First report of *Biecheleriopsis adriatica* in Bolinao, Northwestern Philippines and its wide distribution in Southeast Asia and adjacent waters

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ABSTRACT

Morphology and phylogeny of the marine woloszynskioid dinoflagellate *Biecheleriopsis adriatica*, collected from Philippines, Singapore, Palau and Japan, were examined by using light and scanning electron microscopy (SEM), and molecular phylogeny based on rDNA sequences. Cells of these cultures were ovoid to spherical, measured 11.5–17.3 µm in length, with a displaced cingulum, a sigmoid sulcus and an eyespot. Cells have an elongate apical vesicle (EAV) mostly 3.1–3.8 µm in length with globular knobs up to 32, and straight lower cingular margin in the dorsal side. These morphological characters were identical to those of *B. adriatica* previously reported from the Adriatic Sea, China, Japan and Korea. Molecular phylogeny based on sequences of ITS and LSU rDNA revealed that the culture isolated from Bolinao, Philippines positioned in a clade with *B. adriatica*. Cultures isolated from Japan, Palau and Singapore had the identical morphological characters under light microscopy, and cultures investigated were positioned in *B. adriatica* clade in the phylogenetic tree of ITS and LSU rDNA. *B. adriatica* co-occurred with a bloom of *Takayama* sp. associated with mass mortality of farmed milkfish in May 2016 in Bolinao, Philippines. Based on existing data, this species is unlikely the culprit responsible for the massive fish kill event but the results suggest the need for further study to clarify its role in the overall dynamics of algal blooms in Bolinao. The results also show the wide distribution of *B. adriatica* in Southeast Asia and adjacent waters.

KEYWORDS

Biecheleriopsis adriatica, dinoflagellate, distribution, harmful algal blooms, Southeast Asia, Suessiaceae

INTRODUCTION

The genus *Biecheleriopsis* Moestrup, Lindberg et Daugbjerg is a woloszynskioid dinoflagellate first described from the Adriatic Sea, with the type species *Biecheleriopsis adriatica* Moestrup, Lindberg et Daugbjerg (Moestrup et al. 2009b). Woloszynskioid dinoflagellates have intermediate numbers of amphiesmal vesicles between unarmored and armored dinoflagellates and usually possess thin thecal plates, and are thus often referred to as thin-walled dinoflagellates (Fensome et al. 1993). *Biecheleriopsis* is assigned to the family Suessiaceae, characterized by having a single elongate apical vesicle (EAV) and type E eyespot, which distinguish them from the other woloszynskioid families Borghiellaceae and Tovelliaceae (Moestrup and Daugbjerg 2007; Moestrup et al. 2009a, b). In the Suessiaceae, *B. adriatica* has the superficial resemblance with marine species of other genera, especially *Ansanella* and *Biecheleria*, because of similar cell length and number of latitudinal amphiesmal vesicle (AV) series (Moestrup et al. 2009a, b; Siano et al. 2010; Jang et al. 2017). The diagnostic character of *B. adriatica* from other suessiaceans observed in the original material is the presence of nuclear connective in the flagellar apparatus (Moestrup et al. 2009b). Another feature to identify *B. adriatica* is the straight lower margin of the cingulum, which has been observed as zig-zag shaped in other genera of the family (Takahashi et al. 2014).

Since the first description of *B. adriatica* from the Adriatic Sea, it has so far been isolated from at least thirteen sampling locations in Japan, Korea and China (Takahashi et al. 2014; Jang et al. 2015; Luo et

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al. 2015; Kang and Wang 2017). In China, cells of *B. adriatica* have been obtained by incubation of sediment samples from Bohai Sea, East China Sea, Yellow Sea and South China Sea, which confirm the presence of its resting cyst form, as this has only been observed from culture in the original description (Moestrup et al. 2009b; Luo et al. 2015; Kang and Wang 2017). These reports also indicate the distribution of *B. adriatica* in temperate East Asian coasts. Moreover, molecular data from analyzing environmental DNA from the open ocean area of South China show close similarities to *B. adriatica*, suggesting a more extensive distribution into subtropical pelagic water (Fan et al. 2013). However, this species has not yet been reported from tropical areas in Asia.

