



Title	Ultrastructure and Systematics of Two New Species of Dinoflagellate, <i>Paragymnodinium Asymmetricum</i> sp. nov. and <i>Paragymnodinium Inerme</i> sp. nov. (Gymnodiniales, Dinophyceae)(1)
Author(s)	Yokouchi, Koh; Takahashi, Kazuya; Nguyen, Van Nguyen; Iwataki, Mitsunori; Horiguchi, Takeo
Citation	Journal of phycology, 56(3), 730-746 https://doi.org/10.1111/jpy.12981
Issue Date	2020-06
Doc URL	http://hdl.handle.net/2115/81858
Rights	This is the peer reviewed version of the following article: Journal of Phycology 56(3) June 2020, pp.730-746 which has been published in final form at https://onlinelibrary.wiley.com/doi/full/10.1111/jpy.12981 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Type	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	J. Phycol. 56-3_730-746.pdf



[Instructions for use](#)

19

20

*Takeo Horiguchi*²

21

Department of Biological Sciences, Faculty of Science, Hokkaido University, 060-0810

22

Sapporo, Japan

23

24

²Author for correspondence: e-mail horig@sci.hokudai.ac.jp

25

26

Running title: Two new species of *Paragymnodinium*

27 The genus *Paragymnodinium* currently includes two species, *P. shiwhaense*
28 and *P. stigmaticum* that are characterized by mixotrophic nutrition and the possession of
29 nematocysts. In this study, two new dinoflagellates belonging to this genus were
30 described based on observations using LM, SEM and TEM together with a molecular
31 analysis. Cells of *P. asymmetricum* sp. nov., isolated from Nha Trang beach, Vietnam,
32 were 7.9–12.6 μm long and 4.7–9.0 μm wide. The species showed no evidence of
33 feeding behavior and was able to sustain itself phototrophically. *P. asymmetricum*
34 shared many features with *P. shiwhaense*, including presence of nematocysts, absence of
35 an eyespot and a planktonic lifestyle, but was clearly distinguished by the asymmetric
36 shape of the hyposome, possession of a single chloroplast, and its nutritional mode.
37 Cells of *P. inerme* sp. nov., isolated from Jogashima, Kanagawa Pref, Japan, were 15.3–
38 23.7 μm long and 10.9–19.6 μm wide. This species also showed no evidence of feeding
39 behavior. *P. inerme* was similar to cells of *P. shiwhaense* in shape and planktonic
40 lifestyle, but its nutritional mode was different. The presence of incomplete nematocysts
41 was also a unique feature. A phylogenetic analysis inferred from concatenated SSU and
42 LSU rDNA sequences recovered the two dinoflagellates in a robust clade with
43 *Paragymnodinium* spp., within the clade of *Gymnodinium sensu stricto*. This evidence,
44 together with their morphological similarities, made it reasonable to conclude that these

45 two dinoflagellates are new species of *Paragymnodinium*.

46

47 *Key index words:* chloroplast, flagellar apparatus, *Gymnodinium sensu stricto*,

48 nematocyst, nutritional mode, *Paragymnodinium*, taxonomy

49

50 *Abbreviations:* BBC1-3, basal body connectives 1-3; C1 and 2, connective 1 and 2;

51 DAPI, 4', 6-diamidino-2-phenylindole; LB, longitudinal basal body; ML, maximum

52 likelihood; MSP, microtubular strand of a peduncle; R1-4, root 1-4; SRC, striated root

53 connective; TB, transverse basal body; TMR, transverse microtubular root; TMRE,

54 transverse microtubular root extension; TSR, transverse striated root; TSRM, transverse

55 striated root microtubule; VC, ventral connective

56 The athecate genus *Paragymnodinium* was established by Kang et al. (2010),
57 with a single species, *P. shiwhaense* as the type species. Later, Yokouchi et al. (2018)
58 described another species *P. stigmaticum*. Currently, only these two species of
59 *Paragymnodinium* are known, both of which are marine one, *P. shiwhaense*, planktonic
60 and the other, *P. stigmaticum*, benthic (Kang et al. 2010, Yokouchi et al. 2018). *P.*
61 *stigmaticum* possesses an eyespot, whereas *P. shiwhaense* lacks it (Kang et al. 2010,
62 Yokouchi et al. 2018). Although this genus is robustly included in the clade
63 *Gymnodinium sensu stricto* based on the phylogenetic analysis, both of its species lack
64 the three key characters defining *Gymnodinium*, i.e. a horseshoe-shaped apical groove,
65 nuclear envelope chambers and a nuclear fibrous connective (Daugbjerg et al. 2000,
66 Kang et al. 2010, Yokouchi et al. 2018).

67 Despite the presence of plastids, these two species feed on other prey cells and
68 thus show mixotrophic growth (Yoo et al. 2010, Yokouchi et al. 2018). The mixotrophic
69 nutritional mode is frequently encountered among various eukaryotes, including the
70 dinoflagellates, and it has an important role in aquatic ecosystems (Hansen 2011, Mitra
71 et al. 2016, Stoecker et al. 2017). Mixotrophic dinoflagellates show a variety of
72 strategies to gain nutrients (Hansen 2011), and *P. shiwhaense* is characterized by
73 obligate mixotrophy, where both photosynthesis and phagotrophy are required for its

74 successful growth (Yoo et al. 2010). Interestingly, there is a clear difference in the
75 feeding mechanism between these two species of *Paragymnodinium*: *P. shiwhaense*
76 uses a peduncle to intake a prey cell (Yoo et al. 2010), while the engulfment of the prey
77 cell in *P. stigmaticum* does not involve a peduncle (Yokouchi et al. 2018).

78 *Paragymnodinium* is also characterized by the possession of nematocysts
79 (Kang et al. 2010, Yokouchi et al. 2018). The nematocyst is a kind of extrusome with a
80 complex ultrastructure and has been reported in some other dinoflagellates belonging to
81 the clade *Gymnodinium sensu stricto*, such as *Polykrikos* and *Nematodinium* (Westfall et
82 al. 1983, Gavelis et al. 2017). The nematocysts of *Paragymnodinium* are small and
83 simple relative to those found elsewhere, but the basic structure is the same.
84 Observations of dinoflagellates bearing large nematocysts have shown that nematocysts
85 are used to capture prey cells prior to ingestion (Matsuoka et al. 2000, Lee et al. 2015,
86 Gavelis et al. 2017). In *Paragymnodinium*, this organelle is presumed to function in the
87 same way, although it never has been observed directly (Jeong et al. 2017). In addition,
88 *P. stigmaticum* has been shown to place one of its nematocysts to the tip of the
89 peduncle-like structure (Yokouchi et al. 2018).

90 Successful cultures of two novel dinoflagellates were established and
91 maintained without the need to add any prey organisms. Differences in feeding

92 mechanism are already known in *Paragymnodinium*, and now that strictly phototrophic
93 species have also been found, this taxon provides an opportunity to consider the
94 evolutionary pathways of nutritional strategies. Here, the novel dinoflagellates are
95 described as *Paragymnodinium asymmetricum* sp. nov. and *P. inerme* sp. nov., based on
96 observations using LM, SEM and TEM. We demonstrate their phylogenetic affinities
97 based on concatenated sequences of the SSU and LSU rDNA genes and discuss the
98 evolution of nutritional strategies within the genus.

99

100

101

MATERIALS AND METHODS

102

103

104

105

106

107

108

109

Paragymnodinium asymmetricum (strain vnd299) was isolated from water
samples from Nha Trang beach, Nha Trang, Vietnam (12°14.56'N, 109°11.49'E) on 26
April, 2014. *P. inerme* (strain JGD) was isolated from water samples from Jogashima,
Kanagawa, Japan (35°08.02'N, 139°36.41'E) on 19 November, 2017. Isolated cells
were kept in Daigo's IMK Medium for Marine Microalgae (Nihon Pharmaceutical Co.,
Tokyo, Japan). Cultures of *P. asymmetricum* and *P. inerme* were maintained without
adding any prey. The established monoclonal cultures were incubated at 20°C, with an
illumination of 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ under 16:8 h light:dark cycle. To observe if

110 these dinoflagellates show feeding behavior, cells of *Chroomonas* sp. (strain Ak01),
111 *Rhodomonas* sp. (strain Mr06, Cryptophyceae), two strains of *Amphidinium* aff.
112 *carterae* (strains TH006 and HG286), *Ansanella natalensis* (strain CW-19)
113 (Dinophyceae), *Euglena* sp. (strain ST-11) and an unidentified Raphidophyceae (strain
114 HG316) were added to each culture as candidates of prey and were kept several days.
115 The potential prey organisms were chosen based on reports that *A. carterae* is the
116 appropriate prey for *P. shiwhaense* (Yoo et al. 2010), and that *Chroomonas* sp. is added
117 to the culture of *P. stigmaticum* as a prey (Yokouchi et al. 2018).

