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Heterocapsa bohaiensis sp. nov. (Peridiniales: Dinophyceae): a novel marine dinoflagellate from the Liaodong Bay of Bohai Sea, China

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Abstract

A small armed dinoflagellate bloomed in the aquaculture ponds off the coast of Liaodong Bay, Bohai Sea of China, resulting in heavy mortalities of the cultured prawns (*Penaeus japonicus*) and larvae of Chinese mitten handed crabs (*Eriocheir sinensis*). The bloom-forming species was successfully isolated, and cellular morphology of the specimen was consequently investigated through light, fluorescent and electron microscopy. The small ((14.4±1.6) μm in length) ellipsoid cells show typical *Heterocapsa* thecal plate arrangement (Po, cp, 5′, 3a, 7″, 6c, 5s, 5‴, 2⁗). The episome is evidently bigger than the hyposome. One to three spherical pyrenoids are located above or beside the large elongated nucleus. The body scale is characterized by a triangle basal plate with one central upright and nine peripheral spines. Above all, *Heterocapsa bohaiensis* could be distinguished from other *Heterocapsa* species by the combination of the cell size, morphology, cellular structure and body scale. Sequence analyses of both ITS and LSU regions reveal the significant genetic divergence between *H. bohaiensis* and other established species in this genus, further supporting novelty of this species. Noticeably, different sample treatment methods resulted in morphological variation of the apical pore complex (APC) of *H. bohaiensis*, which needs to be taken into account in future study.

Key words: *Heterocapsa*, harmful algal bloom, thecal plate, body scale, ITS

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1 Introduction

The genus *Heterocapsa* was originally established by Stein in the 1880s based on the type species of *Heterocapsa triquetra* (=*Glenodinium triquetrum* Ehrenberg). A number of species were subsequently transferred into this genus, including *H. pacifica*, *H. niei*, *H. illdefina* and *H. rotundata* (Loeblich III, 1968; Herman and Sweeney, 1976; Hansen, 1995). Since the 1990s, more species were discovered and described, along with *H. circularisquama*, an HAB species blooming off the west coasts of Japan (Horiguchi, 1995, 1997; Matsuyama et al., 2001; Iwataki et al., 2002a, 2003, 2004, 2009; Tamura et al., 2005). So far, the genus *Heterocapsa* comprises about 19 species, characterized with a general plate formula of Po, cp, 5′, 3a, 7″, 6c, 5–8 s, 5‴, 0- lp, 2⁗ (Loeblich III et al., 1981; Morrill and Loeblich III, 1981; Hansen, 1995; Horiguchi, 1995; Iwataki et al., 2002a, 2004; Gómez, 2005, 2012), an elongate anterior sulcal plate and presence of minute three-dimensional body scales (Pennick and Clarke, 1977; Morrill and Loeblich III, 1981, 1983; Iwataki et al., 2004). Although the taxonomic relationship between *Heterocapsa* and its closely-related genera (e.g., *Cachonina*) was still unresolved (Morrill and Loeblich III, 1981; Horiguchi, 1995), species within *Heterocapsa* could be distinguished by cell morphology, relative position and shape of the nucleus and pyrenoids, and the fine structure of the body scales (Iwataki et al., 2004; Iwataki, 2008).

Heterocapsa spp. are distributed widely in the coastal regions around the world, and some bloomed densely causing detrimental ecological impacts in some regions. *Heterocapsa circularisquama* was originally identified in Japanese coast, and intensive blooms of this species occurred in western Japan since 1988, resulting in catastrophic mortalities of the farmed molluscan shellfishes (Matsuyama et al., 1995, 2001). Later, Iwataki et al. (2002b) reported the similar *H. circularisquama* cells in the fixed backup samples of red tides occurred in Hong Kong in the 1980s, speculating a wider distribution of this species along the western Pacific. Although systematic research on toxicity and harmful effects of *H. circularisquama* on various organisms are still ongo-

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ing (Salcedo et al., 2012; Basti et al., 2015, 2016; Nishiguchi et al., 2016), a photosensitizing hemolytic toxin, presumably a novel porphyrin derivative, has been successfully isolated and characterized from *H. circularisquama* (Sato et al., 2002; Miyazaki et al., 2005). *Heterocapsa circularisquama* is the only toxic species reported in this genus so far. Other species, *H. triquetra* as an example, may form dense blooms in the coastal and estuary waters, while no evident harmful impacts have been reported (Kim, 1997; Lindholm and Nummelin, 1999; Kononen et al., 1999).

Except the *H. circularisquama* blooms in Hong Kong (Iwataki et al., 2002b), little was known about *Heterocapsa* spp. along the coasts of China. Two *Heterocapsa* species (*H. minima* and *H. triquetra*) were listed in the historical records as the common dinoflagellates in the East China Sea (ECS; Liu, 2008), while no descriptions on morphology and distribution were provided. Blooms of *H. circularisquama* and *H. rotundata* were also reported sporadically in the Changjiang (Yangzi River) Estuary of ECS, coasts of Dalian and Qingdao in recent years (Du, 2005; Wang et al., 2006; Liu et al., 2014). Whereas, the details on morphology and physiology of the suspicious cells were not reported, resulting in doubtful identity of these blooming cells (Liu et al., 2009; Zheng, 2009). In 2008 and 2012, dense-blooming of a small dinoflagellate occurred in the aquaculture ponds in Panjin, Liaodong Bay of Bohai Sea, causing heavy mortalities of the cultured prawns (*Penaeus japonicus*) and larvae of Chinese mitten handed crabs (*Eriocheir sinensis*; Yang et al., 2015; Liu, 2016). More research further revealed the harmful effects of this dinoflagellate on *Ruditapes philippinarum*, *Brachionus plicatilis* and *Calanus sinicus* (Yang et al., 2015; Liu, 2016). Microscopic examination of the blooming species indicated that it did not resemble any common dinoflagellates in this region, and the true species identity is yet to be determined. Here in this study, we reported the taxonomic research on this blooming species, which may assist future research on toxicity, physiology and assessment on ecological impact.

2 Materials and methods

2.1 *Sample collection*

Vegetative cells were isolated from the aquaculture ponds in the coastal water of Panjin (40°51′N, 121°46′E) in 2008 and 2012. The original cultures were established by the Guanghe Crab Industry Limited Company (Panjin, China). The subset samples were then sent to the First Institute of Oceanography, State Oceanic Administration in Qingdao. They were maintained in f/2 medium with 0.50 mg/L GeO_2 to inhibit diatom growing at 20 °C with an illumination of 100 μmol photons m^{-2} s^{-1} and 12L:12D photoperiod. The cells were transferred to the fresh medium every month to maintain the viability.

