

Newly recorded unarmored dinoflagellates in the family Kareniaceae (Gymnodiniales, Dinophyceae) in brackish and coastal waters of Korea

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Abstract: Unarmored dinoflagellates, in the family Kareniaceae, include harmful or toxic bloom-forming species, which are associated with massive fish kills and mortalities of marine organisms worldwide. The occurrence and distribution of the toxigenic species in the family Kareniaceae were investigated in the brackish and coastal waters of Korea between July 2018 and October 2020. During the survey, we collected seven newly recorded species; *Karenia papilionacea*, *Karlodinium digitatum*, *Karl. veneficum*, *Karl. zhuanum*, *Takayama acrotricha*, *T. helix*, and *T. tasmanica*. A total of fifteen strains of the seven taxa were successfully established as clonal cultures and examined using LM, SEM, and molecular phylogeny inferred from LSU rDNA sequences. Herein, we present the taxonomic information, morphological features, and molecular phylogenetic positions of the unrecorded dinoflagellate species collected from Korean coastal waters.

Keywords: first record, Kareniaceae, LSU rDNA, molecular phylogeny, morphology

INTRODUCTION

The unarmored dinoflagellate genus *Gymnodinium* F. Stein has been recognized to be assemblage of unrelated species for many years. Based on combination of LSU rDNA sequences, chloroplast pigment composition, and outline of apical groove, Daugbjerg *et al.* (2000) proposed that the heterogeneous genus *Gymnodinium sensu lato* divided into four genera: *Gymnodinium sensu stricto* with a horse-shoe-shaped apical groove, *Akashiwo* G.Hansen & Moestrup with a clockwise spiral apical groove, *Karenia* G.Hansen & Moestrup with a straight apical groove, and *Karlodinium* J.Larsen with a short straight apical groove and a ventral pore. The former two genera *Gymnodinium* and *Akashiwo* possess peridinin in the chloroplast as a main accessory pig-

ment as most typical photosynthetic dinoflagellates contain, whereas the latter two genera *Karenia* and *Karlodinium* contain fucoxanthin or its derivatives as their major carotenoids. Later, new fucoxanthin-containing genus *Takayama* de Salas, Bolch, Botes & Hallegraeff with a sigmoid apical groove was erected and three unarmored dinoflagellates described by Larsen (1994, 1996), *Gymnodinium pulchelum* Larsen, *Gyrodinium acrotrichum* Larsen, and *G. cladochroma* Larsen, having a sigmoid apical groove and fucoxanthin, were transferred to the genus (de Salas *et al.* 2003). Those three fucoxanthin-containing genera formed a well-supported monophyletic clade and constituted a separate evolutionary lineage and the new family Kareniaceae comprising those genera was proposed (Bergholtz *et al.* 2005).

Some species in the family Kareniaceae such as *Karenia*

brevis, *K. mikimotoi*, and *Takayama pulchella* have been reported to form harmful algal blooms, causing massive fish kills and fisheries damages worldwide (Steidinger *et al.* 1998; Pierce and Henry 2008; Brand *et al.* 2012). In Asia, harmful red tides by *Karlodinium australe* and *Karl. digitatum* were reported from Johor Strait between Malaysia and Singapore in 2014 and 2015 and from China in 2019, respectively (Lim *et al.* 2014; Leong *et al.* 2015; Cen *et al.* 2019; Sakamoto *et al.* 2021). In Korea, two *Karenia* species have been reported to cause massive harmful blooms, leading to economic losses: one by *K. mikimotoi* from Jinhae Bay in 1992 with economic loss of 5.5 million USD and the other by *Karenia* sp. (as *Gymnodinium* sp.) from Tongyeong in August 1992 with damage of approx. 1.82 M USD, respectively (Sakamoto *et al.* 2021).

In this study, we reported unrecorded dinoflagellate species in the family Kareniaceae from brackish and coastal

waters of Korea and provided their morphological features and molecular phylogenetic relationships inferred from LSU rDNA sequences.

