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FIRST REPORT OF A MARINE DINOFLAGELLATE, *ALEXANDRIUM ANDERSONII* (DINOPHYACEAE) IN MALAYSIAN WATERS

Roziawati Mohd Razali[1,*], Chui-Pin Leaw[2], Hong-Chang Lim[3], Mohd Nor Azman Ayub[1], Masazurah A. Rahim[1] and Po-Teen Lim[2]

[1] Fisheries Research Institute Batu Maung, Department of Fisheries Malaysia, 11960 Batu Maung, Pulau Pinang, Malaysia
[2] Bachok Marine Research Station, Institute of Ocean and Earth Sciences, University of Malaya, Bachok, 16310 Kelantan, Malaysia
[3] Faculty of Applied Sciences and Computing, Tunku Abdul Rahman University College, Johor Branch Campus, 85000 Segamat, Johor, Malaysia
*Corresponding author: email: roziawati_r80@yahoo.com
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ABSTRACT A field survey was carried out at aquaculture areas of Aman Island, Penang to monitor harmful microalgae. In this study, a strain of *Alexandrium* species was established in laboratory culture and identified using morphological criteria and nucleotide sequences of the LSU rDNA to confirm the identification. The strain was observed under epi-fluorescence microscope and the morphological characters resembled to the species description of *A. andersonii*. Based on the nucleotide sequences, the strain also revealed close relationship to *A. andersonii*. HPLC analysis found that *A. andersonii* was not able to produce paralytic shellfish toxins. This is the first report of *A. andersonii* found in Malaysian waters.

ABSTRAK *Kajian lapangan telah dijalankan di kawasan akuakultur Pulau Aman, Pulau Pinang untuk memantau mikroalga berbahaya. Dalam kajian ini, strain spesis* Alexandrium *telah dikultur di makmal dan pengesahan identifikasi spesis adalah berdasarkan morfologi dan jujukan nukleotida, LSU rDNA. Strain tersebut diperhati di bawah mikroskop epi-pendarfluor dan didapati ciri morfologinya adalah menyerupai spesis* A. andersonii. *Berdasarkan jujukan nukleotida, strain ini juga didapati mempunyai hubungan rapat dengan* A. andersonii. *Analisa HPLC mendapati bahawa* A. andersonii *tidak menghasilkan toksin paralitik. Ini merupakan laporan pertama* A. andersonii *dijumpai di perairan Malaysia.*

Keywords: *Alexandrium*, morphology, LSU rDNA, PSP toxins

INTRODUCTION

Harmful algal bloom (HAB) is a natural phenomenon due to the drastic increase of single species of phytoplankton density in the water column resulting from favorable conditions for growth. Rapid growth of these microalgae causes negative impact to marine ecosystem, human health and mariculture. At present, paralytic shellfish poisoning (PSP) is the only HAB-related shellfish poisoning that had been reported in Malaysia. The first report of shellfish poisoning was documented in Brunei Bay, Sabah in 1976 when the PSP producer, *Pyrodinium bahamense* var. *compressum* (Böhm) Steidinger, Tester & Taylor bloomed and poisoned 202 people, with seven casualties [1]. Subsequently, HABs that involved PSP toxic dinoflagellate, *Alexandrium minutum* Halim was reported at East Coast of Peninsular Malaysia [2]. In September 2015, selling and collection of the shellfish at Geting River of Kelantan was banned due to the high level of saxitoxin contamination in the shellfish tissue [3].

Paralytic shellfish poisoning is caused by ingestion of shellfish contaminated with saxitoxin (STX) produced by toxic dinoflagellate species from the genera *Alexandrium* Halim, *Gymnodinium* Stein and *Pyrodinium* Plate [4, 5]. This biotoxin acts on the mammalian nervous system by blocking the sodium channel and thus preventing the transmission of neuron signal. High levels of PSP can lead to severe illness and

death caused by respiratory arrest within a few minutes to a few hours [4].

