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# Species of the thecate dinoflagellate genus *Heterocapsa* (Dinophyceae; Heterocapsaceae) from the tropical Mexican Pacific, with special reference to two new records in the area and the ultrastructure of *Heterocapsa borneoensis*

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**Abstract**

**Background:** The thecate dinoflagellate genus *Heterocapsa* encompasses marine planktonic species, which have been poorly studied in the Mexican Pacific coasts.

**Question:** Is it possible to extend the knowledge of the genus *Heterocapsa* in the Mexican Pacific by means of different study techniques?

**Study sites**: Acapulco Bay, Guerrero, Mexico During November 2021 and March 2024.

**Methods:** Establishment of cultures, morphological analysis by light microscopy and molecular identification using the region D1-D2 (LSU rDNA).

**Results:** Five strains belonging to species of the genus *Heterocapsa* were established. The strains were identified as *H. borneoensis*, *H. iwatakii* and *Heterocapsa* sp., respectively. Each strain was morphologically characterized by light microscopy, highlighting characters such as the overall shape of the cell, proportion of the episome and hyposome, shape and position of the nucleus and number and position of the pyrenoids. Additionally, the morphology of the body scales and the ultrastructure of *H. borneoensis* were studied. Molecular identification of two species was confirmed by analysing the D1-D2 region (LSU rDNA). The phylogeny showed that sequences obtained in this study clustered with previous sequences of *H. borneoensis* and *H. iwatakii*.

**Conclusion:** This study confirmed the first record of *H. borneoensis* and *H. iwatakii* from the eastern Pacific Ocean.

**Key words:** *Heterocapsa*, morphology, phylogeny, ultrastructure.

**Resumen**

**Antecedentes:** El género *Heterocapsa* engloba especies planctónicas marinas, las cuales han sido poco estudiadas en las costas del Pacífico mexicano.

**Pregunta:** ¿Es posible ampliar el conocimiento del género *Heterocapsa* en el Pacífico mexicano mediante diferentes técnicas de estudio?

**Sitio de estudio:** Bahía de Acapulco, Guerrero, México Durante noviembre de 2021 y marzo de 2024.

**Métodos:** Establecimiento de cultivos, análisis morfológico por microscopía de luz e identificación molecular utilizando la región D1-D2 (LSU rDNA).

**Resultados:** Se establecieron cinco cepas pertenecientes a especies del género *Heterocapsa.* Las cepas se identificaron como *H. borneoensis*, *H. iwatakii* y *Heterocapsa* sp., respectivamente. Cada cepa se caracterizó morfológicamente por microscopía de luz, resaltando caracteres como la forma general de la célula, proporción del episoma e hiposoma, forma y posición del núcleo y número y posición de los pirenoides. Además, se estudió la morfología de las escamas corporales y la ultraestructura de *H. borneoensis*. La identificación molecular de dos especies se confirmó mediante el análisis de la región D1-D2 (LSU rDNA). La filogenia mostró que las secuencias obtenidas en este estudio se agruparon con secuencias previas de *H. borneoensis* y *H. iwatakii*.

**Conclusión:** Este estudio confirmó el primer registro de *H. borneoensis* y *H. iwatakii* del Océano Pacífico oriental.

**Palabras clave:** *Heterocapsa*, morfología, filogenia, ultraestructura.

Planktonic dinoflagellates are a very important component of the marine pelagic realm all over the world and they strongly contribute to the diversity, biomass and trophic strategies of the phytoplankton communities (Saldarriaga & Taylor 2017, Gómez 2020). This group is a relevant component in the flora of the Mexican Pacific and many reports of toxic and potentially toxic species in the area have been reported (Hernández-Becerril 1988, Hernández-Becerril *et al*. 2007, 2023, Hernández-Becerril & Vega-Juárez 2022).

*Heterocapsa* Stein is a genus that has been poorly studied on the Mexican coast. It includes thecate forms with a variable size (7.5-45 µm) and very delicate theca (Iwataki 2008), with 26 accepted species (Guiry & Guiry 2024), some of which can produce red tides or harmful algal blooms (HAB), such as *H. circularisquama* Horiguchi, *H. rotundata* (Lochmann) Hansen, *H. triquetra* (Ehrenberg) Stein., and *H. bohaiensis* Xiao & Li (Lee *et al*. 2019, Xiao *et al*. 2018). In the last six years a number of new species have been described, such as *H. bohaiensis*, *H. claromecoensis* Sunesen, Rodríguez, Tillmann & Sar, *H. busaensis* Choi & Kim, *H. philippinensis* Benico, Lum, Takahashi & Iwataki, *H. borneoensis* Teng, Hanifah, Leaw & Lim, *H. limii* Teng, Hanifah & Leaw, *H. iwatakii* Teng, Hanifah, Leaw & Lim (Sunesen *et al*. 2020, Benico *et al*. 2021, Choi & Kim 2021, Hanifah *et al*. 2022).

Potentially toxic species include *Heterocapsa circularisquama*, which has been responsible, since its original finding, in Uranoichi bay, Japan, for the mortality of different shellfish species, along the coasts of the western Pacific, causing considerable economic losses (Yamamoto & Tanaka 1990, Yoshida & Miyamoto 1995, Matsuyama *et al*. 1995, 1996, 1997, Matsuyama 2012). *H. bohaiensis* has shown lethal effects on crustacean larvae of *Penaeus japonicus* and *Eriocheir sinensis* in culture tanks (Yang *et al*. 2015, Xiao *et al*. 2018). More recently, Wu *et al*. (2022) isolated three strains of *Heterocapsa* from a HAB from East China Sea: *H. horiguchii* Iwataki, Takayama & Matsuoka, *Heterocapsa* cf. *niei* Morrill & Loeblich, and *Heterocapsa* cf. *pygamea* Lobelich III, Schmidt & Sherley, which showed toxic effects on brine shrimp and cell-density dependent hemolytic effects on rabbit erythrocytes. Additionally, *H. minima* Pomroy, *H. rotundata* Lohmann and *H. steinii* Tillmann, Gottschling, Hoppenrath, Kusber & Elbrächter are bloom-forming species, although they have not been associated to mortality of marine organisms (Iwataki 2008, Hanifah *et al*. 2022).

Characteristics such as tabulation are not useful to distinguish between species of the genus *Heterocapsa* (Benico *et al*. 2021). Some morphological characters for species identification are the shape of cells, which is stable in each species, proportion of epitheca and hypotheca, number and position of pyrenoids, shape and location of nucleus (Iwataki 2008). However, the most relevant morphological character is the structure of the external body scales, only visible by electron microscopy (Iwataki *et al.* 2003, Benico *et al*. 2021, Iwataki *et al*. 2004).

Studies of this genus in the eastern Pacific Ocean are scarce (Hernández-Becerril *et al*. 2010). In the Mexican Pacific, the state of knowledge of this genus is even more limited; however, three species have been recorded: *Heterocapsa niei* (Loeblich) Morrill et Loeblich., *H. steinii* of which no photographic record or sequences are available, and *H. pygmaea*, which has morphological record, but not molecular sequences (Okolodkov & Garate-Lizárraga 2006, Hernández-Becerril *et al*. 2010). On the other hand, there are limitations that make it difficult to identify these organisms, such as the case of fixed samples, where a low cell abundance is observed and the distinctive characteristics of each species can be modified. Additionally, depending on the fixative used, molecular identification may or may not be carried out.