118 For LM, cells were observed in differential interference contrast (DIC) with a
119 Zeiss Axioskop 2 Plus microscope (Zeiss Japan, Tokyo, Japan), and images were taken
120 using a Canon EOS Kiss X8i digital camera (Canon, Tokyo, Japan). Chlorophyll
121 autofluorescence was observed using a Zeiss Axioskop 2 Plus microscope with a No. 15
122 filter set. The nucleus was stained with 4', 6-diamidino-2-phenylindole (DAPI) after
123 fixation in 2.5% glutaraldehyde (final concentration) and the fluorescence was observed
124 using a Zeiss Axioskop 2 Plus microscope with a No. 49 filter set.

125 For SEM, cells of *P. asymmetricum* were fixed for at least 0.5 h on ice with 1 or
126 2% (final concentration) OsO₄ in distilled water. Cells of *P. inerme* were fixed for 1.5 h
127 on ice with 2 or 3% (final concentration) OsO₄ in distilled water. Fixed cells were

128 placed on the membrane filter (pore size = 5 μm) that was glued on the bottom of a
129 short tube (cut-off proximal part of 1000 μl blue tip), using a pipette. Membrane filters
130 were washed three times with distilled water. Cells were then dehydrated in an ethanol
131 series (30%, 50%, 70%, 80%, 90%, 95%) for 10 min at each concentration, with two
132 subsequent submersions of 30 min each in 100% ethanol. Dehydrated cells were dried
133 with CO_2 using a critical point drier (Leica EM CPD300, Wetzlar, Germany), sputter
134 coated with gold (Hitachi E-1045 sputter coater), and viewed with a Hitachi S-3000N
135 SEM.

136 For TEM, cells were fixed using one of two protocols. In the first protocol,
137 cells were fixed in 2.5% glutaraldehyde (final concentrations) in seawater for 1 h, and
138 washed twice in sea water. Cells were post fixed in 1% OsO_4 (final concentrations) in
139 distilled water for 1 h. In the second, cells were fixed in a mixture of 2% glutaraldehyde
140 and 0.5% OsO_4 (final concentrations) in 0.1 M Na-cacodylate buffer, pH 7.4 for 15 or
141 30 min, and rinsed twice in 0.1 M Na-cacodylate buffer. Cells were post-fixed in 1%
142 OsO_4 (final concentration) in 0.1 M Na-cacodylate buffer, pH 7.4 for 1 h. In both
143 protocols, cells were first attached to the bottom of a polypropylene dish coated with
144 poly-L-lysine. After fixation, cells of both protocols were dehydrated in an acetone
145 series (30%, 50%, 80%, 90%, 95%) for 10 min at each concentration, and submersed

146 twice, each time for 30 min, in 100% acetone. One hundred percent acetone and Agar
147 Low Viscosity Resin (Agar Scientific, Essex, UK) were mixed in ratios of 3:1, 1:1, and
148 1:3 and the samples were introduced into each higher resin concentration sequentially
149 for 15 min each. Finally, cells were infiltrated in 100% resin for 30 min, after which
150 they were polymerized at 65°C for 16 h. Samples were sectioned using a diamond knife
151 on an EM-Ultracut S ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections
152 were placed on formvar-coated one-slot grids and observed with a Hitachi H-7650
153 TEM.

154 For extraction of total DNA, several cells were isolated by capillary pipettes,
155 rinsed several times in serial drops of sterilized culture medium and transferred into 10
156 µl of Quick Extract FFPE RNA Extraction Kit (Epicentre, Wisconsin, USA) to extract
157 DNA according to the manufacturer's protocol. Primers SR1, SR4, SR8TAK, SR9,
158 SR12b and 18SRF were used to amplify SSU rDNA sequences (Nakayama et al. 1996,
159 Takano and Horiguchi 2004, Iritani et al. 2018), and D1RF1, 25R1, D3A and 28-1483R
160 to amplify partial LSU rDNA (Daugbjerg et al. 2000). For SSU rDNA amplification,
161 almost complete gene sequences were obtained using the SR1 and SR12b primers in the
162 first round of PCR, the products of which were used as DNA templates in the second
163 round of PCR. For this, three pairs of primers (SR1-18SRF, SR4-SR12b and

164 SR8TAK-SR12b) were used for *P. asymmetricum*, and three pairs of primers
165 (SR1-18SRF, SR4-SR9 and SR8TAK-SR12b) were used for *P. inermis*. To obtain partial
166 LSU rDNA sequences for both species, D1RF1 and 28-1483R were applied in the first
167 round of PCR and two pairs of primers (D1RF1-25R1 and D3A-28-1483R) were used
168 in the second round of PCR. The PCR conditions for both rounds of amplification
169 consisted of one initial cycle of denaturation at 94°C for 5 min, followed by 35 cycles
170 (in the second round for LSU rDNA, 25 cycles) of denaturation at 94°C for 30 s,
171 annealing at 55°C for 30 s, and extension at 72°C. The time of the extension step was
172 changed by the length of targeting sequences; 2 min for the first round, 1 min 40 s for
173 the two pairs of primers, SR1-18SRF and SR4-SR12b, and 1 min for other pairs of
174 primers. PCR was completed by a final extension cycle at 72°C for 7 min. Purified PCR
175 products were used in a sequencing reaction with ABI BigDye Terminator (Applied
176 Biosystems, Foster City, California, USA) and subsequently purified with ethanol. The
177 products were eluted in 18 µl of Hi-Di Formamide (Applied Biosystems) and sequenced
178 with a 3130 genetic analyzer (Applied Biosystems).

179 Both SSU rDNA sequences and partial LSU rDNA sequences were aligned
180 using MUSCLE (Edgar 2004) together with 45 taxa, including *Perkinsus andrewsi* as an
181 outgroup, and the alignments were modified manually using MEGA7 (Kumar et al.

182 2016). The highly divergent D2 region of LSU rDNA sequences was deleted.

183 Consequently, 1771 positions of SSU rDNA and 1107 positions of LSU rDNA were

184 aligned. Pairwise distance of the two aligned sequences of four *Paragymnodinium* spp.

185 were calculated using MEGA7 with p-distance model. The two aligned sequences for

186 all taxa were concatenated using Kakusan4 (Tanabe 2011). No significant nucleotide

187 compositional heterogeneity was detected for the combined data set ($P = 0.99792$ using

188 the chi-square test in Kakusan4). The appropriate models of substitution ratio for

189 concatenated rDNA sequences were determined using Kakusan4, and resulted in a

190 separate model for maximum likelihood (ML) analysis and a proportional model for

191 Bayesian analysis. The appropriate models of DNA evolution for each rDNA sequences

192 were determined by AIC for ML analysis and by BIC for Bayesian analysis using

193 Kakusan4, and resulted in the selection of the GTR + Gamma model. The parameters in

194 these analyses for SSU rDNA were: assumed nucleotide frequencies $A = 0.264$, $C =$

195 0.198 , $G = 0.262$ and $T = 0.275$; substitution rate matrix with $A \leftrightarrow C = 1.251914$, A

196 $\leftrightarrow G = 3.199366$, $A \leftrightarrow T = 1.376839$, $C \leftrightarrow G = 0.441724$, $C \leftrightarrow T = 8.534122$ and

197 $G \leftrightarrow T = 1.000000$. The proportion of sites were assumed to follow a gamma

198 distribution with the shape parameter = 0.285333 . The parameters for LSU rDNA were:

199 assumed nucleotide frequencies $A = 0.285$, $C = 0.191$, $G = 0.285$ and $T = 0.239$. The

200 substitution rate matrix had A <-> C = 0.651709, A <-> G = 2.112521, A <-> T =
201 0.834059, C <-> G = 0.524352, C <-> T = 5.656149 and G <-> T = 1.000000. The
202 proportion of sites were assumed to follow a gamma distribution with the shape
203 parameter = 0.370529. The ML analysis was performed using the RAxML 8.0.0
204 (Stamatakis 2006). Bootstrap analysis for ML was calculated for 1000 pseudo-replicates.
205 The Bayesian analysis was performed using MrBayes 3.2.6 (Huelsenbeck and Ronquist
206 2001). Markov chain Monte Carlo iterations were carried out until the average standard
207 deviation of split frequency fell below 0.01 (1300000 generations were attained) and
208 trees were sampled every 100 generations. The first 175000 generations were discarded
209 as burn-in. Posterior probabilities were calculated from all post burn-in trees.