In this study, a woloszynskioid dinoflagellate was isolated from Bolinao, Pangasinan, Philippines, which has co-occurred with a bloom of *Takayama* sp. that was associated with mortalities of milkfish *Chanos chanos* in May 2016. The morphology and molecular phylogeny of this Bolinao isolate, along with further isolates from Japan, Palau and Singapore, were examined to discuss the distribution of *B. adriatica* in Southeast Asia and adjacent waters.

MATERIALS AND METHODS

Culture and observation

Eight unialgal cultures of *B. adriatica* were established from seawater samples collected from the Philippines, Singapore, Palau and Japan (Table 1). One Philippine strain (UI16) originated from sampling during a bloom of the unarmored dinoflagellate *Takayama* sp. coinciding with mortalities of farmed milkfish in Bolinao, Pangasinan in May 2016. Another strain (T7) was isolated from another bloom of *Takayama* sp. in May 2017. Cells of *B. adriatica* in May 2016 were enumerated from net-hauled plankton samples, while cells in May 2017 were quantified from seawater samples using a Sedgwick-Rafter counting chamber. Cultures were established by capillary pipetting cells into full or half strength IMK medium (Wako, Tokyo, Japan) with salinity of 30 and maintained at 20ºC in a 12:12h light:dark photoperiod under 40–50 µmol photons m^{-2} s^{-1}.

For light microscopy, cells were observed using a light microscope Zeiss Axioskop 2 (Carl Zeiss, Göttingen, Germany) equipped with a digital camera Zeiss Axiocam HRc (Carl Zeiss, Göttingen, Germany). Cell measurements were done on micrographs.

For scanning electron microscopy, cells were fixed with 1% OsO_4 (w/v) solution on a poly-L-lysine-coated SEM plate for 5 min at room temperature. Fixed cells were rinsed twice in distilled water for 30 min each, dehydrated through an ethanol series of 30%, 50%, 75%, 90%, 95% for 15 min each, followed by pure ethanol twice for 30 min each. It was then replaced with an isoamyl acetate and dried using a critical point dryer JCPD-5 (JEOL, Tokyo, Japan). After coating with gold, cells were observed under the JSM-6390 SEM (JEOL) at 10 kV accelerating voltage.

Molecular analyses

Genomic DNA was extracted using either the 2x hexadecyltrimethylammonium bromide (CTAB) method (Takahashi et al. 2014), or Qiagen Plant MiniKit (Valencia, CA, USA) following the manufacturer's protocol. PCR was used to amplify the ~1.5-kb ITS region (ITS1, 5.8S, ITS2) and LSU rDNA (D1–D3) using primers shown in Takahashi et al. (2015). Amplifications were performed using either TaKaRa Ex Taq or MightyAmp (Takara, Shiga, Japan) as described in Takahashi et al. (2015), with an iCycler (BIO-RAD, Tokyo, Japan) or a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, California, USA). The amplification was confirmed by 1.0% agarose gel electrophoresis. The PCR product was purified using a QIAquick PCR purification kit (Qiagen Genomics, Bothell, Washington, USA). Sequencing was outsourced to Eurofins Genomics Inc. (Tokyo, Japan).

Determined sequences of ITS region and LSU rDNA were manually aligned using Se-Al sequence alignment editor v2.0a11 (Rambaut 1996) with 28 taxa of the Suessiales for ITS and 48 for LSU rDNA obtained from GenBank. Identical sequences from the same locality were treated as a single sequence. Two

Table 1. Strains used in the present study.

Strains	Locations	Coordinates	Dates	Salinity	Temperature	Accession
UI16	Bolinao Channel, Pangasinan, Philippines	16°21'21"N, 119°56'12"E	May 2016	30	31.0ºC	
T7	Bolinao Channel, Pangasinan, Philippines	16°20'04"N, 119°57'26"E	May 2017	30	32.0ºC	LC413946
sgd441-kt	South China Sea, St. John's Island, Singapore	01°13'N, 103°51'E	Jul 2016	30		
sed279-kt	Pacific, Matsushima Bay, Miyagi, Japan	38°16'N, 141°03'E	Jul 2013	28	22.0ºC	LC413947
trd278-kt	Sea of Japan, Tsuruoka, Yamagata, Japan	38°45'46"N, 139°43'39"E	Jul 2013			LC068843
trd280-kt	Sea of Japan, Tsuruoka, Yamagata, Japan	38°45'46"N, 139°43'39"E	Aug 2013			LC413948
pld296-kt	Pacific, Palau Island	07°14'11"N, 134°30'31"E	Nov 2013	34	28.5ºC	LC413949
hmd326-kt	Pacific, Lake Hamana, Shizuoka, Japan	34°41'47"N, 137°35'43"E	Jun 2014	35	21.0ºC	LC413950