MATERIALS AND METHODS

1. Sampling and culture collection

Plankton samples were collected by vertical and horizontal sampling using a 20 µm-mesh plankton net in brackish and coastal waters of Korea from July 2018 to October 2020 (Table 1). Water temperature and salinity were measured *in situ* using a YSI instrument (YSI Inc., OH, USA). The collected samples were kept at 20°C until microscopic observation. Single cells were individually isolated with a capillary pipette under an inverted microscope (Axio Vert. A1; Zeiss, Hallbergmoos, Germany) and washed several

Table 1. List of newly recorded unarmored dinoflagellates in the family Kareniaceae in brackish and coastal waters of Korea

Species	Strain	Date	Locality (Latitude, Longitude)	Temp. (°C)	Sal.	GenBank accession
<i>Karenia papilionacea</i>	KpLomme01	Jul 24, 2018	Yongho, Busan (35°08'00"N, 129°06'55"E)	26.1	32.8	MZ358888
<i>Karlodinium digitatum</i>	KdLomme01	Oct 14, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	20.7	31.2	MZ358887
<i>Karlodinium digitatum</i>	KdLomme02	Oct 16, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	20.0	31.6	MZ358883
<i>Karlodinium digitatum</i>	KdLomme03	Oct 16, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	20.0	31.6	MZ358886
<i>Karlodinium digitatum</i>	KdLomme04	Oct 16, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	20.0	31.6	MZ358884
<i>Karlodinium digitatum</i>	KdLomme05	Oct 16, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	20.0	31.6	MZ358885
<i>Karlodinium veneficum</i>	KvLomme01	May 01, 2020	Jangchun, Changwon (35°07'41"N, 128°41'54"E)	17.0	32.8	MZ358877
<i>Karlodinium veneficum</i>	KvLomme02	May 01, 2020	Jindong, Gosung (35°11'19"N, 128°33'58"E)	18.8	32.7	MZ358875
<i>Karlodinium veneficum</i>	KvLomme03	May 13, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	15.3	36.0	MZ358876
<i>Karlodinium veneficum</i>	KvLomme04	June 10, 2020	Hakri, Gijang (35°15'30"N, 129°14'48"E)	19.1	34.1	MZ358874
<i>Karlodinium zhouanum</i>	KzLomme01	Oct 25, 2019	Yongho, Busan (35°08'00"N, 129°06'55"E)	21.1	31.8	MZ358878
<i>Takayama achrotrocha</i>	TaLomme01	Oct 14, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	20.7	31.2	MZ358881
<i>Takayama helix</i>	ThLomme01	Sep 23, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	21.5	31.0	MZ358882
<i>Takayama tasmanica</i>	TtLomme01	Sep 12, 2020	Jangchun, Changwon (35°07'41"N, 128°41'54"E)	24.3	26.1	MZ358880
<i>Takayama tasmanica</i>	TtLomme02	Oct 14, 2020	Yongho, Busan (35°08'00"N, 129°06'55"E)	20.7	31.2	MZ358879

times in a series of drops of 0.2 μm filtered and sterilized seawater. The specimen was transferred to a 96-well plate filled with ambient filtered seawater. Clonal cultures were maintained in F/2 medium (Guillard and Ryther 1962) at 20°C, with a 14 : 10 light-dark cycle under 100–120 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A pair of gelatin-embedded slides prepared from each clonal culture strain after final conc. 1% glutaraldehyde fixation were deposited at the Nakdonggang National Institute of Biological Resources, Korea (NNIBRPR 17563–NNIBRPR17576).

2. Light microscopy

Live cells were observed with Axio Imager A2 (Zeiss) light microscope, equipped with differential interference illumination. Light micrographs were taken at $\times 1000$ using a AxioCam HRc (Zeiss) photomicrographic system equipped with the microscope. Glutaraldehyde-fixed cells (1% final concentration) were examined to determine the shape and location of nuclei after staining with 4'-6-diamidino-2-phenylindole (DAPI: 0.1 $\mu\text{g mL}^{-1}$ final concentration) under epifluorescence microscope with ultraviolet light (excitation of 360 nm and emission of 460 nm).

3. Scanning electron microscopy

For scanning electron microscopy, 2 mL of culture was fixed with an equal volume of glutaraldehyde (2% final concentration) in 0.2 M cacodylate buffer at pH 7.4 at 4°C for 1 h. Fixed cells were rinsed twice in distilled water for 1 h and dehydrated using ethanol concentration gradient (30, 50, 70, 80, 95, and two changes of 100% ethanol) soaking for 12 min at each step. Dehydrated samples were critical point dried in liquid CO_2 using an HCP-2 (Hitachi, Tokyo, Japan). Finally, the samples were coated with gold-palladium for 3 min and examined under a MIRA3 FE-SEM (Tescan Korea, Seoul, Korea).

