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Balechina and the New Genus Cucumeridinium Gen. Nov. (Dinophyceae), Unarmored Dinoflagellates with Thick Cell Coverings

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Abstract

The genus *Balechina* (=subgenus *Pachydinium*) was established for heterotrophic gymnodinioid dinoflagellates with a thick cell covering. The type species, *B. pachydermata* (= *Gymnodinium pachydermatum*), showed numerous fine longitudinal striae, whereas *B. coerulea* (= *G. coeruleum*) showed ~24 prominent longitudinal surface ridges or furrows and a distinctive blue pigmentation. We have investigated the morphology and molecular phylogeny of these taxa and the species *Gymnodinium cucumis*, *G. lira* and *G. amphora* from the western Mediterranean, Brazil and Japan. Sudden contractions at the cingulum level were seen in *B. pachydermata*, which also showed a high morphological variability which included morphotypes that have been described as *Amphidinium vasculum*, *G. amphora*, *G. dogieli* and *G. gracile* sensu Kofoed and Swezy. Molecular phylogeny based on small subunit rRNA gene sequences revealed that *Balechina coerulea*, *G. cucumis* and *G. lira* formed a clade distantly related to the clade of the type species, *B. pachydermata*, and *G. amphora*. We propose the new genus *Cucumeridinium* for the species with longitudinal ridges and a circular apical groove (*Cucumeridinium coeruleum* comb. nov., *C. lira* comb. nov. and *C. cucumis* comb. nov.), and *Gymnodinium canus* and *G. costatum* are considered synonyms of *C. coeruleum*. The genus *Balechina* remains for the species with a double-layer cell covering, bossed surface with fine striae, and an elongated elliptical apical groove. At present, the genus is monotypic containing only *B. pachydermata*.

Keywords

athecate Dinoflagellata; autotomy; blue pigmentation; *Gymnodinium*; intraspecific variability; Mediterranean Sea; molecular phylogeny; new genus; North Pacific Ocean; South Atlantic Ocean

Two major groups of dinoflagellates can be distinguished based on their cell coverings. Thecate (armored) dinoflagellates with large amphiesmal vesicles filled with cellulosic material, and the athecate (unarmored or “naked”) dinoflagellates that contain hundreds of alveoli lacking cellulosic material (Morrill and Loeblich 1983). The naked dinoflagellates are usually fragile and delicate, the cells easily lyse during the observation of live samples,

lyse due to the fixation, or the fixed cells are too distorted for proper identification. Particularly, gymnodinioid dinoflagellates are notoriously difficult to preserve. The chemical fixatives produce misshapen cells, swollen membranes, and clumping of specimens (Kofoid and Swezy 1921). However, the separation between armored and unarmored species is not clear cut and some gymnodinioid dinoflagellates are characterized by a thick cell covering. This is the case of the genus *Balechina* Loeblich et A.R. Loeblich, which has been placed either in the order Kolkwitiellales (Taylor 1987) or Ptychodiscales (Fensome et al. 1993). Kofoid and Swezy (1921) published a monograph of the unarmored dinoflagellates known at that time. They proposed the subgenus *Pachydinium* Kofoid et Swezy for gymnodinioid species with a thick cell covering. They included three new species lacking surface longitudinal ridges (*Gymnodinium pachydermatum* Kofoid et Swezy, *G. dogieli* Kofoid et Swezy, and *G. amphora* Kofoid et Swezy), and species with a surface covered by longitudinal ridges (*Gymnodinium coeruleum* Dogiel, and the new species *G. canus* Kofoid et Swezy, *G. costatum* Kofoid et Swezy, and *G. lira* Kofoid et Swezy). Despite the large sizes and thick cell covering of these species, only *G. coeruleum* has sporadically been reported in the literature, while the records of the other species are very rarely reported or only known from the original descriptions (Dogiel 1906, Kofoid and Swezy 1921, Wood 1968). This may be due to the fact that *G. coeruleum* is characterized by a striking blue or purple coloration, which is relatively rare in nature, in particular in microbial species. With no known observations, Loeblich and Loeblich (1968) proposed that the subgenus *Pachydinium* should be raised at the genus rank. They proposed the new name *Balechina* because *Pachydinium* Kofoid et Swezy 1921 was a junior homonym of the thecate *Pachydinium* Pavillard 1915. This proposal was followed only by Taylor (1976), who transferred *G. coeruleum* into *Balechina*, and proposed a third species *Balechina marianiae* F.J.R. Taylor (see Appendix S1 in the Supporting Information for a taxonomic, nomenclatural and biogeographical account). The genus *Balechina* was further used in the literature (Lessard and Swift 1986, Taylor 1987, Fensome et al. 1993, Steidinger and Tangen 1997), while other authors considered *Balechina* as a synonym of *Gymnodinium* F. Stein (Sournia 1986, Balech 1988). In the last 15 years our knowledge of the unarmored dinoflagellates has increased with the advances of molecular phylogeny, initially based mostly on photosynthetic cultivable species (Daugbjerg et al. 2000), abundant heterotrophic coastal species (Hansen and Daugbjerg 2004, Takano and Horiguchi 2004) and later, on other less abundant heterotrophic species (Gomez et al. 2009). However, little is known about the unarmored dinoflagellates with a thick cell covering that reach larger sizes (>200 µm long, i.e., *Gymnodinium cucumis* F. Schutt), or highly distinctive species with striking blue or purple pigmentation (i.e., *Balechina coerulea* (Dogiel) F.J.R. Taylor). To the best of our knowledge, the type species of *Balechina*, *B. pachydermata* (Kofoid et Swezy) Loeblich et A.R. Loeblich, remains only known from the original description (Kofoid and Swezy 1921) and Wood (1968). This study was based on observations of live material from several locations of the Mediterranean Sea (Marseille, Banyuls sur Mer, Villefranche sur Mer, Valencia), the South Atlantic Ocean on the coast of Brazil (São Sebastião Channel and off Ubatuba), and the North Pacific Ocean on the coast of Japan (Kure, Hiroshima Prefecture). We provided the first micrographs of the species *B. pachydermata*, *G. cucumis*, *G. dogieli*, *G. amphora* and *Amphidinium vasculum*, and scanning electron microscopy pictures of the species *B. coerulea*, *B. pachydermata*, and *G. lira*, including their apical grooves. We

reported for the first time the phenomenon of the sudden contraction of *B. pachydermata*, and its high intraspecific variability, mainly in the shape of the episome. We propose the species *A. vasculum* Kofoid et Swezy, *G. amphora*, *G. dogieli* and *G. gracile* sensu Kofoid and Swezy as synonyms of *B. pachydermata*. We also illustrated the phenomenon of autotomy in an unarmored dinoflagellate based on our observations of *B. coerulea*. We propose the species *G. canus* and *G. costatum* as synonyms of *B. coerulea*. We provided the first molecular data (SSU rRNA gene sequences) of the species *B. pachydermata*, *B. coerulea*, *Gymnodinium amphora*, *G. cucumis* and *G. lira*. We propose an emended description of the genus *Balechina*, a new genus for the species *B. coerulea*, *G. cucumis*, *G. lira*, and a tentative undescribed species with intermediate characteristics between *B. coerulea* and *G. lira*.