2.2 *Light microscopy*

Cell observation was carried out using a compound microscope (Olympus BX53, Japan) equipped with epifluorescence and differential interference contrast optics. In order to avoid the size reduction during cell fixation and dehydration process (Salas et al., 2014), we measured sizes of live cells. A drop of cultured cells were covered with a cover slip and observed at 1 000× magnification. After the cells slightly settled, images were captured and cell sizes were then measured using the CellSens Imaging software (Olympus, Japan). To examine the thecal plate, cells were fixed in 2% glutaraldehyde (final concentration), stained with Calcofluor White (Fritz and Triemer, 1985) and then checked under a violet excitation light. The shape and location of the chloroplasts were observed under a blue excitation light. The nucleus was stained with 4′-6-diamidino-2-phenylindole (DAPI, 0.1 μg/mL final concentration) and determined under a violet excitation light.

2.3 *Electron microscopy*

For SEM, cultured cells were either directly fixed by OsO_4 or undergone a pretreatment process to clean the out layer debris before they were fixed and dehydrated. About 5 mL of cells at exponential growing phase were fixed with an equal volume of 4% OsO_4 (w/v) for about 40 min. The fixed cells were then filtered onto a nylon membrane (5 μm pore-size) by gravity. Subsequently, the folded membrane with cells was washed by an acetone series (10%, 30%, 50%, 70%, 80%, 90%, 100% and repeat 100% for two times, critical point dried and coated with gold. For another batch of sample, a pretreatment process was performed to clean the out layer before they were fixed. Briefly, cells were collected by centrifugation at 5 000 r/min for 5 min, the supernatant was then removed and cell pellet was re-suspended in 60% ethanol at 4°C for 1 h to strip off the outer cell membrane. Subsequently, cells were pelleted again and re-suspended in 5 mL filtered seawater for 30 min at 4°C. These re-suspended cells were then fixed and dehydrated as described above. The gold-coated cells were viewed under a scanning electron microscope (Hitachi S-3400N, Japan). Some SEM micrographs were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, California, USA).

For the transmission electron microscopy, fixed cells (2% OsO_4) were dehydrated through an acetone series and then embedded in Spurr's resin. Thin sections were cut with a diamond knife, stained with 2% uranyl acetate and observed using Hitachi 7 500.

2.4 *PCR amplification and sequence analysis*

Dinoflagellate cells were pelleted through centrifugation at 10 000 r/min for 5 min, cell pellet was then lysed and extracted using E.Z.N.A.^{TM}HP plant DNA kits (OMEGA Bio-tek Inc., GA, USA) following the manufacturer's protocol. PCR amplifications of ITS and LSU rRNA genes were performed as D'Onofrio et al. (1999) using the primer pairs: ITS1 5′-TCCGTAGGTGAACCTGCGG-3′, ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (White et al., 1990) and D1R 5′-ACCCGCTGAATTTAAGCATA-3′, D2C 5′-CCTTGGTCCGTGTTTCAAGA-3′ (Edvardsen et al., 2003), respectively. Amplicons were ligated into pMD®18-T vectors (TaKaRa Co., Dalian, China) and then transformed into DH5∂ competent cells (TaKaRa) following the manufacture's instructions. Selected clones were screened using M13 primers and those with right insertions were subsequently sequenced bi-directionally by the TsingKe Biological Technology (Qingdao, China).

The resulting sequences were cleaned for the ambiguous reads manually and then aligned with the *Hetercapsa* sequences retrieved from the public database (https://www.ncbi.nlm.nih.gov, up to date of April 7, 2017). Two sequences from *Cochonina hallii* (JQ972674 and AF033867) were also included in the analyses, given its unresolved taxonomic status relevant to *Heterocapsa* (Horiguchi, 1995). Sequences of *Prorocentrum minimum* (AF208244, AF260379) were treated as outgroups in ITS and LSU analyses, respectively. These sequences were aligned using MUSCLE algorithm (Edgar, 2004) implemented in MacVector 15.1.5 (Accelrys, CA, USA). Maximum-likelihood (ML), Neighbour-joining (NJ) and Maximum-parsimony (MP) analyses were performed in MEGA 6 (Tamura et al., 2013) with the best DNA substitution models (Kimura 2-parameter for ITS and Tamura-Nei for LSU) chosen based on Bayesian Information Criterion (BIC). Boot-

strap analyses (1 000 replicates) were then conducted to evaluate the robustness of each clade. Gaps were deleted for the analyses.

3 Results

3.1 *Cell morphology*

Cells are ellipsoidal, elongate with an average length of (14.4±1.6) μm (9.9–16.5 μm, n=102) and width of (10.2±1.4) μm (6.7–12.4 μm). The L:W ratios range from 1.2 to 1.8 with a mean of 1.4. The episome of cells ((8.4±2.1) μm in length, (10.2±1.4) μm in width) is significantly bigger than the hyposome ((4.1±1.1) μm, (8.2±1.2) μm, P<0.01, two-tailed t-test). Cingulum is wide and accounts for about 1/7 to 1/5 of the total cell length, evidently towards the antapex. The large nucleus is ellipsoid, mostly located in the middle of the cell (Figs 1a, b) and the numerous condensed chromosomes are visible when stained with DAPI (Fig. 1c). The spherical pyrenoids (1–3 per cell) are surrounded by starch sheaths, located above or beside the nucleus often in the episome or close to cingulum (Fig. 1c). A red accumulation body with variable shape and size is consistently observed in the hyposome (Figs 1a, b). The chloroplast is visible in the periphery of the cell (Fig. 1d) under the epifluorescence microscope. In the exponentially growing culture, cells are often divided by binary fissions (Figs 1e, f). Temporary cysts or an intermediate stage of planozygote and resting cyst are often observed at the bottom of the culture vessels and surrounded by heavy mucus. They are spherical to oval, significantly larger than the motile cells, and full of pale granules (Fig. 1g).

