *P. reticulatum* (Mertens et al., 2012a). Howard et al. (2009) investigated the phylogenetic relationships of yessotoxin-producing dinoflagellates, including several strains of *P. reticulatum* from different localities. Using Large Sub Unit (LSU) and Internal Transcribed Spacer (ITS) ribosomal DNA (rDNA) sequencing, they showed that the *P. reticulatum* strains formed a monophyletic clade in both phylogenies. One particular strain (CCMP404) isolated from the Salton Sea (California) in 1966 showed significant genetic differences from the other strains in both phylogenies. Despite these genetic differences, Howard et al. (2009) considered all the strains to belong to the species *P. reticulatum*.

The Salton Sea is the largest saline lake in California with a surface area of 980 km (Reifel et al., 2002). It has a mean depth of 8 m and a maximum depth of 15 m (Ferrari and Weghorst, 1997). Although originally composed of relatively freshwater, it has become saline due to a lack of outflow and high evaporation rates. During 1997-1999, the salinity was between 41 and 45 g l^{-1} (Watts et al., 2001), while the temperature varied between about 12 and 40°C seasonally (Watts et al., 2001; Holdren and Montano, 2002). Oxygen at times was supersaturated due to phytoplankton photosynthesis, but was also often severely depleted,

occasionally even in surface waters (Watts et al., 2001; Holdren and Montano, 2002). Reifel et al. (2002) reported *P. reticulatum* from the Salton Sea without illustration or description.

In the present study, through reevaluation of the CCMP404 strain originated from the Salton Sea and observations of recently collected plankton samples from the Salton Sea, it iswe demonstrated that specimens living in the Salton Sea that had previously been identified as *P. reticulatum,* have a different tabulation to that of *P. reticulatum.* To resolve this issue and accommodate these organisms, *Pentaplacodinium saltonense* n. gen. et sp. is erected. From the Salton Sea plankton samples, the morphology of the thecate stage is described, showing significant differences with *P. reticulatum.* Similarly, through incubation of cysts from Salton Sea surface sediments, the corresponding cyst is described. Phylogenetic relationships are explored, including those with several unpublished sequences of *P. reticulatum*, *Ceratocorys armata* (Schutt 1895) Kofoid 1910, *Ceratocorys gourretii* Paulsen 1931, *Ceratocorys horrida* Stein 1883, and several unidentified strains. In addition, both the autecology and fossil record of *Pentaplacodinium saltonense* are examined.

2. Material and Methods

The cyst-theca relationship of *P. saltonense* was established through a germination experiment of a sample from the Salton Sea (CA, USA). To identify differences and similarities between *P. reticulatum* and *P. saltonense,* the morphology of thecate stages of strains present in culture collections and other cells used for sequencing, were compared (Table 1, Suppl. Table 1). In addition, phytogenies were constructed of using LSU, ITS and SSU rDNA based sequences of *P. saltonense* and *P. reticulatum* from several of the same cells or cultured strains, as well as three *Ceratocorys* species isolated from the Mediterranean, and several unidentified

strains from Hawaii and Malaysia (Table 1, Suppl. Table 1).

2.1. Morphological study-imaging of cells in-from plankton samples and strains present in culture collections

Plankton samples were obtained from the Salton Sea (California, U.S.A.; 33.50 °N, 115.91 °W) on 24 Oct. 2013 using a plankton net with a 20 pm mesh size. These samples were fixed with ethanol (50% final concentration) and stored cold. Several strains from previously sequenced strains from culture collections established from several other locations were also studied using transmitted light or scanning electron microscopy (Figure 1, Table 1).

For scanning electron microscopy (SEM) of thecate stages by M.C.C-TM., samples were prepared either by filtering a plankton sample or culture, or isolating a single cell under a Leica™ inverted light microscope (Germany). Samples were filtered by placing an ~300 pL aliquot on a Millipore™ 0.25 mm diameter-5-pm pore-polycarbonate filter at the bottom of a Millipore™ column. Approximately 7 mL of distilled water were added to remove the fixative (ethanol, lugol or formaldehyde) and seawater. A gentle manual vacuum with a 60 cc syringe was used to speed filtration. Cells were removed using a glass micropipette under a Leica inverted light microscope. Individual cells were washed six times with distilled water in double depression microscope slides. After the cells were clean, they were placed on the same kind of filter as for the filtered samples. All filters were air-dried, then affixed to 25 mm diameter aluminium stubs with adhesive tabs (7/16" diameter). The mounted filters were then coated with a mixture of gold-palladium in a Cressington Sputter Coater (U.S.A.) for 60 s.

Observations were performed with a FEI Quanta 3D Dual Beam SEM (Clackamas, Oregon, U.S.A.), at 5 kV. Tilts up to 52° were applied. Digital images were saved in Tiff format (2048

x 1768 pixels). Adobe-Photoshop™ software was used to remove the background while maintaining the integrity of the original image.

For scanning electron microscopy of culture CCMP 3243 by K.N.M., the culture was filtered and washed with distilled water and dehydrated in a graded ethanol series (30 to 100% in six steps). The filters were encased in metallic baskets, critical-point dried with CO_2 (CPD Bal-Tec 030), glued onto stubs, sputter coated with platinum/palladium for 90 s (JEOL JFC-2300 HR) and examined in a JEOL 6330F scanning electron microscope (JEOL, Tokyo, Japan) at the University of Copenhagen.

Measurements of hecae of the newly described species were conducted by M.C.C-TM. under SEM. For each motile cell, the length was measured along the longitudinal axis, the width was measured along the middle of the cingulum, from one lateral margin to the other. All motile cell measurements in the species descriptions cite the minimum, average (in parentheses) and maximum values (in pm), in that order. The standard deviation (SD) is also provided where appropriate.

Labelling of tabulation follows a modified Kofoid system that recognizes homologs (e.g., Fensome et al. 1993b). The sulcal plate labelling accord with Balech (1980).

2.2. Germination experiment of cysts of P. saltonense

Sediment samples were collected from the Salton Sea at the same time plankton sample collection on 24 October. 2013, using a Petite Ponar Grab at shallow water depths (<0.5 m). All samples were stored in plastic bags in a refrigerator at 4°C. *In-situ* sea surface salinities and sea surface temperatures were measured during sampling (Table 1).