In this study, five strains of *Heterocapsa* species, isolated from Acapulco Bay, tropical Mexican Pacific, were established and studied concerning their morphology by Light Microscopy (LM) and Transmission Electron Microscopy (TEM), including observations of the external body scales and ultrastructure of *Heterocapsa borneoensis*, as well molecular characterization of two species, inferred by LSU rDNA, which are considered as new records for the area: *Heterocapsa borneoensis* and *H. iwatakii*.

## Material and methods

*Sampling and culture establishment*. Surface water samples from Acapulco Bay were obtained with a Van Dorn bottle (3L), ("Parque de la Reina", 16° 50' 58.8" N, 99° 54' 1.6" W and "Naval", 16° 50' 18.33" N, 99° 51' 27.16"

W) in November 2021 and March 2024 (Figure 1). Living samples were placed in a dark cooler and transported to the laboratory. Each sample was first filtered through a 20 µm mesh and then passively (by gravity) concentrated using a 3 µm cellulose filter. Isolations of dinoflagellate cells were made with sterile capillary micropipettes using an inverted microscope (Invertoskop, ZEISS) and transferred to a 96 wells plate, previously filled with IMK and GSe culture medium (Daigo´s, Wako, Tokio, Japan). Strains were transferred to larger plates (24 wells), then scaled to 25 ml plastic flasks, to obtain a greater biomass for detailed studies. Culture conditions were temperature 24 °C, salinity 35 and a of 12:12 light/darkness photoperiod.

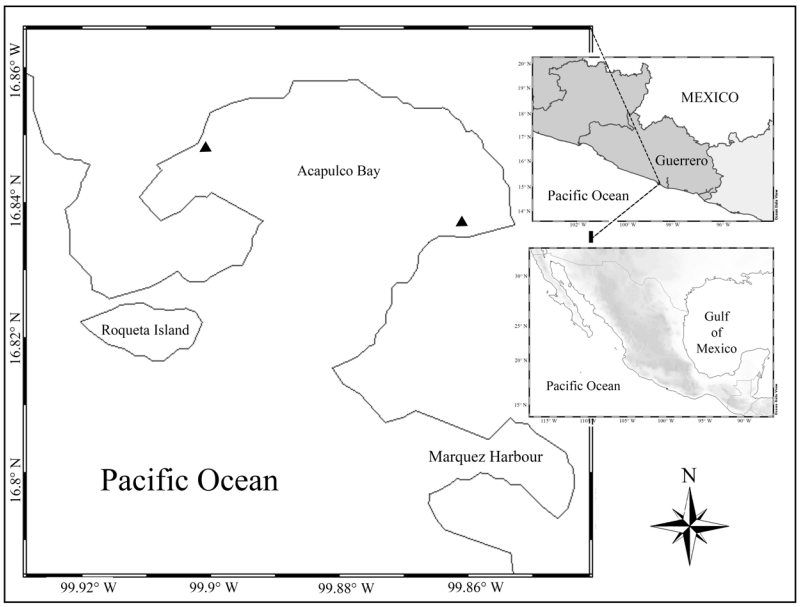


**Figure 1**. Map of the Area of study with sampling stations inside Acapulco Bay. The black dots represent sampling sites.

*Microscopy*. Observations of living cells were carried out with a bright field light microscope (Olympus BX40F-3, Tokyo, Japan and Leica DMLB, Wetzlar, Alemania), at 40X and 100X. Micrographs were taken with a camera Canon G10 and Nikon Z6 (Tokyo, Japan). Additionally, an epifluorescence microscope (Olympus IX71, Tokyo, Japan) (with 470-490 nm excitation and 510-550 nm emission) was used to observe some details such as the thecal tabulation, number and position of chloroplasts, and size and position of nucleus, with the aid of Calcofluor and 4', 6-diamidino-2 phenylindole dihydrochloride (DAPI) (Fisher Scientific, Madrid, Spain).

External body scales were observed by TEM (JEOL-JEM-1200) following the method described by Iwataki *et al.* (2002), which briefly consists in placing 0.05 mL of glutaraldehyde fixed culture on a 100-mesh copper grid, which was rinsed three times with distilled water, and then stained with Uranyl acetate (2 %) for two minutes.

*Ultrastructure*. TEM subsamples were prepared according to Benico *et al*. (2019). Cells were prefixed in 2 % (v/v) glutaraldehyde and post-fixed in 1% (w/v) $OsO_4$ for 8 h, followed by dehydration through an ethanol series. This was followed by embedding with low - viscosity resin, sectioning and staining of the sample with uranyl acetate and lead citrate. Observations were carried out with a JEOL-JEM-1200 at an accelerating voltage of 100 kV.

*DNA extraction*, *PCR and sequencing.* DNA extraction was carried out with a kit (Zymo Research, USA), following the manufacturer's instructions. For the amplification of the extracted DNA, a Master Mix (PROMEGA Go Taq® G2 Hot Start) was used following the PCR conditions of Escarcega-Bata *et al*. (2021). Single-cell PCR was performed directly, following the protocol described by Hernández-Rosas *et al*. (2018), with minor modifications in PCR conditions (Escarcega-Bata *et al*. 2021). Partial LSU rRNA gene sequences were amplified using primer pairs D1R-F and

D2C-R (Scholin & Anderson 1994). The D1-D2 region of LSU was selected due to the coverage of *Heterocapsa* species in GenBank database to compare our specimens. PCR amplificons were confirmed by electrophoresis with 1 % agarose gel for 20 min at 90 V; staining was performed with GelRed (Biotium). The visualization was carried out using a gel documentation system with UV light. PCR products were stored in -20 °C and sent to the Genomic Sequencing Laboratory, Instituto de Biología, Universidad Nacional Autónoma de México (Mexico City) for purification and sequencing. LSU sequences were determined for the two chains, using the referenced primers.

*Sequence alignment and phylogenetic analysis*. The sequences obtained were aligned in BioEdit v. 7.0.5 (Hall 1999) with those of a selected group of species of the genus *Heterocapsa* from the NCBI (GenBank). *Prorocentrum cordatum* (Ostenfeld) Dodge and *Prorocentrum micans* Ehrenberg were used as outgroup. Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian Inference (BI). ML analysis was performed using RAxML software (Stamatakis 2006). Node support was obtained for each branch from 1,000 bootstrap replications. BI analysis was performed using TOPALi version 2 software (Milne *et al*. 2009). The evolutionary model selected was GTR + G (general time reversible + gamma distribution) determined on the basis of the maximum likelihood ratio test implemented by TOPALi v. 2 software (Milne *et al*. 2009). Four strings of the Markov Monte Carlo chain (MCMC) were used, starting with a random tree and sampling the data every 1,000 iterations for $5 \times 10^6$ generations, discarding the first 25 % of the trees (heating the strings). Distance values per pair (*p*-distances) and distances corrected with Jukes Cantor model, were calculated using Mega v. 11 (Tamura *et al*. 2011).