4. DNA extraction, PCR, Sequencing

Genomic DNA was extracted from 1 mL of exponentially growing culture strains using Chelex extraction method (Kim and Park 2014). The purity and quantity were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, DE, USA). Approximately 1050 bp of the LSU rDNA were amplified using the primers D1R and D3B (Nunn *et al.* 1996). PCR was conducted using a C1000 Touch thermal cycler with a commercially available PCR premix (Accu-Power PCR PreMix; BIONEER, Daejeon,

Korea). The thermal cycle condition of PCR was as follows: initial 95°C for 3 min, followed by 39 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were purified using ExoSAP-IT™ Express according to manufacturer's instructions, confirmed by 1% agarose gel electrophoresis. The purified PCR products were sequenced in an ABI model 3730xl DNA Analyzer (Applied Biosystems; Foster City, CA), using the same primers used for PCR in conjunction with a Big-Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). ContigExpress (Vector NTI v. 10.1; Invitrogen, Grand Island, NY) was used to edited out low quality and to assemble the sequence reads and the complete sequences were deposited in GenBank (Table 1).

5. Alignments and phylogenetic analyses

A total of 76 sequences of Kareniacean species including Korean strains and two genera *Gymnodinium* and *Gyrodinium* species as outgroup were aligned with MEGA (Kumar *et al.* 2018) and unambiguously aligned regions (1002 positions) were applied for further phylogenetic analyses. Maximum likelihood analysis was performed with RAxML 8.0 (Stamatakis 2014) using the GTRGAMMA evolution model and rapid bootstrapping of 2,000 replicates. Bayesian analysis was conducted using MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003) running four simultaneous Monte Carlo Markov Chains for 2,000,000 generations and sampling every 100 generations, following a burn in of 100,000 generations.

RESULTS AND DISCUSSION

1. Taxonomic summary

Three genera and seven species in the family Kareniaceae were newly recorded in brackish and coastal waters of Korea (Table 1). The newly recorded species *Karenia papilionacea*, *Karlodinium digitatum*, *Karl. veneficum*, *Karl. zhouanum*, *Takayama acrotrocha*, *T. helix*, and *T. tasmanica* were described based on their morphological characteristics and the LSU rDNA sequences obtained to perform molecular phylogenetic analyses.

Class Dinophyceae Fritsch
 Order Gymnodiniales Apstein
 Family Kareniaceae Bergholtz, Daugbjerg, Moestrup &

Fernández-Tejedor
Genus *Karenia* G.Hansen & Moestrup

***Karenia papilionacea* A.J.Haywood & K.A.Steindinger
(Fig. 1A–E)**

Synonym: *Gymnodinium breve* C.C.Davis (Haywood *et al.* 1996).

Reference: Haywood *et al.* 2004 (Figs. 2e–h and 3a–f).

Specimen examined. Table 1.

Cells are 38.5–44.9 µm long and 22.4–22.9 µm wide and dorsally convex and ventrally concave. The epitheca has a prominent apical carina and a short straight apical groove with extended one third of the dorsal epitheca. The cingulum is displaced by the cingulum width. The nucleus is spherical and located in the left hyposome. Chloroplasts are few and large or several and small, located peripherally, yellow-green in color.

Distribution. Hawke's Bay in New Zealand (Haywood *et al.* 2004), Uranouchi inlet, Nomi Inlet, Sukumo Bay, Saiki Bay, Yatsushiro Sea in western Japan (Yamaguchi *et al.* 2016).

Site of collection. Specimens were collected from Yongho Bay of Busan, Republic of Korea (35°08'00"N, 129°06'55"E) on July 24, 2018.

Voucher slide. NNIBRPR17563–NNIBRPR17564.

Genus *Karlodinium* J.Larsen

***Karlodinium digitatum* Gu, Chan & Lu (Fig. 1F–J)**

Basionym: *Karenia digitata* Z.B.Yang, H.Takayama, K.Matsuoka & I.J.Hodgkiss.

Reference: Cen *et al.* 2019 (Figs. 2 and 3).

Specimen examined. Table 1.

