



Cyst-theca relationships of *Spiniferites bentorii*, *S. hyperacanthus*, *S. ramosus*, *S. scabratus* and molecular phylogenetics of *Spiniferites* and *Tectatodinium* (Gonyaulacales, Dinophyceae)

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ABSTRACT

It is well known that modern resting cysts with morphologies matching those of species of the fossil genus *Spiniferites* germinate into motile cells of the genus *Gonyaulax*. Different *Spiniferites* species have been connected to a single *Gonyaulax* species, raising the question of whether they are over-classified. Through germination experiments of cysts with the morphological features of four species of *Spiniferites*, viz. *S. bentorii*, *S. hyperacanthus*, *S. ramosus* and *S. scabratus*, we established cyst-theca relationships. Cysts with the morphology of *S. bentorii* gave rise to vegetative, motile cells of *Gonyaulax nezaniae* sp. nov., which is characterized by two stout antapical spines. Cysts with *S. hyperacanthus* and *S. ramosus* morphologies germinated into *Gonyaulax whaseongensis* and *G. spinifera*, respectively. Cysts with *S. scabratus* morphology lacked a ventral pore and were attributed to *Gonyaulax* cf. *spinifera*. Gene sequences for SSU, LSU and/or ITS-5.8S rRNA were obtained from these four species, and from cysts with the morphology of *Spiniferites belerius*, *S. mirabilis*, *S. lazus*, *Spiniferites* cf. *bentorii* and *Tectatodinium pellitum*. The maximum likelihood and Bayesian inference analyses based on LSU and SSU rRNA gene sequences revealed that cysts assignable to *Spiniferites* formed a polyphyletic group, intermingled with *Tectatodinium*, *Bitectatodinium*, *Ataxiodinium* and *Impagidinium*, whereas *Gonyaulax* species appeared as monophyletic. From our results we inferred the phylogenetic positions of *S. bentorii*, *S. mirabilis*, *S. lazus*, *S. scabratus*, *Tectatodinium pellitum* and *Gonyaulax digitale* for the first time, supporting the idea that *Spiniferites* species are not over-classified and each of them may correspond to different *Gonyaulax* species.

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INTRODUCTION

The International Code of Nomenclature for algae, fungi and plants (ICN; Turland *et al.* 2018) permits the use of different names for dinoflagellate species with fossil types (generally a cyst; e.g. Head *et al.* 2016) and extant species with non-fossil types (usually based on motile cells) even when the fossil and modern forms are considered conspecific (Turland *et al.* 2018, Art. 11.7 and 11.8). Classic examples of the use of two names for taxa often considered the same species are found in the fossil genus *Spiniferites* Mantell and the extant genus *Gonyaulax* Diesing. The relationship between *Spiniferites* and *Gonyaulax* was first established through germination of a living cyst with the morphological features of *Hystrichosphaera bentorii* M. Rossignol [now known as *Spiniferites bentorii* (M. Rossignol) D. Wall & B. Dale], from which a motile cell emerged. This cell was identified as

G. digitale (C.H.G. Pouchet) Kofoid (Wall & Dale 1966). *Spiniferites* cysts display the tabulation formula 2pr, 4', 6'', 6c, 5–6s, 6''', 1p, 1'''' and possess gonal and/or intergonal processes (the former are always trifurcate and the latter are always bifurcate), sutural ridges or septa connecting their bases, and a precingular archeopyle formed by the loss of plate 3'' (Mertens & Carbonell-Moore 2018). 2pr was first introduced by Lewis *et al.* (1999) and refers to the presence of an apical pore complex and a ventral pore on the epitheca. One hundred and six species of *Spiniferites* are currently accepted (Williams *et al.* 2017). Key features to differentiate *Spiniferites* species include size and shape of central body, number, location and shape of processes, the presence or absence of an apical boss, wall surface ornamentations, and cingulum displacement. However, the morphology of these species may intergrade, which complicates their identification

(Mertens *et al.* 2018b). The type species of *Spiniferites*, *S. ramosus* (Ehrenberg) Mantell was described from the Senonian (Upper Cretaceous; Sarjeant 1970).

Only 13 *Spiniferites* species have been reported from modern material (Zonneveld *et al.* 2013; Price & Pospelova 2014; Mertens & Carbonell-Moore 2018). The cyst-theca relationship of some *Spiniferites* species has been established, e.g. *S. bentorii* and *S. ramosus* have been connected to *Gonyaulax digitale* and *G. spinifera* (Claparède & J. Lachmann) Diesing, respectively (Wall & Dale 1967, 1970). However, *Spiniferites mirabilis* (M. Rossignol) Matsuoka, *S. elongatus* P.C. Reid and *S. membranaceus* (M. Rossignol) Sarjeant have also been connected to *G. spinifera* (Wall & Dale 1968; Dale 1976), although later *S. membranaceus* and *S. elongatus* were transferred to *Gonyaulax* as the two independent species *G. membranacea* (M. Rossignol) Ellegaard, Daugbjerg, Rochon, Jane Lewis & I. Harding and *G. elongata* (P.C. Reid) Ellegaard, Daugbjerg, Rochon, Jane Lewis & I. Harding, respectively (Ellegaard *et al.* 2003). Several other cyst-defined genera were reported to produce motile cells which were similar to *Gonyaulax spinifera*, including *Nematosphaeropsis balcombiana* Deflandre & Cookson, *Ataxiodinium choanum* P.C. Reid and *Tectatodinium pellitum* D. Wall (Wall & Dale 1968; Morquecho *et al.* 2009). Later, *Bitectatodinium tepikiense* G. J. Wilson was also connected to *Gonyaulax digitale* (Lewis *et al.* 2001), which casts doubt on the assignment of *G. digitale* as the parent theca of *S. bentorii*-like cysts (Wall 1965). The assignment of numerous fossil-based species to a single extant species (*G. spinifera*) would suggest that the fossil-based species are over-classified.

The non-fossil genus *Gonyaulax* was established with *G. spinifera* as the type species (Diesing 1866). However, the cell morphology of *Gonyaulax* is rather conservative, and three morphologically similar species have been placed in the *Gonyaulax spinifera* group, namely *G. spinifera*, *G. digitale* and *G. diegensis* Kofoid (Kofoid 1911). The tabulation of the genus *Gonyaulax* was interpreted as Po, 3', 2a, 6'', 6c, 4–8s, 5''', 1p, 1'''' (Dodge 1989), but this was later reinterpreted as 2pr, *4', 6'', 6c, ?s, *6''', 1p, 1'''' (Lewis *et al.* 1999; Carbonell-Moore & Mertens 2019). Lewis *et al.* (1999) interpreted the third apical plate as Cv (Taylor-Evitt notation), thus the two Kofoidian intercalary plates were designated as the third and fourth apical homologues (*3', *4'), as previously proposed by Fensome *et al.* (1993).

Heterospory (one species producing several cyst types) has been proposed for the genus *Gonyaulax* (Wall & Dale 1968, p. 270). This was demonstrated for *Gonyaulax baltica* Ellegaard, Jane Lewis & I. Harding, which was related to two cyst types: one resembling *Spiniferites bulloideus* (Deflandre & Cookson) Sarjeant and another like *Impagidinium caspiense* F. Marret (Ellegaard *et al.* 2002; Mertens *et al.* 2018a). In addition, crossed strains of *Gonyaulax spinifera*-like cells produced cysts similar to both *Spiniferites ramosus* and *Nematosphaeropsis labyrinthus* (Ostenfeld) P.C. Reid, and the expression of a particular phenotype was attributed to the specific salinity conditions (Rochon *et al.* 2009). However, a number of *Gonyaulax* species have been shown not to be heterosporous: *G. elongata*, *G. membranacea* and

G. ellegaardiae K.N. Mertens, H. Aydin, Y. Takano, A. Yamaguchi & Matsuoka were proposed to be the respective motile stages of cysts resembling *Spiniferites elongatus*, *S. membranaceus* and *S. pachydermus* (M. Rossignol) P.C. Reid, with differences in morphology of both cyst and motile stage as well as in LSU rRNA gene sequence (Ellegaard *et al.* 2003; Mertens *et al.* 2015).

Whether the extant material examined herein represents the same species that we know from fossilized cysts, formed tens or hundreds of thousands of years ago, is debatable. However, we assume a close relatedness between fossil and modern species producing cysts with very similar morphologies. To keep the text simple, in the following we refer to the cysts of our modern material that match the morphology of fossilized cysts by the names used for the corresponding fossil species.

It is of note that some species of *Gonyaulax* can produce yessotoxin (YTX), a marine polyether toxin that was first isolated from the scallop *Mizuhopecten yessoensis* Jay (Murata *et al.* 1987). To date, 92 variants of YTX-like ladder shape polyketide toxins have been reported, but most of them have not yet been structurally characterized (Miles *et al.* 2005). Therefore, they are commonly named yessotoxins (YTXs) and adriatoxins (ATX) (Ciminiello *et al.* 1998; Domínguez *et al.* 2010). Some strains identified as *Gonyaulax spinifera* were reported to produce yessotoxins while others did not (Riccardi *et al.* 2009; Chikwililwa *et al.* 2019; Pitcher *et al.* 2019), highlighting the importance of precise identification. In fact, the strain identified as *G. spinifera* producing homoYTX and 45-hydroxyYTX in South Africa (Pitcher *et al.* 2019) was probably *G. membranacea* instead, as inferred from the molecular sequences presented. A strain identified as *Gonyaulax taylorii* Carbonell-Moore was reported to produce YTXs but unfortunately a molecular sequence was not provided (Álvarez *et al.* 2016).

Cyst-theca relationships of *Spiniferites ramosus*, *S. bentorii*, *S. mirabilis* and *Tectatodinium pellitum* have been established (Wall 1965; Lewis *et al.* 1999; Morquecho *et al.* 2009), but since no molecular sequences were obtained, the taxonomic identity of the motile stage needs reassessing. On the other hand, cyst-theca relationships of *S. hyperacanthus* (Deflandre & Cookson) Cookson & Eisenack, *S. lazus* P.C. Reid and *S. scabratus* (D. Wall) Sarjeant have not been examined yet and their molecular sequences are not available. *Spiniferites* and related genera appear as para- or polyphyletic in molecular phylogenies based on small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA) gene sequences (Ellegaard *et al.* 2018; Mertens *et al.* 2018a), suggesting that taxonomic revisions are needed. To address the question of whether *Spiniferites* and related genera are over-classified, we isolated single cysts worldwide and performed germination experiments to obtain SSU, LSU and/or ITS–5.8S rRNA gene sequences of motile cells. Both cyst and theca morphologies were examined in detail with light microscopy (LM) and scanning electron microscopy (SEM) of selected strains, and molecular phylogeny was inferred based on LSU and SSU rRNA gene sequences. In addition, strains of *S. hyperacanthus* and *S. ramosus* were examined for

yessotoxin production by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

MATERIAL AND METHODS

Sample collection and treatment

To isolate cysts, sediment sampling was done using an Ekman grab in coastal waters of China, Diana Lagoon (Corsica, France), Baie de Vilaine (France), Pantan (Croatia), and a Petite Ponar grab in Saanich Inlet (western Canada), between 2011 and 2020 (Table 1). In addition, surficial sediment was collected by a diver at a lagoon located at Isla San José in the Gulf of California on 13 April 2014. The island is at the northern end of Bahía de La Paz (Table 1). For most samples, the top 2 cm of sediment were collected. The samples were stored in the dark at 4°C until further treatment. Approximately 5 g of wet sediment was mixed with 20 ml of filtered seawater and stirred vigorously to dislodge detrital particles. The settled material was subsequently sieved

through 120 µm and 10 µm mesh and washed and collected with filtered seawater. Single cysts were isolated from residues using a micropipette with an inverted Eclipse TS100 (Nikon, Tokyo, Japan) microscope and incubated in small containers with f/2-Si medium (Guillard & Ryther 1962) at 20°C, and 90 µmol m⁻² s⁻¹ under a 12:12 h light:dark cycle (hereafter called standard culture conditions). A water sample was collected in Concarneau Bay, France, on 12 February 2008, from which a motile cell of *Gonyaulax digitale* was isolated (Fig. S1).

Morphological study of thecate stages and cysts

Living cells and cysts of all strains (for which a culture is available) or isolates (without a culture) listed in Table 1 were examined and photographed using a Zeiss Axio Imager light microscope (Zeiss, Göttingen, Germany) equipped with a Zeiss AxioCam HRc digital camera. Cell and cyst size was measured on LM images.

Table 1. Information on *Spiniferites* and *Tectatodinium* strains or isolates used in this study. Species designation, strain/isolate identification, origin, collection date, GenBank accession numbers and yessotoxin (YTX). NA: not available.

