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Prorocentrum shikokuense Hada and *P. donghaiense* Lu are junior synonyms of *P. obtusidens* Schiller, but not of *P. dentatum* Stein (Prorocentrales, Dinophyceae)

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Abstract :

In Japanese, Chinese and Korean coastal waters, recurrent blooms of a small, elongate *Prorocentrum* species have been observed in recent years. In previous studies, this species has been respectively identified as *P. shikokuense*, *P. donghaiense* and *P. dentatum*, despite morphological similarity and identical rDNA sequences. To resolve the confusion, morphological features, including the architectural details of the periflagellar area, were examined and ribosomal DNA (rDNA) sequences were obtained from specimens collected from the East China Sea and Korean coast, and a strain established in the waters off the Canary Islands of Spain. In addition, the descriptions of the three species and allied species were reviewed. Morphological observations and a phylogeny based on the SSU, ITS region and LSU rDNA sequences revealed that the previously confused species and our studied strains are conspecific and that the morphology of strains identified as *P. dentatum* in the phylogenetic trees does not coincide with *P. dentatum sensu stricto*. The confusion can be traced back to Dodge (1975, p. 116), who considered *P. veloi, P. monacense* and *P. obtusidens* as junior heterotypic synonyms of *P. dentatum*. However, Dodge's *P. dentatum* are closer to *P. obtusidens sensu stricto*, rather than *P. dentatum sensu stricto*. *P. obtusidens sensu stricto* can be distinguished from *P. dentatum sensu stricto* by its relatively small size, parallel sides towards the anterior and a blunt anterior extension on one side. This indicates that *P. obtusidens* should not be considered a synonym of *P. dentatum sensu stricto*. In addition, a comparison of the original descriptions of *P. obtusidens* and allied species allowed to conclude that small, elongate *Prorocentrum* from Japanese, Chinese and Korean coastal waters previously identified as *P.*

shikokuense, *P. donghaiense*, *P. dentatum sensu* Yoo and Lee (1986) and the specimens studied herein, which share identical rDNA sequences, morphologically coincide with *P. obtusidens.* Therefore, it is proposed that *P. shikokuense* and *P. donghaiense* should be regarded as junior synonyms of *P. obtusidens.*

Highlights

► *Prorocentrum shikokuense* Hada, *P. donghaiense* Lu and *Prorocentrum obtusidens* Schiller are conspecific. ► *Prorocentrum obtusidens* is a senior to *P. shikokuense* and *P. donghaiense*. ► *Prorocentrum obtusidens* is a separate species from *P. dentatum sensu stricto*, *P. veloi* and *P. monacense*. ► The periflagellar area of *Prorocentrum obtusidens* consists of seven platelets bearing a wing-like structure and two platelets without a wing-like structure, one flagellar pore and one accessory pore.

Keywords : Morphology, Periflagellar area, rDNA, *Prorocentrum veloi, P. monacense*

1. Introduction

The thecate dinoflagellate genus *Prorocentrum* was erected by Ehrenberg (1834), with *Prorocentrum micans* as the type species, and since then about 80 species have been described (Hoppenrath et al., 2009). Marine *Prorocentrum* species are found worldwide in planktonic and benthic habitats, and some of them have attracted special attention because of their harmfulness and toxicity (Faust et al., 1999; Lu et al., 2005; Glibert et al., 2012). *Prorocentrum* species differs distinctly in morphology from other dinoflagellates; they have no cingulum and sulcus, and the flagella are inserted apically. The classification of *Prorocentrum* species has traditionally been based on morphology: cell shape and size, thecal plate surface morphology, intercalary band morphology, and the architectural details of the periflagellar area (Dodge, 1975; Taylor, 1980; Faust et al., 1999; Hoppenrath et al., 2013). In particular, the architectural details of the periflagellar area are very useful for species identification (Hoppenrath et al., 2013), however for many species these details have not been documented, properly.

Several small, elongate *Prorocentrum* species have been described; *P. dentatum* by Stein (1883) from the Atlantic Ocean, *P. obtusidens* by Schiller (1928) from the Adriatic Sea, *P. veloi* by Osorio-Tafall (1942) from Acapulco Bay and the Port of Mazatlán in the Pacific coast, and *P. monacense* by Kufferath (1957) from offshore Monaco, Mediterranean Sea. Dodge (1975) considered *P. dentatum* to be senior to three species: *P. obtusidens* Schiller, *P. veloi* Osorio-Tafall, and *P. monacense* Kufferath. Later, two similar species were described: *P. shikokuense* was described by Hada (1975) from Iwamatsu Bay of the Bungo Channel on the western coast of Shikoku, Japan, and Lu and Goebel (2001) described *P. donghaiense* from Gouqi Island waters (China), in the East China Sea.

Recurrent blooms of similar small elongate *Prorocentrum* species have been observed in Japanese, Chinese and Korean coastal waters. These have been identified by three different names: *P. dentatum* Stein, *P. shikokuense* Hada and *P. donghaiense* Lu (Yoo and Lee, 1986; Lu et al., 2005; Takano and Matsuoka 2011). Lu et al. (2005) provided morpho-molecular observations of *P. donghaiense* and, after morphological comparison with *P. shikokuense*, expressed uncertainty over their conspecificity. However, following a remark by Sournia (1982, p.160), Lu et al. (2005) claimed that *P. shikokuense* is an invalid name under both the International Code of Botanical Nomenclature and the International

Code of Zoological Nomenclature (ICZN), because no Latin diagnosis was provided and no type was designated by Hada (1975). Takano and Matsuoka (2011) disagreed and claimed that *P. shikokuense* does have validity under the ICZN and has priority over *P. donghaiense*, as described by Lu and Goebel (2001). Consequently, the name *P. shikokuense* was used by Japanese and Italian researchers (Roselli et al., 2019), whereas *P. donghaiense* was used by mostly Chinese researchers and the Norwegian Culture Collection of Algae (NORCCA). In addition, based on morphological descriptions of *Prorocentrum* species provided by Dodge (1982), the species was also identified as *P. dentatum* by Korean and Japanese researchers (Yoo and Lee 1986; Onoue and Nozawa 1989).

During a workshop in 2003, the morphology (includingcell size) was compared between respectively strains identified as *P. donghaiense* and *P. dentatum* from the East China Sea, Korean and Japanese coastal waters, and a strain (CCMP 1517) previously identified as *P. dentatum* from the South Pacific of South America.The experts suggested that the small *Prorocentrum* taxon occurring in the East China Sea should be identified as *P. donghaiense* (Qi and Wang, 2003). More recent molecular analyses showed high similarity in the rDNA locus and protein profiles of strains of *P. donghaiense* and the strain (CCMP 1517) and suggest that these are conspecific (Chan et al., 2004; Lu et al., 2005; Lin et al., 2006; Wang et al., 2005). Nevertheless, the controversy still lingers on, because of confusion within the morphological identifications.

Although many *Prorocentrum* species have been described, the morphological description of some species has been confusing, which has led to erroneous identifications of the species (e.g. Dodge 1975; Lin et al., 2006; Hoppenrath et al., 2013). Uncertainties remain about morphological variations in small elongate *Prorocentrum* species occurring around Japanese, Chinese and Korean coasts. To resolve the controversy, morphological features, including the architectural details of the periflagellar area, and the molecular phylogeny of specimens established from the East China Sea and Korean coast and waters off the Canary Islands of Spain were examined and the taxonomic descriptions of small elongate *Prorocentrum* species were reviewed. Based on this information, it is evaluated whether *P. donghaiense*, *P. shikokuense*, *P. dentatum sensu* Yoo and Lee (1986) and the strains studied herein should be considered as one or more species, and which name(s) should be used to designate them.

2. Study area

The East China Sea (ECS) is connected to the East Sea (Sea of Japan) via the Korea Strait and opens to the north into the Yellow Sea (Fig. 1A). The Kuroshio Current flows along the continental slope of the ECS, and constitutes the western boundary current of the subtropical North Pacific circulation. The Kuroshio Current has a branch current entering into Korean coastal area: the Tsushima Current (TC) (Fig. 1A). The TC and Taiwan Warm Current (TWC) have a strong influence on water circulation and the physical properties of seawater in the ECS and Korean coastal area (Lie and Cho, 1994; Hu and Yang 2001; Yuan et al., 2008). Movement of the TWC has an impact on the nutrient concentrations within the ECS (Dai et al. 2013).

The Changjiang River, which is the largest river in China and the third largest in the world, is the primary source of freshwater into the ECS, and the Korean coastal area is also affected by the intrusion of freshwater (Hu and Yang, 2001; Yuan et al., 2008). Red tides occur more frequently in the Changjiang River estuary and its adjacent area (Zhou et al., 2003). In the 1980s and early 1990s, the main causative species of the red tides were *Skeletonema costatum* (Greville) Cleve, *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle and *Noctiluca scintillans* (Macartney) Kofoid & Swezy. However, the blooms caused by the organisms have been replaced by extensive *Prorocentrum* blooms in 2000-2006 (Lu and Goebel, 2001; Lu et al., 2005). The blooms have had an impact on the aquaculture and marine ecosystem along the Chinese coast (Li et al., 2009), despite the fact that the causative species are not toxigenic (Yan et al., 2007).

3. Materials and methods

3.1. Sampling and culture

On June 24, 2016, a dark-brown discoloration of the water in Gamak Bay located in the southern coastal area of Korea was observed (Fig. 1B-C), water samples were collected and a small *Prorocentrum* species was observed using a light microscope. *Prorocentrum* cells were directly isolated

for single-cell analysis from the plankton samples, and another part of the plankton sample was fixed and used for scanning electron microscopy (see below).

Between 15 and 22 May 2017, small *Prorocentrum* specimens were collected at seven stations of the Korean coastal area and the East China Sea using a plankton net (20 µm mesh) (Table 1) and cells were then isolated using a light microscope on the research vessel. The isolated cells were inoculated into individual wells of 48-well tissue culture plates filled with f/2 culture medium (Guillard, 1975) at a salinity 32, which were then cultured at an appropriate temperature (20 °C) under white illumination in a mini digital incubator (H220-HC), prior to further analysis in the laboratory. The cultured cells were then transferred into individual wells of six- well tissue plates, and then into 30 ml culture flasks by micropipetting using a capillary pipette. They were then maintained at 20 °C and ca 100 µmol photons m^{-2} s^{-1} cool-white illumination under a 14L:10D photo-cycle. After the strains had sufficiently divided, the cells were isolated from the culture flask, their morphological features were observed using an inverted microscope (Primo Vert; Carl Zeiss, Germany), and they were photographed using an AxioCam MRc digital camera on an upright microscope (Axio Imager 2; Carl Zeiss, Germany).