Cells are 16.1–22.7 µm long and 14.5–20.2 µm wide and globular or oval in shape. A straight apical groove extends from the dorsal apex to the ventral epicone. The structure of the curve knot is present in hypocone. The sulcus invades the epicone slightly as a small finger-like intrusion. The cingulum is descended with a displacement of approx. 25% of cell length. The nucleus is spherical and located in the posterior. Chloroplast is spherical in shape and distributed irregularly in cells.

Distribution. Western coastal waters of Japan (Yang *et al.* 2000), Silver Mine Bay in Hong Kong (Lee *et al.* 2011), Fujian Province in China (Cen *et al.* 2019).

Site of collection. Specimens were collected from Yongho Bay of Busan, Republic of Korea (35°08'00"N, 129°06'55"E) on October 16, 2020.

Voucher slide. NNIBRPR17565–NNIBRPR17566.

***Karlodinium veneficum* (Ballantine) J.Larsen (Fig. 1K–O)**

Basionym: *Gymnodinium veneficum* (Ballantine 1956) p. 469.

Synonym: *Karlodinium micrum* (Leadbeater & Dodge) J.Larsen, *Gymnodinium galatheanum* Braarud sensu Kite & Dodge, *Gymnodinium micrum* (Leadbeater & Dodge) Loeblich III, *Gyrodinium galatheanum* (Braarud) Taylor sensu Taylor.

References: Bergholtz *et al.* 2005 (Figs. 13 and 14).

Specimen examined. Table 1.

Cells are 10.6–14.7 µm long and 6.4–10.6 µm wide and ovoid in shape. The cingulum is displaced about two cingulum width. The sulcus extends to the left epicone. A short straight apical groove is visible. The nucleus is spherical and located centrally or in the posterior. Four chloroplasts are located peripherally and two in epicone and two in the hypocone.

Distribution. Australia, New Caledonia, New Zealand, Asia (China and Japan, and Qatar), Europe (England, France, Germany, Italy, and Norway), and USA.

Site of collection. Specimens were collected at Jangchun of Changwon (35°07'41"N, 128°41'54"E) and Jindong of Gosung-gun (35°11'19"N, 128°33'58"E) on May 01, 2020 and Yongho Bay of Busan (35°08'00"N, 129°06'55"E) on May 13, 2020 and Hakri harbor of Gijang-gun (35°15'30"N, 129°14'48"E) on June 10, 2020, respectively.

Voucher slide. NNIBRPR17567–NNIBRPR17568.

***Karlodinium zhouanum* Z.Luo & H.Gu (Fig. 1P–T)**

References: Luo *et al.* 2018 (Figs. 1–4).

Specimen examined. Table 1.

Cells are 10.4–14.9 µm long and 7.7–11.9 µm wide and ovoid with a conical epicone and hemispherical hypocone in shape. The cingulum is descended with a displacement of 25% of total cell length. The sulcus intruded into the epicone as a fingerlike projection. A straight apical groove extends from above the sulcal intrusion to the dorsal epicone. The nucleus is spherical and situated in the epicone. Six chloroplasts are peripherally located in the cell with internal

