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A new small thecate dinoflagellate *Azadinium anteroporum sp. nov*. (Amphidomataceae, Dinophyceae) isolated from the Asian Pacific

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RUNNING TITLE

Azadinium anteroporum sp. nov. (Dinophyceae)

ABSTRACT

The marine thecate dinoflagellate *Azadinium* includes species known to produce the diarrhetic shellfish toxins known as azaspiracids (AZAs). In this study, the morphology of a thecate dinoflagellate isolated from Mutsu Bay, Japan, was examined by LM and SEM, and its phylogenetic position was inferred from ITS and LSU rDNA sequences. The production of AZAs was examined by liquid chromatography-triple quadrupole mass spectrometer (LC-MS/MS). Cells were elliptical, 15.9–22.4 µm long and 11.1–17.1 µm wide. A chloroplast was positioned peripherally, with a pyrenoid near the cingulum, and the nucleus was placed in the hyposome. The hyposome was smaller than the episome, with an antapical spine on plate 2′′′′. Thecal tabulation was Po, cp, X, 4′, 3a, 6′′, 6C, 5S, 6′′′, 2′′′′. The apical pore complex consisted of a symmetrical apical pore plate (Po), a cover plate (cp) and a small canal plate (X), and was surrounded by a prominent collar formed by the apical plates. A ventral pore was located on the mid-anterior edge of plate 1′ and contacted with the X-plate and/or the ventral left of plate Po. Plate 1′′ was in contact with plate 1a. AZA was not detected in the cultures. Molecular phylogeny showed that the species was related to *Azadinium* species that possess the ventral pore on the left side of plate 1′, i.e. *A. cuneatum*, *A. dalianense*, *A. obesum*, *A. poporum*, *A. spinosum* and *A. trinitatum*, but that it was not included in these species. The new species *Azadinium anteroporum sp. nov.* differs in the ventral pore position (mid-anterior edge of plate 1′) from these related species (which have the ventral pore on the left side of plate 1′), and from *A. polongum* (with ventral pore on the posterior left side of 1′) and all other *Azadinium* species (with ventral pore on the right side of 1′).

KEYWORDS

Antapical spine; Azaspiracids (AZAs); Harmful algal blooms; Shellfish poisoning; Ventral pore

INTRODUCTION

The small thecate dinoflagellate genus *Azadinium* Elbrächter & Tillmann, which includes species producing diarrhetic shellfish toxins known as azaspiracids (AZAs), was described from the North Sea off the coast of Scotland with the type species *Azadinium spinosum* Elbrächter & Tillmann (Tillmann *et al.* 2009). After the discovery of this AZA-producing dinoflagellate, rigorous surveys of the small marine dinoflagellates have been carried out, which resulted in the description of 16 species of *Azadinium* species thus far (Tillmann *et al.* 2009, 2010, 2011, 2012b, 2014, 2020; Nézan *et al.* 2012; Percopo *et al.* 2013; Luo *et al.* 2013, 2017; Tillmann & Akselman 2016; Tillmann 2018; Salas *et al.* 2021). Among them, AZAs have been detected in three species, *A. dexteroporum* Percopo & Zingone, *A. poporum* Tillmann & Elbrächter and *A. spinosum* (Tillmann *et al.* 2009, 2011, 2017; Percopo *et al.* 2013; Kilcoyne *et al.* 2014; Krock *et al.* 2015, 2019; Rossi *et al.* 2017). In the genus *Amphidoma*, a sister genus of *Azadinium*, *Amphidoma languida* Tillmann, Rafael Salas & Elbrächter is also known to produce AZAs (Tillmann *et al.* 2012a). Species diversity and distribution of dinoflagellates in the Amphidomataceae (i.e. *Azadinium* and *Amphidoma* spp) have been reported mainly from the Atlantic, e.g. 14 *Azadinium* species up to now.

To detect AZA producers in the Amphidomataceae, several attempts have been carried out in the North Pacific. To date, all four amphidomatacean species potentially producing AZAs (*A. poporum*, *A. spinosum*, *A. dexteroporum* and *Am. languida*) and four non-AZA producers (*A. dalianense* Z. Luo, H. Gu & Tillmann, *A. cuneatum* Tillmann & Nézan, *A. trinitatum* Tillmann & Nézan and *A. zhuanum* Z. Luo, Tillmann & H. Gu) have been detected (Potvin *et al.* 2012; Gu *et al.* 2013; Luo *et al.* 2013, 2017; Kim *et al.* 2017; Sildever *et al.* 2019; Adams *et al.* 2020; Fu *et al.* 2021; Takahashi *et al.* 2021). Determining the species composition of *Azadinium* is therefore an important undertaking in the detection of AZA-producers in this region. Among them, only one AZA-producer, *A. poporum*, and the four non-AZA producers have been identified by using cultures, with comprehensive investigation

of electron microscopic morphology, rDNA phylogeny and rates of AZA production (Krock *et al.* 2012, 2014, 2019; Potvin *et al.* 2012; Gu *et al.* 2013; Luo *et al.* 2013, 2017; Kim *et al.* 2017; Takahashi *et al.* 2021; Ozawa *et al.* 2021, 2023). Meanwhile, the other three AZA producers have been detected only by qPCR and DNA-based metabarcoding analyses in the North Pacific, i.e. *A. dexteroporum*, *A. spinosum* and *Am. languida* from the Sea of Okhotsk, Japan (Sildever *et al.* 2019), *A. spinosum* from the Gulf of Thailand and Washington State, USA (Adams *et al.* 2020; Fu *et al.* 2021). Even though these AZA producers have cryptic and unexplored diversity, many species may have been overlooked because of their small size.