## Results

*Morphology.* i) *Heterocapsa borneoensis* Teng, Hanifah, Leaw & Lim (Figures 2A-I, 3A-J). References: Hanifah *et al*. 2022, p. 4, Figures 1A-L, 2A-O.

Cells solitary and motile with a very thin and transparent theca. Usually, the cells are ovoid to elongate ovoid in shape, with episome and hyposome of nearly equal length, although sometimes the episome may be slightly larger and wider. Measurements are: 10-15 µm in length and 6-10 µm in width ($n$ = 50). The cingulum is wide and excavated, occupying about ¼ of the total length. The episome may be ovoid, showing a very slight or no protuberance in the apical end, at the apical pore complex level, whereas the hyposome is more rounded (Figures 2A-I). The nucleus is subspherical to elongate and is situated near the centre of the cell, more toward the hyposome (Figures 2C, G, H, 3A, F). Photosynthetic, with several (4-8) golden to brown elongate plate-like chloroplasts, and two to four large pyrenoids per cell (Figures 2A-F, 3A-D, F). Various red bodies were also found in the cells (Figures 2A-B, D-F). Few details of the thecal tabulation were seen in epifluorescence microscopy, showing some main plates in ventral view, such as the apical plates 1′, 2′ and 5’, the precingular plates 1″ and 7″, the cingular plates $C_1$, $C_6$, the postcingular plates 1‴, 5 ‴, the antapical plates 1⁗ and 2 ⁗, and two sulcal plates Sa and Sp (Figure 2I).

The general outline of the cells was also observed by TEM, with subequal epi- and hyposome, and wide and excavated cingulum (Figures 3A, F). Observations of the nucleus confirmed its position in the cell, slightly eccentric, toward the hyposome, and elongate shape (Figures 3A, F). Elongate plate-like chloroplasts were observed, distributed in the periphery of the cell, and showing some details of the thylakoids (Figures 3A-D, F). Observation of pyrenoids was especially interesting, as they were large and connected to the chloroplasts, always surrounded by almost continuous starch sheaths, whereas tubular invaginations were absent (Figures 3B-C, F); cells showed trichocysts (Figure 3E).

Body scales showed a pattern with a roundish to hexagonal basal plate finely perforated, ornamented by a central spine and six peripheral uprights, interconnected by radial and peripheral bars; the connections between peripheral uprights form three radial pentagons. Perforation of the basal plate was highly variable, with coarse to finer perforations were also present in the basal plate. The diameter of the scales ranged from 320 to 440 nm (Figures 3G-J).

ii) *Heterocapsa iwatakii* Teng, Hanifah, Leaw & Lim (Figures 4A-D). References: Hanifah *et al*. 2022, p. 10, Figures 5A-E, 6A-K.

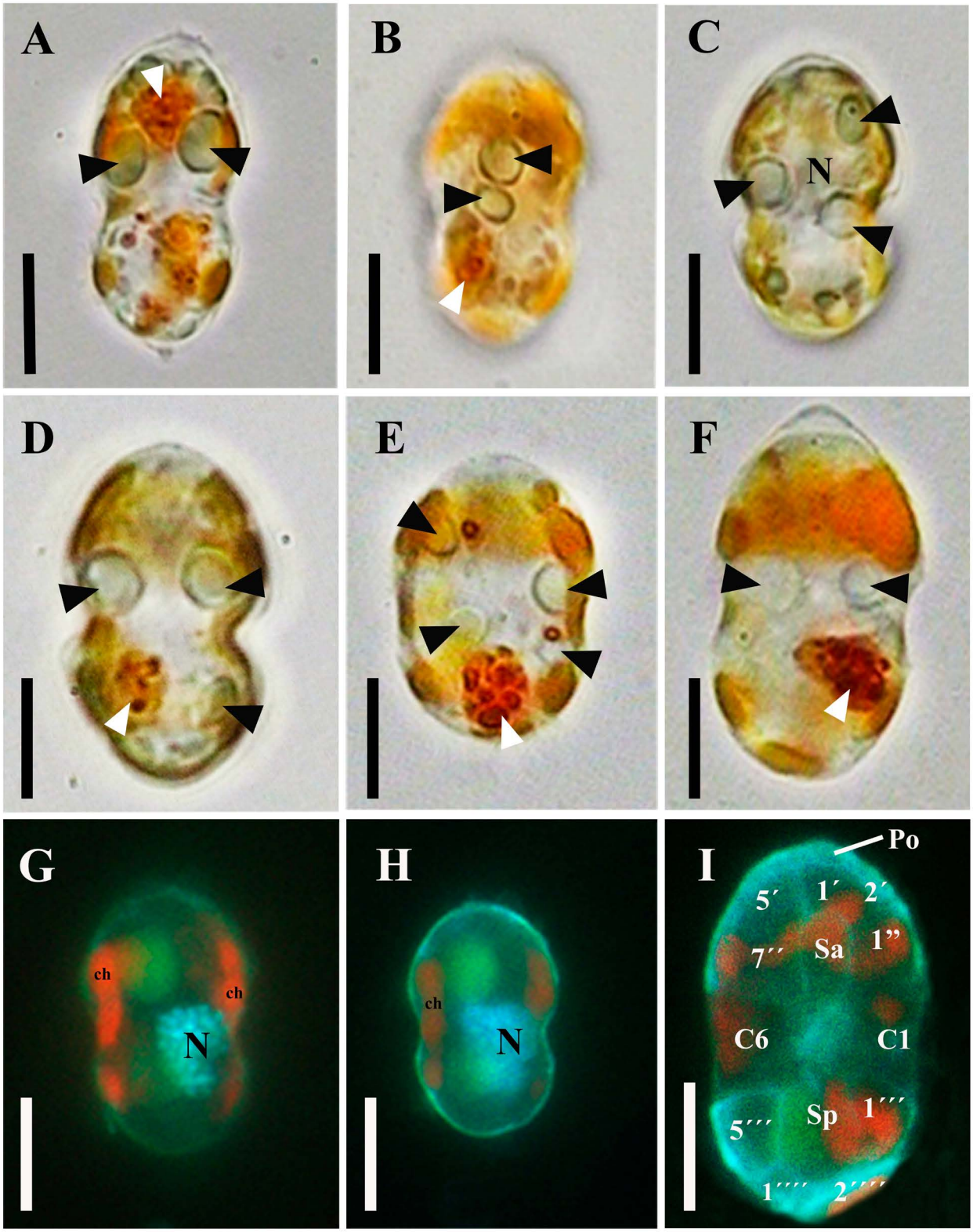


**Figure 2** A-I. *Heterocapsa borneoensis*, light and epifluorescence microscopy. A-F. Live cells showing the general outline of the species; pyrenoids (black arrows) and red bodies (white arrows) are shown. G-I. Cells stained with calcofluor and DAPI showing nucleus (N), chloroplast (Cl) and some thecal plates in ventral view. Scale bar: A-I = 5 µm.