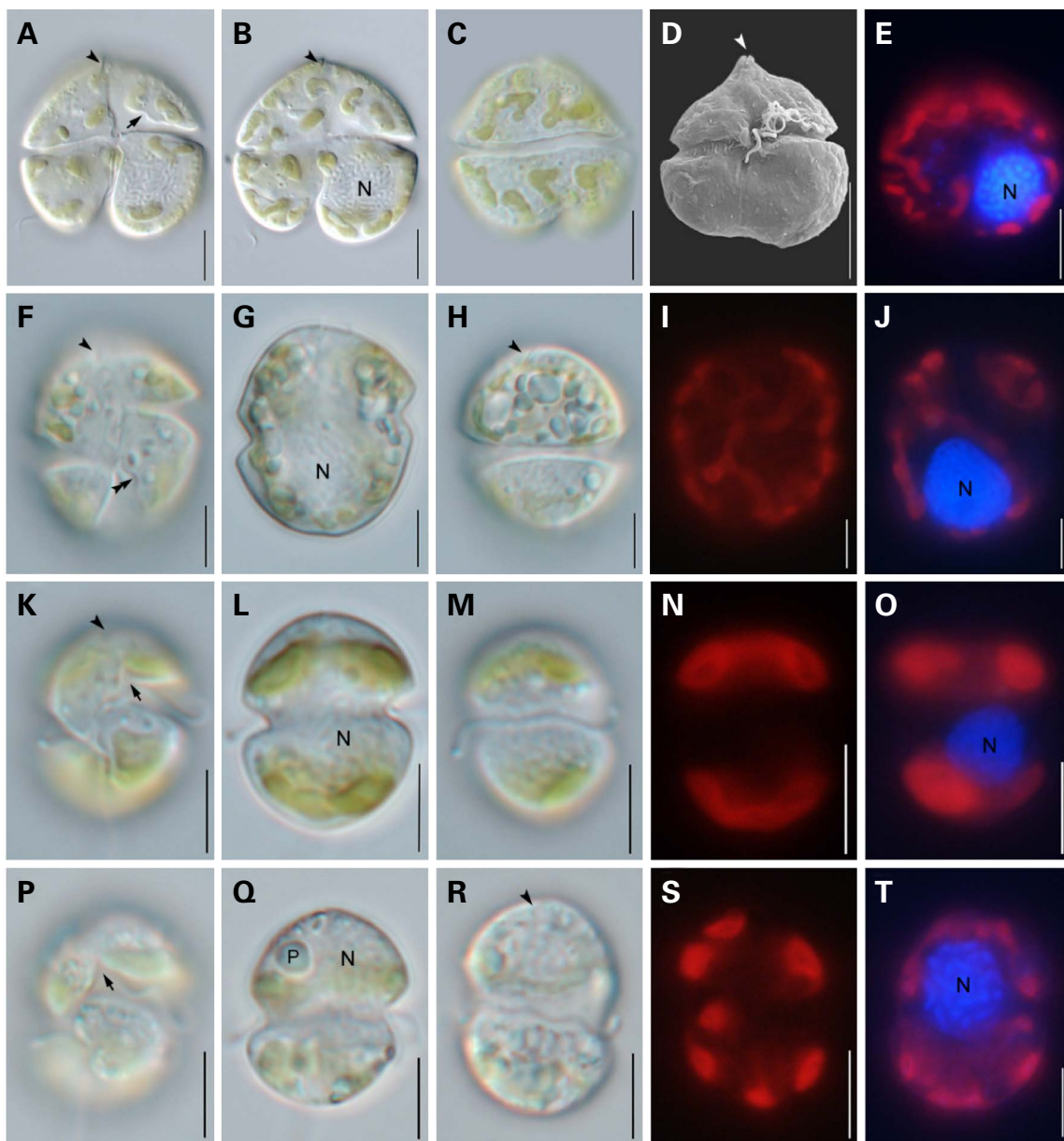


Fig. 1. Light and scanning electron micrographs of four species in the two genera *Karenia* and *Karlodinium*. (A–E) *Karenia papilionacea*. (F–J) *Karlodinium digitatum*. (K–O) *Karlodinium veneficum*. (P–T) *Karlodinium zhouanum*. Cells showing sulcal intrusion into epicone (arrow); apical groove (arrowhead); structure of curve knot (double arrowheads); nucleus (N); pyrenoid (P). Scale bars represent 10 μm in (A–E) and 5 μm in (F–T), respectively.

lenticular pyrenoids.

Distribution. South China Sea, Yellow Sea of China (Luo *et al.* 2018).

Site of collection. The species was observed from Yongho Bay of Busan in Korea (35°08'00"N, 129°06'55"E) on October 25, 2019.

Voucher slide. NNIBRPR17569–NNIBRPR17570.

Genus *Takayama* de Salas, Bolch, Botes & Hallegraeff

***Takayama acrotrocha* (Larsen) de Salas, Bolch and Hallegraeff (Fig. 2A–E)**

Basionym: *Gyrodinium acrotrochum* Larsen.

References: Larsen 1996 (Figs. 2–4 and 35).