In Japanese coastal waters, a survey of amphidomatacean dinoflagellates has shown the presence of *A. poporum*, *A. trinitatum* and *A. zhuanum* (Takahashi *et al.* 2021). To date, a total of five species of *Azadinium* have been reported, including the molecular detection of *A. dexteroporum* and *A. spinosum* (Sildever *et al.* 2019), suggesting undetected species diversity of amphidomatacean dinoflagellates in Japanese coastal waters. In this study, a new non-toxic species of *Azadinium* isolated from Mutsu Bay, Aomori, Japan, was described based on its morphology, phylogeny and toxin content.

MATERIAL AND METHODS

Cultures and light microscopy

Two unialgal culture strains of *Azadinium* examined in this study were isolated from the seawaters of Mutsu Bay, Aomori, Japan. Strain AsAz544 was isolated from Asamushi (40°54′N, 140°51′E) in August 2018, and AmAz661 was from Noheji (40°55′N, 141°07′E) in September 2020. The temperature and salinity of the seawaters were 23.5°C and 30 psu (AsAz544), and 22°C and 33 psu (AmAz661), respectively. Cells were isolated by capillary pipetting under an Olympus CKX53 inverted microscope (Olympus, Tokyo, Japan), and maintained in 1/10 K medium (Wako, Tokyo, Japan) containing soil extract, under the conditions of 23°C, 30 psu and a 12:12 h light:dark cycle with 40–50 μmol photons m^{-2} s^{-1}

illumination. Both cultures were used for molecular phylogeny, but unfortunately strain AsAz544 was lost prior to the conduct of morphological observation by light and scanning electron microscopy. All morphological observations were conducted using strain AmAz661.

Cells were observed using a Zeiss Axioskop 2 light microscope (Carl Zeiss, Göttingen, Germany) equipped with a Zeiss Axiocam 305 colour digital camera and an Olympus IX71 inverted microscope with a DP73 digital camera (Olympus). Autofluorescence of chloroplast, DAPI-stained nucleus, and thecal plates stained with calcofluor (Fluorescent Brightener 28) were observed under UV excitation (Zeiss filter set 02), according to Takahashi *et al.* (2014).

Scanning electron microscopy (SEM)

For SEM observation, cells were fixed in 60% ethanol for 30 min on a poly-L-lysine-coated glass plate at room temperature, rinsed twice with distilled water for 15 min each, and post-fixed with 5% glutaraldehyde for 30 min. Cells were dehydrated by immersing in an increasing series of ethanol solutions (10%, 30%, 50%, 75%, 90% and 95%) for 15 min each, followed by 100% ethanol twice for 30 min and finally replaced with isoamyl acetate for 15 min. Cells were dried using a JCPD-5 critical point dryer (JEOL, Tokyo, Japan) and sputter-coated with platinum-palladium before being observed using an S-4800 SEM (JEOL, Tokyo, Japan) operated at 3.0–5.0 kV.

DNA sequencing and phylogenetic analyses

For rDNA sequencing, isolated cells were either directly transferred into PCR tubes after rinsing with distilled water, or DNA was extracted using a 2× hexadecyltrimethylammonium bromide (CTAB) method (Takahashi *et al.* 2019). For the amplification and sequencing of ITS region and LSU rDNA, primers reported in previous studies were used (Kawami *et al.* 2006; Iwataki *et al.* 2007; Takahashi *et al.* 2015). Extracted DNA was amplified in a reaction containing 1 µl of Ex Taq polymerase (Takara, Shiga, Japan), according to the