About 0.5-1.0 cm of wet sediment was immersed in filtered seawater and, after one

minute of ultrasonication using an ultrasonic bath, the sediment was rinsed through a 20 pm nylon mesh sieve using filtered seawater. From this residue, the cyst fraction was separated using the heavy-liquid sodium polytungstate (SPT) at a density of 1.3 g cm^{-1} (Bolch, 1997). Single cysts were then transferred to Orange Scientific 0.5 mL microwells subjected to an irradiance of 100 pmol photons m^{-} s^{-} and 24-hour light, and filled with f/2 medium at room temperature and a salinity of 35 psu. Cysts were regularly checked for germination, and observations were performed under a Leitz DM IL inverted light microscope. Encysted and excysted cysts, as well as motile cells, were photographed and measured using a Leica DM5000B light microscope with 100x oil immersion objectives.

2.3. Morphological study of cysts extracted from surface sediments with using light microscopy and SEM

Surface sediment samples were collected from several Salton Sea sites to study f cysts of *Pentaplacodinium saltonense* (Table 1). Palynological techniques were used for processing (e.g., Pospelova et al., 2010; Mertens et al., 2012b). Material was rinsed twice with distilled water to remove salts. The samples were oven-dried at 40°C and then treated with 10% hydrochloric acid (HCl) at room temperature to remove calcium carbonate particles. To dissolve silicate particles, samples were treated with 48-50% hydrofluoric acid (HF) at room temperature for two days, and then treated for 10 min with room-temperature HCl (10%) to remove fluorosilicates. The residue was rinsed twice with distilled water, ultrasonicated for ~30 sec and finally collected on a 15 pm mesh. Aliquots of residue were mounted on microscope slides using glycerine jelly.

All measurements and light photomicrographs were obtained by K.N.M- and V.P.,

respectively using an Olympus BX51 with a Nikon digital sight DS-1L 1 module, and a Nikon Eclipse 80i transmitting light microscope with a DS-L2 module, all with 100x oil immersion objectives.

For each cyst, the lengths of the three longest visible processes with the corresponding widths at their base were measured within the focal plane. Process length was measured from the middle of the process base to the process tip. The average distance between processes was determined by measuring the distance between a process on the upper surface of the cyst near the centre and the five processes nearest to it, as measured between the middle of the process bases as seen from the surface of the cyst. The central body wall thickness was measured at two to three positions around the cross section of each cyst. The central body maximum and minimum diameters were also measured unless specimens were overly compressed or broken. Fragments representing less than half of a cyst, and cysts with mostly broken processes, were not measured. All cyst measurements in the species descriptions cite the minimum, average (in parentheses) and maximum values (in pm), in that order. The standard deviation (SD) is also provided where appropriate.

For SEM observation of cysts at Geotop (the Universite du Quebec a Montreal, Canada), single specimens were picked under an inverted microscope with a micropipette, sputter coated with platinum/palladium for 60 s and observed using a scanning electron microscope (Hitachi S-3400N SEM).

Figure 14

2.4. Single-cell polymerase chain reaction (PCR) amplification and sequencing of Salton Sea culture

Isolated cells were washed three times in serial drops of 0.22 pm filtered and sterilized seawater

by micropipette. Each cell was transferred to a 200 pm PCR tube containing 10 pL of Quick Extract FFPE DNA Extraction Solution (Epicentre, Madison, WI, USA) and incubated for 1h at 56°C, then for 2 min at 90°C. The resulting extract was used as a DNA template for the initial PCR amplification. Sequences of SSU and partial LSU rDNA were determined from single cells of *P. saltonense.* The PCR was performed with EconoTaq 2X Master Mix (Lucigen, Middleton, WI, USA) following the manufacture's protocols. The external primers (SR1 and LSU R2) were used for the initial PCR. The first PCR product was used as a DNA template for the second PCR. The following combinations of primer pairs were used separately for the second PCR: SR1 and SR12, 25F1 and LSU R2. Using the second PCR products as the template DNA, the third PCR was performed by the following combinations of primer pairs: SR1b and SR3, SR1b and SR5TAK, SR4 and SR7TAK, SR6 and SR9p, SR8p and SR12, 25F1 and 25R1, D3A and LSU R2. The details of the primers are described in Takano and Horiguchi (2004) and Yamaguchi et al. (2016). The PCR protocols and sequencing are described in Yamaguchi et al. (2016).

2.5. Sequencing of single cells of Protoceratium reticulatum *from Elands Bay (South Africa), originally described as* Gonyaulax grindleyi *by Reinecke (1967)*

Isolated cells were washed three times in serial drops of 0.22 pm filtered and sterilized distilled water and then transferred to a 0.2 mL PCR tube. Cells were subjected to three rounds of heating to 95°C for 5 minutes and cooling on ice for 5 minutes to induce cellular lysis. 5 pL of the cell lysate was then used as a template for PCR using primers to amplify a 168 bp region of the SSU, encompassing the V9 region, V9 For (5'-GTACACACCGCCCGTC-3') V9 Rev (5'-TGATCCTTCTGCAGGTTCACCTAC-3')

(Lane, 1991; Medlin et al., 1988). PCR reactions were carried out in 25 gL volumes containing 5 gL DNA template, 10 pmol each primer, 1 x buffer, 1 mM $MgCl_2$, 0.0025 mM dNTPs, 0.5 Unit Gotaq polymerase (Promega). PCR reactions proceeded with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 20 seconds and extension at 72°C for 20 seconds and a final extension step of 72°C for 5 minutes. PCR products were sequenced directly in both directions using the respective primers (Source Bioscience).

2.6. Sequencing of unidentified cultured strains

For strains from Hawaii and Malaysia, single cells were isolated from plankton samples (Suppl. Table 1) and washed three times with sterilized bi-distillate water and were used as the template to amplify about 1,430 bp of the LSU rRNA gene (D1-D6 domains), using the primers D1R (forward, 5' -ACCCGCTGAATTTAAGCATA-3') (Scholin et al., 1994), 28- 1483R (reverse, 5' -GCTACTACCACCAAGATCTGC-3') (Daugbjerg et al., 2000), 1740 bp of the SSU rRNA gene, using the primers SR1 (forward, *5'* - TACCTGGTTGATCCTGCCAG-3') and SR12b (reverse, 5' -CGGAAACCTTGTTACGACTTCTCC-3') (Takano & Horiguchi, 2006), and 600 bp of the total ITS1-5.8S-ITS2, using the primers ITSA (forward, 5' -CCTCGTAAC AAGGHTCCGTAGGT-3'), ITSB (reverse, 5' -CAGATGCTTAARTTCAGCRGG) (Adachi et al., 1996). A 50 gL PCR cocktail containing 0.2 gM forward and reverse primer, PCR buffer, 50 gM dNTP, 1U of Taq DNA polymerase (Takara, Dalian, China) was subjected to 35 cycles using a Mastercycler PCR (Eppendorf, Hamburg, Germany). The PCR reaction procedure was 4 min at 94 °C, followed by 25 cycles of 1 min at 94 °C, 2 min at 45 °C, 3 min at 72 °C, and

final extension of 7 min at 72 °C. PCR products were sequenced directly in both directions using the ABI Big-Dye dye-terminator technique (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations.