ITS sequences of *Pelagodinium bei* (KP342301, DQ195362) and a LSU rDNA sequence of *Dactylodinium pterobelotum* (LC272997) were selected as an outgroup. The software Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) was used for the analyses (Tamura et al. 2013). The best-fit model of evolutionary sequence substitution determined by MEGA6 was general time reversible (GTR) plus a discrete gamma distribution for both ITS (G = 0.2532) and LSU rDNA (G = 0.3676) data sets. Neighbor-joining (NJ) and maximum likelihood (ML) analyses were conducted using MEGA6 with 1,000 and 500 bootstrap replicates, respectively. The initial tree was generated by the program BioNJ under the nearest-neighbor interchange (NNI) and subtree pruning and regrafting (SPR) heuristic methods. Sampling location and accession numbers of OTUs are given in Figs. 3 and 4.

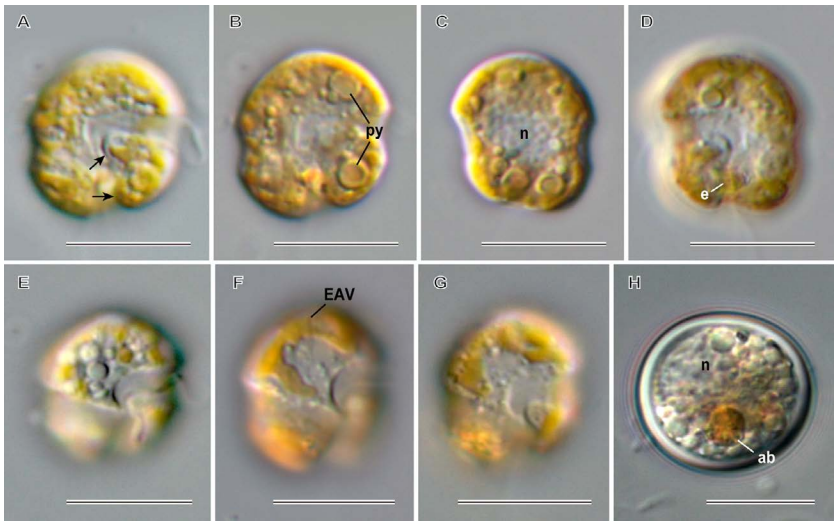

Figure 1. Light microscopy of *Biecheleriopsis adriatica*. A–D. T7 strain. E. trd278-kt strain. F. sgd441-kt strain. G. pld296-kt strain. H. sed279-kt strain. (A) Ventral surface view showing boomerang shape of the left sulcal area (arrows). (B) Slightly deeper focus showing two pyrenoids (py) surrounded by starch sheath. (C) Dorsal view showing a dinokaryotic nucleus (n). (D) Dorsal deeper view from (C) showing an eyespot (e). (E, F) Ventral view of different strains, an elongate apical vesicle (EAV) is also shown. (H) Resting cyst like cell covered by transparent thick wall, cytoplasm with no apparent chloroplast, nucleus (n) and accumulation body (ab). Scale bars = 10 µm.