210

211

RESULTS

212 *Paragymnodinium asymmetricum* K.Yokouchi, K.Takahashi, Nguyen, Iwataki et

213 T.Horiguchi sp. nov.

214 *Description.* Marine, athecate dinoflagellate. Cells with almost equal-sized episomes

215 and hyposomes, 7.9–12.6 µm long and 4.7–9.0 µm wide. Episome hemispherical or

216 conical. Hyposome asymmetric with larger right side. Cingulum wide and well

217 excavated, descending 1/4 to 1/2 of its own width. Sulcus straight, reaching to, and

218 widening slightly at, the antapex. Sulcal extension-like furrow straight. Eyespot absent.
219 Nucleus spherical, in center of episome. Chloroplast single, mainly in hyposome and
220 with lateral lobes extending into episome. Amphiesmal vesicles arranged in five to
221 seven rows on the episome, in five rows in the cingulum. Nematocysts present.
222 Pyrenoid and pusule not observed. Phototrophic. GenBank accession numbers are
223 LC516501 for 18S rDNA sequence and LC516500 for 28S rDNA sequence.

224 *Holotype*: SEM stub was deposited in the herbarium of the Faculty of Science,
225 Hokkaido University as SAP 115483. Fig. 1, J and K were taken from that stub.

226 *Collection date*: 26 April 2014.

227 *Type locality*: Nha Trang beach, Nha Trang, Vietnam (12°14.56'N,
228 109°11.49'E).

229 *Etymology*: Latin *asymmetricum*, refers to the asymmetric shape of hyposome.

230 *LM and SEM*: Cells small, 7.9–12.6 μm ($9.6 \pm 1.0 \mu\text{m}$, mean \pm SD, n = 55)

231 long and 4.7–9.0 μm ($6.9 \pm 1.0 \mu\text{m}$, n = 55) wide. Episome and hyposome were almost
232 equal in size (Fig. 1, A and B). Episome was conical (Fig. 1, A and B); hyposome was
233 asymmetric; right side larger than left side (Fig. 1, A and B). Cingulum was wide, well
234 excavated and descended by a distance one quarter to a half its own width (Fig. 1, A and
235 B). Sulcus was straight and widened slightly before reaching the antapex (Fig. 1A).

236 Eyespot was not observed. A straight sulcal extension-like furrow (SEF, *sensu* Kang et
237 al. 2010) ran from the right end of the cingulum toward the apex (Fig. 1A). Chloroplast
238 was single and yellow-brown (Fig. 1C), mainly occupying posterior area of hyposome,
239 but with lateral lobes extending anteriorly into episome but not reaching the apex (Fig. 1,
240 C and D). Nucleus was located in the central area of episome (Fig. 1, B, C and E). DAPI
241 staining confirmed the single nucleus occupied almost the entire episome (cf. Fig. 1, C
242 and E). The motile cell was planktonic and free-swimming. Cells encysted during the
243 dark period. Cysts were spherical and covered with a wall (Fig. 1F). The organism grew
244 in complete isolation from other eukaryotes and did not show feeding behavior when
245 co-cultured with potential prey organisms.

246 SEM observations showed cells were covered by small polygonal amphiesmal
247 vesicles (AVs) (Fig. 2, G-O). These AVs in the episome were arranged in anything from
248 5-7 lateral rows (Fig. 1, G-L). Such variation was not observed in the cingulum and the
249 sulcus. The AVs in the cingulum were arranged in 5 rows (Fig. 1J). The sulcus was
250 deeply incised but the exact boundary of sulcus with the remainder of the cell was not
251 sharply defined (Fig. 1, G, J and M). The SEF was less incised than the sulcus and
252 consisted of nine elongate AVs (Fig. 1, G, H, K, L, N and O). The hyposome was also
253 covered with AVs arranged in approximately 4 lateral rows, but the exact number was

254 difficult to ascertain because of its asymmetric shape (Fig. 1M).

255 *TEM*: The positioning and morphology of the nucleus and chloroplast in motile
256 cells were confirmed in thin-sectioned material (Fig. 2A). The nucleus was a typical
257 dinokaryon with condensed chromosomes, and occupied most of the episome (Fig. 2B).
258 It was surrounded by numerous mitochondria (Fig. 2B). The nuclear envelope possessed
259 nuclear pores but lacked nuclear envelope chambers (Fig. 2C). Trichocysts were typical
260 for dinoflagellates and were peripherally arranged (Fig. 2, D and E). Cells were covered
261 by a typical amphiesma, the vesicles of which had no thecal plates or other plate-like
262 structures (Fig. 2F). A microtubular strand of a peduncle (MSP) ran from the right side
263 of the flagellar apparatus (Fig. 2, G-J). There were some electron-opaque vesicles near
264 the MSP (Fig. 2, G-J). Chloroplast was surrounded by three membranes. The posterior
265 mass contained condensed thylakoids (Fig. 3A), most of which were double stacked,
266 and the distance between adjacent thylakoid bands was approximately 6–10 nm (Fig.
267 3B). On the other hand, the lateral lobes contained double or triple stacked thylakoid
268 bands, and the distance between bands was relatively greater and more variable (Fig. 3,
269 C and D). The boundary between the more condensed thylakoids of the posterior mass
270 and the less condensed thylakoids of the lateral lobes was obvious (Fig. 3E).

271 Cells each contained at most four nematocysts (Fig. 4). Each nematocyst was

272 composed of an oval posterior body and an anterior operculum. The posterior body was
273 covered by a capsule and a posterior chamber, and contained a fibrous strand. The
274 anterior region of the posterior body was occupied by an anterior chamber with a stylet
275 (*sensu* Westfall et al. 1983). A central filament-like structure was observed in the central
276 axis of the fibrous strand (Fig. 4B), but could not be resolved in the transverse serial
277 sections (Fig. 4, J-L). The length and width of nematocysts were approximately 0.8 μm
278 and 0.5 μm , respectively. Taeniocysts and posterior vacuoles were not observed.

279 The flagellar apparatus of *P. asymmetricum* was re-constructed (Fig. 5) from
280 serial sections (Figs. S1 and S2). The transverse basal body (TB) and the longitudinal
281 basal body (LB) were connected, at an oblique angle of about 150° to one another, by a
282 basal body connective (BBC) (Figs. S1, F-H; and S2C). Root 1 (R1) consisted of 12
283 microtubules and was inserted on the dorsal side of LB (Figs. S1, A-I; and S2, D-H). R1
284 and LB were linked by the connective C1 (Figs. S1E; and S2D). Root 3 (R3) was
285 comprised of a transverse microtubular root (TMR) and a transverse microtubular root
286 extension (TMRE) (Figs. S1, C-J; and S2, A, B, K and L). TMR was a single
287 microtubular root and inserted on the right side of TB (Figs. S1, D-F; and S2, A and K).
288 The TMRE consisted of six microtubules nucleated by the TMR (Figs. S1, C-J; and S2,
289 A, B, K and L). Root 4 (R4), comprising a transverse striated root (TSR) and TSR

290 microtubule (TSRM), was inserted on the left side of the TB (Figs. S1, G-L; and S2,
291 D-J). R1 and R4 were linked by a striated root connective (SRC) (Figs. S1, H and I; and
292 S2, E-G and J). Despite our observations of the flagellar apparatus being made from 5
293 different cells, the expected root 2 and a nuclear fibrous connective were not observed.

294

295 *Paragymnodinium inerme* K.Yokouchi, K.Takahashi, Nguyen, Iwataki et T.Horiguchi

296 sp. nov.

297 *Description.* Marine, athecate dinoflagellate. Cells with almost equally-sized episomes
298 and hyposomes, 15.3–23.7 μm long and 10.9–19.6 μm wide. Episome hemispherical or
299 conical and hyposome hemispherical. Cingulum wide and well excavated, descending
300 1/2 to once its own width. Sulcus straight, reaching to, and widening slightly at, the
301 antapex. Sulcal extension-like furrow slightly curved. Eyespot absent. Nucleus spherical,
302 in the center of the dorsal side of cell. 20-30 chloroplasts, some of which are connected
303 by narrow bridges. Amphiesmal vesicles arranged in 19 or 20 lateral rows (eight or nine
304 rows to the episome, five rows to the cingulum, and six rows to the hyposome).