DNA extracts from strains collected in Spain (processed at IRTA) were prepared according to the protocol described in Andree et al. (2011). The extracted DNA was used in the amplification of ITS-1, 5.8S, ITS-2 sequences utilizing primers described in Andree et al. (2011), and apartial LSU sequence was amplified utilizing the primers described in Hansen et al. (2000). The amplification reactions were carried out in 25pL volume containing: 10 mM Tris-HC1 pH 8.3 (at 25 °C), 50 mM KCl, 2 mM $MgC1_2$, 0.001 % w/v gelatin, 400 pM dNTP's, 1 pM of each primer, and 1 U Taq polymerase. Amplifications were performed using the following parameters: 94 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. The PCR products were purified using Qiagen spin columns (Qiagen PCR Purification Kit) and sent for bi-directional sequencing by a commercial company (Sistemas Genomicos, Valencia, Spain) utilizing the same primers as those used in the original amplification. The resulting nucleic acid sequence data was manually proofed using BioEdit (Hall et al., 1999) to confirm the consensus sequence.

The strain 091223-38_M16 from Helgoland (North Sea) was sequenced by M.H.. The Epicentre MasterPure complete DNA & RNA Purification Kit was used for the DNA extraction. puReTaq ready-to-go PCR beats were used; annealing temperature was 50°C; 33 cycles; primers: ITS1 (forward) 5' GGTGAACCTGAGGAAGGAT 3'; ITS4 (reverse) 5' TCCTCCGCTTATTGATATGC 3'. The PCR product of the correct size was gel isolated (QIAquick Gel Extraction Kit). Sequencing was done by Macrogen with the ITS1 primer.

Strains and single cells from Japan, sequenced by Yoshihito Takano and Kazuhiko Koike, were sequenced using methods mentioned in Mertens et al. (2012a).

DNA was extracted from cultures (strain references K1474, 1476, 1477, 1478, 1479 and 0976) acquired from the NCMA (National Centre for Marine Algae) using the DNeasy DNA extraction kit (Qiagen) according to manufacturers' instructions. The 760 bp region of the LSU rRNA gene was amplified using 2 pL DNA in PCR reactions spanning the D1-D2 variable region D1R (forward, 5'-ACCCGCTGAATTTAAGCATA-3'), D2C (reverse, 5'-GCTTGGTCCGTGTTTCAAGA-3') (Scholin et al., 1994) a 168 bp region of the SSU rRNA gene (V9) V9 For (5'-GTACACACCGCCCGTC-3') V9 Rev (5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Lane, 1991; Medlin et al., 1988) and a 710 bp intergenic region ITS1, 5.8S, ITS2, EITS2 For (5' -GTAGGTGAACCTGCVGAAGA-3') EITS2 Rev (5'-TGGGGATCCTGTTTAGTTTC-3') (Guillou et al. 2002). PCR for V9 is detailed in section 2.5. For LSU and ITS, PCR reactions were carried out in 50 pL volumes containing 2 pL DNA, 20 pmol each primer, 1 x buffer, 1.5 mM $MgCl_2$, 0.0025 mM dNTPs, 1 Unit Gotaq polymerase (Promega). PCR reactions proceeded with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 1 min and a final extension step of 72°C for 5 minutes. PCR products were sequenced directly in both directions using the respective primers (Source Bioscience) and sequences were manually verified using Chromas (Technelysium Pty Ltd) prior to phylogenetic analysis.

Novel sequences were deposited in Genbank under accession numbers G646283-MG646333.

2.7. Sequence alignments and phylogenetic analyses

Multiple sequence alignments were constructed for sequences generated for the variable regions V9 (SSU), D1-D2 (LSU) and partial ITS1, 5.8S, ITS2 (intergenic region), respectively, in

BioEdit 7.0 (Hall 1999) using ClustalW along with other available sequences from Genbank. Alignments were trimmed accordingly based on the lengths of the sequences acquired and to allow for a sufficient number of sequences to be included in the phylogeny. Phylogenetic analysis based on neighbour-joining and maximum likelihood was undertaken using MEGA 6 (Tamura et al., 2013) using the default parameters. Bootstrap values were retrieved from 1000 replicates and are indicated on the nodes of the trees.

3. Results

3.1. Study of plankton samples, culture strains, germination experiments, and surface sediments

Investigation of plankton samples from the Salton Sea revealed the presence of a species that is superficially similar to *P. reticulatum* and is here assigned to *Pentaplacodinium saltonense* gen. et sp. nov.^sp Three process-bearing cysts (Plate 1) were isolated from surface sediments of the Salton Sea (Table 1) and identical morphologies emerged from these cysts (Plate 2). These cells started dividing after germination, and one strain was maintained. The cells were identical in morphology to specimens observed in plankton samples from the Salton Sea (Plate 3), as well as to specimens from several culture strains (Plate 4, Suppl. Table 1), as described below.

3.2. Systematics

Division DINOFLAGELLATA (Butschli 1885) Fensome et al. 1993b

Class DINOPHYCEAE Pascher 1914

Subclass PERIDINIPHYCIDAE Fensome et al. 1993b

Order GONYAULACALES Taylor 1980

Suborder Gonyaulacineae autonym

Family uncertain

Genus *Pentaplacodinium* Mertens, Carbonell-Moore, Pospelova et Head gen. n. (Plate 3) *Type:* Plate 3A, the holotype of *Pentaplacodinium saltonense* gen. et sp. nov.

Diagnosis: A gonyaulacinean genus with roundish to polyhedral thecae with-bearing heavily reticulated plates without appendages. The tabulation is Po, Pt, 4' or 2'+*2'. 5", 6C6c, 6S6s, *65"', 1p, 1"", cover plate is oval.

Etymology: The name is derived from the Greek words*penta* meaning five, *plax* plate, and *dino* whirling; with reference to the five precingular plates that characterize this dinoflagellate genus.