manufacturer's protocol. The thermal cycling conditions included an initial denaturation at 95°C (3 min), followed by 35 cycles of the following 3 steps: 95°C (1 min), 48°C (1 min) and 72°C (3 min), and finally an elongation step of 72°C (6 min). Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen Genomics, Bothell, Washington, USA), following the manufacturer's protocol. Amplicons were sequenced by Eurofins Genomics Inc. (Tokyo, Japan). ITS and LSU rDNA sequences were aligned with sequences from GenBank using a default setting in MAFFT online v7 and were then manually corrected. Phylogenetic trees were constructed based on maximum likelihood (ML) using MEGA 11 (Stecher *et al.* 2020; Tamura *et al.* 2021) with the best substitution model selected by the software: the general time-reversible (GTR) model plus gamma distribution (G = 1.0700) plus the proportion of invariable sites (I = 0.2307) for ITS, and Tamura-Nei (TN93) model plus gamma distribution (G = 0.4840) plus the proportion of invariable sites (I = 0.3085) for LSU rDNA. Bootstrap support (BS) values of ML and neighbour-joining (NJ) analyses for ITS and LSU rDNA trees were estimated using 500 replicates each. Posterior probabilities (PP) of Bayesian inference were calculated using 5,000,000 Markov chain Monte Carlo generations with four chains and trees were sampled every 100 generations with a sample frequency of 10, and set to a burn-in of 10%. Convergence of the chains was confirmed when the average standard deviations of the split frequencies were below 0.01 after calculations. Outgroups selected were *Heterocapsa steinii* Tillmann, Gottschling, Hoppenrath, Kusber & Elbrächter (MF423346), *Heterocapsa circularisquama* T. Horiguchi (AB084091) and *Scrippsiella trochoidea* (F. Stein) A.R. Loeblich (HM483396). The calculation of the pairwise genetic distance was conducted using MEGA 11 with 585 bases in ITS and 672 bases in LSU rDNA. ITS and LSU rDNA sequences are available for AsAz544 (LC757006) and AmAz661 (LC756461).

AZA analysis

Analysis of AZAs was carried out based on a previous method (Ozawa *et al.* 2021; Takahashi *et al.* 2021) with slight modifications. Cells were harvested by centrifugation at 1,200× *g* for 3 min and frozen at –20°C until extraction. The thawed cell pellets were extracted twice with 400 µl acetone. The extract was centrifuged at 2,000× *g* for 5 min and the supernatant was collected. The combined supernatant was filled up to 1 ml with acetone in a 1-ml volumetric flask.

AZAs were analysed by selected reaction monitoring (SRM) LC-MS/MS system, consisting of the LC system Nexera XR-20 (Shimadzu, Kyoto, Japan) coupled to a hybrid triple quadrupole-linear ion trap mass spectrometer QTRAP-4500 (Sciex, Massachusetts, USA). AZA analogues were separated with a HPLC column (Hypersil Gold C8, Thermo Fischer Scientific, Massachusetts, USA). Analytical conditions were the same as those reported in a previous method (Ozawa *et al.* 2021). The LC-MS/MS was operated in electrospray ionization positive mode. Certified reference materials of AZAs (CRM-AZA-1, -2 and -3) were purchased from the National Research Council of Canada. Each AZA standard was diluted 100 times with methanol and mixed.

RESULTS

Azadinium anteroporum* Kuwata, Kazuya Takahashi, W.M. Lum & Iwataki *sp. nov.

Figs 1–25

DESCRIPTION: Marine photosynthetic thecate dinoflagellate. Cells ellipsoidal, 15.9–22.4 µm long and 11.1–17.1 µm wide. A brownish chloroplast, peripherally located, with a pyrenoid near cingulum. Nucleus spherical in hyposome. Cingulum wide and occupying one-fifth of the cell length. Episome conical with a conspicuous apical pore complex (APC). Hyposome hemispherical, smaller than the episome, with an antapical spine on plate 2‴, slightly to the right-hand side of the antapex. Thecal tabulation: Po, cp, X, 4′, 3a, 6″, 6C, 5S, 6‴, 2⁗. Po symmetrical. A ventral pore located on the anterior end of 1′, and contacting with the X-plate and/or the ventral left side of plate Po. X-plate smaller than ventral pore. Plate 1″ in contact with plate 1a.

HOLOTYPE: Cells of AmAz661 strain, preserved in a metabolically inactive state and mounted on an SEM stub, are deposited at the Department of Botany, National Museum of Nature and Science, Tsukuba, as TNS-AL-58995sta.

ISOTYPE: Preserved cells of AmAz661 strain on an SEM stub are deposited at the Department of Botany, National Museum of Nature and Science, Tsukuba, as TNS-AL-58995stb.

TYPE LOCALITY: Mutsu Bay (40°55′N, 141°07′E), Aomori, Japan.

ETYMOLOGY: The species epithet *anteroporum* refers to the position of ventral pore on the anterior edge of plate 1′.

GENBANK ACCESSION NUMBER: LC756461 (AmAz661).

Light microscopy (LM)

Cells of *A. anteroporum* ranged 15.9–22.4 µm in length (mean 18.5 ± 1.6 µm, n = 30) and 11.1–17.1 µm in width (mean 13.9 ± 1.3 µm, n = 30), with a mean length:width ratio of 1.3 (Figs 1–10; Table S1). Cells were elliptical and slightly dorsoventrally flattened, with the conical episome larger than the hemispherical hyposome (Figs 1–5). The apical pore complex (APC) was conspicuous at the cell apex, and a small antapical spine was visible in LM (Figs 2–5). The wide cingulum (2.7–3.8 µm; n = 30) was excavated, positioned slightly posterior to the cell middle, and descended about half its own width (Fig. 1). A large dinokaryotic nucleus was spherical and located in the hyposome (Figs 2, 3, 6). A single brownish chloroplast was lobate or reticulate in the periphery of the cell (Figs 1–5). A spherical pyrenoid, covered with a starch sheath, was located near the cingulum, and was usually observed on the right side of the cell (Figs 2, 4). Thecal plates were made visible by calcofluor staining (Figs 7–10), and their detailed arrangements were examined by SEM.