Species	Strains/ isolates	Collection date	Location	Latitude	Longitude	GenBank No. (SSU/ITS/LSU)	YTX
<i>S. belerius</i>	-/18-896	27 Jul. 2018	Baie de Vilaine, France	2°39.40'N	47°27.60'W	-/MW775689	NA
<i>S. bentorii</i>	Pantan/-	04 May 2017	Pantan, Croatia (Mediterranean)	43°31.26'N	16°16.38'E	MW793450/-/MW775690	NA
<i>S. bentorii</i>	-/HSN114	04 Nov. 2020	Bohai Sea, China	38°29.51'N	118°32.80'E	MW775713/-/	NA
<i>S. cf. bentorii</i>	-/WN20	08 Mar. 2016	Panjin, Bohai Sea, China	42°34.85'N	122°0.10'E	-/MW775691	NA
<i>S. cf. bentorii</i>	-/WN21	08 Mar. 2016	Panjin, Bohai Sea, China	42°34.85'N	122°0.10'E	-/MW775692	NA
<i>S. cf. bentorii</i>	-/WN22	08 Mar. 2016	Panjin, Bohai Sea, China	42°34.85'N	122°0.10'E	-/MW775693	NA
<i>S. hyperacanthus</i>	TIO712/-	27 Feb. 2018	Xiamen Bay, East China Sea, China	24°35.568'N	118°9.198'E	MW775714/-/MW775694	none
<i>S. hyperacanthus</i>	TIO260/-	31 Mar. 2016	Taiwan Strait, China	24°39.60'N	118°57.00'E	MW775715/-/MW775695	none
<i>S. hyperacanthus</i>	GSJZ01/-	28 Apr. 2011	Jinzhou, Bohai Sea, China	40°43.60'N	121°2.50'E	-/MW775696	NA
<i>S. hyperacanthus</i>	TIO1416/-	13 Oct. 2019	Sanmen, East China Sea, China	29°0.16'N	121°45.27'E	-/MW775697	NA
<i>S. lazus</i>	-/18-898	27 Jul. 2018	Baie de Vilaine, France	2°39.40'N	47°27.60'W	-/MW775698	NA
<i>S. mirabilis</i>	-/20-188	13 Apr. 2014	Bahía de La Paz, Gulf of California	24° 52.54'N	110° 33.50'W	MW775716/MW775678/ MW775699	NA
<i>S. ramosus</i>	TIO700/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	MW775717/MW775679/ MW775700	none
<i>S. ramosus</i>	TIO701/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	MW775718/MW775680/ MW775701	none
<i>S. ramosus</i>	TIO702/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	-/MW775681/MW775702	none
<i>S. ramosus</i>	TIO703/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	-/MW775682/MW775703	none
<i>S. ramosus</i>	TIO704/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	-/MW775683/MW775704	none
<i>S. ramosus</i>	TIO705/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	-/MW775684/MW775705	none
<i>S. ramosus</i>	TIO904/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	-/MW775706	none
<i>S. ramosus</i>	TIO905/-	05 Mar. 2017	Saanich Inlet, Canada	48°39.30'N	123°28.31'W	-/MW775685/MW775707	NA
<i>S. ramosus</i>	TIO1400/-	05 Mar. 2017	Saanich Inlet, Canada	48°40.23'N	123°28.03'W	-/MW775708	NA
<i>S. ramosus</i>	-/WYJ	13 Nov. 2017	Xiamen Bay, East China Sea, China	24°35.57'N	118°9.20'E	-/MW775709	NA
<i>S. ramosus</i>	TIO1218/-	12 Sep. 2019	Jiaozhou Bay, Qingdao, China	36°0.15'N	120°21.27'E	-/MW775686/MW775710	NA
<i>S. scabratus</i>	TIO706/-	18 Jan. 2016	Diane Lagoon, Corsica, France (Mediterranean)	42°7.66'N	9°31.72'E	MW775719/MW775687/ MW775711	NA
<i>T. pellitum</i>	-/20-179	13 Apr. 2014	Bahía de La Paz, Gulf of California	24°52.54'N	110°33.50'W	MW775720/MW775688/ MW775712	NA

For scanning electron microscopy (SEM), mid-exponential batch cultures of selected strains were concentrated with a Universal 320 R centrifuge (Hettich-Zentrifugen, Tuttlingen, Germany) at 850 g for 10 min at room temperature. Cells were fixed with 2.5% glutaraldehyde for 3 h at 8°C, and rinsed twice with deionized water. The supernatant was removed and the settled cells were transferred to a coverslip coated with poly-L-lysine (molecular weight 70,000–150,000). The cells attached to the coverslip were rinsed twice in deionized water and dehydrated through a graded ethanol series (10%, 30%, 50%, 70%, 90% and 3 × in 100%, 10 min at each step), critical-point-dried using a K850 Critical-Point Dryer (Quorum/Emitech, West Sussex, UK), sputter-coated with gold using a Hitachi JFC-1600 sputter-coater (Hitachi, Japan), and examined with a Zeiss Sigma FE (Zeiss, Oberkochen, Germany) scanning electron microscope at Xiamen University, China.

For SEM of cultures obtained from cysts with the morphologies of *Spiniferites bentorii* and *S. belerius* P.C. Reid, single specimens were isolated using a micropipette on a IX70 (Olympus) inverted microscope. The cells were deposited on polycarbonate membrane filters (GTTP Isopore, 0.22 µm pore size; Millipore, Billerica, MA, USA), which were rinsed with distilled water. The filters were processed following the methods described in Chomérat & Couté (2008). They were dehydrated in a graded series of ethanol baths (15–100%), critical-point-dried, attached to a stub using double-sided adhesive tape and coated with gold. The stubs were examined at the Station of Marine Biology in Concarneau, France, using a Sigma 300 Gemini (Zeiss, Oberkochen, Germany) field-emission SEM equipped with both a conventional Everhart-Thornley and an in-lens secondary electron detector at 1.5 kV. Tabulation labelling follows the Kofoidian system (Kofoid 1909, 1911). Sulcal plate labelling follows Balech (1980).

PCR amplifications and sequencing

Single cells or cysts were isolated and washed several times with sterile distilled water. They were broken by applying gentle force on the coverslip and used for templates. Various regions of the ribosomal RNA (rRNA) genes including the SSU, partial LSU (D1–D6) and ITS1–5.8S–ITS2 were amplified using primer pairs specified previously and following standard protocols (Luo *et al.* 2019). The SSU rRNA gene of *G. digitale* collected in Concarneau Bay was sequenced following the procedures described in Wang *et al.* (2019). Newly obtained sequences were deposited in GenBank with accession numbers MW775678 to MW775720, MW795366 and MW793450.

Sequence alignment and phylogenetic analyses

Newly obtained LSU rRNA (*c.* 1300 bp) and SSU rRNA (*c.* 1700 bp) gene sequences were aligned with sequences of *Gonyaulax* species and related taxa available in GenBank. Sequences were aligned using MAFFT v7.110 (Katoh & Standley 2013) online program (<http://mafft.cbrc.jp/alignment/server/>) with default settings. Alignments were

manually checked with BioEdit v7.0.5 (Hall 1999). The final alignment consisted of 1370 (LSU) and 1853 (SSU) base pairs including introduced gaps. For Bayesian inference (BI), the program jModelTest (Posada 2008) was used to select the most appropriate model of molecular evolution with Akaike Information Criterion (AIC). Bayesian reconstruction of the data matrix was performed using MrBayes 3.2 (Ronquist & Huelsenbeck 2003) with the best-fitting substitution model (GTR+G). Four Markov chain Monte Carlo (MCMC) chains ran for 2,000,000 generations, sampling every 1000 generations. The first 10% of burn-in trees were discarded. A majority rule consensus tree was created in order to examine the posterior probabilities (BPP) of each clade. Maximum likelihood (ML) analyses were conducted with RaxML v7.2.6 (Stamatakis 2006) on the T-REX web server (Boc *et al.* 2012) using the model GTR+G. Bootstrap support (BS) was assessed with 1000 replicates.

Yessotoxin analysis

Cultures of two strains of *G. whaseongensis* and seven strains of *G. spinifera* were grown in 200 ml Erlenmeyer flasks under standard culture conditions. At stationary phase, ~10⁵ cells were concentrated with a Universal 320 R centrifuge at 850 g for 10 min at room temperature. Stationary phase was determined via linear regression of log-transformed cell count time series. Algal pellets for quantification of intracellular YTX were transferred to 2 ml microcentrifuge tubes and stored at –20°C until analysed. Measurements were carried out by liquid chromatography (LC 1100, Agilent, Waldbronn Germany) coupled to tandem mass spectrometry (API 4000 QTrap, Sciex, Darmstadt Germany) as detailed in Wang *et al.* (2019). In brief, separation was performed on a reversed phase column with gradient elution from 40% aqueous methanol–acetonitrile mixture to 100% methanol–acetonitrile. Yessotoxins were screened in the negative mode by selected reaction monitoring (SRM).

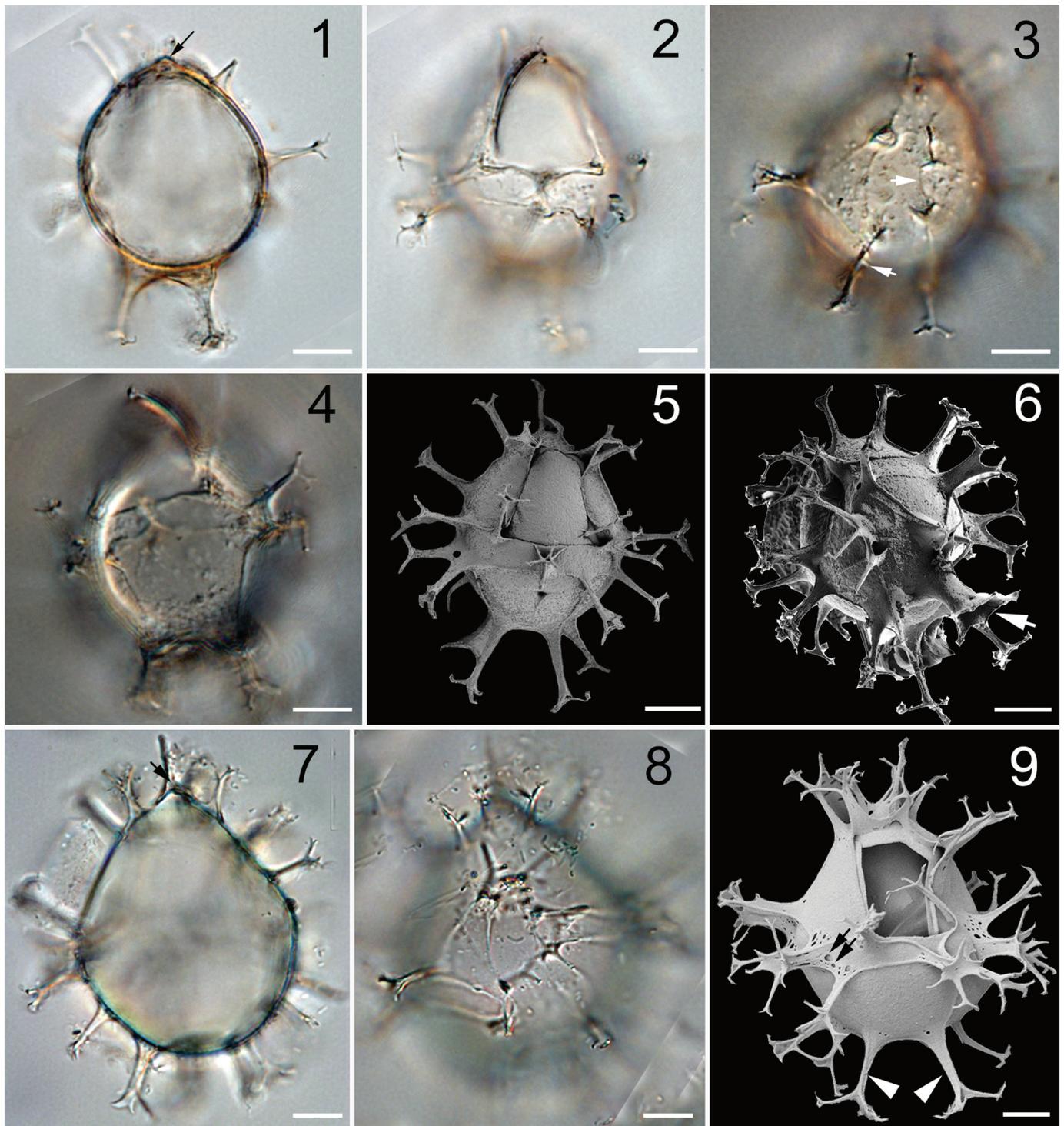
RESULTS

Morphology

For species not established in culture only the cyst stages are described, whereas both cyst and thecate stages are described from the cultures available. Cyst morphologies are indicated by the names of the fossil species which they match. In the following, dimensions given for the cyst central body do not include the processes.

SPINIFERITES BELERIUS

The cyst from Baie de Vilaine, France, from which the hatchling (isolate 18–896, Table 1) was sequenced, was oval, with a central body 37.5 µm long and 32.5 µm wide, with a characteristic, small apical boss (Fig. 1). The archeopyle was not reduced, and was formed by the loss of plate 3'' (Figs 2, 5). The operculum was monoplacate and free. The paracingulum descended with a displacement of twice its width (Fig. 3). The parasulcus was weakly expressed. The cyst had a thin and finely granular wall ornamented with exclusively gonal,



Figs 1–9. Light (LM) and scanning electron micrographs (SEM) of modern cysts with the morphologies of *Spiniferites belerius* from Baie de Vilaine, France, and *Spiniferites bentorii* from Pantan, Croatia. Scale bars = 10 μ m.

Fig. 1. *Spiniferites belerius*, LM, mid focus of ventral view of an empty cyst, showing the apical protuberance (arrow).

Fig. 2. *Spiniferites belerius*, LM, high focus of dorsal view of an empty cyst, showing the archeopyle.

Fig. 3. *Spiniferites belerius*, LM, high focus of ventral view of an empty cyst, showing the cingulum (arrows).

Fig. 4. *Spiniferites belerius*, LM, high focus of dorsal view of an empty cyst.

Fig. 5. *Spiniferites belerius*, SEM, dorsal view of cyst, showing the archeopyle.

Fig. 6. *Spiniferites belerius*, SEM, antapical view of cyst, showing the trumpet-like process (arrow).

Fig. 7. *Spiniferites bentorii*, LM, mid focus of ventral view of an empty cyst, showing the apical boss (arrow).

Fig. 8. *Spiniferites bentorii*, LM, high focus of ventral view of an empty cyst, showing the cingulum displacement.

Fig. 9. *Spiniferites bentorii*, SEM, dorsal view of an empty cyst, showing the reduced archeopyle, the fenestrations at the base of processes (arrows) and two antapical processes (arrowheads).

trifurcate processes 5.8–13.3 μm long (Figs 3–5). There was one petaloid antapical trumpet-shaped process (Fig. 6).

SPINIFERITES BENTORII

The cyst from Pantan, Croatia, that originated the culture (strain Pantan, Table 1) was pear-shaped, with a central body 53.3 μm long and 43.8 μm wide, with a pronounced apical protuberance (Fig. 7). The paracingulum descended with a displacement of three times its width with no overhang (Fig. 8). The parasulcus was weakly expressed. Processes were exclusively gonal with trifurcate distal ends 9.6–11.7 μm long, with fenestrate bases (Fig. 9). The wall surface was finely granulated. The archeopyle was reduced, formed by the loss of plate 3'' (Fig. 9). The operculum was monoplacate and free. There were two pronounced antapical processes *c.* 14.6 μm in length (Fig. 9). The cyst from Bohai Sea, China (isolate HSN114; Table 1) was pear-shaped, with a central body 62.7 μm long and 64.2 μm wide, with a pronounced apical protuberance (Fig. S2). Processes were exclusively gonal with trifurcate distal ends 9.0–10.5 μm long. The cells of strain Pantan were 36.7–67.5 μm ($46.8 \pm 6.9 \mu\text{m}$, $n = 25$) long and 33.3–51.7 μm ($41.6 \pm 4.7 \mu\text{m}$, $n = 25$) wide with two stout antapical spines 2.3–10.7 μm ($6.8 \pm 2.5 \mu\text{m}$, $n = 25$) long (Fig. 10). The nucleus was elongated and located in the hyposome. The cingulum was located in the middle of the cell and descended about three times its width with an overhang of four times of its width (Fig. 11). These cells displayed a plate formula of 2pr, 4', 6'', 6C, 6S, 5''', 1p, 1'''''. The thecae had a sexiform gonyaulacoid tabulation (Fensome *et al.* 1993, text-fig. 64) with an S-type ventral organization (Fensome *et al.* 1993, text-figs 82B, D) and neutral torsion (Fensome *et al.* 1993, text-fig. 83B) (Figs 12, 13, 15). The epitheca was conical with pronounced shoulders whereas the hypotheca was trapezoidal. There were six cingular plates of similar size (Fig. 19). The cell surface was thick and reticulated, with numerous trichocyst pores, often at the junctions of the reticulations. The apical pore complex (APC) comprised a pore plate with a lenticular pore inside, surrounded by raised ridges of neighbouring plates, with a small Q plate neighbouring the APC at the right side (Fig. 18).