Pentaplacodinium saltonense Mertens, Carbonell-Moore, Pospelova et Head gen. et sp. n. (Plates 3, 4, Figs 2A, 3, 4A)

Synonymy:

1970 *Protoceratium reticulatum* (Claparede et Lachmann); Steidinger and Williams, p. 62, plate 38, fig. 140a-c.

1991 *Protoceratium reticulatum* (Claparede et Lachmann); Al-Muftah, pp. 180-181, figs. 246-247.

? 2002 *Protoceratium reticulatum* (Claparede et Lachmann); Reifel et al., p. 275.

2005 Gonyaulax grindleyi Reinecke; Faust et al., p. 110, figs. 2-4.

? 2007 *Gonyaulax grindleyi* Reinecke; Tiffany et al., p. 582.

? 2009 "*Protoceratium globosum*" Kofoid etand Michener; Morquecho et al., p. 18, 20, figs. 13-17.

Diagnosis: Theca roundish to somewhat polyhedral with tabulation Po, Pt, 2'+*2', 35"~~+*2"~~, 6C6c, 6S6s, *65"', 1p, 1"", with 3" interpreted as *(3"+4"). The theca has an L-type ventral organization and dextral torsion. The plates are heavily reticulated with one pore inside each

reticulation, although two or more pores might be found in reticulations next to a suture. The ends of the descending cingulum are displaced by ~2.0 widths. The cysts have an approximately spherical central body with a thin pedium and thicker spongy-fibrous luxuria. Process distribution apparently intrantabular. Processes fibrous and distally tapering, and have acuminate to minutely expanded distal ends. The archeopyle corresponds to the tfed-*(3"+4") precingular plate and has a smooth margin with rounded angles. The operculum is free.

Etymology: The specific epithet refers to the type locality for this species.

Type locality: The Salton Sea, California, U.S.A. (station 1 at 33°30.192" N, 115°54.869" W).

Gene sequence: The 28S and 18S gene sequence of the cell isolated from culture 2E3, established from a cyst extracted from surface sediment from station 2 in the Salton Sea (Table 1). GenBank Accession No. MG646301 (18S) and MG646323 (28S). Several other strains are considered to belong to the same species (Suppl. Table 1).

Holotype: Plate 3A. The specimen illustrated is on an SEM stub (designated CEDiT2017H62) curated at the Senckenberg Research Institute and Natural History Museum, Centre of Excellence for Dinophyte Taxonomy, Germany.

Description: Motile cells observed in the Salton Sea plankton samples (Plate 3, except D).

The cae have a roundish to somewhat polyhedral shape (Plate 3A, C) and a typical sexiform gonyaulacoid tabulation(sensu Fensome et al., 1993b, tText-Fig. 64B) with an L-type ventral organization (sensu Fensome et al., 1993b, Text-Figs. 82A, C) and dextral torsion (sensu Fensome et al., 1993b, Text-Fig. 83C). The epitheca was often somewhat shorter in length than the hypotheca. The plates are reticulated with one pore inside each reticulation, although two or more pores may occur in reticulations next to a suture. All pores each contain ~3 minute pores (small arrowhead in Plate 3B). The reticulations are faintly expressed on the sulcus and cingulum (Plate 3C). The cell content is brownish-red owing to the presence of chloroplasts

(Plate 2A). Several red bodies are present (Plate 2A-C).

The apical pore complex consists of a cover plate surrounded by a pore plate (Plate 7F, H). The oval cover plate, which is often absent (Plate 3B), is relatively broad and is urrounded by the pore plate. The pore plate is perforated by 5-7 large pores. A low apical collar may encompass the pore plate and is formed by the raised edges of the first and second apical plates, and the fourth apical homolog as well (Plate 3F, H). The first and second apical plates (1' and 2') and the fourth apical homolog (*4') are elongated. The first apical plate (1') is rectangular, while the second apical plate (2') and the fourth apical homolog (*4') are six-sided and irregularly shaped (Plate 3B). The third apical homolog (*3') is small and contacts 2' and *4', but in the specimens observed it never contacted the apical pore plates (Plate 3B). There is a large ventral pore located posteriorly between 1' and *4' (Plates 3A, B, 4A-C). The precingular series consists of five large plates, where 2" is the largest, *(3"+4") forms the keystone plate, and *6" is the smallest. Plates 1", *(3"+4"), and *5" are five-sided, 2" is four-sided, while *6" is six-sided (the suture with the anterior right sulcal is very small (Fig. 3) (Plates 3B, 4A-C). External views of the theca could suggest that there would be no contact between the anterior sulcal plate and 1' (e.g., Plate 3A, B).

Properly oriented external views and internal views, however, show a narrow contact between both plates (Plate 3D). This contact between the anterior sulcal plate and 1', in combination with the contact between *6" and 1' therefore results in an insert configuration (sensu Fensome et al. 1993b, Text-Fig. 62A). The cingulum is left-handed (descending), lined with narrow lists, andcomprises six cingular plates. The ends of the cingulum do not overhang, and are displaced by~2.0 widths (Plates 3A, 4B).

The sulcus is narrow anteriorly and slightly widens posteriorly. It consists of six plates (Plate 3D, Fig. 3) the first postcingular plate 1'" is treated as a sulcal and labeled the anterior

left sulcal plate (Ssa). The anterior sulcal plate (Sa) is relatively large and anteriorly intruded between plates 1" and *6" and barely contacts 1' (Plate 3D). The anterior left sulcal plate (Ssa) is similar in size to the anterior right sulcal plate (Sda). Immediately below these two plates, lay the small posterior right sulcal (Sdp) and a much larger plate, the left posterior sulcal. Finally, there occurs the large posterior sulcal (Sp), which presents lines of pores around its sutures with the adjacent non-sulcal plates (Plate 3D, Fig. 3).

The hypotheca is asymmetrical as a consequence of dextral torsion (Plates 3A). There are five homolog postcingular plates. Plate *2''' is irregularly shaped and the smallest in the series. All other postcingular plates are large, though *6''' is relatively smaller; in addition, they are trapezoidal and four-sided (Plates 3E, 4E). The posterior intercalary plate (1p) bears a conspicuous flange on its right margin (Plates 3A, 4C). The plate overlap is typical for gonyaulacoids, with 3" (in our case *(3"+4") forming the keystone plate (the plate that overlaps all adjacent plates) in the epitheca, and *4''' forming the keystone plate in the hypotheca (Fig. 4, Plate 3E).