3.2. Scanning electron microscopy

For scanning electron microscopy (SEM), plankton samples from the bloom in Gamak Bay were fixed with acidic Lugol's Iodine solution (0.1% final concentration).

For SEM of the established strains, 5 mL of mid-exponential batch cultures were fixed with 2% acidic Lugol's solution (Sigma-Aldrich, Korea) for two hours at room temperature, then rinsed with deionized water. After fixation, the samples were dehydrated in a graded ethanol series (10–99.9% in eight steps) for 15 min at each step, and finally replaced by isoamyl acetate for critical point drying (Spi-Dry Regular CPD, USA) with liquid CO_2. The filters were mounted on stubs, coated with platinum–palladium and examined in a field SEM microscope JEOL JSM 7600F.

3.3. PCR and sequencing

From the sampled bloom from Gamak Bay, single-cell PCR was performed on a cell isolated from

the plankton sample following the method of Takano and Horiguchi (2004). The single cell was transferred into a 200 µL tube.

For the other strains, genomic DNA was extracted from 1 mL of exponentially growing cultures of seven strains established from seven stations of the Korean coastal area and the East China Sea, and a strain of *P. donghaiense* (K-1260) provided by the NORCCA using the DNeasy Plant mini kit (QIAGEN Inc., Valencia, California, USA) following the manufacturer's instructions. The K-1260 strain was established from oceanic water obtained offshore the Canary Islands, Spain. Sequences of SSU and part of LSU rDNA were amplified using the primer pairs SR1 and SR12b, and LSU D1R and LSU R2 (Takano and Horiguchi, 2006).

PCR amplification was carried out in a 50 µL reaction volume with KOD-plus-DNA Polymerase in two rounds (Sarai et al., 2013). PCRs were performed on a Mastercycler nexus (Eppendorf, Hamburg, Germany) in a 25 µL PCR mix containing 1 × *Ex Taq* Buffer (20 mM Mg2+ plus, Takara, Japan), 5.0 mM of each dNTP, 15 pmol forward and reverse primers, 0.75 U of *TaKaRa Ex Taq* (Takara, Japan), and the following cycles: 95 °C for 4 min; then 35 cycles at 95 °C for 30 s, 55°C for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min. The PCR-amplified products were confirmed through 1.0% agarose gel electrophoresis. For direct DNA sequencing, the PCR products were purified using a QIAquick PCR purification kit (Qiagen). The DNA sequencing reactions were performed using the ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies Corporation, Carlsbad, California, USA).

3.4. Sequence alignments and phylogenetic analyses

Sequences were observed and assembled in DNABaser version 4.36 (http://www.dnabaser.com). Contigs were imported into MEGA version 7.0 (Kumar et al., 2016) and aligned using ClustalW with default settings. The final alignment of the SSU rDNA dataset contained 42 taxa and had 1,603 characters, including gaps introduced for alignment. Two sequences of *Scrippsiella acuminata* (AY421792 and EF492513) were used as outgroups to root the phylogenetic tree. The TIM3+I substitution model was selected using the Akaike information criterion as implemented in jModelTest

version 2.1.4 (Darriba et al., 2012).

For the analysis of the ITS region (ITS1-5.8S-ITS2) sequence, the dataset contained 47 taxa and consisted of 574 characters (including gaps inserted for alignment). Two sequences of *Scrippsiella acuminata* (AF527121 and HQ658160) were used as outgroups. The GTR+G model of nucleotide substitution was chosen.

For the analysis of LSU sequences, the dataset contained 51 taxa and consisted of 1,223 characters (including gaps inserted for alignment). Sequences of *Scrippsiella acuminata* (HQ670228 and EF613366) were also used as outgroup. The TIM3+I+G model of nucleotide substitution was chosen. Phylogenetic trees for this sequence dataset were constructed using maximum likelihood (ML) analysis and Bayesian inference, respectively. The ML analyses were performed using PhyML 3.0 (Guindon et al., 2010). The starting tree was generated using BIONJ with optimization for the topology, branch lengths, and rate parameters selected. Bootstrap analyses were employed for the ML analysis, with 1000 replicates. The Bayesian inference was conducted using the MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Four Markov chain Monte Carlo chains were run for 10 million generations, with sampling every 100 generations. A majority rule consensus tree was created in order to examine the posterior probability of each clade. The final tree was visualized using TreeView v1.6.6.

4. Results

4.1. Morphology of Prorocentrum obtusidens

All established strains, including a strain K-1260 provided by the NORCCA, are hereafter designated as *Prorocentrum obtusidens* (see discussion for further details, and their names, localities and sizes are shown in Table 1. The morphological features of *P. obtusidens* are shown in Figs 2, 3, 4, 5, and Supplementary Fig. S1.

All strains were morphologically identical. Cells were elongated, slightly asymmetric, and narrow towards the posterior end, and most of them had parallel sides towards the anterior. The posterior end of the cells was generally rounded; however, it was sometimes attenuated (Fig. 2A-C). The cells generally occurred as single cells but two-celled and four-celled chains were also observed (Figs. 2G-

I). In blooms, chain forming cells were frequently observed. In lateral view, one side of the anterior end was slightly extended and blunt (Figs 2A- B), however, in chain forming cells the slight extension was not observed and the anterior end was rounded (Figs 2G-I). The cells were brownish-yellow in color, and the size for all specimens ranged from 10. 9 to 22.9 µm in length and 6.1 to 12.3 µm in depth (n = 50) (Table 1). A peanut-shaped or spherical pusule was visible at the anterior part of the cell (Fig. 2A, C, G) and occupied about one-third of the cell. The chloroplast was visible in the center and along the margins of the cell however the number of chloroplasts was not visible using a light microscope (Fig 2D-F). A rounded nucleus was located in the posterior region of the cell (Fig. 2E-F).

SEM observations showed that most of the cells were characterized by parallel sides toward the anterior; however, a lack of parallelism was sometimes observed. Knob-like spines were densely distributed on the surface of the thecal plates (Fig. 4C). The V-shaped periflagellar area was visible on the right thecal plate (Fig. 3A, B, E, F). Interior views of theca showed that the periflagellar area was prominent on the right thecal plate (Fig. 4D). Intercalary bands were well developed with dense rows of tiny knobs (Fig. 4A, B). Small thecal pores were irregularly distributed on the thecal plates (Fig. 4C), their sizes were approximately 0.15 µm (n=50) and there were no different types of thecal pore present. On the inner surface of a thecal plate, these small pores were surrounded by a circle on the inside of the thecal plates, which correspond to the thecal pores (Figs 4F-I). There were many depressions on the inside of the thecal plates (Figs 4G-I), and these likely reflect the knob-like spines on the outer side of the thecal plates.

The periflagellar area consisted of seven platelets bearing the wing-like structure, two platelet without such structure, one flagellar pore and one accessory pore (Fig. 5). The flagellar pore (fp) was larger than the accessory pore (ap). Of all the wing-like structures on the periflagellar area, the wing-like structure of platelet 1 was the largest (Fig. 5) and the wing-like structure of platelet 2 was the smallest (Fig. 5A-B). Platelets 4 and 7 bore no such structures (Fig. 5B and E). Platelet 7 was hidden by platelet 1 (Fig. 5B and E), and was difficult to observe. The two pores were separated by platelet 8 bearing the wing-like structure on both sides (Fig. 5B, C, E). The ap was surrounded by platelets 7 and 8, whereas the fp was surrounded by platelets 3, 5a, 5b, 6 and 8 (Figs. 5A-C). In conclusion, based on

the SEM observations of periflagellar area, the platelet formula was 1, 2, 3, 4, 5a, 5b, 6, 7, 8 (Fig. 5F).

4.2. Phylogeny of Prorocentrum obtusidens

The rDNA sequences (SSU, ITS region and LSU rDNA sequences) of all strains of *Prorocentrum obtusidens* obtained in this study were identical. The sequences grouped with those of *P. donghaiense*, *P. shikokuense* and *P. dentatum* recorded from Korea, Japan, China (East China Sea), the Pacific and South America (Figs. 6-8).

The ML and Bayesian inference analyses based on the SSU rDNA sequences showed that the strains of *P. obtusidens* (accession numbers: MH729037-MH729044 and MK713637) formed a clade with the Korean (AJ841810 and DQ336054) and Chinese (AY551272, AY551273 and AY803743) strains of *P. donghaiense*, the Japanese strains (AB781324, AB781325 and AB781326) of *P. shikokuense* and the South American strains (AY803742 and DQ336057) labelled as *P. dentatum* (Fig. 6). This clade was sister to *P. cordatum* (Ostenfeld) Dodge (AY803740, AY421791 and DQ336066), which formed a strongly supported clade with 100% ML bootstrap support and 1.00 Bayesian posterior probability (PP).

In the phylogenetic tree based on ITS region sequences, the strains of *P. obtusidens* established in this study (accession number: MK217267-MK217274 and MK713638) and Chinese (KF032445, KF998562, HQ833324, JN595869, JN595870, AY465116 and KY290718) strains of *P. donghaiense*, Japanese strains of *P. shikokuense* (AB781328 and AB781329) and Pacific strains labelled as *P. dentatum* (FJ823581 and EU927560) formed a clade with 87% ML bootstrap support and 1.00 Bayesian PP (Fig. 7). This clade showed a close phylogenetic relationship with *P. balticum* (Lohmann) Loeblich (EU927547), although the relationship had relatively weak support (60% ML bootstrap support and 0.99 Bayesian PP). In addition, the clade and *P. balticum* were sister to *P. cordatum* (AF208244 and EU927538).