Materials and Methods

Sampling and isolation of material

Specimens were collected from the Mediterranean Sea by slowly filtering surface seawater taken from the pier of the Station Marine d'Endoume at Marseille (43°16'04.05" N, 5°20'56.22" E, bottom depth 3 m) from October 2007 to September 2008. A strainer of 20, 40, or 60- μ m mesh size (Millipore Inc., St. Quentin-Yveline, France) was used to collect planktonic organisms from water volumes ranging between 10 and 100 L, depending on particle concentration. The plankton concentrate was scanned in settling chambers at 9100 magnification with an inverted microscope (Nikon Eclipse TE200; Nikon Inc., Tokyo, Japan). Cells were photographed alive at 9200 or 9400 magnifications with a Nikon Coolpix E995 digital camera. Further specimens were collected using the same method from October 2008 to August 2009 in the surface waters (depth of 2 m) of the port of Banyuls sur Mer, France (42°28'50" N, 3°08'09" E). The concentrated sample was examined in Utermohl chambers with an inverted microscope (Olympus IX51; Olympus Inc., Tokyo, Japan) and photographed with an Olympus DP71 digital camera. Sampling continued from September 2009 to February 2010 in the Bay of Villefranche sur Mer, Ligurian Sea. For this location, sampling was performed at the long-term monitoring site Point B (43°41'10" N, 7°19'00" E, water column depth ~80 m). Water column samples (0–80 m) were obtained using a phytoplankton net (53 μ m mesh size, 54 cm diameter, 280 cm length). Samples were prepared according to the same procedure as described above and specimens were observed with an inverted microscope (Olympus IX51; Olympus Inc.) and photographed with an Olympus DP71 digital camera. Sampling continued from May 2012 to February 2013 in the port of Valencia, Spain (39°27'38.13" N, 0°19'21.29" W, water column depth of 4 m). Specimens were obtained using a phytoplankton net (20 μ m mesh size). Samples were prepared according to the same procedure as described above and specimens were observed with an inverted microscope (Nikon Eclipse T2000; Nikon Inc.) and photographed with an Olympus DP71 digital camera. In the South Atlantic Ocean, sampling continued after March 2013 in São Sebastião Channel (23°50'04.05" S, 45°24'28.82" W), and from December 2013 to December 2014 off Ubatuba (23°32'20.15" S, 45°50'58.94" W). The Brazilian specimens were obtained using a phytoplankton net (20 μ m mesh size) in surface waters. The living concentrated samples were examined in Utermohl chambers at magnification of 9200 with inverted microscopes (Nikon Diaphot-300 at São Sebastião, and Nikon Eclipse

TS-100 at Ubatuba), and photographed with a digital camera (Cyber-shot DSC-W300; Sony, Tokyo, Japan) mounted on the microscope's eyepiece. In the North Pacific Ocean, samples were collected with a plankton net (30 µm mesh size) from the coastal Inland Sea of Japan at Kure (34°100 30" N, 132°330 21.6" E) in December 2014. The living concentrated samples were observed at magnification of 9400 and 91,000 with an upright microscope (Olympus BH2; Olympus Inc.), and photographed with a digital camera (Canon EOS Kiss F; Canon Inc., Tokyo, Japan). In all cases, each specimen was photographed and then micropipetted individually with a fine capillary into a clean chamber and washed several times in a series of drops of 0.2 µm-filtered and sterilized seawater. Finally, the specimen was placed in a 0.2 mL tubes (ABgene; Thermo Fisher Scientific Inc., Courtaboeuf, France) filled with several drops of absolute ethanol. The sample was kept at room temperature and in darkness until the molecular analysis could be performed. After obtaining samples for DNA analysis, the subsequent specimens of *B. coerulea* and *B. pachydermata* from Brazil were isolated with the aim to establish cultures. These specimens were individually placed in 6 or 12-well tissue culture plates with 0.2 µm-filtered seawater. To have controlled environmental conditions, the plates were placed in an incubator used for microalgae culturing, at 23°C, 100 µmol photons m⁻² s⁻¹ from cool-white tubes and photoperiod 12:12 L:D. To feed *Balechina* spp., we added aliquots of cultures of the cryptophyte *Rhodomonas* sp., the haptophyte *Isochrysis* sp., the dinoflagellates *Heterocapsa* sp. and *Prorocentrum* sp., and several centric diatoms isolated from field samples (*Chaetoceros* spp., *Thalassiosira* spp., etc.). None of the potential preys that were available in our culture collection contained blue pigments.

Scanning electron microscopy

Seawater samples were collected with a bucket from the coastal areas of the Inland Sea of Japan along Hiroshima Prefecture in 1980–1985 as described in Takayama (1998). For scanning electron microscopy, dinoflagellate cells were pipetted individually, rinsed three times in filtered seawater and placed on poly-lysine coated coverslips. They were fixed in 2% osmium tetroxide in seawater for 20 min. After washing in distilled water for 30 min, cells were dehydrated in an ethanol series, 10 min in each change of 30%, 50%, 70%, 90%, and 95%, followed by two 30 min changes in absolute ethanol, and finally transferred to amyl acetate. The cells were critical-point-dried using liquid carbon dioxide and ion sputter coated with gold. They were observed using a scanning electron microscope (Hitachi S-430; Hitachi Ltd, Tokyo, Japan) operated at 15 kV. The method is explained in detail in Takayama (1998). Pictures were scanned and presented on a black background using Adobe Photoshop CS3 (Adobe Systems Inc., San José, CA, USA). PCR amplification of small subunit rRNA genes (SSU rDNAs) and sequencing. The specimens fixed in ethanol were centrifuged for 5 min at 504 g. Ethanol was then evaporated in a vacuum desiccator, and single cells were resuspended directly in 25 µL of Ex TaKaRa buffer (TaKaRa, distributed by Lonza, Levallois-Perret, France). PCRs were done in a volume of 30–50 µL reaction mix containing 10–20 pmol of the eukaryotic-specific SSU rDNA primers EK-42F (5'-CTCAARGAYTAAGCCATGCA- 30) and EK-1520R (5'-CYGCAGGTTTCACCTAC- 30) (Lopez-Garcia et al. 2001). PCRs were performed under the following conditions: 2 min denaturation at 94°C; 10 cycles of "touch-down" PCR (denaturation at 94°C for 15 s; a 30 s annealing step at decreasing temperature from 65 down to 55°C, employing a 1°C decrease