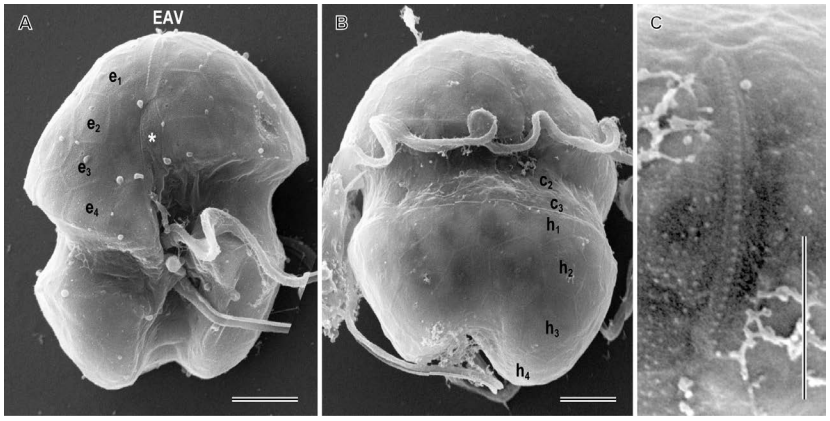

Figure 2. Scanning electron microscopy of *Biecheleriopsis adriatica*, trd278-kt strain. (A) Ventral view showing an elongate apical vesicle (EAV) at the apex and four latitudinal amphiesmal vesicle (AV) series in the epicone (e_1–e_4). Longitudinally elongated AV upper the sulcus (asterisk) is also shown. (B) Dorsal view with four latitudinal vesicle series in the hypocone (h_1–h_4), and the second (c_2) and third series in the cingulum (c_3). (C) Apical view showing 32 knobs on the EAV. Scale bars = 2 µm.

RESULTS

Morphology

Morphological characters of strains isolated from Bolinao, Philippines were identical to *B. adriatica* under light microscopy (Fig. 1). Cells were ovoid to spherical and measured 11.5–17.3 µm (mean 14.7 µm, n = 30) in length and 7.3–11.0 µm (mean 8.9 µm, n = 30) in width for UI16, and 11.9–18.5 µm (mean 15.4 µm, n = 30) in length and 7.9–13.5 µm (mean 10.9 µm, n = 30) in width for T7 strain (Figs. 1A–D). The episome was roundish and the hyposome was asymmetric with the right posterior end slightly bulging (Figs. 1A–D). The cingulum has displacement more than its own width, and the sulcus was sigmoid in shape (Fig. 1A). The nucleus was located in the middle of the cell (Fig. 1C), and chloroplast was situated in the periphery (Figs. 1A–G). Several pyrenoids, usually 2–4, surrounded by a starch sheath were evident (Figs. 1B–D). An eyespot was located at the sulcal region (Fig 1D). The straight elongate apical vesicle (EAV) was sometimes observed under a light microscope (Fig. 1F). Resting cyst-like cells covered by a transparent thick wall with a distinct red accumulation body (ab) and nucleus was found in cultures (Fig. 1H). The spines were not observed from the surface of cysts as seen in Moestrup et al. (2009b). All other cultures showed morphological characters identical to cultures from Bolinao (Figs. 1E–G). A strain from Singapore (sgd441-kt) also had the obvious sigmoid sulcus and long EAV seen in *B. adriatica*, unlike species of *Ansanella* and *Biecheleria* of similar size which have less curving sigmoid sulcus and shorter EAV (e.g., Takahashi et al. 2014; Jeong et al. 2014).