As for the phylogenetic trees based on the SSU rDNA and ITS region, the LSU-based phylogenetic tree showed that the strains of *P. obtusidens* (MH729045-MH729051, KX656893 and MK713639) established in this study and Chinese (AY863006, AY833516, AY822610 and EU856259) strains of *P.*

donghaiense formed a strongly supported clade with 100% ML bootstrap support and 1.00 Bayesian PP (Fig. 8). Unfortunately, no LSU rDNA sequences of *P. shikokuense* from Japan and *P. dentatum* from the Pacific were reported. In this phylogenetic tree, the clade consisting of strains from this study, and Chinese strains were closely related to the strains of *P. cordatum* (EU532479 and EU780639) and *P. balticum* (AF042816) (100% ML bootstrap support and 1.00 Bayesian PP).

5. Discussion

5.1. Prorocentrum obtusidens *is a separate species from* P. dentatum sensu stricto, P. veloi *and* P. monacense

Line drawings and morphological characteristics of *Prorocentrum obtusidens*, *P. dentatum* and allied species recorded in previous studies are shown in Fig. 9 and Table 2. According to the taxonomic division of the genus *Prorocentrum* suggested by Dodge (1975), *P. obtusidens* described in this study belongs to Section E defined by "spiny thecal plates present, trichocyst pores and anterior spine may also occur". This section also includes *P. dentatum*, however in the original description of *P. dentatum*, the spiny thecal plates were not recorded (Stein 1883). Dodge (1975) suggested that *P. obtusidens* and *P. dentatum* are varieties of the same species, characterized by an anterior extension at one side which may be pointed or blunt, and thus regarded *P. obtusidens* as a junior heterotypic synonym of *P. dentatum*. Dodge (1975) displayed drawings of *P. obtusidens* and *P. dentatum* (Figs. 9S- T), however, in Dodge (1982, his Fig. 2), he only showed a drawing similar to *P. obtusidens* and recorded it as *P. dentatum*. (Fig. 9S). It is possible that the unclear descriptions and classification of *P. dentatum* by Dodge (1975, 1982) have created the confusion for taxonomists in the identification of these and related species. Dodge (1975) also mentioned 36-60 µm as the range in cell length for *P. dentatum* to include the size of *P. obtusidens* originally recorded by Schiller (1928) (36 µm in length). However, Throndsen (1983) pointed out that Dodge (1975) made an error in his measurements; the sizes of the specimens provided by Dodge (1975) are 28 µm (Dodge, 1975, his plate 4B), and 23 and 24 µm in length (Dodge, 1975, his plate 4A).These sizes are much smaller than those of *P. dentatum* stated by Schiller (1931) (50-60 µm length), although in the original description of *P. dentatum*, the size is not mentioned (Stein, 1883).

In addition, the cells in the plates provided by Dodge (1975) correspond to the morphology of *P. obtusidens* in having a blunt anterior extension at one side and parallel sides toward the anterior. Consequently, the morphological description of the specimen provided by Dodge (1975) differs from *P. dentatum sensu stricto* which is characterized by a leaf shaped outline with a tapered posterior side (heart-shaped outline in the description of Dodge, 1975), 50-60 µm in cell length and sharply pointed anterior extension at one side in the description of Schiller (1931), and it is likely that the specimen identified as *P. dentatum* by Dodge (1975) matches *P. obtusidens*, rather than *P. dentatum.*

Although morphological details of *P. obtusidens sensu stricto* were not sufficiently elucidated by Schiller (1928), the original description by Schiller (1928) showed that this species was characterized by an elongated cell shape, parallel sides towards the anterior, a distinct blunt extension at one side, the presence of thecal plate pores, and 36 µm in length and 16-20 µm in depth (Fig. 9D). Except for the difference in cell size, the morphological features of *P. obtusidens sensu stricto* are almost identical to those of the studied specimens, however they clearly differ from those of *P. dentatum sensu stricto* (Stein 1883, Figs. 9A- B). After the original description of *P. dentatum* from the Atlantic Ocean (Stein, 1883), this species was recorded by Lohmann (1920), with the morphological description of his specimen characterized by an asymmetric cell outline and almond shape in lateral view, 39 µm in length, a sharp pointed anterior extension at one side and a round nucleus located in the center of the cell (Fig. 9C). Based on the recorded morphological descriptions, *P. dentatum* can be distinguished from *P. obtusidens* by its lack of parallel sides, with differences in anterior extension and size (e.g. Stein, 1883; Schiller, 1928, 1931). In addition, Halim (1960) recorded the occurrence of *P. dentatum* from the Mediterranean Sea, with a drawing of cell outline (Fig. 9L), and Rampi (1969) provided a photograph of *P. dentatum* that seems to be morphologically identical to the original description of *P. dentatum* (see Pl. 1, Fig. 1 in Rampi, 1969). Nevertheless, Dodge (1975) considered that *P. obtusidens* and *P. dentatum* are varieties of the same species, without a detailed discussion on morphological comparisons and similarities. According to Osorio-Tafall (1942), *P. veloi* is close to both *P. dentatum* and *P. obtusidens*. However, in the line drawing of Osorio-Tafall (1942) the species, which is characterized by 33 µm in length, a leaf shape outline with a tapered posterior, a sharply pointed anterior extension at

one side and a rounded nucleus located in the center of the cell (Figs. 9G-I), it is here considered closer to *P. dentatum* than *P. obtusidens*. Taylor (1976) recorded that two specimens collected from the Indian Ocean agree with *P. veloi* in length and in general shape and concluded that *P. veloi* and *P. obtusidens* may be conspecific (Figs. 9D and X). However, the specimens lack parallel sides towards the anterior. Consequently, *P. obtusidens* can be distinguished from *P. dentatum* and *P. veloi* by a relatively small size, parallel sides towards the anterior and a blunt anterior extension on one side.

According to the taxonomic classification suggested by Dodge (1975), *P. monacense* Kufferath is also a synonym of *P. dentatum*. According to Kufferath (1957), *P. monacense* is very close to *P. obtusidens*, however it is distinguished by a marked asymmetry and no parallel sides (Fig. 9J). In the original description of *P. monacense*, the pores are densely distributed in the center of the thecal plates (Figs. 9J-K). This feature is not visible in *P. obtusidens*. In addition, the cell size of *P. monacense* is 42 µm in length and 19 µm in depth, which makes it larger than *P. obtusidens sensu stricto* and smaller than *P. dentatum sensu stricto*. In particular, in ventral or dorsal view of *P. monacense* pores can be seen to be arranged on both sides of the median line, which are not shown in *P. obtusidens* and *P. dentatum*. These morphological differences indicate that *P. monacense* cannot be considered to be a synonym of either *P. obtusidens* or *P. dentatum*.

In the phylogenetic trees based on the SSU rDNA and ITS region in this study, all established strains share identical sequences with the South American (AY803742 and DQ336057) and the Pacific strains (FJ823581 and EU927560) of *P. dentatum* provided by the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA (strain no. CCMP 1517) and the Roscoff Culture Collection, France (strain no. RCC 848). The morphological features of these specimens are characterized by their small size (14-17 µm length and 8-12 µm depth), the parallel sides towards the anterior, a blunt extension at one side and a round nucleus located in the posterior region of the cell (e.g., Lü et al., 2003; Lu et al., 2005; Le Gall et al., 2007). These characteristics can also be observed in specimens identified as *P. dentatum* collected from the Mexican Pacific (Hernández-Becerril et al., 2000), however, the characteristics do not coincide with the morphological description of *P. dentatum sensu stricto*. This indicates that the South American and the Pacific strains are not *P. dentatum*. In conclusion, *P.*

obtusidens is a separate species from *P. dentatum sensu stricto*, *P. veloi* and *P. monacense*.

5.2. Prorocentrum obtusidens *is senior to* P. shikokuense, P. donghaiense *and* P. dentatum sensu Yoo and Lee (1986)

Based on the morpho-molecular data, Lu et al. (2005) reported that specimens previously reported as *P. dentatum sensu* Yoo and Lee (1986) and *P. donghaiense* are conspecific, and Takano and Matsuoka (2011) reported that *P. donghaiense* and *P. shikokuense* can be considered to be the same species. Because these species are also similar, morphologically, to *P. obtusidens*, the morphological similarities are discussed here.

In the phylogenetic trees based on the SSU, ITS region and LSU rDNA sequences, *Prorocentrum obtusidens* established in this study shared identical sequences with *P. shikokuense* and *P. donghaiense* established from Japan and China, and *P. dentatum* established from South America, the Pacific and France. The morphological features of *P. shikokuense* and *P. donghaiense* (e.g. Hada, 1975; Lu and Goebel, 2001; Lu et al., 2005; Takano and Matsuoka, 2011), such as the shape and size of the cell, a blunt extension at one side, the presence of thecal pores, knob-like spines on the surface of the thecal plates and a round nucleus located in the posterior region of the cell, also coincide with those of *P. obtusidens* described in this study (Table 2). In addition, the morphological features are identical to those of *P. shikokuense* reported from the southern Adriatic Sea (Roselli et al., 2019, their figs. 2-3). Consequently, previously recorded *P. shikokuense*, *P. donghaiense*, and *P. dentatum sensu* Yoo and Lee (1986) and *P. obtusidens* described in this study seem to be conspecific and are distributed worldwide. In particular, it is notable that *P. shikokuense* from the southern Adriatic Sea, which is the type locality of *P. obtusidens*, share identical morphological features with specimen studied here.

Although Lu and Goebel (2001) and Lu et al. (2005) reported *P. donghaiense* as an independent species, they recorded the morphological similarity between *P. donghaiense* and *P. obtusidens sensu stricto*; the outlines of *P. donghaiense* characterized by parallel sides towards the anterior are quite similar to those of *P. obtusidens*, rather than of *P. dentatum* (see Lu et al., 2005, their figs. 2-3). However, the line drawing of *P. donghaiense* provided by Lu et al. (2005) is somewhat unclear (Fig. 9Z), possibly

due to the observations of the species having been made fixed samples. In addition, they realized that *P.* aff. *obtusidens* Schiller sensu Throndsen (1983) is closer to *P. donghaiense* in cell shape and size (Fig. 9Y). Besides the latter features, both *P. donghaiense* and *P.* aff. *obtusidens* have a round nucleus located in the posterior region of the cell and a similar appearance of the chloroplasts. Although Throndsen (1983) used 'affinity' to suggest the difference in cell size and shape of posterior end with *P. obtusidens*, the morphological features of *P.* aff. *obtusidens* are identical to those of *P. obtusidens* described in this study (Fig. 9Y).

