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Identification of the Gene *sxtA* (Domains *sxtA1* and *sxtA4*) in Mexican Strains of *Gymnodinium catenatum* (Dinophyceae) and Their Evolution

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Saxitoxin (STX) and its analogs are a broad group of natural neurotoxic alkaloids, commonly known as paralytic shellfish toxins. *SxtA* is the initial gene in the biosynthesis of saxitoxin. It has been proposed that the genes for STX biosynthesis had a bacterial origin and were acquired in the dinoflagellates by a horizontal gene transfer (HGT). In *Gymnodinium catenatum,* the origin of the STX genes is not well established. In this paper, we sequenced *sxtA* gene (domains *sxtA1* and *sxtA4*) and determined the gene copy number in the genome in four Mexican strains of *G. catenatum*. We compare them with sequences of *G. catenatum, Pyrodinium bahamense,* and *Alexandrium* spp. from other geographic regions, and non-toxic producing dinoflagellates. Amplifications were performed for domains *sxtA1* and *sxtA4* from strains of *G. catenatum* and the phylogenetic analyses was done by maximum likelihood and Bayesian inference. The copy number determination was carried out using qPCR. The phylogenetic tree of domain *sxtA4* showed the formation of two clades where *G. catenatum* sequences separated from the *Alexandrium/Pyrodinium* clade. The domain *sxtA1* formed a higher number of clades than *sxtA4.* Sequences of *G. catenatum* were grouped together with sequences of *Alexandrium*. Dinoflagellates sequences that do not produce saxitoxin formed a separate clade. The gene copy number was 64 ± 30 and 110 ± 50 copies of *sxtA1* and *sxtA4* respectively. The identification of the gene *sxtA* of *G. catenatum* shows that the sequences are similar to those of *Alexandrium* species with low variations between species. These results may indicate that the acquisition of the gene *sxtA* was an early HGT event in the evolution of dinoflagellates. The possible loss of the ability to produce STX in some species suggests that the HGT from *Alexandrium* species toward *G. catenatum* is not possible.

Keywords: dinoflagellate, *Gymnodinium catenatum*, horizontal gene transfer, paralytic shellfish toxins, gene copy number

INTRODUCTION

Saxitoxin (STX) and its analogs are a broad group of natural neurotoxic alkaloids, commonly known as paralytic shellfish toxins (PSTs). PSTs are the causative agents of paralytic shellfish poisoning (PSP) and are mostly associated with marine dinoflagellates (eukaryotes) and freshwater cyanobacteria (prokaryotes), which form extensive blooms around the world (Wiese et al., 2010). The biosynthetic pathway and genes responsible for STX synthesis have been characterized in the cyanobacteria *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju T3. This biosynthesis pathway is encoded by a fragment larger than 35 kb, comparative sequences analysis assigns 30 catalytic functions that correspond to 26 proteins (Kellmann and Neilan, 2007; Kellmann et al., 2008). Subsequently, the STX gene cluster was characterized in the cyanobacteria *Anabaena circinalis* Rabenhorst ex Bornet & Flahault, *Aphanizomenon* sp. Morren ex Bornet & Flahault, *Rhaphidiopsis brookii* Hill and *Lyngbya wollei* Farlow ex Gomont (Mihali et al., 2009, 2011; Moustafa et al., 2009; Murray et al., 2011).

The origin of the STX genes in cyanobacteria involved multiple events of horizontal gene transfer (HGT) from different sources, followed presumably by the coordination of the expression of foreign and native genes in the common ancestor. The possible ancestral source for some STX genes was a proteobacteria; suggesting that the key PSP-toxin biosynthesis gene may have evolved in a prokaryotic organism (Kellmann et al., 2008; Moustafa et al., 2009).

SxtA is the unique starting gene of STX-synthesis in cyanobacteria. This gene has a polyketide synthase (PKS)-like structure characterized by four catalytic domains with activities of a *S*-adenosyl-methionine- (SAM) dependent methyltransferase (*sxtA1),* GCN5-related *N*-acetyltransferase (*sxtA2),* acyl carrier protein (*sxtA3*), and a class II aminotransferase (*sxtA4*) (Kellmann et al., 2008).

Some dinoflagellate species of the genus *Alexandrium* Halim, as well as *Pyrodinium bahamense* Plate and *Gymnodinium catenatum* Graham, are known for producing PST (Scholin et al., 1995; Blackburn et al., 2001). Homologs of the genes putatively associated for the STX-synthesis have been identified in *A. tamarense* (Lebour) Balech Group IV, *A. fundyense* Balech, and *A. minutum* Halim (Stüken et al., 2011; Hackett et al., 2013). The transcripts of *A. tamarense* and *A. minutum* show that the dinoflagellate transcripts of *sxtA* have the same domain structure as the cyanobacterial *sxtA* genes. In contrast to the bacterial homologs, the dinoflagellate transcripts are monocistronic, have a higher guanine–cytosine (GC) content, occur in multiple copies, contain typical dinoflagellate spliced-leader sequences, and eukaryotic polyA-tails (Stüken et al., 2011).

