**Taxonomy and molecular phylogenetics of Ensiculiferaceae, fam. nov. (Peridiniales, Dinophyceae), with consideration of their life-history**

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Please note that there was a mistake with SEM images. The SEM images from the P. imariense strain were not from France, but from New Zealand. I’ve made the modifications. You also need to include the sequence and publish it here:

>Ensiculifera imariensis Opoa Bay D1\_D3 (18-719)

GATTCCCTCAGTAATGGCGAATGAACAGGGATAAGCTCAGCATGGAAATTGGGGCCTGCGGCCTTGAATTGTAATCTGGAGATGTATTGCCAACGGAGGCGCAGATGTAAGCCTCTTGGAAAAGAGCATCATCGAGGGTGAGAATCCCGTTTGTCATCTGCAGTCCCCCGTGCACGGCATACCTTCTAAGAGTCACGTTCCTCGGGATTGGAGCGCAAAGTGGGTGGTAAATTTCATCTAAAGCTAAATATTGGTTTGAGACCGATAGCAAACAAGTACCATGAGGGAAAGGTGAAAAGGACTTTGAAAAGAGAGTTAAAAGTGCCTGAAATTGTTGAAAGGGAAGCGAATGGAACCAGTGTGTCGTGGCGAGATTGTTGCATGCCAATGTGATGATCTGCTGTTTCAGCGCAAGTGTGGCAGTAGGTTTTGATCAGGATGTGTGCAATGCTTCTTGCCTTGTGTGTCAACTTCAATTCGCATTTGAAGAAAACTCCAAGGACATGGTAACTTGCCTTCGGGTGTGTGAATGTGTTTGGCTGAATTCATATGTGTATTGATCGTTGACTAAATGGTTCCTTTCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATATGTGCGAGTTCACGGGTGGGTAAACCTGCTTGCGCAATGAAAGTGACTGCTGGGATTCTTGCACCAGCAACCGACCAATCAATTGAGAGAGGTTTGAGTATGAGCATATCTGTTAGGACCCGAAAGATGGTGAACTATGCCTGTGAAGGGCAAACTCAGGGGAAACTTCTGATGGAGGCTCGTAGCGATACTGACGTGCAAATCGTTCGTCATAACATGGGTAT

Note also:

* There were a lot of “fam. nov.” in the text. Reviewer 2 remarked to remove a lot of these. This is now done.
* Note also

Shin and Li can you manage these remarks?

* Haifeng remarks: P.11, line 256, Sa was not indicated in Fig. 2A; line 261, Sp was not indicated in Fig. 2I.

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**ABSTRACT**

In the current circumscription, the Thoracosphaeraceae comprise all dinophytes exhibiting calcified coccoid cells produced during their life-history. Species hitherto assigned to *Ensiculifera* are mostly based on the monadoid stage of their life-history, while species based on the coccoid stage are often unresolved. We combined the study of different life-history stages and DNA sequence data of *Ensiculifera mexicana* and other species using samples collected from all over the world. Based on concatenated rRNA gene sequences Ensiculiferaceae represented a distinct peridinalean branch, which showed a distant relationship to other calcareous dinophytes. Both molecular and morphological data (particularly of the coccoid stage) revealed the presence of three distinct clades within Ensiculiferaceae, which may include other dinophytes exhibiting a parasitic life-history stage. At a higher taxonomic level, Ensiculiferaceae showed relationships to parasites and endosymbionts (i.e., *Blastodinium* and *Zooxanthella*) as well as to dinophytes harbouring diatoms instead of chloroplasts. The unexpected phylogenetic relationships are corroborated by the presence of five cingular plates in all such taxa, which differs from the six cingular plates of most other Thoracosphaeraceae. We describe Ensiculiferaceae, emend the descriptions of *Ensiculifera* and *Pentapharsodinium*, erect *Matsuokaea* and provide several new combinations at the species level*.*

**Key words**: dinoflagellate, molecular phylogenetics, parasite, plate overlap, rRNA, taxonomic delimitation, thecal plate

**Introduction**

The life-history of the predominantly unicellular dinophytes is complex and frequently comprises at least two morphologically and ecologically distinct stages: an actively swimming monadoid stage in the water column and a benthic coccoid stage that is commonly deposited in the sediment. Coccoid cells have diverse functions (Dale 1983; Fensome et al. 1993), including dormancy to survive unfavourable ecological (e.g., seasonal) conditions, in which case they are colloquially termed ‘resting cysts.’ In addition to monadoid cells, some dinophytes include parasitic stages during their life-history, whose biodiversity began to be recognised a century ago (Chatton 1920; Chatton and Grassé 1952; Cachon and Cachon 1987; Coats 1999). The host range of parasitic dinophytes is broad and comprises fish, copepods, flatworms and corals, as well as unicellular organisms such as ciliates, radiolarians and other dinophytes. Molecular sequence data have shown that parasitic dinophytes are a polyphyletic assemblage, nesting within subordinate groups of dinophytes such as Gymnodiniales (Gómez et al. 2009), Peridiniales (Litaker et al. 1999; Coats et al. 2010) and †Suessiales (here considered to include modern taxa such as Zooxanthella, Polarella, etc.) (Levy et al. 2007). However, parasitic dinophytes remain poorly known, and precise information about host specificity, infective process, life-history, ploidy level and phylogenetic relationship is available for only a limited number of species.

The group of calcareous dinophytes is difficult to circumscribe. They are characterised by the presence of calcareous coccoid cells produced during their life-history (Elbrächter et al. 2008; Gottschling and Söhner 2013). Because of their high potential to fossilise, they are well documented in the fossil record (e.g., Wanner 1940; Deflandre 1949; Bolli 1974; Keupp 1981, 1991; Zonneveld et al. 1999; Hildebrand-Habel & Streng 2003; Streng et al. 2004). The trait of calcification is unique among extant alveolates and was considered apomorphic for calcareous dinophytes (Wall and Dale 1968; Janofske 1992; Elbrächter et al. 2008). It was surprising to discover from subsequent molecular studies that many calcareous dinophytes have close relatives, among which the potential for calcification is not known (Gottschling et al. 2005a, 2012; Gottschling and Söhner 2013). Calcareous dinophytes, together with their non-calcareous relatives, have been unified within the Thoracosphaeraceae family (Peridiniales, Dinophyceae; Elbrächter et al. 2008; Gottschling and Söhner 2013), which is subdivided into three major groups: 1) the E/Pe-clade consisting of *Ensiculifera* Balech and *Pentapharsodinium* Indel. & A.R. Loebl.; 2) the T/Pf-clade comprising Pfiesteria Steid. & J.M. Burkh., Thoracosphaera Kamptner and their relatives; and 3) the Scrippsiella Balech *sensu lato* (*s.l.*) clade (Gottschling et al. 2005a, 2012; Gu et al. 2013a). The last two clades are inferred from molecular phylogenetics; however, the phylogenetic placement of the E/Pe clade within the Peridiniales still remains unclear. A closer relationship to a clade composed of endosymbionts (i.e., *Zooxanthella* K.Brandt), parasites (i.e., *Blastodinium* Chatton) and the Kryptoperidiniaceae harbouring diatoms as endosymbionts, rather than to the other Thoracosphaeraceae, appears more likely (Gottschling and McLean 2013; Gottschling et al. 2017; Kretschmann et al. 2018a).