After the original description of *P. obtusidens* by Schiller (1928), this species has been recorded by Böhm (1936) and Wood (1963), whom provided simple drawings of the cell outline (Figs. 9E, F, M and N). In the descriptions, the specimens are characterized by parallel sides towards the anterior and a distinct blunt extension at one side, which are also seen in *P. donghaiense*, *P. shikokuense* and *P. obtusidens* in this study. However, the size of *P. obtusidens* recorded by Wood (1963), which is 50 µm in length, is much larger than the cell lengths reported for *P. donghaiense, P. shikokuense* and *P. obtusidens* in this study. *P. obtusidens* has also been described by Adachi (1972), based on the specimens collected from the Ago and Ofunato Bays of Japan (Figs. 9O-R). Adachi (1972) recorded detailed morphological features of the specimens characterized by 15-30 µm in length and 6-9 µm in depth, the presence of the fp in periflagellar area, parallel sides toward the anterior and a distinct blunt extension at one side. The morphological description is almost identical to that of *P. shikokuense* by Hada (1975) (Figs. 9U-V), indicating that *P. obtusidens* and *P. shikokuense* recorded from the Japanese coast are conspecific. Nevertheless, Hada (1975) did not compare *P. obtusidens* with *P. shikokuense*. Takano and Matsuoka (2011) recorded *P. obtusidens* described by Adachi (1972), however, they did not discuss the morphological similarities or differences between *P. shikokuense* and *P. obtusidens* recorded from Japanese coastal waters.

Excluding *P. obtusidens* described by Adachi (1972), the major difference between *P. shikokuense*, *P. donghaiense* and *P. obtusidens* described in this study, and previously described *P. obtusidens* is the cell size. The sizes of *P. shikokuense, P. donghaiense,* and *P. obtusidens* described in this study are generally smaller than those of previously recorded for *P. obtusidens* (Table 2). In particular, the size

(50 µm in length) of *P. obtusidens* recorded by Wood (1963) is exceptionally large. Wood (1963) showed two figures to describe *P. obtusidens*. However, although one resembles *P. obtusidens* in cell outline (Fig. 9N), the other is similar to *P. dentatum* and characterized by a leaf shaped outline, a slightly pointed anterior extension, and a tapered posterior, compared with *P. obtusidens* (Fig. 9M). This indicates that Wood's figures cannot be attributed to either *P. obtusidens* or *P. dentatum*. Besides *P. obtusidens* recorded by Wood (1963), the sizes of *P. obtusidens* described by Schiller (1928) and Böhm (1936), which are 36 and 34 µm in length, respectively, are slightly larger than those of *P. shikokuense*, *P. donghaiense* and *P. obtusidens* described in this study. However, as the maximum size of *P. shikokuense* from Iwamatsu Bay of Japan is 31.7 µm in length (Takano and Matsuoka, 2011), the sizes recorded by Schiller (1928) and Böhm (1936) are not much larger. In addition, given that cell size in *Prorocentrum* species can change under different environmental conditions of sampling areas (Hulburt, 1965), other taxonomic characteristics as well as the cell size of the target species should also be considered to allow species identification.

5.3. The periflagellar area of Prorocentrum obtusidens

The external shapes of the periflagellar area of *P. donghaiense* and *P. shikokuense* have been reported (Lu et al., 2005; Takano and Matsuoka, 2011), however, its architectural details have not been described. This study first provides the details of the periflagellar area.

According to Lu et al. (2005), *P. donghaiense* has a V-shaped periflagellar area with an "ear-shaped" extension (Lu et al., 2005, their fig. 6). In their paper, the extension corresponds to the wing-like structure of the platelet 1 described in this study. Platelet 1 is the largest, which is also shown in benthic and planktonic *Prorocentrum* species (Hoppenrath et al., 2013; Han et al., 2016; Ndhlovu et al., 2017). In benthic *Prorocentrum* species, a very narrow and small platelet (platelet 7 in the labelling system of Hoppenrath et al., 2013) always contacts the accessory pore (ap), and in many species the ap is surrounded by platelets 7 and 8 (Hoppenrath et al., 2013). This feature is identical to what is seen in *P. obtusidens*, however the platelet 7 is difficult to detect in the periflagellar area of *P. obtusidens*.

Platelets bearing the wing-like structure were also recorded in benthic *Prorocentrum* species

(Hoppenrath et al., 2013), however the number of platelets having a wing-like structure is different from *P. obtusidens*; benthic *P. emarginatum* have wing-like structures in one or two platelets (platelet 1 or 1 and 4), whereas the wing-like structures of *P. obtusidens* are observed in seven platelets (the platelet 1, 2, 3, 5a, 5b, 6, 8). In addition, planktonic *Prorocentrum* species, such as *P. triestinum*, *P. koreanum* Han, Cho & Wang and *P. micans* Ehrenberg, bear a spine on the platelet 1 (Han et al., 2016; Ndhlovu et al., 2017; Tillman et al., 2019). Consequently, the number of platelets bearing a wing-like structure and the difference in the structure of platelet 1 may be prominent features for distinguishing *P. obtusidens* from other *Prorocentrum* species.

Taxonomic considerations

***Prorocentrum obtusidens* Schiller 1928: p. 57, fig. 15**

Heterotypic synonyms: *P. shikokuense* Hada [Hada, 1975, p. 37, figs 1, 2 (as '*shikokuensis*')]; *P. donghaiense* Lu (Lu & Goebel, 2001 p. 338, fig. 2)

Emended diagnosis: Cells are elongated, slightly asymmetric and narrow toward the posterior end. Cells are solitary, or sometimes form two- and four-celled chains. The cells are brownish-yellow in color, and parallel sides toward the anterior, a blunt extension at one side, the presence of thecal plate pores and knob-like spines on the surface of the thecal plates, and a rounded nucleus located in the posterior region of the cell. The chloroplast is visible in the center and along the margins of the cell. The V-shaped periflagellar area is visible on the right thecal plate, and the thecal pores are randomly distributed near the periflagellar area. The periflagellar area of *Prorocentrum obtusidens* consists of seven platelets bearing the wing-like structure and two platelets without the wing-like structure, one flagellar pore, and one accessory pore.

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Table 1. Information on strains of *P. obtusidens* used in this study.

Strain No.	Year of isolation	Locality	Length (µm)	Depth (µm)	GenBank No(s)		
			mean ± SD* min–max	mean ± SD* min–max	SSU	ITS	LSU
LIMS-PS-2540	2017	East China Sea	19.9±1.6 17.3–22.9	9.1±1.3 6.4–12.3	MH729037	MK217267	MH729045
LIMS-PS-2533	2017	East China Sea	17.8±2.0 12.7–21.0	8.9±1.1 6.8–11.0	MH729038	MK217268	MH729046
LIMS-PS-2530	2017	East China Sea	17.4±1.4 15.5–20.7	9.4±1.0 7.4–11.1	MH729039	MK217269	MH729047
LIMS-PS-2534	2017	East China Sea	18.8±1.9 14.7–22.1	8.7±1.1 7.1–11.5	MH729040	MK217270	MH729048
LIMS-PS-2542	2017	Korean coast	17.7±1.3 15.5–20.7	9.1±1.1 6.1–12.1	MH729041	MK217271	MH729049
LIMS-PS-2529	2017	Korean coast	18.2±1.7 12.3–22.1	8.4±1.2 7.1–12.0	MH729042	MK217272	MH729050
LIMS-PS-2536	2017	East China Sea	17.8±1.3 15.7–21.2	8.9±1.3 6.7–10.9	MH729043	MK217273	MH729051
-*	2016	Gamak Bay, Korea	18.3±1.7 17.1–22.0	9.1±1.1 7.9–12.1	MH729044	MK217274	KX656893
K-1260	2009	Canary Islands, Spain	15.33±2.1 10.9–20.1	8.1±1.3 6.1–11.2	MK713637	MK713638	MK713639

Number for size measurements = 50; * = sequences were obtained from this sample by single-cell analysis of a cell directly isolated from the bloom, whilst measurements were done on cells from a fixed plankton sample.

Table 2. Morphological characteristics of *Prorocentrum obtusidens* and related *Prorocentrum* species

	P. obtusidens	***P. obtusidens***	***P. obtusidens***	***P.* aff. *obtusidens***	***P. donghaiense***	***P. shikokuense***	***P. shikokuense***	***P. dentatum***	***P. dentatum***	***P. dentatum***	***P. veloi***	***P. monacense***
Cell size	17.3–22.9 μm (L); 6.4–12.3 μm (D)	36 μm (L); 16–20 μm (D)	50 μm (L)	21–22 μm (L); 8.5–9 μm (D)	18.6–21.6 μm (L); 9.6–13.0 μm (D)	20–27 μm (L); 7–10 μm (D)	20.2–31.7 μm (L); 8.0–14.3 μm (D)	ND	50–60 μm (L)	36–60 μm (L); 15–20 μm (D)	33 μm (L); 12 μm (D)	42 μm (L); 19 μm (D)
Parallel side	Visible	Visible	Visible	Visible	Visible	Visible	Visible	Invisible	Invisible	Visible	Invisible	Invisible
Extension of anterior end	Blunt	Blunt	Blunt	Blunt	Blunt	Blunt	Blunt	Sharp	Sharp	Blunt	Sharp	A point thick, a little rounded
Knob-like spine on the surface	Visible	ND	Visible	ND	Visible	ND	Visible	ND	ND	Visible	Visible	ND
Pore size	0.15 μm	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pusule	Peanut-shaped or round	ND	ND	ND	ND	Round	ND	ND	ND	ND	ND	ND
Shape and location of nucleus	Round, posterior	ND	ND	Round, posterior	Round, Posterior	Round, posterior	Round, posterior	ND	ND	ND	Round, center	ND
Chloroplast	Visible in center and margin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Periflagellar area	V-shaped (9 platelets)	ND	ND	ND	V-shaped	ND	V-shaped	ND	ND	ND	ND	ND
Reference	This study	Schiller (1928)	Wood (1963)	Throndsen (1983)	Lu et al. (2005)	Hada (1975)	Takano and Matsuoka (2011)	Stein (1883)	Schiller (1931)	Dodge (1975)	Osorio-Tafall (1942)	Kufferath (1957)

L= length; D = depth; ND= no data available

Figure legends

Fig. 1. (A) Circulation pattern modified from Hu and Yang (2001) and Yuan et al. (2008), (B) sampling sites in the East China Sea and Korean coastal area and (C) the dark-brown discoloration of the water caused by *Prorocentrum obtusidens* in Gamak Bay, Korea. KC: Kuroshio Current; TWC: Taiwan Warm Current; ECSCC: East China Sea Coastal Current; CDW: Changjiang Diluted Water; TC: Tsushima Current; KCC: Korean Coastal Current.