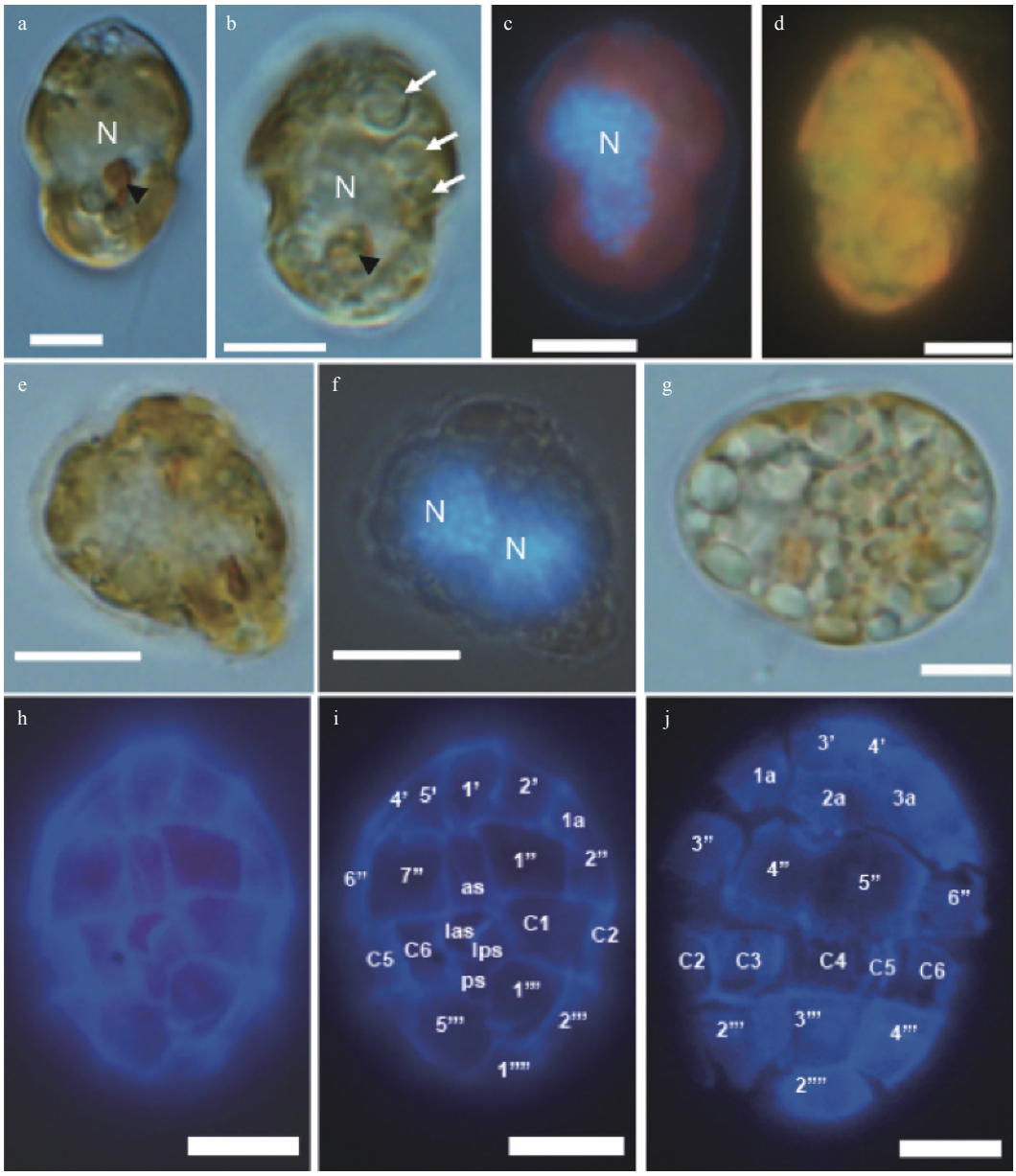

Fig. 1. Light microscopic images of *Heterocapsa bohaiensis*. a and b. Live cells showing the large nucleus (N), multiple pyrenoids (white arrows) and a red accumulation body (black arrows); c. DAPI stained cells with visible nucleus (N); d. chloroplast under epifluorescence LM; e and f. a DAPI stained dividing cell under white (e) and UV light (f), two dividing nuclei (N) are visible in f; g. a temporary cyst or an intermediate stage of planozygote with pale granules; h–j. ventral (h and i) and dorsal (j) views of the thecal plates, plate pattern is labeled in i and j. Scale bars: 5 μm (a–d and g–j), and 10 μm (e and f).

3.2 *Thecal plate morphology*

After being stained with Calcofluor White, the thecal plates of *H. bohaiensis* are visible (Fig. 1h) and consistent with the observations from SEM (Fig. 2). The plate pattern is schematized as in Fig. 3. Consistent with known *Heterocapsa* spp., the thecal plate configuration of *H. bohaiensis* is Po, cp, 5′, 3a, 7″, 6C, 5s, 5‴, 2⁗. As shown in the diagram (Fig. 3), the epitheca consists 5 apical plates, 3 anterior intercalary plates and 7 precingular plates. The cingulum composes of 6 plates, and the hypotheca has 8 plates. The body scale (300–350 nm) is outlined by a triangle basal plate (Fig. 2e). A central upright and nine peripheral (three at each ridge) spines are visible on the basal plate. Three radiating spines (one at each side of the triangle) are probably connected with the central upright by the radiating bars. Interestingly, the morphology of apical pore complex (APC) varies when cells were treated with different methods. When cells are directly fixed with OsO_4 without pretreatment, a spherical (0.8 μm in diameter) protruded cover plate (cp) is consistently observed above the pore plate (po, Fig. 2c), and there are visible radical rod-like decorations on cp. Whereas, when cells are pretreated using ethanol to strip off the outer layer, the cover plate (cp) turns to be flat, not protruded, and no decorations could be observed on the cp (Fig. 2d). The APC morphology of pretreated *H. bohaiensis* cells is similar to that of *H. minima* (Salas et al., 2014).

3.3 *Ultrastructure*

The ultrastructure of the cell shows a large dinokaryon, a presumably single chloroplast, a spherical pyrenoid, mitcondria, trichocysts and an accumulation body (Fig. 4a). The chloroplast with lamellae of three appressed thylakoids is located in periphery of the cell and connects with the pyrenoid. The pyrenoid is often surrounded by the starch sheath, and the thylakoids do not penetrate into the pyrenoid (Fig. 4b). A large accumulation body is quite visible in the hyposome.