with each cycle, extension at 72°C for 2 min); 20 additional cycles at 55°C annealing temperature; and a final elongation step of 7 min at 72°C. A nested PCR was then carried out using 2–5 µL of the first PCR products in a GoTaq (Promega, Lyon, France) polymerase reaction mix containing the eukaryotic-specific primers EK-82F (50-GAAACTGCGAATGGCTC-30) and EK-1498R (50-CACCTACGGAAACCTTGTTA-30; Lopez-García et al. 2001) and similar PCR conditions as described above. A third, semi-nested PCR was carried out using the dinoflagellate specific primer DIN464F (50-TAACAATACAGGGCATC CAT-30; Gomez et al. 2009) and the reverse primer EK-1498R. Negative controls without template DNA were used at all amplification steps. Amplicons of the expected size (~1,200 bp [base pairs]) were then sequenced bidirectionally using primers DIN464F and EK-1498R using an automated 96-capillary ABI PRISM 3730xl sequencer (BC Genomics, Takeley, UK). Phylogenetic analyses. The new SSU rDNA sequences were aligned to a large multiple sequence alignment containing ~1,500 publicly available complete or nearly complete (>1,300 bp) dinoflagellate sequences using the profile alignment option of MUSCLE 3.7 (Edgar 2004). The resulting alignment was manually inspected using the program ED of the MUST package (Philippe 1993). Ambiguously aligned regions and gaps were excluded in phylogenetic analyses. Preliminary phylogenetic trees with all sequences were constructed using the Neighbor-Joining method (Saitou and Nei 1987) implemented in the MUST package (Philippe 1993). These trees allowed identification of the closest relatives of our sequences together with a sample of other dinoflagellate species, which were selected to carry out more computationally intensive Bayesian Inference analyses. These were done with the program MrBayes 3.2.3 (Ronquist et al. 2012) applying a GTR + Γ 4 model of nucleotide substitution, taking into account a Γ -shaped distribution of substitution rates with four rate categories. Our sequences were deposited in DDBJ/ EMBL/GenBank under accession numbers #KR139785– KR139792 (Table 1).

Results

Balechina coerulea

This species is highly distinctive due to its striking blue or more rarely purple pigmentation. In previous observations based on Lugol's solution fixed samples, the specimens were easily overlooked as *Gyrodinium*-like species. However, since 2007 and due to the observation of fresh live samples, *B. coerulea* appeared in all the sampling areas examined in warm waters such as in the Mediterranean Sea (Marseille, Banyuls sur Mer, Villefranche sur Mer, Valencia), the Caribbean Sea (La Parguera and Bah_ía Fosforescente, Puerto Rico), and the South Atlantic Ocean (São Sebastião Channel and off Ubatuba, São Paulo State, Brazil). The coloration of the specimens ranged from navy blue (Fig. 1, a–c), cobalt blue (Figs. 1, d, e and 2, a–e), plum (Fig. 1, f–i), purple (Fig. 1, j–n), periwinkle (Fig. 1p), blue–green (Fig. 1, o, q–s, u) to colorless (Fig. 1, t–w). As a general trend, the specimens from the same sample showed the same coloration (Fig. 1s). The specimens with the most intense blue pigmentation were observed in Marseille and Banyuls sur Mer. However, this phenomenon was very likely related to the sampling stress and time lapse between the collection and the microscopic observations rather than to true differences between the populations of different geographic areas. The specimens from Marseille and Banyuls sur Mer were collected in

front of the laboratory using a bucket and a soft filtration using a strainer, and observed just a few minutes after collection. That procedure reduced the stress and specimens showed a more natural pigmentation. In other sampling areas, the samples were collected using plankton nets and with a longer delay between collection and observation (at least 2 h as in Valencia). Under the microscope, the blue color is easily bleached due to the stress of sampling and observation (Fig. 2f). For example, the isolated cell FG764 (GenBank accession number #KR139786) showed an intense blue pigmentation when first observed, but the pigmentation disappeared when it was transferred into a clean chamber for isolation (Fig. 2, i, j). In other stressed specimens, the blue substance was released around the cells and it is bleached (Fig. 2, l–n, see Video S1 in the Supporting Information, <https://youtu.be/eLK5FMGNtTI>). The cell division of *B. coerulea* was by oblique binary fission (Fig. 2a). During cell division, the apex of the episome of one of the daughter cells was elongated (Fig. 2, b, c). After the cell division, both daughter cells remained joined (Fig. 2, d, e), and exceptionally they may appear forming two pairs of dividing cells (Fig. 2g). One of the daughter cells showed an elongated episome with a blunt truncated apex (Fig. 2d). This morphology corresponded typically to the species described as *G. canus*. The size of the specimens usually ranged from 90–120 µm long and 45–65 µm wide, and we exceptionally observed specimens that reached 150 µm. This coincided with the morphology of *G. costatum* (Fig. 1s). In fact, the cells of *B. coerulea* showed different shapes. The shape was biconical in the less stressed specimens (Fig. 1, a, b, d–i) or ellipsoidal (Fig. 1, c, j–n). Other specimens showed a conical episome and a wide antapex (Fig. 1r). Other specimens showed a dome-shaped episome, and a slightly bifurcated antapex in ventral or dorsal views (Fig. 1, q, t, u). Other specimens showed a conical episome and a reduced episome (Fig. 1, v, w). When a cell was stressed, the pigmentation disappeared (see Video S1, <https://youtu.be/eLK5FMGNtTI>). The shape also changed, usually being rounder (Fig. 2i) and later ellipsoidal (Fig. 2j); the most extended phenomenon being that the cell changed progressively from biconical to ellipsoidal (Fig. 2, l–n). Specimens also showed other responses during the microscopic observations. At first, the blue pigmentation disappeared and the apex became rounder (Fig. 2o). A constriction encircled the middle of the episome and hyposome (Fig. 2p) and this constriction progressed until the cell was separated into three parts, which did not lyse during the process (Fig. 2, q, r). The central section kept the nucleus and the transversal flagellum that remained beating (Fig. 2, r). The complete process required ~5 min, and it was similar in the Mediterranean and Brazilian specimens (Fig. 2s). In the plankton samples, cells with a complete hyposome and a reduced episome were observed (Fig. 2, t, u). This suggests that after the autotomy, at least the part of the cell that contained the nucleus was able to regenerate, and the hyposome was the first part of cell to recover the normal size and shape. Despite a context of high intraspecific variability in color and shape, the specimens of *B. coerulea* showed several common morphological characters. The cingulum was median and had a cingulum descending ~4 times its width. In ventral view, the hyposome showed a shallow depression at the antapex. The sulcus was well marked and extended from the antapex to the base to the apex. The cell surface was covered with well-marked longitudinal equidistant ridges (Fig. 1n). The nucleus was spherical and small (~20 µm in diameter), and situated in the central part of the hyposome, more visible in dorsal view. The nucleus showed a well-developed perinuclear membrane (Fig. 1, p, s, u). The cells showed prominent food vacuoles, mainly located in the middle cell and in the