Scanning electron microscopy (SEM)

SEM showed the typical thecal arrangement of *A. anteroporum* as Po, cp, X, 4′, 3a, 6″, 6C, 5S, 6‴, 2⁗ (Figs 11–25). The apical pore was round or teardrop-shaped with a pointed

192 ventral side, and was covered by a cover plate (cp; Figs 14, 16–19). From the surface, the
193 apical pore plate (Po) was C-shaped and bilaterally symmetrical, with an opening at the
194 ventral side, where a small canal plate (X) was situated (Figs 16–19). The dorsal and lateral
195 margins of the APC, including plates Po, cp and X, were surrounded by a prominent collar
196 composed of the anterior rims of apical plates 2′–4′ (Figs 16–19). A large ventral pore was
197 located on the anterior end of plate 1′, and an anterior ridge of plate 1′ was present below the
198 ventral pore (Figs 16–19). The ventral pore was in contact with plate X and/or the ventral left
199 margin of plate Po (Figs 16–19, S1–S4). Among thirteen ventral pores clearly observed, six
200 contacted both plates X and Po, six only plate X, and one only Po. The ventral pore lacked a
201 platelet-like surrounding structure, which has been observed from ventral pores of *Azadinium*
202 (Figs 19, S1–S4). Plate X was notably short (mean 0.36 ± 0.09 μm in length; n = 9) and
203 usually smaller than the ventral pore (mean 0.43 ± 0.04 μm in diameter; n = 9).
204 In the apical plate series, the first apical plate (1′) was long and narrow, while the other
205 three (2′–4′) were small (Figs 11–15). Plate 1′ was almost symmetrical and contacted with
206 seven plates; Po, X, 2′, 4′, 1′′, 6′′ and Sa (Figs 11, 12, 14–19). Plates 2′ and 4′ were
207 pentagonal and hexagonal, respectively, and similar in size. Plate 3′ was hexagonal and
208 slightly smaller than plates 2′ and 4′ (Fig. 14). The margins of plates 2′ and 4′ that contacted
209 with plate 3′ (sutures between the 2′ and 3′, and the 3′ and 4′) were slightly ridged and were
210 connected to the prominent collar of APC (Figs 14, 16–19, S1–S4). Three anterior intercalary
211 plates (1a–3a) were arranged symmetrically, of which the second (2a) was the smallest (Figs
212 13, 14). Plate 2a was surrounded by four plates (3′, 1a, 3a and 3′′), but appeared to be
213 hexagonal due to the angular sutures with plates 1a and 3a (Figs 13, 14). The precingular
214 series consisted of six plates similar in size, with the third plate (3′′) relatively large and bell-
215 shaped (Figs 13, 14). Plate 1′′ was in contact with the plate 1a, whereas 6′′ was separated
216 from the 3a (Figs 14, 15).

The cingular series was composed of six plates, of which C1 and C6 were situated on the ventral side and were smaller than other cingular plates (Figs 11–13, 20, 22, 23, 25). The five sulcal plates consisted of the anterior sulcal (Sa), median sulcal (Sm), right sulcal (Sd), left sulcal (Ss) and posterior sulcal (Sp) plates (Figs 11, 12, 20–22). Plate Sa was triangular, and the anterior end slightly invaded the epitheca and contacted with the plate 1′ (Figs 11, 12). Plate Ss was laterally elongated, and the plate Sd was situated above the Ss (Figs 20, 21). The median sulcal plate (Sm) was difficult to observe, due to its position beneath plate Sa (Figs 12, 21). Plate Sp was surrounded by five plates (Ss, 1‴, 6‴, 1⁗ and 2⁗), and the anterior suture towards the Ss plate was ridged (Figs 20–22).

The hypotheca consisted of six postcingular and two antapical plates (Figs 20–25). Of the six postcingular plates, the first (1‴) and sixth (6‴) were smaller than the other four (2‴–5‴; Figs 20, 22–25). The anterior margins of the postcingular plates formed the posterior cingular list (Figs 22–25). In the antapical series, plate 2⁗ was apparently larger than 1⁗ (Figs 24, 25). An antapical spine was located at the centre of plate 2⁗ and associated with several small pores (Fig. 24).

Morphological variations, probably due to the culture conditions, were present. In the antapical plates, small finger-like protrusions into the adjacent postcingulars 2‴–5‴ were occasionally observed (Figs 23, 25). Variations of the antapical spine in number, shape and location, e.g. cells lacking a spine or possessing two spines, were observed (Figs 24, 25, S5–S10). Deformity of the thecal plates in shape and number, e.g. plate enlargement, plate splits, plate insertions, different plate numbers and plate fusions were also observed (Figs S11–S19).