3.4 *Phylogeny of H. bohaiensis*

The ITS sequences form about 15 distinct clades (Fig. 5) including the one representative of *H. bohaiensis* from this study. The eight monophyletic clades with high bootstrapping support values represent taxa of *H. horiguchii*, *H. circularisquama*, *H. tri-*

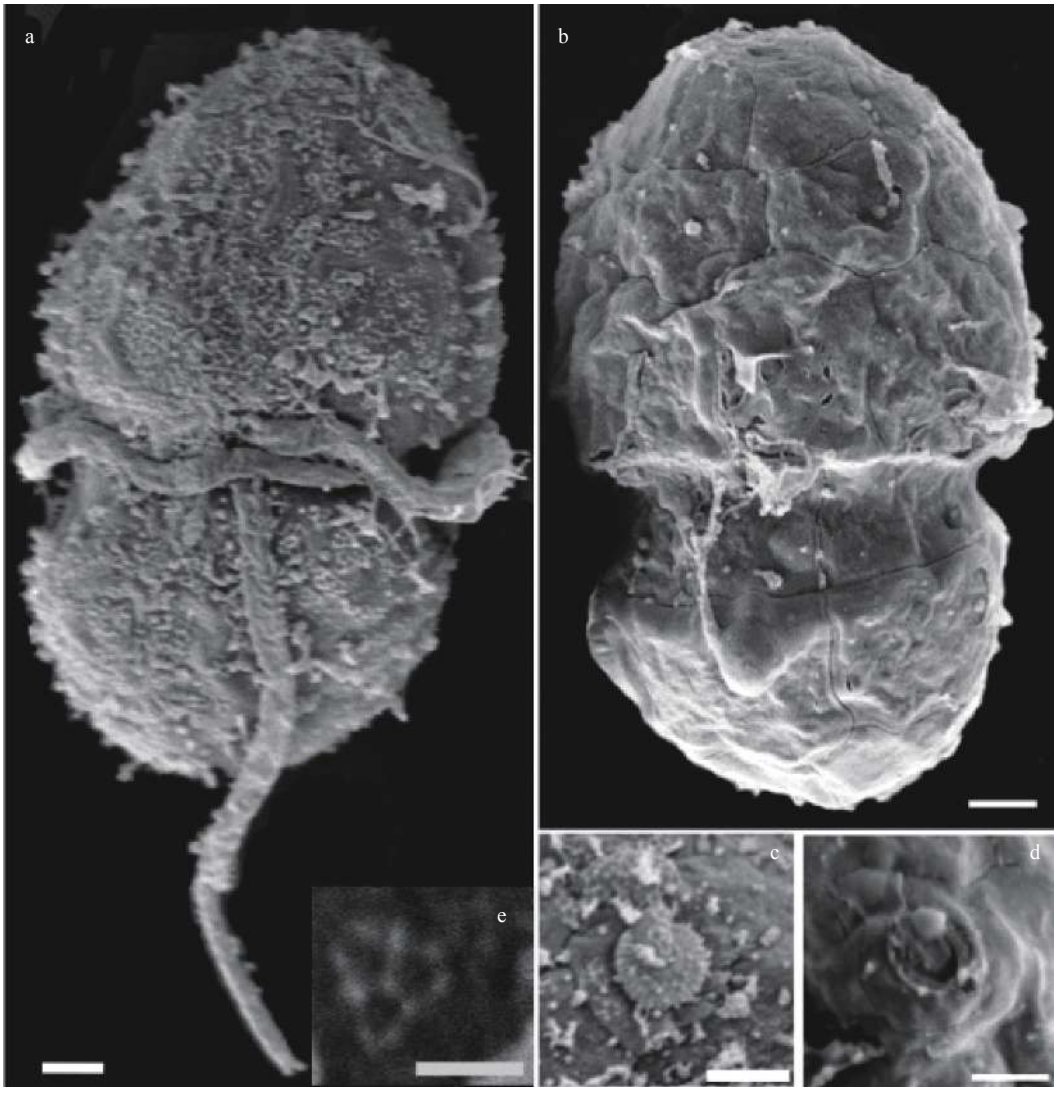

Fig. 2. SEM images of *Heterocapsa bohaiensis*. a. Ventral view of an *H. hohaiensis* cell directly fixed with OsO_4, b. dorsal view of an *H. hohaiensis* cell pretreated, c. apical pore complex (APC) without pretreatment, d. APC with pretreatment, and e. body scale. Scale bars 1 μm (a–d), and 0.3 μm (e).