The taxonomy and nomenclature of *Ensiculifera* and *Pentapharsodinium* are complex. *Ensiculifera* was established by Balech (1967), with *Ensiculifera mexicana* Balech as type and delimited from *Scrippsiella* by the presence of five (versus six) cingular plates and an internal spine (‘*ensiculum*’) attached to the first cingular (c1) plate. Later, *E. loeblichii* El.R. Cox & H.J.Arn. was described; it also has five cingular plates but lacks a long spine on the c1 plate (Cox and Arnott 1971), broadening the original taxonomic concept of *Ensiculifera*. The presence of a cingular spine is not restricted to *Ensiculifera* among calcareous dinophytes. A similar structure, though attached to the anterior sulcal (Sa) not the c1 plate, is described for *Scrippsiella trochoidea* var. *aciculifera* Montresor (Montresor et al. 2003) from the *Scrippsiella s.l.* clade and *Pentapharsodinium dalei* var. *aciculiferum* H.Gu(Gu et al. 2013b) and *P. jinhaense* Zhun Li, M.S. Han & H.H. Shin (Li et al. 2015b) from the E/Pe-clade*.* Thus, the presence or absence of spines as a distinguishing feature is not restricted to *Ensiculifera*, *Pentapharsodinium* or *Scrippsiella* and they do not clearly correspond to monophyletic groups.

Indelicato and Loeblich III (1986) considered *Ensiculifera* not validly published, because a Latin description or diagnosis was not provided by Balech (1967). Therefore, they rejected the name *Ensiculifera* and erected the new generic name *Pentapharsodinium*, with *P. dalei* Indel. & A.R. Loebl. as type. They also incorporated *E. loeblichii* in *Pentapharsodinium*, while *E. mexicana* was transferred to *Scrippsiella*. However, Enrique Balech (1912–2007) consistently treated dinophytes under the International Code of Zoological Nomenclature (ICZN; Elbrächter et al. 2008), in which a Latin description is not a condition for the equivalent of valid publication. If all requirements of another relevant code are satisfied, Article 45.1 of the International Code of Nomenclature for algae, fungi, and plants (ICN; Turland et al. 2017) explains that such names can be considered validly published also in botany. A very similar case has been made for *Scrippsiella* (Andersen, 2018 and references therein), and can also be made for *Fragilidinium* Balech.

Knowledge of the relations between the different life-history stages of calcareous dinophytes has greatly improved in recent decades (e.g., Dale 1983; Lewis 1991; Gu et al. 2013c; Li et al. 2015a, 2015b; Matsuoka et al. 1990; Zinßmeister et al. 2012), and both monadoid and coccoid cells have been recognised in species of *Ensiculifera* and *Pentapharsodinium*. Identification of coccoid cells is generally based on the shape, size, colour, operculum and archaeopyle, and the constitution of the wall (not necessarily a variation on, or homologue of, the cellulosic cell wall: Elbrächter et al. 2008) in terms of ornamentation, ultrastructure (the orientation of the crystallographic c-axis of the calcitic crystals) and geochemical composition (Dale 1983; Keupp 1991; Fensome et al 1993; Janofske 1996; Karwath 2000; Streng et al. 2004; Kohring et al. 2005; Li et al. 2005a; Elbrächter et al. 2008; Mertens et al. 2013). The existence of these two life-history stages has led to the erection of separate neontological and palaeontological nomenclatures. As a result, the two heterotypic names *P. tyrrhenicum* (Balech) Montresor, Zingone & D.Marino ex Head and †*Calcicarpinum bivalvum* G. Versteegh may represent the same biological species (Montresor et al. 1993). Other species exhibiting two different life-history stages are currently treated under a single name (e.g., *P. dalei*: Dale 1977; *E. carinata*: Matsuoka et al. 1990; *E. imariensis*: Kobayashi and Matsuoka 1995), or havecoccoid cells that have remained unknown until now (e.g., *E. mexicana* Balech, *E. loeblichii*).

Eight extant species and a variety are readily identified as belonging to the E/Pe-clade, though not all taxa are yet characterised by molecular phylogenetics (e.g., *E. mexicana,* *E. carinata*, *E. mexicana*). The lack of information regarding taxonomic availability and typification of scientific names, the degree of morphological variation, particularly regarding the coccoid cells, and DNA sequence data for many critical species hinders the development of a clarified and integrative taxonomy within the E/Pe-clade. In the present study, we investigate the type species of *Ensiculifera*, namely *E. mexicana*,on the basis of morphological and molecular data. This taxonomic clarification allows for a clear differentiation between *Ensiculifera* and *Pentapharsodinium*, and leads to the erection of *Matsuokaea*, gen. nov. The three lineages represented by these genera can be distinguished by the morphology of their coccoid cells. In the taxon sampling for molecular phylogenetics we include a sequence gained from a ctenophoran parasite that nests with sequences of the E/Pe-clade. The parasite is attached to the ectoderm or embedded in the mesoglea of the ctenophore, where it causes localised collapse of the mesoglea, particularly in regions near the aboral pole (Smith et al. 2007; Smith 2011). Overall, we aim at a better understanding of calcareous dinophytes, particularly regarding their life-history and the morphology of coccoid cells.

**Materials and methods**

**Sampling, cultivation and light microscopy**

Surface sediment samples were collected from the Tongyeong coastal area and Jinhae Bay (Korea), Nagasaki Bay (Japan), Comau Fjord (Chile) and Opua Bay (New Zealand). The sediment sample analysis was conducted using the methods of Li et al. (2015a). The isolated coccoid cells were identified at ×400 magnification with an inverted transmitted light microscope Primo Vert (Zeiss, Germany) and transferred onto a 96-well tissue culture plate (Eppendorf, Germany) containing 200 μL of sterile F/2-Si culture medium (Marine Water Enrichment Solution; Sigma-Aldrich, USA−MO; Guillard 1975) with a salinity of 32. The inoculated coccoid cells were incubated at 20 °C under 100 μmol photons m–2 s–1 provided by cool-white fluorescent tubes on a 14:10 h light: dark cycle and were checked daily until vegetative cells were observed. Plankton samples were collected with a plankton net (mesh size 20 μm) in a shallow natural reservoir used for oyster cleansing before commercialization (water depth 1.2–1.5 m)close to Meyran (France), off the coast of Liverpool (United Kingdom), in the western North Atlantic, the Gulf of Mexico off the coast of Louisiana (USA), the East China Sea, along the south coast of Korea and East Sea/Sea of Japan (Table S1). Single cells were isolated using a capillary pipette and inoculated into individual wells of 48-well culture plates (Eppendorf) filled with sterile F/2-Si culture medium. The cells were incubated at 20 °C and ca. 100 μmol photons m-2 s-1 cool-white illumination under a 14:10 h light: dark photo-cycle. All strains are currently held in the culture collection at the Library of Marine Samples (Korea Institute of Ocean Science and Technology; <http://lims.kiost.ac.kr>).

Light micrographs were taken using an ultra-high resolution digital camera (DS-Ri2; Nikon, Japan) on an upright microscope (ECLIPSE Ni; Nikon). For fluorescence microscopy, approximately 1 mL of live, healthy cell cultures were transferred to a 1.5 mL microcentrifuge tube, and SYTOX® Green Nucleic Acid Stain (Molecular Probes, USA−OR) was added at a final concentration of 1.0 μM. The cells were incubated in a dark at room temperature for 30 min, observed through a Zeiss Filterset (emission: BP 450–490; beam splitter: FT 510) and photographed at ×400 or ×1000 magnification using an AxioCam MRc digital camera on an upright microscope (Axio Imager 2, Zeiss). The Kofoidean system was used to designate thecal plates (Taylor 1980, Fensome et al. 1993). The sulcal plate labelling follows Balech (1980).

