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ULTRASTRUCTURE AND SYSTEMATICS OF TWO NEW SPECIES OF DINOFLAGELLATE, *PARAGYMNODINIUM ASYMMETRICUM* SP. NOV. AND *PARAGYMNODINIUM INERME* SP. NOV. (GYMNODINIALES, DINOPHYCEAE)[1]

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Running title: Two new species of *Paragymnodinium*

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

two dinoflagellates are new species of *Paragymnodinium*.

Key index words: chloroplast, flagellar apparatus, *Gymnodinium sensu stricto*, nematocyst, nutritional mode, *Paragymnodinium*, taxonomy

Abbreviations: BBC1-3, basal body connectives 1-3; C1 and 2, connective 1 and 2; DAPI, 4', 6-diamidino-2-phenylindole; LB, longitudinal basal body; ML, maximum likelihood; MSP, microtubular strand of a peduncle; R1-4, root 1-4; SRC, striated root connective; TB, transverse basal body; TMR, transverse microtubular root; TMRE, transverse microtubular root extension; TSR, transverse striated root; TSRM, transverse striated root microtubule; VC, ventral connective

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

Despite the presence of plastids, these two species feed on other prey cells and thus show mixotrophic growth (Yoo et al. 2010, Yokouchi et al. 2018). The mixotrophic nutritional mode is frequently encountered among various eukaryotes, including the dinoflagellates, and it has an important role in aquatic ecosystems (Hansen 2011, Mitra et al. 2016, Stoecker et al. 2017). Mixotrophic dinoflagellates show a variety of strategies to gain nutrients (Hansen 2011), and *P. shiwhaense* is characterized by 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 *Paragymnodinium asymmetricum* (strain vnd299) was isolated from water
103 samples from Nha Trang beach, Nha Trang, Vietnam (12°14.56'N, 109°11.49'E) on 26
104 April, 2014. *P. inerme* (strain JGD) was isolated from water samples from Jogashima,
105 Kanagawa, Japan (35°08.02'N, 139°36.41'E) on 19 November, 2017. Isolated cells
106 were kept in Daigo's IMK Medium for Marine Microalgae (Nihon Pharmaceutical Co.,
107 Tokyo, Japan). Cultures of *P. asymmetricum* and *P. inerme* were maintained without
108 adding any prey. The established monoclonal cultures were incubated at 20˚C, with an
109 illumination of 50 μmol photons m^{-2} 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) OsO_4 in distilled water. Cells of *P. inerme* were fixed for 1.5 h
127 on ice with 2 or 3% (final concentration) OsO_4 in distilled water. Fixed cells were

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

For TEM, cells were fixed using one of two protocols. In the first protocol, cells were fixed in 2.5% glutaraldehyde (final concentrations) in seawater for 1 h, and washed twice in sea water. Cells were post fixed in 1% OsO_4 (final concentrations) in distilled water for 1 h. In the second, cells were fixed in a mixture of 2% glutaraldehyde and 0.5% OsO_4 (final concentrations) in 0.1 M Na-cacodylate buffer, pH 7.4 for 15 or 30 min, and rinsed twice in 0.1 M Na-cacodylate buffer. Cells were post-fixed in 1% OsO_4 (final concentration) in 0.1 M Na-cacodylate buffer, pH 7.4 for 1 h. In both protocols, cells were first attached to the bottom of a polypropylene dish coated with poly-L-lysine. After fixation, cells of both protocols were dehydrated in an acetone 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 65ºC 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

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

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

2016). The highly divergent D2 region of LSU rDNA sequences was deleted. Consequently, 1771 positions of SSU rDNA and 1107 positions of LSU rDNA were aligned. Pairwise distance of the two aligned sequences of four *Paragymnodinium* spp. were calculated using MEGA7 with p-distance model. The two aligned sequences for all taxa were concatenated using Kakusan4 (Tanabe 2011). No significant nucleotide compositional heterogeneity was detected for the combined data set (P = 0.99792 using the chi-square test in Kakusan4). The appropriate models of substitution ratio for concatenated rDNA sequences were determined using Kakusan4, and resulted in a separate model for maximum likelihood (ML) analysis and a proportional model for Bayesian analysis. The appropriate models of DNA evolution for each rDNA sequences were determined by AIC for ML analysis and by BIC for Bayesian analysis using Kakusan4, and resulted in the selection of the GTR + Gamma model. The parameters in these analyses for SSU rDNA were: assumed nucleotide frequencies A = 0.264, C = 0.198, G = 0.262 and T = 0.275; substitution rate matrix with A <-> C = 1.251914, A <-> G = 3.199366, A <-> T = 1.376839, C <-> G = 0.441724, C <-> T = 8.534122 and G <-> T = 1.000000. The proportion of sites were assumed to follow a gamma distribution with the shape parameter = 0.285333. The parameters for LSU rDNA were: assumed nucleotide frequencies A = 0.285, C = 0.191, G = 0.285 and T = 0.239. The

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

RESULTS

Paragymnodinium asymmetricum K.Yokouchi, K.Takahashi, Nguyen, Iwataki et T.Horiguchi sp. nov.