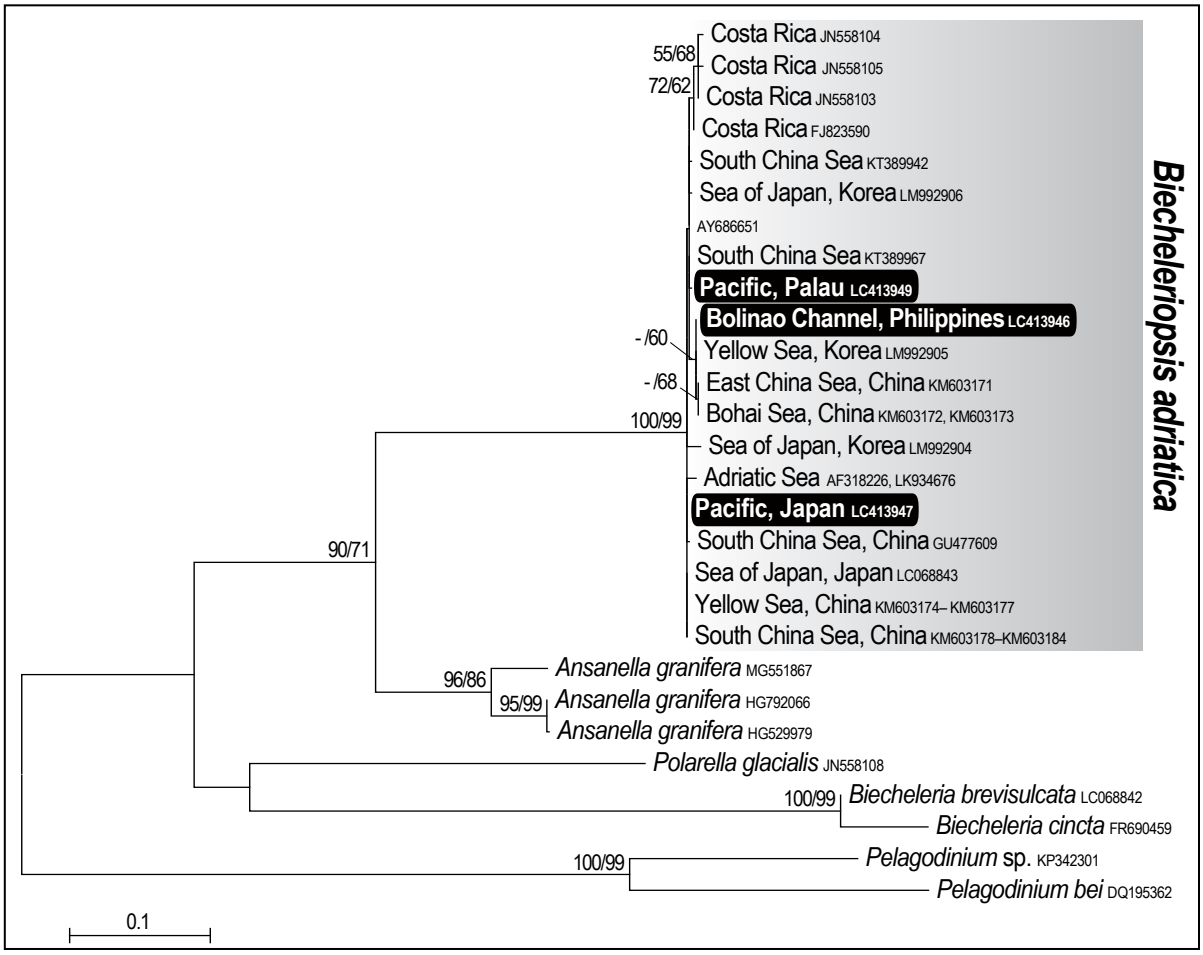

Figure 3. Maximum likelihood (ML) tree inferred from sequences of ITS region (ITS1, 5.8S and ITS2, 752 bp). Bootstrap support values of neighbor-joining (left) and ML (right) are shown. *Biecheleriopsis adriatica* from Bolinao, Philippines analyzed in this study is highlighted in black box.

Under SEM, the episome was slightly larger than the hyposome (Fig. 2A). The elongate apical vesicle (EAV) and the four latitudinal amphiesmal vesicle (AV) series in the episome were present (Fig. 2A). The longitudinal elongated AV located above the sulcus was pronounced. In dorsal view, four latitudinal vesicle series in the hyposome, and the second and third series in the cingulum were observed (Fig. 2B). The lower margin of the cingulum was straight in the dorsal side (Fig. 2B). In the EAV, the elongate amphiesmal vesicle possessed up to 32 knobs arranged in a straight row (Fig. 3C). The length of EAV varied mostly from 3.1–3.8 µm, but in small cells EAV length was only 2.4 µm, with 24 knobs.

Molecular phylogeny

Three sequences of ITS region were obtained from cultures from Bolinao, Japan and Palau (T7, sed279-kt and pld296-kt), and four sequences of LSU rDNA were determined from cultures from Japan and Palau (sed279-kt, trd280-kt, pld296-kt and hmd326-kt). The aligned ITS sequences of 26 OTUs were 752 bps, of which 286 sites (38.0%) were variable and 211 sites (28.0%) were parsimonious informative. Its average base compositions were A = 0.193, C = 0.254, G = 0.248 and T = 0.305. A total of 48 aligned LSU rDNA sequences which is 1,009 nucleotides, of which 516 sites (51.1%) were polymorphic and 345 sites (34.2%) were parsimonious informative. Its average base compositions were A = 0.255, C = 0.199, G = 0.295 and T = 0.251.