Fig. 2. Light and fluorescence micrographs of *Prorocentrum obtusidens* collected from Gamak Bay, Korea and East China Sea (Strain LIMS-PS-2540). (A-C) Cells collected from Gamak Bay, Korea, showing pusules (black arrows); (D) fluorescence micrograph of a cell collected from Gamak Bay, Korea, showing chloroplasts; (E-F) SYTOX Green-stained cells collected from Gamak Bay, Korea and East China Sea, showing the position of the round nucleus; (G) two-celled chain collected from Gamak Bay, Korea, showing pusule (black arrows); (H-I) four-celled chains collected from the East China Sea. Scale bars = 10 μm.

Fig. 3. Scanning electron microscope images of *Prorocentrum obtusidens* collected from Gamak Bay of Korea (A-D) and East China Sea (Strain LIMS-PS-2540) (E-H). (A, B, E-F) Right thecal plates and (C, D, G-H) left thecal plates of the cells. The V-shaped excavations of the periflagellar area (white arrowheads) are visible on the right thecal plates of the cells. Scale bars = 5 μm.

Fig. 4. Scanning electron microscope images of *Prorocentrum obtusidens* (Strain LIMS-PS-2540). (A) Ventral view of cell showing the intercalary band; (B) detail of the intercalary band showing rows of tiny knobs; (C) posterior region of the cell showing thecal pores (white arrows) and knob-like spines (black arrows); (D) inner surface of the right thecal plates showing the periflagellar area (black arrow); (E-F) inner surface of the left thecal plates showing small hollows (white arrows); (G-H) details of small hollows on inner surface of the cell. The internal small hollows probably correspond to the thecal pores on the outside. Scale bars = 5 μm (A, C, D-F and I).

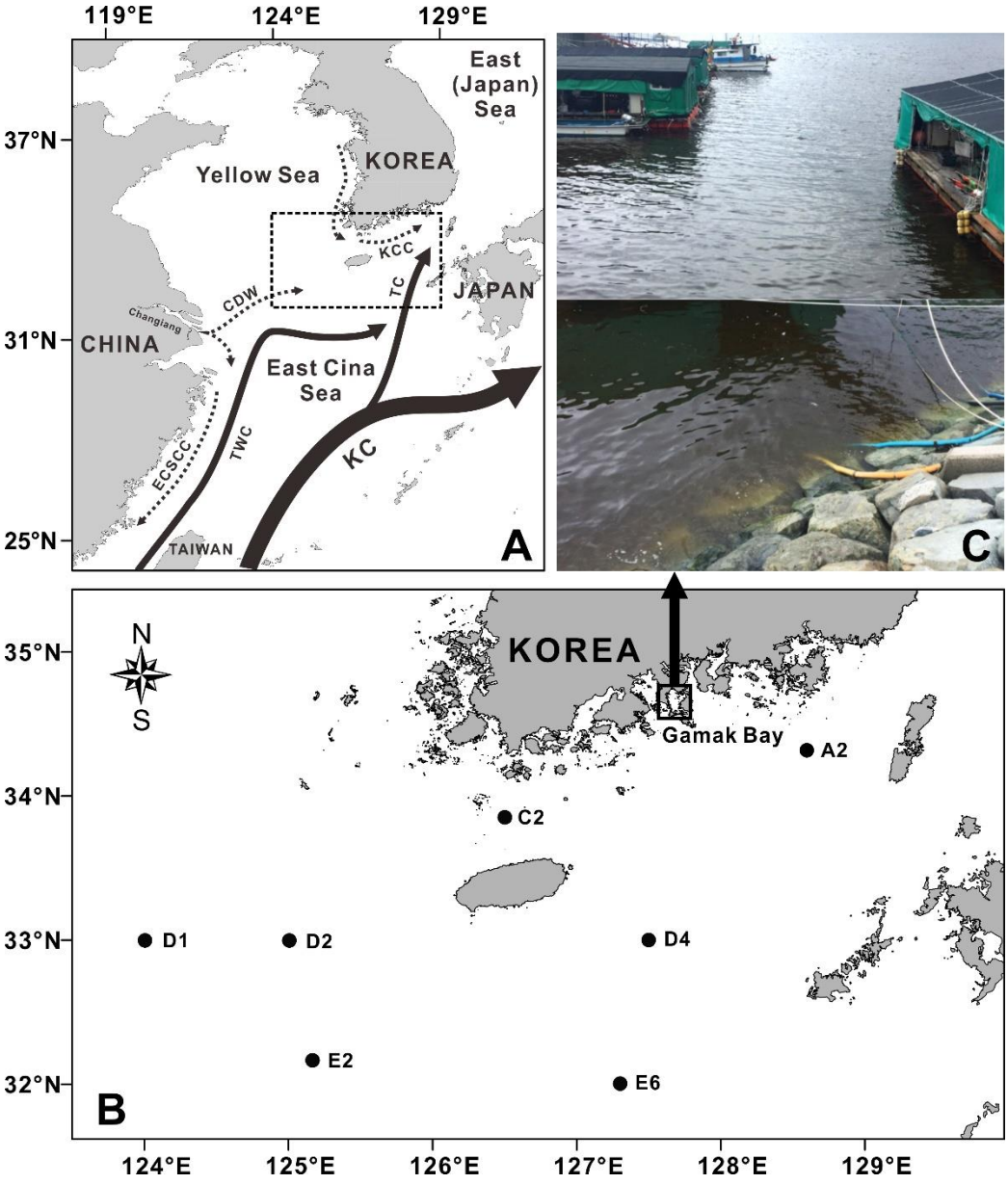
Fig. 5. Scanning electron microscope micrographs and line drawing of the periflagellar area of *Prorocentrum obtusidens* (Strain LIMS-PS-2540). (A-D) Details of the labelled platelets, accessory pore (white arrows) and flagellar pore (white arrowheads). ap = accessory pore, fp = flagellar pore. Scale bars = 1 μm.

Fig. 6. Maximum likelihood (ML) tree showing the phylogenetic positions of *Prorocentrum obtusidens* (in bold) based on nuclear-encoded SSU rDNA sequences (1603 bp). Two isolates of *Scrippsiella acuminata* (AY421792 and EF492513) were used as the outgroup. The numbers at each node are the bootstrap value (%) followed by the Bayesian posterior probability (PP). Only bootstrap values above 50% and PP above 0.7 are shown. The GenBank accession number follows each taxon name. Scale bar = 0.1 nucleotide substitutions per site.

Fig. 7. Maximum likelihood (ML) tree showing the phylogenetic positions of *Prorocentrum obtusidens* (in bold) based on ITS1+5.8S+ITS2 sequences (574 bp). Two isolates of *Scrippsiella acuminata* (AY421792 and EF492513) were used as the outgroup. The numbers at each node are the bootstrap value (%) followed by the Bayesian posterior probability (PP). Only bootstrap values above 50% and PP above 0.7 are shown. The GenBank accession number follows each taxon name. Scale bar = 0.5 nucleotide substitutions per site.

Fig. 8. Maximum likelihood (ML) tree showing the phylogenetic positions of *Prorocentrum obtusidens* (in bold) based on partial nuclear-encoded LSU rDNA sequences (1223 bp). Two isolates of *Scrippsiella acuminata* (AY421792 and EF492513) were used as the outgroup. The numbers on each node are the bootstrap values (%) followed by the Bayesian posterior probability (PP). Only bootstrap values above 50% and PP above 0.7 are shown. The GenBank accession number follows taxon name. Scale bar = 0.1 nucleotide substitutions per site.

Fig. 9. Line drawings of *Prorocentrum obtusidens* and related species. (A-B) *P. dentatum* (Stein 1883); (C) *P. dentatum* (Lohmann 1920); (D) *P. obtusidens* (Schiller 1928); (E-F) *P. obtusidens* (Böhm 1936); (G-I) *P. veloi* (Osorio-Tafall 1942); (J-K) *P. monacense* (Kufferath 1957); (L) *P. dentatum* (Halim 1960); (M-N) *P. obtusidens* (Wood 1963); (O-R) *P. obtusidens* (Adachi 1972); (S-T) *P. dentatum* (Dodge 1975); (U-V) *P. shikokuensis* (=*P. shikokuense*) (Hada 1975); (W-X) *P. veloi* (Taylor 1976); (Y) *P.* aff. *obtusidens* (Throndsen 1983); (Z) *P. donghaiense* (Lu et al. 2005). Drawings are not to scale.