The thecal surface was usually smooth with small pores of similar size, presumably trichocyst pores (Figs 11–25). In the epitheca, rows of 2–4 pores were distributed especially in the apical series, whereas pores were not observed in plate 2a (Figs 13, 14). Pores were relatively scarce in the hypothecal plates, except for the row of pores arranged immediately below the posterior cingular list (Figs 20, 23–25).

Diagrammatic illustrations of the thecal arrangement are given in Figs 26–29.

Molecular phylogeny

Both ITS and LSU rDNA phylogenies showed that *A. anteroporum* did not belong to any clade of previously described *Azadinium* species.

The ML phylogeny inferred from ITS sequences showed monophyly of the Amphidomataceae (ML/NJ/BI = 100%/100%/1.00), which consisted of clades of *Amphidoma* (100/100/1.00) and *Azadinium* (76/<70/0.99) species (Fig. 30). Two strains of *A. anteroporum* (AmAz661 and AsAz544) formed a clade with maximum support (100/100/1.00), although their sequence divergence was 4.1% in ITS region. This *A. anteroporum* clade belonged to the clade of six other *Azadinium* species (98/<70/0.84), including *A. cuneatum* (one taxon), *A. dalianense* (100/98/1.00), *A. obesum* (100/100/1.00), *A. poporum* (100/100/1.00), *A. trinitatum* (91/93/1.00) and *A. spinosum* (99/79/0.84), with an undescribed *Azadinium* cf. *spinosum* (77/90/1.00). In this clade, *A. anteroporum* was most closely related to the clade of *A. trinitatum* (<70/<70/1.00), followed by *Azadinium* cf. *spinosum* and *A. obesum* with no support. This branching order was similar in the ITS tree constructed by NJ method (Fig. S20). The clade of *A. anteroporum* was not accommodated in the clades of other *Azadinium* species.

The ML phylogeny inferred from partial LSU rDNA (D1–D3) sequences showed monophyly of the clades of *Azadinium* (72/74/1.00) and *Amphidoma* (99/100/1.00) (Fig. 31). Two strains of *A. anteroporum* formed a supported clade (100/100/1.00), with a sequence divergence of 1.7% in LSU rDNA. Similar to ITS phylogeny, *A. anteroporum* was positioned in the clade that consisted of six *Azadinium* species (89/93/0.96), including *A. cuneatum* (100/100/1.00), *A. dalianense* (100/100/1.00), *A. obesum* (100/100/1.00), *A. poporum* (98/97/1.00), *A. trinitatum* (100/100/1.00) and *A. spinosum* (100/99/1.00), plus *Azadinium* cf. *spinosum* (99/100/1.00). Topology in the clade was identical to the NJ tree of LSU rDNA

(Fig. S21). In the clade, *A. anteroporum* was related to the clade of *Azadinium* cf. *spinosum* and *A. trinitatum* (<70/<70/<0.80).

Azaspiracid production

Azaspiracids, including putative novel azaspiracids, were not detected in two strains of *A. anteroporum*, with the detection limits of <0.53 fg cell^{-1} for strain AsAz544 and <3.97 fg cell^{-1} for strain AmAz661.

DISCUSSION

Morphological comparison

The new *Azadinium* species from the Asian Pacific, *A. anteroporum sp. nov.*, is a small thecate dinoflagellate with a thecal arrangement of Po, cp, X, 4′, 3a, 6′′, 6C, 5S, 6′′′, 2′′′′, which is consistent with those reported from *Azadinium*, but different from *Amphidoma* which possesses six apical plates and lacks anterior intercalary plates (Kofoid & Michener 1911; Tillmann *et al.* 2009, 2012a). In the genus *Azadinium*, species have been described with their morphological characters, such as thecal arrangements, position of ventral pore and presence or absence of antapical spine, as well as phylogenetic positions inferred from ITS and LSU rDNA sequences (e.g. Tillmann *et al.* 2009, 2014; Nézan *et al.* 2012; Tillmann & Akselman 2016; Salas *et al.* 2021).

Cells of *A. anteroporum* have plate 1′′ in contact with plate 1a, a ventral pore located on the anterior end of plate 1′, a small antapical spine on plate 2′′′′. *Azadinium anteroporum* is phylogenetically related to *A. cuneatum*, *A. dalianense*, *A. obesum*, *A. poporum*, *A. spinosum* and *A. trinitatum* as reflected in ITS and LSU rDNA phylogenies. Its morphology differs from those of related species, which all possess the ventral pore on the left side of plate 1′ (Table 1). The position of the ventral pore on the anterior end of plate 1′ has never been reported from any previously described *Azadinium* species; hence, the position is unique to *A.*