The first and fourth apical (1', 4') plates were narrow and elongated, whereas plates 2' and 3' were large and occupied the dorsal part (Fig. 13). There was a ventral pore at the junction of plates 1', 4' and Q (Fig. 14). The angle between the major axis and a line joining the ends of the cingulum was approximately 20°. The precingular plates were similar in size except that plate 6'' was triangular and the smallest (Fig. 13). The postcingular plates were similar in size (Figs 15, 16). The antapical (1''''') plate was seven-sided and asymmetrical, wider than long, located in the middle of the hypotheca (Fig. 15). The posterior intercalary (1p) plate was narrow and elongated, contacting the left side of plate Sp (Fig. 15). The sulcus comprised the anterior sulcal plate (Sa), anterior left sulcal plate (Ssa), posterior left sulcal plate (Ssp), anterior right sulcal plate (Sda), posterior right sulcal plate (Sdp) and posterior sulcal plate (Sp) (Figs 16, 17). A schematic drawing of the motile cells is provided in Figs (20–23).

SPINIFERITES CF. BENTORII

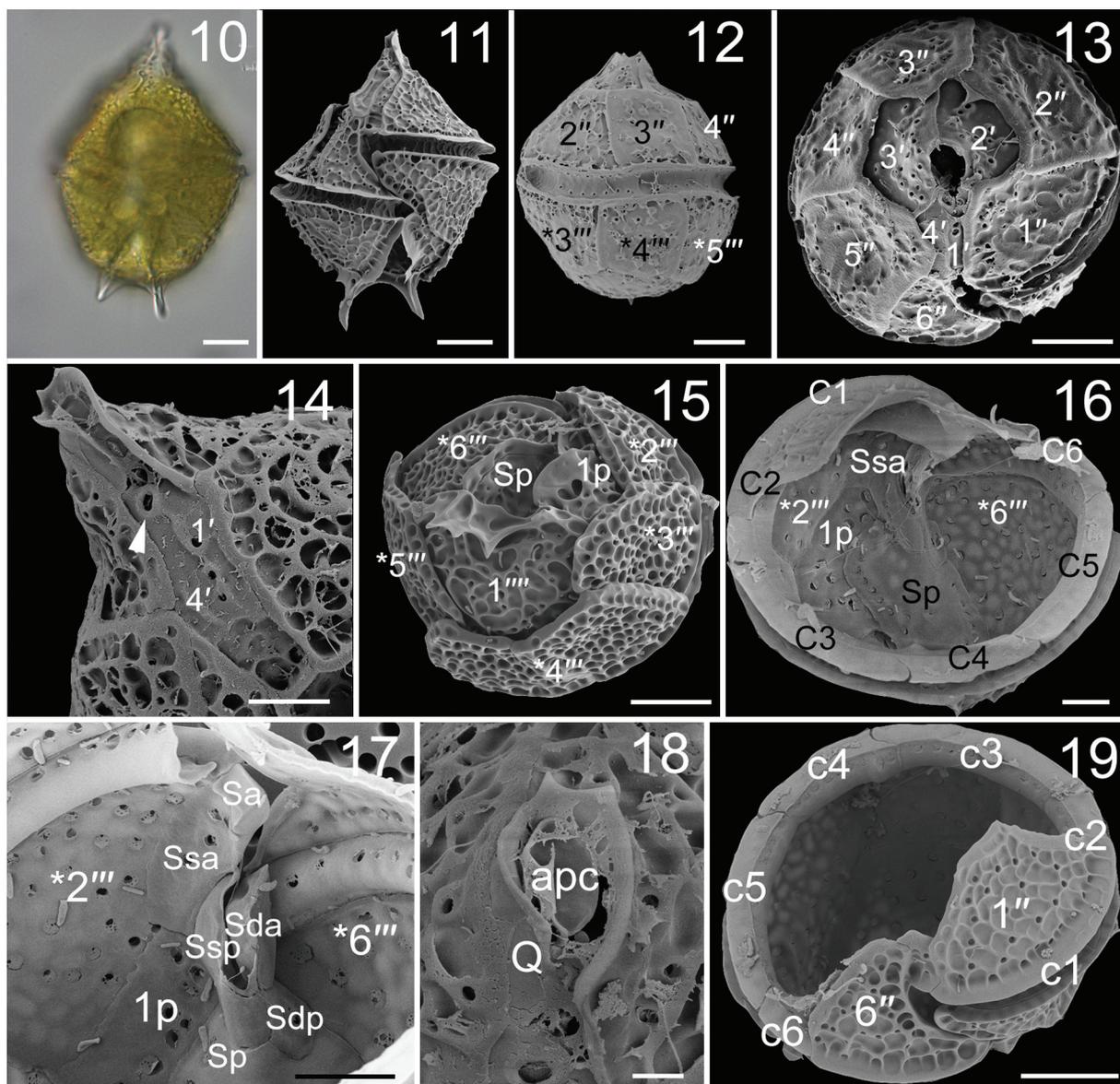
Cysts from the Bohai Sea (isolates WN20, WN21, WN22, Table 1) were pear-shaped, with a central body 49.8–63.9 μm ($54.5 \pm 6.4 \mu\text{m}$, $n = 4$) long and 43.5–52.8 μm ($47.0 \pm 4.1 \mu\text{m}$, $n = 4$) wide, with a pronounced apical protuberance (Fig. 24). The wall surface was finely granular. One or two bifurcate intergonal processes were found between each pair of trifurcate gonal processes (Figs 25, 28). The processes were 4.2–7.3 μm long. Process bases were not fenestrate (Figs 27–29). The paracingulum descended with a displacement of three times its width with no overhang (Fig. 27). The parasulcus was weakly expressed. The archeopyle was reduced, formed by the loss of plate 3'' (Fig. 28). The operculum was monoplacate and free.

SPINIFERITES HYPERACANTHUS

The ovoid cyst from the East China Sea that yielded the strain TIO712 (Table 1) had a central body 41.8 μm long and 37.7 μm wide. The cyst had a smooth to finely granular surface. One bifurcate intergonal process was found between each pair of trifurcate gonal processes (Figs 30, 31). Processes were hollow (Fig. 32), 9.0–11.2 μm long, with bases connected by low ridges about 1.5 μm high. Sometimes fenestrate bases could be observed (Fig. 34). The paracingulum descended with a displacement of its own width. The parasulcus was somewhat expressed. The archeopyle was reduced and precingular, corresponding to plate 3'' (Figs 31, 33). The operculum was monoplacate and free. Cysts formed spontaneously in culture of strain TIO260, which was established through the germination of a single cyst. They were 34.5–40.5 μm long and 31.5–37.0 μm wide, with processes 5.4–12.0 μm long (Figs 33–35).

Thecate cells of strain TIO712 were 27.5–48.3 μm ($36.9 \pm 7.8 \mu\text{m}$, $n = 20$) long and 22.4–36.6 μm ($28.6 \pm 5.3 \mu\text{m}$, $n = 20$) wide. The epitheca was conical and the hypotheca was trapezoidal. The cingulum was situated in the equatorial part of the cell and descended about twice its width with a slight overhang (Fig. 36). The angle between the major axis and a line joining the ends of the cingulum was approximately 15°. There were numerous banded chloroplasts in the periphery of the cell and a curved nucleus in the hyposome (Fig. 37). Cells had a plate formula of 2pr, 4', 6'', 6C, 6S, 5''', 1p, 1'''''. The theca had a sexiform gonyaulacoid tabulation (Fensome *et al.* 1993, text-fig. 64) with an S-type ventral organization (Fensome *et al.* 1993, text-figs 82B, D) and dextral torsion (Fensome *et al.* 1993, text-fig. 83C) (Figs 38, 39). Most cells had a long and a short antapical spine on the left and right of posterior sulcal rim, respectively (Fig. 38). Each cell had a short apical horn and a slightly angled shoulder. The cell surface was thick and reticulated with numerous trichocyst pores, often at the junctions of the reticulations. The apical pore complex (APC) comprised a pore plate with a lenticular pore inside, surrounded by raised ridges of neighbouring plates with a small Q plate abutting the APC at the right side (Fig. 42).

Plates 1' and 4' were narrow and elongated, with a row of small pores at the right margin of 1', whereas plates 2' and 3'



Figs 10–19. Motile cells from culture originated from *Spiniferites bentorii*-like cysts from Croatia, LM and SEM.

Fig. 10. Mid focus of a living cell, showing two stout antapical spines (LM). Scale bar = 10 μ m.

Fig. 11. Ventral view of a cell showing cingulum displacement (SEM). Scale bar = 10 μ m.

Fig. 12. A cell in dorsal view showing neutral torsion (SEM). Scale bar = 10 μ m.

Fig. 13. Apical view of a cell showing four apical plates (1''–4'') and six precingular plates (1'''–6''') (SEM). Scale bar = 10 μ m.

Fig. 14. Ventral view of a cell showing a ventral pore (arrow) (SEM). Scale bar = 5 μ m.

Fig. 15. Antapical view of a cell showing the five postcingular plates (*2'''–6'''), one antapical plate (1''''') and one posterior intercalary plate (1p, SEM). Scale bar = 10 μ m.

Figs 16, 17. Internal view showing the anterior sulcal plate (Sa), left anterior sulcal plate (Ssa), left posterior sulcal plate (Ssp), anterior right sulcal plate (Sda), posterior right sulcal plate (Sdp) and posterior sulcal plate (Sp, SEM). Scale bars = 5 μ m.

Fig. 18. Apical view showing the apical pore complex (apc) and a Q plate (SEM). Scale bar = 2 μ m.

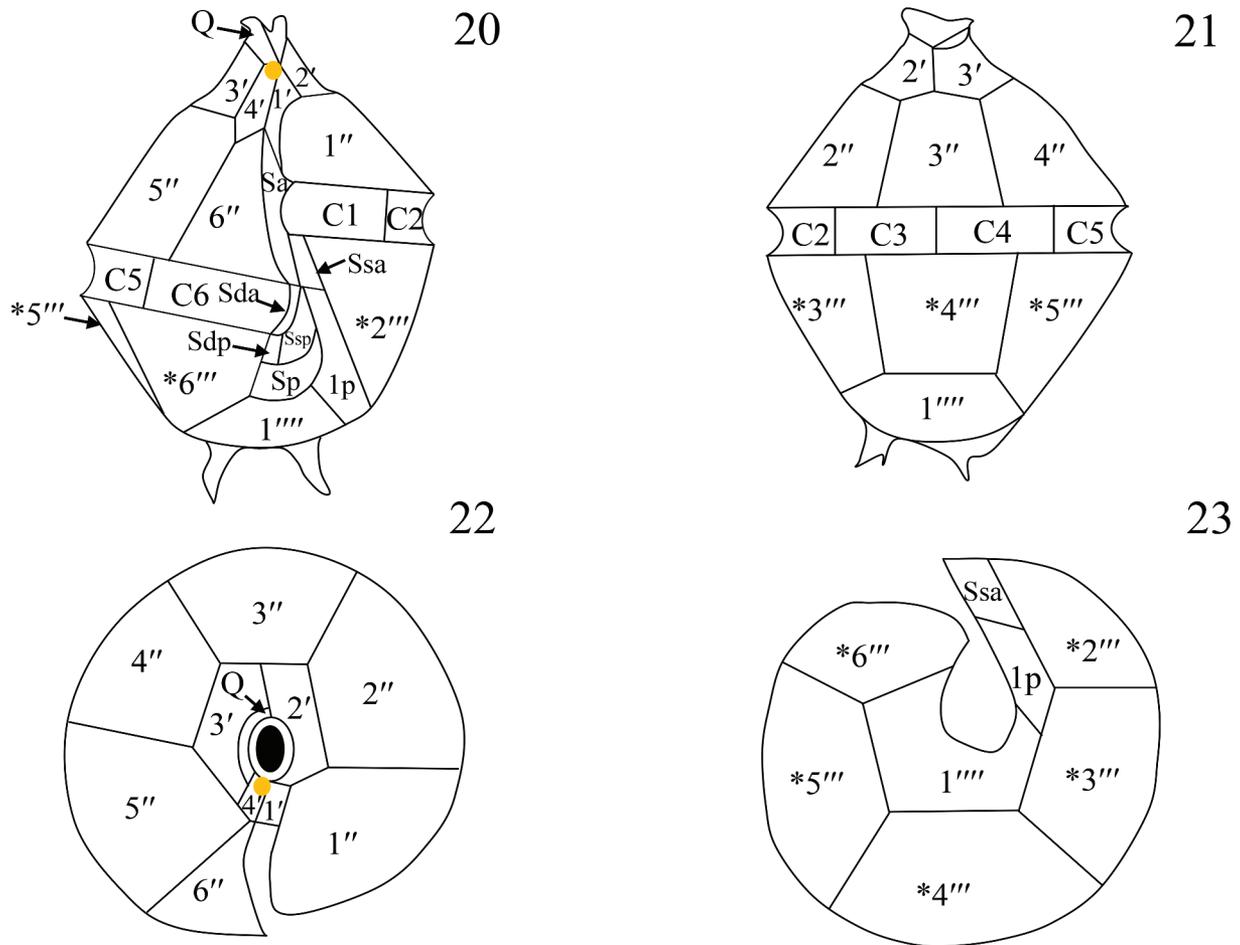
Fig. 19. A broken cell showing six cingular plates (SEM). Scale bar = 10 μ m.

were large and occupied the dorsal part (Figs 40, 41). There was a ventral pore at the junction of plates 4' and Q (Fig. 41). Plates 1'' and 2'' were larger than the other precingular plates, with plate 6'' quadrangular and the smallest (Figs 40, 42). The postcingular plates were similar in size (Fig. 43). Plate 1'''' was hexagonal and asymmetrical, wider than long, located in the middle of the hypotheca. Plate 1p was narrow and elongated, and contacted the left side of plate Sp (Fig. 43). The sulcus comprised plates Sa, Ssa, Ssp, Sda, Sdp and Sp (Fig. 44). The morphology of the cultured strains (GSJZ01, TIO260) was

similar to that of strain TIO712 and is not shown here. The morphology of strain TIO1416 was not studied.

