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CHARACTERIZATION OF THE SAXITOXIN BIOSYNTHETIC STARTING GENE, *sxtA*, IN THE TOXIC DINOFLAGELLATE, *Pyrodinium bahamense* var. *compressum*

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Abstract: *Pyrodinium bahamense* var. *compressum*, a saxitoxin-producing dinoflagellate, frequently blooms on the west coast of Sabah. According to previous studies, saxitoxin from cyanobacteria and dinoflagellates is manufactured from similar precursors (three arginines, one methionine via S-adenosylmethionine and one acetate) through identical biochemical pathways. The saxitoxin biosynthetic starting gene, *sxtA* is essential for synthesizing the final compound. The genes associated with saxitoxin synthesis in *Alexandrium* spp. and cyanobacteria have been previously identified; in spite of this, limited information is known about *P. bahamense* var. *compressum*, the principal tropical saxitoxin-producing dinoflagellate. In this study, the exclusive starting gene for saxitoxin biosynthesis, *sxtA*, specifically the SAM-dependent methyltransferase, *sxtA1* and the class II aminotransferase coding gene, *sxtA4* of *P. bahamense* was studied. Comparative sequence analysis revealed that *sxtA1* and *sxtA4* genes in *P. bahamense* exhibited high sequence similarity with other toxic dinoflagellates such as *Alexandrium fundyense* and *Alexandrium tamarense*. This study provides a genetic insight into saxitoxin biosynthesis in *P. bahamense*, which will be helpful in future investigations such as the development of genetic markers to study the expression of the *sxtA* gene and the identification of potential molecular targets for bloom characterization.

Keywords: Cyanobacteria, dinoflagellate, harmful algal blooms, saxitoxin, *sxtA1, sxtA4.*

Introduction

Saxitoxin (STX) and its analogues are potent neurotoxic alkaloids that causes paralytic shellfish poisoning (PSP) in humans from ingestion of mussels, oysters and other shellfish contaminated with the toxin (Perini *et al*., 2014). These are carbamate alkaloid compounds that are produced by a rare chemical reaction in metabolic pathways where Claisen condensation occurred with amino acid and polyketide synthase-like enzyme (Shimizu *et al.*, 1984; Shimizu, 1993). Saxitoxin compounds are Na^+ channel blockers that inhibit the formation of neural impulses in cell membranes and prolong the gating duration of the K^+ channels in cardiac muscle cells, resulting in cardiovascular problems (Pearson *et al*., 2010; Leal & Cristiano, 2021). Saxitoxin compounds appear to be synthesized in a similar biosynthetic route by microorganisms from two kingdoms: Prokaryotic cyanobacteria and eukaryotic dinoflagellates (Kellmann *et al.*, 2008a; Hackett *et al*., 2013).

The STX biosynthetic gene cluster identified in STX-producing cyanobacteria strain *Cylindrospermopsis raciborskii* T3 was investigated based on functional homology analysis and liquid chromatography-mass spectrometry study. More than 35 kb of the STX biosynthesis pathway is encoded, with 30 catalytic functions allocated to 26 genes (*sxtA* – *sxtZ*) (Kellmann *et al*., 2008b). Stüken *et al*. (2011) has reported that the presence of the *sxtA1* and *sxtA4* domains of the starting gene, *sxtA* are essential for the synthesis of STX and is a significant indicator for effective STX production. Dinoflagellates that produce saxitoxins are *Pyrodinium, Gonyaulax, Alexandrium* and *Gymnodinium.* Most of the

STX-producing dinoflagellate strains were found to encode both *sxtA1* and *sxtA4* domains on their genome. However, in non-STX-producing dinoflagellate strains, it could be either the last domain *sxtA4* or both the domains are absent. The dinoflagellate *Alexandrium ostenfeldii* appeared to be devoid of the *sxtA4* motifs, which contributed to the dinoflagellate's inability to produce STX (Suikkanen *et al.*, 2013). Murray *et al.* (2015) found that the *sxtA4* domain has significant sequence conservation in STX-producing *Pyrodinium*, *Alexandrium* and *Gymnodinium* species, but not in non-STX-producing species. However, there were some exceptions that *sxtA1* and *sxtA4* genes had been amplified from the genome of non-STX-producing *Alexandrium tamarense* strain CCMP1771. It was stipulated that the amount of STX produced in this strain had to be less than the detection limit of the high-performance liquid chromatography/mass spectrometry, or else exceptional isoforms may have been created instead (Stüken *et al*., 2015).