episome. Food vacuoles were colorless or exhibited a brownish color (Figs. 1 and 2). Under SEM, the longitudinal ridges showed different lengths. There were more ridges on the hyposome than the episome (Fig. 3a). However, not all the ridges that emerged from the cingulum reached the antapex or the base of the apex. In the episome, there were 24 ridges that extended anteriorly from the upper cingular groove, and about one half extended anteriorly more than $2/3$ of the length of the episome, and ending at the base of the apical groove. In the hyposome, there were 48 ridges that extended from the base of the cingulum, and only about one half of them reached the antapex (Fig. 3a). The separation between the ridges was ~ 6 and $3 \mu\text{m}$ in the junction of the sulcus and episome and hyposome, respectively. The apex was free of ridges and showed an apical groove with a shape almost circular that encircled the apex (Fig. 3b). The diameter of the apical groove was $\sim 14 \mu\text{m}$ (Fig. 3c). In dorsal view, the groove was not dissected by any ridge (Fig. 3c). We tried to culture *B. coerulea* by feeding it with different microalgae under laboratory conditions. The specimens showed the typical biconical shape of the non-stressed specimens and even they divided during the first days. However, after the first day, the specimens became colorless and the large food masses were absent. We were able to increase survival time, up to 1 week, when *B. coerulea* was fed a mix of diatoms from the seawater samples enriched with nutrients and other contaminant smaller microalgae in the culture. In contrast, *B. coerulea* did not survive more than 2 d when it was fed with clonal cultures of *Rhodomonas* sp., *Isochysis* sp., dinoflagellates or diatoms. *Gymnodinium lira*. The cell shape of *G. lira* was ellipsoidal ($95 \mu\text{m}$ long, $60 \mu\text{m}$ wide, Fig. 4, a–e) or round ($70 \mu\text{m}$ long, $65 \mu\text{m}$ wide, Fig. 4, f–i), with a dome-shaped or hemispherical episome. The sulcus extended from the antapex to the base of the apex (Fig. 4, b and g). The surface was covered with longitudinal equidistant ridges. In contrast to the other species, the nucleus was located in the episome (Fig. 4c). Brownish food vacuoles were observed in the hyposome (Fig. 4b). The cell was colorless and the main distinctive character was the presence of red or pink corpuscles in the periphery, especially at both sides of the cingulum. These corpuscles or body inclusions were more or less globular, with heterogeneous sizes and shapes, and located in the middle longitudinal ridges (Fig. 4i). These corpuscles did not disappear when the cells were stressed. Under SEM, the cell had 24 and 36 ridges in the episome and hyposome, respectively (Fig. 3d). The ridges were separated 9 and $6 \mu\text{m}$ in the episome and hyposome, respectively. The apex was free of ridges, except the sulcus that finished more anteriorly than the ridges (Fig. 3e). The apical groove was almost circular with a diameter that ranged from 10 to $13 \mu\text{m}$. The apical groove was not visible just in the point of junction with the anterior end of the sulcus (Fig. 3f). We have obtained the sequence of a specimen illustrated in Figure 4, a–c (isolated cell FG1601, GenBank accession number #KR139787). *Gymnodinium* cf. *lira*. These specimens were more slender than *G. lira* (95 – $110 \mu\text{m}$ long, 40 – $45 \mu\text{m}$ wide), the cell body was biconical with a pointed apex (Fig. 4, j–n). The cells showed peripheral red corpuscles and the nucleus was located in the hyposome. Brownish food vacuoles were observed in the episome (Fig. 4n). During the microscopic observations, *Gymnodinium* cf. *lira* was overlooked with *G. lira*. However, a more careful observation suggests significant differences with *G. lira* and that these specimens likely corresponded to an undescribed species with intermediate characteristics between *B. coerulea* and *G. lira*. *Gymnodinium* cf. *lira* shared the cell shape, size and nucleus position with *B. coerulea*, and it shared the presence of red or pink corpuscles and lack of blue pigmentation with *G. lira*.

Unfortunately, we did not isolate specimens for single-cell PCR. *Gymnodinium cucumis*. The vertical hauls with a 53 µm mesh size plankton net taken at the Bay of Villefranche sur Mer from 80 m depth to the surface were mainly dominated by large thecate dinoflagellates. The few exceptions were large species with a thick cell covering such as *B. coerulea*, *G. lira*, or *G. cucumis* that resisted to this sample treatment that is drastic for many unarmored dinoflagellates. *G. cucumis* is a distinctive species and it could not go unnoticed, when present in the surface samples collected from other locations examined in this study. Consequently, the paucity of records could be associated with a preferential deep water distribution of this species. The specimens of *G. cucumis* showed a slender fusiform body of 190 µm long and 65 µm wide, slightly wider posteriorly, and tapering at both ends (Fig. 5, a–f). The episome was conical, slightly asymmetrical with a narrow blunt apex. The hyposome was broader with a narrow blunt antapex (Fig. 5, a–f). The hyposome was slightly bifurcated with a shallow depression at the antapex (Fig. 5, c and e). The median cingulum had a descending left spiral course. The sulcus extended from the apex to the antapex (Fig. 5d). The cell surface was covered with longitudinal equidistant ridges, ~18–20 of which crossed the ventral face of the episome (Fig. 5d). The specimens showed yellow-grayish pigmentation. The nucleus was located in the hyposome (Fig. 5a).

A swollen cell shape was observed in moribund specimens (Fig. 5g). In contrast to other gymnodinioid dinoflagellates, this species did not lyse when it was fixed with ethanol. The cell contour was swollen, with tentatively food masses and a well-marked rounder organelle in the middle of the hyposome that corresponded to the nucleus (Fig. 5h). We obtained the sequence of the specimen shown in Figure 5, a–c (isolated cell FG1602, GenBank accession number #KR139788).

Balechina pachydermata

In all the sampling areas, we observed specimens with common characteristics: large size, hyposome larger than the episome, thick cell covering with a double-layer structure, cingular displacement of ~4 cingular widths, sulcus extended to the antapex and slightly intruding onto the episome as an anterior sulcal notch, nucleus placed in the hyposome, distinctive ochre pigmentation and prominent food vacuoles. It was problematic to assign these specimens to *A. vasculum*, *G. amphora*, *G. dogielii*, *B. pachydermata* or *Gymnodinium gracile* sensu Kofoed and Swezy because of the occurrence of intermediate forms between these species, and the only available illustrations are restricted to the original line drawings (see Appendix S1). Our specimens showed a sudden contraction at the cingulum level (Figs. 6, s–y; 7, g–h and k–n; see Video S2 in the Supporting Information, <http://youtu.be/FDytvHEJsFg>). Consequently, the specimens changed from the morphology of one described species to another in 1 s. Within this context, we assigned the specimens to the five morphotypes described mainly based on the shape of the episome. The specimens with a reduced and almost low conical episome and a conical hyposome were assigned to *G. amphora* (Figs. 6, a–j; 7, a, b, d–i, o). The isolated cells FGB8, FGB9, and FGH11 corresponded to the Figure 7, e–g, respectively (GenBank accession numbers #KR139790, #KR139791, #KR139792). Specimens from the same sample had different sizes (Fig. 7g). When this species was under division, the daughter cells showed the shape typical of *G. amphora* (Figs. 6h and 7j). We assigned the specimens with a triangular episome and a wide

