

# Evolutionary Acquisition and Loss of Saxitoxin Biosynthesis in Dinoflagellates: the Second “Core” Gene, *sxtG*

Russell J. S. Orr,<sup>a,b</sup> Anke Stüken,<sup>a</sup> Shauna A. Murray,<sup>c,d</sup> Kjetill S. Jakobsen<sup>a,b</sup>

MERG (Microbial Evolution Research Group), Department of Biosciences, University of Oslo, Oslo, Norway<sup>a</sup>; CEES (Centre for Ecological and Evolutionary Synthesis), Department of Biosciences, University of Oslo, Oslo, Norway<sup>b</sup>; Ecology and Evolution Research Centre and School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia<sup>c</sup>; Sydney Institute of Marine Sciences, Mosman, NSW, Australia<sup>d</sup>

Saxitoxin and its derivatives are potent neurotoxins produced by several cyanobacteria and dinoflagellate species. SxtA is the initial enzyme in the biosynthesis of saxitoxin. The dinoflagellate full mRNA and partial genomic sequences have previously been characterized, and it appears that *sxtA* originated in dinoflagellates through a horizontal gene transfer from a bacterium. So far, little is known about the remaining genes involved in this pathway in dinoflagellates. Here we characterize *sxtG*, an amidinotransferase enzyme gene that putatively encodes the second step in saxitoxin biosynthesis. In this study, the entire *sxtG* transcripts from *Alexandrium fundyense* CCMP1719 and *Alexandrium minutum* CCMP113 were amplified and sequenced. The transcripts contained typical dinoflagellate spliced leader sequences and eukaryotic poly(A) tails. In addition, partial *sxtG* transcript fragments were amplified from four additional *Alexandrium* species and *Gymnodinium catenatum*. The phylogenetic inference of dinoflagellate *sxtG*, congruent with *sxtA*, revealed a bacterial origin. However, it is not known if *sxtG* was acquired independently of *sxtA*. Amplification and sequencing of the corresponding genomic *sxtG* region revealed noncanonical introns. These introns show a high interspecies and low intraspecies variance, suggesting multiple independent acquisitions and losses. Unlike *sxtA*, *sxtG* was also amplified from *Alexandrium* species not known to synthesize saxitoxin. However, amplification was not observed for 22 non-saxitoxin-producing dinoflagellate species other than those of the genus *Alexandrium* or *G. catenatum*. This result strengthens our hypothesis that saxitoxin synthesis has been secondarily lost in conjunction with *sxtA* for some descendant species.

Dinoflagellates are a diverse group of unicellular protists that play important ecological roles in marine and freshwater habitats. Approximately 2,000 species of extant dinoflagellates are known to date, most of which are found in marine habitats (1). Dinoflagellates are known for the array of toxic compounds they produce, even though fewer than 100 species have been identified as synthesizing compounds toxic to humans (2). One group of toxins, saxitoxin (STX) and its documented 58 analogues, are potent environmental neurotoxins that can cause the severe human illness paralytic shellfish poisoning (PSP) upon consumption of vector species such as mussels, clams, and oysters (3, 4). Within the dinoflagellates, STX is synthesized by species of the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium* (see Orr et al., 2011 [5], and references therein). However, the same compounds are also produced by several species of freshwater cyanobacteria (6–8). In both eukaryotes and prokaryotes, STX appears to be synthesized by similar processes; precursor incorporation patterns and stereochemistry are identical in cyanobacteria and dinoflagellates (9–11). The biosynthetic pathway and genes responsible for STX synthesis have been characterized in numerous cyanobacterial species (12–17). The STX biosynthetic pathway in *Cylindrospermopsis raciborskii* T3, for example, is encoded by more than 35 kb, with 30 catalytic functions being assigned to 26 proteins (14). A complement of 14 genes (*sxtA-sxtI*, *sxtP-sxtR*, *sxtS*, and *sxtU*) is common between the *sxt* clusters of five cyanobacterial STX-producing strains (17). These have been subsequently defined as “core” genes (4, 17). Eight of these genes (*sxtA*, *sxtB*, *sxtD*, *sxtG*, *sxtH* or *sxtT*, *sxtI*, *sxtS*, and *sxtU*) seem to be directly implicated in STX synthesis (14). The majority of these have seemingly originated in cyanobacteria via horizontal gene transfers (HGTs) from other bacteria (13).