305 Nematocysts rare and, if present, abnormal. Pyrenoid and pusule not observed.

306 Phototrophic. GenBank accession numbers are LC516503 for 18S rDNA sequence and

307 LC516502 for 28S rDNA sequence.

308 *Holotype*: SEM stub was deposited in the herbarium of the Faculty of Science,
309 Hokkaido University as SAP 115484. Fig. 6, L and M were taken from that stub.

310 *Collection date*: 19 November 2017.

311 *Type locality*: Jogashima, Kanagawa, Japan (35°08.02'N, 139°36.41'E).

312 *Etymology*: Latin *inermis*, (= unarmed) refers to absence of nematocyst.

313 *LM and SEM*: Cells were 15.3–23.7 μm ($19.4 \pm 2.0 \mu\text{m}$, mean \pm SD, $n = 28$)

314 long and 10.9–19.6 μm ($14.9 \pm 2.1 \mu\text{m}$, $n = 28$) wide. Episome and hyposome were

315 almost equal in size (Fig. 6, A and B). Episome was conical to hemispherical, and the

316 hyposome was hemispherical (Fig. 6, A and B). Cingulum was wide, well excavated and

317 descended by a distance half to equal of its own width (Fig. 6, A and B). Sulcus was

318 straight and widened slightly before reaching the antapex (Fig. 6A). No eyespot was

319 observed. A slightly curved sulcal extension-like furrow (SEF) ran from the right end of

320 the cingulum toward the apex (Fig. 6A). Chloroplasts were yellow brown and

321 distributed throughout the cell (Fig. 6A-D). Analysis of autofluorescence images

322 demonstrated the presence of multiple chloroplasts in each cell (Fig. 6, C and D). The

323 nucleus was central on the dorsal side of the cell (Fig. 6, B, E and F). DAPI staining

324 showed a single nucleus in the central or dorsal of cell (Fig. 6, E and F). The motile cell

325 was planktonic and free-swimming. Cells encysted during the dark period. Shape of the

326 cysts was similar to that of motile cells but each was covered with a wall. Cell division
327 took place during the walled cyst stage (Fig. 6G). Some motile daughters released from
328 germinating cells remained connected at their ventral surfaces (Fig. 6H). Cultures of this
329 species grew in the absence of other eukaryotes and did not show feeding behavior
330 when grown together with selected strains of other organisms.

331 SEM observations showed cells covered by small polygonal amphiesmal
332 vesicles (AVs) (Fig. 6, I-O). AVs were arranged in 19 or 20 lateral rows, i.e. eight or
333 nine rows to the episome, five rows to the cingulum, and six rows to the hyposome (Fig.
334 6, J-M). The SEF was slightly incised and consisted of nine AVs (Fig. 6, N and O). The
335 sulcal AVs can be distinguished from surrounding ones, but the absolute number could
336 not be determined (Fig. 6, I-K and M). Cells with doubled flagella were common in
337 culture (Fig. 6L and M).

338 *TEM*: Positioning and morphology of the organelles in motile cells were
339 confirmed in thin-sectioned material (Fig. 7A). The nucleus was a typical dinokaryon
340 with condensed chromosomes (Fig. 7B) and a nuclear envelope interrupted by nuclear
341 pores but lacking nuclear envelope chambers (Fig. 7C). Trichocysts were typical for
342 dinoflagellates and were peripheral (Fig. 7, D and E). Cells were covered by a typical
343 amphiesma (Fig. 7F), the vesicles of which had no thecal plates or other plate-like

344 structures (Fig. 7F). A microtubular strand of the peduncle ran from the right side of the
345 flagellar apparatus (Fig. 7, G-J), but electron-opaque vesicles in its vicinity were not
346 observed (Fig. 7, G-J). The cell contained approximately 20-30 oval chloroplast masses
347 (Fig. 8A). Chloroplasts were surrounded by three membranes (Fig. 8B) and contained
348 multiple thylakoids forming double- or triple-stacked thylakoid lamellae (Fig. 8C) that
349 were evenly distributed throughout all chloroplast masses. Some of these masses were
350 interconnected by narrow bridges (Fig. 8, D-F), making the actual number of
351 chloroplasts fewer than apparent. Serial sections through two whole cells of *P. inermis*,
352 revealed that one had only three chloroplasts while the other had 15 (Video S1).

353 Cells rarely contained nematocysts (Fig. 9): only three of 15 entire cells
354 investigated by serial sectioning were found to have them. Where present, the anterior
355 operculum was almost completely collapsed, leaving the organelles composed solely of
356 the oval posterior bodies. Each posterior body consisted of an anterior chamber and a
357 capsule-covered, posterior chamber, containing multiple (approximately three) fibrous
358 strands. A stylet was not observed.

359 The flagellar apparatus of *P. inermis* was re-constructed (Fig. 10) from serial
360 sections (Figs. S3 and S4). The transverse basal body (TB) and the longitudinal basal
361 body (LB) were held at an oblique angle of about 150° relative to one another by three

362 basal body connectives (BBC1-3) (Fig. S4, E-H). Root 1 (R1) consisted of 18
363 microtubules and was inserted on the dorsal side of the LB (Figs. S3, A-F; and S4, A-D).
364 R1 and the LB were linked by two connectives, C1 and C2 (Fig. S3, C and D). Root 3
365 (R3) was comprised of a transverse microtubular root (TMR) and a transverse
366 microtubular root extension (TMRE) (Figs. S3, H-L; and S4, I-K). The TMR was
367 comprised of a single microtubule inserted on the right side of the TB (Figs. S3, H-L;
368 and S4, I-K). The TMRE consisted of several (presumably less than 10) microtubules
369 nucleated by the TMR, but the precise number could not be determined (Figs. S3, K and
370 L; and S4, J and K). Root 4 (R4), comprising a transverse striated root (TSR) and a TSR
371 microtubule (TSRM), was inserted on the left side of the TB (Figs. S3, H-L; and S4,
372 E-H). R1 and R4 were linked by a striated root connective (SRC) (Figs. S3, G and H;
373 and S4, D and E). Root 2 and a nuclear fibrous connective were not observed in any
374 serial sections through the flagellar apparatus of eight different cells.

375 *Phylogenetic analysis.* The topologies resulting from ML and Bayesian
376 analyses were only slightly different, and only the ML tree is shown (Fig. 11). Both
377 strains studied here were included in the clade *Gymnodinium sensu stricto*, and formed
378 a robust clade with *Paragymnodinium* spp. Within the *Paragymnodinium* clade, *P.*
379 *inermis* was shown to be sister to *P. shiwhaense* (Table 1), and *P. asymmetricum* was

380 sister to the *P. shiwhaense*/*P. inerme* clade with high support. *P. stigmaticum* was sister
381 to the *P. shiwhaense*/*P. inerme*/*P. asymmetricum* clade. Although the *Paragymnodinium*
382 clade was basal in the *Gymnodinium sensu stricto* clade in both the ML and Bayesian
383 analyses, its position did not enjoy convincing support. Species with nematocysts were
384 restricted to some members of the clade *Gymnodinium sensu stricto*, notably *Polykrikos*,
385 *Nematodinium*, *Gyrodiniellum* and *Paragymnodinium* (denoted by stars in Fig. 11), but
386 the character of possession of nematocysts was not monophyletic.

387

388 DISCUSSION

389 *Taxonomy.* *Paragymnodinium asymmetricum* has characteristics shared by
390 other species of the genus *Paragymnodinium*, such as the possession of nematocysts,
391 polygonal amphiesmal vesicles and a SEF (Kang et al. 2010, Yokouchi et al. 2018). It is
392 more affiliated with *P. shiwhaense* than with *P. stigmaticum* in that it lacks an eyespot,
393 has double- or triple-stacked thylakoid lamellae and a planktonic lifestyle. This
394 relationship is supported by the topology of the molecular tree. On the other hand, *P.*
395 *asymmetricum* is clearly distinguished from *P. shiwhaense* by the cell size, the
396 asymmetric shape of hyposome (larger right than left side) and the anterior position of
397 the nucleus rather than central or dorsal position seen in *P. shiwhaense* (Kang et al.