hyposome to *A. vasculum* (Fig. 7c), and we assigned the specimens with a dome-shaped or hemispherical episome to *B. pachydermata* (Fig. 6, p–r), which included the isolated cell FGB22 (GenBank accession number #KR139789; Fig. 6r). The specimens with an elongate episome were assigned to *Gymnodinium dogielii* (Fig. 6, l–n). Specimens with dome-shaped episome specimens corresponded to *G. gracile* sensu Kofoed and Swezy (Fig. 6k). Other specimens were intermediate forms between *Gymnodinium dogielii* and *G. amphora* (Fig. 6o). The cell lengths varied from the smaller forms of *B. pachydermata* (110 µm long, Fig. 6r) to the larger morphotypes of *G. dogielii*, *G. gracile*, or *G. amphora* (180 µm long, Fig. 7g). When *B. pachydermata* entered in contact with another cell, it showed a sudden contraction, ~1 s, at the cingulum level, with a fast change toward a round shape (Figs. 6, s–y; 7, h, i and k–n; see Video S2, <http://youtu.be/FDytvHEJsFg>). We examined at high magnification the morphology of *B. pachydermata* (Fig. 8). This allowed observing that the cell was covered of numerous thin longitudinal striae (Fig. 8, c and h). The transverse flagellum emerged from a cavity in the hyposome (Fig. 8h). The apex showed a discontinuity in the contour that corresponded to the apical groove (Fig. 8, e and f). The hyposome showed a kind of rod-shaped structures that were almost radially distributed, named rodlets by Kofoed and Swezy (Fig. 8, j–k). We observed under SEM two specimens of *B. pachydermata* from different samples (Fig. 9). These specimens showed a high effect of shrinkage compared to other species. The cell surface was bossed and the fine longitudinal striae were visible at high magnification. The striae were separated 3–4 µm one each other (Fig. 9d). The cells showed an anterior sulcal notch (Fig. 9, c and d). In both specimens, the cells showed a shrunk apex and apical groove (Fig. 9, b and e) compared to the observations of live specimens (Fig. 8, e and f). Taken into account the shrinkage of the cells, the apical groove had an elongate elliptical shape (Fig. 9, b and e). Under culture conditions with different microalgae, the cells of *B. pachydermata* did not survive more than 2 d. We did not observe the mechanism of prey capture. Molecular phylogeny. The SSU rDNA sequences of the three isolated cells (FGB8, FGB9 and FGB11) of *G. amphora* from the South Atlantic Ocean (Fig. 7, e–g) were identical among them and to the sequence of an isolated cell (FGB22) of *B. pachydermata* from the Mediterranean Sea (Fig. 6r). The sequences of the two specimens of *B. coerulea* were identical. *G. lira* and *G. cucumis* sequences were 98% and 95% identical to that of *B. coerulea*, respectively. The sequences of the type species, *B. pachydermata*, and of *B. coerulea* were 92% identical. We examined the phylogenetic position of *G. amphora*, *G. cucumis*, *G. lira*, *B. coerulea* and *B. pachydermata* using a data set including a variety of dinoflagellate SSU rDNA sequences and rooted using syndinean sequences as the outgroup. The Bayesian phylogenetic tree showed that *B. coerulea* and *B. pachydermata* branched in two clades distantly related; one with short branches for the identical sequences of *B. pachydermata* and *G. amphora*, and another with long branches for the sequences of *G. cucumis*, *G. lira*, and *B. coerulea* (posterior probability of 1). In this clade, *G. lira* and *B. coerulea* were sister lineages and *G. cucumis* occupied a basal position (Fig. 10). However, these two clades did not show any well-supported close affiliation to other dinoflagellate groups present in public sequence databases. In fact, the new sequences branched within the large lineage comprising Gymnodiniales, Peridiniales, Dinophysales, and Prorocentrales but with poor support, making it difficult to infer the affinity with any of these orders. Nevertheless, the molecular phylogeny clearly supported that *B. pachydermata* and *B. coerulea* should not be placed in

the same genus or even in the same family (Fig. 10). The taxonomic affinity of these two genera remains unclear at the moment.

Taxonomic revisions

Our morphological and molecular data strongly support the separation of the species *B. pachydermata* and *B. coerulea* into two distinct genera that are not related even at the family level. As *B. pachydermata* is the type species, the genus *Balechina* remains for the species with the characteristics of the type. We provide an emended description of the genus *Balechina* and we propose the new genus *Cucumeridinium* to accommodate the species with the characteristics of *G. cucumis*, *G. lira*, and *B. coerulea*.

F. Gomez, P. Lopez-Garcia, H. Takayama et D. Moreira. Original publication: Loeblich and Loeblich (1968, p. 210). Diagnosis: Unarmored heterotrophic dinoflagellates with a double-layer thick cell covering. The cingulum is descending ~4 times its width. The sulcus extends to the antapex and slightly intrudes onto the episome as an anterior sulcal notch. The apical groove is elongated and elliptical. The cell surface is bossed with fine longitudinal striae, and lacked prominent ridges or furrows. Type species: *B. pachydermata* (Kofoid et Swezy 1921) Loeblich et A.R. Loeblich 1968. Basionym: *G. pachydermatum* Kofoid et Swezy (1921, pp. 239–240, plate 3, fig. 32, text-figure AA 5). Synonyms: *A. vasculum* Kofoid et Swezy 1921, *G. amphora* Kofoid et Swezy 1921, *Gymnodinium dogieli* Kofoid et Swezy 1921 and *G. gracile* sensu Kofoid and Swezy. The species *B. coerulea* (Dogiel) F.J.R. Taylor and *B. marianiae* F.J.R. Taylor do not belong to the genus *Balechina*. Iconotype: Fig. 11, a, b *Cucumeridinium* F. Gomez, P. Lopez-Garcia, H. Takayama et D. Moreira, gen. nov. Diagnosis: Unarmored heterotrophic dinoflagellates with prominent longitudinal ridges or furrows in the cell surface covering. The cingulum is descending ~4–7 times its width, and the sulcus extends from the antapex to the base of the apex. The apex is free of ridges, and the apical groove is almost circular. Synonyms: *Balechina* sensu Taylor 1976, auct. mult. Non: *Balechina* Loeblich et A.R. Loeblich 1968. Etymology: *cucumis*, *cucumeris*; Latin: cucumber. The surface furrows or ridges resemble the skin of some fruits of the plant family Cucurbitaceae. The gender is neuter. Type species: *Cucumeridinium coeruleum* (Dogiel 1906) F. Gomez, P. Lopez-Garcia, H. Takayama et D. Moreira, comb. nov., hic designatus. Basionym: *Gymnodinium coeruleum* Dogiel 1906, p. 35, figs. 46, 47. Non: *G. coeruleum* N.L. Antipova 1955. Homotypic synonym: *B. coerulea* (Dogiel) F.J.R. Taylor 1976. Heterotypic synonyms: *G. cucumis* sensu Schütt 1895, fig. 64.1,3,4; *Balechina marianiae* F.J.R. Taylor 1976; *G. costatum* Kofoid et Swezy 1921; *G. canus* Kofoid et Swezy 1921. Iconotype: Fig. 11, c, d Other species of the genus: *Cucumeridinium cucumis* (F. Schütt 1895) F. Gomez, P. Lopez-Garcia, H. Takayama et D. Moreira, comb. nov. Basionym: *G. cucumis* F. Schütt 1895, p. 116, pl. 21, fig. 64.2. Schütt, F. 1895. Die Peridineen der Plankton- Expedition. Ergebnisse der Plankton-Expedition der Humboldt-Stiftung 4:1–170, Lipsius and Teicher, Kiel. Non: *G. cucumis* F. Schütt 1895, pl. 21, figs. 64.1, 64.3, 64.4. Heterotypic synonym: *B. coerulea* sensu Taylor 1976. *Cucumeridinium lira* (Kofoid et Swezy 1921) F. Gomez, P. Lopez-Garcia, H. Takayama et D. Moreira, comb. nov. Basionym: *G. lira* Kofoid et Swezy 1921, p. 227, text-figure Z 11, pl. 3, fig. 30. Kofoid, C.A. & Swezy, O. 1921. The free-living unarmored Dinoflagellata. Memoirs of the University of California 5: 1–562. Non: *G. lira* Kofoid et Swezy 1921, p. 160, 162, text-fig. W1, 2. An undescribed