The cyanobacterial genome's distinct starting gene *sxtA* has the same domain structure as dinoflagellate transcripts, with the exception that dinoflagellates have higher GC content. In addition, there is also presence of poly(A)-tails at the 3'-end and spliced leader sequences at the 5'-end in dinoflagellates (Stüken *et al*., 2011). In cyanobacteria, *sxtA1* (SAM-dependent methyltransferase), *sxtA2* (GCN5-related N-acetyltransferase), *sxtA3* (acyl carrier protein) and *sxtA4* (class II aminotransferase) are the four catalytic *sxtA* domains (Kellmann *et al*., 2008b). Dinoflagellate transcripts may contain *sxtA1-sxtA3* (short isoform) or contain all four (*sxtA1-sxtA4*) catalytic domains (long isoform) (Stüken *et al*., 2011). Amplifications have been successfully carried out on dinoflagellate genomes of *sxtA1* and *sxtA4* fragments but not the entirety of the *sxtA* gene (Stüken *et al*., 2011).

Presently, the characterization of the *sxtA* gene was reported in Alexandrium species (*A. minutum*, *A. fundyense*, *A. catanella*, *A. tamarense* and *A. tamiyavanichii*) and *Gymnodinium catenatum* (Hii *et al*., 2012; Hackett *et al*., 2013; Zhang *et al*., 2014; Mendoza-Flores *et al*., 2018), but the gene from one of the significant tropical STX-producing harmful algae *P. bahamense* var. *compressum* (Usup *et al*., 1989; Mertens *et al*., 2015) is notably scarce. More human illnesses and deaths have been caused by this species than any other STX-producing dinoflagellates. In 1972, the first confirmed toxic *P. bahamense* bloom was reported in Papua New Guinea (Maclean *et al.,* 1989), followed by incidences of toxic blooms in Asia; Brunei and Sabah in 1976, the Philippines (Manila Bay and Mindanao) in 1983, Indonesia (Ambon) in 1994 and the Philippines (Palawan Island) in 1998 (Wiadnyana, 1996; Sombrito *et al.,* 2004; Azanza *et al*., 2010; Usup *et al*., 2012).

Plate (1906) was first to describe the Atlantic *P. bahamense* at New Providence Island, Bahamas. Two variants of this species, P. *bahamense* var. *bahamense* Plate and *P. bahamense* var. *compressum* (Böhm, 1931) were identified by Steidinger *et al*. (1980), both of which have minor morphological variations. One of their distinguishing characteristics is that *P. bahamense* var. *bahamense* does not produces toxin while *P. bahamense* var. *compressum* produces toxin (Azanza & Taylor, 2001). Yet, a previous study showed that *P. bahamense* var. *bahamense* cultures can occasionally produce STX in the laboratory (Landsberg *et al*., 2006). *P. bahamense* var. *compressum* microalgae are found in tropical Indo-West Pacific coastal waters (Hallegraeff, 1991; Steidinger, 1996) whereas var. *bahamense* has been found in the western North Atlantic (Steidinger *et al.,* 1980; Phlips & Badylak, 1996; Badylak & Phlips, 2004; Phlips *et al*., 2006).

The saxitoxin content of *P. bahamense* peak when the cells are actively dividing (mid-exponential growth phase), remained constant during the stationary growth phase and rapidly declined as the microalgae approached death (Gedaria *et al*., 2007). Physiological studies revealed a definite connection between *P. bahamense* growth rate and toxin synthesis was due to arginine production, an amino acid precursor to the STX formation. Arginine

concentrations were very low during the highest toxin level and rapidly increased as the cultures approached death (Gedaria *et al*., 2007). The findings led to the hypothesis that toxin concentration increases at the beginning of the growth phase could be due to the presence of excess free arginine being transformed into STX following the initial surge in nitrogen uptake (Usup *et al*., 1994). However, it was discovered that when the toxin level was decreased, the growth rate of *P. bahamense* was shown to be higher. This correlation has also been confirmed when other *Alexandrium s*pecies were subjected to diverse environmental stressors in batches of culture, although not all culture conditions were used (Anderson *et al*., 1990). In this scenario, the build-up of toxins occurred at a quicker pace than toxin transmission to daughter cells during cell division (Gedaria *et al*., 2007).

Recently, Cusick and Duran (2021) had employed *sxtA4* as a molecular proxy to assess the genetic potential of *P. bahamense* to produce STX in a natural population. The *P. bahamense* strain used in their study was associated with the Atlantic-Caribbean variety 'bahamense' whereas this study characterized the *sxtA* gene from the tropical 'compressum' variety. Although the *P. bahamense* var. *bahamense* was commonly known as a non-toxic strain, interestingly, Cusick and Duran (2021) were able to demonstrate the existence of both *sxtA4+* and *sxtA4-* genotypes variants within *P. bahamense* Atlantic-Caribbean subpopulations. While the *sxtA4* gene may or may not be present in 'non-toxic' strains, its absence is a strong indication of lack of toxicity.