398 2010). The SEF of *P. asymmetricum* is straight as opposed to the curved equivalent in
399 other members of the genus (Kang et al. 2010, Yokouchi et al. 2018). It also shows
400 variation in the number of AV rows of its episome. Intraspecific variation of AVs is seen
401 in some other dinoflagellates (e.g. Pandeirada et al. 2014), but has not been reported in
402 the genus *Paragymnodinium*. If the number of AVs is mutable, this morphological
403 character is not appropriate as a taxonomic criterion. In addition, *P. asymmetricum* can
404 be distinguished from the mixotrophic *P. shiwhaense* (Yoo et al. 2010) because it shows
405 no evidence of feeding behavior and can sustain itself phototrophically. DAPI staining
406 shows that DNA is focused in one area (the nucleus) without subsidiary satellite
407 fluorescence as would be expected had ingested bacteria. In addition to this, no
408 intracellular bacteria were ever observed by TEM. It is conceded that *P. asymmetricum*
409 has the potential to be mixotrophic because it retains structures related to feeding
410 behavior, such as a peduncle and nematocysts. However, it is clearly not an obligate
411 mixotroph that requires both feeding and photosynthesis as is the case for *P. shiwhaense*
412 (Yoo et al. 2010).

413 Asymmetry of the hyposome, as seen in *P. asymmetricum*, is rare in athecate
414 dinoflagellates. The hyposome of some species of the genus *Gyrodinium*, such as *G.*
415 *dominans*, are similarly asymmetric, but *P. asymmetricum* is clearly not a member of

416 this genus because it does not have longitudinal striations, and it is not heterotrophic
417 (Hoppenrath et al. 2014). The phylogenetic analysis also recovered *Gyrodinium* spp. in
418 a distantly-related clade to that of *Paragymnodinium* spp. Therefore, *P. asymmetricum*
419 can be distinguished from any other dinoflagellates described to date, and we conclude
420 that this dinoflagellate is a new species.

421 *Paragymnodinium inerme* is similar to *P. shiwhaense* in shape, and in the
422 possession of polygonal AVs, a slightly curved SEF, a planktonic lifestyle and the
423 absence of an eyespot. Although the number of AVs of the two species is different, the
424 arrangement of AVs within the SEF is the same (Kang et al. 2010). The genetic distance
425 between these two species is also small. However, the nutritional strategy of *P. inerme*
426 differs from that of *P. shiwhaense*: *P. inerme* can grow without any supplementation to
427 phototrophy and does not feed when provided with cells of *Amphidinium* aff. *carterae*
428 despite the fact that *A. carterae* was identified as the most appropriate prey for *P.*
429 *shiwhaense* (Yoo et al. 2010). In addition, although we also provided unicellular algae
430 belonging to different classes as possible prey, *P. inerme* did not feed any of these algal
431 cells. DAPI staining and TEM observations showed no evidence of ingested bacteria in
432 *P. inerme*. The abnormality or degeneration of nematocysts in *P. inerme* is also a clear
433 difference from *P. shiwhaense* and in *P. inerme* there is no evidence of the plate-like

434 structures that found in the amphiesmal vesicles of *P. shiwhaense* (Kang et al. 2010).
435 The presence of a transverse microtubular root extension (TMRE) of R3 and of the
436 ventral connective (VC) in the flagellar apparatus of *P. inerme* also represent differences
437 from *P. shiwhaense* (Kang et al. 2010). While it is conceded that the TMRE and VC
438 might have been overlooked in *P. shiwhaense* (see below), there are a suite of clear
439 morphological differences between *P. inerme* and *P. shiwhaense*, despite their close
440 phylogenetic relationship, and the two organisms can be regarded as different species.

441 There are some dinoflagellates which morphologically resemble *P. inerme*.
442 *Aureodinium pigmentosum* is similar in size and shape to *P. inerme*, but has pyrenoids
443 (Dodge 1967, 1982), which are lacking in *P. inerme*. *Gymnodinium incertum* is also
444 similar, but the SEF or apical groove-like structure has not been described in this
445 species (Dodge 1982). *Gymnodinium pygmaeum* is also similar in size and has a furrow
446 in its episome, but this species is rounder than *P. inerme*, and both the apex and antapex
447 are notched, so it is distinguishable from *P. inerme* (Dodge 1982, Hansen and Larsen
448 1992). Therefore, *P. inerme* can be distinguished from any other morphologically
449 similar species described to date, and we conclude that this dinoflagellate is a new
450 species.

451 *Chloroplasts and nutritional mode.* The chloroplast of *Paragymnodinium*

452 *asymmetricum* is single, unlike the multiple chloroplasts seen in other
453 *Paragymnodinium* spp. (Kang et al. 2010, Yokouchi et al. 2018, this study). In addition,
454 it is composed of two distinctive parts; an ‘antapical mass’ and anterior ‘lateral lobes.’
455 The antapical mass in the hyposome contains densely-stacked, double thylakoids
456 resembling the grana-like thylakoids seen in some dinoflagellates such as *Ansanella*
457 *granifera* (Jeong et al. 2014) or *Dactylocladus pterobellus* (Takahashi et al. 2017).
458 However, the double-stacked thylakoids of this region of the chloroplast of *P.*
459 *asymmetricum* are not attached to each other. Thus, the thylakoids cannot be likened to a
460 true granum, but are rather a tighter packing of the thylakoid lamellae relative to the
461 lateral lobes, which are an extension of the antapical mass. The variability in the
462 numbers (two or three) of thylakoids stacked together, and in the packing density of
463 these stacks, in different regions of a chloroplast has not been reported in any other
464 dinoflagellates. *P. inerme* also has double- or triple-stacked thylakoids but there is no
465 difference in its packing density or stacking thylakoid number by region of the
466 chloroplast. In addition, *P. inerme* contains numerous oval masses of chloroplasts,
467 which is similar to the condition in *P. shiwaense* (Kang et al. 2010). However, some of
468 these masses are directly connected to each other by thin bridges.

469 In the genus *Paragymnodinium*, mixotrophy is only recognized in *P.*

470 *shiwhaense* and *P. stigmaticum*. The two new species, *P. asymmetricum* and *P. inerme*,
471 do not show phagotrophy and thus are entirely phototrophic, rather than mixotrophic.
472 Interestingly, the close phylogenetic relationship between *P. shiwhaense* and *P. inerme*,
473 is not reflected in their nutritional mode and thus, the diversification of nutritional mode
474 is thought to have occurred quite recently. The evolution pattern of nutritional mode can
475 be explained by two hypotheses. (1) The common ancestor of this clade was
476 phototrophic, and *P. shiwhaense* and *P. stigmaticum* has acquired mixotrophic strategy
477 independently. This hypothesis is parsimonious on the nutritional mode, but cannot
478 explain why the phototrophic species possess some structures related to feeding, such as
479 nematocysts and a peduncle. (2) The common ancestor of this clade had a mixotrophic
480 strategy, and *P. asymmetricum* and *P. inerme* lost phagotrophic capability independently.
481 Based on this hypothesis, the abnormal nematocyst in *P. inerme* (discussed below) is
482 thought to represent a degeneration of the organelle as a result of the loss of the
483 requirement for phagotrophy. To determine which of these hypotheses is correct, the
484 nutritional mode of the common ancestor of the genus *Paragymnodinium* needs to be
485 estimated, and thus, the symplesiomorphic character among these species and the
486 closest related taxa should be confirmed, however, this requires improved statistical
487 support of the entire topology of the phylogenetic tree for the clade *Gymnodinium sensu*

488 *stricto*.