SPINIFERITES LAZUS

The cyst from Baie de Vilaine, France, that originated the hatching (isolate 18–898, Table 1) that was sequenced was oval, with a central body 43.3 μ m long and 39.2 μ m wide (Fig. 45). The cyst wall was thick with a microgranular surface. Processes were exclusively gonal, 13.3–15.0 μ m long, with wide fenestrate bases and connected by low sutural crests. The archeopyle was



Figs 20–23. Schematic representation of motile cells originated from *Spiniferites bentorii*-like cysts.

Fig. 20. Ventral view.

Fig. 21. Dorsal view.

Fig. 22. Apical view.

Fig. 23. Antapical view.

reduced, formed by the loss of plate 3'' (Fig. 46). The operculum was monoplate and free. The paracingulum descended with a displacement of three times its width and there was no overhang (Fig. 47). The parasulcus was weakly expressed.

SPINIFERITES MIRABILIS

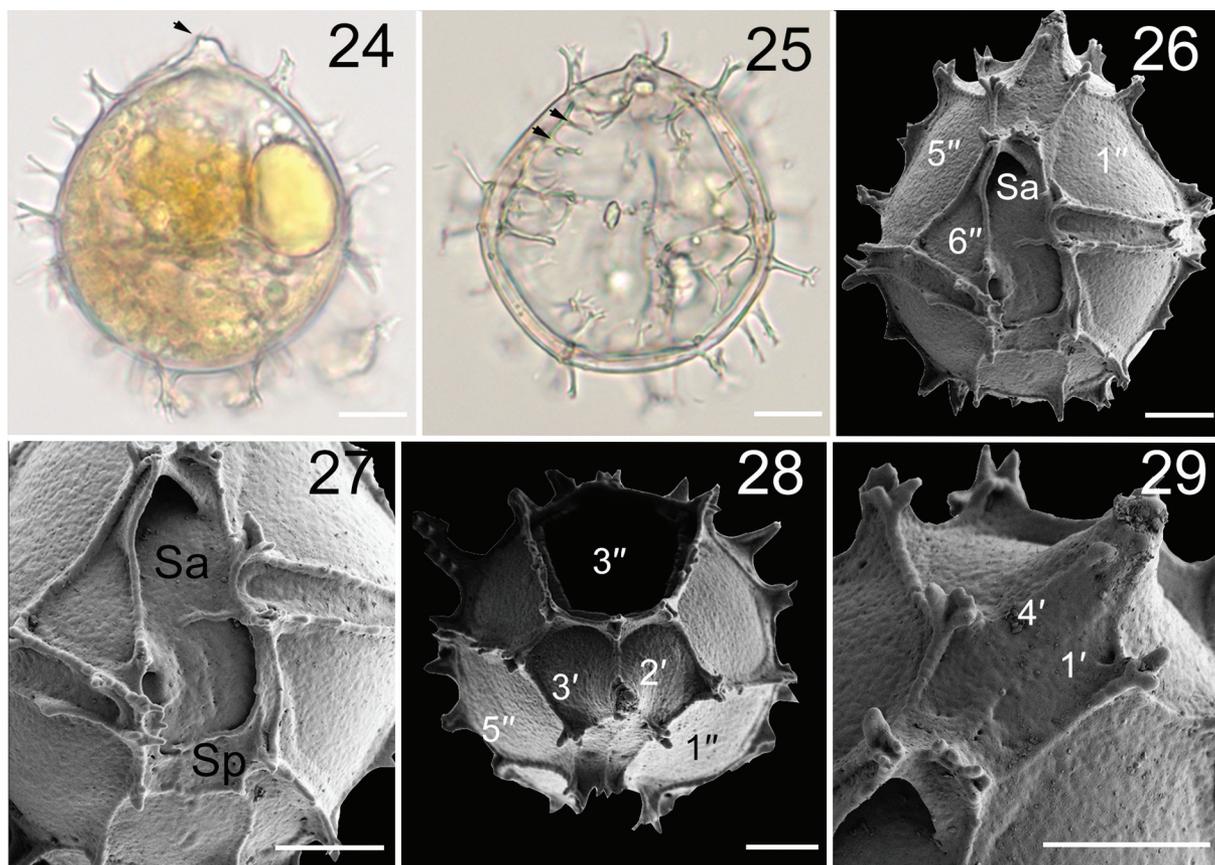
The cyst from the Gulf of California from which the hatchling (isolate 20-188, Table 1) was sequenced was oval, with a central body 35.4 μm long and 30.4 μm wide (Fig. 48). The cyst wall was microgranular. Gonial processes were 6.7–8.3 μm long, slender and connected by low crests. One or two intergonal processes were present between two adjacent gonial processes (Fig. 49). A prominent antapical flange connected the two antapical processes, and was approximately 12.1 μm in length (Fig. 48). The paracingulum descended with a displacement of twice its width and there was no overhang (Fig. 49). The parasulcus was weakly expressed. The archeopyle was reduced, formed by the loss of plate 3'' (Fig. 50).

SPINIFERITES RAMOSUS SENSU ROCHON ET AL. (1999)

Cysts from the Canadian Pacific giving rise to strains TIO700–TIO705, TIO904, TIO905 (Table 1) were ovoid to spheroidal,

with a central body 28.1–46.1 μm ($34.9 \pm 6.2 \mu\text{m}$, $n = 8$) long and 23.4–36.1 μm ($29.3 \pm 4.6 \mu\text{m}$, $n = 8$) wide with processes 8.6–13.5 μm long (Fig. 51). Typical gonial processes were 2.8–10.0 μm long and trifurcate with bifurcate tips. The paracingulum descended with a displacement of its own width, without overhang. The parasulcus was weakly expressed. Cysts formed in culture of strain TIO1400 (Table 1) were 27.5–37.5 μm long and 25.0–33.9 μm wide (Figs 52–56). The cyst surface was coarsely granular (Figs 53–55). Occasionally intergonal processes were observed (Fig. 55). The archeopyle was reduced, corresponding to plate 3'' (Fig. 56).

Cells of strain TIO701 had a pronounced apical horn and slightly angled shoulders. The epitheca was conical and the hypotheca was trapezoidal (Fig. 57). The cingulum was located in the equatorial part of the cell and descended about twice of its width with an overhang of one to two widths. The angle between the major axis and a line joining the ends of the cingulum was approximately 15°. There were numerous banded chloroplasts in the periphery and a curved nucleus in the cingular region (Fig. 58). Cells of strain TIO904 were 26.9–41.0 μm ($34.8 \pm 3.8 \mu\text{m}$, $n = 31$) long and 22.3–32.5 μm ($28.8 \pm 3.4 \mu\text{m}$, $n = 31$) wide. The plate formula was 2pr, 4', 6'', 6C, 6S, 5''', 1p, 1'''. Cells often had two antapical spines of similar size, but some cells



Figs 24–29. *Spiniferites* cf. *bentorii* from Bohai Sea, China, LM and SEM. Scale bars = 10 μ m.

Fig. 24. Mid focus of a living cyst showing the apical boss (arrow) (LM).

Fig. 25. Mid focus of an empty cyst showing the intergonal processes (arrows) (LM).

Fig. 26. Ventral view of an empty cyst (SEM).

Fig. 27. Detail of the sulcus showing anterior sulcal plate (Sa) and posterior sulcal plate (Sp) (SEM).

Fig. 28. Apical view of an empty cyst showing the archeopyle (SEM).

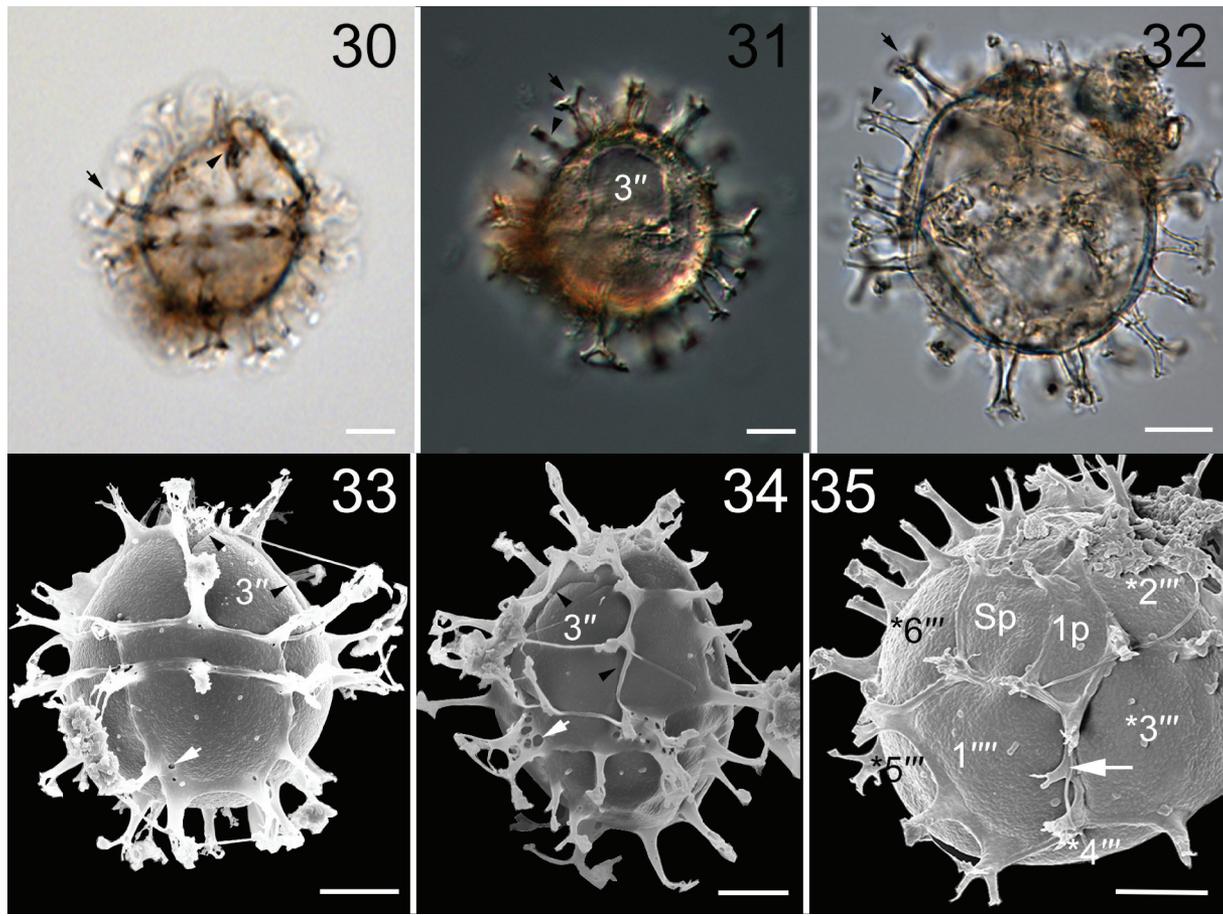
Fig. 29. Apical-ventral view of an empty cyst showing the first and fourth apical plates (SEM).

lacked such spines or had numerous smaller spines. The theca had a sexiform gonyaulacoid tabulation (Fensome *et al.* 1993, text-fig. 64) with an S-type ventral organization (Fensome *et al.* 1993, text-figs 82B, D) and neutral torsion (Fensome *et al.* 1993, text-fig. 83B) (Figs 59, 60). The cell surface was thick and reticulated, with many trichocyst pores. The apical pore complex comprised a pore plate with a lenticular pore inside, surrounded by raised ridges of neighbouring plates, with a small Q plate abutting the APC at its right side (Fig. 63). Plates 1' and 4' were narrow and elongated, whereas plates 2' and 3' were large and occupied the dorsal part (Figs 59–61). There was a ventral pore at the junction of plates 4' and Q (Fig. 63). The precingular plates were similar in size except that plate 6'' was much smaller (Figs 59–62). The postcingular plates were similar in size (Figs 62, 64). Plate 1''' was hexagonal and symmetrical, located in the middle of the hypotheca. Plate 1p was narrow and elongated, and contacted the left side of plate Sp (Fig. 64). The sulcus comprised plates Sa, Ssa, Ssp, Sda, Sdp and Sp (Fig. 62). The morphology of the cultured strain TIO1400 was similar to that of strain TIO701 and is not shown here. The morphology of other strains (TIO700, TIO702–TIO705, TIO904, TIO905, TIO1218, WYJ) was not studied.

SPINIFERITES SCABRATUS

The cyst from the Mediterranean Sea from which strain TIO706 (Table 1) was established was oval, with a central body 32.6 μ m long and 26.8 μ m wide (Fig. 65). There was no apical protuberance. The paracingulum was displaced its own width and the archeopyle was not reduced, formed by the loss of plate 3'' (Fig. 66). The operculum was free. The processes were hollow and wide at their base, c. 8.3–11.0 μ m in length. The processes were exclusively gonal, and trifurcate with bifurcate tips (Figs 65, 66). The wall surface was coarsely granulate (Fig. 67).

Cells of strain TIO706 had a pronounced apical horn and a slightly angled shoulder. The epitheca was conical and the hypotheca was trapezoidal. There were numerous banded chloroplasts in the periphery and a curved nucleus in the hyposome (Fig. 68). The cingulum was located in the equatorial part of the cell and descended about twice its width with an overhang of two widths (Figs 69, 70). The angle between the major axis and a line joining the ends of the cingulum was approximately 10°. Most cells had two antapical spines of similar size (Fig. 70). Cells were 23.6–35.7 μ m ($28.1 \pm 3.2 \mu$ m, $n = 19$) long and 20.9–33.4 μ m



Figs 30–35. *Spiniferites hyperacanthus* from the East China Sea, LM and SEM. Scale bars = 10 μ m.

Fig. 30. High focus of an empty cyst that originated strain TIO712, showing gonial (arrow) and intergonal (arrowhead) processes (LM).

Fig. 31. High focus of the same cyst shown in Fig. 30, showing gonial (arrow) and intergonal (arrowhead) processes and the reduced archeopyle (LM).

Fig. 32. Mid focus of the same cyst shown in Fig. 30, showing gonial (arrow) and intergonal (arrowhead) processes (LM).