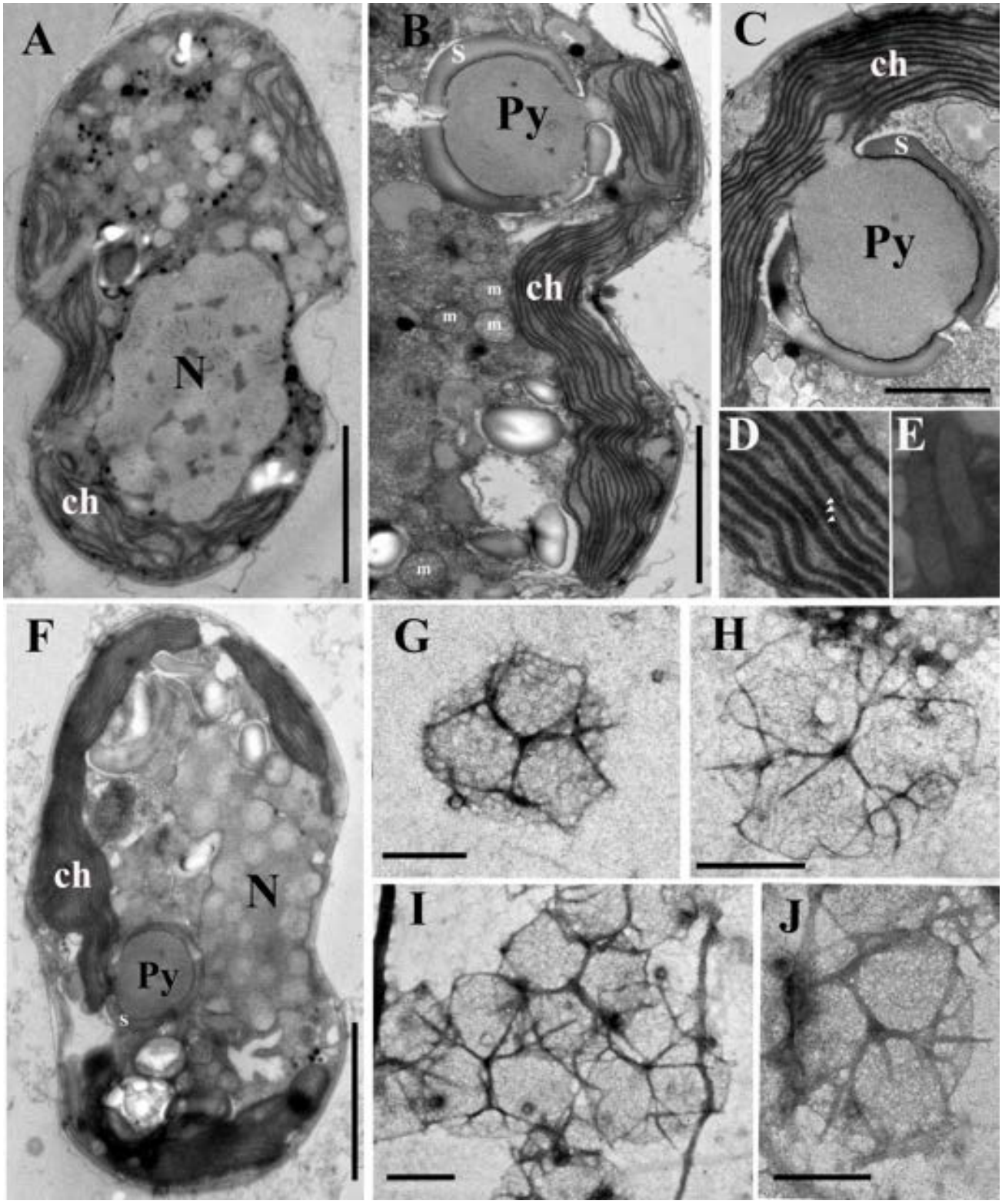


**Figure 3** A-J**.** *Heterocapsa borneoensis*, transmission electron microscopy. A, B, F. Longitudinal section with nucleus (N), Chloroplast (ch), Pyrenoids (Py), Starch grain (S) and mithocondria (m). C. Details of pyrenoid connection with chloroplast. D. Details of the chloroplast with thylakoids in stacks of three (white arrows). E. Trychocyst in longitudinal section. G-J. Body scale morphology. Scale bar: A, F = 3 µm; B = 2 µm; C = 1 µm; G-J = 200 nm.

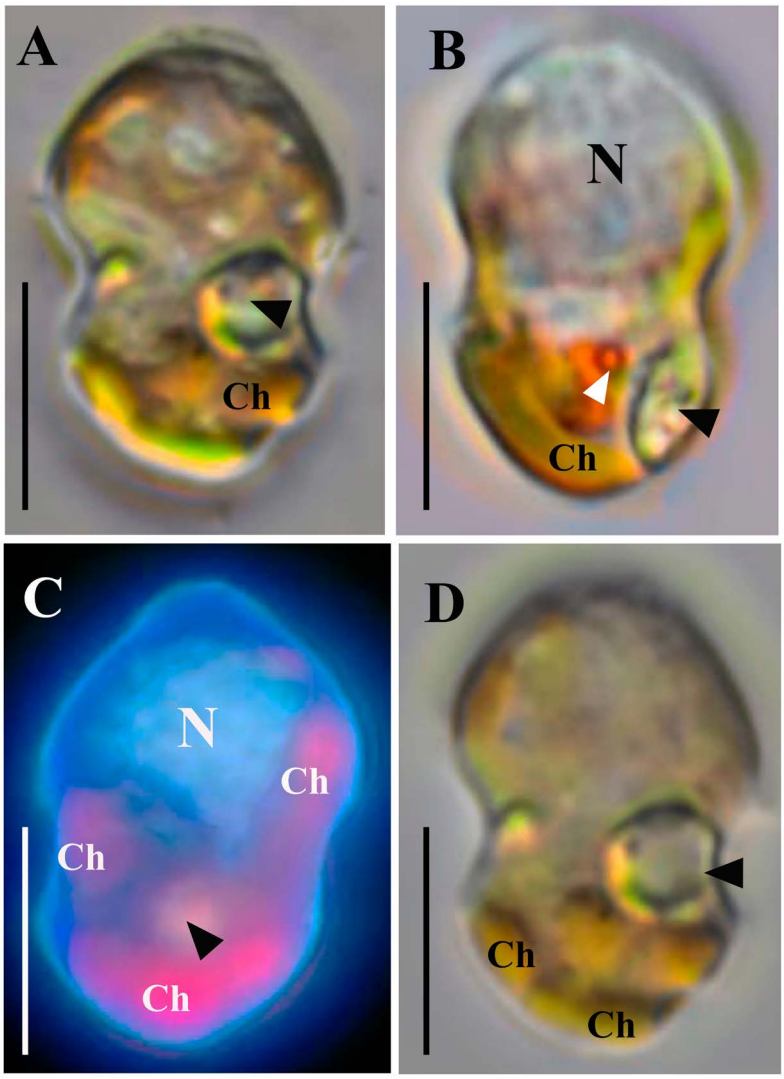


**Figure 4.** A-D. *Heterocapsa iwatakii,* light and epifluorescence microscopy. A, B, D. Live cells showing the general outline of the species; pyrenoids (black arrows), red bodies (white arrows) and chloroplast (Ch) are shown. B and C. Spherical nucleus (N) in episome. Scale bar: A-D = 5 µm.