489 *Nematocysts*. Some dinoflagellates included in the clade *Gymnodinium sensu*
490 *stricto*, e.g. polykrikoids, warnowiids, *Gyrodiniellum* and *Paragymnodinium* contain
491 nematocysts (Marshall 1925, Westfall et al. 1983, Greuet 1987, Hoppenrath and
492 Leander 2007a, b, Hoppenrath et al. 2009, 2010, Kang et al. 2010, 2011, Yokouchi et al.
493 2018). The nematocyst-bearing taxa did not form a clade in our phylogenetic analysis,
494 indicating the multiple gain or loss of nematocyst in this clade. However, since the
495 topology is not supported well, it is difficult to discuss how the nematocysts have
496 evolved within the *Gymnodinium sensu stricto* clade. The nematocyst is thought to be
497 used to capture prey cells prior to ingestion, as observed in the relatively large
498 nematocyst-bearing dinoflagellates, such as *Polykrikos* and *Nematodinium* (Matsuoka et
499 al. 2000, Lee et al. 2015, Gavelis et al. 2017). This is also the case with
500 *Paragymnodinium* despite the lack of direct evidence (Jeong et al. 2017).
501 *Paragymnodinium asymmetricum* contains multiple nematocysts with basically the
502 same structure as those of other *Paragymnodinium* spp. apart from their relatively small
503 size (Kang et al. 2010, Yokouchi et al. 2018). However, we were unable to demonstrate
504 phagotrophy in *P. asymmetricum*. Thus, the function of this organelle remains elusive.
505 The ultrastructure of the nematocysts of *Paragymnodinium inerme* is abnormal

506 and has never been observed before in any other dinoflagellates. The nematocyst is rare
507 in this species (only found in three of 15 entire cells that were serially sectioned and in
508 none of the other random sections observed). It is possible that the abnormality of
509 nematocyst shows its developing stage seen in other nematocyst bearing dinoflagellates
510 (Gavelis et al. 2017), or is a result of external factors, such as poor fixation, but the
511 larger number of fibrous strands relative to the single fibrous strand of nematocysts in
512 other *Paragymnodinium* spp. could be incurred by such factors (Kang et al. 2010,
513 Yokouchi et al. 2018, this study). Therefore, the ultrastructure of the nematocyst of *P.*
514 *inerme* is clearly different to those of other *Paragymnodinium* spp. A paucity of
515 nematocysts is also unique to the genus *Paragymnodinium*. The original description of *P.*
516 *shiwhaense* by Kang et al. (2010) does not mention the number of nematocysts per cell,
517 but at least 6 nematocysts can be identified in a single TEM image (figs 73-75 in Kang
518 et al. 2010). While the degree of nematocyst production may be influenced by nutrition,
519 especially the presence/absence of prey, this is unlikely because a cell of *P.*
520 *asymmetricum* contains numerous nematocysts under the same culture conditions as *P.*
521 *inerme*. If nematocysts are commonly used by *Paragymnodinium* spp. to capture prey
522 cells, it is reasonable to assume that there is some link between the reduction of
523 nematocysts and the loss of phagotrophy in *P. inerme*. However, as mentioned above,

524 the function of nematocysts in this genus still requires definitive evidence. To confirm
525 the role of nematocysts, more direct observation of the behavior of nematocyst-bearing
526 species is needed.

527 *Flagellar apparatus.* The flagellar apparatuses of *Paragymnodinium*
528 *asymmetricum* and *P. inerme* share basic features with the other known species of the
529 genus, although the number of microtubules comprising R1 is variable (Kang et al. 2010,
530 Yokouchi et al. 2018). The absence of the nuclear fibrous connective (NFC), one of the
531 key characters of the genus *Gymnodinium* (Daugbjerg et al. 2000), is also common to all
532 the species of *Paragymnodinium*. However, the two new species of *Paragymnodinium*
533 have a ventral connective (VC) linking R1 to the plasma membrane, which has not been
534 reported before for *Paragymnodinium*. The VC is often observed in other
535 dinoflagellates (e.g. Iwataki et al. 2010). In the original description of *P. shiwhaense* and
536 *P. stigmaticum*, an elongate object can be seen near the R1 (Fig. 32 in Kang et al. 2010,
537 Fig. 39 in Yokouchi et al. 2018). That of *P. stigmaticum* in particular is quite similar to
538 the VC of *P. asymmetricum* and *P. inerme*, although its direction differs. Therefore, it is
539 possible that the presence of a VC has been overlooked in *P. shiwhaense* and *P.*
540 *stigmaticum*. In addition, *P. asymmetricum* and *P. inerme* contain a TMRE nucleating
541 from the TMR. The TMRE is also reported in *P. stigmaticum*, but not in *P. shiwhaense*

542 (Kang et al. 2010, Yokouchi et al. 2018). It is possible that the TMRE of *P. shiwhaense*
543 was overlooked due to its small size, as mentioned by Yokouchi et al. (2018). The
544 number of microtubules comprising the TMRE in this group is small relative to other
545 dinoflagellates, such as *Gymnodinium fuscum* (Hansen et al. 2000) which have
546 numerous microtubules. There are six in *P. asymmetricum*, less than 10 in *P. inerme*, and
547 4 in *P. stigmaticum* (Yokouchi et al. 2018, this study).

548

549 The authors wish to thank Dr. Stuart D. Sym for reading the manuscript. We also
550 express our thanks to Dr. Kevin Wakeman for his technical advice. Strains Ak01 and
551 Mr06 were provided by Dr. Ryo Onuma, and strains TH006 was provided by Mr.
552 Hirono Tsuchida, all used as prey candidates. Critical point drying was performed at the
553 Electron Microscope Laboratory, Research Faculty of Agriculture, Hokkaido University.
554 This work was supported by Grant-in-Aid for JSPS Fellows Grant Number JP19J20893.
555 The authors declare no conflict of interest.

556

557 Daugbjerg, N., Hansen, G., Larsen, J. & Moestrup, Ø. 2000. Phylogeny of some of the
558 major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence
559 data, including the erection of three new genera of unarmoured dinoflagellates.

560 *Phycologia* 39:302–17.

561 Dodge, J. D. 1967. Fine structure of the dinoflagellate *Aureodinium pigmentosum* gen.
562 et sp. nov. *Br. Phycol. Bull.* 3:327–36.

563 Dodge J. D. 1982. *Marine dinoflagellates of the British Isles*. Her Majesty's Stationery
564 Office, London. 303 pp.

565 Edgar, R. C. 2004. MUSCLE: a multiple sequence alignment method with reduced time
566 and space complexity. *BMC Bioinformatics* 5:113.

567 Gavelis, G. S., Wakeman, K. C., Tillmann, U., Ripken, C., Mitarai, S., Herranz, M.,
568 Özbek, S., Holstein, T., Keeling, P. J. & Leander, B. S. 2017. Microbial arms race:
569 Ballistic “nematocysts” in dinoflagellates represent a new extreme in organelle
570 complexity. *Sci. Adv.* 3: 1–7.

571 Greuet, C. 1987. Complex organelles. In Taylor, F. J. R. [Eds.] *The biology of*
572 *dinoflagellates*. Blackwell Science, Oxford, UK. pp. 119–42.

573 Hansen, G. & Larsen, J. 1992. Dinoflagellater i danske farvande. In Thomsen, H. A.
574 [Eds.] *Plankton i de indre danske farvande*. Havforskning fra Miljøstyrelsen,
575 Copenhagen. pp. 45–155.

576 Hansen, G., Moestrup, Ø. & Roberts, K. R. 2000. Light and electron microscopical
577 observations on the type species of *Gymnodinium*, *G. fuscum* (Dinophyceae).

578 *Phycologia* 39:365–76.

579 Hansen, P. J. 2011. The role of photosynthesis and food uptake for the growth of marine
580 mixotrophic dinoflagellates. *J. Eukaryot. Microbiol.* 58:203–14.

581 Hoppenrath, M. & Leander, B. S. 2007a. Character evolution in polykrikoid
582 dinoflagellates. *J. Phycol.* 43:366–77.

583 Hoppenrath, M. & Leander, B. S. 2007b. Morphology and phylogeny of the
584 pseudocolonial dinoflagellates *Polykrikos lebourae* and *Polykrikos herdmanae* n. sp.
585 *Protist* 158:209–27.

586 Hoppenrath, M., Bachvaroff, T. R., Handy, S. M., Delwiche, C. F. & Leander, B. S.
587 2009. Molecular phylogeny of ocelloid-bearing dinoflagellates (Warnowiaceae) as
588 inferred from SSU and LSU rDNA sequences. *BMC Evol. Biol.* 9:116.

589 Hoppenrath, M., Yubuki, N., Bachvaroff, T. R. & Leander, B. S. 2010. Re-classification
590 of *Pheopolykrikos hartmannii* as *Polykrikos* (Dinophyceae) based partly on the
591 ultrastructure of complex extrusomes. *Eur. J. Protistol.* 46:29–37.

592 Hoppenrath, M., Murray, S. A., Chomérat, N. & Horiguchi, T. 2014. *Marine benthic*
593 *dinoflagellates – unveiling their worldwide biodiversity*. Schweizerbart, Stuttgart,
594 Germany. 276 pp.

595 Huelsenbeck, J. P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of

596 phylogenetic trees. *Bioinformatics* 17:754–5.

597 Iritani, D., Horiguchi, T & Wakeman, K. 2018. Molecular phylogenetic positions and
598 ultrastructure of marine gregarines (Apicomplexa) *Cuspidella ishkariensis* n. gen., n. sp.
599 and *Loxomorpha* cf. *harmothoe* from western pacific scaleworms (Polynoidae). *J.*
600 *Eukaryot. Microbiol.* 65:637–47.