Description. Marine, athecate dinoflagellate. Cells with almost equal-sized episomes and hyposomes, 7.9–12.6 µm long and 4.7–9.0 µm wide. Episome hemispherical or conical. Hyposome asymmetric with larger right side. Cingulum wide and well excavated, descending 1/4 to 1/2 of its own width. Sulcus straight, reaching to, and

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

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

Collection date: 26 April 2014.

Type locality: Nha Trang beach, Nha Trang, Vietnam (12°14.56’N, 109°11.49’E).

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

LM and SEM: Cells small, 7.9–12.6 µm (9.6 ± 1.0 µm, mean ± SD, n = 55) long and 4.7–9.0 µm (6.9 ± 1.0 µm, n = 55) wide. Episome and hyposome were almost equal in size (Fig. 1, A and B). Episome was conical (Fig. 1, A and B); hyposome was asymmetric; right side larger than left side (Fig. 1, A and B). Cingulum was wide, well excavated and descended by a distance one quarter to a half its own width (Fig. 1, A and B). Sulcus was straight and widened slightly before reaching the antapex (Fig. 1A).

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

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

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

Collection date: 19 November 2017.

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

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

LM and SEM: Cells were 15.3–23.7 µm (19.4 ± 2.0 µm, mean ± SD, n = 28) long and 10.9–19.6 µm (14.9 ± 2.1 µm, n = 28) wide. Episome and hyposome were almost equal in size (Fig. 6, A and B). Episome was conical to hemispherical, and the hyposome was hemispherical (Fig. 6, A and B). Cingulum was wide, well excavated and descended by a distance half to equal of its own width (Fig. 6, A and B). Sulcus was straight and widened slightly before reaching the antapex (Fig. 6A). No eyespot was observed. A slightly curved sulcal extension-like furrow (SEF) ran from the right end of the cingulum toward the apex (Fig. 6A). Chloroplasts were yellow brown and distributed throughout the cell (Fig. 6A-D). Analysis of autofluorescence images demonstrated the presence of multiple chloroplasts in each cell (Fig. 6, C and D). The nucleus was central on the dorsal side of the cell (Fig. 6, B, E and F). DAPI staining showed a single nucleus in the central or dorsal of cell (Fig. 6, E and F). The motile cell 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

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

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

The flagellar apparatus of *P. inerme* was re-constructed (Fig. 10) from serial sections (Figs. S3 and S4). The transverse basal body (TB) and the longitudinal basal 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 *inerme* was shown to be sister to *P. shiwhaense* (Table 1), and *P. asymmetricum* was

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

DISCUSSION

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

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

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

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

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

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

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

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

stricto.

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

The ultrastructure of the nematocysts of *Paragymnodinium inerme* is abnormal

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

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

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667 FIG. 1. (A-F) Differential interference contrast (DIC) and fluorescence light
668 micrographs of *Paragymnodinium asymmetricum* sp. nov. Scale bars = 5 μm. 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 μm 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 μm. (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

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

FIG. 3. TEM micrographs of chloroplast of *Paragymnodinium asymmetricum* sp. nov. Cells fixed using first protocol. (A) Antapical mass of chloroplast with densely-packed thylakoids. Scale bar = 1 µm. (B) Detail of antapical mass. Each thylakoid band is double-stacked (double-headed arrows). Scale bar = 100 nm. (C) Lateral lobe of chloroplast with less-dense packing of thylakoids. Scale bar = 1 µm. (D) Detail of lateral lobe, showing each thylakoid band as double- or triple-stacked

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

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

FIG. 5 3D reconstruction of flagellar apparatus of *Paragymnodinium asymmetricum* sp. nov. (not to scale). LB, longitudinal basal body; TB, transverse basal body; R1, root 1; R3, root 3; R4, root 4; SRC, striated root connective; VC, ventral connective; C1, connective 1 linking LB and R1; BBC, basal body connective; TMR, transverse microtubular root; TMRE, transverse microtubular root extension; TSR, 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 µm. 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 µm 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 µm. (O) Schematic illustration
738 of SEF showing arrangement of AVs.

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

FIG. 8. TEM micrographs of the chloroplast of *Paragymnodinium inerme* sp. nov. C is a cell fixed using the first fixation protocol, while others were fixed using the second protocol. (A) A mass of chloroplast. Scale bar = 2 μm. (B) Detail of chloroplast envelope comprised of three membranes (arrowheads). Scale bar = 50 nm. (C) Detail of 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 μm.
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. *P. shiwhaense*		0.1338	0.0405	0.0075
2. *P. stigmaticum*	0.0919		0.0905	0.0985
3. *P. asymmetricum*	0.0151	0.0901		0.0254
4. *P. inerme*	0.0006	0.0914	0.0145	

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