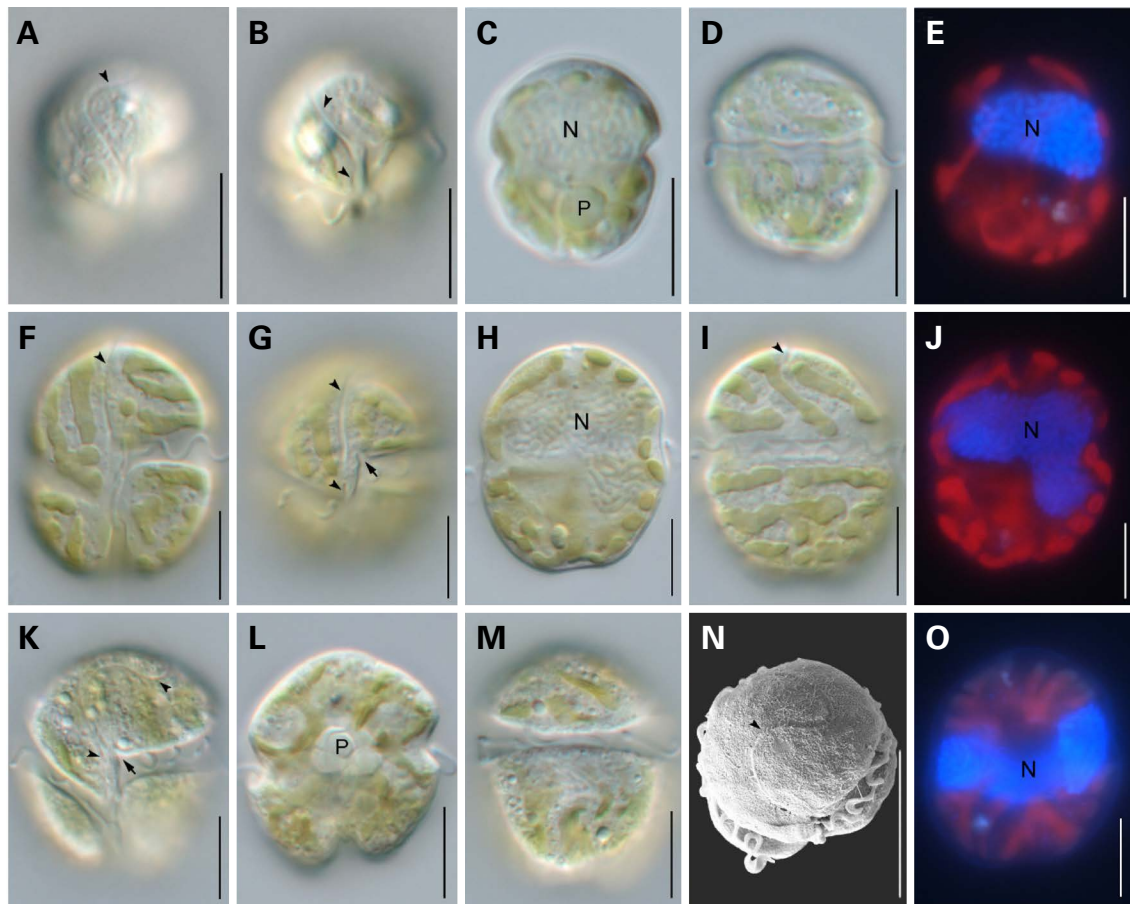


Fig. 2. Light and scanning electron micrographs of three species in the genus *Takayama*. (A–E) *Takayama acrotrocha*. (F–J) *Takayama helix*. (K–O) *Takayama tasmanica*. Cells showing sulcal intrusion into epicone (arrow); apical groove (arrowhead); nucleus (N); pyrenoid (P). Scale bars = 10 μm .

Specimen examined. Table 1.

Cells are 14.9–21.7 μm wide and 12.9–20.2 μm wide and spherical in shape. The cingulum is descended with a displacement of one fourth of the cell length. A sigmoid apical groove extends from the proximal end of girdle around the apex. The nucleus occupies much of the episome. Several disc-shaped chloroplasts are present.

Distribution. Hobsons Bay in Australia (Larsen 1996).

Site of collection. The species was observed from Yongho Bay of Busan in Korea (35°08'00"N, 129°06'55"E) on October 14, 2020.

Voucher slide. NNIBRPR17571–NNIBRPR17572.

Takayama helix de Salas, Bolch, Botes and Hallegraeff
(Fig. 2F–J)

Synonym: *Gymnodinium* sp. 6 (*Takayama* 1998, plate 6,

Figs. 8 and 9).

References: de Salas *et al.* 2003 (Figs. 7–10, 13C and D).

Specimen examined. Table 1.

Cells are 21.0–32.4 μm long and 17.8–31.9 μm wide and rhomboidal to spherical in shape with a conical epicone and truncated hypocone. The cingulum is deeply excavated with a displacement of approximately 25% of total cell length. A shallow sigmoidal apical groove extends from below the right of the sulcus, passes to left of the cell apex, to about one third of the way down the dorsal epicone. The nucleus is large, variable in shape and located in the epicone or centrally. The sulcus extends into the epicone as a finger-like protrusion. Chloroplasts are thin, elongated and arranged in spiraling bands.