Cysts from the Salton Sea surface sediments (Plates 1, 5). The central body is approximately spherical. The wall is thick, consisting of a thin, solid pedium that has a smooth inner surface, and a thicker spongy-fibrous luxuria that appears loosely granular in surface view. Processes are numerous and are solid and fibrous along their entire length, often loosely fibrous at the base. Process bases are expanded, and larger processes may be concave in lateral profile for at least half of their length. Some closely adjacent processes are joined at the base. Most processes usually have a minute distal expansion, observed under SEM as a concave platform ~1.0 pm or less in diameter with strongly irregular margins that may be approximately perpendicular to the shaft. Alongside these, some processes on most specimens taper to distal points, and such

processes occasionally predominate on cyst individual specimens. Processes are mostly of even height, but shorter and thinner processes may be interspersed. The process length/central body ratio is about 0.06. Processes are not evenly spaced, and their parallel alignment and bands devoid of processes observed in many specimens suggest intratabular distribution. There is however no clear evidence of tabulation except for the archeopyle and often parallel alignment along the cingular margins. The archeopyle that is moderately wide and reflects the precingular thecal plate *(3"+4"), where as the operculum is released as a single piece, and has well defined to moderately rounded angles and straight margins, as illustrated on Plate 1G-H. -A reentrant angle along the anterior margin of the archeopyle, signaling the fusion of plates 3" and 4", was not seen in the thecal or cyst tabulation of *P. saltonense* although this might not faet be expected (see Below, 1987, p. 36, fig. 18a; translated in Fensome et al., 1993a, p. 844). An unusually wide archeopyle that seems to reflect two adjacent precingular thecal plates, *(3"+4") and 2", where the operculum is again released as a single piece, is illustrated on Plate 1C-E. If this interpretation is correct, then the component representing 2" in the archeopyle/operculum is reduced in size, because on the theca the second precingular plate is actually similar or larger in size than the *(3"+4") plate.

Dimensions: The cell illustrated in Plate 3A, the holotype figure, is 44 pm in length, 41pm in width and 38 pm in depth. Germinated motile cells: length, 48.1 (53.7) 63.4 pm (SD=6.0, n=5); width, 38.5 (42.4) 47.5 pm (SD= 3.2, n=5). Cells observed in plankton from St. 2 in the Salton Sea: length, 37.8 (46.1) 59.8 pm (SD = 5.5, n=28); width, 31.0 (39.5) 48.5 pm (SD=4.2, n=28). Two single cysts germinated to give the identifiable thecae: maximum central body diameter, 52.3 (53.5) 54.7 pm (SD=1.7, n=2); minimum central body diameter, 51.1 (52.2) 53.3 pm (SD=1.6, n=2); average length of three randomly chosen processes per cyst, 2.4 (3.0) 3.6 pm (SD=0.4, n=6); process width at base 1.4 (2.2) 2.7 (SD=0.6, n=6) and wall thickness 1.3 (1.7)

2.1 (SD=0.3, n=6). Palynologically treated cysts from surface sediments of the Salton Sea: maximum central body diameter, 48.6 (56.3) 70.9 pm (SD=5.3, n=23); minimum central body diameter, 45.7 (52.1) 61.4 pm (SD=3.8, n=22); average length of three processes per cyst, 1.0 (3.1) 5.7 pm (SD=1.2, n=66); process width at base 1.0 (2.2) 3.9 (SD=0.6, n=66) and wall thickness 0.9 (1.6) 2.4 (SD=0.4, n=66).

Comments: Pentaplacodinium saltonense n. gen et sp. is defined primarily from the characters of the motile stage, these distinguishing it from species of the genus *Protoceratium.* The morphology of several thecae observed from off Yucatan (Gulf of Mexico), the Indian River Lagoon (Florida, USA), and off Qatar (Persian Gulf) (Table 1; Plate 2) and from cultures established from cells from Biscayne Bay (Florida, USA) (CCMP1720, CCPM1721), the Indian River Lagoon (Florida, USA) (CCMP3241, CCMP3243) and the Salton Sea (California, USA) (CCMP404) (Suppl. Table 13, Plate 1) agree with the description of *P. saltonense* given above. Cysts formed from cultures established from a strain from the Indian River Lagoon (Florida, USA) (CCMP3243) have the same morphologies (Plate 6). The observed cysts correspond most closely to the fossil based taxon-species *Operculodinium israelianum* (Rossignol 1962) Wall 1967 described from the Pleistocene of Israel, and *Operculodinium psilatum* Wall 1967 described from the postglacial (Holocene) of the Caribbean. However, *Operculodinium israelianum* has longer processes (6-10 nm; Rossignol, 1964), and *O. psilatum* has a psilate surface interrupted by minute and sparsely distributed processes, and a pronounced cingulum (Wall, 1967). Both have archeopyles that are less wide than for the cyst of *P. saltonense.*

3.3. *Phylogenetic position of* P. saltonense *and other studied strains*

The SSU rDNA sequences for all *P. reticulatum* strains analysed were identical, forming a distinct clade separated from the *P. saltonense* sequences which were identical to the

unidentified Malaysian sequences (Fig. 5). *P. reticulatum* and *P. saltonense* sequences shared 92% nucleotide identity for the V9 region analysed.

For the LSU rDNA V4 analysis (Figure 6), *P. reticulatum* sequences were identical apart from a couple of sporadic nucleotide substitutions which were identified as ambiguous bases by the sequencing software. The unidentified strain from Hawaii had 12 nucleotide substitutions across the 570 bp multiple sequence alignment compared to *P. reticulatum. P. saltonense* sequences shared more similarity with the unidentified GgSm strains from Malaysia (96%) compared to that of *P. reticulatum* (94%).

The ITS (intergenic region between ITS1 and 2) was the only marker to resolve intraspecific diversity within the *P. reticulatum* species, with strain E12 (Baffin Bay, Arctic) sharing 98% nucleotide similarity with strain VG0757 isolated from Spain. The phylogeny separates *P. reticulatum* into two large subclades: subclade 1A that regroups several strains from warmer waters, and subclade 1B that regroups several strains from colder waters (Fig 7).