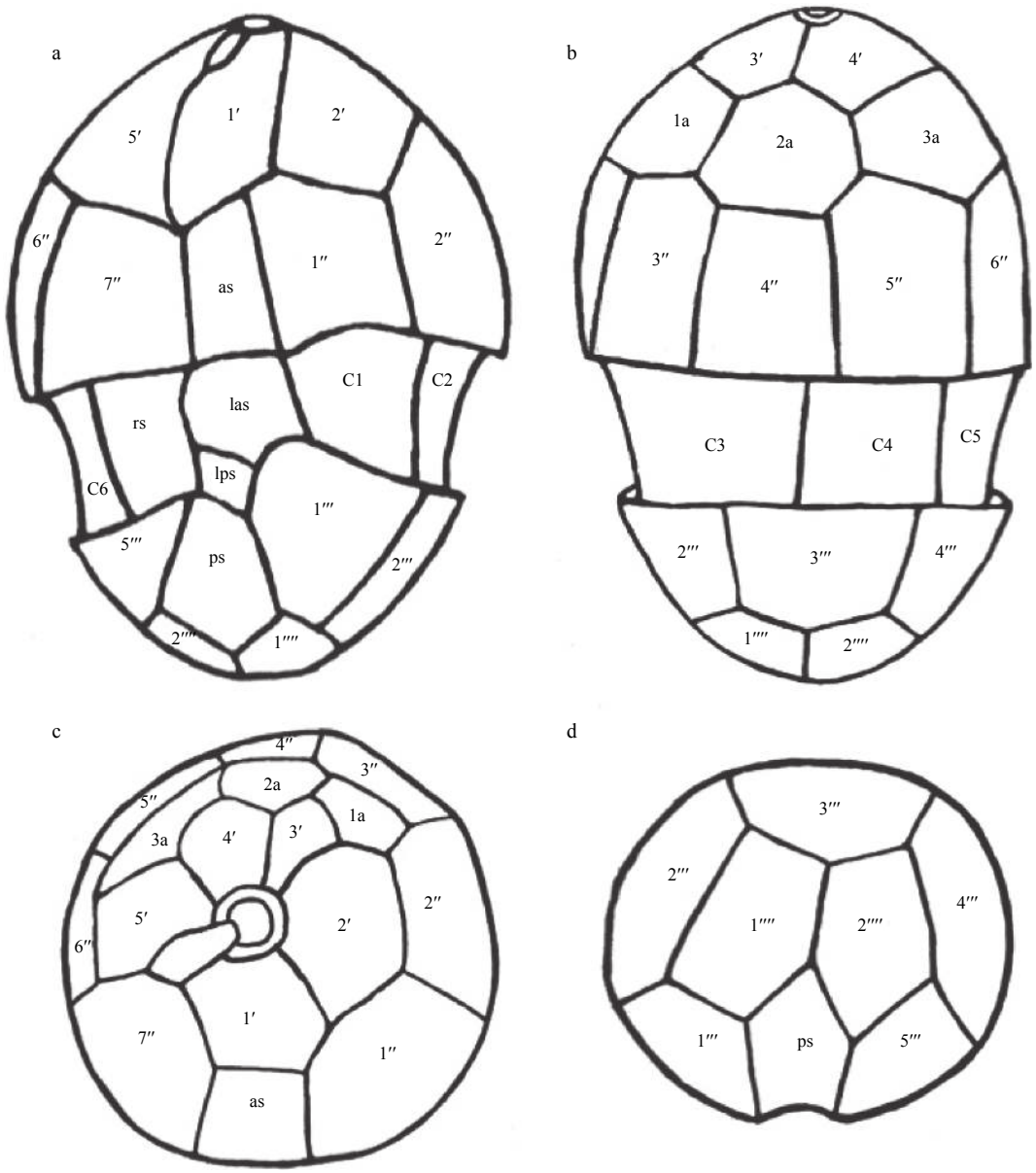

Fig. 3. Diagrams showing the thecal plate arragement of *Heterocapsa bohaiensis*. a. Ventral view, b. dorsal view, c. apical view, and d. antapical view.

quetra, *H. illdefina*, *H. arctica*, *H. lanceolata*, *H. minima*, *H. pygmaea*, respectively. Additional three single sequences (AB084098, KF240777 and AB445394) are genetically divergent with their sister taxa and formed three individual clades, standing for the species of *H. ovata*, *H. rotundata* and *H. huensis*, respectively. Interestingly, the sequences of FJ823556 and FJ823557, both designated as *H. niei*, fall into two distinct groups. The sequence FJ823556 (isolate UTEX 2722, Stern et al., 2012) is loosely clustered with AY499509 (*Heterocapsa* sp. GeoB 222, Gottschling et al., 2005) forming a sister clade to *H. pseudotriquetra* (AB084100), while the other (FJ823557, CS-36, Stern et al., 2012) is clustered with accessions FJ823557, JN020158 (*H. niei* identified in Iran; Attaran-Fariman and Javid, 2013) and JQ991005 (*H.* sp. CCMP424 collected from Australia) forming a strongly supported clade. Another well-supported clade (labeled as *Heterocapsa* sp. in Fig. 5) consists of four sequences (KX853194, KX853193, KX853191 and JQ9726681) with unknown identities, one (AB084093) designated as *H. pygmaea* (CCMP 1322), and one (JQ972674) as *Cachonina hallii* (CCMP 2770). The p-distances between *H. bohaiensis* and the closest lineages (*H. pygmaea*, *H.* sp., *H. huensis* and *H. niei*) are 0.059–0.087, which is comparable to the levels between *H. pygmaea* and *H. huensis* (0.099), *H. rotundata* and *H. lanceolata* (0.087), *H. lanceolata* and *H. arctica* (0.062), *H. minima* and *H. arctica* (0.095).

A total of 33 LSU sequences, including 28 retrieved from NCBI GenBank and 5 obtained in the study, form two reciprocally monophyletic clades (Fig. 6). *Heterocapsa bohaiensis* is located within the clade comprising *H. pygmeae* (EU165306), one undetermined strain (KT860562) isolated from the Mediterranean Sea, a few strains from the Gulf of Mexico, Florida of USA (FJ939577, EU165271 and EU165312), Iran (JN119844) and the Arabian Gulf of Turkey (KX853175, 77, 78, 87). The other clade with low bootstrapping support consists of the rest 18 sequences representing various strains from east coast of USA, the Baltic Sea, the Mediterranean Sea, and off the coasts of Australia, New Zealand, South Korea, etc.. Noticeably, both ITS and LSU sequences

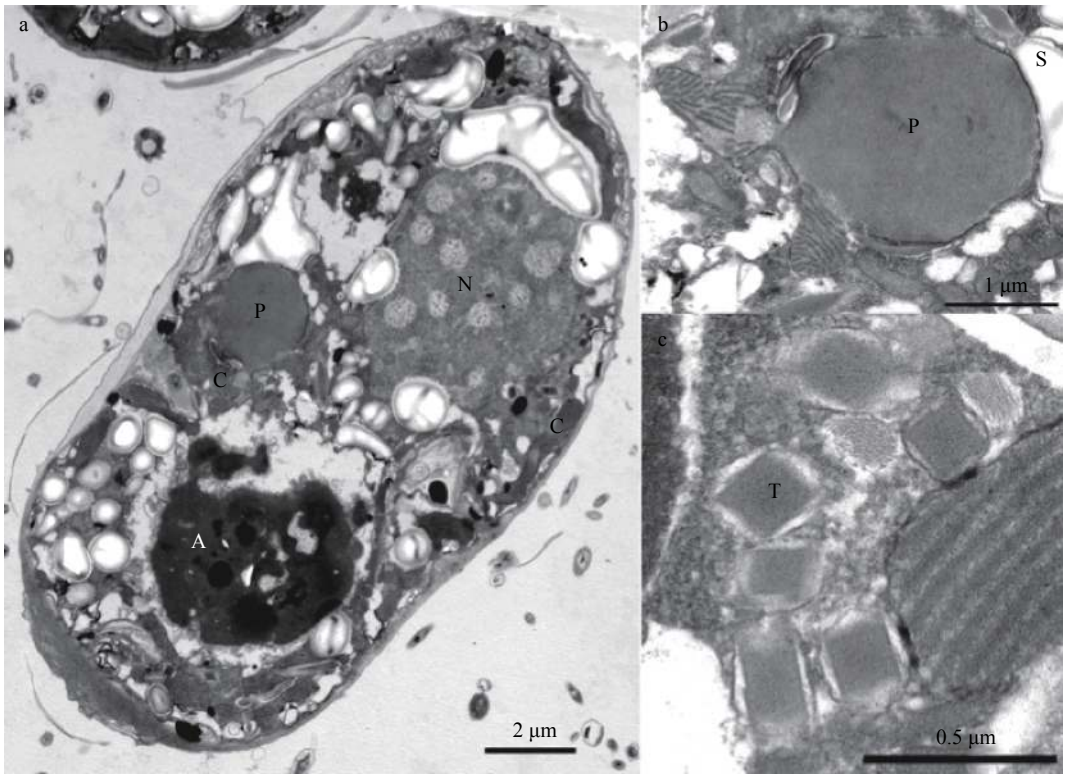

Fig. 4. TEM images of *Heterocapsa bohaiensis*. a. Longitudinal section of the cell, b. detailed structure of a pyrenoid, and c. details of trichocysts. N represents nucleus, P pyrenoid, C chloroplast, S starch sheath, T trichocyst, and A accumulation body.