Figs 33, 34. Dorsal view of cysts produced in culture of strain TIO260, showing the archeopyle (arrowheads) and the fenestrations at the base of processes (arrows) (SEM).

Fig. 35. Antapical view of a cyst produced in culture of strain TIO260, showing the intergonal process (arrow) (SEM).

($24.6 \pm 3.2 \mu\text{m}$, $n = 19$) wide. Cells had the plate formula 1pr, 4', 6'', 6C, 6S, 5''', 1p, 1'''. The theca had a sexiform gonyaulacoid tabulation (Fensome *et al.* 1993, text-fig. 64) with an S-type ventral organization (Fensome *et al.* 1993, text-figs 82B, D) and neutral torsion (Fensome *et al.* 1993, text-fig. 83B) (Figs 69, 71). The cell surface was thick and reticulated with many trichocyst pores. The apical pore complex comprised a pore plate with a lenticular pore inside, surrounded by raised ridges of neighbouring plates with a small Q plate abutting the APC at the right side (Fig. 72). The sulcus comprised plates Sa, Ssa, Ssp, Sda, Sdp and Sp.

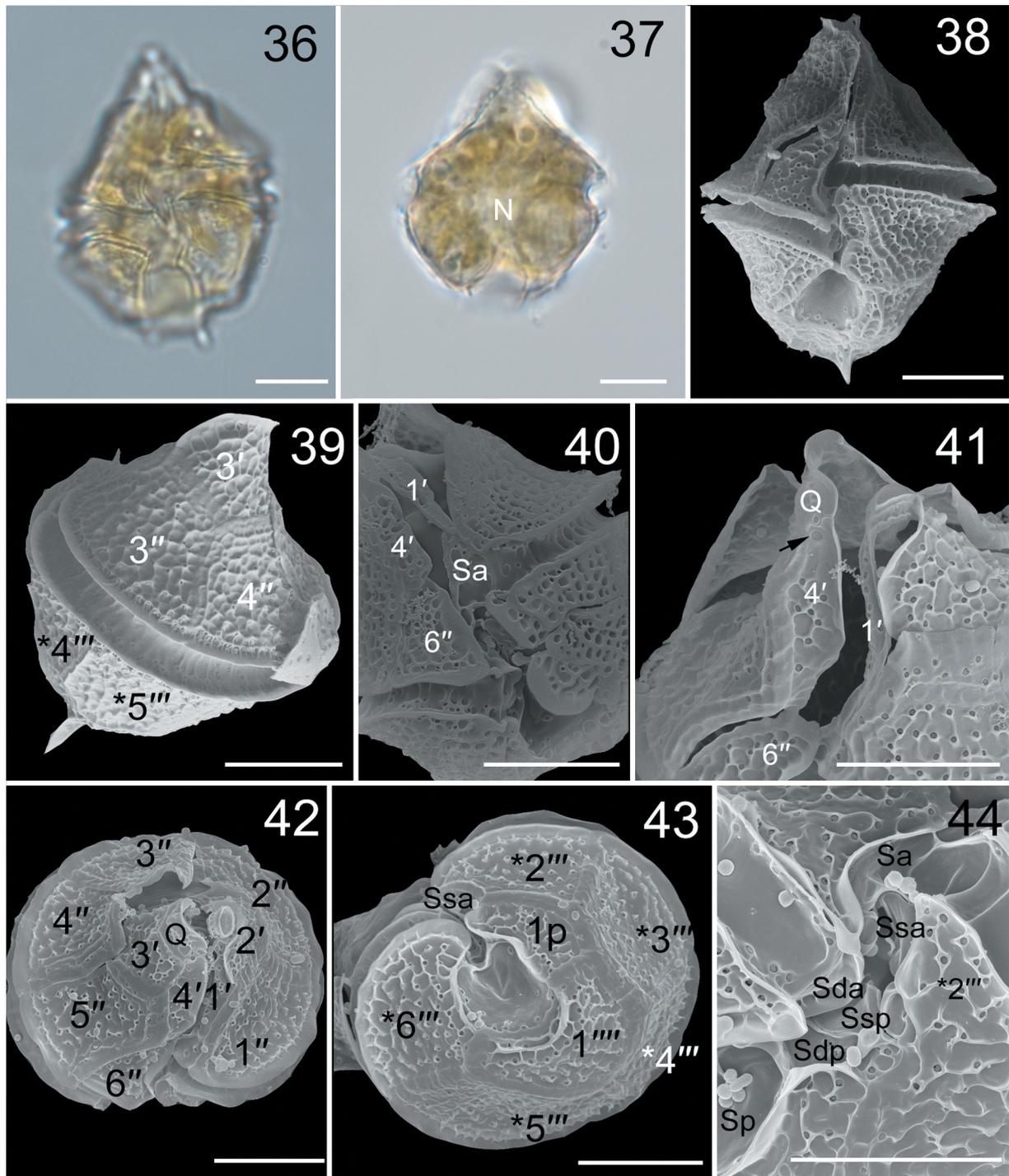
Plates 1' and 4' were narrow and elongated without a ventral pore, whereas plates 2' and 3' were large and occupied the dorsal part (Figs 69–72). The precingular plates were similar in size except that plate 6'' was much smaller. The postcingular plates were similar in size (Figs 70, 73). Plate 1'' was hexagonal and symmetrical, located in the middle of the hypotheca. Plate 1p was five-sided and contacted the left side of plate Sp (Fig. 73).

TECTATODINIUM PELLITUM

The cyst from the Gulf of California from which the hatchling (isolate 20–179, Table 1) was sequenced was oval, with a central body $49.6 \mu\text{m}$ long and $43.8 \mu\text{m}$ wide (Fig. 74). The cyst had an apical protuberance (Fig. 74) and a coarsely granular surface (Fig. 75). The inner wall was thin whereas the outer wall was spongy and $2.9 \mu\text{m}$ thick, formed by numerous, finely interwoven fibrils (Fig. 76). The archeopyle was large and trapezoidal (Fig. 75).

Molecular phylogeny

All strain and isolate numbers and the corresponding GenBank accession numbers are summarized in Table 1. *Spiniferites hyperacanthus* strains TIO260 and TIO1416 had identical LSU rRNA gene sequences. Sequences of *S. hyperacanthus* strains TIO712 and GSJZ01 were identical to those of *Gonyaulax whaseongensis* A.S. Lim, H.J. Jeong & Ji



Figs 36–44. *Spiniferites hyperacanthus* strain TIO712 from the East China Sea, LM and SEM. Scale bars = 10 μ m.

Fig. 36. High focus of a living cell showing the cingulum displacement (LM).

Fig. 37. Mid focus of a living cell showing the nucleus (N) (LM).

Fig. 38. Ventral view of a cell showing the two unequal antapical spines (SEM).

Fig. 39. Dorsal view of a cell showing the dextral torsion (SEM).

Figs 40, 41. Ventral view of a cell showing the first and fourth apical plates (1', 4'), a ventral pore (arrow), the quadrangular sixth precingular plate (6'') and the anterior sulcal plate (Sa) (SEM).

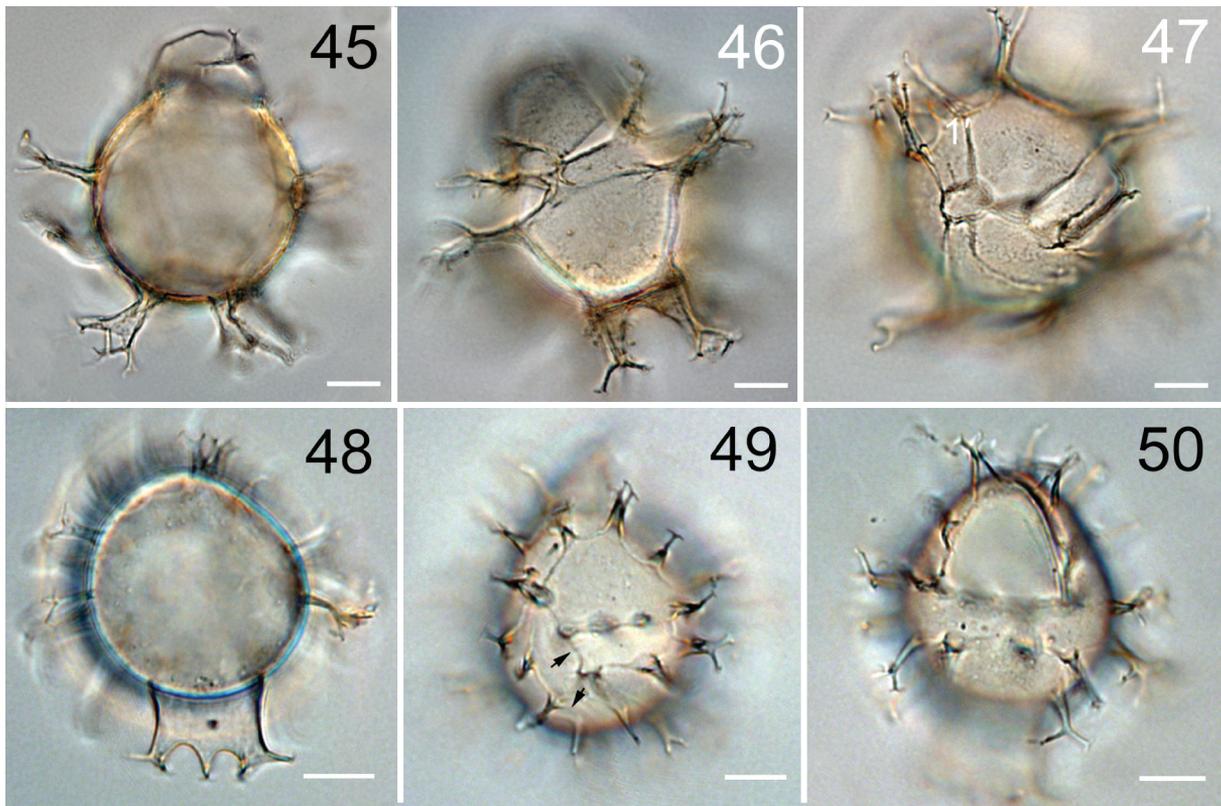
Fig. 42. Apical view of a cell showing four apical plates (1'–4'), Q plate and six precingular plates (1''–6'') (SEM).

Fig. 43. Antapical view of a cell showing five postcingular plates (*2'''–*6'''), one antapical plate (1''') and one posterior intercalary plate (1p) (SEM).

Fig. 44. The sulcus showing the anterior sulcal plate (Sa), anterior left sulcal plate (Ssa), posterior left sulcal plate (Ssp), anterior right sulcal plate (Sda), posterior right sulcal plate (Sdp) and posterior sulcal plate (Sp) (SEM).

Hye Kim (GenBank accession: LS481152), but differed from strains TIO260 and TIO1416 in nine positions (99.33% similarity). *Spiniferites ramosus* strains from the Canadian Pacific,

the Yellow Sea of China and the East China Sea shared identical sequences but they differed from strain UW323 from the U.K. (Lewis *et al.* 1999; Ellegaard *et al.* 2003;



Figs 45–50. *Spiniferites lazus* from Baie de Vilaine, France, and *Spiniferites mirabilis* from the Gulf of California, LM. Scale bars = 10 μ m.

Fig. 45. Mid focus of an empty cyst of *S. lazus* showing the archeopyle.

Fig. 46. High focus of an empty cyst of *S. lazus* showing two more developed antapical processes.

Fig. 47. High focus of an empty cyst of *S. lazus* showing the cingulum displacement.

Fig. 48. Mid focus of an empty cyst of *S. mirabilis* showing the antapical flange.

Fig. 49. High focus of an empty cyst of *S. mirabilis* showing cingulum displacement (arrows).

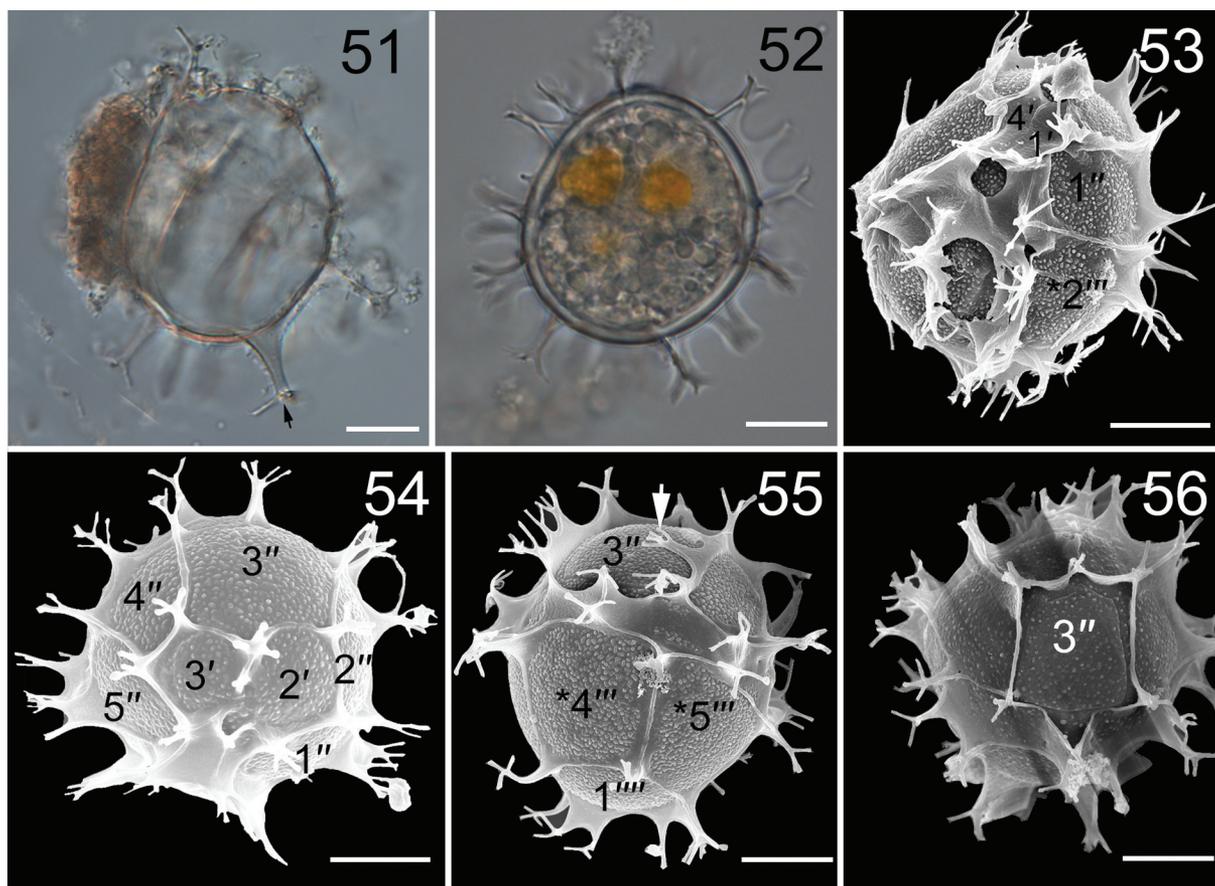
Fig. 50. High focus of an empty cyst of *S. mirabilis* showing the reduced archeopyle.