species of this genus could be *Gymnodinium* cf. *lira* (Fig. 4, j–n) that may correspond to *G. lira* sensu Kofoid et Swezy 1921, p. 160, p. 162, text-fig. W1, 2 (non *G. lira* Kofoid et Swezy 1921, p. 227, text-figure Z: 11, plate 3, fig. 30). *Gymnodinium* cf. *lira* differed from *Cucumeridinium lira* in the larger and slender cell body, conical episome with pointed apex and the nucleus placed in the hyposome. *Gymnodinium* cf. *lira* differed from *C. coeruleum* in the lack of blue or purple pigmentation, and the presence of red or pink peripheral corpuscles. We refrain to describe this species until the molecular data will confirm the distinction from *C. coeruleum* and *C. lira*.

Discussion

The combination of microscopic observations of live specimens from different locations and the molecular data was able to shed light on this group of large species with a thick cell covering and an unusual resistance to sampling and fixation. Species such as *C. coeruleum* showed a striking pigmentation that could easily receive the attention of the observer. Despite these features that could facilitate the recognition of the species, the records in the literature have been scarce for most of the species. In some cases, the observations were restricted to the original descriptions (see Appendix S1). In some genera, the paucity of observations could be attributed to the deep ocean distributions (i.e., *Heterodinium* Kofoid; Gomez et al. 2012). However, with the exception of *C. cucumis*, all the species of *Balechina* and *Cucumeridinium* can also be found in surface waters in coastal areas. *Cucumeridinium cucumis* and *C. coeruleum* were described from the Bay of Naples (Schütt 1895, Dogiel 1906), where up to date there is a high tradition of dinoflagellate studies. *Cucumeridinium lira*, *B. pachydermata* and its synonyms were described from the pier and waters near the Scripps Institute of Oceanography at San Diego, California (Kofoid and Swezy 1921). *B. pachydermata* was still present at San Diego, the type locality, as revealed by recent environmental sequencing surveys (GenBank accession number #KJ763266, Lie et al. 2014). All species can be found in the French Marine Stations along the Mediterranean coasts. Consequently, we cannot attribute the lack of studies to a remote distribution of these species far of well-equipped laboratories. An alternative explanation may be that, in addition to the general paucity of taxonomists interested in non-toxic species, the high polymorphism and the lack of micrographs make difficult the identification at the species level and many previous observations have simply been pooled as *Gymnodinium* sp. The errors in the original descriptions of the species, often based on fixed specimens, or based on the observation of a single or few specimens also contributed to the difficulties in the identification. We provided a brief summary of the problems in the species descriptions (see details in Appendix S1). In the earlier publication of these species based on preserved material, Schütt (1895) illustrated at least two species collected from an indeterminate place under the name *Gymnodinium cucumis*. The illustrations of smaller specimens corresponded to *G. coeruleum*. In other cases, Schütt (1895) also described different taxa under the same species name (i.e., *Dissodinium pseudolunula* E.V. Swift and *Pyrocystis lunula* (F. Schütt) F. Schütt). A few years later, Dogiel (1906) described *G. coeruleum* from the Bay of Naples, which is probably the same place where Schütt (1895) collected *G. cucumis*. Dogiel examined live specimens, and he was right when he described *G. coeruleum* under two different morphologies: biconical specimens with blue pigmentation and the stressed

specimens that showed an ellipsoidal contour and lacked the pigmentation. Kofoed showed a trend to be a splitter taxonomist and he often described new species based on the observation of single specimens and, consequently, ignored the intraspecific variability. Kofoed and Swezy (1921) reported that they observed a high number of specimens of *G. coeruleum*. However, they did not illustrate any specimen, and only reproduced the illustration of the ellipsoidal specimen described by Dogiel (1906). This anomaly did not allow us to know the intraspecific morphological variability in *G. coeruleum*. Kofoed and Swezy (1921) described *G. canus* from a single specimen with bluish pigmentation. This species was considered to be one of the daughter cells of *G. coeruleum* (Fig. 2, b–d). They also described *G. costatum* that likely corresponds to a large specimen of *G. coeruleum* which blue color is bleaching (Fig. 1s). These authors described *G. lira* with round apex and nucleus in the episome. However, in other part of the text they also used the name *G. lira* for a specimen with a pointed apex and the nucleus in the hyposome. They described five other species (*A. vasculum*, *G. pachydermatum*, *G. amphora*, *G. dogieli*, and *G. gracile* sensu Kofoed and Swezy) with several common characters: large size, a distinct thick doublelayer cell covering, low cingular displacement (descending ~4 times its width), surface lacking prominent longitudinal ridges, sulcus extended to the antapex, and an anterior sulcal notch in the episome, radial rod-shaped structures, distinctive yellow or ochre pigmentation, nucleus in the hyposome, prominent food vacuoles, etc. The main difference of these species was the shape of the episome. However, Kofoed and Swezy (1921) did not observe that the specimens were able of a sudden contraction at the cingulum level, so that the shape of the episome can change from that of one species to another. Schiller (1933) created more confusion when he illustrated *G. pachydermatum* with important differences in the cell shape and nucleus position when compared to the original description (see Appendix S1). Loeblich and Loeblich (1968) did not contribute to the taxonomy of these species, and they only proposed the new genus *Balechina* for the type species of the subgenus *Pachydinium*. They did not transfer other species of the subgenus *Pachydinium* into *Balechina*. The genus was defined based on the characteristics of *G. pachydermatum*, such as the lack of prominent longitudinal ridges. Taylor (1976) examined net samples of formalin fixed specimens. He proposed to class the species *G. coeruleum*, with prominent surface ridges, into the genus *Balechina*, and he described a new species, *B. marianiae*. However, he proposed *B. marianiae* based on specimens of *G. coeruleum*, and he confused specimens of *G. cucumis* with *G. coeruleum*. Balech (1988), to whom the genus *Balechina* was dedicated, did not place *G. coeruleum* into *Balechina*. In his classification, Taylor (1987) placed the genus *Balechina* into the family Kolkwiziellaceae that contains thecate species such as *Herdmania* J.D. Dodge and *Kolkwiziella* Er. Lindem. Later, Fensome et al. (1993) placed *Balechina* into the order Ptychodiscales, a mixing bag of genera with no relation among them. For example, *Brachidinium* F.J.R. Taylor is included in that order based on its thick cell covering. However, *Brachidinium* and *Karenia* Gert Hansen et Moestrup are closely related genera, if not synonyms (Gómez et al. 2005, Henrichs et al. 2011) but up to date, no study has reported a thick cell covering for *Karenia*. *C. coeruleum* is an amazing dinoflagellate, mainly due to its striking blue or purple pigmentation and its capacity of autotomy. The blue corpuscles, cyanophores, of similar size accumulated in the periphery of cell. The blue color fades when the cell is stressed, and the blue substance is released around the cell (see Video S1, <https://youtu.be/eLK5FMGNtTI>). The origin and function of the blue