of *Cachonina hallii* (JQ972674, AF033867) are clustered within the *Heterocapsa* clades and show little sequence divergence (<0.1%) with the neighbor sequences. This indicates a problematic taxonomic status of *Cachonina*, which requires more research.

4 Discussion

Taxonomy of *Heterocapsa* is difficult due to the small cell size and similarity of cellular morphology. The diagnostic characters included cell size and shape, shape and position of nucleus, number and position of pyrenoid, etc. (Iwataki et al., 2003; Iwataki, 2008). The newly found species described as *Heterocapsa bohaiensis* in this study is morphologically similar to *H. pygmaea* and *H. huensis*, both have ovoid cells (about 12–22 μm in length) and multiple pyrenoids. *H. bohaiensis*, however, posseses evidently larger epitheca compared to its hypotheca (Fig. 1), which is different from the almost equal size of epi-, hypotheca of *H. pygmaea* and *H. huensis* (Loeblich III et al., 1981; Iwataki et al., 2009). According to a review by Iwataki (2008), four species (*H. arctica*, *H. lanceolata*, *H. minima* and *H. rotundata*) reported to have a relatively larger epitheca, while they could be distinguished from *H. bohaiensis* by the cell size, shape and number of pyrenoids, as summarized in Table 1. Cells of *H. arctica* are quite elongated and were only observed in Arctic and the Baltic Sea where water temperatures are lower than 5°C (Horiguchi, 1997; Rintala et al., 2010). *Heterocapsa lanceolata* is featured by the unique lanceolate shapes (Horiguchi, 1997; Iwataki, 2008). Cells of *H. minima* and *H. rotundata* are relatively small (*H. minima*: 10–13 μm in length, mean=11.8 μm, Salas et al., 2014; *H. rotundata*: 9–12 μm, 10.5 μm, Hansen, 1995). All the four species have only one pyrenoid per cell (Iwataki, 2008; Rintala et al., 2010; Salas et al., 2014), which is distinct from *H. bohaiensis* (1–3 pyrenoids). The body scales have long been recognized present on the cell surface of *Heterocapsa*. Iwataki et al. (2004) compared and summarized the ultrastructure of body scales for 12 *Heterocapsa* species, and suggested its utility in the taxonomy of *Heterocapsa* spp. (Tamura et al., 2005; Iwataki et al., 2004). Although we failed to reveal the detailed structure through TEM, observation under SEM indicated that body scales of *H. bohaiensis* are outlined by a triangle basal plate (300–350 nm). The central upright and 9 peripheral spines are present on the basal plate (Fig. 2e). The body scale of *H. bohaiensis* is morphologically similar to that of *H. huensis* (Iwataki et al., 2009). The sequence phylogeny of both ITS and LSU further supports that *H. bohaiensis* is a novel species divergent from other described *Heterocapsa* spp.. Therefore, we classified it as a new species with the formal description as following:

Heterocapsa bohaiensis Xiao and Li sp. nov.

(Figs 1–4)

Cellula ellipsoideae, 9.9–16.5 μm longae, et 6.7–12.4 μm latae; ex epitheca grandi et hypotheca parva constans; tabulatio thecalis Po, cp, 5′, 3a, 7″, 6c, 5s, 5‴, 2⁗; Nucleus elongatus, in parte hypoconi ad mediam cellulae situs.; pyrenoides singularis ad tres, sphaerica, cum incursionibus cytoplasmatis et amylo cingente; haec squama structura similaris Heterocapsa huensis Iwataki.

Description: Cells are ellipsoidal, 9.9–16.5 μm long, 6.7–12.4 μm wide; epitheca larger than hypotheca; thecal plate arrangement po, cp, 5′, 3a, 7″, 6c, 5s, 5‴, 2⁗; Nucleus elongated and centrally located; pyrenoids one to three, spherical, surrounded by starch sheaths. The structure of body scale is similar to that of *Heterocapsa huensis* Iwataki.

Holotype: Fig. 1.

Type locality: Panjin (40°51′N, 121°46′E), Liaodong Bay, Bohai Sea, China.