**Scanning electron microscopy (SEM)**

For SEM of thecate cells and coccoid stages of ………., 2 mL of mid-exponential batch strains were fixed at 4 °C for 5 h with Lugol’s iodine solution (2.0% final concentration). Next, the samples were washed twice with deionised water. After rinsing, the samples were dehydrated in a graded ethanol series (10–99.9% in eight steps) for 15 min at each step. The samples were then critical point dried using a critical point dryer (SPI-Dry Regular Critical Point Dryer, SPI Supplies, USA−PA) with liquid CO2. Finally, the samples were coated with platinum and examined at a voltage of 5kV under a JEOL JSM 7600F field emission scanning electron microscope (JEOL, Japan). Coccoid cells were collected with a micropipette from the sediment samples and cultures, transferred onto graphite-covered stubs and left to dry at room temperature. Energy-dispersive X-ray spectroscopy (EDS) of these uncoated coccoid cells was performed in a field emission SEM JEOL JSM 7600F equipped with an Energy Dispersive Spectroscopy System (Oxford Instruments, Germany).

For SEM of thecate cells and coccoid stages from Opoa Bay (New Zealand) (41° 16' 2.1198'' S, 174° 12' 7.722'' E), cells and coccoid stages were isolated using a micropipette to polycarbonate membrane filters (Millipore, Billerica, MA, USA, GTTP Isopore, 0.22 μm pore size). These were rinsed with distilled water and dehydrated in a graded ethanol series (30 to 100% in six steps), critical-point-dried with CO2 (CPDBal-Tec 030), glued onto a stub, sputter-coated with gold. The images were made with a Zeiss SIGMA300 Gemini field emission SEM at the Station de Biologie Marine (Concarneau, France).

**DNA extraction, PCR conditions and DNA sequencing**

Genomic DNA was extracted from 1 mL of exponentially growing cultures of strains using the Dneasy Plant mini kit (Qiagen, USA−CA) following the manufacturer’s instructions. Small subunit (SSU) rRNA gene sequences were amplified in three overlapping fragments using the primer pairs SR1 and SR9p, SR4 and SR12b as well as DIN464F and EK-1498R (Gómez et al. 2010; Yamaguchi and Horiguchi 2005), respectively. The internal transcribed spacer (ITS) region sequence (ITS1-5.8S rRNA-ITS2) was amplified using the primer pairs ITS1 and ITS4 (White et al. 1990). D1-D3 large subunit (LSU) rRNA gene sequences were amplified in two overlapping fragments using the primer pairs D1R and R2 (Takano and Horiguchi 2006) as well as 25F1 and 25R1 (Kogame et al. 1999; Yamaguchi and Horiguchi 2005), respectively (Table S2). PCR was carried out in a 1×PCR buffer which contained less than 0.1 μg genomic DNA template, 0.3 μM of each primer, 1.25 U PrimeSTAR® GXL DNA polymerase (Takara Bio, Japan), and PCR-grade water to a final volume of 50 μL. The PCR was conducted using a thermoblock (T100™ Thermal Cycler, Bio-Rad, USA−CA) at 95 °C for 4 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 68 °C for 2 min. The reaction was completed with a final elongation at 72 °C for 5 min. PCR-amplified product was confirmed by 1.0% agarose gel electrophoresis. The PCR products were purified with a QIAquick PCR purification kit (Qiagen). The direct sequencing reaction was performed using the ABI PRISM® Big DyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA−CA).

**Alignment and phylogenetic analyses**

Sequences were viewed and assembled in DNABaser version 4.36 (<http://www>.dnabaser.com) and aligned using MAFFT v6.624b online version 7 and the L-INS-I algorithm [(https://maff](C:\\Users\\Vera\\AppData\\Local\\Temp\\Temp1_wetransfer-bc38d3.zip\\(https:\\maff)t.cbrc.jp/alignment/server/; Katoh et al. 2017). The alignment was manually edited, and ambiguously aligned characters were excluded using MEGA version 7.0 (Kumar et al. 2016). The similarity matrix of pairwise *p*-distance was also calculated using MEGA version 7.0 (Kumar et al. 2016). The final alignment of the ITS dataset consisted of 82 taxa. The sequences of other Thoracosphaeraceae, Kryptoperidiniaceae, Amphidomataceaeand Heterocapsaceae were used as outgroups. The separate alignments were then checked and concatenated using SequenceMatrix version 1.8 (Vaidya et al. 2011). The alignment of the concatenated dataset consisted of 59 taxa for SSU / ITS / LSU (Table S1). Sequences of Amphidomataceaeand Heterocapsaceae species were used as outgroups.

Phylogenetic trees for both datasets were constructed using maximum likelihood (ML) analyses and Bayesian inference, respectively. ML analyses of both alignments were carried out using the program RaxML version 8 (Stamatakis 2014). The general time reversible (GTR) model with parameters accounting for γ-distributed rate variation across sites (G) was used in all analyses. Bootstrap analyses for both datasets were carried out for ML with 1,000 replicates to evaluate statistical reliability. Bayesian inference was conducted on both datasets using the MrBayes program version 3.2 (Ronquist et al. 2012) under the GTR+G model taking into account 6-class gamma. The GTR+G substitution model was selected using the Akaike information criterion as implemented in jModelTest version 2.1.4 (Darriba et al. 2012). The Markov chain Monte Carlo method was used with four runs for 10 million generations, sampling every 100 generations. A majority rule consensus tree was created in order to examine the posterior probabilities of each clade. The final trees were visualised with FigTree v1.4.4.

**Results**

**Morphology**

***Ensiculifera mexicana***

The monadoid cells of strain LMBE-TY1 are solitary, ovoid and yellow in colour (Fig. 1A–F). The cells are 30.7–49.6 µm (average :  41.7 µm, n=50) in length and 25.5–38.9 µm (average :  34.2 µm, n= 50) in width. The length of the epitheca is generally the same as the hypotheca (Fig. 1A, C). The hypotheca is rounded and lacks horns or projections (Fig. 1A–F). The cingulum is subequatorial and descending, its ends displaced by one-half cingulum width (Fig. 1A, E). Cells contain reticular chloroplasts and a pyrenoid (Fig. 1B, G). The nucleus is elongated and located centrally in the posterior part of the cell (Fig. 1H–I).

Motile cells display a plate tabulation formula of Po, x, 4′, 3a, 7″, 5c, 5S, 5′′′, 2′′′′ (Fig. 2A–I). Many pores and small granules or bumps are randomly distributed on the thecal surface (Fig. 2A–I). The epitheca is conical and composed of the apical pore plate (Po), an elongated ventral canal plate (x), four apical plates (4′), three anterior intercalary plates (3a) and seven precingular plates (7″) (Fig. 2A–E). Plate Po is surrounded by a small collar located centrally at the apex (Fig. 2E) and has a round apical pore (Fig. 2E). The first apical plate (1′) is longer than wide and contacts plates x, 2′, 4′, 1′′ and 7′′ (Fig. 2A, D, E). Plates 2′ and 4′ are hexagonal in shape and larger than plate 3′ (Fig. 2E). Plate 3′ is hexagonal in shape and contacts plates Po, 2′ and 4′ as well as the three anterior intercalary plates (Fig. 2C, E). Plate 2a is a symmetrical hexagon (Fig. 2) and is larger than plates 1a and 3a. Seven precingular plates are arranged symmetrically. Plates 1″ and 4″ are rectangular in shape (Fig. 2A–B, D–E). Plates 2″, 3″, 5″ and 6″ are pentagonal in shape (Fig. 2B–E). Plate 4″ is smaller than plates 3″ and 5″ (Fig. 2C, E). On the hypotheca, there are five postcingular plates (5′′′) and two antapical plates (2′′′′) (Fig. 2G). The postcingular plates are arranged symmetrically (Fig. 2G). Plate 3′′′ is the smallest postcingular plate and pentagonal in shape (Fig. 2C, G); the other postcingular plates are trapezoidal in shape (Fig. 2G). The antapical plates, 1′′′′ and 2′′′′, are pentagonal in shape, dissimilar in size, larger than the five postcingular plates, and in contact with the posterior sulcal plate (Fig. 2G).