GenBank accession: AY154960) in six positions (99% similarity). Three Chinese isolates of *S. cf. bentorii* shared identical sequences but they differed from that of *S. bentorii* from the Adriatic Sea at 14 positions (98.38% similarity). *Spiniferites belerius* from Baie de Vilaine, France differed from Japanese *S. belerius* (GenBank accession: LC222310) at 85 positions (88.09% similarity) and from *Impagidinium caspiense* (GenBank accession: LC222302) at 20 positions (97.14% similarity).

ML and BI analyses based on LSU rRNA gene sequences yielded similar phylogenetic trees. The BI tree is illustrated in Fig. 77, showing five well-resolved clades, corresponding to families Ceratiaceae, Protoceratiaceae, Pyrophacaceae, Gonyaulacaceae and Lingulodiniaceae. Gonyaulacaceae comprised the fossil genera *Spiniferites*, *Impagidinium* Stover & Evitt, *Ataxiodinium* P.C. Reid and *Tectatodinium* D. Wall, and the extant genus *Gonyaulax* with maximal support (BPP:1.0, ML BS:100). Species assigned to *Spiniferites* and *Impagidinium* did not form monophyletic groups. The new French *S. belerius* isolate grouped together with *Gonyaulax baltica*/*Impagidinium caspiense* with strong support (BPP:0.95, BS:96), and in turn formed a sister clade to the Japanese *S. belerius* with maximal support. *Spiniferites ramosus* isolates grouped together with maximal support and they formed a sister clade to *S. scabratus* strain TIO706

with maximal support. *Spiniferites hyperacanthus* grouped together with *G. whaseongensis* with maximal support. *Tectatodinium pellitum* formed a clade with *Ataxiodinium choane* with low support. *Spiniferites mirabilis* was a sister clade of *Gonyaulax ellegaardiae* with moderate support (BPP:0.98, BS:79). *Spiniferites bentorii* and *Spiniferites cf. bentorii* grouped together with maximal support, and formed a sister clade to *Gonyaulax elongata* with low support.

The SSU rRNA gene sequences of *Spiniferites hyperacanthus* strains TIO260 and TIO712 differed in two positions, and differed from *Gonyaulax whaseongensis* (GenBank accession number: LS481152) in two and four positions respectively (99.80% similarity). *S. bentorii* from the Adriatic Sea differed from isolate HSN114 from Bohai Sea in 112 positions (93.29% similarity), and differed from *G. spinifera* strain GSTL1 from Malaysia (GenBank accession: AF052190) in 148 positions (91.51% similarity) and from *G. digitale* in 312 positions (82.07% similarity). ML and BI analyses based on SSU rRNA gene sequences yielded similar phylogenetic trees. The BI tree is illustrated in Fig. 78 showing five well-resolved clades as LSU rRNA gene sequence-based tree. *Spiniferites bentorii* grouped together with Malaysian strain GSTL1 with maximal support, and *Gonyaulax digitale* formed a clade with several strains of *Gonyaulax ellegaardiae* from the Mediterranean Sea with maximal support.



Figs 51–56. *Spiniferites ramosus* from the Canadian Pacific, LM and SEM. Scale bars = 10 μm .

Fig. 51. High focus of an empty cyst from which strain TIO904 was established, showing gonial processes (arrow) (LM).

Fig. 52. A living cyst produced in culture of strain TIO700, showing two red bodies (LM).

Fig. 53. Ventral view of a cyst produced in culture of strain TIO1400 (SEM).

Fig. 54. Apical view of a cyst produced in culture of strain TIO1400 (SEM).

Fig. 55. Dorso-antapical view of a cyst produced in culture of strain TIO1400 (SEM).

Fig. 56. A cyst produced in culture of strain TIO1400, showing the reduced archeopyle (SEM).

Yessotoxins

Two strains of *G. whaseongensis* and seven strains of *G. spinifera* were studied for YTXs. None of the YTXs were detected in these strains. The detection limits ranged from 0.1 fg cell⁻¹ (*G. spinifera*, TIO904) to 11 fg cell⁻¹ (*G. spinifera*, TIO705) depending on the available biomass (Table 2).

DISCUSSION

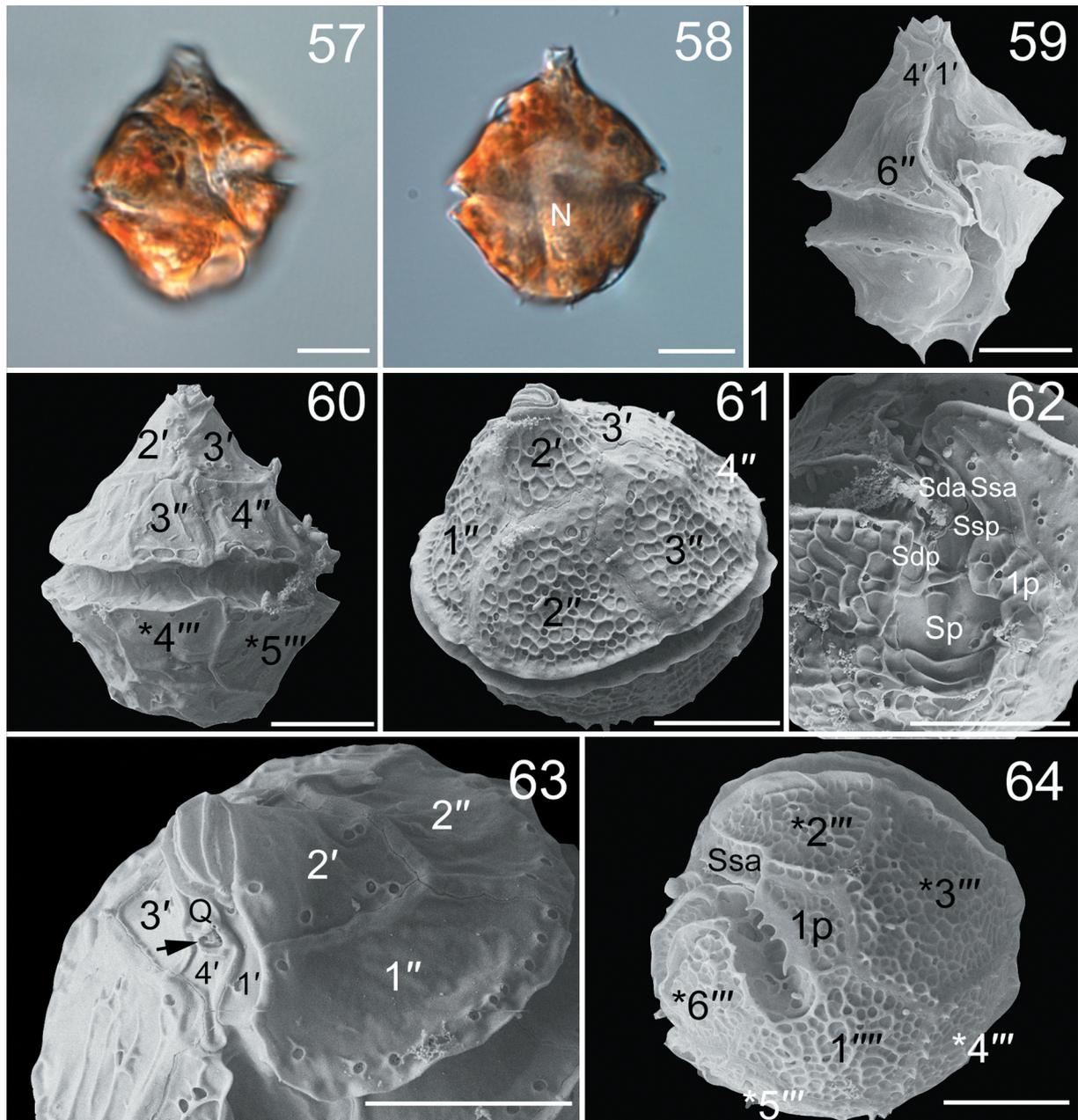
Morphology

SPINIFERITES BELERIUS AND *S. BENTORII*

Spiniferites belerius is characterized by a relatively small size (35–42 μm long and 28–37 μm wide), bearing a small apical boss and one high antapical, trumpet-shaped process; it is considered to have a wide distribution ranging from tropical to subpolar regions (e.g. Reid 1974; Gurdebeke *et al.* 2018; Limoges *et al.* 2018). Prior to our study, *S. belerius* had been reported from Japanese waters (Kojima 1989) but only one sequence from a Japanese *S. belerius* was reported (Mertens *et al.* 2018a). It is surprising that our new sequence of *S. belerius* from coastal waters of France differs markedly

from the Japanese sequence, although no appreciable morphological separation was observed between them. This suggests that they might belong to a species complex. *Spiniferites belerius* has been related to *Gonyaulax scrippsae* Kofoid (Wall & Dale 1968; Reid 1974). *Gonyaulax scrippsae*, which was described from the San Diego region, California, USA (Kofoid 1911), needs to be restudied from the type locality to establish its resting stage.

Spiniferites bentorii has a pronounced apical boss, a pear shape and generally two more developed antapical processes (e.g. Rossignol 1964; Limoges *et al.* 2018). Intergonal processes are only occasionally present in *S. bentorii*, but in our specimens from Bohai Sea, China, they are constantly present, and for that reason these specimens were identified as *Spiniferites cf. bentorii*. *Gonyaulax digitale* is an obscure species described by Pouchet (1883) from Concarneau Bay, France. No size was given by Pouchet, but a significant displacement of the cingulum and two antapical spines can be observed in his illustrations. *Gonyaulax digitale* has been connected previously to *Spiniferites bentorii* (Wall & Dale 1968, 1970). However, the SSU rRNA gene sequences of *G. digitale* from the type locality (Concarneau Bay) is reported



Figs 57–64. *Spiniferites ramosus* strain T10701 from the Canadian Pacific, LM and SEM. Scale bars = 10 μ m.

Fig. 57. High focus of a living cell showing cingulum displacement (LM).

Fig. 58. Mid focus of a living cell showing the nucleus (N) (LM).

Fig. 59. Ventral view of a cell showing the two equal antapical spines (SEM).

Fig. 60. Dorsal view of a cell showing neutral torsion (SEM).

Fig. 61. Apical-dorsal view of a cell showing two apical plates and four precingular plates (SEM).

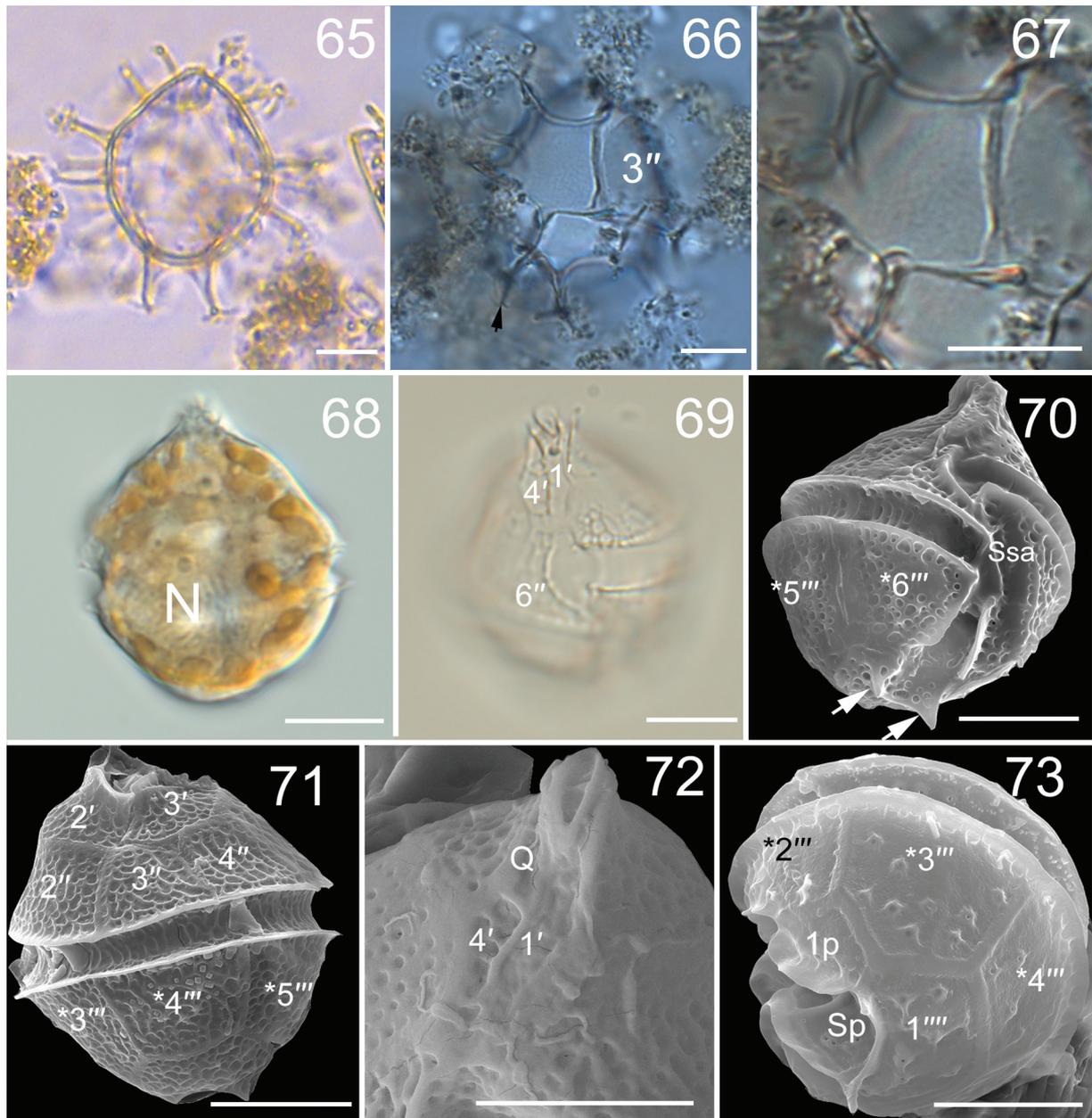
Fig. 62. The sulcus showing the anterior sulcal plate (Sa), anterior left sulcal plate (Ssa), posterior left sulcal plate (Ssp), anterior right sulcal plate (Sda), posterior right sulcal plate (Sdp) and posterior sulcal plate (Sp) (SEM).