Aman Island, located in the northern part of the Straits of Malacca, is a very important marine finfish (grouper, snapper and sea bass) aquaculture area and blood cockle farming. The presence of potentially harmful microalgae especially toxin producer in aquaculture areas can pose potential threats to seafood safety and economic losses [6, 7]. Blooms of harmful microalgae can cause massive fish kills in the fish farming areas due to either hypoxia or anoxia in the surrounding environments or excretion of toxic bioactive compounds. Harmful algae bloom can also cause toxin contamination in seafood and this has lead to human intoxication via consumption of contaminated shellfish by toxic microalgae [8]. Thus, the baseline data on the assemblage of potentially harmful microalgae in the area is important to provide further information to the Department of Fisheries for monitoring programs. This could provide additional information on HABs species distribution in the region and contribute to the species inventory for country monitoring purposes.

In this study, a marine dinoflagellate from the genus *Alexandrium* was morphologically and molecularly characterized. Clonal cultures of *Alexandrium* were established and morphology of each species was investigated by fluorescence and further analyzed based on the nucleotide sequences of ribosomal DNA genes (rDNA). In contrast to morphological techniques, DNA sequencing methods have improved greatly in recent years, both in terms of accuracy and efficiency, making molecular characterization of cells technically easier and less expensive to perform [9, 10]. Genomic DNA was extracted, and the rDNA was amplified prior to sequencing. The sequences obtained were analyzed and used for phylogenetic reconstruction. The toxicity of the clone was also documented.

MATERIALS AND METHODS

Isolation and Establishment of clonal cultures

Phytoplankton samples were collected from Aman Island, Penang during high tide using 20 µm mesh size plankton net. Some samples were preserved in Lugol's iodine solution and some were brought back live to the laboratory for cell isolation and culturing. For culturing, individual cell was isolated using a Pasteur pipette with very fine tip. Cells were rinsed a few times in sterile filtered seawater before transferring into a 24-well plate containing sterile filtered seawater. Filtered natural seawater with salinity of 30 PSU was used as medium base. One drop of ES-DK medium [11] was added into the wells daily to avoid nutrients depletion. Growth of cells were monitored daily and let to divide for a few weeks. Cells were transferred into sterilized test tubes containing ES-DK medium when reached desired concentration. Cultures were maintained in ES-DK medium at 25 °C under a light:dark cycle of 16:8.

Morphology Observation

Species identification was based on morphology descriptions given by Balech [12, 13] and Fukuyo [14]. Thecal morphology and plate tabulation was observed under an epifluorescence microscope. Fixed samples were stained with 1% calcofluor white (Fluka, Japan) and viewed under an Olympus IX51 inverted research microscope (Olympus, Tokyo, Japan) at 100× magnification with UV filter sets. Micrographs of each dinoflagellate species were captured with an attached cooled CCD camera and the digital images were analyzed by

Analysis® software (Soft Imaging System Inc., USA).

DNA extraction, rDNA amplification and sequencing

Exponentially growing cultures were pooled and harvested by centrifugation at 3000 × g for 5 minutes and cell pellets were kept at -80°C until DNA extraction. Genomic DNA was extracted using DNeasy Plant Kit (Qiagen, USA), according to the manufacturer's instruction. The D1/D3 region of the large subunit (LSU) rDNA gene was amplified by polymerase chain reaction (PCR) with primer pair D1R (5'ACCCGCTGAATTTAAGCATA-3') and D3Ca (5'CTTGGTCCGTGTTTCAAGA-3') [15]. The protocol for gene amplification and DNA sequencing were as described previously by Leaw et al. [16, 17]. In brief, PCR was performed with 25 µl reaction mixtures, using following program: 4 min at 94°C followed by 35 cycles of 35 s at 94°C, 50 s at 55°C, 35 s at 72°C and finally an elongation step of 7 min at 72°C. The size of the PCR products were estimated by 1 % agarose gel electrophoresis, stained with SYBR Safe DNA Stain (Invitrogen, Life Technologies, Carlsbad, CA, USA) and visualized under UV light. The PCR products were purified using QIAquick purification columns (Qiagen, USA) according to the manufacturer's instructions. The purified products were directly sequenced for both directions by First Base Laboratories (Selangor, Malaysia), using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). The estimated sequence length from the amplicon was about 900 bp.