The cingulum is slightly excavated and is formed by five plates (Fig. 2A–F). The c1 plate of the cingular series is the smallest and encroaches on the left-hand side of the anterior sulcal area (Fig. 2F, H, I). A spine is attached to the c1 plate and is hidden behind plate 1′ (Fig. 2F, H, I). Plates c3 and c4 are larger than the c2 and c5 plates (Fig. 2F). The sulcus is narrow and consists of five plates (Fig. 2A, G–I). The anterior sulcal plate (Sa) is small and located between plates 1″ and 7″ (Fig. 2A, H). The left sulcal (Ss) and right sulcal (Sd) plates are narrow, larger than plate Sa and connect to plates c1 and Sa, respectively ([Fig.](javascript:popRefFull('i0031-8884-46-5-572-f02')) 2H–I). The median sulcal plate (Sm) is very small, almost completely hidden by the wing attached to the posterior sulcal (Sp) plate ([Fig.](javascript:popRefFull('i0031-8884-46-5-572-f02')) 2A, G), and is seen only in internal views (Fig. 2I). The Sp plate is short, pentagonal, extends into the hypotheca without reaching the antapex and does not contact plate c1 ([Fig.](javascript:popRefFull('i0031-8884-46-5-572-f02')) 2A, G, I).

Coccoid cells are brown, with a prominent red internal body (Fig. 3A–B, D–E). Cell size ranges from 57.5 to 68.4 μm (average :  62.5 μm, n= 6) in length and 48.5 to 58.7 μm (average:  56.2 μm, n= 6) in width. The coccoid cell is roughly pentagonal in apical view and subrectangular in lateral view (Fig. 3A–C). The calcareous wall is thin between the thick crests (Fig. 3A). The strong equatorial ridges correspond to cingular sutural structures. The endocoel is subspherical, 42.9 to 47.2 μm in diameter (average :  44.6 μm, n= 6) (Fig. 3D). The archaeopyle is subcircular (Fig. 3F) and is located at the centre of the apical face (Fig. 3F–I). The operculum may remain attached after excystment (Fig. 3G–I). The operculum is subcircular (22.5 μm in diameter, n= 2) with a smooth edge (Fig. 3G–I). The coccoid cells were not observed in the cultivated strain.

***Ensiculifera carinata***

The monadoid cells of strain I09 are 20.5–51.2 µm long (average :  42.3 µm, n= 20) and 16.8–36.5 µm wide (average: 30.3 µm, n= 20). The epitheca is convex-conical and the hypotheca is hemispherical (Fig. S1). The cells display a plate formula of Po, x, 4′, 3a, 7″, 5c, 5S, 5′′′, 2′′′′ (Fig. S1). A spine approximately 13 µm long is attached to the c1 plate and is hidden behind plate 1′. The thecal plates are covered with many pores and small granules or bumps (Fig. S1). A single hollow spine is observed at the triple junction of plates Sp, 1′′′′ and 2′′′′ (Fig. S1).

Coccoid cells of *E. carinata* are spherical and brown (Fig. 4A). The cell body diameter ranges from 41.6 to 58.5 µm (average:  50.3 µm, n= 10). The calcareous wall is ridged and covered by numerous needle-shaped calcareous crystals (Fig. 4B–D). The cingular and sulcal equivalents are observed on the calcareous wall. When the outer calcareous layer was dissolved at a low pH using a CO2 incubator (Fig. 4E–F) a prominent reddish pigment body was always visible (Fig. 4E). The naked coccoid cell has a smooth organic wall that is distinctly visible (Fig. 4F). The archaeopyle is theropylic intercalary (Fig. 4F). The coccoid cells were not observed in the cultivated strain.

***Pentapharsodinium imariense*, comb. nov.**

The monadoid cells of strain TIO278a are ovoid, 18.1–21.3 µm long (average :  20.2 µm, n=20) and 13.2–17.5 µm wide (average :  14.5 µm, n= 20). The epitheca is conical, whereas the hypotheca is hemispherical (Fig. 5). The plate formula is Po, x, 4′, 3a, 7″, 5c, 5S, 5′′′, 2′′′′ (Fig. 5 A–F). The thecal plates (except plates Po and x) are covered with numerous trichocyst pores, where each pore is surrounded by one or two concentric circles. The pore plate is surrounded by a small collar (Fig. 5A, C). Three intercalary plates of similar size are present on the dorsal part of the epitheca (Fig. 5B–C). The cingulum is deep and descending by one-half of the cingulum width (Fig. 5A, D–E). The cingulum consists of five plates. A long spine (>10 µm) is attached to plate c1 and is hidden behind plate 1′. Plate 3′′′ is pentagonal in shape and is the smallest of the postcingular plates. The two antapical plates (1′′′′ and 2′′′′) are nearly the same size and are symmetrical (Fig. 5A, D, F). The sulcal series comprises five plates: Sa, Sd, Sm, Ss and Sp (Fig. 5D–E). The Sp plate is short and does not contact c1 (Fig. 5A, E).

The coccoid cells are spherical, 19.6 to 23.5 µm (average :  20.8 µm, n=10) in diameter and transparent to greenish-brown (Fig. 6A–D). Live coccoid cells contain several greenish granules. The cell wall is thick and bears numerous processes (average :  4.3 µm long, n=10) (Fig. 6A–F). The processes bear small bumps, and the distal end of these processes range from simple to branched at the apex, with or without capitate tips (Fig. 6 E–H). No archaeopyle was observed. The coccoid cells were also observed in the cultivated strain.