Solitary and motile cells, with an ellipsoid to elongated ellipsoid shape, the episome is larger than the hyposome, the episome is conical and the hyposome is hemispheric (Figures 4A-D). Measurements are: 8-12 µm in length and 6-8 µm in width ($n$ = 50). Deep and excavated cingulum. The nucleus is spherical and located in the episome (Figures 4B-C). The species is photosynthetic, with several golden and parietal chloroplasts (Figures 4A-B, D). Cells have a single spherical pyrenoid, located in the central part of the cell or in the hyposome (Figures 4A-D). The presence of red bodies was recorded (Figure 4B), however, this was not found in all cells, only in those that were growing exponentially.

iii) *Heterocapsa* sp. (Figures 5A-F). Cells solitary and motile, ellipsoidal to ovoid in shape, with a slightly conical episome and hemispheric hyposome (Figures 5A-F). Measurements are: 15-18 µm in length and 9-12 µm in width ($n$ = 50). The episome is slightly larger than the hyposome and the cingulum is excavated. Photosynthetic species with several golden and parietal chloroplasts (Figures 5A-B). One to three spherical pyrenoids located in the episome (Figure 5A-C). Nucleus ellipsoidal to ovoid centrally located, between the episome and the hyposome (Figures 5C, F). Occasional presence of red bodies, variable in shape and size (Figure 5 B).

**Table 1**. Morphological comparision betwen *Heterocapsa borneoensis*, *Heterocapsa iwatakii* and related species.

| **Morphological features** | ***Heterocapsa borneoensis**** | ***Heterocapsa iwatakii**** | ***Heterocapsa borneoensis***[a] | ***Heterocapsa bohaiensis***[a] | ***Heterocapsa limii***[a] | ***Heterocapsa iwatakii***[a] | ***Heterocapsa pygmea***[bc] | ***Heterocapsa busaensis***[d] |
|---|---|---|---|---|---|---|---|---|
| Lenght (µm) | 10-15 | 8-12 | 10-19 | 10-17 | 11-19 | 7-12 | 12-19 | 17-27 |
| Width (µm) | 6-10 | 6-8 | 6-15 | 6-12 | 6-13 | 5-8 | 8-9 | 10-16 |
| Cell shape | Ellipsoidal, episome and hyposome in equal size | Ellipsoidal, episome larger than hyposome | Ellipsoidal episome and hyposome in equal size | Ellipsoidal, larger epi-some | Ellipsoidal, episome and hypo-some in equal size | Elongated, ellipsoidal, larger epi-some | Ellipsoidal, episome and hyposome in equal size | Ellipsoidal, with conical episome and round hyposome, larger epi-some |
| Nucleus | Ovoid to ellipsoid, located in hyposome and central part | Espherical located in episome | Ovoid to ellipsoid, located in hyposome | Elongated ovoid toel-lipsoid, located across the episome and hyposome | Prolate spherical or spherical, located in hyposome. | Spherical, located in episome. | Spherical, located in hyposome. | Elongated, located in episome on the left side of the cell |
| Pyrenoids | 2-4 | 1 | 2-4 | 4 | 2 | 1 | 2 | 1 |
| Red body | Present | Present | Present | Present | Absent | Present | Absent | Present |

*This study; [a]Hanifah *et al*. (2022), [b]Iwataki *et al*. (2004), [c]Iwataki (2008), [d]Choi & Kim (2021).

The ventral view of the species observed with calcofluor showed some thecal plates, confirming the arrangement of them, the presence of five apical plates and seven precingular plates and the prominence of the anterior and posterior sulcal plates (Sa and Sp) (Figures 5D-E).

*Phylogenetic analysis*. Four partial LSU rDNA sequences were obtained for *Heterocapsa. borneoensis* and *H. iwatakii*. The final LSU alignment included 47 sequences, with a length of 900 bp. Phylogenetic reconstruction from the LSU dataset (with maximum phylogenetic support ML = 100 %, BI = 1), was made with sequences of species of the genus *Heterocapsa.* The resulting phylogeny showed three main clades (I-III, Figure 6). Clade I, with high phylogenetic support (ML = 78 %, BI = 0.84). Clade II, with low phylogenetic support (ML = 78 %, BI = 0.60), and Clade III, with low phylogenetic support (ML = 0.59 %, BI = 0.61). The sequences generated in this study were grouped in two subclades of Clade III (A and B). Subclade A, with high support (ML = 99 %, BI = 0.97) where sequences of *H. iwatakii* from the Acapulco Bay and those of the type species clustered together. Subclade B (ML = 81 %, BI = 0.80) clustered *H. borneoensis* together with several clades belonging to *H. bohaiensis*, *H. limii*, *H. pygmaea* and *Heterocapsa* sp. sequences.

The genetic distances found between sequences of *H. borneoensis* and species of the genus *Heterocapsa* can be seen in Table 2, where uncorrected distances and corrected distances are shown. Distance values between the sequences of *H. borneoensis*, were not included because the variation between them was close to zero (0.001 %), the same as in *H. iwatakii*.

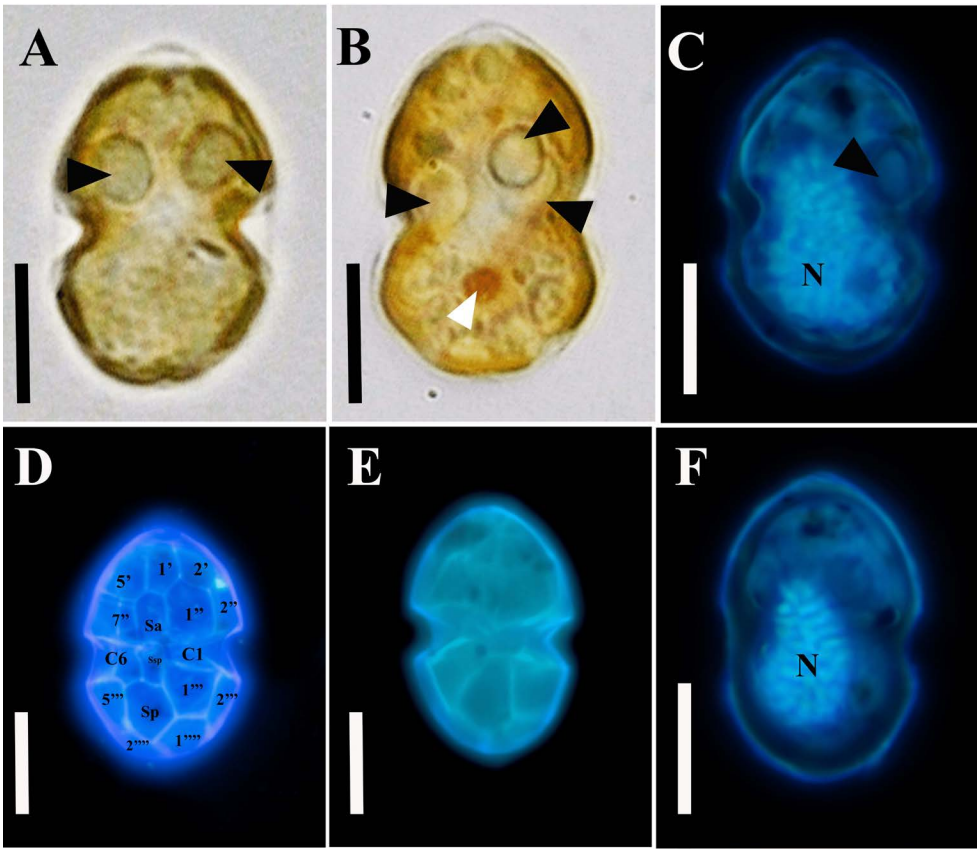


**Figure 5**. A-F *Heterocapsa* sp. A-B. Light microscopy. General morphology of live cells with pyrenoids (black arrows) and red bodies (white arrows). C, F. Fixed cells in epifluorescence with elongated nucleus (N). D-E. Plate pattern in ventral view. Scale bar: A-F = 5 µm.