The genes responsible for the STX-synthesis were not exchanged directly between cyanobacteria and dinoflagellates. The strong monophyly of *sxtA*-related proteins from STX^+ dinoflagellates, and separately from cyanobacteria, supports the hypothesis that precursors of these *sxtA* genes were acquired independently by each linage (Hackett et al., 2013). Based on the *sxtA* data, the origin of the STX gene cluster within the dinoflagellates may have occurred between an ancestral STX^+ bacterium and the common ancestor of *Alexandrium* and *Pyrodinium* (Orr et al., 2013).

Gymnodinium catenatum, a chain-forming dinoflagellate is the only species that can produce PST within the genus (Attaran-Fariman et al., 2007; Negri et al., 2007; Hallegraef et al., 2012; Gu et al., 2013). The origin of SXT genes in this species is not well established, but the distribution of the *sxtA* and *sxtG* genes suggest that *G. catenatum* acquired STX genes independently from a secondary dinoflagellate-dinoflagellate transfer (Orr et al., 2013). Another hypothesis, proposing that a single horizontal transfer event occurred early in dinoflagellate evolution, is the most parsimonious explanation for the origin of STX in dinoflagellates (Murray et al., 2015).

In this paper, we analyse the identification, the genomic copies number and the evolution of the *sxtA* gene in *G. catenatum* using Mexican strains and comparing them with sequences of *G. catenatum, P. bahamense,* and *Alexandrium* species from other geographic regions, as well other non-toxic producing dinoflagellates.

MATERIALS AND METHODS

Strains and Culture Maintenance

The strains of *G. catenatum* in our study were isolated from several localities along the Mexican Pacific and the Gulf of California (**Figure 1**, **Table 1**). The strains were grown in modified GSe media (Bustillos-Guzmán et al., 2015) at 24 ± 1°C under a 12:12 h light:dark photoperiod and a photon irradiance of 150 μE $m^{-2}s^{-1}$ from white cool fluorescent lights.

DNA Extraction and PCR

For the DNA extraction, 30 mL of each strain were harvested during the exponential growth phase by centrifugation at 1,000 × g for 10 min; the supernatant was removed by decantation. The pellet was frozen until extraction of DNA, using the CTAB method (Band-Schmidt et al., 2012). Quantity and quality of DNA were determined using nanodrop instrument (NanoDrop, Thermo Fisher Scientific, Waltham, MA) and by agarose gel electrophoresis.

The genomic DNA was amplified by PCR in a PCR thermal-cycler system (MJ Mini, Bio-Rad Laboratories, Hercules, CA). The PCR reactions to amplify *sxtA1* and *sxtA4* were carried out in a final volume of 25 μl, the reaction mix consisted of 1 x PCR buffer, 0.5% DMSO, 1 mM $MgCl_2$, 0.5 μM of each primer (**Table 2**), 200 μM dNTP's, and 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The *sxtA1* was amplified as follows: 1 cycle of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 61°C, and 1 min at 72°C, and 1 cycle of 7 min at 72°C. The *sxtA4* was amplified as follows: 1 cycle of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C, and 1 cycle of 7 min at 72°C. The fragments were visualized on an agarose gel by electrophoresis.