***Matsuokaea loeblichii*, comb. nov.**

The monadoid cells of strain LMBE-JH2 are solitary, pyriform and yellow-green (Fig. 7A–D). The cells are 18.6–31.3 µm (average:  24.4 µm, n=50) in length and 14.6–22.5 µm (average:  18.2 µm, n= 50) in width. The length of the epitheca is generally similar to that of the hypotheca (Fig. 7A). The cingulum is subequatorial and descending, displaced by one half the cingulum width (Fig. 7A–C). A rod-shaped eyespot is located on the right side of the sulcus (Fig. 7A, B). The elliptical nucleus in the hypotheca is prominent (Fig. 7D, E). The cells contain ribbon-like chloroplasts (Fig. 7F). The cells have a plate formula of Po, x, 4′, 3a, 7″, 5c, 5S, 5′′′, 2′′′′ (Fig. 8A–J). Many pores and small bumps are randomly distributed on the thecal surface. Plate Po is surrounded by a small collar and together they form a conspicuous horn (Fig. 8A–C). The first apical plate (1′) contacts seven plates: x, 2′, 4′, 1′′, 7′′, Sa and c1 (Fig. 8A, G, J). Three intercalary plates (1a, 2a and 3a) are of similar size and are present on the dorsal part of the epitheca (Fig. 8F). The precingular plates are symmetrically distributed. Plates 1′′ and 7′′ are larger than the others. Plates 1′′, 3′′ and 7′′ are trapezoidal, and the other precingular plates are pentagonal. The hypotheca is composed of five postcingular plates (5′′′) and two antapical plates (2′′′′), which are symmetrically distributed. Plates 1′′′, 2′′′, 3′′′, 4′′′ are tetragonal, nearly equal in size and longer than they are wide, whereas plate 3′′′ is small and pentagonal in shape (Fig. 8D, H). Two antapical plates (1′′′′ and 2′′′′) are similar in size and have pentagonal shapes (Fig. 8H). The cingulum is regularly descending but not deeply excavated and is formed by five plates (Fig. 8A–B, I). Plate c1 bears no spine (Fig. 8I). The sulcus is narrow with inconspicuous lists and does not contact the antapex (Fig. 8A, B). The sulcus consists of four major plates: plate Sa is small, located between plates 1′ and 7′′; plates Ss and Sd are short (Fig. 8J); and plate Sp is the largest sulcal plate and does not contact plate c1 (Fig. 8J).

The coccoid cells are spherical in shape, the wall is transparent, consisting of two layers, an outer thin layer and a thicker endospore layer below, and cell-contents are yellow-green with a prominent red body (Fig. 7G, H). The diameter of coccoid cells range from 19.5 to 25.2 µm (average :  21.6 µm, n= 10). The live coccoid cell contains abundant yellowish granules (Fig. 7G, H). The organic wall is thick and smooth without any distinguishing features on the surface (Fig. 7I). The coccoid cells were also observed in the cultivated strain.

**Plate overlap**

The overlap patterns of *E. mexicana*, *P. imariense*, comb. nov., and *M. loeblichii*, comb. nov., have been determined from differential marginal growth in the sutures of two adjacent plates (Fig. 9). These three species have an identical plate overlap pattern. Plate overlap in epithecal, cingular and hypothecal plate series follow two general gradients: from dorsal to ventral and from the cingulum to the two poles. The fourth precingular (4′′), the third postcingular (3′′′) and the third cingular (3c) plates were identified as keystone plates, which overlap all their adjacent plates. On the epitheca, the middle intercalary plate 2a overlaps the other intercalary plates. On the hypotheca, the second antapical plate 2′′′′ overlaps plate 1′′′′. Among the sulcal plates, plate Sp is overlapped by all hypothecal plates. Plates Sd and Ss are overlapped by 5′′′ and 1′′′, respectively.

**Molecular phylogeny**

The SSU + ITS + LSU alignment was 1,726 + 934 + 1,112 bp long and comprised 302 + 557 + 427 parsimony informative sites as well as 1,901 distinct rAxML alignment patterns. In the phylogenetic tree (Fig. 10), peridinialean dinophytes segregated into *Blastodinium* Chatton (100LBS, 1.00BPP), Ensiculiferaceae (97LBS, 1.00BPP), Kryptoperidiniaceae (99LBS, 1.00BPP), Peridiniaceae (100LBS, 1.00BPP), Peridiniopsidaceae (97LBS, 1.00BPP), Thoracosphaeraceae (98LBS, 1.00BPP) and *Zooxanthella*. Calcareous dinophytes were distributed over three lineages: Ensiculiferaceae, *Scrippsiella s.l.* (84LBS, 1.00BPP) and the T/Pf-clade (76LBS, 0.97BPP), but did not constitute a monophyletic group. Rather, Ensiculiferaceae, showed a closer relationship to *Blastodinium*, Kryptoperidiniaceae and *Zooxanthella*, but the support was low (53LBS).

The ITS + 5.8S alignment was 1,114 bp long and comprised 579 parsimony informative sites as well as 846 distinct rAxML alignment patterns. Bayesian and ML analyses generated similar trees (Fig. 11), and Ensiculiferaceae constituted a well-supported group (60LBS, 1.00BPP). They segregated into the three major clades: *Ensiculifera* (50LBS, 0.92BPP), *Matsuokaea*, gen. nov. (95LBS, 1.00BPP) and *Pentapharsodinium* (85LBS, 1.00BPP). The *Ensiculifera* and *Pentapharsodinium* clades appeared more closely related to each other (0.91BPP) than to the *Matsuokaea*, gen. nov. clade. *Ensiculifera* includes four previously named species: the type, *E. mexicana* (90LBS, 1.00BPP), *E. carinata* (94LBS, 1.00BPP), *E.* *jinhaensis*, comb. nov. (single accession), and *E. tyrrhenica*, comb. nov. (99LBS, 1.00BPP). Additionally, *Ensiculifera* includes the sequences of a parasite that was almost identical to a sequence from an unidentified calcified coccoid cell (Fig. S2; 100LBS, 1.00BPP). *Pentapharsodinium* segregated into four clades, including the type, *P. dalei* (98LBS, 1.00BPP), *P. dalei* var. *aciculiferum* (100LBS, 1.00BPP), *P. imariense*, comb. nov. (100LBS, 1.00BPP), and an unnamed species (strain SSND22 from China). Sequence divergences of Ensiculiferaceae are provided in Table S3

**DISCUSSION**

**Correlations between molecular phylogenetics and morphological traits**

The phylogenetic analyses presented here accord with previous studies (Gottschling et al. 2005a, 2012; Gu et al. 2013a; Gottschling and Söhner 2013): the E/Pe-clade is reliably monophyletic and segregates into three lineages. Strikingly, the three lineages are characterised by differences in the coccoid cell’s wall composition and/or ornamentation (Fig. 11): *Ensiculifera* is characterised by calcareous coccoid cells of various shapes with an operculum corresponding to a single plate equivalent (Matsuoka et al. 1990; Montresor et al. 1993; Gottschling et al. 2005a; Li et al. 2015b); *Pentapharsodinium* comprises organic-walled coccoid cells, centrifugal processes of similar length and termination, and an apical split as an archaeopyle (Gu et al. 2013b; Kobayashi and Matsuoka 1995); and *Matsuokaea*, gen. nov., exhibits smooth, organic-walled coccoid cells with an unknown archaeopyle. In the phylogenetic tree based on ITS-based sequences, an undetermined species of *Pentapharsodinium* (EU728696; strain SSND22 fromChina) produces a coccoid cell characterised by short organic-walled processes on the surface. As the coccoid cell is morphologically similar to those of *P. dalei* and *P. imariense*, comb. nov. (Gu and Wang 2007), this unnamedspecies can be assigned to *Pentapharsodinium*.