The bacteria communities associated with the same *P. bahamense* var. *compressum* strain were examined using high-throughput sequencing of the 16S metagenomes in prior research by Law *et al.* (2020). *P. bahamense* was found to be associated with a number of interesting bacterial species, some of which had been previously reported associated with saxitoxin production. To complement the previous study, we conducted a study on the characterization of *sxt* genes in *P. bahamense* (this study) and its associated bacteria (ongoing study). The present study reports the characterization of the two domains of the STX starting gene, *sxtA1* and *sxtA4*, from the toxic Sabah dinoflagellate *P. bahamense* var. *compressum* and compares the genes to those from other toxic dinoflagellates and STX-producing organisms. Our study revealed that both *sxtA1* and *sxtA4* from *P. bahamense* exhibited high sequence similarity and identity with *sxtA* domains of toxic *Alexandrium* species and *Gymnodinium catenatum*. The findings of this study will better understand the saxitoxin biosynthesis pathway in *P. bahamense*, as there is very minimal information available on the saxitoxin genes of this species. The information from this study could assist in the molecular detection of the *sxtA* genes of *P. bahamense*, which may result in more significant efforts such as long-term management of HABs and mitigation of PSP cases in local communities.

Materials and Methods

Culture Establishment

Pyrodinium bahamense var. *compressum* (*P. bahamense*) strain CC-UHABS-040(M) was isolated from Sepanggar Bay (6.08° N, 116.12° E) in December 2012 (Table 1). This was also the time when high levels of STX were identified in the seawater, reaching up to 3,300 Mouse Units (MU). The *P. bahamense* cells were cultured in sterile seawater-based enriched f/2 medium (Guillard & Ryther, 1962). The *P. bahamense* culture was maintained at 25°C with a 12:12 hour light/dark cycle and a light intensity of 150 µmol photons m^{-2} s^{-1} in a culture chamber.

Toxicity Determination

The toxicity of the *P. bahamense* culture was determined using a high-performance liquid chromatography method established by Oshima (1995) at the Fisheries Research Institute in Batu Maung, Penang, Malaysia. The sample was resolved on a Supercoil C-18 column by using the isocratic elution profile. The mobile phase for the STX was 2 mM heptanesulfonate in 30 mM ammonium phosphate buffer and acetonitrile, pH 7.1. For the GTX 1 to 6, the

Table 1: The details of the Sabah *Pyrodinium bahamense* var. *compressum* CC-UHABS-040(M) strain

Strain	Place and Date of Isolation	Isolator	GenBank Accession Number	
			sxtA1	*sxtA4*
CC-UHABS-040(M)	Sepanggar Bay, Sabah, December 2012	Yusdi Ismail	MN431957	

mobile phase was 2 mM heptanesulfonate in 10 mM ammonium sulphate buffer, pH 7.1. The injection volume of the sample was 10 µL. For each post-column reagent, the mobile phase flow rates were 0.8 and 0.4 mL/min. All of the columns were set to a temperature of 30°C. For detection, wavelengths used was 330 nm for excitation and 390 nm for emission.

DNA Extraction and PCR Amplification

Pyrodinium bahamense culture was harvested during the exponential phase using a 0.45 µm sterile membrane filter (Whatman). The filtered *P. bahamense* cells were washed with sterile seawater in a 15 mL centrifuge tube and centrifuged at 3,000 x g for 5 minutes at room temperature. The genomic DNA extraction was carried out using the NucleoSpin® Plant II kit (Macherey-Nagel) based on the manufacturer's protocol.

PCR amplifications were carried out in 20 µL volumes consisting of DNA template of approximately 1 ng/µL, 1 unit of 2x Phusion® High-Fidelity Master Mix with HF Buffer (New England Biolabs) and 0.5 µM of each the forward and reverse primer pair (Table 2). *Pyrodinium bahamense*-specific 18S ribosomal RNA (rRNA) gene primers, TPL1F and CWL1R (Chin *et al*., 2010) were also used alongside with the *sxtA* primers as a positive control and to indicate that both primer sets have the ability to bind to and amplify their respective targets when employed with the same PCR conditions.

The PCR amplification was performed on a PTC-200 Thermal Cycler (MJ Research, USA), with the following conditions: Initial denaturation at 98°C for 10 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 8 seconds, with a final extension at 72°C for 1 minute. The PCR amplicons were visualized on a 1% agarose gel by electrophoresis, where the PCR products that contained the DNA band corresponding to the expected size were sent to First Base Laboratories for DNA Sequencing Plus service, which included PCR product purification and sequencing.

Both sequences, *sxtA1* and *sxtA4* were deposited in the National Center for Biotechnology Information (NCBI) Genbank with the accession number of MN431957. However, the *P. bahamense* 18S rRNA amplicon was not included in the sequencing since the 18S rRNA primer sets used for the validation were designed to amplify *P. bahamense* dinoflagellate (Chin *et al*., 2010). The amplification was carried out to ensure the presence of *P. bahamense* cells in the reaction tubes and to ensure that any potential contaminants did not hinder the PCR amplifications.