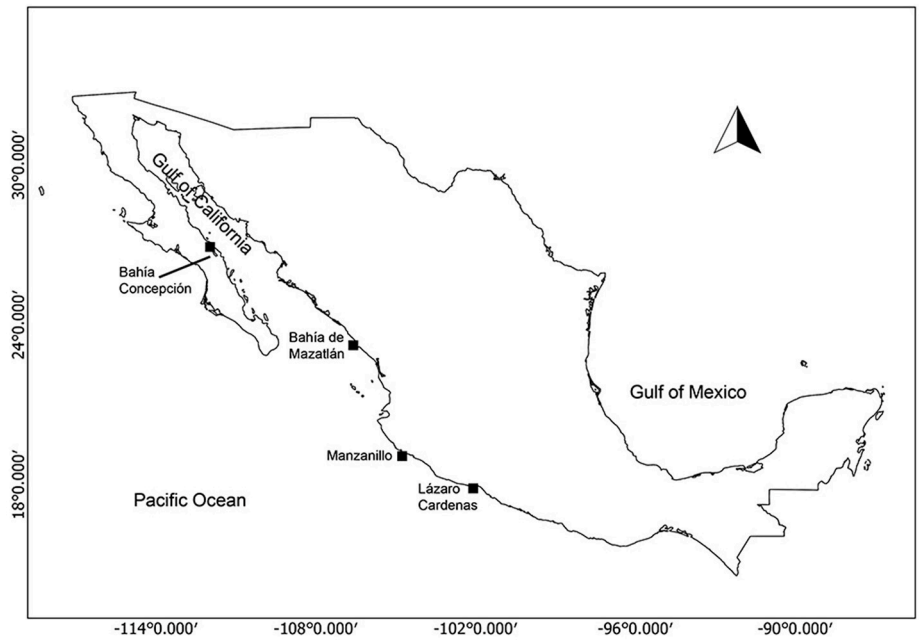

FIGURE 1 | Isolation localities of *Gymnodinium catenatum* strains in the Gulf of California and the Mexican Pacific.

TABLE 1 | Strains, place and year of isolation, and GenBank accession number of the Mexican *Gymnodinium catenatum*.

Strain	Place and date of isolation	Isolator	GenBank accession number	
			sxtA1	***sxtA4***
GCMV-7*	Bahía de Mazatlán, Sinaloa, 2007	C. Band	KY575974	KY575969
LC-62	Lázaro Cardenas, Michoacán, 2005	M. Rodríguez	KY575972	KY575968
G7 new	Manzanillo, Colima, 2010	S. Quijano	KY575971	KY575969
GCCV-6*	Bahía Concepción, B.C.S., 2000	C. Band	KY575973	

**Provided by the Colección de Dinoflagelados Marinos of CIBNOR (CODIMAR).*

TABLE 2 | List of primers of each domain, name, sequence and the reference, were used in the study.

Domain	Primer	Sequence 5′–3′	References
sxtA1	Sxt001F	TGCAGCGMTGCTACTCCTACTAC	Stüken et al., 2011
	Sxt002R	GGTCGTGGTCYAGGAAGGAG	Stüken et al., 2011
sxtA4	Sxt007F	ATGCTCAACATGGGAGTCATCC	Stüken et al., 2011
	Sxt008R	GGGTCCAGTAGATGTTGACGATG	Stüken et al., 2011
*sxtA4*qPCR	sxtA4Gcat	CGTACGCTTGAAGCACAATG	This study
	sxtA4Gcat	TGCGAGTCGTCAACGAGTAT	This study
*sxtA1*qPCR	sxtA1Gcat	AGCACCAGACGTTCTTCACT	This study
	sxtA1Gcat	CTTCACGTGCTCGTAGATGC	This study

Sequencing and Phylogenetic Analyses

PCR products were purified by precipitating with ethanol and sent for sequencing in Macrogen (Seoul, South Korea) using an automatic DNA sequencer (ABI 3730 × 1).

For the phylogenetic analysis of the domain *sxtA1* the EST from different species of dinoflagellates deposited in the Marine Eukaryote Transcriptome Sequencing Project were used. To obtain the domain from these sequences BLAST searches were done using the software BLAST+ 2.7.1 (National Center for Biotechnology Information, USA).

Local databases were created using the peptides sequences and comparing them with *Alexandrium* protein sequence as a query, for the searches blastp was used, hits with *e*-value$<1e^{-3}$ were included in the phylogenetic analyses. Sequences of toxic and non-toxic *Alexandrium* species deposited in GenBank were also included in the analyses.

For the phylogenetic analyses of the *sxtA4* domain, only sequences of *Alexandrium* species and *Pyrodinium bahamense* deposited in GenBank were included (**Table 3**). In both cases (*sxtA1* and *sxtA4*) the sequences were compared with cyanobacterial sequences of the gen *sxtA*.

The sequences were examined and edited with Sequencher 4.1.4 software (Gene Codes, Ann Arbor, MI, USA). The sequences of domain *sxtA1* of *G. catenatum, P. bahamense* and

TABLE 3 | Strains and GenBank accession numbers of species used in phylogenetic analyses for the *sxtA1* and *sxtA4* domains.