pigmentation is uncertain. Some dinoflagellates show a blue–green pigmentation due to the presence of phycobilin from cryptophyte kleptoplastids (Hu et al. 1980, Takano et al. 2014). However, *C. coeruleum* has no plastids. In June 2008 at Marseille, we observed a high abundance of *C. coeruleum* with specimens that exhibited an intense blue pigmentation, also coinciding with blue nauplii (see Fig. S1 in the Supporting Information). The observation of parallel non-concentrated samples revealed the bottom of the Utermohl chamber covered with the massive presence of a non-motile small microalgae with globular or ellipsoidal shapes (4–7 µm long) and blue–green pigmentation. Unfortunately, the limitations of the microscope and camera did not allow obtaining better quality images (see Fig. S1). We have not observed the phenomenon again and we cannot demonstrate that the presence of that microalga could be related to the increase in the abundance and intense blue pigmentation of *C. coeruleum*. In metazoans such as chameleons the color shift is due to guanine nanocrystals (Teyssier et al. 2015). The closest relatives of *C. coeruleum*, i.e., *C. lira*, *C. cucumis* and *Gymnodinium* cf. *lira*, did not show the blue or purple pigmentation. *Cucumeridinium* *lira* and *Gymnodinium* cf. *lira* showed red or pink corpuscles of different size. Their color and brightness were similar to those of corpuscles found in some species of *Kofooidinium* Pavillard (Gómez and Furuya 2007). The accumulation of reddish corpuscles is common in heterotrophic dinoflagellates such as *Protoperidinium* Bergh (Neveux and Soyer 1976, Carreto 1985). We observed prominent food vacuoles, most of them with a distinctive brownish color, in specimens of *C. coeruleum* from field samples. However, we did not observe the feeding behavior and the nature of the preys. Taylor (1976) reported that the specimen observed of *C. cucumis* (misidentified as *C. coeruleum*) had ingested diatoms. Our cultures of *C. coeruleum* only survived for 1 week when fed with a mix of diatoms, and the cells remained colorless since the first day. We collected the specimens using a plankton net (≥ 20 µm mesh pore size) and the samples were concentrated in settling samples. This procedure excluded the observation of the smaller size fraction of organisms (i.e., blue–green cyanobacteria, cryptophyte), so that we could not observe the full range of potential prey for *C. coeruleum* in the natural samples. Another amazing phenomenon in *C. coeruleum* was the ability of autotomy, which to the best of our knowledge was unknown in other unarmored dinoflagellates. The autotomy was known in thecate dinoflagellates such as *Tripos* Bory, which is able to cut their antapical and apical horns (Kofoid 1908). However, in this case the autotomy was only the voluntary shedding of the horns (i.e., appendages or body extensions), and it did not affect to the main body of the cell. By contrast, *C. coeruleum*, that does not have body extensions, was able to separate one part of the hyposome, and sometimes also one part of the episome, and the cell did not lyse during the process (Video S1, <https://youtu.be/eLK5FMGNtI>). The observation of incomplete specimens in healthy conditions revealed that the cell is able to regenerate after autotomy (Fig. 2, t, u). The autotomy seems to be a moderate response to stress. When the cells were highly stressed, they lysed as the typical gymnodinoid dinoflagellates. However, in contrast to other unarmored dinoflagellates, the lysis was slower and the cell membrane seemed to be more resistant. In terrestrial animal ecology, autotomy is considered an anti-predator mechanism, which is literally left holding a part of the intended victim's body. For example, some rodents, salamanders, and lizards autotomize their tails, and the thrashing tail often distracts the predator while the prey escapes (Fleming et al. 2007). However, the possible advantage of this phenomenon of autotomy in this unarmored dinoflagellate was uncertain.

These unique characteristics, the combination of the blue or purple pigmentation and the autotomy, may be related to its ecological success because *C. coeruleum* is more ubiquitous and abundant than its congeneric species. *B. pachydermata* showed a sudden contraction at the cingulum level, with a fast change in cell shape (see Video S2, <http://youtu.be/FDytvHEJsFg>). The contraction of *B. pachydermata* seemed to be a defensive strategy in response to the approach of a potential predator. *Balechina pachydermatum* is characterized by a thick double-layer cell covering with a rough surface and fine longitudinal striae. This ultrastructural feature may facilitate the sudden contraction without damage or lysis of the cell. This contraction was common in some noctiluroid dinoflagellates such as the leptodiscaceans (i.e., *Scaphodinium* Margalef, *Abedinium* Loeblich et A.R. Loeblich; Gómez and Furuya 2004, Gómez et al. 2010). These noctiluroid dinoflagellates possessed a net of myofibrils that facilitated the change in shape (Cachon and Cachon 1967). Kofoid and Swezy (1921) illustrated *A. vasculum*, *G. pachydermatum*, *G. dogieli*, *G. amphora*, and *G. gracile* with a kind of almost concentric radial filaments that they described as long slender rodlets or radial canals. Kofoid and Swezy did not refer to the nature of these organelles. This organelle was visible in *B. pachydermata* under high magnification (Fig. 8, j, k). The shape and position, mainly around the nucleus, resembled those observed in *Abedinium* (Gómez et al. 2010). These structures in leptodiscaceans were ejectile bodies used for prey capture (Cachon and Cachon 1969). In *B. pachydermata*, these organelles were considered to be ejectile bodies used for the prey capture, more so than myofibril-like structures. This study summarized the observations from several regions of the world's oceans. Studies will continue to obtain information of the ultrastructure, especially the thick cell covering, and ecological significance of the pigmentation, autotomy, contraction, or ejectile bodies of these amazing dinoflagellates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Light micrographs of *Balechina coerulea*. (a–e) Specimens from Banyuls sur Mer. (a and b) Isolated cell FG754 (GenBank accession number #KR139785). (f–i, o) Specimens from Marseille. (j–n) Specimen from Valencia. (p–w) Specimens from São Sebastião Channel. (p) Note the different of size between the specimens. n = nucleus; scale bars, 50 µm.