The maximum likelihood (ML) trees inferred from the ITS and LSU rDNA are shown in Figs. 3 and 4, respectively. Bootstrap support values derived from ML and neighbor-joining (NJ) analyses were given. The ITS tree showed that the Bolinao strain in addition to isolates from Palau and Japan was positioned in the *Biecheleriopsis* clade with strong supports (NJ/ML = 100/99%), with *B. adriatica* from the Adriatic Sea, China, Costa Rica, Japan and Korea (Fig. 3). The ML tree based on LSU rDNA sequences showed three isolates from Japan and one from Palau branched in a

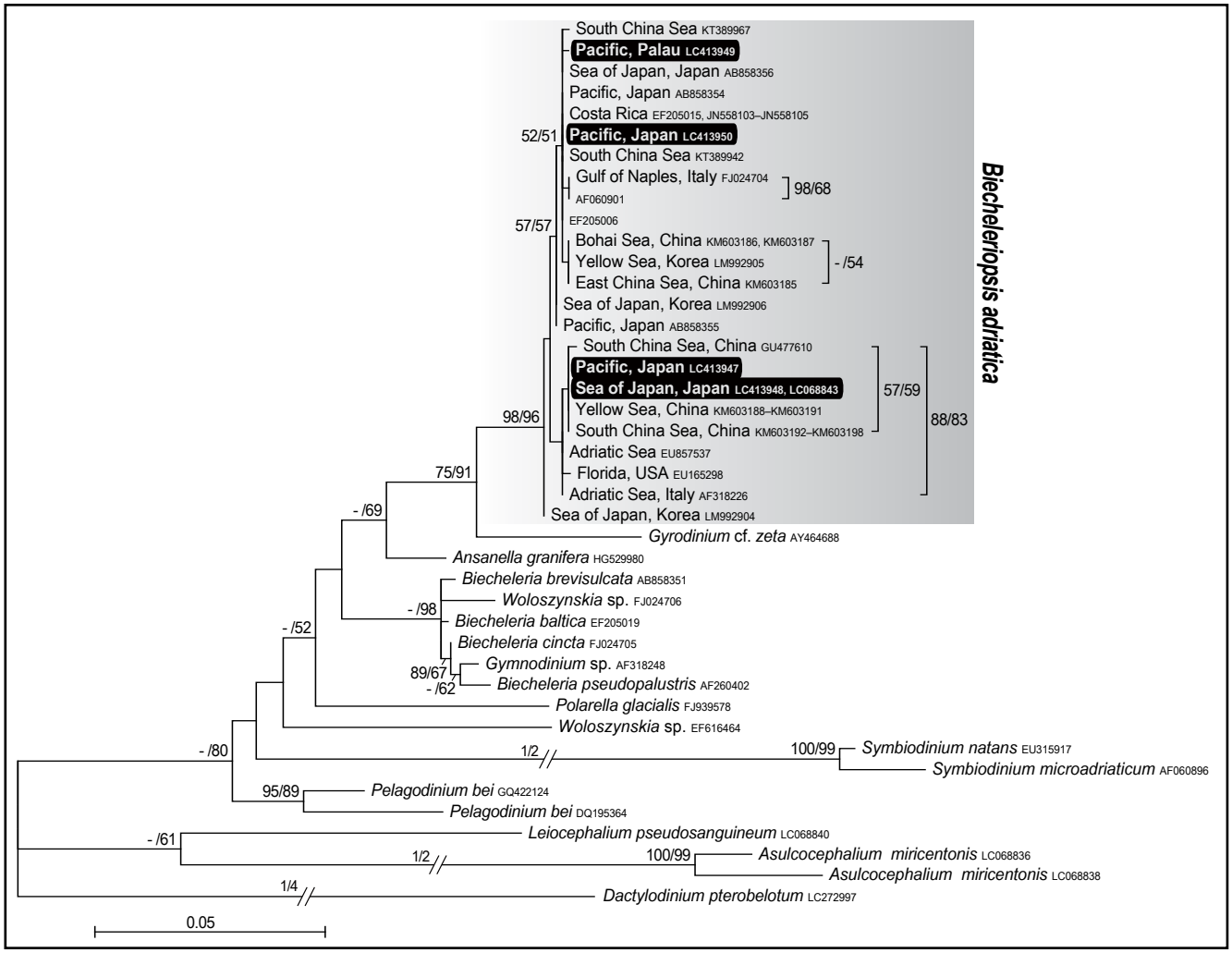

Figure 4. Maximum likelihood (ML) tree inferred from LSU rDNA sequences (D1–D3 region, 1,009 bp). Bootstrap support values of neighbor-joining (left) and ML (right) are given. *Biecheleriopsis adriatica* cultures analyzed in this study are highlighted in black boxes.