Members of the Ensiculiferaceae have a morphology distinct from other calcareous dinophytes. Most notably, *Ensiculifera*, *Matsuokaea*, gen. nov., and *Pentapharsodinium* have five cingular plates, which distinguishes them from most other members of the Thoracosphaeraceae, which have six cingular plates (Dale 1978; Fensome et al. 1993). Their distinctiveness is also expressed by recent molecular phylogenetics, which show a closer relationship of Ensiculiferaceae not with the other Thoracosphaeraceae but to an ecologically heterogeneous assemblage comprising of *Blastodinium*, Kryptoperidiniaceae and *Zooxanthella* (Gottschling and McLean 2013; Gottschling et al. 2017; Kretschmann et al. 2018a); strikingly, these taxa also have five cingular plates (Probert et al. 2014; Kretschmann et al. 2018a; Skovgaard and Salomonsen 2009), supporting a close relationship with the Ensiculiferaceae, The Peridiniaceae (in a strict sense: Gottschling et al. 2017) also have three intercalary plates and five cingular plates, but can be distinguished from the Ensiculiferaceae based on habitat preference (freshwater versus marine). Furthermore, the position of Ensiculiferaceae is distinct from Peridiniaceae and other peridinialean dinophytes in molecular phylogenetics (Fig. 11). Thus, we propose the erection of Ensiculiferaceae, fam. nov., within the Peridiniales for the species of the E/Pe-clade (see below).

Usually, the tabulation, with its highly diverse arrangements and plate shapes, is considered diagnostic of groups of armoured dinophytes at different taxonomic levels (Taylor 1980; Fensome et al. 1993). However, thecate cells of taxa within the Ensiculiferaceae have indistinguishable tabulation patterns, although the ornamentation might be different. Thus, the morphology of the coccoid cells has to be used to determine taxa within the Ensiculiferaceae in addition to diagnostic sequences. A greater diversification of the coccoid cells compared to the thecate cells applies in other dinophyte lineages, such as the Gonyaulacaceae, Protoperidiniaceae and Thoracosphaeraceae (Wall and Dale 1968; Ellegaard et al. 2003; Elbrächter et al. 2008; Matsuoka and Head 2013; Li et al. 2015a, 2015b; Shin et al. 2014). The morphological features of coccoid cells assigned to Ensiculiferaceae, are summarised in Table 1 (Gu et al. 2013b; Kobayashi and Matsuoka 1995; Dale 1977; Li et al. 2015b; Matsuoka et al. 1990; Montresor et al. 1993). However, coccoid cell diversity and function(s) are still poorly understood.

The correlation between molecular phylogenetics and coccoid cell morphology is straightforward, but the presence and position of spines associated with cingular plates on the thecate cells are difficult to interpret. Three states can be distinguished within the E/Pe-clade; ‘spine on the c1 plate’ (in *E. carinata*, *E. mexicana*, *P. imariense*, comb. nov.: Balech, 1967; Kobayashi and Matsuoka 1995; Matsuoka et al. 1990); ‘spine on the Sa plate’ (in *E.* *jinhaensis*,comb. nov., *P. dalei* var. *aciculiferum*: Gu et al. 2013b; Li et al. 2015b); and ‘no spine’ (in *E. tyrrhenica*, comb. nov., *M. loeblichii*, comb. nov., *P. dalei*: Cox and Arnott 1971; D’'Onofrio et al. 1999). However, the groups with each of these states do not group together in the DNA tree (Fig. 11), and the trait cannot be used to distinguish *Ensiculifera* from *Pentapharsodinium*. However, the trait may help to distinguish species.

That the presence of spines does not accord with clades in the DNA tree prompts the question as to whether observations of the exact position of the spines in the literature are correct and, if not, whether such spines may be homologues across Ensiculiferaceae. Further research is necessary to explain the confusion regarding presence and position of the spine associated with cingular plates. At least the cingular spine described for *S. trochoidea* var. *aciculifera* Montresor (D’'Onofrio et al. 1999) appears as an independent development as inferred from the DNA trees of calcareous dinophytes (Gottschling et al. 2005a; Gu et al. 2013b). In addition, *E.* *jinhaensis*, comb. nov., *E.mexicana*, *E.* *tyrrhenica*, comb. nov., *E. carinata* and *M. loeblichii*, comb. nov., have many pores and bumps on the thecal surface, whereas the thecal surface of all species of *Pentapharsodinium* is smooth (Gu et al. 2013b; Kobayashi and Matsuoka 1995; Li et al. 2015b; Matsuoka et al. 1990; Montresor et al. 1993).

Plate overlap patterns are generally considered to be conserved at higher taxonomic levels (Netzel and Dürr 1984). This assumption is supported by the Ensiculiferaceae which show a consistent plate overlap pattern (Figs 9 and S3). The epithecal plate overlap patterns are the same as in most peridinioid dinophytes (e.g., *Durinskia* Carty & El.R.Cox and *Scrippsiella*: Kretschmann et al. 2015, 2018a), with the fourth precingular plate forming the keystone plate. This differs from gonyaulacoid dinophytes, in which the third precingular plate is identified as the keystone plate in *Pyrrhotriadinium sphaericum* (G.Murray & Whitting) Nakada and *Lingulodinium polyedra* (F.Stein) J.D.Dodge (Fensome et al. 1993). In Ensiculiferaceae plate 3c is identified as the keystone plate for the cingular series, which has been previously reported for *Durinskia oculata* (F.Stein) Hansen & Flaim (Kretschmann et al. 2018a). In contrast, plate 4c is the keystone plate in many other peridinioid species including *Scrippsiella acuminata* (Ehrenb.) Kretschmann, Zinssmeister, S.Soehner, Elbr., Kusber & Gottschling (Kretschmann et al. 2015), *Heterocapsa steinii* Tillmann, Gottschling, Hoppenrath, Kusber & Elbr. (Tillmann et al. 2017), *Parvodinium travinskii* Kretschmann, Owsianny, Zerdoner & Gottschling and *Parvodinium mixtum* Wołosz. ex Kretschmann, Owsianny, Zerdoner & Gottschling (Kretschmann et al. 2018b). Consequently, the plate overlap pattern suggests that Ensiculiferaceae has a close relationship with the Peridiniopsidaceae and Heterocapsaceae (e.g., Kretschmann et al. 2018b).

**The link between calcareous dinophytes and parasites**

Highly specialised organisms, such as parasites and endosymbionts, have evolved repeatedly and independently within various organismal groups in general and within dinophytes in particular. Because of many lost traits as well as many autapomorphies, the determination of their closest relatives is frequently only possible through comparison of molecular sequences. It is particularly surprising that in our molecular phylogenetics, the closest relative of an organismparasiting the ctenophore *Mnemiopsis* Agassiz, 1865 (Smith et al. 2007; Smith 2011) is a dinophyte that has a calcareous coccoid stage during its life-history. Moreover, the organism shows no phylogenetic affinities to other parasite groups, not even the Peridiniales.

The parasite nesting with *Ensiculifera* is not the only one that shows phylogenetic affinities to the calcareous dinophytes: *Amyloodinium* E.-M.Br. & Hovasse and *Paulsenella* Chatton are closely related to the pfiesterians of the T/Pf-clade (Litaker et al. 1999; Kühn et al. 2005), while *Dubscquodinium* Grassé is nested within *Scrippsiella s.l.*, and *Tintinnophagus* Coats and may represent an independent lineage within the complex relationships of the Thoracosphaeraceae (Coats et al. 2010). Two interpretations of this scattered distribution of parasites and other unusual life forms in the dinophyte molecular trees are conceivable: 1. evolution from phototrophic cells towards more specialised dinophytes with heterotrophic, endosymbiontic or parasitic life-styles, has occurred several times independently; 2. the life-history of particular dinophyte species is incompletely known and also includes parasitic stages not discovered so far. Particularly for the case of the *Scrippsiellaacuminata* species complex, including morphologically indistinguishable but molecularly distinct (‘cryptic’) species (Montresor et al. 2003; Gottschling et al. 2005; Zinßmeister et al. 2011; Söhner et al. 2012; Gottschling and Söhner 2013), it is tempting to speculate that the reproductively isolated units are differentiated not based on their morphology but on their hosts (*pers. comm.* K.J.S. Meier; Kiel, Germany). Further research is required to enlighten the complex biology and evolution of the calcareous dinophytes.