601 Iwataki, M., Hansen, G., Moestrup, Ø. & Matsuoka, K. 2010. Ultrastructure of the
602 harmful unarmored dinoflagellate *Cochlodinium polykrikoides* (Dinophyceae) with
603 reference to the apical groove and flagellar apparatus. *J. Eukaryot. Microbiol.* 57:308–
604 21.

605 Jeong, H. J., Jang, S. H., Moestrup, Ø., Kang, N. S., Lee, S. Y., Potvin, É. & Noh, J. H.
606 2014. *Ansanella granifera* gen. et sp. nov. (Dinophyceae), a new dinoflagellate from the
607 coastal waters of Korea. *Algae* 29:75–99.

608 Jeong, H. J., Kim, J. S., Lee, K. H., Seong, K. A., Yoo, Y. D., Kang, N. S., Kim, T. H.,
609 Song, J. Y. & Kwon, J. E. 2017. Differential interactions between the
610 nematocyst-bearing mixotrophic dinoflagellate *Paragymnodinium shiwhaense* and
611 common heterotrophic protists and copepods: killer or prey. *Harmful Algae* 62:37–51.

612 Kang, N. S., Jeong, H. J., Moestrup, Ø., Shin, W., Nam, S. W., Park, J. Y., de Salas, M.
613 F., Kim, K. W. & Noh, J. H. 2010. Description of a new planktonic mixotrophic

614 dinoflagellate *Paragymnodinium shiwhaense* n. gen., n. sp. from the coastal waters off
615 western Korea: morphology, pigments, and ribosomal DNA gene sequence. *J. Eukaryot.*
616 *Microbiol.* 57:121–44.

617 Kang, N. S., Jeong, H. J., Moestrup, Ø. & Park, T. G. 2011. *Gyrodiniellum shiwhaense* n.
618 gen., n. sp., a new planktonic heterotrophic dinoflagellate from the coastal waters of
619 western Korea: morphology and ribosomal DNA gene sequence. *J. Eukaryot. Microbiol.*
620 58:284–309.

621 Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular evolutionary genetics
622 analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:1870–74.

623 Marshall, S. M. 1925. On *Proterythropsis vigilans*, n. sp. *Q. J. Microsc. Sci.* 69:177–84.

624 Lee, M. J., Jeong, H. J., Lee, K. H., Jang, S. H., Kim, J. H. & Kim, K. Y. 2015.
625 Mixotrophy in the nematocyst-taeniocyst complex-bearing phototrophic dinoflagellate
626 *Polykrikos hartmannii*. *Harmful Algae* 49:124–34.

627 Matsuoka, K., Cho, H. & Jacobson, D. M. 2000. Observations of the feeding behavior
628 and growth rates of the heterotrophic dinoflagellate *Polykrikos kofoidii* (Polykrikaceae,
629 Dinophyceae). *Phycologia* 39:82–6.

630 Mitra, A., Flynn, K. J., Tillmann, U., Raven, J. A., Caron, D., Stoecker, D. K., Not, F.,
631 Hansen, P. J., Hallegraeff, G., Sanders, R., Wilken, S., McManus, G., Johnson, M., Pitta,

632 P., Våge, S., Berge, T., Calbet, A., Thingstad, F., Jeong, H. J., Burkholder, J. A., Glibert,
633 P. M., Granéli, E. & Lundgren, V. 2016. Defining planktonic protist functional groups
634 on mechanisms for energy and nutrient acquisition: Incorporation of diverse
635 mixotrophic strategies. *Protist* 167:106–20.

636 Nakayama, T., Watanabe, S., Mitsui, K., Uchida, H. & Inouye, I. 1996. The
637 phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred
638 from 18S rDNA sequence data. *Phycol. Res.* 44:47–55.

639 Pandeirada, M. S., Craveiro, S. C., Daugbjerg, N., Moestrup, Ø. & Calado, A. J. 2014.
640 Studies on woloszynskioid dinoflagellates VI: description of *Tovellia aveirensis* sp. nov.
641 (Dinophyceae), a new species of Tovelliaceae with spiny cysts. *Eur. J. Phycol.* 49:230–
642 43.

643 Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic
644 analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–90.

645 Stoecker, D. K., Hansen, P. J., Caron, D. A. & Mitra, A. 2017. Mixotrophy in the marine
646 plankton. *Annu. Rev. Mar. Sci.* 9:311–35.

647 Takahashi, K., Moestrup, Ø., Wada, M., Ishimatsu, A., Nguyen, V. N., Fukuyo, Y. &
648 Iwataki, M. 2017. *Dactylocladus pterobelotum* gen. et sp. nov., a new marine
649 woloszynskioid dinoflagellate positioned between the two families Borghiellaceae and

650 Suessiaceae. *J. Phycol.* 53:1223–40.

651 Takano, Y. & Horiguchi, T. 2004. Surface ultrastructure and molecular phylogenetics of
652 four unarmoured heterotrophic dinoflagellates, including the type species of the genus
653 *Gyrodinium* (Dinophyceae). *Phycol. Res.* 52:107–16.

654 Tanabe, A. S. 2011. Kakusan4 and Aminosan: two programs for comparing
655 nonpartitioned, proportional and separate models for combined molecular phylogenetic
656 analyses of multilocus sequence data. *Mol. Ecol. Resour.* 11:914–21.

657 Westfall, J. A., Bradbury, P. C. & Townsend, J. W. 1983. Ultrastructure of the
658 dinoflagellate *Polykrikos*. I. Development of the nematocyst-taeniocyst complex and
659 morphology of the site for extrusion. *J. Cell Sci.* 63:245–61.

660 Yokouchi, K., Onuma, R. & Horiguchi, T. 2018. Ultrastructure and phylogeny of a new
661 species of mixotrophic dinoflagellate, *Paragymnodinium stigmaticum* sp. nov.
662 (Gymnodiniales, Dinophyceae). *Phycologia* 57:539–54.

663 Yoo, Y. D., Jeong, H. J., Kang, N. S., Song, J. Y., Kim, K. Y., Lee, G. & Kim, J. H. 2010.
664 Feeding by the newly described mixotrophic dinoflagellate *Paragymnodinium*
665 *shiwhaense*: feeding mechanism, prey species, and effect of prey concentration. *J.*
666 *Eukaryot. Microbiol.* 57:145–58.

667 FIG. 1. (A-F) Differential interference contrast (DIC) and fluorescence light
668 micrographs of *Paragymnodinium asymmetricum* sp. nov. Scale bars = 5 μ m. Ch,
669 chloroplast; Ci, cingulum; Nu, nucleus; SEF, sulcal extension-like furrow; Su, sulcus.
670 (A) Ventral view. (B) Dorsal view. (C-E) Same cell showing DIC morphology (C),
671 autofluorescence of chloroplasts (D) and nucleus stained by DAPI (E). (F) Cyst with
672 outer wall (arrowheads). (G-O) Scanning electron micrographs of *Paragymnodinium*
673 *asymmetricum* sp. nov., showing arrangement of polygonal amphiesmal vesicles (AVs)
674 on cell surface. Scale bars = 3 μ m except where otherwise indicated. (G and H) Ventral
675 view. Vesicles in episome arranged in seven rows (E1-E7). (I) Dorsal view. Vesicles in
676 episome arranged in five rows (E1-E5). (J) Left lateral view. Vesicles in cingulum
677 arranged in five rows (C1-C5); those in episome arranged in five rows (E1-E5). (K and
678 L) Apical view, showing episome and its vesicles arranged in seven (K) or five (L) rows.
679 (M) Antapical view, showing hyposome, its vesicles and sulcus. (N) Detail of SEF
680 comprising some elongate AVs (asterisks). Scale bar = 1 μ m. (O) Schematic illustration
681 of SEF showing arrangement of AVs.

682

683 FIG. 2. Transmission electron micrographs (TEMs) of *Paragymnodinium*
684 *asymmetricum* sp. nov. A-D cells fixed using first fixation protocol; others fixed using

685 second protocol (see material and methods). Ch_{LL}, lateral lobe of chloroplast; Ch_{AM},
686 antapical mass of chloroplast; Mt, mitochondrion; Nu, nucleus. (A) Longitudinal section
687 of cell. Scale bar = 2 μ m. (B) Nucleus containing condensed chromosomes and
688 surrounded by numerous mitochondria. Scale bar = 1 μ m. (C) Detail of nuclear
689 envelope comprising two membranes and nucleopore (arrowheads). Scale bar = 100 nm.
690 (D) Longitudinal section of trichocyst. Scale bar = 200 nm. (E) Transverse section of
691 trichocyst. Scale bar = 100 nm. (F) Detail of amphiesmal vesicle. No plate-like structure
692 observed. Scale bar = 200 nm. (G-J) Serial, non-consecutive sections of extended
693 peduncle. Microtubular strand of peduncle (arrows); electron-opaque vesicles
694 (arrowheads) indicated. Numbers of selected serial sections indicated in circles. Scale
695 bars = 200 nm.