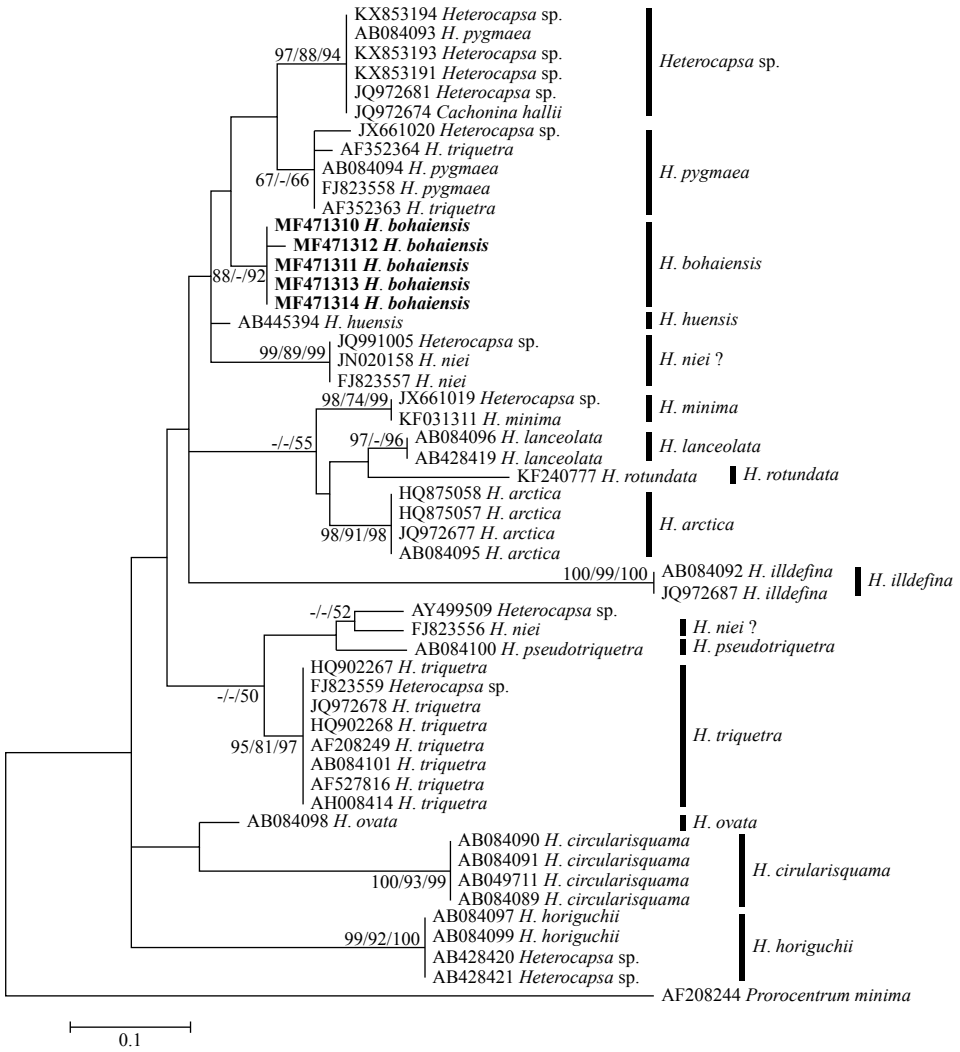

Fig. 5. Phylogenetic tree based on the ITS sequences. Numbers at the nodes are bootstrapping support values larger than 50% after 1 000 replicates in maximum-likelihood/neighbor-joining/maximum-parsimony analyses. *Prorocentrum minimum* (AF208244) is treated as the outgroup. The inferred clades are labeled. Bold fonts indicate sequences from this study.

Etymology: The species name refers to the Bohai Sea, the innermost gulf in northern China, where this species was firstly detected and isolated.

As described above, the morphology of APC varied when cells were treated using different methods. A protruded cover plate with radical rod-like decorations was consistently observed for the cells directly fixed with OsO_4. Similar protruded cover plate was observed in *H. pygmaea*, which was also fixed directly (Roberts et al., 1987). In contrast, there was a flat cover plate with no visible ornaments (Fig. 3) when cells of *H. bohaiensis* were pretreated. This morphology is consistent with the observation of *H. minima* (Salas et al., 2014), which were also pretreated similarly with ethanol. Apparently, the pretreatment procedure not only tripped off the outer layer of the dinoflagellate cells, but also could affect the appearance of the cover plate. Apical structure has been recognized as an important feature for the taxonomy of dinoflagellate (Dodge and Hermes, 1981; Toriumi and Dodge, 1993; Hoppenrath, 2017). Dodge and Hermes (1981) compared the apical pore structures of marine dinoflagellates, suggesting ornamentation and shape of cover plate may vary among taxa in certain genera. Unfortunately, there was few research reported the APC structure of *Heterocapsa*, even less the shape and ornament of the cover plate, so that we could not compare the APCs among *Heterocapsa* spp.. Given the distinct morphology of APCs among dinoflagellates (Dodge and Hermes, 1981; Toriumi and Dodge, 1993; Hoppenrath, 2017), more research is needed on the APCs of *Heterocapsa*, which may provide valuable information for intra- and inter-generic taxonomy studies.

So far, *H. bohaiensis* was only confirmed occurring in Panjin, the Bohai Sea, while the distribution of this species has not been fully investigated. Both ITS and LSU sequences reveal a monophyletic clade of *H. bohaiensis*, which differs from other *Heterocapsa* species. Based on the ITS sequences, the genetic distances between *H. bohaiensis* and its closely-related taxa *H. huensis*, *H. pygmaea* and *H. niei* are comparable to those among the sister taxa. The sequence phylogenetic analyses support the findings from the morphological data. A recent survey detected multiple ribosomal sequences with high identity (>99%) with SSU sequences of *H. bohaiensis* in the coastal water of Qinghuangdao, the Bohai Sea (data not shown), suggesting this species may distribute more broadly. In addition, blooms of *H. circularisquama* and *H. rotundata* were recorded in the Changjiang Estuary and