Table 2: The details of primers targeting the *sxtA1* and *sxtA4* domains

Domain	Primer	Orientation	Sequence 5' - 3'	Reference
sxtA1	sxt001	Forward	TGCAGCGMTGCTACTCCTACTAC	Stüken *et al*., 2011
	sxt002	Reverse	GGTCGTGGTCYAGGAAGGAG	
sxtA4	sxt007	Forward	ATGCTCAACATGGGAGTCATCC	
	sxt008	Reverse	GGGTCCAGTAGATGTTGACGATG	

Phylogenetic Analyses

The raw sequences of *sxtA1* and *sxtA4* were pre-analyzed using GeneStudio™ Professional (Sequence analysis software). The chromatogram file was referred to as a comparison to trim and edit the sequences, which was subsequently compiled into FASTA format. The nucleotide sequence was then translated into amino acids using an online translation tool (insilico.ehu.es). The correct reading frame was determined by aligning each open reading frame (ORF) generated in the NCBI BLASTP.

For phylogenetic analysis, related sequences were acquired from the NCBI GenBank. The multiple protein sequence alignment was generated using MAFFT version 7, with the auto model selection based on the data size (Katoh *et al*., 2019). GBlock was applied to the multiple alignments to remove the poorly aligned amino acids' positions and consequently, avoid systematic error such as the long-branch effect (Castresana, 2000; Talavera & Castresana, 2007; Dereeper *et al*., 2008). The best fit model for the amino acid sequences was selected using MEGAX (Kumar *et al*., 2018) where LG being the optimal evolutionary model selected. Using the LG model, the maximum likelihood (ML) analysis was performed using raxmlGUI 2.0 (Edler *et al*., 2021) with 1,000 bootstrap replicates. Bayesian inference was run under MrBayes 3.2.7a (Ronquist *et al*., 2012) using the same model. After running 200,000 generations, the generation was appended until the standard deviation of split sequences was less than 0.01.

Results and Discussion

Toxin Analysis

Saxitoxin is a tricyclic perhydropurine alkaloid that can have different substituents at different locations, resulting in more than 30 naturally occurring STX analogues (Kellmann *et al.,* 2008b; Wiese *et al*., 2010). Based on the toxin analysis of *P. bahamense* culture, Figure 1 (A) showed that the strain was actively producing toxins, in which STX, neosaxitoxin (neoSTX) and dicarbamoyl-saxitoxin (dcSTX) were detected with neoSTX as the primary compound detected. As shown in Figure 1 (B), four sets of gonyautoxins, GTX1, GTX3, GTX4 and GTX5

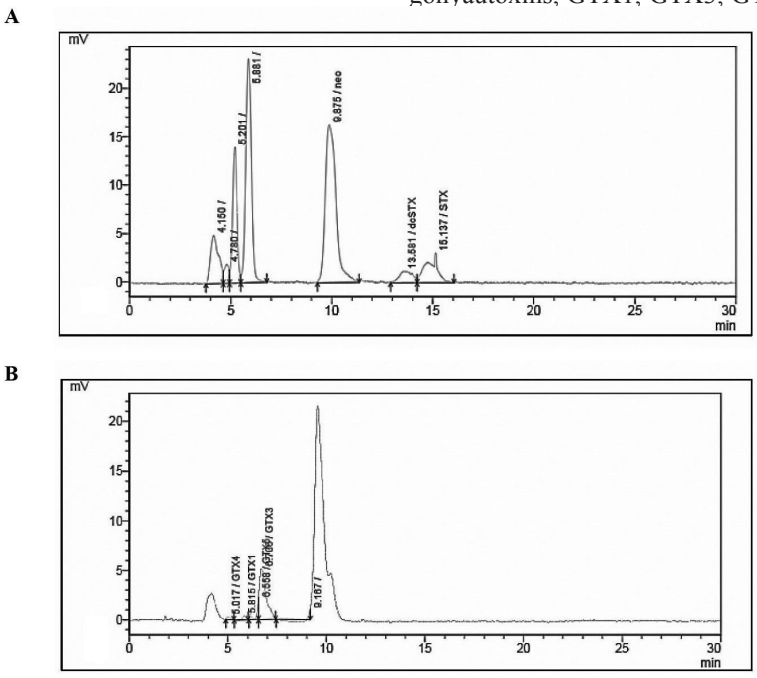

Figure 1: HPLC chromatogram profile of the cultured *P. bahamense* (A) for STX detection and (B) for GTX1-6 detection during the exponential phase

were also detected, among which GTX3 was the primary toxin compound presents in the culture of *P. bahamense*.