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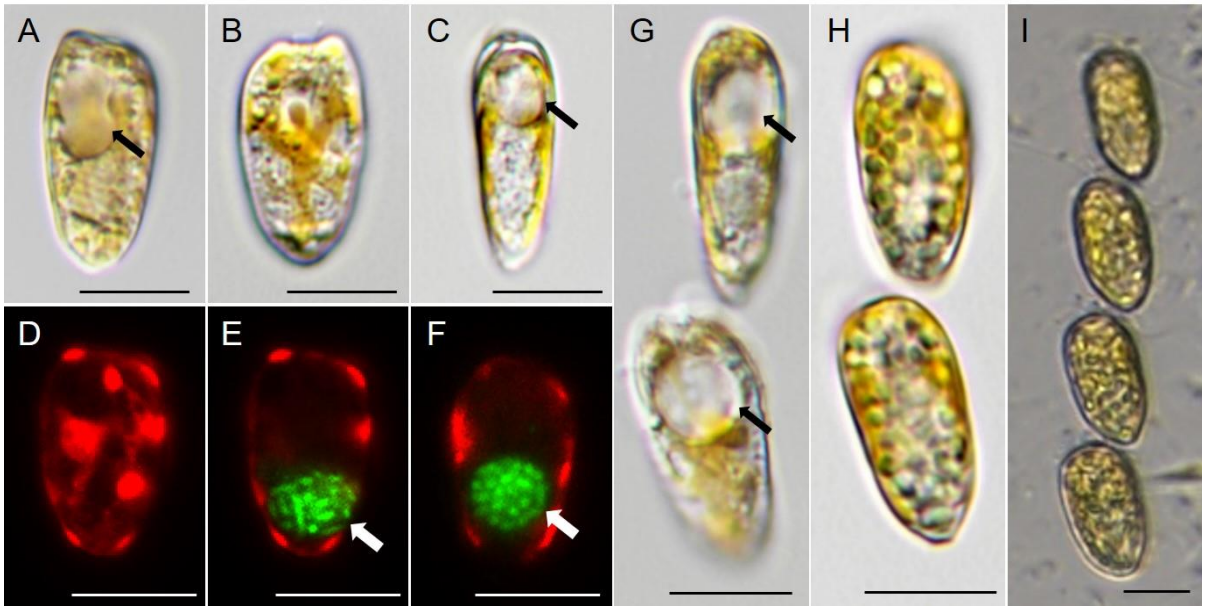

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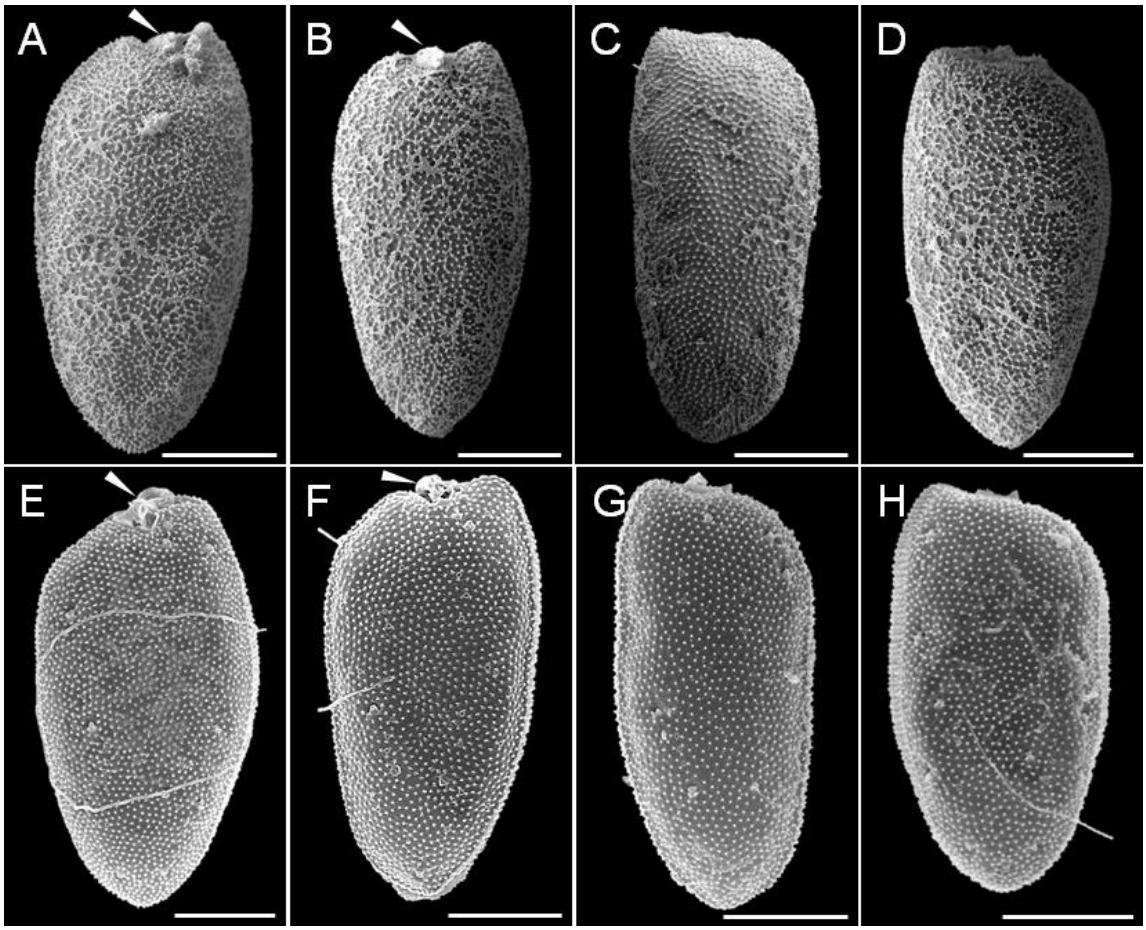

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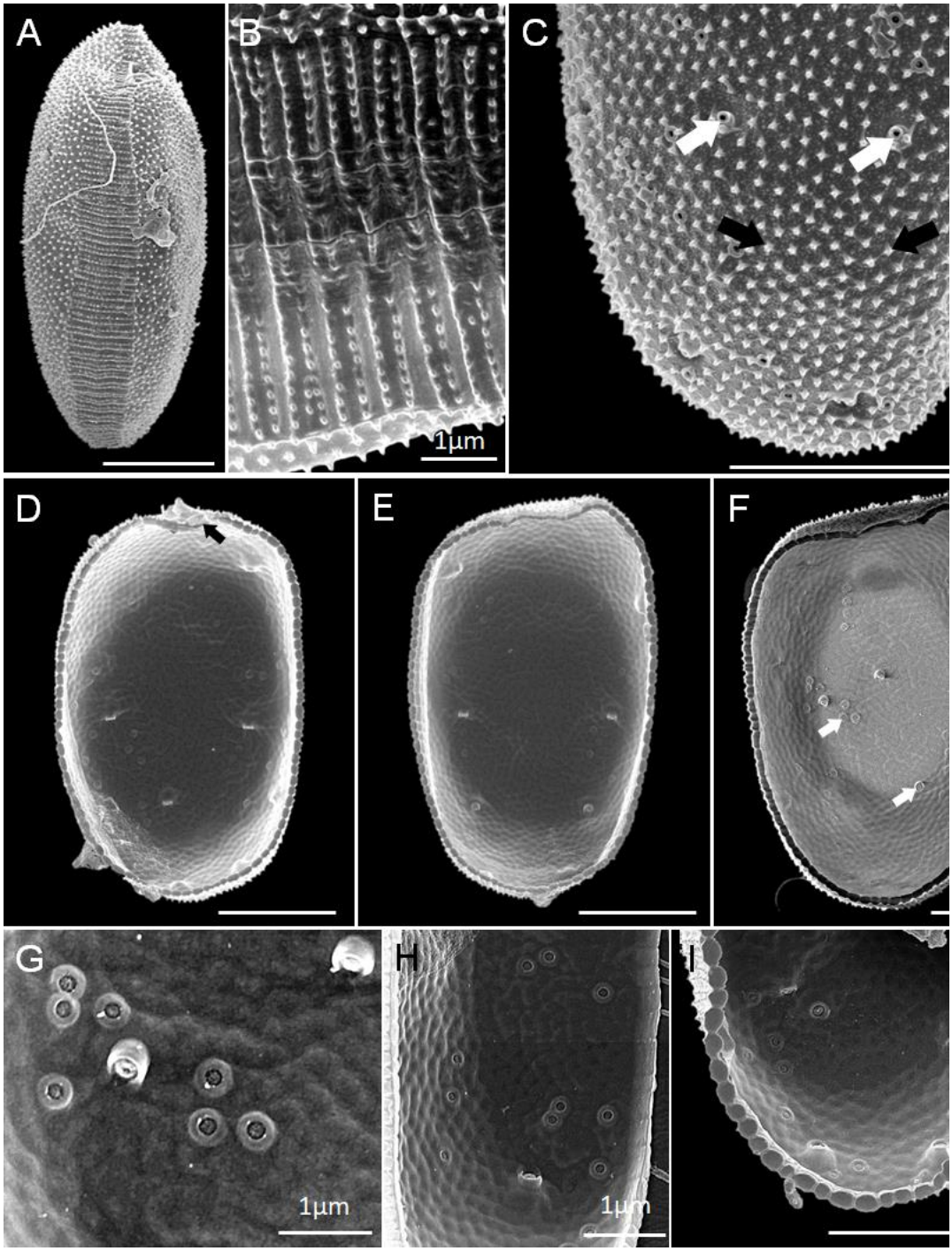

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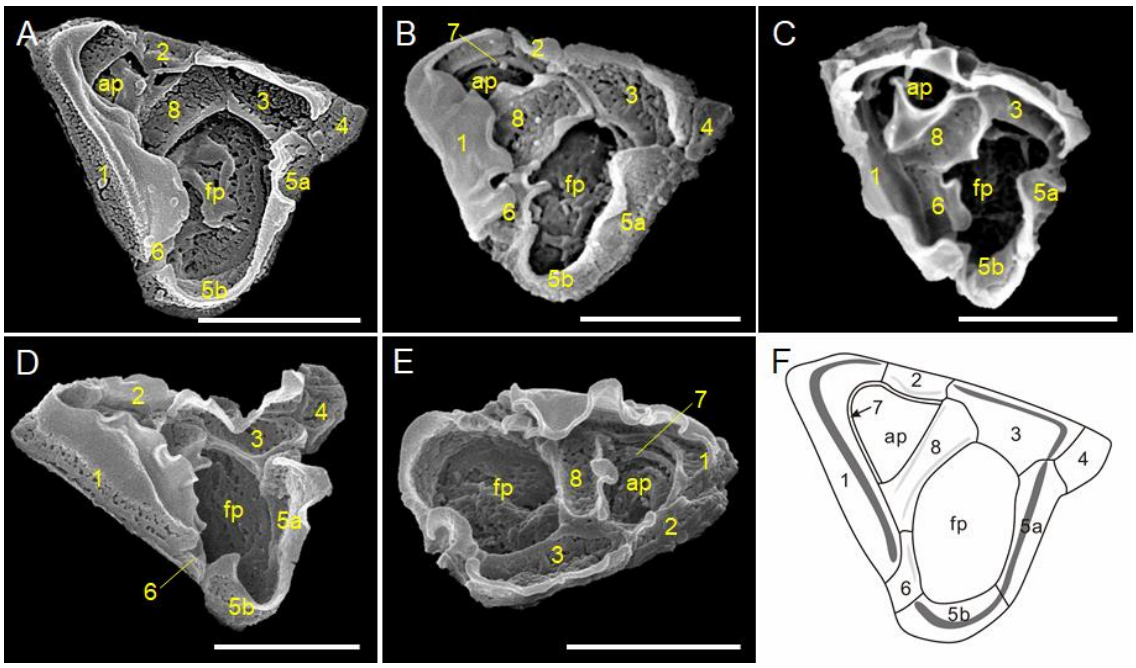