Species	Strain designation	GenBank accession number		Isolation place
		sxtA1	*sxtA4*	
Alexandrium fundyense	CCMP-1979		JF343354	Bay of Fundy, Canada
Alexandrium fundyense	CCMP-1719	JF343371	JF343337	Portsmouth, USA
Alexandrium fundyense	CCMP-1979	JF343309	JF343352	Bay of Fundy, Canada
Alexandrium fundyense	CCMP-1719		JF343345	Portsmouth, USA
*Alexandrium pacificum**	ACSH-02		JF343260	Syndey, Australia
*Alexandrium pacificum**	ACCC-01	JF343243	JF343259	NSW, Australia
*Alexandrium pacificum**	CCMP-1493	JF343283	JF343329	Hong Kong, China
*Alexandrium pacificum**	ACTRA-02	JF343246	JF343261	Tasmania, Australia
Alexandrium pacificum	OF-101		KF985179	
Alexandrium pacificum	ATTL-01		KF985178	Thau Lagoon, France
Alexandrium pacificum	ACQH-01		KF985182	Washington, USA
Alexandrium tamarense	CCMP-1771	JF343285	JF343331	Plymouth, England
Alexandrium pacificum^{+}	ACSH-02		KM104250	Sydney, Australia
Alexandrium pacificum^{+}	CS-315	KM104305	KM104289	Victoria, Australia
Alexandrium australiense^{+}	ATBB-01	JF343291	JF343333	Tasmania, Australia
Alexandrium minutum	AMAD-16	JF343278	JF343328	Port River, Australia
Alexenadrium minutum	CCMP-1888	JF343307	JF343348	Laguna Obidos, Portugal
Alexandrium minutum	CCMP-113	JF353269	JF343313	Vigo, Spain
Alexandrium minutum	ALSP-02		JF343318	Vigo, Spain
Alexandrium ostenfeldii	AOF-0927	KC835400	KC835401	Aland, Baltic Sea
Alexandrium ostenfeldii	NCH-85	KC835499	KC835402	Skagerrak, North Sea
Alexandrium margalefii	CS-322	KM104228		
Alexandrium fraterculus	CAWD52	KM104312		Coromandel, New Zeland
Alexandrium pseudogoniaulax	CAWD54	KM104226		Tamaki Strait, New Zealand
Gymnodinium catenatum	GCTRA-02	JF343257	JF343266	Australia
Gymnodinium catenatum	CS-395	JF343253		Australia
Pyrodinium bahamense			GBX01000001	
Cylindrospermopsis raciborski	T3		EU629178	
Anabaena circinalis	AWQC118	ACF94633	EU629177	
Aphanizomenon flos-aquae	NH-5	ACF94632	EU629175	
Lyngbya wollei			EU629174	

**Before named Alexandrium catenella.* $^{+}$*Before named Alexandrium tamarense.*

Alexandrium was translated to amino acid sequences using the program CLC Sequence Viewer 7 (CLC Bio, Qiagen, Denmark). The dinoflagellate amino acid sequences were aligned with MAFFTv7.313 (Yamada et al., 2016) with the L-INS-I model using the default parameters. Poorly aligned positions of amino acid alignments were removed with Gblocks set to the least stringent trimming options.

The best fit model for the amino acid sequences was selected using MEGA6 (Tamura et al., 2013); that WAG model was the optimal evolutionary model. Maximum Likelihood analyses were performed with RAxMLGUI (Silvestro and Michalak, 2012), PROTWAG model was used, with 100 bootstrap replicates. Bayesian inference was performed with the software MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) using the same model; one million generations were run until the standard deviation of split sequences was less than 0.01.

The nucleotide sequences of domain *sxtA4* were aligned with MAFFTv7.313 with the G-INS-I model using the default parameters. Poorly aligned positions of nucleotide alignments were removed with Gblocks. Maximum Likelihood analyses were performed with RAxMLGUI (Silvestro and Michalak, 2012). The GTR model with gamma distribution was used for the analysis with 500 bootstrap replicates. The Bayesian Inference was performed using GTR model; one million generations were run until the deviation standard was lower than 0.01. Trees were sampled every 200 generations with a burning of 1000 trees.

Copy Number Determination of the Domains *sxtA1* and *sxtA4*

For the determination of the copy number *G. catenatum* (strain 62L) we cultivated the strain under the culture conditions previously described, using batch cultures in triplicate in 250 mL

Erlenmeyer flasks with 150 mL of media. For DNA extraction 100 mL were harvested by centrifugation in the lag phase, 50 mL in the early exponential phase and 30 mL in the late exponential and stationary phase; in each phase a 2 mL sample was taken to determine the cell abundance using a 1 mL Sedgewick–Rafter chamber, the counts were done in an inverted microscope Carl Zeiss Axio Vert.A1. For cell counts cells were fixed with lugol.

Primers for qPCR were designed from conserved regions of *G. catenatum* to amplify partial sequences *sxtA1* (150 bp) and *sxtA4* (180 pb) using the software Primer 3 (Untergasser et al., 2012). The qPCR reactions were performed in triplicate in 10 μL reactions mixtures containing, 0.5 μM of each primer, 2.5 mM $MgCl_2$, 1x PCR buffer, 200 μM dNTPs, 1U Taq Platinum (Invitrogen), and 1 × EvaGreen dye. Amplifications were carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad), with an initial denaturing step at 95°C for 4 minutes and 45 cycles of 95°C for 15 s, 56 or 55°C (to *sxtA1* and *sxtA4* primers, respectively) for 30 s. A melting-curve analysis was performed at the end of each cycle to confirm amplification specificity.