Fig. 2.

Light micrographs of *Balechina coerulea*. (a–e, h–n) Specimens from Banyuls sur Mer. (f) Specimens from Marseille. (m–q) Specimens from Valencia. (g, s–u) Specimens from São Sebastião Channel. (a–f) Dividing cells by oblique binary fission. (b, c) Specimen with an elongate apex, similar to *Gymnodinium canus*. (e, f) Two daughter cells before separation. (f) Depigmentation. Note that the colored spheres remained in the apex and antapex. (g) Two pairs of daughter cells. The arrow points the elongate episome attached to the episome of the daughter cell. (h) Blue color is bleached. (i, j) Change in coloration and shape of a single specimen. Isolated cell FG764 (GenBank accession number #KR139786). (k–m) Serial micrographs of the depigmentation and shape change in a single specimen. (m) Note the

dissolution of the pigment in the surrounding water. (m–r) Serial pictures of the autotomy of a single specimen. (o) The arrow points the place where the autotomy begins in the episome and hyposome. (q) The arrow points the transversal flagellum. (s) Specimen beginning the autotomy. (t, u) Cells under regeneration after autotomy. n, nucleus; tf, transversal flagellum; scale bars, 50 μ m.

Fig. 3. Scanning electron microscopy pictures of *Balechina coerulea* (a–c) and *Gymnodinium lira* (d–f) from South Japan. (a) Dorsal view. (b) Detail of the episome. (c) The arrow points the apical groove. (d) Ventral view. (e) Apical view. (f) Detail of the apical groove; scale bars, 50 μm , b, c, f; scale bars, 5 μm .

Fig. 4.

Light micrographs of *Gymnodinium lira* (a–i) and *Gymnodinium cf. lira* (j–n). (a–c) Isolated cell FG1601 of *Gymnodinium lira* (GenBank accession number #KR139787) from Villefranche sur Mer. (b) The arrows point the brownish food vacuoles. (d, e) *G. lira* from São Sebastião Channel. (f–i) *G. lira* from Villefranche sur Mer. (i) Note the different size of the red corpuscles. (j, k) *Gymnodinium cf. lira* from Marseille. (l, m) *G. cf. lira* from São Sebastião Channel. (n) *G. cf. lira* from Ubatuba. The arrow points the brownish food vacuole in the episome. n, nucleus; scale bars, 50 µm.

Fig. 5. Light micrographs of *Gymnodinium cucumis* from the Bay of Villefranche sur Mer. (a– c) Live specimen used for single cell PCR, isolated cell FG1602 (GenBank accession number #KR139788). (d–f) Another live specimen. (g) A moribund specimen. (h) Specimen fixed in ethanol. n, nucleus; scale bars, 50 μ m.

Fig. 6.

Light microscopy pictures of *Balechina* spp. from the Mediterranean Sea. (a–g) Specimens from Banyuls sur Mer. (h) Specimens from Marseille. (i–y) Specimens from Valencia. (a–j) *Gymnodinium amphora*. (h) *G. amphora* under division. (k–n) *Gymnodinium dogieli*. (o) ***Gymnodinium amphora***. (p–r) *Balechina pachydermata*. (r) Isolated cell FGB22 (GenBank accession number #KR139789). (s–y) Serial micrographs of *G. amphora* during the cell contraction. n, nucleus; scale bars, 50 μ m.

Fig. 7. Light microscopy pictures of *Balechina* spp. from the South Atlantic Ocean. (a–j) Specimens from São Sebastião Channel. (k–o) Specimens from Ubatuba. (a, b) *Gymnodinium amphora*. (c) *Amphidinium vasculum*. (d–i) *Gymnodinium amphora*. (d) Isolated cell FGB8 (GenBank accession number #KR139790). (e) Isolated cell FG9 (GenBank accession number #KR139791). (f) Note the different size. Isolated cell FGB11 (GenBank accession number #KR139792). (g, h) Serial micrographs of a cell contraction. (j) *Gymnodinium amphora* under division. (k–n) Serial micrographs of a cell contraction. (o) *Gymnodinium amphora*. n, nucleus; scale bars, 50 μ m.

Fig. 8.

Light microscopy pictures of *Balechina pachydermata* from South Japan. (a, b) Dorsal view. Note the brownish food vacuoles. (b, c) Ventral view. (e, f) Detail of the apex. (g) Detail of the cingular displacement. (h) Detail of the cavity of the transversal flagellum. See arrow. (i) Detail of the nucleus in the hypotheca. (j, k) Detail of the tentative ejectile bodies in the hyposome. c, cingular groove; eb, ejectile body; fv, food vacuole; lf, longitudinal flagellum; n, nucleus; s, sulcal groove; scale bars, 50 μ m (a–d), 10 μ m (e–k).

Fig. 9.

Scanning electron microscopy pictures of two specimens of *Balechina pachydermata* from South Japan. (a) Ventral view. (b) Detail of the episome. The arrow points the apical groove. The inset shows the apical groove. (c) Another specimen in ventral view. (d) Inset of the cingular and sulcal grooves. The arrows point the fine longitudinal striae. (e) The arrow points the apical groove. The inset shows the apical groove; scale bars, 50 μm (a, c), 10 μm bars (b, d, e).

Fig. 10.

Fig. 11.
Line drawings of *Balechina pachydermata* (a, b) and *Cucumeridinium coeruleum* gen. et
comb. nov. (c, d). (a, c) Ventral view. (b, d) Apical view.

Table 1

List of new SSU rDNA sequences used for the phylogenetic analysis. Accession numbers, geographic origin, and collection dates are provided.

Taxa	GenBank No.	Geographical origin (date)	Figure
<i>Balechina coerulea</i> FG754	KR139785	Banyuls sur Mer (28 Jul 2009)	Fig. 1, a and b
<i>Balechina coerulea</i> FG764	KR139786	Banyuls sur Mer (2 Jul 2009)	Fig. 2, i and j
<i>Gymnodinium lira</i> FG1601	KR139787	Villefranche sur Mer (7 Jan 2010)	Fig. 4a-c
<i>Gymnodinium cucumis</i> FG1602	KR139788	Villefranche sur Mer (21 Jan 2010)	Fig. 5a-c
<i>Balechina pachydermata</i> FGB22	KR139789	Valencia (11 May 2012)	Fig. 6r
<i>Gymnodinium amphora</i> FGB8	KR139790	São Sebastião Channel (5 Aug 2013)	Fig. 7e
<i>Gymnodinium amphora</i> FGB9	KR139791	São Sebastião Channel (9 Aug 2013)	Fig. 7f
<i>Gymnodinium amphora</i> FGB11	KR139792	São Sebastião Channel (21 Jun 2013)	Fig. 7g