## Discussion

*Morphology.* Four strains corresponding to the thecate dinoflagellate species *Heterocapsa borneoensis* (Strains: C9HBAB-C10HBAB), *H. iwatakii* (Strain: FPMBA01) and *Heterocapsa* sp. (Strain: B8HSPAB), were obtained from the Acapulco Bay, Mexico, which represents the first record of the species *Heterocapsa borneoensis* and *H. iwatakii* since their original description from the coast of Sarawak, Borneo, Malaysia (Hanifah *et al*. 2022). The general and detailed morphology of these two species, studied by light microscopy, and in the case of *H. borneoensis* also by electron microscopy (*e.g*, the body scales), described in this study completely agree with the morphological characters described by Hanifah *et al*. (2022).

Morphologically, *H. borneoensis* is similar to *H. bohaiensis*, overlapping in size, (10-15/ 10-17 µm long and 6-10/ 6-12 µm wide, respectively), and the main difference between the two species is the shape of the hyposome (Figures 1A-B; Xiao *et al*. 2018). However, in the strain established by Hanifah *et al*. (2022), the ellipsoidal shape of *H. bohaniensis* strongly resembled *H. borneoensis*. In this study, the shape and size match the original description of *H. borneoensis*. The presence of multiple pyrenoids is another shared character, although in the description of Xiao *et al*. (2018), *H. bohaiensis* showed 1 to 3 pyrenoids, whereas Hanifah *et al*. (2022) reported 1 to 4 pyrenoids, the same number of pyrenoids reported for *H. borneoensis* (2-4 pyrenoids).

Other species show multiple pyrenoids, as *H. pygmaea* (2 pyrenoids; Iwataki *et al.* 2004, 2008, Hernández-Becerril *et al*. 2010), which differs from *H. borneoensis* in the displacement of the cingulum and the shape and location of the nucleus, *H. limii* (2 pyrenoids; Hanifah *et al*. 2022), which has a close relationship with *H. borneoensis.* However, the differences are the shape of the nucleus, the invariable presence of 2 pyrenoids and the absence of red bodies in *H. limii*. Finally, *H. huensis* Iwataki & Matsuok has 1-3 pyrenoids, but differs in the general form of the cell (Iwataki *et al*. 2009, Hanifah *et al*. 2022).

**Table 2.** Genetic divergence (percentage) between sequences of genus *Heterocapsa*. Uncorrected distances are shown below the diagonal, and Jukes Cantor distance values are located above the diagonal. GenBank accession numbers are shown below each taxon, bold highlights correspond to the sequences generated in this study.

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1. *Heterocapsa borneoensis* OQ946954** | - | 4.7 | 0 | 4.7 | 6.4 | 3.9 | 2.3 | 3.9 | 3.9 | 7.3 | 4.7 | 2.3 | 4.7 | 4.7 | 2.3 | 7.3 | 1 | 4.7 | 4.7 | 1.9 |
| **2. *Heterocapsa iwatakii* PP911072** | 4.4 | - | 4.7 | 0 | 7.3 | 7.3 | 5.6 | 3.9 | 7.3 | 9.1 | 6.4 | 5.6 | 6.4 | 4.7 | 5.6 | 9.1 | 4.7 | 8.2 | 8.2 | 4.7 |
| 3. *Heterocapsa borneoensis* MZ774028 | 0 | 4.4 | - | 4.7 | 6.4 | 3.9 | 2.3 | 3.9 | 3.9 | 7.3 | 4.7 | 2.3 | 4.7 | 4.7 | 2.3 | 7.3 | 1.4 | 4.7 | 4.7 | 1.9 |
| 4. *Heterocapsa iwatakii* MZ774038 | 4.4 | 0 | 4.4 | - | 7.3 | 7.3 | 5.6 | 3.9 | 7.3 | 9.1 | 6.4 | 5.6 | 6.4 | 4.7 | 5.6 | 9.1 | 4.7 | 8.2 | 8.2 | 4.7 |
| 5. *Heterocapsa limii* MZ774031 | 5.9 | 6.6 | 5.9 | 6.6 | - | 7.3 | 9.1 | 8.2 | 7.3 | 9.1 | 6.4 | 9.1 | 4.7 | 6.4 | 9.1 | 9.1 | 6.4 | 5.6 | 5.6 | 6.4 |
| 6. *Heterocapsa huensis* MZ774047 | 3.7 | 6.6 | 3.7 | 6.6 | 6.6 | - | 3.1 | 4.7 | 3.1 | 4.7 | 3.9 | 3.1 | 3.9 | 2.3 | 3.1 | 4.7 | 3.9 | 5.6 | 5.6 | 3.8 |
| 7. *Heterocapsa rotundata* MZ774039 | 2.2 | 5.1 | 2.2 | 5.1 | 8.1 | 2.9 | - | 1.5 | 4.7 | 6.4 | 5.6 | 0.0 | 5.6 | 3.9 | 0.0 | 6.4 | 2.3 | 7.3 | 7.3 | 2.2 |
| 8. *Heterocapsa minima* MW626888 | 3.7 | 3.7 | 3.7 | 3.7 | 7.4 | 4.4 | 1.4 | - | 6.4 | 8.2 | 5.6 | 1.5 | 5.6 | 3.9 | 1.5 | 8.2 | 3.9 | 7.3 | 7.3 | 3.8 |
| 9. *Heterocapsa busanensis* MW003725 | 3.7 | 6.6 | 3.7 | 6.6 | 6.6 | 2.9 | 4.4 | 5.9 | - | 4.7 | 3.9 | 4.7 | 2.3 | 3.9 | 4.7 | 4.7 | 3.9 | 5.6 | 5.6 | 3.8 |
| 10. *Heterocapsa claromecoensis* MN509451 | 6.6 | 8.1 | 6.6 | 8.1 | 8.0 | 4.4 | 5.9 | 7.4 | 4.4 | - | 7.3 | 6.4 | 5.6 | 3.9 | 6.4 | 0.0 | 7.3 | 8.2 | 8.2 | 7.3 |
| 11. *Heterocapsa pseudotriquetra* MF423367 | 4.4 | 5.9 | 4.4 | 5.9 | 5.9 | 3.7 | 5.1 | 5.1 | 3.7 | 6.6 | - | 5.6 | 3.1 | 3.1 | 5.6 | 7.3 | 4.7 | 4.7 | 4.7 | 4.7 |
| 12. *Heterocapsa lanceolata* OQ383719 | 2.2 | 5.1 | 2.2 | 5.1 | 8.1 | 2.9 | 0 | 1.4 | 4.4 | 5.9 | 5.1 | - | 5.6 | 3.9 | 0.0 | 6.4 | 2.3 | 7.3 | 7.3 | 2.2 |
| 13. *Heterocapsa horiguchii* OP970968 | 4.4 | 5.9 | 4.4 | 5.9 | 4.4 | 3.7 | 5.1 | 5.1 | 2.2 | 5.1 | 2.9 | 5.1 | - | 3.1 | 5.6 | 5.6 | 4.7 | 3.9 | 3.9 | 4.7 |
| 14. *Heterocapsa triquetra* MK660144 | 4.4 | 4.4 | 4.4 | 4.4 | 5.9 | 2.2 | 3.7 | 3.7 | 3.7 | 3.7 | 2.9 | 3.7 | 2.9 | - | 3.9 | 3.9 | 4.7 | 4.7 | 4.7 | 4.7 |
| 15. *Heterocapsa arctica* AY571372 | 2.2 | 5.1 | 2.2 | 5.1 | 8.1 | 2.9 | 0 | 1.4 | 4.4 | 5.9 | 5.1 | 0.0 | 5.1 | 3.7 | - | 6.4 | 2.3 | 7.3 | 7.3 | 2.2 |
| 16. *Heterocapsa orientalis* AY464690 | 6.6 | 8.1 | 6.6 | 8.1 | 8.1 | 4.4 | 5.9 | 7.4 | 4.4 | 0 | 6.6 | 5.9 | 5.1 | 3.7 | 5.9 | - | 7.3 | 8.2 | 8.2 | 7.3 |
| 17. *Heterocapsa pygmaea* LC621347 | 1.1 | 4.4 | 1.1 | 4.4 | 5.9 | 3.7 | 2.2 | 3.7 | 3.7 | 6.6 | 4.4 | 2.2 | 4.4 | 4.4 | 2.2 | 6.6 | - | 4.7 | 4.7 | 2.3 |
| 18. *Heterocapsa pygmaea* OR194153 | 4.4 | 7.4 | 4.4 | 7.4 | 5.1 | 5.1 | 6.6 | 6.6 | 5.1 | 7.4 | 4.4 | 6.6 | 3.7 | 4.4 | 6.6 | 7.4 | 4.4 | - | 0 | 4.7 |
| 19. *Heterocapsa* sp. KX853175 | 4 | 7.4 | 4.4 | 7.4 | 5.1 | 5.1 | 6.6 | 6.6 | 5.1 | 7.4 | 4.4 | 6.6 | 3.7 | 4.4 | 6.6 | 7.4 | 4.4 | 0 | - | 4.7 |
| 20. *Heterocapsa bohaiensis* MZ774040 | 1.5 | 4.4 | 1.5 | 4.4 | 5.9 | 3.7 | 2.2 | 3.7 | 3.7 | 6.6 | 4.4 | 2.2 | 4.4 | 4.4 | 2.2 | 6.6 | 2 | 4.4 | 4.4 | - |