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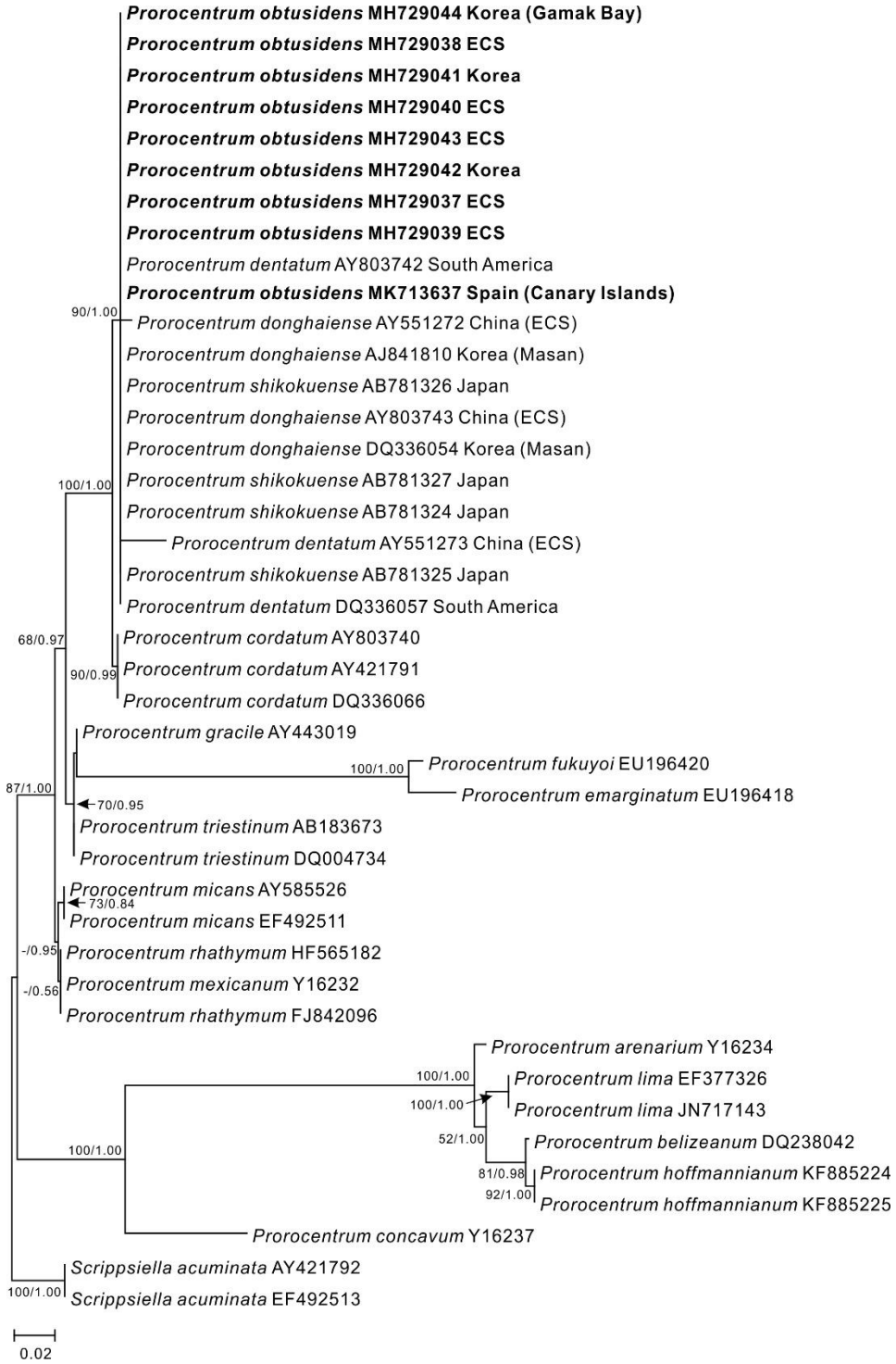

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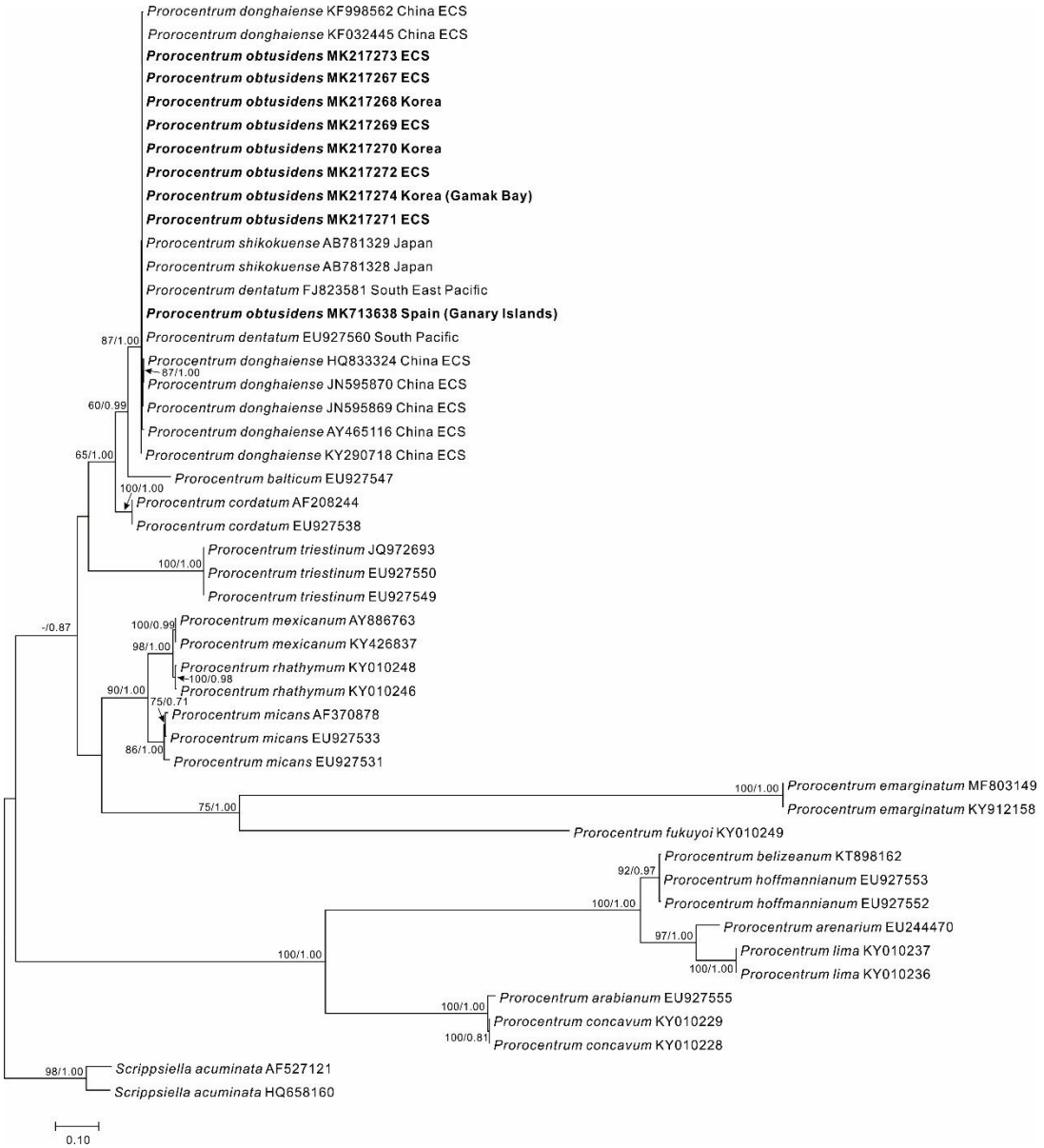