292 *anteroporum*. Each *Azadinium* species possesses a distinctive structure of the apical pore
293 complex (APC), and the structures of related species are illustrated in Fig. 32. The ventral
294 pore is located on the apex of plate 1′ (on the left side of a strongly asymmetric Po) in *A.*
295 *cuneatum*, on the left lateral margin of plate 1′ in *A. obesum* and *A. spinosum*, and on the
296 ventral left of plate Po in *A. dalianense*, *A. poporum* and *A. trinitatum* (Tillmann *et al.* 2009,
297 2010, 2011, 2014; Luo *et al.* 2013; Takahashi *et al.* 2021). In the ventral pore, a platelet-like
298 structure has been reported from the related *Azadinium* species (Luo *et al.* 2017; Tillmann
299 2018; Tillmann *et al.* 2020; Salas *et al.* 2021), but was not observed in *A. anteroporum*.
300 Although the large ventral pore lacking the platelet appears to be a difference from other
301 species, it is unclear whether this absence is stable in this species or whether it was merely
302 lost during SEM preparation. Nonetheless, the position of ventral pore was stable and no
303 other pores larger than presumed trichocyst pores were observed, and we therefore recognize
304 this as a ventral pore of *A. anteroporum*. In the APC, plate X of *A. anteroporum* was
305 considerably smaller than those of other *Azadinium* species (Fig. 32), probably due to the
306 position of the ventral pore occupying the ventral side of X. The ventral pore in contact with
307 plate X is also a unique arrangement in *A. anteroporum*, and has not been reported from other
308 *Azadinium* species previously described.
309 In addition to the morphology, ITS and LSU rDNA sequences of *A. anteroporum*
310 differed from all *Azadinium* species. There are two species described without molecular data,
311 i.e. *Azadinium asperum* Tillmann and *A. luciferelloides* Tillmann & Akselman found in the
312 preserved specimens of the algal bloom in the continental shelf of Argentina in 1990–1991
313 (Akselman & Negri 2012; Tillmann & Akselman 2016; Tillmann 2018). The ventral pore of
314 *A. asperum* is located on the anterior left side of plate 1′, where it is distant from the Po
315 (Tillmann 2018). That of *A. luciferelloides* is located on the right side of APC, similar to *A.*
316 *caudatum* var. *margalefii* Nézan & Chomérat, *A. concinnum* Tillmann & Nézan, *A.*
317 *dexteroporum*, *A. galwayense* Tillmann & Rafael Salas, *A. perforatum* Tillmann, Wietkamp

318 & H. Gu, *A. perfusorium* Tillmann & Rafael Salas and *A. zhuanum* (Luo *et al.* 2013; Percopo
319 *et al.* 2013; Tillmann *et al.* 2014, 2020; Tillmann & Akselman 2016; Salas *et al.* 2021). Aside
320 from *A. asperum* and *A. luciferelloides*, Tillmann (2018) reported the thecal morphology of
321 three undescribed *Azadinium* spp 1–3 from the northern Argentine Shelf in 1991, and the
322 positions of their ventral pore also differed from *A. anteroporum*. The ventral pore is located
323 on the posterior left of plate 1′ in *Azadinium* sp. 1 and sp. 2, and almost in the middle of plate
324 1′ in *Azadinium* sp. 3 (Tillmann 2018). An *Azadinium* cf. *spinosum* has also been reported
325 from the west coast of Ireland, which is phylogenetically distant and morphologically
326 indiscernible from *A. spinosum* (Tillmann *et al.* 2021). The ventral pore of *Azadinium* cf.
327 *spinosum* is located on the posterior left suture of plate 1′ (Tillmann *et al.* 2021), in contrast
328 to *A. anteroporum*.

329 Recently, Liu *et al.* (2023) reported environmental ITS1 sequences of undescribed
330 *Azadinium* species from the Taiwan Strait. The ITS1 sequence of *A. anteroporum* differed
331 also from *Azadinium* spp 1–8 (Liu *et al.* 2023).

332 **Molecular phylogeny and morphological traits**

333 The phylogenetic relationship has not been fully resolved in *Azadinium*, and the relative
334 positions of species were slightly different in previous studies. Our ITS phylogeny showed an
335 affinity of *A. anteroporum* to *A. trinitatum*, *Azadinium* cf. *spinosum* and *A. obesum*, and the
336 LSU rDNA tree showed an affinity to *A. trinitatum* and *Azadinium* cf. *spinosum*. Although
337 the topologies were slightly different between ITS and LSU rDNA phylogenies, *A.*
338 *anteroporum* resolved in the clade consisting of six species: *A. cuneatum*, *A. dalianense*, *A.*
339 *obesum*, *A. poporum*, *A. spinosum* and *A. trinitatum*, plus the undescribed species *Azadinium*
340 cf. *spinosum*. This clade has been recovered in recent molecular phylogenies (e.g. Wietkamp
341 *et al.* 2019; Salas *et al.* 2021; Takahashi *et al.* 2021; Tillmann *et al.* 2021).