Sequences analysis and taxon sampling

The sequences obtained were then confirmed using BLAST (Basic Local Alignment Search Tool) at NCBI (National Center of Biotechnology Institute). Taxon sampling was performed and selected *Alexandrium* LSU sequences were retrieved from GenBank Database. A total of 34 taxa were compiled, with *Ostreopsis ovata* Fukuyo, *Ostreopsis* sp. and *Coolia* sp. were used as outgroup. Sequences obtained in this study were deposited in GenBank Database and the details of sequences used for analyses were compiled in Table 1. Only D1/D2 region of the sequence was analyzed. Multiple sequence alignments were performed by using ClustalX [18] and subsequently checked and edited manually using BioEdit [19].

Table 1: LSU nucleotide sequences of *Alexandrium* species obtained in this study and sequences retrieved from Genbank. Cultured strains obtained in this study were in boldface.

Taxon	Strain designation	Genbank	Location
Ostreopsis ovata	OS-07BR	FM994900	-
Ostreopsis sp.	s0733	AB674860	West Pacific Coast, Japan
Coolia sp.	CMJJ2	FR845221	-
A. affine	AS-1	JF906997	South China Sea
A. affine	JHW0210	AY438015	-
A. affine	A10	DQ287852	-
A. affine	AABB0101	JF521616	Bell Bay, Tasmania
A. affine	**AaPA01**	KR188517	Aman Island, Penang,
A. concavum	-	AF032348	North Island, New Zealand
A. tamarense	AT-6	JF906995	-

Taxon	Strain designation	Genbank	Location
A. tamarense	JHW0003	GQ120507	-
A. excavatum	GEV1	AY056824	-
A. catenella	ACDH	JF906989	East China Sea
A. catenella	Axsp-K03	DQ785886	Jangmok, South Korea
A. fundyense	CCMP1719	JF521624	New Hampshire, USA
A. fraterculus	CAWD97	AY338750	New Zealand
A. taylori	AY1T	AJ535347	Italy
A. andersonii	CCMP2222	JF521621	Tyrrhenian Sea, Italy
A. andersonii	CCMP1597	JF521619	Massachusetts, USA
A. andersonii	CCMP1718	JF521620	Massachusetts, USA
A. andersonii	**AspPA01**	KR188518	Aman Island, Penang,
A. ostenfeldii	CCMP1773	JF21636	Kattegat, Denmark
A. ostenfeldii	IMRV062007	JF521637	Flodevigen, Norway
A. peruvianum	AP19	JF921197	New River, NC, USA
A. peruvianum	AP17	JF921195	New River, NC, USA
A. tamutum	AL2T	EU707459	-
A. insuetum	X6	AF318233	Corsica, France
A. insuetum	CCMP2082	JF521630	Japan
A. minutum	AL-1	JF906999	Portugal
A. minutum	CCMP113	JF521634	Ria de Vigo, Spain
A. leei	AIMS03	AY566184	-
A. leei	AT2	AY959942	Singapore
A. leei	JHW0006-2	AY438019	-
A. leei	**AlPA01**	KR188516	Aman Island, Penang, Malaysia