Fig. 63. Apical-ventral view of a cell showing the ventral pore (arrow) at the junction of plates 4' and Q (SEM).

Fig. 64. Antapical view of a cell showing five postcingular plates, one antapical plate and one posterior intercalary plate (SEM).

here for the first time and is phylogenetically separated from the strain originated from cysts with the morphology of *S. bentorii* (Fig. 78); we describe this strain as a new species, *Gonyaulax nezaniae* sp. nov. Another cyst-defined species, *Bitectatodinium tepikiense* has also been related with *Gonyaulax digitale* (Lewis et al. 2001), but the cyst has not been found in Concarneau (K.N. Mertens, personal observations) and the two species appear distantly related (Figs 77,

78). This suggests that modern cysts with *B. tepikiense* morphology may correspond to an undescribed species of *Gonyaulax*, perhaps related to the specimen of *G. digitale* described by Kofoid (1911) from the Faeroe Islands. Differences between *G. nezaniae*, *G. digitale sensu stricto* and the motile stage derived from *B. tepikiense*-like cysts are summarized in Table 3. *Gonyaulax digitale sensu stricto* is not sufficiently known and needs further study.



Figs 65–73. *Spiniferites scabratus* strain TIO706 from the Mediterranean Sea, LM and SEM. Scale bars = 10 μ m.

Fig. 65. Mid focus of the empty cyst from which strain TIO706 was established (LM).

Fig. 66. High focus of the same cyst shown in Fig. 65, with the archeopyle and bifurcate tips of processes (arrow) (LM).

Fig. 67. High focus of the same cyst shown in Fig. 65, showing the granular surface (LM).

Fig. 68. Mid focus of a living cell showing the nucleus (N) (LM).

Fig. 69. High focus of a theca showing plates 1', 4' and 6'' (LM).

Fig. 70. Ventral view of a cell showing two equal antapical spines (arrows) (SEM).

Fig. 71. Dorsal view of a cell showing neutral torsion (SEM).

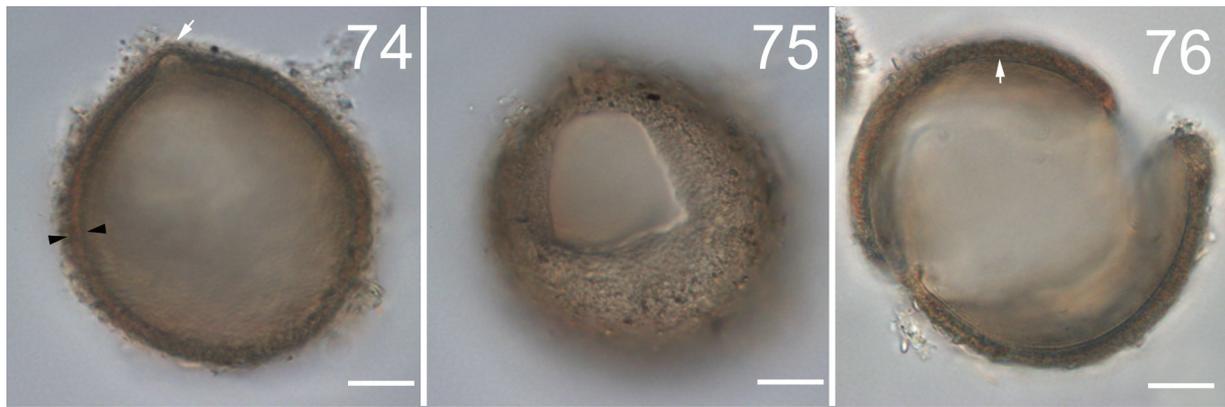
Fig. 72. Apical view of a cell showing plates 1', 4' and Q without a ventral pore (SEM).

Fig. 73. Antapical view of a cell showing three postcingular plates, one antapical plate and one posterior intercalary plate (SEM).

SPINIFERITES HYPERACANTHUS AND *S. MIRABILIS*

Spiniferites hyperacanthus and *S. mirabilis* are morphologically similar. Both of them are spherical, with a variable number of intergonal processes, but differ in the absence of a well-developed flange along the suture between the antapical plate and plate 3''' in *S. hyperacanthus* (Fig. 30), which is present in *S. mirabilis* (Fig. 48). Although both species have

sometimes been grouped in palynological counts (e.g. Limoges *et al.* 2013), our study demonstrates that they are clearly distinct species. *Spiniferites hyperacanthus* was originally described from the Middle Miocene of Balcombe Bay by Deflandre & Cookson (1955). Their holotype is significantly larger than our specimen, having a central body diameter of 54–59 μ m and processes 13–20 μ m long. However, other



Figs 74–76. *Tectatodinium pellitum* from Gulf of California, LM. Scale bars = 10 μ m.

Fig. 74. Mid focus of the empty cyst showing an apical protuberance (arrow) and two walls (arrowheads).

Fig. 75. High focus of the empty cyst showing the archeopyle.

Fig. 76. High focus of the empty cyst showing the spongy wall (arrow).

specimen identified as *S. hyperacanthus* from Japan by Matsuoka (1985) have similar sizes to our specimen (38.2–40.3 μ m long and 34–38.5 μ m wide; process length 12.0–18.5 μ m). *Spiniferites hyperacanthus* from Tokyo Bay has been connected to the *Gonyaulax spinifera* complex (Matsuoka et al. 2003), but here we demonstrate that the motile stages correspond to *Gonyaulax whaseongensis*, which is characterized by two unequal antapical spines, a quadrangular plate 6'' and a ventral pore at the junction of plates 4' and Q (Lim et al. 2018). In addition, our cells show a dextral torsion, also reported in *G. whaseongensis* (Lim et al. 2018), whereas in *G. digitale* and *G. spinifera* there is a neutral torsion (Kofoid 1911). *Spiniferites hyperacanthus* from Chinese coastal waters and *G. whaseongensis* also have identical molecular sequences, further indicating that they are conspecific. Our strain TIO260 produces cysts in culture spontaneously, but the Korean strain did not generate cysts in culture (Lim et al. 2018), suggesting that this species might be heterothallic. Our strain was established from an assumed sexual resting cyst that produced daughter cells through meiosis (Elbrächter 2003) whereas the Korean strain is from a single, presumably vegetative cell (Lim et al. 2018). Our specimen of *S. mirabilis* has one or two intergonal processes along each major suture, and may therefore be identified as *S. mirabilis* subsp. *mirabilis* (Rossignol 1964). *Spiniferites mirabilis* has been connected to *Gonyaulax spinifera*, which has a variable number of small antapical spines, with two of them prominent (Wall & Dale 1968; Morquecho et al. 2009).

SPINIFERITES LAZUS AND SPINIFERITES SCABRATUS

Spiniferites lazus is characterized by having exclusively gonal processes with wide fenestrate bases (Reid 1974), and our specimen fits the original description well. The cyst of *Spiniferites scabratus* from Corsica matches the original description of *S. scabratus* in terms of microgranular septa with exclusively gonal and hollow processes, and an archeopyle that is not reduced (Mertens et al. 2018b). The motile cells of strain TIO706 are morphologically similar to *Gonyaulax spinifera* but they are smaller and lack a ventral

pore. A resting cyst identified as *S. scabratus* from the Gulf of Paria yielded a motile cell without antapical spines and with very indistinct ornamentation (Wall & Dale 1968).

SPINIFERITES RAMOSUS SENSU ROCHON ET AL. (1999)

The taxonomic history of *S. ramosus* is complicated and we refer to Mertens et al. (2018b, p. 34) for more information. For modern cysts, the informal taxon *S. ramosus sensu* Rochon et al. (1999) is characterized by possessing exclusively gonal processes and a smooth wall. Our studied specimens had a granular wall, but were nearly identical molecularly (99% similarity) with smooth-walled *S. ramosus* cysts documented by Ellegaard et al. (2003), who connected this species to *Gonyaulax* cf. *spinifera*. Our cysts are similar to *Spiniferites ramosus* from the North Sea, which shows a weakly granulate surface, occasional intergonal process and a reduced archeopyle (Lewis et al. 1999). Our motile cells are also morphologically similar to those of Lewis et al. (1999) in having a pronounced apical horn, a girdle displacement of two cingulum widths, a ventral pore at the junction of plates 4' and Q, and two antapical spines.

TECTATODINIUM PELLITUM

Living cysts with the morphology of *T. pellitum* have been reported in sediments of Massachusetts, USA, and of the Gulf of California, Mexico; the germinated cells had two antapical spines and were connected to *Gonyaulax spinifera* (Wall & Dale 1967, 1968; Morquecho et al. 2009). The identity of the motile stage, however, remains to be determined as its molecular sequence is quite different from that of *G. spinifera*. *Tectatodinium pellitum* is a coastal subtropical to equatorial species (Zonneveld et al. 2013) but it can be occasionally found in estuarine temperate waters (e.g. Pospelova et al. 2004).

Molecular phylogeny

Our results suggest that species of the fossil genera *Spiniferites* and *Impagidinium*, as currently circumscribed, are

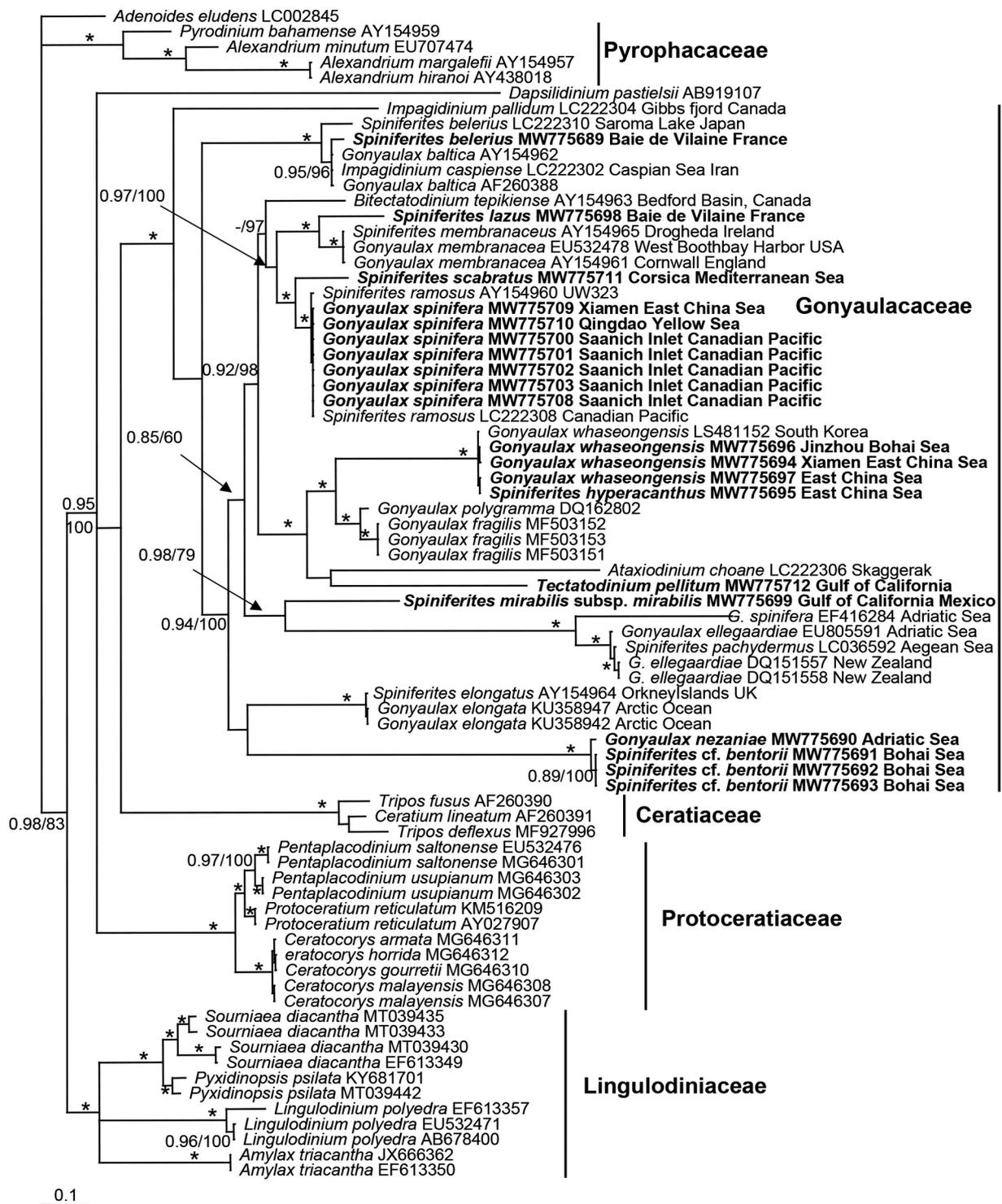


Fig. 77. Phylogeny of *Spiniferites* and *Tectatodinium* inferred from partial LSU rRNA gene sequences using Bayesian inference (BI). New sequences indicated in bold. Five families labelled and marked with vertical lines on the right. Branch lengths drawn to scale, with scale bar indicating number of nucleotide substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (left: Bayesian posterior probabilities; right: maximum likelihood (ML) bootstrap support values). Only Bayesian posterior probabilities above 0.9 and ML bootstrap support values above 50 are shown. * indicates maximal support (BI posterior probability: 1.0, ML bootstrap support: 100).

polyphyletic (Mertens *et al.* 2018a) because species of *Bitectatodinium*, *Ataxiodinium* and *Tectatodinium* group with those of *Spiniferites* and *Impagidinium*. The phylogenetic positions of *S. bentorii*, *S. mirabilis*, *S. lazus*, *S. scabratus* and *S. hyperacanthus* are inferred here for the first time. Each of these species forms a distinct clade, supporting the idea that

there is a large diversity within *Spiniferites*. The extant genus *Gonyaulax* appears to be related to several fossil genera, and the tabulation of *Gonyaulax* fits well with the paratabulation of *Spiniferites* and *Impagidinium*, suggesting that it has changed very little during evolution. In contrast, species of *Bitectatodinium*, *Ataxiodinium* and *Tectatodinium* do not