well-supported monophyletic clade of *Biecheleriopsis* (98/96%), with *B. adriatica* from China, Japan, Korea, Gulf of Naples, Italy and Adriatic Sea (Fig. 4). In comparison within sequences of *Biecheleriopsis* clade, base substitutions were found at 20 (ITS) and 11 (LSU rDNA) loci, representing 2.66% (ITS) and 1.09% (LSU rDNA) sequence divergences.

Abundance of *B. adriatica* in Bolinao

During the fish kill event in Bolinao, Pangasinan in May 2016, an unidentified *Gymnodinium*-like dinoflagellate (= *B. adriatica*, UI16 strain) was the second dominant species in the bloom caused mainly by *Takayama* sp. Cell density of *Takayama* sp. recorded 1,710 cells/mL with a relative abundance of 84%, and *B. adriatica* reached 299 cells/mL (15%) during the event. Other phytoplankton species, e.g. *Skeletonema* spp., *Protoperidinium* spp., *Gonyaulax* spp., *Prorocentrum micans*, accounted only for 1% of the total phytoplankton community. Environmental conditions were typical of tropical coasts, with sea surface temperature of 31ºC and salinity of 30 psu.

A similar bloom composed mainly of *Takayama* sp. associated with *B. adriatica* recurred in May 2017, although no fish kill was documented then. Maximum cell density of *Takayama* sp. was 1,030 cells/mL (73%) and *B. adriatica* was 305 cells/mL (22%). Other phytoplankton species, e.g. *Alexandrium* spp., *Peridinium quinquecorne*, *P. micans*, *Scrippsiella* spp. and *Skeletonema* spp., comprised 4% of the community. For this period, the sea surface temperature was 32ºC and salinity was 30 psu.

DISCUSSION

The morphology observed from culture strains mostly coincided with the original description of *B. adriatica* provided by Moestrup et al. (2009b), i.e. cell length, sigmoid sulcus representing boomerang shape of left sulcal area, the length of an elongate

apical vesicle (EAV, ca. 2.8 µm from Fig. 12 in Moestrup et al. 2009). Takahashi et al. (2014) suggested that the straight lower cingular margin in the dorsal side of the cell is an informative feature to identify *B. adriatica*, and this was also observed in the present study. Phylogenetic analysis inferred from ITS and LSU rDNA sequences supported the morphological identification by forming a well-supported clade including the sequence from the original description. Some strains (e.g., CCMP419 in Moestrup and Daugbjerg 2007; CCMP420 in Wilcox 1998; G31, GJZ01, CGJ02 in Luo et al. 2015) positioned in the same clade have been identified as *Protodinium simplex* Lohmann (= *Gymnodinium simplex* (Lohmann) Kofoid et Swezy). Takahashi et al. (2014) briefly summarized the taxonomic history of *P. simplex* and discussed the ambiguity of taxonomic identity with *B. adriatica*. The presence of an eyespot and sigmoid sulcus are characteristic features of members of the family Suessiaceae, but are unclear in *P. simplex*. We therefore recognized all species in the clade as *B. adriatica.*

A range of knob numbers in EAV has so far been reported from *B. adriatica*. In the original description, 18 knobs were reported from the Adriatic Sea strain, with approximately 350 nm and 450 nm of the proximal and distal parts of an EAV lacking knobs (Moestrup et al. 2009b). Subsequently, up to 32 knobs was reported in the Japanese strain, 20 knobs were illustrated from *B.* cf. *adriatica* from the South China Sea, and a range of 16–30 knobs was observed in the Korean strain (Takahashi et al. 2014; Jang et al. 2015; Luo et al. 2015). Similar variability was also observed in this study, where approximately 32 knobs were commonly found, while smaller cells with shorter EAV had 24 knobs.