Phylogenetic analyses

Phylogenetic analyses of maximum parsimony (MP) and maximum likehood (ML) were performed using Phylogenetic Analysis Using Parsimony* (PAUP*) ver. 4.0b10 [20]. A heuristic search and tree-bisection-reconnection (TBR) swapping with 1,000 bootstrap replications were conducted for the MP analysis. For ML analysis, a substitution and rate heterogeneity model was calculated in jModelTest2 [21] through Akaike information criterion (AIC). ML analysis was then performed in PAUP with 100 random-addition replicates and heuristic searches with stepwise-addition plus TBR branch-swapping and 500 bootstrap replications. Bayesian analysis was performed using MrBayes v3.2.5 [22] with 500,000 Markov chain Monte Carlo (MCMC) generations and 50% majority rule consensus tree was conducted using a burn-in of 25% and posterior probabilities was calculated.

PSP toxin analysis

Mid exponential phase cells were harvested in triplicate by centrifugation at 2000 x g for 5 min. Cells were re-suspended in 0.05 M acetic acid and lysed by sonication. The sample was then centrifuged at 10,000 × g for 10 minutes and subsequently the supernatant was collected. The sample extract was filtered through a 0.45 µm nylon membrane filter and the filtrate was analyzed by using HPLC. Analysis of the PSP toxins was carried out using the post column

oxidative fluorescence method of Oshima et al. [23]. Liquid chromatography was performed using a Shimadzu UFLC HPLC system fitted with a Pickering post-column reaction system with a fluorescence detector. Toxins were separated by reversed phase chromatography using an Ascentis C18 column (4.6 mm i.d. x 25 cm, 120Å, 5 µm) with a flow rate of 0.8 ml min^{-1}. The column temperature was kept at 27ºC, while the post-column temperature was set at 65ºC. All reagents used were of HPLC grade. The chromatographic conditions were performed with mobile phase for STXs consisting of 2mM heptanesulfonate in 30mM ammonium phosphate buffer and 6% acetonitrile (v/v), pH 7.1 and mobile phase for gonyautoxins (GTXs) contained 2mM heptanesulfonate in 30mM ammonium phosphate buffer, pH 7.1. The post-column oxidizing reagent was 7mM periodic acid in 80mM sodium phosphate buffer, pH 9.0, and the acidifier was 0.5M acetic acid with a flow rate of 0.4 ml min^{-1}. Sample injection volume was 10 µl. The monitoring of fluorescence was observed at 330 nm excitation and 390 nm emissions. PSP toxin profile was identified and quantified by comparing chromatograms of certified toxins standards (NRC, Halifax, Canada).

RESULT AND DISCUSSION

Some dinoflagellates of the genus *Alexandrium* Halim are known to produce neurotoxin saxitoxin and its' congeners that causes paralytic shellfish poisoning (PSP). Three *Alexandrium* species have been implicated in paralytic shellfish poisoning (PSP) incidents in Malaysia, namely *Pyrodinium bahamense var. compressum, Alexandrium tamiyavanichii* Balech and *A. minutum* [2, 24-25]. The identification of species within *Alexandrium* was based on fine details of the thecal plates pattern such as shape and size of first apical plate (1′), sixth precingular (6′), anterior sulcal (s.a.) and posterior sulcal plate (s.p), the presence and location of the ventral pore (v.p) [13]. In this study, three clonal cultures of *Alexandrium* species were established from Aman Island, Penang, Malaysia. The species were identified as *Alexandrium leei* Balech, *A. affine* (Inoue and Fukuyo) Balech and *A. andersonii* Balech. The morphology of the collected *Alexandrium leei* and *A. affine* resembled with those previously documented in Strait of Malacca [25]. This represents a new record of *A. andersonii* species occurrence in Malaysia.