**Taxonomic clarifications in Ensiculiferaceae**

Since its first description by Balech (1967), *E. mexicana* has been rarely reported in the scientific literature (Wall and Dale 1968; Wall et al. 1970; Licea et al. 2004; Okolodkov and Gárate-Lizárraga 2006). To the best of our knowledge, no studies or illustrations exist that improve the initial description of this species. Unfortunately, Balech (1988) created more confusion when he presented illustrations of *E. mexicana* with six cingular plates, contradicting his original description of five such plates. One of the most important results of our present study is thus to demonstrate that *E. mexicana* has a combination of five cingular plates and a distinct spine on the first cingular plate. The only differences between the specimens shown in our SEM images and those in original drawings provided by Balech (1967) are that, in ours, the second intercalary plate is narrower and the apical elongation of the anterior sulcal plate is missing. Nevertheless, the morphology of the monadoid cells is consistent with the protologue of *E. mexicana* (Balech 1967) to the extent that we are confident to have recollected the species for the first time after half a century. A comparison of *E. mexicana* with related species is shown in Table 1.

Here, we report on *E. mexicana* from the western Pacific and not from the western Atlantic where it was originally described, indicating a possible broad distribution of the species. To support the present work, we plan to provide epitypification in the future based on material from the Gulf of Mexico, the type locality of *E. mexicana* (Balech 1967). The importance of this approach has been discussed in various previous studies (Kretschmann et al. 2015, 2018a, b; Tillmann et al. 2017). Nevertheless, this work provides a taxonomic clarification of *Ensiculifera* at the generic level..

Molecular phylogenetics indicate that *P. imariense*, comb. nov., is not closely related to *Ensiculifera*, but to *Pentapharsodinium* and its type, *P. dalei* (Figs 10 and 11). This is corroborated by morphology: the thecal surface has numerous trichocyst pores surrounded by concentric circles, and the organic-walled coccoid cell has capitate processes (Gu et al. 2013b; Indelicato and Loeblich III. 1986; Kobayashi and Matsuoka 1995). However, *P. imariensis*, comb. nov.,has a long spine on the c1 plate, and such a spine has never been observed in *P. dalei* (Gu et al. 2013b; Kobayashi and Matsuoka 1995). Furthermore, a contact between plates Sp and c1 was described by Kobayashi and Matsuoka (1995), resembling the situation in *Scrippsiella* (Lewis 1991), although *P. imariensis* is not closely related to the latter. Our SEM observations of *P. imariensis*, comb. nov., show that plate Sp is short and does not contact plate c1 (Fig. 6E). Thus, we cannot exclude an incorrect observation by Kobayashi and Matsuoka (1995: fig. 23). Kobayashi and Matsuoka (1995) also described the coccoid cell of *P. imariensis*, comb. nov., as characterised by wider processes (1.5–2.2 µm wide) and pointed out that this feature may be key to distinguishing coccoid cells of *P. imariensis*, comb. nov., from *P. dalei*. However, the processes of *P. imariensis*, comb. nov., vary (Fig. 5E–H). Thus, morphological traits of the coccoid cells do not allow for the unequivocal species distinction of *P. imariensis*, comb. nov., *P. dalei*, and *P. dalei* var. *aciculiferum*; molecular studies are needed to identify those dinophytes to the species and subspecies rank.

**The fossil record of Ensiculiferaceae**

Two extant species of Ensiculiferaceae have a fossil record. The first occurrence of cysts of *P. dalei* is observed in the Upper Miocene of the Norwegian Sea (De Schepper et al. 2015, 2017), whilst there is a more or less continuous documentation of *E.* *tyrrhenica*, comb. nov. (= †*C. bivalvum*) since the Upper Pliocene from the Mediterranean (Versteegh 1993). This evidence, and the short branch lengths in the phylogenies, suggests that Ensiculiferaceae evolved relatively late compared to other marine dinophytes (Žerdoner Čalasan et al. 2019). Moreover, there are a number of Neogene fossils that are morphologically similar to coccoid cells of extant *Ensiculifera*, such as †*Calciconus* Streng, Banasová, D. Reháková & H. Willems and †*Cylindratus* Banasová, Kopčáková & D.Rehaková ex Streng, Banasová, D. Reháková & H. Willems, which have an apical operculum that corresponds to a single plate equivalent (Streng et al. 2009). At least some of these Neogene forms have survived until today, including †*Follisdinellum splendidum* G. Versteegh, †*Melodomuncula berlinensis* G. Versteegh and †*Praecalcigonellum schizosaeptum* G. Versteegh (Montresor et al. 1994; Zonneveld et al. 1999; Rubino et al. 2013, 2017). However, none of these forms have been successfully cultivated and reliable phylogenetic data are therefore not available.

The coccoid cells of *E. mexicana* are similar to significantly older fossils such as †*Bicarinellum* Deflandre (Keupp 1991; Zinßmeister et al. 2012), which exhibits a tabulation and a circular apical operculum equivalent to plate 3´ (Streng et al. 2004). This archaeopyle type has been considered as ancestral to the combination operculum type present in *Scrippsiella s.l.* (Gottschling et al. 2008). The coccoid cells of *E. mexicana* with †*Bicarinellum* *cristatum* Keupp from the lowermost Barremian to early Albian (Keupp 1982) possess other similarities to each other, such as a pentagonal outline in apical view, a prominent carinae, and a more or less flattened hypocyst. However, the coccoid cell of *E. mexicana* has a strongly oblate autophragm and a single-layered calcareous wall in contrast to †*Bicarinellum*, which has a double-layered calcareous wall (Keupp 1991). We cannot preclude the possibility that more fossils (particularly obliquipithonelloids: Kohring et al. 2005) are assignable to the E/Pe-clade, but this requires further careful taxonomic revision and comprehensive phylogenetic analysis.

The uniform characteristic tabulation and plate overlap pattern in the Ensiculiferaceaereflect evolutionary stability in these taxa, in contrast to the extreme variation known for *Peridinium* and *Protoperidinium* Bergh. The plate overlap pattern in Ensiculiferaceae and Thoracosphaeraceae is similar to that of Triassic through Jurassic †Phallocysteae Below (Below 1987), but even more so to the Cretaceous †*Subtilisphaera terrula* (Palaeoperidinioidea; Harding 1988). This suggests that the Ensiculiferaceaeevolved from these fossil taxa, and that their tabulation and overlap pattern has been conserved since then. However, we advise caution since sole morphological similarity can be misleading, as *Caladoa arcachonensis* Z.Luo, K.N.Mertens & H.Gualso has identical tabulation and overlap as *Scrippsiella* but it is genetically distinct (Luo et al. 2019). The morphology of the coccoid cells of Ensiculiferaceaeis known to be much more variable than the equivalent thecate monadoid cells. Interestingly, similar conclusions can be drawn for *Gonyaulax* Diesing and *Scrippsiella*, which may indicate similar adaptive evolutionary mechanisms.