Distribution. East coast of Tasmania (Australia), Port Lincoln (South Australia), Port Phillip Bay (Victoria, Australia).

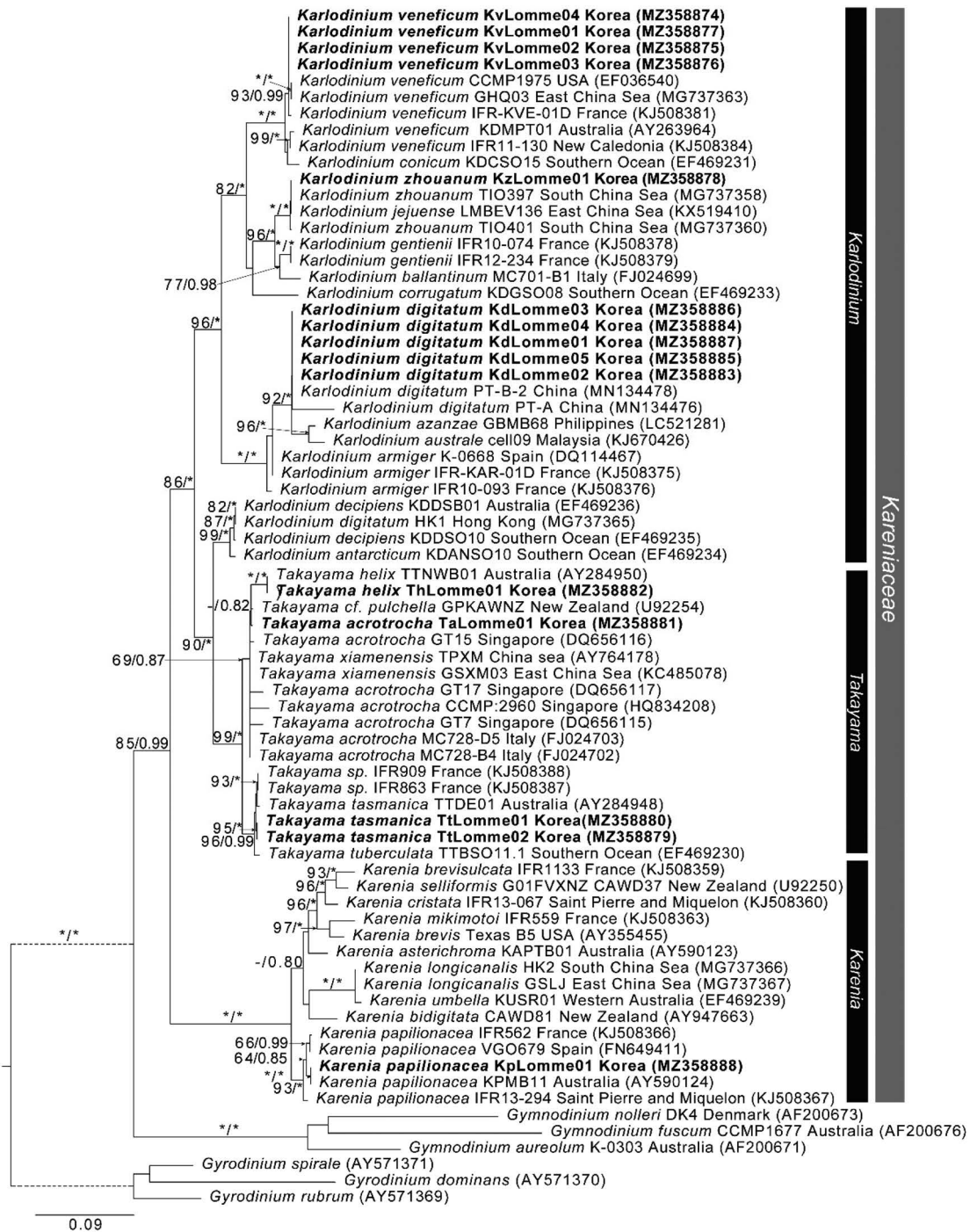


Fig. 3. Phylogenetic tree inferred from LSU rRNA gene sequences including the family Kareniaceae and two genera of *Gymnodinium* and *Gyrodinium* as outgroup. Numbers above the nodes represent ML bootstrap supports (left, LBS) and Bayesian posterior probabilities (right, BPP) higher than 60% and 0.7, respectively. Robust statistical supports (100 of LBS or 1.0 of BPP) are shown as an asterisk (*).

lia), South Africa, and Japan. (de Salas *et al.* 2003).
Site of collection. Specimens were collected from Yongho Bay of Busan in Korea (35°08'00"N, 129°06'55"E) on Sep-

tember 23, 2020.
Voucher slide. NNIBRPR17573–NNIBRPR17574.