During the bloom event in 2013, a massive *P. bahamense* outbreak had caused four human casualties and 58 PSP cases in Kota Kinabalu (Suleimen *et al*., 2017). Many people were ill after eating toxin-contaminated shellfish bought from markets or gathered from the beach. Sabah areas which included Gaya Island, Sepanggar Bay and Likas Bay (Kota Kinabalu district), as well as Papar, Putatan and Tuaran districts, were all affected (Sabah Fisheries Department, 2013). The toxic samples reported from Papar northward to Tuaran were as high as 6,000 MU; meanwhile, the toxins detected in Sepanggar Bay reached 3,300 MU (Daily Express, 2013). The toxin level of 400 MU is already toxic and dangerous to consumers (Visciano *et al*., 2016). Between 2000 and 2017, Jipanin *et al*. (2019) reported a total of 3,279 HAB events in Sabah, with *P. bahamense* blooms accounting for more than 50% (1,752 events) of those HAB occurrences. Although there were many *P. bahamense* cases reported, not all *P. bahamense* blooms produced toxins.

Previously, Usup *et al*. (1994) had detected neoSTX, GTX 5, STX, GTX 6 and dcSTX, with neoSTX and GTX5 being the primary toxin produced in *P. bahamense* isolated from the west coast of Sabah, Malaysia. They obtained these results by observing the impact of various growth parameters such as temperature, salinity and light intensity in different growth phases of the *P. bahamense* culture. In addition, Palau's *P. bahamense* isolates in the Philippines were high in GTX4 and GTX5, with STX and neoSTX at relatively low concentrations (Harada *et al.*, 1983). The primary toxin of *P. bahamense* isolated from Masinloc Bay, the Philippines, in 1993 to 1995 was neoSTX, followed by STX and small amounts of dcSTX, though, higher STX concentration was reported in the 1995 *P. bahamense* isolates (Azanza *et al*., 2010). In addition, *P. bahamense* isolated from Bamban Bay, Zambales, the Philippines, produced three types of toxins: STX, dcSTX and B1, with STX being the prevalent toxin produced (Gedaria *et al*., 2007).

The different proportions of toxins produced by the same dinoflagellate, *P. bahamense*, in the various locations mentioned previously were due to different growth conditions, which could be influenced by the environmental conditions of the seawater such as temperature, nutrients, salinity, carbon dioxide level and other factors (Murray *et al*., 2020). Nevertheless, these reports showed that toxin compositions of *P. bahamense* differ through its global distribution, with no conclusive correlation between the toxin profiles and biogeography of the same species. Most importantly, the cultivated Sabah *P. bahamense* used in this study has shown to be capable of producing the major toxin, STX.

Phylogenetic Analysis of sxtA1 and sxtA4 Domains

Based on the PCR sequencing analysis result, the partial coding sequences of *P. bahamense* domain *sxtA1* were 493 bp with deduced amino acid sequences of 164 polypeptides and G+C content of 66.7%. The NCBI BLASTX similarity search showed that the amplified *sxtA1* sequences of *P. bahamense* generated 136 organism hits, with 41 hits (30%) from eukaryotes, dinoflagellates (28%), pelagophytes (1%) and fungi (0.7%) and the remaining 70% from bacteria, terrabacteria (57%) and proteobacteria (12%). Meanwhile, the results from BLAST obtained 95 hits that corresponded to the sequences of other STX-producing dinoflagellates, *Alexandrium* spp. (89%) and *G. catenatum* (11%). *Alexandrium fundyense* strain CCMP1719 partial sequence *sxtA* (Accession number: JF343359) was the second best match, with 95.74% identities and 0.0 *e*-value.

The phylogenetic analysis of the *sxtAl* domain revealed a well-supported distinction of two sub-clusters, specifically, dinoflagellate and bacterial clades (Figure 2). The bacterial clade then separated into two sister clades: Cyanobacterial *sxtA1* and proteobacterial polyketide synthases. This study's *P. bahamense* (indicated in red rectangle) was positioned

within the dinoflagellate clade. There were two clearly delineated branches, one for the short isoform *sxtA* and the other for the long isoform *sxtA*. The dinoflagellate clade's sub-clusters comprised non-STX and STX-producing dinoflagellates. The *sxtA1* gene of *P. bahamense* isolated from this study formed a sister clade with *Alexandrium* spp. long isoform group, with *G. catenatum* as its closest relative.

Additionally, the partial coding sequences of *sxtA4* domain of *P. bahamense* were 632 bp with deduced amino acid sequences of 210 polypeptides and G+C content of 62.8%. The NCBI BLASTX similarity search generated 113 organism hits, which corresponded to other STX-producing harmful algae such as *Alexandrium* spp. (97%) and *G. catenatum* (3%). The second best match was the *A. fundyense* strain A8 partial sequence *sxtA* (Accession number: KJ87912) with 95.40% identities and 0.0 *e*-value.