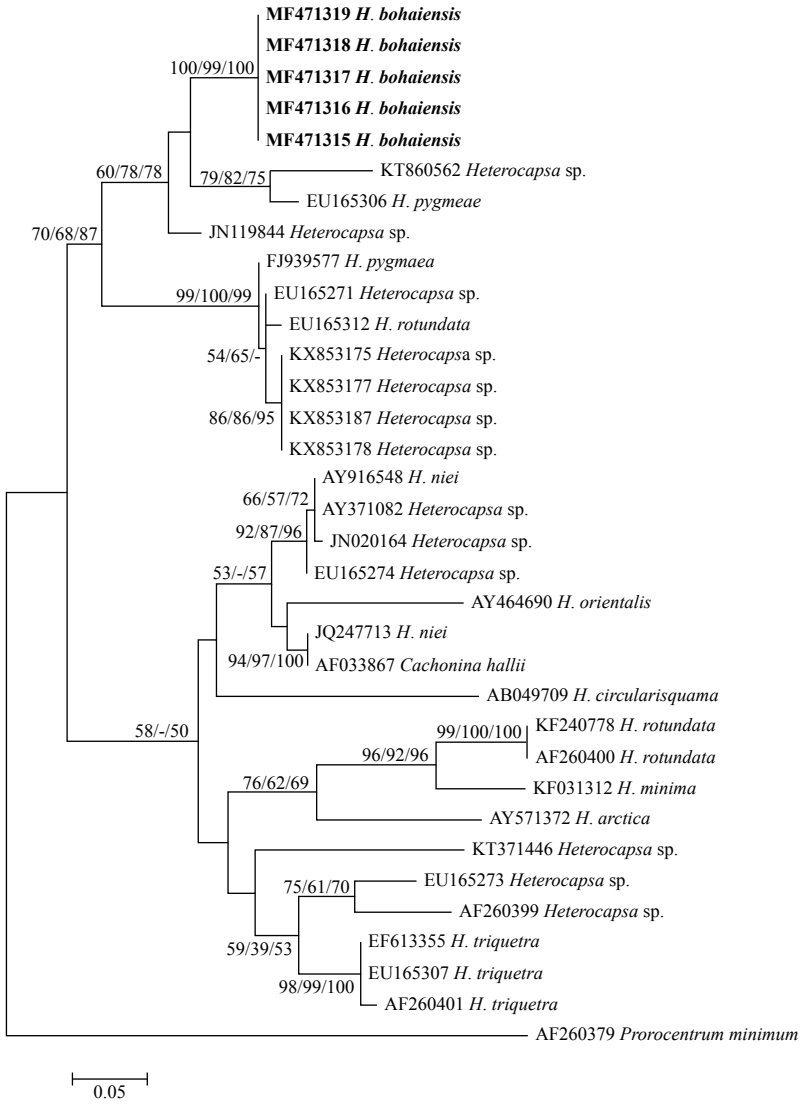

Fig. 6. Phylogenetic tree based on the LSU sequences. Numbers at the nodes are bootstrapping support values larger than 50% after 1 000 replicates in maximum-likelihood/neighbor-joining/maximum-parsimony analyses. *Prorocentrum minimum* (AF260379) is treated as the outgroup. Bold fonts indicate sequences from this study.

the coast of Qingdao in 2003, 2005 and 2008 (Wang et al., 2006), although no informative morphological and sequence data were provided. *Heterocapsa*-like sequences were also detected in the South China Sea as indicated by the deposited sequence accessions (KT389857, KT389866, KT389965, KT389969, KT389981 and KT389989). These sequences, however, were not included in the phylogenetic analyses of this study due to their un-alignability of multiple gaps and insertions in these sequences (data not shown). Nonetheless, all the information above implied probably a high species diversity of *Heterocapsa* spp. off the coasts of China, and further research is needed to elucidate the diversity and distribution of *Heterocapsa* spp. in China, including the newly found *H. bohaiensis*.

Although the bloom of *H. bohaiensis* in natural environment was not reported so far, significant mortalities of cultured *Penaeus japonicus* and *Eriocheir sinensis* lavae were observed in aquaculture ponds with *H. bohaiensis* cell abundance of 10^5 cells/mL (salinity 26). More laboratory testing suggested the harmful effects of *H. bohaiensis* on various marine organisms. Preliminary tests showed that high density (2×10^5 cells/mL) of *H. bohaiensis* could hamper the reproduction of rotifer *Brachionus plicatilis* (the major predator of cultured *Eriocheir sinensis* larvae) and metamorphosis of *Eriocheir sinensis* zoea (Yang et al., 2015). The mortality (cumulative mortality of 98.3%) of testing Manila clams (*Ruditapes philippinarum*) cultured with *H. bohaiensis* was significantly higher than that of the control groups (14.2% mortality, cultured with *Chlorella vulgaris*, Liu, 2016). In one toxicity screening, about 0.37 μg/g GTX4 (one component of PSP toxins) was detected from the *Ruditapes philippinarum* tissues fed with *H. bohaiensis* (Liu, 2016), while no toxins (e.g., PSP) were identified in the cultured *H. bohaiensis* cells after multiple trials (Yang et al., 2015; Gao Chunlei, personal communication). Furthermore, no tests have been conducted on the photosensitizing hemolytic toxin which has been recently isolated from *H. circularisquama* (Sato et al., 2002; Miyazaki et al., 2005), a red tide dinoflagellate harmful to bivalves in Japan (Horiguchi, 1995; Matsuyama et al., 1995, 1997, 2001). Apparently, more research is needed to clarify the harmful effect or toxicity of *H. bohaiensis*.

Table 1. Summary of morphological characters of 17 *Heterocapsa* species

Species[1)]	Size/μm	Cell shape	Nucleus	Pyrenoid	Body scale	
					Basal plate	Spines
H. arctica	12–30	ellipsoid, larger epitheca	elongated	1, below nucleus	350–400 nm, triangular	9 or 12
H. circularisquama	16–27	ellipsoid, epi- and hypotheca equal size	elongated	1, below nucleus	400 nm, circular	6
H. horiguchii	13–21	ellipsoid, epi- and hypotheca equal size	spherical, in anterior	1, below nucleus	310 nm, circular	6
H. huensis	13–25	ellipsoid, epi- and hypotheca equal size	spherical or oblong laterally	multiple, above nucleus	550 nm, triangular	9
H. illdefina	23–36	ellipsoid, epi- and hypotheca equal size	elongated	1, above nucleus	430 nm, triangular	9
H. minima	10–13	elongated ellipsoid, larger epitheca	oval to ellipsoid	1, above nucleus	400 nm, circular	6
H. niei	18–28	ellipsoid, epi- and hypotheca equal size	spherical, in posterior	1, above nucleus	300 nm, triangular	15
H. orientalis	18–35	spherical, epi- and hypotheca equal size	spherical, in posterior	1, above nucleus	300 nm, triangular	9
H. ovata	23–34	spherical, epi- and hypotheca equal size	spherical, in anterior	1, below nucleus	220 nm, triangular	6
H. psammophila	9–12	ellipsoid, epi- and hypotheca equal size	spherical, in posterior	1, above nucleus	230 nm, triangular	9
H. pygmaea	12–19	ellipsoid, epi- and hypotheca equal size	spherical, in posterior	multiple, above nucleus	400 nm, circular	6
H. rotundata	9–14	ellipsoid, larger epitheca	elongated	1, above nucleus	350 nm, triangular	9
H. triquetra	>15	hypotheca with an antapical horn, epi- and hypotheca equal size	spherical	1, below nucleus	250 nm, triangular	9
H. pseudotriquetra	18–28	spherical, epi- and hypotheca equal size	spherical, in anterior	1, below nucleus	240 nm, triangular	9
H. lanceolata	>15	lanceolate, antapical end slightly pointed, larger epitheca	elongated	1, above nucleus	500 nm, hexagonal	9
H. pacifica	>15	hypotheca with an antapical horn, epi- and hypotheca equal size	oval to ellipsoid	1, above nucleus	n.d.[2)]	n.d.
H. bohaiensis	9–17	ellipsoid, larger epitheca	oval to ellipsoid	1–3, above or aside nucleus	300–350 nm, triangular	9

Note: [1)] Three *Heterocapsa* species (*H. chattonii* P. H. Campbell, 1973, *H. kollmeriana* M. J. Swift et McLaughlin, 1970, and *H. umbilicata* Stein, 1883) are not included in this summary due to little data and doubtful validity of their taxonomy; [2)] n.d. means no data.

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