Morphology characterization of *Alexandrium andersonii*

Cells are non-chain forming, round to slightly oval in shape and small sized species (Figure 1A & 1B). Cells were 14-25 µm long and 16-24 µm wide. Our observed found that the cells have relatively thin thecal plate, easily collapsed. First plate (1′) is narrow with small ventral pore, adjacent to a characteristic s.a and characteristic 6′′ (Figure 1B). Plate 2′ comparatively broad (Figure 1C). Plate 3′ is hexagonal and almost symmetric (Figure 1C). The 6′′ plate is arrow-shaped left margin and the s.a is trapezoidal (Figure 1B). A ventral pore was located midway on the right margin of 1′ (Figure 1B), although sometimes it was observed inside 1′ (Figure 1C). The posterior sulcal plate (s.p) is wider than long (Figure 1D). The descriptions reported here agree well with the original description of *A. andersonii* from coastal waters off Cape Cod on the Atlantic coast of North America [12]. *Alexandrium andersonii* is one of the members in *A. minutum* group [13]. The features of the species (*A. minutum, A. lusitanicum, A. angustitabulatum and A. andersonii*) in *A. minutum* group are small size, predominantly oval shape and posterior sulcal plate is wider than long, not quite symmetrical [13]. *Alexandrium andersonii* is clearly distinguished with the

other species in genus by the shape of plate 6′′and s.a plate [13, 24-26]. Based on Figure 2, *A. andersonii* was shown to be closely related to *A. ostenfeldii* and *A. peruvianum* phylogenetically. In contrast to *A. andersonii* that is in *A. minutum* group, *A. ostenfeldii* and *A. peruvianum* were placed in the *ostenfeldii* group by Balech [13] with medium to large cell-size; narrow 1' and very oblique with large ventral pore. The distinct morphological features that can readily differentiate *A. andersonii* with *A. ostenfeldii* and *A. peruvianum* were cell size and characteristic of ventral pore between 1' plate [13, 27, 28]. Other than Malaysia, *Alexandrium andersonii* was also been found in the Irish coastal waters [27], Gulf of Naples, Italy [29], Aegean Sea, Greece [26, 30] and along the coast of China [31].