The phylogenetic topology analysis of *sxtA4* (Figure 3) showed that the bacterial sequences formed a well-supported clade that diverged into two sub-clusters, separating *sxtA4* genes from two kingdoms: Eukaryote (dinoflagellate) and prokaryote (bacteria). The bacterial cluster was split into two sister clades, namely actinobacterial aminotransferases and cyanobacterial *sxtA4* genes. Meanwhile, this study's *P. bahamense sxtA4* gene (indicated in red rectangle) was clustered together with other non-STX and STX-producing dinoflagellates, with *G. catenatum* as its closest relative as both shared a common ancestor at the end terminal.

Structurally, *P. bahamense sxtA* transcript has higher G+C content (~60%) than the STX-producing cyanobacteria, which contain approximately 40% (Kellmann *et al*., 2008a; 2008b; Mihali *et al*., 2009). Nonetheless, this trait was likely linked to their particular chromosome structure as a universal trait of core dinoflagellates and was most likely not an essential mechanism for gene expression regulation (Williams *et al*., 2017). The G+C content of *P. bahamense sxtA* in this study were comparable to those of other toxic microalgae such as *Alexandrium* spp. and *G. catenatum*, *sxtA1* (64–69%) and *sxtA4* (60–63%) (Stüken *et al*., 2011; Hii *et al*., 2012; Mendoza-Flores *et al*., 2018).

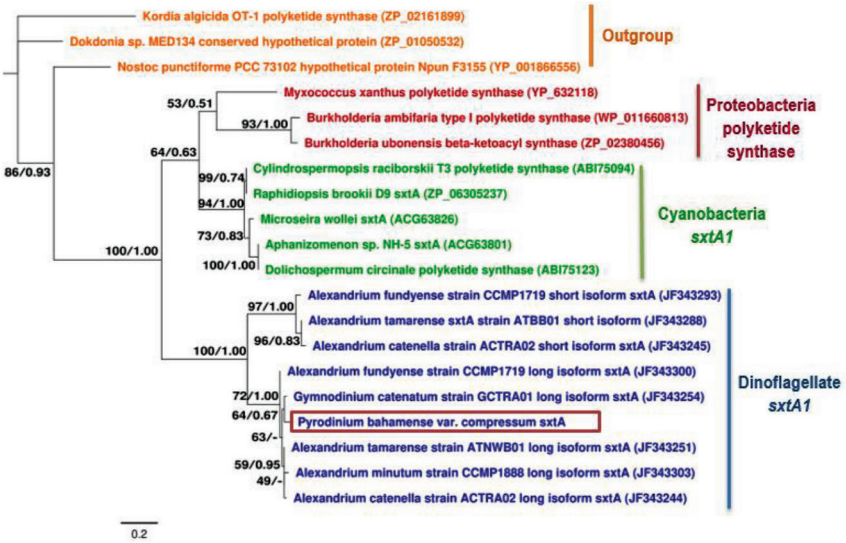

Figure 2: Phylogenetic tree of the *sxtA1* domain from amino acids sequences of STX-producing organisms. Values at nodes indicate clade support from ML bootstrap analysis and the Bayesian posterior probabilities from Bayesian analysis. Sequence obtained in this study indicated in the red rectangle. The scale bar represents the length of a branch equating to 0.2 substitutions per site

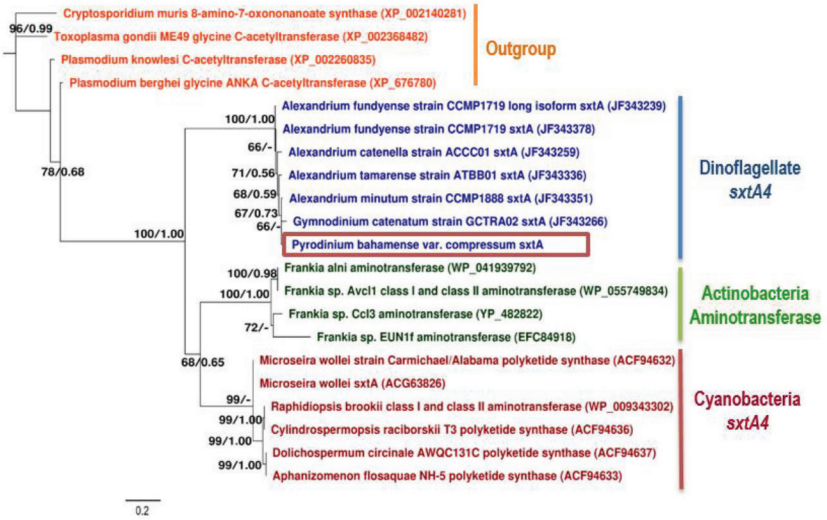

Figure 3: Phylogenetic tree of the domain *sxtA4* from amino acids sequences of STX-producing organisms. Values at nodes indicate clade support from ML bootstrap analysis and the Bayesian posterior probabilities from Bayesian analysis. Sequence obtained in this study indicated in the red rectangle. The scale bar represents the length of a branch equating to 0.2 substitutions per site