Iwataki (2008) provided a general identification guide, in which the first character to consider is the proportions of the episome and hyposome, as well as the size and shape of the cell. However, the main character that allows differentiation between species of the genus *Heterocapsa* is the morphology of external body scales (Iwataki 2008). Considering this fact, we found the difference between *H. borneoensis* and *H. bohaiensis* to be in the morphology of the scales, which differ in size (320-460 nm and 300-350 nm), outline (triangular and circular), crests (six and three), spines and peripheral arches respectively. Additionally, the identification of *H. borneoensis* is also supported by LSU rDNA phylogenetic inference.

The observations of the ultrastructure of *H. borneoensis* made in this study coincided with the ultrastructure of other species of the genus: peripheral chloroplasts with pyrenoid connection, pyrenoid surrounded by starch grain, mitochondria, nucleus with condensed chromosomes and the presence of trichocysts (Horiguchi 1997, Iwataki *et al*. 2003, Rintala *et al*. 2010, Tillmann *et al*. 2017, Xiao *et al*. 2018, Sunesen *et al*. 2020, Benico *et al*. 2021). In terms of ultrastructure, within the genus *Heterocapsa*, some species have tubular invaginations in the pyrenoid matrix, which have an unclear function (Iwataki *et al*. 2003, Benico *et al*. 2021). In *H. borneoensis*, a relatively small species, tubular invaginations are absent, as these structures are thought to occur only in large species (Iwataki *et al*. 2003).

*Heterocapsa iwatakii* is a small species of the genus, Hanifah *et al*. (2022) described in detail the characteristics that distinguish it from those morphologically similar species, *H. horiguchii*, *H. busaensis* and *H. ovata*, in which, despite sharing characters, *H. iwatakii* can be distinguished due to its size (7-12 um length and 5-8 um width) as it does not overlap with any of the above-mentioned species. The strain from Acapulco Bay coincides with the original description of Hanifah *et al*. (2022): episome larger than hyposome, round nucleus located in the episome, a pyrenoid located in the hyposome and the presence of red bodies. Unfortunately, it was not possible to observe the morphology of the scales, but its identification could be corroborated by the LSU rDNA phylogenetic inference.

The strain (B8HSPBA) was studied only by LM, using live and fixed material. This strain is morphologically close to *H. borneoensis*, *H. bohaiensis* and *H. limii*, all these species overlap in size and overall cell shape. *Heterocapsa* sp. has a higher resemblance to *H. bohaiensis* (Figures 5A-B; Hanifah *et al*. 2022) since both species have a larger episome. The next character to consider is the number and position of the pyrenoids, in which the strain showed 2 pyrenoids located in the episome and only one cell was observed with 3 (Figure 5B). Following the identification guide provided by Hanifah *et al*. 2022 (Figures 12A-B), only five species have multiple pyrenoids: *H. pygmaea*, which has a spherical nucleus and has no red bodies (Loeblich *et al*. 1981, Morril & Loeblich 1981, Iwataki *et al*. 2004, Hernández-Becerril *et al*. 2010), *H. limii*, which differs from *Heterocapsa* sp. by the presence of a third pyrenoid and the presence of red bodies (Hanifah *et al*. 2022), *H. huensis*, which is larger and differs in cell shape (Loeblich *et al*. 1981, Hanifah *et al*. 2022), whereas *H. borneoensis* and *H. bohaiensis* have 1 to 4 pyrenoids. *Heterocapsa* sp. differs from *H. borneoensis* in the proportion of episome to hyposome and in the position and shape of the nucleus, which is found in the episome in *H. borneoensis* and is centrally located in *Heterocapsa* sp. (Figure 5F), although in some cells it can be seen covering more area of the hyposome (Figure 5C). The plate pattern of *Heterocapsa* sp. coincides with *H. borneoensis* (Hanifah *et al*. 2022). The morphological resemblance and overlap of characters from *Heterocapsa* sp. with other species of the genus have made identification difficult and unfortunately it has not been possible to obtain sequences or observe scale morphology. This overlap of characters can be explained by the deterioration or modification of the morphology of *Heterocapsa* sp. in fixed samples.