***Takayama tasmanica* de Salas, Bolch & Hallegraeff
(Fig. 2K–O)**

References: de Salas *et al.* 2003 (Figs. 2–4, 13A and B).

Specimen examined. Table 1.

Cells are 20.9–33.2 µm long and 17.5–30.1 µm wide and obovate to spherical in shape with a hemispherical epicone and truncated hypocone. The sulcus is wide and extends shortly into the epicone as a finger-like projection. The cingulum is wide and displaced approximately one fourth of the total cell length. A sigmoid apical groove extends from below the right of the sulcal extension, detours around the cell apex, to two third of the way down the dorsal epicone. The nucleus is cup-shaped and located centrally. Chloroplasts radiate from a central pyrenoid, through the nucleus, and branch peripherally.

Distribution. North- and southeastern Tasmania (Australia) (de Salas *et al.* 2003).

Site of collection. The species was observed from Yongho Bay of Busan and Jangchun harbor of Masan in Republic of Korea (35°08'00"N, 129°06'55"E) on September 12 and October 14, 2020, respectively.

Voucher slide. NNIBRPR17575–NNIBRPR17576.

2. Molecular phylogeny

ML tree inferred from LSU rDNA sequences (D1–D3 regions, 1002 aligned positions) showed that all newly recorded seven species of three genera *Karenia*, *Karlodinium*, and *Takayama* obtained from Korea nested within the family Kareniaceae, forming a monophyletic group with strong bootstrap supports and Bayesian posterior probability (LBP/BPP = 100/1.00) (Fig. 3).

All sequences of Korean *Karl. veneficum* strains were identical based on LSU rDNA region (988 bp) and tightly clustered with the *Karl. veneficum* strains from USA, China, and France (EF036540, MG737363, and KJS08381). The sequence of Korean *Karl. zhouanum* (Kz-Lomme01) strain was identical with that from South China Sea (MG737358) and formed a highly supported clade. All sequences of the Korean *Karl. digitatum* strains were identical and formed a clade with the isolates from China (Cen *et al.* 2019) and a sister group of the clade of *Karl. azanzae* and *Karl. australe*. However, the sequence of *Karl. digitatum* HK1 (MG737365) from Hongkong (Luo *et al.* 2018) was distantly related from the clade of other *Karl. digitatum* strains and formed a clade with *Karl. decipiens* strains from Australia

and Southern Ocean, suggesting that *Karl. digitatum* strain HK1 may be misidentified. Additionally, this clade consisting of *Karl. decipiens* and *Karl. digitatum* HK1 was more closely related to *Takayama* species with moderate statistical supports (LBP/BPP = 90/1.00).

Sequences of our two Korean strains of *T. tasmanica* were identical and formed a clade with *T. tasmanica* (AY284948) from Australia and two sequences of *Takayama* sp. from France. *Takayama tuberculata* (EF469230) from the Southern Ocean branched as a sister lineage for the clade. Sequence of Korean *T. helix* strain was identical with *T. helix* (AY284950) from Australia. The Korean *T. acrotrocha* strain was closely related with *Takayama cf. pulchella* from New Zealand (U92254) and *T. acrotrocha* GT15 from Singapore (DQ656116).

All LSU rDNA sequences of *Karenia papilionacea* strains including the Korean strain KpLomme 01 formed a monophyletic clade with moderate statistical supports (LBS/BPP of 93/1.00). Recent report revealed that LSU rDNA sequences of *K. papilionacea* exhibited some degree of divergence as original *K. papilionacea* phylotype and its novel sister phylotype I (Yamaguchi *et al.* 2016). The sequence of the Korean strain *K. papilionacea* (Kp-Lomme01) clustered with the original *K. papilionacea* phylotype from Australia.

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