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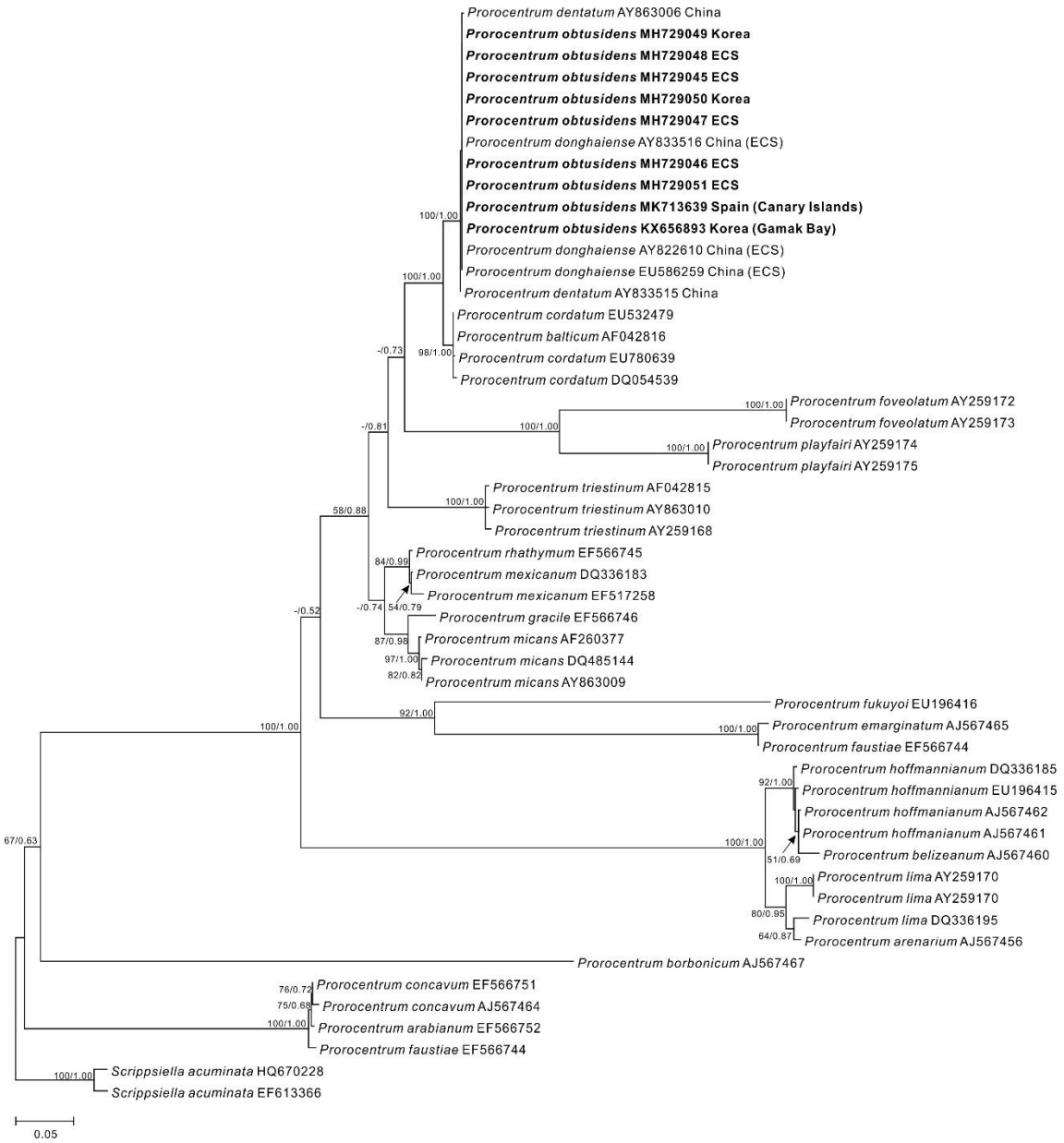

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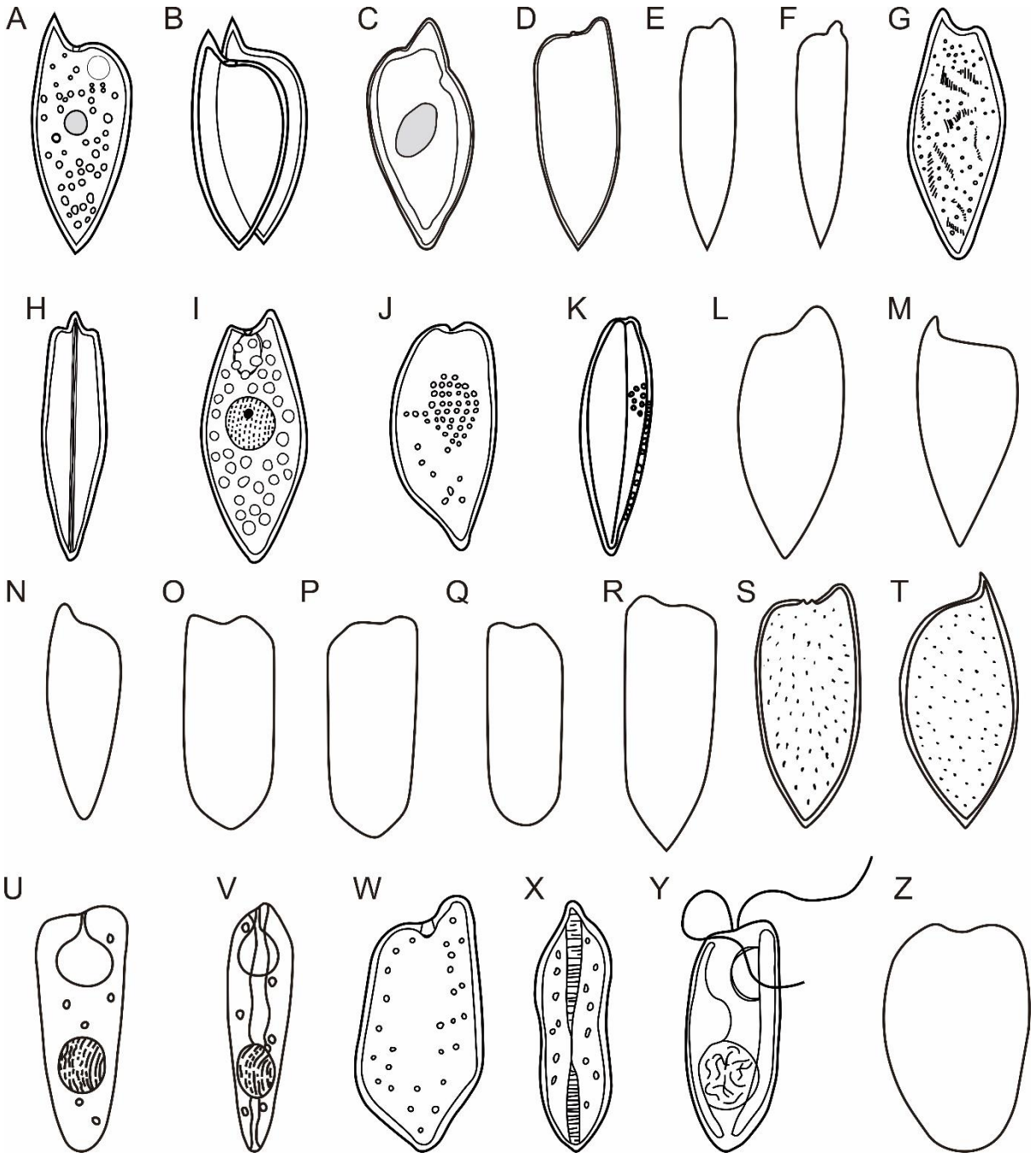

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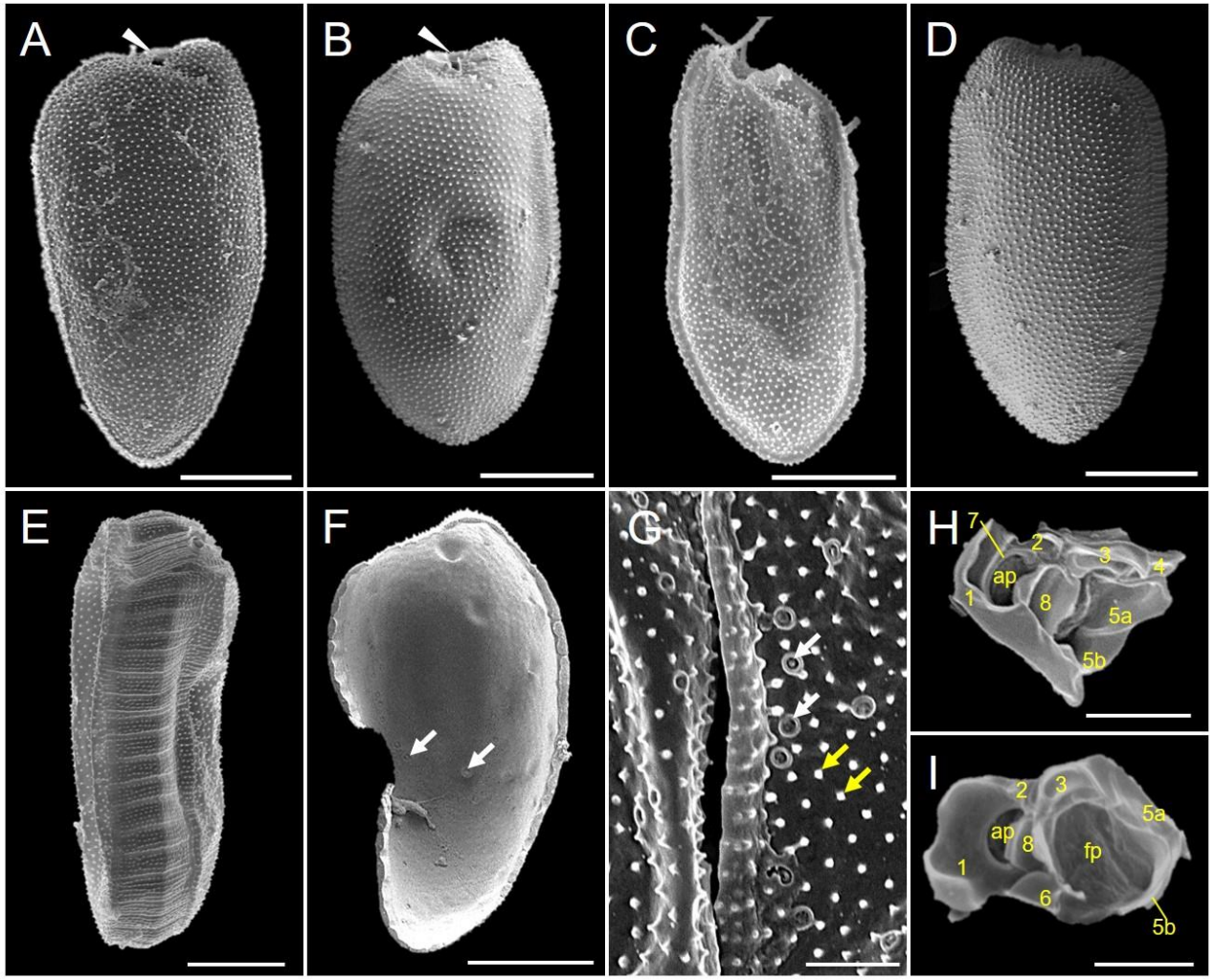

Fig. S1. Scanning electron microscope images of *Prorocentrum donghaiense* collected from waters off the Canary Islands of Spain (strain K-1260). (A, B) Right thecal plates and (C, D) left thecal plates of the cells. The V-shaped excavation of the periflagellar area (white arrowheads) is visible on the right thecal plate of the cells. (E) Ventral or dorsal view of cell showing the intercalary band. (F) Inner surface of the left thecal plate showing small hollows (white arrows). (G) Outer surface of the thecal plate showing thecal pores(white arrows) and knob-like spines (yellow arrows). (H, I) Details of the periflagellar area. Scale bars: 5 μm (A-F); 1 μm (G-I).