The three phylogenies (Figures. 5-7) show that strains identified as *Protoceratium reticulatum* form a monophyletic group (Clade 1), as well as strains identified as *P. saltonense* that form a clade with the unidentified GgSm strains from Malaysia (Clade 2), as well as the *Ceratocorys* species that form a clade with PrTT strains from Malaysia (Clade 3) supported by high bootstrap values (>70). The unidentified strain from Hawaii does not group with the *Protoceratium reticulatum* or *Pentaplacodinium saltonense* clades. The topology of the trees are not consistent between the three phylogenies (i.e. the relatedness between clades), but the three clades identified are consistently formed. The trees furthermore highlight the unexplored diversity within this group of dinoflagellates, and further incubation and plankton studies from these locations should reveal whether the unidentified strains are new species or not.

In addition, the phylogenies show that V9 SSU sequences from cells from Elands Bay

(South Africa), (bootstrap values >80%), that have been previously identified as *G. grindleyi* by Reinecke (1967), clusters with *Protoceratium reticulatum* (Figure 5).

The three studied species of *Ceratocorys (C. armata, C. gourreti, C. horrida)* share high nucleotide similarity for the SSU (100%) and LSU sequences (>99% identity) (Figure 6).

4. Discussion

4.1. Comparison of theeeU-thecapf P. saltonense

Pentaplacodinium saltonense differs from *Protoceratium reticulatum* because it bears five precingular plates, whereas *P. reticulatum* has six. Furthermore, *P. saltonense* has a larger cingular displacement (2 widths vs. 1 width respectively) and an oval cover plate, as opposed to a sigmoidal cover plate in *P. reticulatum*. In addition, the theca of *P. saltonense* is mostly roundish, whereas in *P. reticulatum* it is always polyhedral. Both species have an insert configuration, but in *P. saltonense* the contact

between Sa and 1' is very narrow whereas in *P. reticulatum* this contact is wide this causes a conspicuous separation between 1" and 6" in *P. reticulatum*, when in *P. saltonense* there is almost a small point of contact between those two plates (Plate 1D3D). *Gonyaulax grindleyi* Reinecke 1967 is shown to be a synonym of *P. reticulatum*, as already suggested by von Stosch (1969) and Hansen et al. (1997), and is now confirmed by the LSU rDNA phylogeny in this study (see below).

Several other *Protoceratium* species have been described (e.g. Schiller, 1937, p. 322-326). *Protoceratium splendens* Meunier 1910 from the Kara Sea has six precingular plates; it is possibly a junior synonym of *Protoceratium reticulatum*, as suggested by Gomez (2012).

Protoceratium aculeatum (Stein 1883) Schiller 1937 bears antapical spines and an apical horn. *Protoceratium areolatum* Kofoid 1907 and *Protoceratium spinulosum* (Murray and Whitting 1899) Schiller 1937 have fewer reticulations in both the epitheca and hypotheca than *P. saltonense.* Of the five species described by Kofoid and Michener (1911) Protoceratium *cancellorum, Protoceratium pellucidissimum, Protoceratium pepo, Protoceratium globosum* and *Protoceratium promissum,* none has illustrations and it is therefore if impossible to compare them to *P. saltonense.*

Pentaplacodinium saltonense differs from *Ceratocorys anacantha* Carbonell-Moore 1996 because it is not as polyhedral. In addition, in contrast to the insert epithecal configuration of *P. saltonense, C. anacantha* has an episert type I epithecal configuration, meaning that 1' does not contact the anterior sulcal plate and that 1" and 6" are in contact (Paez-Reyes and Head, 2013).

4.2. Comparison of the cyst of P. saltonense

The cyst of *Pentaplacodinium saltonense* compares with *Operculodinium psilatum* because its cysts display an alignment of processes along the cingulum, it bears short processes (1.0-5.7 gm, its body diameter is of similar size (45.7-70.9 gm vs. 50-60 gm (Wall, 1967) or 62-79 gm (Head, 1996b) and its wall thickness (0.9-2.4 vs. 1.4-2.2 gm (Head, 1996b) is similar. *Operculodinium psilatum* differs, however, in having processes that in general are shorter (2 gm, Wall, 1967; 0.0-2.9 gm, Head, 1996b) and sparsely distributed. The cingulum and sulcus are also more conspicuously expressed in *Operculodinium psilatum* (Wall, 1967; Head et al., 1996b), and *O.psilatum* lacks the wide archeopyle of *P. saltonense.*

The cyst of *P. saltonense* is also similar to *Operculodinium israelianum* (Rossignol 1962) Wall 1967 as described by Rossignol (1964, as *Baltisphaeridium israelianum*); although

the processes of the latter species are longer (6-10 gm) than for *P. saltonense* (1.0-5.7 gm). It is not presently known whether variation in process length is related to variations in ecology, teas demonstrated for the cysts of *Lingulodinium polyedra (=Lingulodinium machaerophorum)* (Mertens et al., 2009), cysts of *Protoceratium reticulatum* (Mertens et al., 2011) and cysts of *Pyrodinium bahamense (=Polysphaeridium zoharyi)* (Mertens et al., 2015). The process distribution appears to be is-intratabular for the cysts of *P. saltonense*, and this is likely to be the case also for-on- *O. israelianum* (e.g., O. cf. *israelianum* of Head, 1997, fig. 17.2), although the study of topotype material will be needed for confirmation. The rounded angles of the archeopyle in *O. israelianum*, *O. psilatum* and the cysts of *Pentaplacodinium saltonense*, and the shared presence of a spongy-fibrous to fibroreticulate luxuria, accentuate the overall similarities between these cysts, although the relatively wider archeopyle in *P.saltonense* cysts distinguishes them from these other species.

Operculodinium israelianum resembles the Miocene *Operculodinium centrocarpum* (Deflandre et Cookson 1955) Wall 1967, which is also has a spongy-fibrous luxuria, although it is somewhat larger (54-80 gmH Deflandre and Cookson, 1955) and has longer processes. Head (1996b) noted an intergradation in size and process length between *O. israelianum* and *O. centrocarpum* in Pleistocene assemblages of eastern England. A restudy of topotype material is needed to confirm the range of variability within each species.

The cysts of *P. saltonense* differs from hose of *P. reticulatum*, in having a thick spongy-fibrous luxuria (vs. thin, fibrous luxuria), less developed distal ends of the processes, larger central rts-body size-diameter (48.6-70.9 um vs. 33-48 um; Rochon et al., 1999), and generally shorter process length (1.0-5.7 um vs. typically 7-14 um; Rochon et al., 1999); although the cysts of *P. reticulatum* vary widely, with some being completely bald (e.g., Mertens et al., 2012a; Jansson et al., 2014).