Although fish kills caused by *Takayama* spp. have been reported in Japan, USA and Australia (Onoue et al. 1985; Larsen 1994; Steidinger et al. 1998; Hallegraeff 2002), harmful effects due to suessiacean dinoflagellates, including *B. adriatica*, remain uncertain. According to Jang et al. (2015), the Korean strain of *B. adriatica* is non-toxic to brine shrimp *Artemia salina* even at the concentration of 60,000 cells/mL. As it is well known what is harmful to invertebrates (such as *Artemia*) is quite different to what harms vertebrates (such as milkfish), potency must be thoroughly studied.

Occurrences of *B. adriatica* in Asia, including previous records (Takahashi et al. 2014, 2015; Fan et al. 2015; Jang et al. 2015; Luo et al. 2015; Kang and Wang 2017), are shown in Fig. 5. In the present study, although molecular analysis was not conducted for the Singapore strain, the occurrence of *B. adriatica* with molecular and morphological information is the first report in tropical Asia (Philippines) and Oceania (Palau). These new sequences, along with previous reports, enable phylogeography of *B. adriatica* in the Asia Pacific. However, there was no obvious correlation between genotypes and sampling locations in *B. adriatica*. The widespread distribution of *B. adriatica,* and the environmental sequences (KT389967 and KT389942) detected from tropical oceanic areas in the South China Sea (Fan et al. 2015), suggest the role of transport of *B. adriatica* population into oceanic waters.

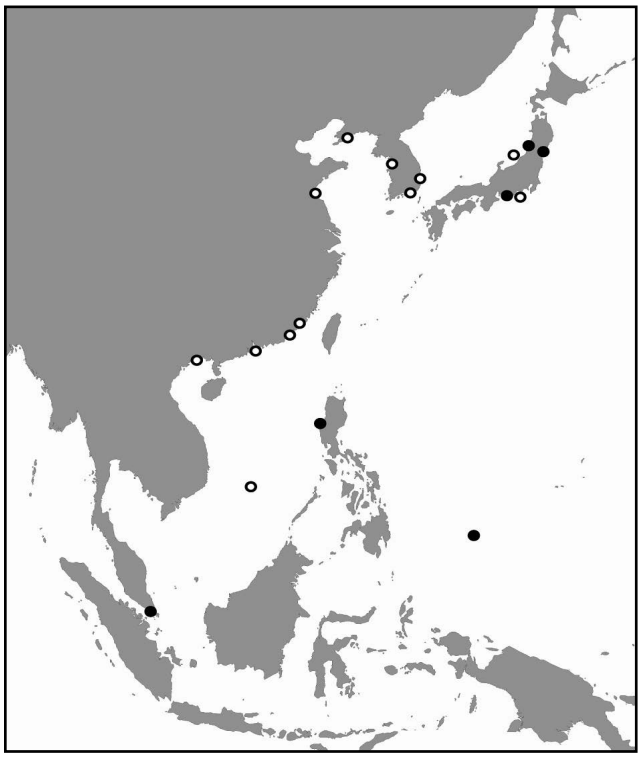

Figure 5. Occurrences of *Biecheleriopsis adriatica* in Asia Pacific. Black dots are from the present study, and white dots refer to previous studies (Takahashi et al. 2014, 2015; Fan et al. 2015; Jang et al. 2015; Luo et al. 2015; Kang and Wang 2017).

Over the years, reports on the occurrence of small woloszynskioid dinoflagellates have been increasing. In Southeast Asia, a woloszynskioid species related to the Suessiaceae, *Dactylodinium pterobelotum* Kazuya Takahashi, Moestrup et Iwataki, was also recently found and described as a new species from south Vietnam (Takahashi et al. 2017). Environmental sequences assigned to the Suessiaceae were detected from Singapore (Leong et al. 2015). This implies the presence of various uninvestigated woloszynskioids in tropical waters. They might have been present but were overlooked because of their small size and subtle morphological features superficially resembling unarmored species.

This study confirms the presence of *B. adriatica* and describes its geographical distribution of *B. adriatica* in Southeast Asian and adjacent waters.

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