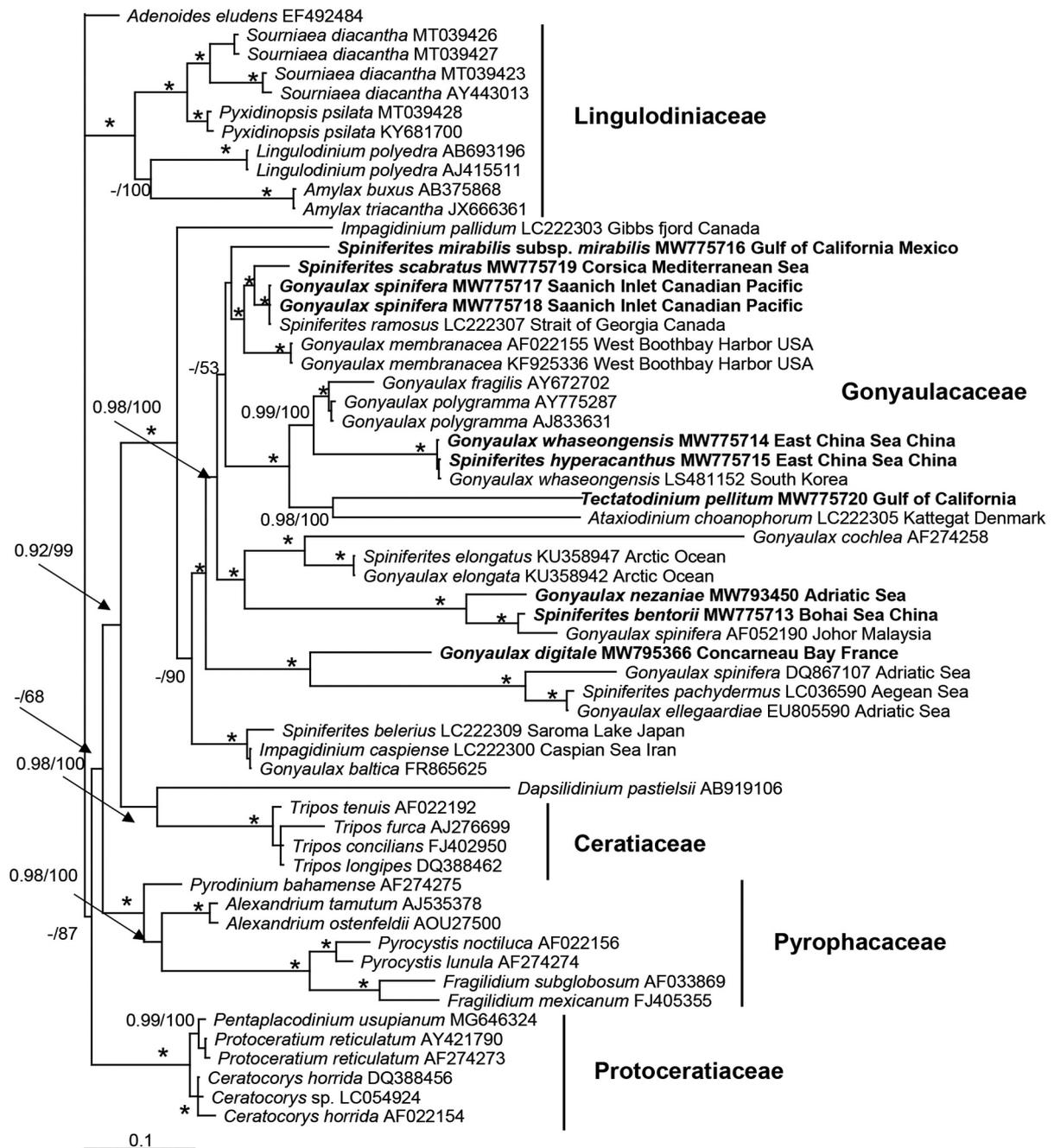


Fig. 78. Phylogeny of *Spiniferites* and *Tectatodinium* inferred from SSU rRNA gene sequences using Bayesian inference (BI). New sequences indicated in bold. Five families labelled and marked with vertical lines on the right. Branch lengths drawn to scale, with scale bar indicating number of nucleotide substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (left: Bayesian posterior probabilities; right: maximum likelihood (ML) bootstrap support values). Only Bayesian posterior probabilities above 0.9 and ML bootstrap support values above 50 are shown. * indicates maximal support (BI posterior probability: 1.0, ML bootstrap support: 100).

display a paratabulation (Reid 1974). These genera do not group together in the molecular phylogeny, suggesting that paratabulation has been lost several times during evolution.

It has been suggested to unite the biological and palaeobiological nomenclatures by retaining *Gonyaulax* as a very large genus and subdividing it (e.g. subgenera) using fossil names such as *Spiniferites*, *Impagidinium*, and *Bitectatodinium* (Ellegaard et al. 2018). However, with their current circumscription, the subgenera remain polyphyletic. The presence or absence of an apical boss and intergonal processes are useful to differentiate

Spiniferites species. However, *S. belerius* and *S. bentorii* do not group together although they share an apical boss. On the other hand, *S. bentorii* with and without intergonal processes group together suggesting that presence or absence of intergonal processes alone is not enough to differentiate species. An integrated analysis of multiple morphological features might be necessary to classify this group of dinoflagellate species.

The genus *Gonyaulax* was initially proposed to be subdivided into four subgenera, i.e. *Gonyaulax* subg. *Gonyaulax*, *G.* subg. *Fusigonyaulax* Kofoid, *G.* subg. *Steiniella* (F. Schütt)

Table 2. Limit of detection (LoD) of yessotoxin in motile cells of *Gonyaulax spinifera* and *G. whaseongensis*. EV: extraction volume.

Species	Strain	Cell count	EV (μl)	LoD (fg cell ⁻¹)
<i>Gonyaulax spinifera</i>	TIO904	6,300,000	250	0.10
<i>G. spinifera</i>	TIO700	378,000	200	7.22
<i>G. spinifera</i>	TIO701	1,000,000	200	2.73
<i>G. spinifera</i>	TIO704	830,000	200	3.29
<i>G. spinifera</i>	TIO705	250,000	200	10.91
<i>G. whaseongensis</i>	TIO260	324,000	150	6.94
<i>G. whaseongensis</i>	TIO712	434,000	250	1.44

Kofoid and *G.* subg. *Acanthogonyaulax* Kofoid, based upon the general shape of the motile cells (Kofoid 1911). *Gonyaulax* subg. *Gonyaulax* was further subdivided into the *spinifera*, *polygramma* and *sphaeroidea* groups. Unfortunately, neither the subgenera nor the groups appear monophyletic. However, few sequences are presently available for the subgenus *Steiniella*, and so far none is available from subgenera *Fusigonyaulax* and *Acanthogonyaulax*. Plate 4' comprises two parts, named 4'a and 4'p, in *G. fragilis* (F. Schütt) Kofoid and *G. hyalina* Ostenfeld & E.J. Schmidt (Carbonell-Moore & Mertens 2019), which have been classified within *Gonyaulax* subg. *Steiniella*, but plate 4' is not divided in other subgenera. Whether molecular phylogenies support the subgenus *Steiniella* remains to be determined.

Although Wall & Dale (1968) linked *Gonyaulax spinifera* and *G. digitale* to at least five cyst morphotypes, they observed that a naturally occurring population of motile cells produced only one cyst morphotype. It is highly likely that several genetically different species exist with morphologies very similar to *G. spinifera* and *G. digitale*. *Gonyaulax spinifera* needs to be more narrowly characterized, preferably from the analysis of populations from the type locality (western coast of Norway). With narrowly defined extant species it may become possible to find connections to cysts corresponding in morphology to *S. belerius*, *S. lazus*, *S. mirabilis*, *S. scabratus* and *T. pellitum*, as has been exemplified for *Gonyaulax elongata* (basonym *Spiniferites elongatus*) and *Gonyaulax membranacea* (previously known as *Spiniferites membranaceus*) (Ellegaard *et al.* 2003). Alternatively, for names based on fossil cysts, a new name may be created, e.g. *Gonyaulax ellegaardiae* was described based on motile cells germinated from *S. pachydermus*-like cysts (Mertens

et al. 2015). Two analogous situations are suggested by our observations on *G. whaseongensis* and *S. hyperacanthus*-like cysts, and on *G. nezaniae* and *S. bentorii*-like cysts.

Yessotoxin

Some strains designated as *Gonyaulax spinifera* are known as producers of yessotoxins. For example, a strain of *G. spinifera* from New Zealand produces YTXs at levels approximately 20-fold higher than *Protoceratium reticulatum* (Claparède & J. Lachmann) Buetschli (Rhodes *et al.* 2006), but molecular phylogeny suggests that this strain in fact is closer to *Gonyaulax ellegaardiae* (Mertens *et al.* 2015), which might explain why we failed to detect yessotoxins in our strains of *G. spinifera*. Similar remarks can be made concerning a *G. spinifera* strain reported by Pitcher *et al.* (2019). Even though the authors described the species as *G. spinifera* based on morphological characteristics, the phylogeny clearly locates the species in the *G. membranacea* clade (Pitcher *et al.* 2019), which leads to conclusion that these two reports of YTX-producing *G. spinifera* are based on misidentifications. The lack of YTX-production of *G. spinifera* found in this study is consistent with other reports of non-toxicogenic *G. spinifera* in the North (Krock *et al.* 2006) and South Atlantic (Krock *et al.* 2015). This raises the question of whether *G. spinifera sensu stricto* is erroneously considered an YTX-producer or if this species comprises toxicogenic and non-toxicogenic genotypes.

Formal taxonomic description

Gonyaulax nezaniae H. Gu & K.N. Mertens *sp. nov.*

Figs 10–23

DESCRIPTION: Cells were 37–68 μm long and 33–52 μm wide with two stout antapical spines 2–11 μm long. The epitheca was conical with pronounced shoulders. The cell surface was thick and reticulated. The cingulum was located in the equatorial part of the cell and descended about three times its width. Cells displayed a plate formula of 2pr, 4', 6'', 6C, 6S, 5''', 1p, 1'''''. There was a ventral pore at the junction of plates 1', 4' and Q. The angle between the major axis and a line joining the ends of the cingulum was approximately 20°. The cyst was pear-shaped, 53–63 μm long and 44–64 μm wide with a pronounced apical

Table 3. Comparisons of motile cell features of *Gonyaulax digitale*, *Gonyaulax nezaniae* and *Bitectadinium tepikiense*.

Character	<i>Gonyaulax digitale sensu stricto</i>	<i>Gonyaulax nezaniae</i>	Motile cells of <i>Bitectadinium tepikiense</i>
Length	52.6 μm	37–68 μm	40–63 μm
Width	49.7 μm	33–52 μm	26–43 μm
Width of girdle	4.5 μm	3 μm	3–5 μm
Girdle displacement	?	3.0	2
Girdle overhang	?	1.7	0.5–1.5
Angle	?	20°	10–19°
Shape of 6''	?	Approx. equal sides	Approx. equal sides
Antapical spines	2	2(3)	2(3)
Length of spines	4.3–5.4 μm	2–11 μm	1–8 μm
Form of sulcus	?	Abruptly widened	Not abruptly widened
Apical horn	Prominent, 2 girdle widths in length	Prominent, 1.3–2 girdle widths in length	Prominent, 2–4 girdle widths in length
4'	?	Not separated	Not separated
References	This study	This study	Lewis <i>et al.</i> 2001

Table 4. List of currently inferred cyst-theca relationships of *Spiniferites* and related species.

Cyst	Motile stage	References
<i>Spiniferites bentorii</i>	<i>Gonyaulax nezaniae</i>	This study
<i>Spiniferites bulloideus</i>	<i>Gonyaulax baltica</i>	Ellegaard et al. (2002)
<i>Spiniferites elongatus</i>	<i>Gonyaulax elongata</i>	Ellegaard et al. (2003)
<i>Spiniferites hyperacanthus</i>	<i>Gonyaulax whaseongensis</i>	This study
<i>Spiniferites membranaceus</i>	<i>Gonyaulax membranacea</i>	Ellegaard et al. (2003)
<i>Spiniferites pachydermus</i>	<i>Gonyaulax ellegaardiae</i>	Mertens et al. (2015)
<i>Spiniferites ramosus</i>	<i>Gonyaulax spinifera</i>	Ellegaard et al. (2003); this study
<i>Spiniferites scabratus</i>	<i>Gonyaulax cf. spinifera</i>	This study
<i>Bitectatodinium tepikiense</i>	<i>Gonyaulax cf. digitale</i>	Lewis et al. (2001)
<i>Impagidinium caspiense</i>	<i>Gonyaulax baltica</i>	Mertens et al. (2018a)

protuberance. The paracingulum descended with a displacement of three times its width. Processes were exclusively gonal, 10–12 µm long, and had fenestrate bases. The archeopyle was reduced and corresponded to the third precingular plate.

HOLOTYPE: SEM stub of thecate cells from a culture established from a cyst extracted from sediment of Pantan, Croatia, stored at the CEDiT (Centre of Excellence for Dinophyte Taxonomy) dinoflagellate type collection, Wilhelmshaven, Germany with the code CEDiT2021H123.

TYPE LOCALITY: Pantan, Croatia (Adriatic Sea; 43°31.26'N, 16°16.38'E). Collection date: 4 May 2017.

HABITAT: Marine and planktonic.

ETYMOLOGY: The epithet ‘*nezaniae*’ is in honour of Dr. Elisabeth Nézan for her significant contributions to dinoflagellate taxonomy.

GENBANK ACCESSION: MW793450 and MW775690, respectively nuclear-encoded SSU and LSU rRNA gene sequence.

CONCLUSION

Seven out of 13 known extant cysts with morphologies matching *Spiniferites* species were examined in the present study. The motile stage of cysts morphologically resembling *S. bentorii* was assigned to a new species, *Gonyaulax nezaniae*. Although the cyst-theca relationships of *Spiniferites scabratus* was established, the identity of motile stages remains to be determined. The current cyst-theca relationships inferred for *Spiniferites* and related species is summarized in Table 4, but those of *S. belerius*, *S. mirabilis*, *S. lazus*, *Spiniferites cf. bentorii* and *Tectatodinium pelltium* have not yet been resolved. A detailed investigation of *Gonyaulax spinifera* and *G. digitale* from their type localities is essential to fully understand the stages of the life cycle of *Spiniferites* and related genera. Future efforts to investigate the relation of these fossil species to *Gonyaulax* are urgently needed.

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