342 The common morphological trait shared among these six related species is the position
343 of ventral pore on the left side of plate 1′ (Tillmann *et al.* 2014, 2017; Salas *et al.* 2021). In
344 this clade, only *A. anteroporum* has a ventral pore located on the middle-anterior edge of
345 plate 1′; the pore contacts with plate X and/or the left ventral margin of plate Po, and no cells
346 showed a pore contacting with the right side of plate 1′. The ventral pore in contact with the
347 left ventral side of plate Po resembles those of related *Azadinium* species. All other
348 *Azadinium* species outside the clade possess the pore located on the right-hand side of plate 1′
349 (Nézan *et al.* 2012; Percopo *et al.* 2013; Tillmann & Akselman 2016; Luo *et al.* 2017;
350 Tillmann *et al.* 2020; Salas *et al.* 2021), except for *A. polongum*, which possesses the pore on
351 the left suture of plate 1′, bordering on plate 1′′ (Tillmann *et al.* 2012b). The phylogeny of *A.*
352 *polongum* is relatively close to the *Azadinium* clade with the left-oriented ventral pore (e.g.
353 Tillmann *et al.* 2020; this study), although the relationship is not always supported in the
354 previous studies (e.g. Salas *et al.* 2021). The affinity of *Azadinium* species having a left-
355 oriented ventral pore also raises the interest of determining the phylogenetic positions of two
356 *Azadinium* species described without molecular data from preserved bloom specimens. The
357 ventral pore of *A. asperum* is located on the left margin of plate 1′, whereas that of *A.*
358 *luciferelloides* is on the anterior right of plate 1′ (Tillmann & Akselman 2016; Tillmann
359 2018).

360 The other character observed in *A. anteroporum* is the contact between plates 1′′ and
361 1a. Of all 16 *Azadinium* species, only five species have been reported to have plate 1′′ not
362 contacting with plate 1a (e.g. Tillmann *et al.* 2020, and references therein). This character
363 seems stable in each species and applicable for their identification, but the species displaying
364 the character have no strong affinity in the genus. Among the six species phylogenetically
365 related to *A. anteroporum*, the plate 1′′ of four species (*A. poporum*, *A. spinosum*, *A.*
366 *trinitatum* and *A. zhuanum*) borders on plate 1a, whereas in other two species (*A. cuneatum*
367 and *A. obesum*) it does not (Tillmann *et al.* 2009, 2011, 2014; Luo *et al.* 2013).

Azadinium species and AZAs in the Asian Pacific

AZAs were not detected in the two cultures of *A. anteroporum* isolated from Aomori, Japan. In the Asian Pacific, five *Azadinium* species have so far been reported with their morphology, phylogeny and potential AZA production, i.e. *A. dalianense*, *A. poporum*, *A. trinitatum*, *A. zhuanum* and *A. anteroporum*, and AZAs have been detected only from *A. poporum* (Potvin *et al.* 2012; Gu *et al.* 2013; Luo *et al.* 2013, 2017; Ozawa *et al.* 2021; Takahashi *et al.* 2021; this study). Two other *Azadinium* species, *A. dexteroporum* and *A. spinosum*, have been detected by rDNA-based metabarcoding analyses (Sildever *et al.* 2019; Fu *et al.* 2021), although their morphology and toxin content have not been investigated. These molecular detections of *Azadinium* species also suggest the limitation of present knowledge on species composition and their AZA productivity in the Asian Pacific. Morpho-molecular identification of culture strains, coupled with toxin analysis, will help exploring the toxigenic amphidomatacean dinoflagellates present in this region.

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TABLES

Table 1. Features of *Azadinium anteroporum sp. nov.* and six related *Azadinium* species possessing the ventral pore on the left side of plate 1′.

	A. anteroporum	*A. cuneatum*	*A. dalianense*	*A. obesum*	*A. poporum*	*A. spinosum*	*A. trinitatum*
Cell length (mean) (µm)	15.9–22.4 (18.5)	11.2–16.9 (14.2)	11.9–18.0 (13.9)	13.3–17.7 (15.3)	11.3–16.3 (13.0)	12.3–15.7 (13.8)	11.5–16.7 (14.1)
Cell width (mean) (µm)	11.1–17.1 (13.9)	8.3–12.7 (10.8)	8.3–12.7 (10.1)	10.0–14.3 (11.7)	8.0–11.6 (9.8)	7.4–10.3 (8.8)	7.3–11.5 (9.2)
Pyrenoid	1	1 (up to two)	up to two	none	up to four	1	1 (up to two)
Ventral pore, relative position to 1′ and Po	anterior of 1′, ventral center of Po	anterior of 1′, left lateral of Po	anterior of 1′, left ventral of Po	left lateral of 1′	anterior of 1′, left ventral of Po	left lateral of 1′	anterior of 1′, left ventral of Po
Apical pore plate (Po)	symmetric	strongly asymmetric	almost symmetric	slightly asymmetric	slightly asymmetric	slightly asymmetric	asymmetric
Shape of 1′	narrow posteriorly	wide posteriorly, anteriorly copped	wide posteriorly	narrow posteriorly	wide posteriorly	wide posteriorly	narrow posteriorly
Size of apical plates (2′–4′)	medium	large	medium	medium	medium	medium	small
1'' adjacent to 1a	yes	no	yes	no	yes	yes	yes
Antapical spine	present	absent	rare, short	absent	absent	present	present (unstable)
AZA production	no	no	no	no	yes	yes	no
References	This study	Tillmann *et al.* (2014)	Luo *et al.* (2013)	Tillmann *et al.* (2010)	Tillmann *et al.* (2011)	Tillmann *et al.* (2009)	Tillmann *et al.* (2014)

.