For both domains standard curves were constructed, from a 10-fold dilution series using fresh PCR product of each domain, ranging from 5 to 5×10^{-5} ng. The molecules of PCR product were determined: $(A \times 6.022 \times 10^{23}) \times (660 \times B)^{-1}$, where A is the concentration of the PCR product, 6.022×10^{23} Avogadro's number, 660: average molecular weight per base pair and B is the length of the PCR product.

RESULTS

Phylogenetic Analysis Domain *stxA1*

The sequences length of domain *sxtA1* of *G. catenatum* were among 525 and 541 bp, with an agreement between predicted and observed PCR amplifications. The GC content of the sequences was 64%. The search performed in BLAST, shows that the amplified sequences of *G. catenatum* correspond to sequences of the same species and domain (98% identities and 0.0 *e*-value). The second best match was related to *Alexandrium fundyense* and gene *sxtA* (91% identities and 0.0 *e*-value).

The phylogenetic inference of domain *sxtA1* showed the separation of the cyanobacteria group, named cyanobacteria clade. Dinoflagellates were included in a separate group, named dinoflagellate clade. With this clade two well-defined branches were observed, one branch grouping dinoflagellates from different genera and some non-PSP producer species of *Alexandrium* (*A. ostenfeldii, A. monilatum,* and *A. fratercolum*) and the second branch including toxic and non-PSP producer species of *Alexandrium,* as well strains of *G. catenatum* (**Figure 2**).

Within the dinoflagellate clade two subclades were formed. One contained sequences of *G. catenatum* strains from Australia and Mexico, without any differentiation as a result of the low differences between *G. catenatum* strains (0.2%), and another subclade formed by *Alexandrium* spp. sequences. The sequences used in the phylogenetic analysis of *Alexandrium* generally were distributed without species- or strain-related patterns. The pairwise distances between *Alexandrium* subclade and *Gymnodinium* subclade were between 7.1 and 10.7%.

Phylogenetic Analysis Domain *sxtA4*

PCR amplification of the domain *sxtA4* of *G. catenatum* strains resulted in one product of approximately 658 bp; there was agreement between predicted and observed PCR amplifications with a GC content of the sequences of 60%. For this domain, sequences from the three dinoflagellate genera/species that produce STX (*Alexandrium, G. catenatum,* and *P. bahamense*) were used. BLAST searches indicated that the amplified sequences of Mexican strains of *G. catenatum* aligned in the first place with the sequence of the *sxtA4* domain of a strain of *G. catenatum* (99% identity); the second alignment in BLAST corresponded to the species *A. fundyense* (90% identity).

The cyanobacterial sequences formed a well-supported clade separate from the dinoflagellate clade (**Figure 3A**). The phylogenetic tree constructed by maximum-likelihood and Bayesian inference showed that *G. catenatum* sequences form a fully supported cluster (100 bootstrap value and 1.0 posterior probabilities), separated from *Alexandrium* and *Pyrodinium* species. In this case, within the *Gymnodinium* clade one Mexican strain was grouped with the Australian strain and the other two Mexican strains were grouped in a second branch (**Figure 3B**). The differences within *Gymnodinium* strains $was 3%.

The only available sequence of *P. bahamense* was placed together with *Alexandrium* species, forming their own cluster (100-bootstrap value and 1.0 posterior probabilities), which was designated as the *Alexandrium/Pyrodinium* cluster. Similar to the *sxtA1* domain, the sequences of *Alexandrium* do not show a species- or strains-related pattern. The differences between the sequences of *Alexandrium* and *G. catenatum* were of 13%.

Copy Number Determination

Between 75 and 32 genomic copies of *sxtA1,* and 143–74 genomic copies of *sxtA4* in *G. catenatum* were found in triplicate batch cultures of strain 62L collected in different phases of the growth curve, based on the qPCR assay (**Figure 4**). There was no difference in the number of genomic copies among the different growth phases of the culture.

DISCUSSION

Horizontal Gene Transfer (HGT) is a common process in eukaryotes and plays an important role in the genome evolution in these organisms (Andersson, 2005; Keeling and Palmer, 2008). In dinoflagellates, HGT has been reported in various species, especially in genes involved in photosynthesis and the Calvin Cycle. The source of these genes differs, being transferred mainly between eukaryotes (Takishita et al., 2003, 2008; Nosenko et al., 2006; Patron et al., 2006; Minge et al., 2010). In these cases the HGT correspond to tertiary endosymbiosis where the endosymbionts transferred the genes to the genome of the host.