*Molecular phylogeny.* Following both the morphological observations and the molecular phylogeny obtained (LSU rDNA sequences), the occurrence of the species *H. borneoensis* and *H. iwatakii* in the eastern Pacific coast was confirmed. The phylogeny of the analyzed data set showed that the four sequences obtained in this study (OQ946954-OQ946955 and PP911072-PP911073), were grouped into separate clades with sequences from *H. borneoensis* and *H. iwatakii* respectively, collected from coasts of Malaysia (Hanifah *et al*. 2022). However, the topology of the LSU tree in Hanifah *et al*. (2022) *H. borneoensis*, showed to be paraphyletic. In our tree, the clade of *H. borneoensis* (subclade B, Figure 6) is well differentiated with maximum support and in the same way as in Hanifah *et al*. (2022), the sequence GBHetBol (LC621347, Genebank access number) of Benico *et al*. (2021) clustered together with the

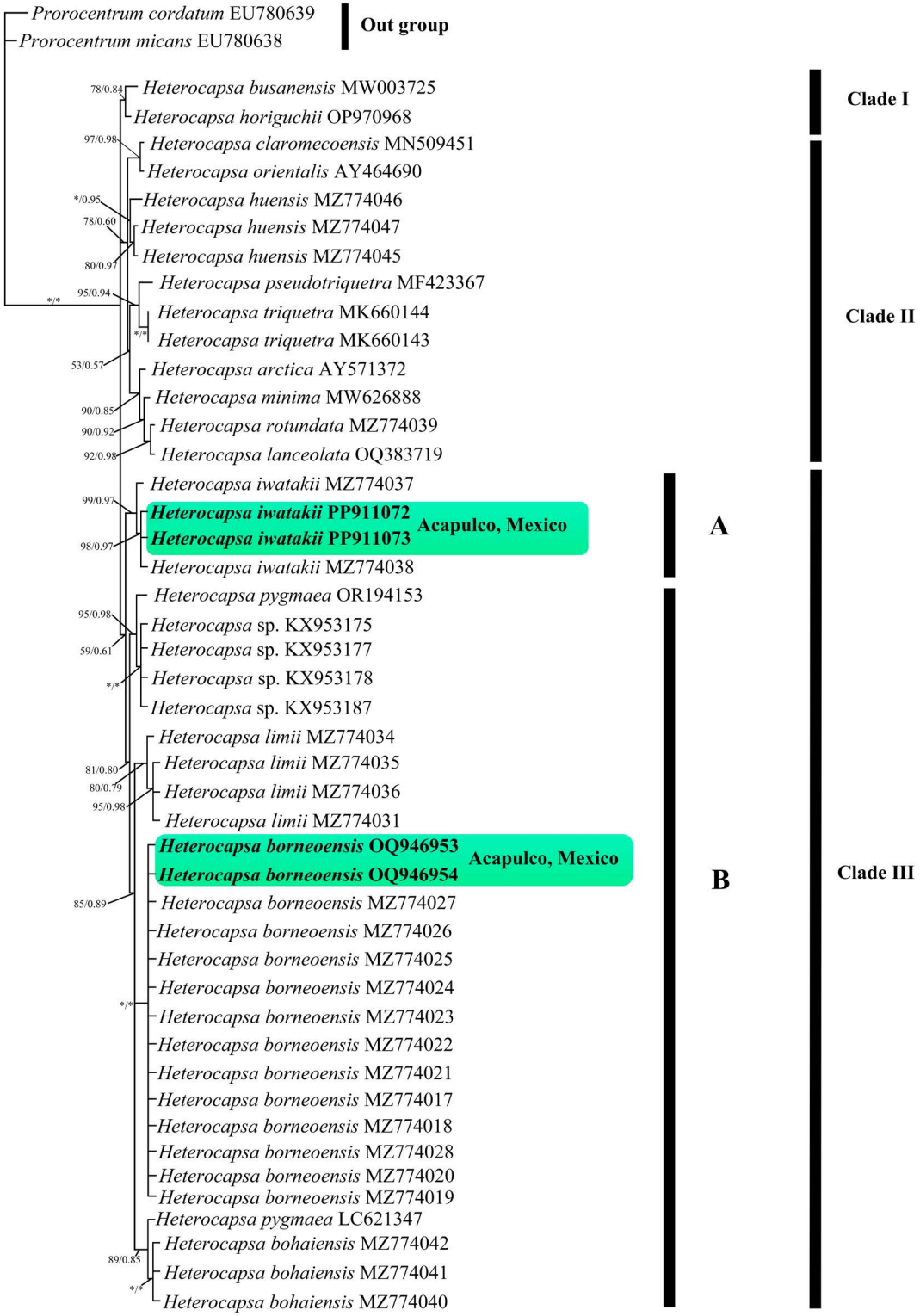


**Figure 6.** Bayesian inference (BI) and Maximum likelihood (ML) tree based on LSU rADN sequences from species of *Heterocapsa*. BI (right) followed by ML bootstrap values (left) on branches. Asterisk indicated full support (ML = 100 %, BI= 1). Sequences generated in this study are highlighted in a green box.

sequences of *H. bohaiensis*. The divergence of the sequences of *H. borneoensis* from this study compared to those provided by Hanifah *et al*. (2022) is zero, implying the same species.

In *H. iwatakii*, our sequences are grouped together with those provided by Hanifah *et al*. (2022) (subclade A, Figure 6), and the divergence of the sequences compared to those of the type species is zero. Therefore we interpreted the correspondence of one and the same species. The position of *H. iwatakii* in the LSU tree is provisional as shown by Hanifah *et al*. (2022), given the lack of sequences of closely related species, this information is sufficient to determine the identity of the species using the LSU marker.

*Distribution*. The genus *Heterocapsa* has a wide global distribution (Hanifah *et al*. 2022, Tillmann *et al*. 2017, Xiao *et al*. 2018, Sunesen *et al*. 2020), some potentially toxic species such as *H. rotundata* and *H. triquetra*, usually have a greater range of distribution (Lee *et al*. 2019). In the case of the western Pacific there is a greater number of studies, given the ecological importance of this genus due to the potential of some species to form harmful algal blooms (HAB's) (*e.g*, *H. circularisquama*, *H. bohaiensis*; Matsuyama *et al*. 1995, Iwataki 2008, Choi & Kim 2021). Studies of the genus in Mexican coasts are scarce, and it may be justified that there are no records of algal blooms that have been harmful or generated economic losses. For *H. borneoensis* and *H. iwatakii*, current information is limited, so it is not possible to determine its global distribution, because only two records are known yet: Borneo, Malaysia and Acapulco, Mexico (Hanifah *et al*. 2022 and this study).

The confirmation of the species *Heterocapsa borneoensis* and *H. iwatakii* in the eastern Pacific Ocean by morphological and molecular identification suggests a wide range of distribution of this potentially harmful genus, which has been little studied along this coast. The information provided in this study contributes to the current knowledge of this genus, and it is necessary to maintain monitoring and research on these species, so that future analyses can provide a more accurate estimate of the diversity in Mexican waters.

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