**Conclusions**

Our study clarifies the relationships of an important, though previously enigmatic, dinophyte species, *E. mexicana*, and provides evidence that Ensiculiferaceaeare composed of three lineages. The assignment of species to generic names was inconsistent in the past, and a possible solution was to accept a single generic name, *Ensiculifera*. However, that solution does not acknowledge the notable diversity of coccoid cells that corresponds to the three lineages: 1. *Ensiculifera*, characterised by a calcareous coccoid stage with an apical operculum; 2. *Pentapharsodinium*, having a coccoid stage with an organic wall and processes of varying lengths; and 3. *Matsuokaea*, gen. nov., with a smooth organic-walled coccoid stage. As *M. loeblichii*, comb. nov., is not closely related to either *Ensiculifera* or *Pentapharsodinium*, we propose the name *Matsuokaea*, gen. nov. This solution is preferred by those who study primarily the coccoid stage of dinoflagellates, but will be challenging for field phycologists who have to attribute monadoid cells to one of the three taxa recognised here.

**Systematics**

**Dinophyceae Pascher**

**Peridiniales Haeckel**

**Ensiculiferaceae** **Zhun Li, Gottschling, K.N.Mertens, H.Gu & H.H.Shin,** **fam. nov.**—Type: *Ensiculifera* Balech, Revista del Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” (Hidrobiología) 2: 122. 1967.

**Description:** Dinophytes free-living, solitary, thecate. Monadoid cells bipesoid, orthoperidinioid with Kofoidean plate formula: Po, x, 4′, 3a, 7″, 5c, 5S, 5′′′, 2′′′′; spine absent or present on the anterior sulcal plate or first cingular plate; chloroplasts present. Coccoid cells calcified or organic-walled.

[http://phycobank.org/102091]

***Ensiculifera* Balech emended Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin** —Type: *Ensiculifera mexicana* Balech, Revista del Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” (Hidrobiología) 2: 120–122, figs 136–144. 1967.

**Description:** Thecal surface of monadoid cell with many pores and small bumps; cingulum subequatorial, descending, ends displaced by one cingulum width. Coccoid cells with an inner organic layer, surrounded by a calcareous outer layer.

***Ensiculifera mexicana*****Balech emended Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin**

**Description:** Monadoid cell broad, slightly flattened dorsoventrally, without antapical horns or posterior notch; the thecal surface with many pores and small bumps; the cingulum subequatorial and descending, ends displaced by one cingulum width; the epitheca conical, with somewhat convex flanks, bipesoid, orthoperidinioid; the Po with a round apical pore, located centrally at the apex; the 1′ plate rhombic, narrow; the 2’' and ’' plates hexagonal; the spine attached to the first cingular plate. Coccoid cells roughly pentagonal in apical view, subrectangular in lateral view, with an inner organic layer, surrounded by a calcareous layer.

**Remarks:** This species has been related to “*Pentadinellum oblatum*” Keupp, a name not validly published (ICN Art. 44.1), whose type is based on a coccoid cell. rRNA and ITS gene sequences of strains LMBE-JH1, LMBE-TY1, LMBE-TY2 and LMBE-UL1 (Table S1) were deposited in GenBank as entries MN821536–MN821539, MN821542–MN821545 and MN821565–MN821568.

***Ensiculifera tyrrhenica* (Balech)Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin, comb. nov.**

**Basionym:** *Peridinium tyrrhenicum* Balech, Helgoländer wissenschaftliche Meeresuntersuchungen 44: 390–391, figs 12–18. 1990.

**Synonym:** *Pentapharsodinium tyrrhenicum* (Balech) Montresor, Zingone & D.Marino ex Head

**Remarks:** This species has been related to †*Calcicarpinum bivalvum* G.Versteegh, whose type is based on a coccoid cell.

[http://phycobank.org/102092]

***Ensiculifera jinhaensis* (Zhun Li, M.S.Han & H.H.Shin) Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin, comb. nov.**

**Basionym:** *Pentapharsodinium jinhaense* Zhun Li, M.S.Han & H.H.Shin in Zhun Li, H.H.Shin, W.A.Lim, T.Lee, Y.H.Yoon & M.S.Han, Phycologia 54: 568–574, figs 1–27. 2015.

[http://phycobank.org/102093]

***Pentapharsodinium* Indel. & A.R.Loebl. emended Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin**—Type: *Pentapharsodinium dalei* Indel. & A.R.Loebl., Japanese Journal of Phycology (Sôrui) 34: 158–159, figs 1–5, 7–8. 1986.

**Description**: Thecal surface of monadoid cell smooth. Coccoid cell spherical to ovoid, acapsulate; the surface with many capitate processes; an outer organic layer.

***Pentapharsodinium* *imariense* (S.Kobayashi & Matsuoka) Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin, comb. nov.**

**Basionym:** *Ensiculifera imariensis* S.Kobayashi & Matsuoka, Journal of Phycology 31: 147–151, figs 1–23. 1995.

[http://phycobank.org/102094]

***Matsuokaea* Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin, gen. nov**.—Type: *Matsuokaea loeblichii* (El.R.Cox & H.J.Arn.) Zhun Li, K.N.Mertens, H.Gu & H.H. Shin, comb. nov.

**Description:** Thecal surface of monadoid cell with many pores and small bumps; the cingulum subequatorial, descending, displaced one cingulum width; the spine absent. Coccoid cells spherical to ovoid; the surface smooth; an outer layer organic.

**Etymology:** In honour of Prof. Kazumi Matsuoka (Nagasaki), a pioneer in the study of armoured dinophyte taxonomy and the biological relationships between monadoid and coccoid cells.

[http://phycobank.org/102095]

***Matsuokaea loeblichii* (El.R.Cox & H.J.Arn.) Zhun Li, K.N.Mertens, Gottschling, H.Gu & H.H.Shin, comb. nov.**

**Basionym:** *Ensiculifera loeblichii* El.R.Cox & H.J.Arn. in P.C.Parker & R.M.Br., Contributions in Phycology: 121, 123–124, figs 1–34. 1971.

**Synonyms:** *Pentapharsodinium trachodium* Indel. & A.R.Loebl.; *Peridinium loeblichii* (El.R.Cox & H.J.Arn.) Dale

[http://phycobank.org/102096]

**Key**

Based on molecular phylogenetics and life-history morphologies, the following key includes the 8 species of Ensiculiferaceae, fam. nov., that we currently accept and of which sequence data are available.

|  |  |  |
| --- | --- | --- |
| 1a. | Coccoid cell with calcareous wall | 2 |
| 1b. | Coccoid cell with organic wall | 3 |
| 2a. | Archaeopyle circular or subcircular | 4 |
| 2b. | Archaeopyle theropylic | 5 |
| 3a. | Coccoid cells with longer or shorter protuberances | 6 |
| 3b. | Coccoid cells with smooth wall | *Matsuokaea loeblichii* |
| 4a. | Spine attached to plate c1 of monadoid cell | *Ensiculifera mexicana* |
| 4b. | No spine attached to plate c1 of monadoid cell | *Ensiculifera tyrrhenica* |
| 5a. | Calcareous wall ridged | *Ensiculifera carinata* |
| 5b. | Calcareous wall not ridged | *Ensiculifera jinhaensis* |
| 6a. | Spine attached to plate c1 or plate Sa of monadoid cell | 7 |
| 6b. | No spine attached to plate c1 or plate Sa of monadoid cell | *Pentapharsodinium dalei* |
| 7a. | Spine attached to plate c1 of monadoid cell | *Pentapharsodinium* *imariense* |
| 7b. | Spine attached to plate Sa of monadoid cell | *Pentapharsodinium dalei* var. *aciculiferum* |

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**Conflict of Interest**

The authors declare no conflicts of interest associated with this manuscript.

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