Murray et al. (2015) propose that the most parsimonious explanation for the origin of STX in dinoflagellates is a single horizontal transfer event that occurred early in dinoflagellate evolution. The presence of paralogs of the *sxtA1* domain in species that do not produce STX may support this hypothesis. If HGT occurred in an early stage in the evolution of dinoflagellates,

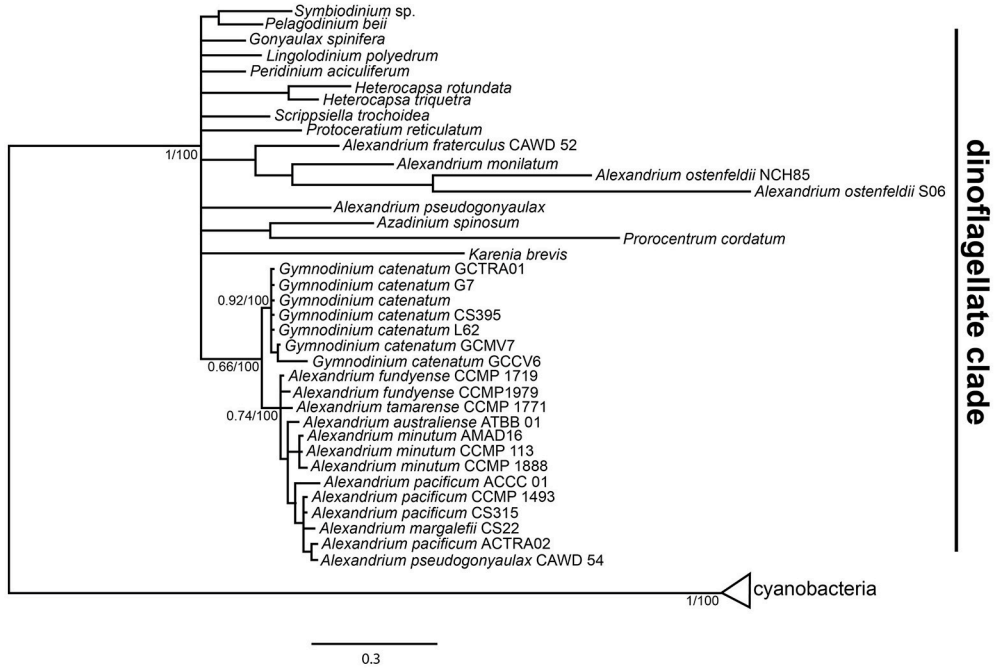

FIGURE 2 | Phylogenetic tree of the domain *sxtA1* from amino acids sequences of dinoflagellates and cyanobacteria. The tree is reconstructed with Bayesian Inference, the numbers in the internal nodes represent the posterior probabilities/boostrap values.

the horizontal transfer from *Alexandrium* to *G. catenatum* proposed by Orr et al. (2013) may not have been possible.

Ingestion of prey or phagocytosis has been described as the mechanism of foreign gene acquisition in eukaryotes (Doolittle, 1998). If *Alexandrium* was a prey of *G. catenatum* and transferred its genes, it would be expected that *G. catenatum* was included within the same clade with *Alexandrium* and *Pyrodinium* species. Since *G. catenatum* in both domains form a well-supported clade, that separates from *Alexandrium* and *Pyrodinium* species, this could indicate that the HGT from *Alexandrium* to *Gymnodinium* probably did not take place. It is possible that HGT occurred during early evolution of dinoflagellates, with loss of genes in other species of the genus. Moreover, *G. catenatum* can only feed on small phytoplankton species $<12\mu$m (Jeong et al., 2005). Feeding of *G. catenatum* on *Alexandrium* spp. has hitherto not been reported.

The genus *Alexandrium* originated around the late Cretaceous 77 MYA (John et al., 2003), while *G. catenatum*, may have evolved around of 150–140 MYA from *Gymnodinium* microreticulate complex (Bolch, 1999). The presence of paralogs of the *sxtA1* domain in two species of the order Gymnodiniales (*Karenia brevis* and *Pelagodinium beii*), suggest that HGT occurred before the divergence of Gymnodiniales and Gonyaulacales, with the subsequent loss in the capacity of PST biosynthesis in other dinoflagellates in the order Gymnodiniales; especially the loss of the domain *sxtA4,* which is present only in species that can produce PST.

In cyanobacteria, STX production genes originated from multiple HGT. The *sxtA* gene has a chimeric origin with the fusion of two different sources in one gene (Kellmann et al., 2008; Moustafa et al., 2009). The presence of a single domain of the *sxtA* gene in the dinoflagellates that do not produce STX can be explained by the loss of the domain that is found in the STX-producing species or that there were multiple transfers and in this case the transfer of the *sxtA4* domain in non-toxic dinoflagellates has not occurred.