Pyrodinium bahamense sxtA transcripts were observed to cluster together with other long *sxtA1* transcripts of other dinoflagellates in Figure 2, suggesting that the dinoflagellate encodes all four *sxtA* domains. The shorter transcript was reported as lacking the terminal aminotransferase (*sxtA4*) while longer transcripts contain all four domains (Stüken *et al*., 2011). In comparison, Stüken *et al*. (2011) reported that the dinoflagellate *A. fundyense* CCMP 1719 comprised the two distinct transcript types. The longer transcript of *sxtA* has been hypothesized to be directly associated with toxin biosynthesis, as its deficiency results in the inability to synthesize STX in the non-toxic mutant of dinoflagellate *Alexandrium catanella* (Zhang *et al*., 2014). Interestingly, a recent study by Cusick & Duran (2021) revealed that the *P. bahamense sxtA4+* (indicating the presence of *sxtA4* gene in the genome) and *sxtA4-* (indicating the absence of *sxtA4*) genotypes coexisted within the wild sub-populations of *Pyrodinium bahamense* from the western Atlantic, although the *sxtA4+* genotype predominates.

In this study, *sxtA1* and *sxtA4* of *P. bahamense* were observed to be related to other STX-producing dinoflagellates. Based on the phylogenetic analyses of *sxtA1* and *sxtA4*, the cyanobacterial *sxtA* and bacterial putative toxin-related genes consistently formed sister clades to the dinoflagellate *sxtA*, which was similar with previous findings (Stüken *et al*., 2011; Hii *et al*., 2012; Hackett *et al*., 2013; Mendoza-Flores *et al*., 2018). Moustafa *et al*. (2009) also presented similar findings, suggesting that the chimeric origin in the cyanobacterial *sxtA1* N-terminus contained the acetyltransferase and ACP domain with a phosphopantetheinyl-attachment site (PP-binding), which were isolated from the genome of a proteobacterium such as *Myxococcus xanthus*. The C-terminus, on the other hand, featured a Class II aminotransferase domain and was most likely acquired from the actinobacterium *Frankia* sp.

Meanwhile, the strong monophyla observed in the *sxtA* phylogenetic topology of STX-producing dinoflagellates, with apparent separation from cyanobacteria (Figures 2 and 3), suggested that the precursors of these *sxtA* genes were independently attained by each lineage (Hacket *et al*., 2013). The STX genes in dinoflagellates were proposed to have originated

early in dinoflagellate evolution through a single horizontal event (Murray *et al*., 2015; Verma *et al*., 2019). The presence of *sxtA* paralogs in species that do not synthesize STX may lend credence to this theory. Interestingly, *P. bahamense* var. *bahamense* was previously thought to be incapable of synthesizing STX or causing PSP syndrome (Steidinger *et al*., 1980), however, saxitoxin was discovered in pufferfish captured from the Indian River Lagoon (Florida, USA) in 2002, coinciding with a *P. bahamense* var. *bahamense* bloom. Saxitoxin was detected in cultures established from these bloom waters (Landsberg *et al*., 2006). Although no molecular data on the unique starting gene in var. *bahamense* was established, it is most likely that var. *bahamense* genome also contains the *sxtA* transcript, based on its ability to produce saxitoxin under some circumstances, which support the occurrence of *sxtA* paralogs in *P. bahamense*.

Both phylogenetic analyses (Figures 2 and 3) showed that both domains shared a similar ancestor, *G. catenatum*. According to several studies, *Pyrodinium sxtA* domains mostly have closer affiliation with *Alexandrium* spp. than other dinoflagellates (Leaw *et al*., 2005; Orr *et al*., 2013; Mendoza-Flores *et al*., 2018). However, the *P. bahamense* strain from Sabah, Malaysia is more closely related to *G. catenatum* strain GCTRA01.

Three *P. bahamense sxtA4* strains: Sepanggar Bay, Malaysia, MY strain (MN431957), Sorsogon Bay, the Philippines, PLP strain (GBXF01000001) and Indian River Lagoon, Florida, USA strain (MZ234675) were aligned using MEGAX. However, due to the scarcity of information in the NCBI database, the *sxtA1* sequences are unavailable for comparison. Based on the sequence alignment of the nucleotide and amino acid of difference strains of *P. bahamense* in Figure 4, *P. bahamense* (MY strain) in this study was identical to the *P. bahamense* from Sorsogon Bay, the Philippines (PLP strain). Meanwhile, the USA strain has a slight difference by single nucleotide substitution in three locations (base pair (bp) 41, 240 and 524) (Figure 4). In the USA strain, a nucleotide substitution of G to C at 41 bp (Figure 4 (a)) has led to the difference in the translation of codon 14, from glycine to alanine (Figure 4 (b)). In contrast, there is no amino acid difference in the nucleotide substitution at 240 bp (Figure 4 (c)) as the nucleotide sequence translated into the same amino acid, phenylalanine (Figure 4 (d)). The final nucleotide substitution at 524 bp (Figure 4 (e)) occurred from A to G in the USA strain, which has caused codon 175 (Figure 4 (f)) to translate into glycine instead of glutamic acid. The significant similarity between MY and PLP *sxtA4* sequence could be likely to be the result of convergent evolution, or both may as well be likely from the same strain. Furthermore, the slight difference between the Pacific and Atlantic strains may suggest fixation to a specific location or environmental condition.