Numerous other *Operculodinium* species have been described and a detailed comparison is given by Marret and Kim (2009) none of these closely resembles the cysts of *P. saltonense.*

4.3. Phylogenetics, evolution and position and relationships of Protoceratium, Pentaplacodinium, *and* Ceratocorys

Several morphological characteristics of the theca thaFare important to understanding the evolution of *Protoceratium, Pentaplacodinium,* and *Ceratocorys* (Plate 7). The shape of the cover plate of *Ceratocorys* is more similar to that of the cover plate of *Pentaplacodinium,* but less similar to the sigmoidal cover plate of *Protoceratium.*

Pentaplacodinium and *Ceratocorys* can be considered closer to *Gonyaulax* than *Protoceratium,* because the anterior intercalary is always well-separated from the apical pore plates, whereas in *Protoceratium reticulatum* it is closer and has even been suggested to contact the apical pore plates (Hansen et al. 1997). It should be noted, however, that *Protoceratium* and *Gonyaulax* have six precingular plates, whereas *Ceratocorys* and *Pentaplacodinium* have five. So it is not surprising that in the molecular phylogenies, *Pentaplacodinium* has an intermediate position between *Ceratocorys* and *Protoceratium* (Figs. 5-7); the relation to other gonyaulacoids at this time is unclear and further molecular studies of related genera are required, particularly to understand how to resolve the position of *Protoceratium* at family level. Another issue regards a conflict in the dual nomenclature: the cyst of *P. reticulatum* and *P. saltonense* both are considered to belong to the cyst-defined genus *Operculodinium,* whereas the thecate stages belong to two different genera; further cyst-theca experiments within this group of related species should help to understand how the genus-generic concepts can be

rationalized.

In addition, the ITS marker was able to separate two large subclades within *P. reticulatum*: strains that are predominantly associated with warmer waters (Sub-clade 1A), and other strains largely associated with colder waters (Sub-clade 1B) (Fig. 7). Do these subclades reflect pseudocryptic speciation in *Protoceratium reticulatum* as previously suggested by Mertens et al. (2012a)?

Other morphological characteristics of the theca are conserved in *Protoceratium, Pentaplacodinium,* and *Ceratocorys* and other gonyaulacoids. For instance, there is no difference in overlap pattern between *Protoceratium, Pentaplacodinium, Ceratocorys, Gonyaulax* and *Lingulodinium* (Fig. 4).

4.4. Biogeography and ecology of P. saltonense

According to the plankton observations, *P. saltonense* can be found in tropical to subtropical regions. *P. saltonense* and *P. reticulatum* have not been observed in the same samples, which suggests that both species inhabit different niches, where *P. saltonense* has a preference for higher temperatures and salinities, and *P. reticulatum* for somewhat lower temperatures and salinities. This difference would need to be quantified through culture experiments.

4.5. Toxicity

Strains identified as *Pentaplacodinium saltonense* (CCMP404, CCMP1720 and CCMP1721), have been identified as yessotoxin producers using fluorescence HPLC (Paz et al., 2004). A later toxin analysis by LC-MS of the same strains was negative (Paz et al., 2007), and the

authors considered that these strains had lost their toxicity after a number of years in culture. The toxins produced by these strains of *Pentaplacodinium saltonense* are similar to toxins produced by strains we identified as *Protoceratium reticulatum,* all of which are yessotoxin producers, such as strains from Chile (Alvarez et al., 2011), Jervis Inlet, British Columbia, Canada (Cassis, 2005), German Bight, North Sea (Roder et al., 2011, 2012), Okkirai Bay, Japan (Koike et al., 2006) and Spain (Paz et al., 2007, 2013). *P. saltonense* has been considered a potential causative agent of mortality events in the Salton Sea (Reifel et al., 2002, whom identified it as *Protoceratium reticulatum).* However, there have not been reports of toxic events knowingly involving *P. saltonense.*

Several other studies have investigated the toxicity of strains that they designate as *Protoceratium reticulatum,* but for which the identifications could not be verified (e.g., Satake et al., 1999; Ciminiello et al., 2003; Samdal et al., 2004; Finch et al., 2005; Eiki et al., 2005; Mitrovic et al., 2005; Guerrini et al., 2007; Suzuki et al., 2007).

5. Conclusions

Pentaplacodinium saltonense gen. et sp. nov. is described from the Salton Sea (CA, USA). The distinct cover plate (similar to *Ceratocorys,* but sigmoidal in *Protoceratium),* five precingular plates (as in *Ceratocorys,* but six in *Protoceratium),* the very narrow contact between 1' and Sa (wide contact in *Protoceratium,* no contact in *Ceratocorys),* a more rounded eeH-thecal shape, the displacement of the cingulum by two widths (vs one with in *Protoceratium*), as well as the clear separation and distances seen in the three phylogenies, justifies the creation of a new genus. The chorate cysts of *P. saltonense* bear short processes often with parallel alignments. These cysts- correspond to the cyst-defined genus *Operculodinium* Wall 1967, and are most

similar to *O. israelianum* and *O psilatum.* Motile stages of *Pentaplacodinium saltonense* was confirmed infrom four widely dispersed locations, suggesting a subtropical to tropical distribution for this species. *Protoceratium reticulatum* and *Pentaplacodinium saltonense* are not known to inhabit the same environments. As with the -yessotoxin-producing *Protoceratium reticulatum, Pentaplacodinium saltonense* is potentially a yessotoxin producer, as shown by previous studies.

Note added: While this paper has been going through the process of final acceptance to Harmful Algae, another study was accepted (Salgado et al., accepted) that addresses similar scientific questions.

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Figure captions

Figure 1. Sites of studied plankton samples and cultured strains containing thecate stages of *Pentaplacodinium saltonense* (in red) and *Protoceratium reticulatum* (in blue). The locations are listed in Table 1.

Figure 2. Line drawings of extant members of the subfamily *Cribroperidinioideae* in dorsal view to show the dextral torsion typical of these gonyaulacoids. A. *Pentaplacodinium saltonense.* B. *Protoceratium reticulatum.* C. *Lingulodiniumpolyedra.* Labeling of tabulation follows a modified Kofoid system that recognizes homologs.

Figure 3. Line drawing of sulcal area of *Pentaplacodinium saltonense.* FP: flagellar pore; Sa: anterior sulcal plate; Sda: right anterior sulcal plate; Sdp: right posterior sulcal plate; Ssa: anterior left sulcal plate; Ssp: posterior left sulcal plate; Sp: posterior sulcal plate; c: cingular plates.