Within the *G. catenatum* strains, the Mexican and Australian sequences group together, but the evolutionary distance between the strains of both regions was greater in the *sxtA4* domain than the *sxtA1* domain. One possible evolutionary scenario is that both domains have different mutation rates. Between *Alexandrium* and *G. catenatum* a similar pattern of evolutionary distance was observed.

The difference in the sequences within the *G. catenatum* strain in each domain may be explained since these domains are present in multiple copies inside the genome, and this modification corresponds to the variance between the copies without a relationship within their geographic origin. One characteristic of the dinoflagellates is that they have a large genome and most of the genes families are present in multiple copies (Bachvaroff and Place, 2008; Lee et al., 2014) and they normally occur in 30–5000 copies per genome, indicating that a high gene copy number is widespread in dinoflagellates (Hou and Lin, 2009). In *Alexandrium* species, there are differences in the sequences in clones of the same strain, which may result from the presence of multiple copies of the same gene (Stüken et al., 2011; Wiese et al., 2014; Murray et al., 2015). This is not only observed in STX genes, but also by the presence of many copies of the actin gene inside

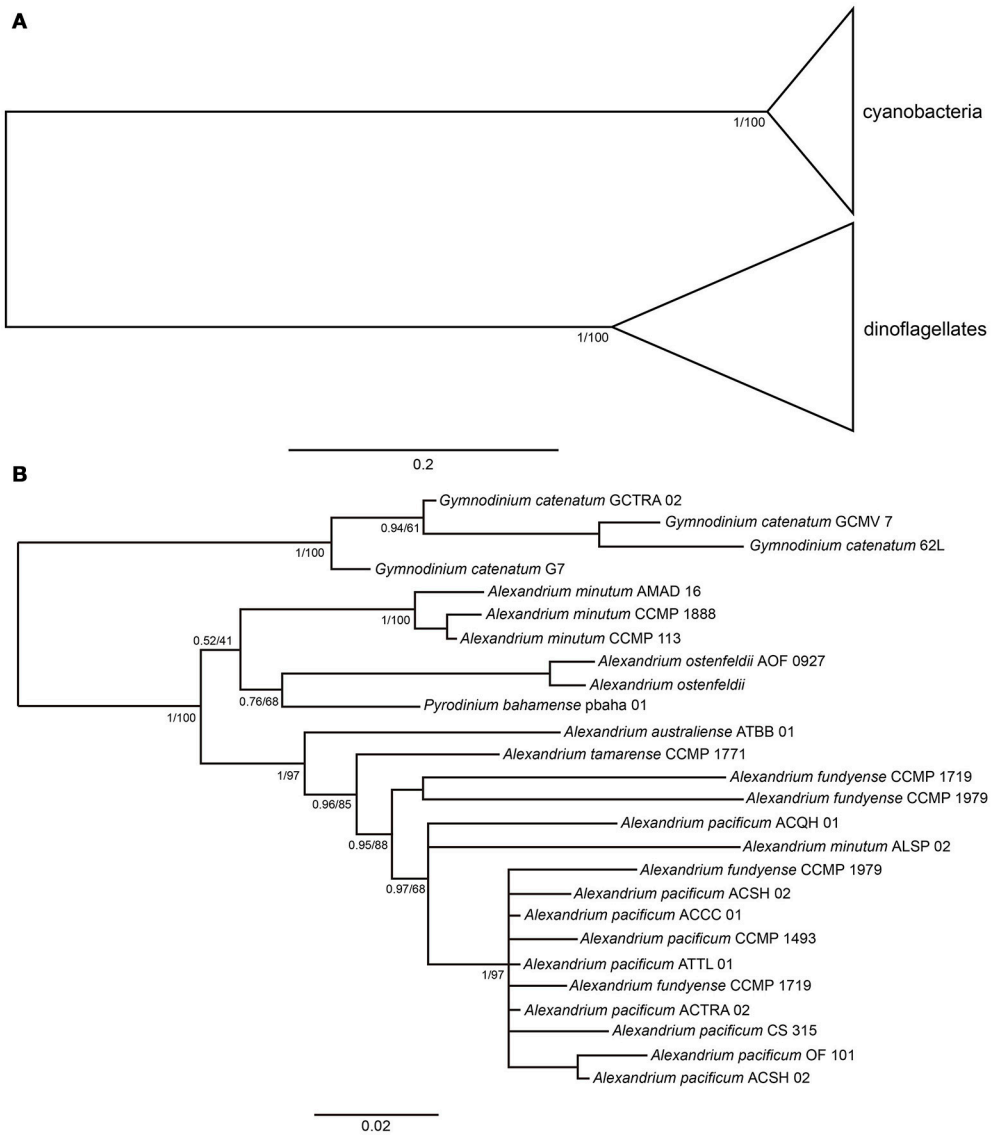

FIGURE 3 | (A) Phylogenetic tree of *sxtA4* domain of cyanobacteria and dinoflagellates, the node labels indicate the posterior probabilities and bootstrap values. **(B)** Phylogenetic tree of *sxtA4* domain of dinoflagellates, the tree was reconstructed by Bayesian Inference, the node labels indicate the posterior probabilities and bootstrap values.

the genome of *Amphidinium* and the changes between copies (Bachvaroff and Place, 2008).