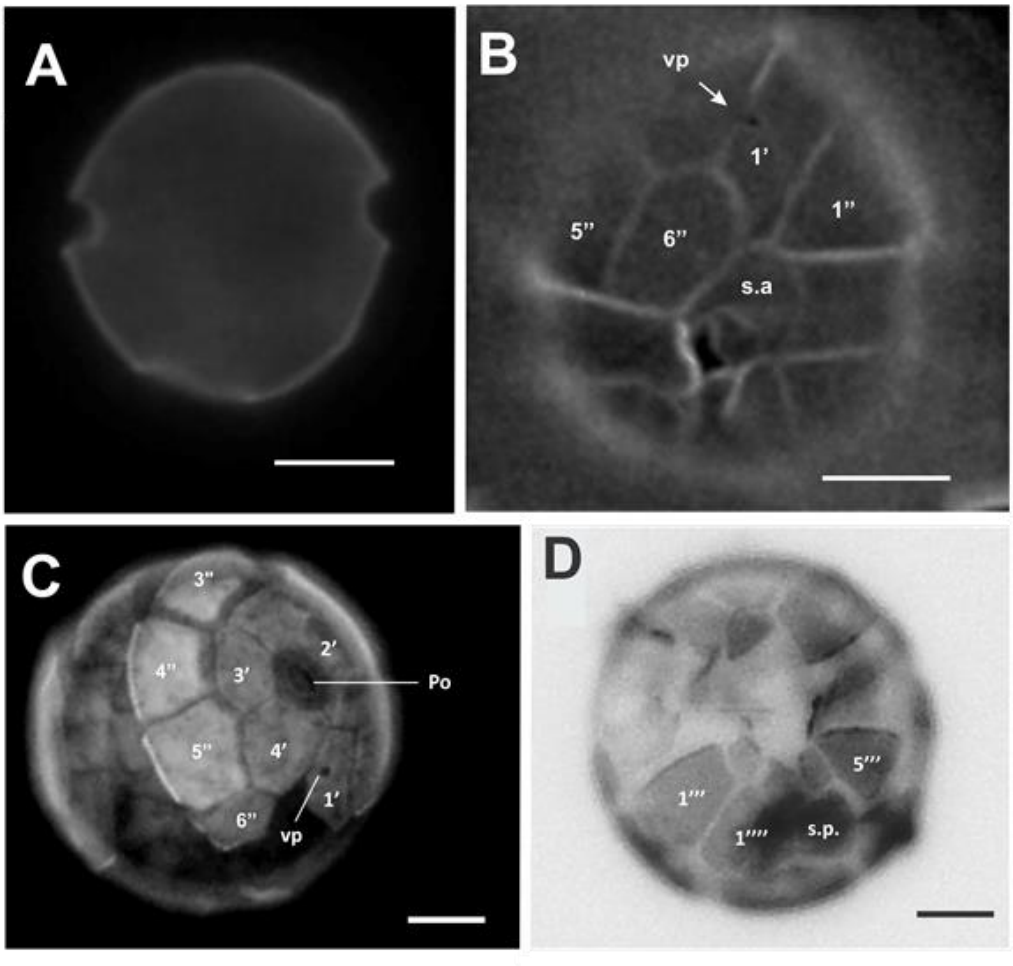

Figure 1: *Alexandrium andersonii.* (A) a roundish vegetative cell; (B) ventral view of a cell showing the first apical plate (1), precingular plates 1′′, 5′′ and arrow-shaped of sixth precingular plate (6′′), location of the ventral pore (v.p) (arrow) and wide anterior sulcal plate (s.a); (C) the apical view showing apical pore (Po), apical plates 1′- 4′, precingular plates 3′′- 6′′ and v.p inside the 1′ plate; (D) antapical view showing sulcal plate (s.p), postcingular plates 1′′′ and 5′′′ and antapical plate 1′′′′. Scale bars = 10µm.

Molecular phylogeny of *Alexandrium* species

A total of 565 characters (including gaps) were obtained from the aligned sequences, of which 95 were constant, 297 were parsimony informative and 173 variable characters were parsimony uninformative. Based on the nucleotide sequences of the LSU rDNA, AaPA01 strain grouped together with other *A. affine*, AlPA01 grouped together with other *A. leei*, both highly supported by bootstraps and posterior probabilities (MP/ML/BI: 100/100/1.0 and 100/100/0/99, respectively) (Figure 2). The strain of AspPA01 revealed close relationship to other *A. andersonii* with strong node support (MP/ML/BI, 100/100/1.00) (Figure 2)

