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
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 cultures 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 woloszynskiioid 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 Takayama sp. in May 2016. 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 Sedgewick-Rafter counting chamber. Cultures were established by capillary pipetting cells into full or half strength IM medium (Wako, Tokyo, Japan) with salinity of 30 and maintained at 20°C in a 12:12 h 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% OsO4 (v/v) solution on a polylysine-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 Giagen Plant MiniKit (Valencia, CA, USA) following the manufacturer’s protocol. PCR was used to amplify the ~1.5 kb ITS region (ITS1, 5.8S LSU rDNA (D1–D3)) using primers 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 Bioticsystems, Carlsbad, California, USA). The amplification was confirmed by 1% agarose gel electrophoresis. The PCR products were purified using a Qiagen PCR purification kit (Qiagen Genomics, Bothell, Washington, USA). Sequencing was outsourced to Eurofin 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 ITS sequences of Pelagodinium bei (KP342301, DQ195362) and a LSU rDNA sequence of Dactyloidiun pterobolitum (LC282979) 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 = 0.3670) 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, using the program BioNJ under the nearest-neighbor interchange (NNI) and subtree pruning and regrafting (SPR) heuristic methods. Sampling location and accession numbers are given in Figures 3 and 4.

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 15.5–17.3 µm (mean 16.7, 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 77 strain (Figs. 1A–D). The epime is roundish and the hypome was asymetrical 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 apical 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 restorad body (ab) and nucleus were 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-k) also had the obvious sigmoid sulcus and long EAV seen in B. adriatica, unlike species of Anneselia and Biecheleria of similar size which have less curving sigmoid sulcus and shorter EAV (e.g., Takahashi et al. 2014; Jeong et al. 2014).
Under SEM, the episphere was slightly larger than the hypopyse (Fig. 2A). The elongate apical vesicle (EAV) and the four latitudinal amphiesmal vesicles (AV) series in the episphere were present (Fig. 2A). The longitudinal elongated AV located above the sulcus was pronounced. In dorsal view, four latitudinal AV series in the episome were present (Fig. 2A). The longitudinal elongated AV located above the sulcus was pronounced. In dorsal view, the second and third series in the cingulum were well supported, respectively. Bootstrap support values of neighbor-joining (left) and ML (right) are shown. 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 given.

**DISCUSSION**

During the fish kill event in Bolinao, Pangasian 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. was 1,030 cells/mL (22%). Other phytoplankton species, e.g. Alexandrium spp., Peridinium quinquecorne, P. micans, Scoposula spp. and Skeletonema spp., comprised 4% of the community. For this period, the 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, Scoposula spp. and Skeletonema spp., comprised 4% of the community. For this period, the sea surface temperature was 32°C and salinity was 30 psu.
simplex positioned in the same clade have been identified in Moestrup and Daugbjerg 2007; CCMP420 in original description. Some strains (e.g., CCMP419 the morphological identification by forming a well- in the present study. Phylogenetic analysis inferred 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 recognize 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 260 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; Steidenger et al. 1996; Hallegaard 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 molecular report in tropical Asia (Philippines) and Oceania (Palau). These new sequences, along with previous reports, enable phylogography 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 (KT339967 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, Dactylopodium pterobolatum Kuzuya Takahashi, Moestrup et lwataki, 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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