Conclusion

The established phylogenetic analysis of the two domains of the STX starting gene, *sxtA1* and *sxtA4,* of *P. bahamense* from Sabah, Malaysia, revealed a close relationship between *P. bahamense* saxitoxin biosynthesis starting genes and those of well-known STX-producing species. Additionally, the genes were found to frequently establish sister clades with other STX-producing organisms, such as cyanobacteria. This study reveals that horizontal gene transfer occurred early in the evolution of *P. bahamense* and may have occurred via a single horizontal event involving *sxtA* paralogs between the two varieties of *P. bahamense*. The comparison of the *sxtA4* sequences of Pacific and Atlantic *P. bahamense* variants revealed a slight difference in the *sxtA4* protein caused by a few base-pair substitutions, which could be a consequence of different environmental conditions. Further research will focus on the expression of the *sxtA* gene in *P. bahamense*. Additional research on the remaining 25 saxitoxin biosynthesis genes could be initiated due to the lack of GenBank reference sequences. The discovery of the essential starting *sxtA* gene will aid in the development of genetic

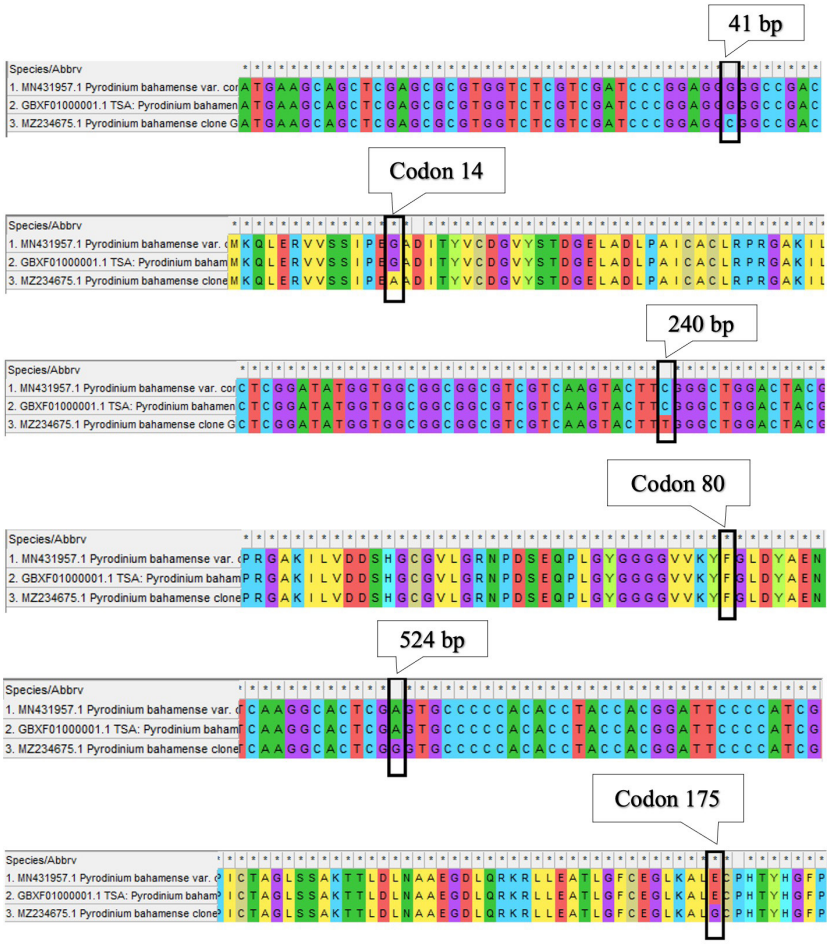

Figure 4: The comparison of aligned nucleotide and amino acid sequences of different strains of *P. bahamense* using MegaX

markers that will be valuable for early bloom detection and, ultimately, mitigation measures against deadly PSP cases. The findings of this study contribute to our knowledge of bloom ecology by presenting critical information on the saxitoxin biosynthetic starting genes of one of Malaysia's most significant STX-producing species, *P. bahamense* var. *compressum*.

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