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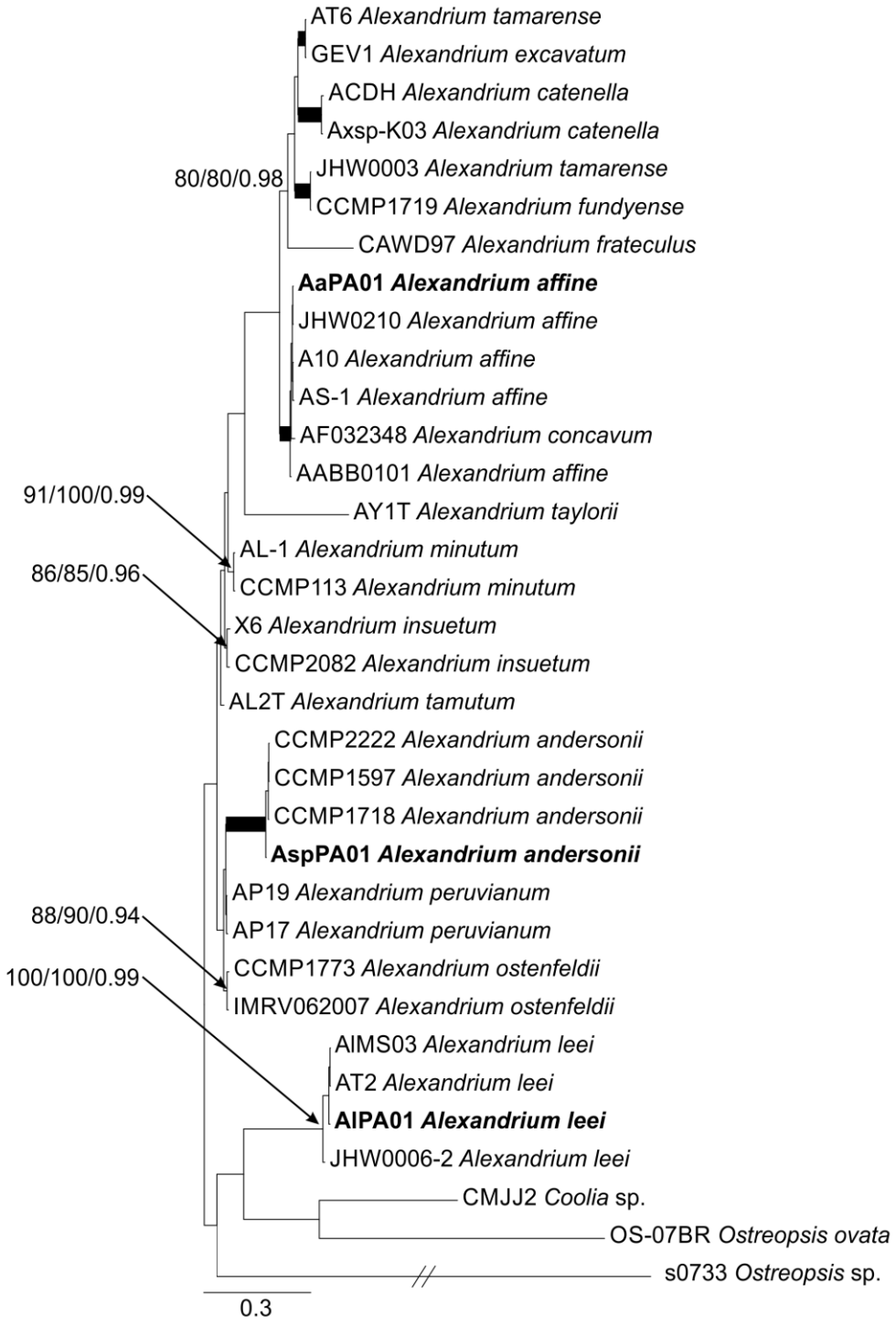

Figure 2: Pylogenetic tree inferred from Bayesian Inference (BI) of *Alexandrium* species based on LSU rDNA sequences. *Alexandrium* strains obtained from Aman Island were in boldface. Nodes are annotated with MP/ML bootstrap values of > 80% and BI posterior probabilities of >0.90.

PSP toxin

In this study, PSP toxin was not detected from the cultured strain of *A. andersonii* (AspPA01); similarly, it was found to be non-toxic based on previous records [14, 29, 31]. In contrast, *A. andersonii* from the Gulf of Naples, Italy was found positive for PSP which also consists of saxitoxin (STX) and neosaxitoxin (neoSTX) [29]. However, Sampedro et al., [32] further proved that the same strain from Gulf of Naples, Italy was non-toxic.

CONCLUSION

The occurrence of *Alexandrium andersonii* was recorded for the first time in Malaysian waters. The presence of the potentially toxins-producing microalgae in the aquaculture area especially in the cockles farming is alarming even though in low concentration, it may pose a potential threat. Factors contributing to the abundance and occurrence of the potentially harmful microalgae in Aman Island are also required for further investigation. Thus, regular monitoring of plankton in the aquaculture area of Aman Island is essential to ensure that cockles harvested from the area are safe to consume. Molecular data obtained from this study for species of *Alexandrium* will serve as the preliminary data for DNA barcoding of the potentially harmful dinoflagellates.

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