The genomic copy number of the domain *sxtA4* of *G. catenatum* is lower than the reported for *A. catenella* from 100 to 280 cell^{-1} (Murray et al., 2011; Stüken et al., 2011); and higher than the values reported for *A. minutum* and *A. ostenfeldii* with 1.5–10.8 and 1–15 copies cell^{-1} respectively (Stüken et al., 2015; Savela et al., 2016). There is no information about the genomic copy number of the domain *sxtA1* in species of the genus *Alexandrium,* but in this study the gene copy number of *G. catenatum* of domain *sxtA1* is lower than number for the *sxtA4* domain. Stüken et al. (2015) proposed that gene copy number are not related to the genome size in *A. minutum* when comparing many strains of the species. Within the *Alexandrium* species, *A. catenella* and *A. ostenfeldii* have a similar genome size; 100 pg cell^{-1} and 115 pg cell^{-1} respectively (Figueroa et al., 2010), where the difference in the *sxA4* copy number is great. Within dinoflagellates the genome size exhibits a positive correlation with cell size (Lin, 2011). The size of *G. catenatum* varies from 31 to 41 μm long and from 27 to 33 μm wide (Blackburn et al., 1989), while *A. catenella* size varies from 21 to 23.5 μm long and from 23 to 25 μm wide (Kim et al., 2002). Although the genome size of *G. catenatum* is unknow; it can be assumed that *G. catenatum* has a higher genome than *A. catenella*. The copy number in *sxtA4* domain is higher than the copy number in *G. catenatum,* therefore, it could be suggested that

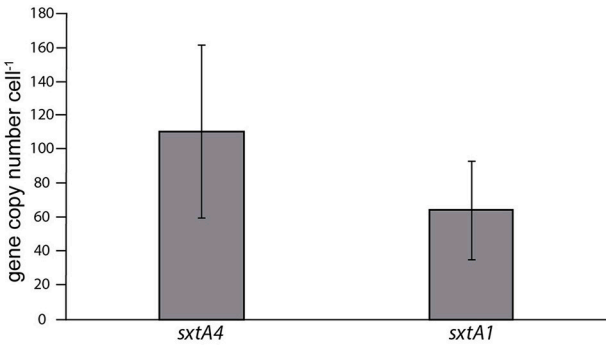

FIGURE 4 | Average gene copy number cell^{-1} of domains *sxtA4* and *sxtA1* in *G. catenatum* L62.

the genome size is not related to the *sxtA4* copy number in dinoflagellates.

Here it is shown for the first time the gene copy number of the domain *sxtA4* of *G. catenatum*, as well as for the domain *sxtA1*, and a variation in the domain *sxtA4* with respect to *Alexandrium* species. Murray et al. (2015) mention that following the acquisition of the genes trough HGT, a duplication process of the genes occurred. However, this duplication process was not the same within dinoflagellates that produce STX when the genes were acquired, and this could be the reason of the difference in gene copy numbers within dinoflagellates, and the selection pressure can act in various ways over the STX genes.

This study establishes that HGT of the gene *sxtA* did not occur from *Alexandrium* to *G. catenatum,* and that the HGT in *G. catenatum* was an early evolutionary event that probably occurred before this species diverged. The posterior loss of the capacity to produce STX in other members of the microreticulate complex, like in the *Alexandrium* genus, resulted in a the unique species of the genus *Gymnodinium* that has the capacity to produce STX.

If the HGT occurred in an ancestor of *G. catenatum* and *Alexandrium,* it is important to observe the presence of the paralogs of the *sxtA1* domain in more species of the order Gymnodiniales; especially in the *Gymnodinium* microreticulate complex, in order to gain a better understanding of how the transfer and losses of STX genes has been within the complex, and how the evolution of these genes occurred in dinoflagellates.

AUTHOR CONTRIBUTIONS

AM-F carried out all experimental work, acquired, analyzed, and interpreted data and drafted the manuscript. IL-V, CB-S participated in drafting the manuscript and analyzed and interpretation of phylogenetic data and supervised the overall progress of this project. CG-S participated in the experimental design of the qPCR analysis and drafting the manuscript. JB-G contributed with strains and commented and approved the manuscript.

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