696

697 FIG. 3. TEM micrographs of chloroplast of *Paragymnodinium asymmetricum* sp.

698 nov. Cells fixed using first protocol. (A) Antapical mass of chloroplast with
699 densely-packed thylakoids. Scale bar = 1 μ m. (B) Detail of antapical mass. Each
700 thylakoid band is double-stacked (double-headed arrows). Scale bar = 100 nm. (C)
701 Lateral lobe of chloroplast with less-dense packing of thylakoids. Scale bar = 1 μ m. (D)
702 Detail of lateral lobe, showing each thylakoid band as double- or triple-stacked

703 (double-headed arrows). Scale bar = 100 nm. (E) Boundary between antapical mass and
704 lateral lobe, demonstrating difference in stacking density of thylakoids. Scale bar = 200
705 nm.

706

707 FIG. 4. Serial TEM sections of nematocysts of *Paragymnodinium*
708 *asymmetricum* sp. nov. Cells fixed using second fixation protocol. Section numbers are
709 indicated by circled numbers. Scale bars = 200 nm. AC, anterior chamber; CA, capsule;
710 FS, fibrous strand; OP, operculum; PB, posterior body; PC, posterior chamber; ST,
711 stylet. (A-D) Longitudinal sections of entire nematocyst. (E-L) Selected transverse
712 sections from anterior (E) to posterior extremes (L). (M) Schematic illustration of
713 nematocyst of *Paragymnodinium asymmetricum* sp. nov. Scale bar = 200 nm.

714

715 FIG. 5 3D reconstruction of flagellar apparatus of *Paragymnodinium*
716 *asymmetricum* sp. nov. (not to scale). LB, longitudinal basal body; TB, transverse basal
717 body; R1, root 1; R3, root 3; R4, root 4; SRC, striated root connective; VC, ventral
718 connective; C1, connective 1 linking LB and R1; BBC, basal body connective; TMR,
719 transverse microtubular root; TMRE, transverse microtubular root extension; TSR,
720 transverse striated root; TSRM, transverse striated root microtubule.

721

722 FIG. 6. (A-H) Differential interference contrast (DIC) and fluorescence light
723 micrographs of *Paragymnodinium inerme* sp. nov. Scale bars = 5 μ m. Ch, chloroplast;
724 Ci, cingulum; Nu, nucleus; SEF, sulcal extension-like furrow; Su, sulcus. (A) Ventral
725 view. (B) Dorsal view. (C and D) Same cell showing autofluorescence of chloroplasts.
726 (E and F) Same cell showing fluorescence of nucleus stained by DAPI. (G) Division
727 cyst with outer wall (arrowheads). (H) Two motile cells connected to each other. (I-O)
728 Scanning electron micrographs of *Paragymnodinium inerme* sp. nov., showing
729 arrangement of numerous polygonal amphiesmal vesicles (AVs) on cell surface. Scale
730 bar = 3 μ m except where otherwise indicated. (I) Ventral view. (J) Dorsal view, showing
731 episome and its vesicles arranged in eight rows (E1-E8), hyposome and its vesicles
732 arranged in six rows (H1-H6). (K) Left lateral view, showing cingulum and its vesicles
733 arranged in five rows (C1-C5). (L) Apical view, showing episome and its vesicles
734 arranged in nine rows (E1-E9). Cell possesses double transverse flagella (arrowheads).
735 (M) Antapical view, showing hyposome and its vesicles arranged in six rows (H1-H6)
736 and sulcus. Note double longitudinal flagella (arrowheads). (N) Detail of SEF
737 comprising nine elongate AVs (asterisks). Scale bar = 1 μ m. (O) Schematic illustration
738 of SEF showing arrangement of AVs.

739

740 FIG. 7. Transmission electron micrographs (TEMs) of *Paragymnodinium*
741 *inerme* sp. nov. B and C are cells fixed using first fixation protocol; others fixed using
742 second protocol. (A) Longitudinal section of cell. AV, amphiesmal vesicle; Ch,
743 chloroplast; Mt, mitochondrion; Nu, nucleus. Scale bar = 2 μ m. (B) Nucleus containing
744 condensed chromosomes. Scale bar = 2 μ m. (C) Detail of nuclear envelope comprising
745 two membranes and nucleopore (arrows). Scale bar = 200 nm. (D) Longitudinal section
746 of trichocyst. Scale bars = 200 nm. (E) Transverse section of trichocyst. Scale bars =
747 100 nm. (F) Detail of amphiesmal vesicle. Scale bar = 500 nm. (G-J) Serial,
748 non-consecutive sections of peduncle. Microtubular strand of peduncle (arrows)
749 indicated. Section numbers circled with direction of sectioning from left to right. Scale
750 bars = 200 nm.

751

752 FIG. 8. TEM micrographs of the chloroplast of *Paragymnodinium inerme* sp.
753 nov. C is a cell fixed using the first fixation protocol, while others were fixed using the
754 second protocol. (A) A mass of chloroplast. Scale bar = 2 μ m. (B) Detail of chloroplast
755 envelope comprised of three membranes (arrowheads). Scale bar = 50 nm. (C) Detail of
756 chloroplast with double- or triple-stacked thylakoid bands, indicated by the

757 double-headed arrows. Scale bar = 100 nm. (D-F) Many masses of chloroplast are

758 connected by narrow bridges (arrows). Scale bars = 1 μ m.

759

760 FIG. 9. Serial TEM sections of nematocysts of *Paragymnodinium inerme* sp.

761 nov. Cells fixed using second protocol. Section numbers indicated in circles. Scale bars

762 = 200 nm. AC, anterior chamber; CA, capsule; FS, fibrous strand; OP, operculum; PB,

763 posterior body; PC, posterior chamber. (A-F) Transverse sections from anterior part (A)

764 to posterior part (F). (G-M) Longitudinal sections.

765

766 FIG. 10. Reconstruction of flagellar apparatus of *Paragymnodinium inerme* sp.

767 nov. LB, longitudinal basal body; TB, transverse basal body; R1, root 1; R3, root 3; R4,

768 root 4; SRC, striated root connective; VC, ventral connective; C1, connective 1 linking

769 LB and R1; C2, connective 2 linking LB and R1; BBC1, basal body connective 1;

770 BBC2, basal body connective 2; BBC3, basal body connective 3; TMR, transverse

771 microtubular root; TMRE, transverse microtubular root extension; TSR, transverse

772 striated root; TSRM, transverse striated root microtubule.

773

774 FIG. 11. Maximum-likelihood phylogenetic tree of selected dinoflagellates,

775 including *Paragymnodinium asymmetricum* sp. nov. and *P. inerme* sp. nov., based on
776 concatenated SSU rDNA and partial LSU rDNA sequences. Each species name is
777 followed by its GenBank accession numbers for SSU rDNA and partial LSU rDNA
778 sequences respectively. Only one accession number indicates that sequence includes
779 both SSU rDNA and partial LSU rDNA sequences. Numbers at each node are ML
780 bootstrap values and Bayesian posterior probabilities respectively. Only values > 50%
781 (bootstrap) and > 0.7 (PP) are indicated. Stars indicate dinoflagellates with nematocysts.
782 *P. inerme* is marked by white star because of abnormality of nematocysts.

783

784 Video S1. Serial TEM sections of a whole cell of *Paragymnodinium inerme* sp.
785 nov. showing more than 20 masses of chloroplasts and only some of them are connected
786 to each other by the thin bridges. The total number of discrete chloroplasts in this
787 individual is three (indicated by A-C).

788 TABLE 1. Pairwise distance matrix of the 18S (lower left) and 28S (upper right) rDNA
 789 sequences of *Paragymodinium* spp. calculated using p-distance model.

Strain	1	2	3	4
1. <i>P. shiwhaense</i>		0.1338	0.0405	0.0075
2. <i>P. stigmaticum</i>	0.0919		0.0905	0.0985
3. <i>P. asymmetricum</i>	0.0151	0.0901		0.0254
4. <i>P. inerme</i>	0.0006	0.0914	0.0145	