Figure 4. Line drawings of epithecal overlapping plate patterns of gonyaulacoids discussed in this paper. Arrows indicate direction of overlap. A. *Pentaplacodinium saltonense.* B. *Protoceratium reticulatum.* C. *Lingulodinium polyedra.* D. *Ceratocorys horrida.* E. *Gonyaulax spinifera.*

Figure 5. Neighbour-joining tree of *P. reticulatum, P. saltonense* and related strains sequenced in this study and sequences from Genbank based on an 80 bp alignment of the V9 region of the SSU gene. Bootstrap values were retrieved from 1000 replicates and those >70% are indicated at the nodes for neighbour-joining and maximum likelihood respectively.
Strain names are indicated followed by their geographic origin and accession number (Genbank).

Figure 6. Neighbour-joining tree of *P. reticulatum, P. saltonense* and related strains sequenced in this study and sequences from Genbank based on a 571 bp alignment of the V4 region of the LSU gene. Bootstrap values were retrieved from 1000 replicates and those >70% are indicated at the nodes for neighbour-joining and maximum likelihood respectively. Strain names are indicated followed by their geographic origin and accession number (Genbank).

Figure 7. Neighbour-joining tree of *P. reticulatum, P. saltonense* and related strains sequenced in this study and sequences from Genbank based on a 356 bp alignment of the ITS 1-2 region. Bootstrap values were retrieved from 1000 replicates and those >70% are indicated at the nodes for neighbour-joining and maximum likelihood respectively. Strain names are indicated followed by their geographic origin and accession number (Genbank).

Plate Captions

Plate 1. Light microscope images of *Pentaplacodinium saltonense* based on cyst-theca experiment from the Salton Sea. A. Living cyst from the Salton Sea St. 1. B-F. Germinated cyst from St. 2 (culture 2E3 used for single-cell PCR). B. Cross section, showing attached operculum. C. Focus on elongated simple operculum reflecting plates 2"+*(3"+4"). D-E. Focus on archeopyle, after removal of operculum. F. Cross section showing processes. G-I. Germinated cyst from St. 2 (culture 1A7 used for single-cell PCR). G. Focus on precingular archeopyle reflecting plate *(3"+4"), showing attached operculum. H. Focus on operculum. I. Cross section, showing opened operculum. Scale bars = 20 pm.

Plate 2. Light microscope images of cyst-theca experiment from the Salton Sea. A-I. Images of living cells of *Pentaplacodinium saltonense* germinated from cyst depicted in Plate 1, Figs. B-F (culture 2E3). A. Globular cell. B. Angular cell. C. Fusiform cell. D. Epitheca. E. Hypotheca. F. Ventral view showing configuration of apical plates. G-I. Sulcal plates. Scale bars = 20 pm.

Plate 3. Scanning electron microscope images of *Pentaplacodinium saltonense,* all different cells from the Salton Sea, except D. A. Holotype. Ventral view. Arrowhead points to ventral pore between plates 1' and *4'. Arrow shows flange on plate 1p. B. Apical view, missing the cover plate. Small arrowhead points to small pores inside the thecal pores. Large arrowhead points to ventral pore between plates 1' and *4'. Small arrowhead points to the three minute pores inside most pores. C. Dorsal view, showing dextral torsion. Note the cell roundness. D. Sulcal plates of a cell from culture SSCAP K-1479 (Indian River Lagoon, Florida). Arrowhead shows the narrow point of contact between the Sa and 1' plates. E. Antapical view. Scale bars A-C, E = 10 pm; D = 5 pm.

Plate 4. Scanning electron microscope images of *Pentaplacodinium saltonense* from the Indian River Lagoon. A. Apical view of a cell from culture SSCAP K-1479. Arrowhead points to ventral pore between plates 1' and *4'. B. Same specimen as in A. Ventral view. Arrowhead points to ventral pore between plates 1' and *4'. C. A different cell from a plankton sample courtesy of Paul Hargraves. Ventral view. Arrowhead points to ventral pore between plates 1' and *4'. Arrow shows flange on plate 1p. D. Ventral view of a cell from a culture established by Paul Hargraves. E. Antapical view of a cell from the same culture as in

D. E. Apical view of a cell from the same culture as in D. D-F: SEMs by Paul Hargraves. Scale bars = 10 pm.

Plate 5. Scanning electron microscope images of cysts of *Pentaplacodinium saltonense* extracted from Salton Sea sediment (St. 2) using palynological methods. A-C. Views showing shape of archeopyle, reflecting plate *(3"+4"). D. Specimen that is torn along the cingulum. E. Specimen showing alignment of processes along the cingulum. F. Specimen with relatively large openings in cyst wall. G. Specimen with distinct intratabular processes. H. Specimen with relatively coarsely reticulated wall surface. I. Internal view of smooth cyst wall. Scale bars = 10 pm.

Plate 6. Scanning electron microscope images of cysts of *Pentaplacodinium saltonense* formed in culture of strain 3243 (Indian River Lagoon). A. Specimen showing preformed archeopyle and margins of principal archeopyle suture with reduced ornament. B. Specimen with attached thecal plate. C. Specimen with partly developed processes. D. Specimen with processes clearly reflecting tabulation. E. Specimen with preformed archeopyle. F. Specimen showing reflection of the sulcus. G-H. Specimen with well-developped wall texture. I. Wall texture of specimen

with ‘spider-web’ microreticulation. Scale bars = 10 pm, except H, I, scale bars = 1 pm.

Plate 7. Scanning electron microscope images of *Protoceratium reticulatum* cells and of the apical pore plates of the gonyaulacoids discussed in this study. A. *Protoceratium reticulatum.* Cell from Greenland, ventral view. B. Same cell in apical view. C. *Protoceratium reticulatum.* Cell from Elands Bay, South Africa. Dorsal view, note the dextral torsion. D. Apical pore plates of a different cell of *Protoceratium reticulatum* (Greenland). E. Apical pore plates of a cell of *Ceratocorys horrida* (Central equatorial Pacific). F. Apical pore plates of a cell of *Pentaplacodinium saltonense* from culture SSCAP K-1479 (Indian River Lagoon). G. Apical pore plates of a cell of *Ceratocorys gourretii.* H. Apical pore plates of another cell of *Pentaplacodinium saltonense* from culture SSCAP K-1479 (Indian River Lagoon). Scale bars