LEGENDS FOR FIGURES

Figs 1–10. Light and fluorescence microscopy of *Azadinium anteroporum sp. nov.* (strain AmAz661). Scale bars = 5 µm.

Fig. 1. Ventral view.

Figs 2, 3. Deeper focus from dorsal views, showing the nucleus (Nu), pyrenoid (Py), apical pore complex (APC) and antapical spine (arrowhead).

Figs 4, 5. Lateral view from the right side of a cell showing pyrenoid (Py), apical pore complex (APC) and antapical spine (arrowhead).

Fig. 6. Fluorescence microscopy with DAPI staining, showing the spherical nucleus (Nu) and chloroplast.

Figs 7–10. Fluorescence microscopy with calcofluor staining.

Figs 7–9. Ventral views.

Fig. 10. Dorsal view.

Figs 11–15. *Azadinium anteroporum sp. nov.* strain AmAz661, SEM. Canal plate (X), apical plates (1′–4′), anterior intercalary plates (1a–3a), precingular plates (1″–6″), cingular plates (C1–C6), anterior and posterior sulcal plates (Sa and Sp), postcingular plates (1‴–6‴) and antapical plates (1⁗ and 2⁗).

Figs 11, 12. Ventral view of cells showing the thecal plates. Scale bars = 5 µm.

Fig. 13. Dorsal view. Scale bar = 5 µm.

Fig. 14. Apical view. Scale bar = 5 µm.

Fig. 15. Epithecal plates in ventral view. Scale bar = 5 µm.

Figs 16–19. Apical pore complex of *Azadinium anteroporum sp. nov.* strain AmAz661, SEM. Thecal plates are apical pore plate (Po), cover plate (cp), canal plate (X) and apical plates (1′–4′). Scale bars = 1 µm.

Figs 16, 17. Apical pore complex. Note ventral pores (vp) contacting both with plate X and the ventral left margin of plate Po.

Fig. 18. Ventral pore (vp) contacting only with the ventral left margin of plate Po.

578 **Fig. 19**. Ventral pore (vp) contacting only with plate X.

579 Figs 20–25. Sulcal and hypothecal plates of *Azadinium anteroporum sp. nov.* strain
580 AmAz661, SEM.

581 **Fig. 20**. Hypothecal plates in ventral view. Scale bar = 5 µm.

582 **Fig. 21**. Detail of the sulcus. Scale bar = 3 µm.

583 **Fig. 22**. Hypothecal plates in ventral view. Scale bar = 5 µm.

584 **Figs 23–25**. Hypothecal plates in antapical view. Scale bar = 5 µm.

585 Figs 26–29. *Azadinium anteroporum sp. nov.*, schematic illustrations of thecal tabulation.
586 Plate labels according to Kofoidian system.

587 **Fig. 26**. Ventral view.

588 **Fig. 27**. Dorsal view.

589 **Fig. 28**. Apical view.

590 **Fig. 29**. Antapical view.

591 **Fig. 30**. Maximum likelihood (ML) phylogeny of *Azadinium* and *Amphidoma* species
592 inferred from ITS sequences. Bootstrap support values (>70%) of ML, and neighbour-joining
593 (NJ) and posterior probabilities (>0.80) of Bayesian inference (BI) are given at nodes, and
594 black dots indicate maximum supports (ML/NJ/BI = 100%/100%/1.00). Ribotypes of *A.*
595 *poporum* (A1–C2) are shown. Sequences determined in this study are highlighted in black.
596 Asterisks indicate *Azadinium* species possessing the ventral pore on the left side of the cell.

597 **Fig. 31**. Maximum likelihood (ML) phylogeny of *Azadinium* and *Amphidoma* species
598 inferred from LSU rDNA (D1–D3 region) sequences. Bootstrap support values (>70%) of
599 ML, and neighbour-joining (NJ) and posterior probabilities (>0.80) of Bayesian inference
600 (BI) are given at nodes. Black dots indicate maximum supports (ML/NJ/BI =
601 100%/100%/1.00). Ribotypes of *A. poporum* (A1–C2) are shown. Sequences determined in
602 this study are highlighted in black. Asterisks indicate *Azadinium* species possessing the
603 ventral pore on the left side of the cell.

604 **Fig. 32**. Schematic illustrations of apical pore complex of *Azadinium anteroporum sp. nov.*
605 and related species, with apical pore plate (Po), cover plate (cp), canal plate (X), apical plates

606 (1′–4′) and ventral pore (vp). The arrangements of related species are given according to
607 previous studies (Tillmann *et al.* 2009, 2010, 2011, 2012b, 2014; Luo *et al.* 2013).

608