In dinoflagellates, expressed sequence tags (ESTs) homologous to several cyanobacterial *sxt* genes have recently been identified in *Gymnodinium catenatum* and multiple species within the genus *Alexandrium* (18). In addition, *sxtA*, the unique starting gene of STX synthesis, has been characterized in detail for *Alexandrium fundyense* CCMP1719 and *Alexandrium minutum* CCMP113 (18). The dinoflagellate *sxtA* gene has a typical dinoflagellate organization (18), it is present in multiple copies in the nuclear genome, its mRNA transcripts are monocistronic as opposed to the cyanobacterial polycistronic *sxt* transcripts, and it has spliced leader sequences (19) and a eukaryotic polyadenylated tail. Additionally, the GC content of the *Alexandrium sxtA* transcripts was higher than that of the cyanobacterial *sxtA* homolog and thus more typical of *Alexandrium* transcriptomes. These results clearly showed that dinoflagellates carry STX genes themselves and that STX synthesis in dinoflagellates is not due to cocultured bacteria, as previously suggested (20–22).

From a phylogenetic point of view, it appears that STX production is paraphyletic within the genus *Alexandrium* and that STX-producing (STX<sup>+</sup>) and -nonproducing (STX<sup>-</sup>) strains of the same species coexist (5). The presence of *sxtA* correlates well with

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Address correspondence to Kjetill S. Jakobsen, k.s.jakobsen@ibv.uio.no.

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**TABLE 1** List of dinoflagellate species/strains used in this study and their characteristics regarding production of STX and amplification of *sxtA1*, *sxtA4*, and *sxtG*<sup>a</sup>

Order and species	Strain	Production of STX (reference or source)	Amplification of:		
			<i>sxtA1</i> and <i>sxtA4</i> (reference[s] or source)	<i>sxtG</i> (this study)	<i>sxtG</i> intron length (bp)
<b>Gonyaulacales</b>					
<i>A. affine</i>	CCMP112	ND (5)	ND (18)	+	260
<i>A. andersoni</i>	CCMP2222	ND (5)	ND (18)	+	
<i>A. insuetum</i>	CCMP2082	ND (5)	ND (this study)	+	No intron
<i>A. minutum</i>	CCMP113	+ (5)	+ (18)	+	740
<i>A. minutum</i>	CS320/01	+ (5)	+ (18)	+	740
<i>A. minutum</i>	CS324/16	+ (5)	+ (18)	+	750
<i>A. tamarensis</i> complex					
<i>A. fundyense</i> (group 1)	CCMP1719	+ (5)	+ (18)	+	No intron
<i>A. tamarensis</i> (group 3)	CCMP1771	ND (5)	+ (18)	+	No intron
<i>A. catenella</i> (group 4)	CCMP1493	+ (5)	+ (18)	+	487
<i>A. catenella</i> (group 4)	ACCC01	+ (24)	+ (18)	+	507
<i>A. tamarensis</i> (group 5)	ATEB01	+ (23)	+ (18, 23)	+	407
<i>Ceratium longipes</i>	CCMP1770		ND (50)	ND	
<i>Coolia monotis</i>	CAWD98		ND (50)	ND	
<i>Gambierdiscus australes</i>	CAWD149		ND (50)	ND	
<i>Lingulodinium polyedrum</i>	CCMP1931		ND (50)	ND	
<i>Protoceratium reticulatum</i>	CAWD99		ND (50)	ND	
<i>Pyrocystis noctiluca</i>	CCMP732		ND (50)	ND	
<i>Thecadinium kofoidii</i>	SCCAP K-1504		ND (50)	ND	
<b>Gymnodiniales</b>					
<i>Amphidinium carteri</i>	UIO081		ND (50)	ND	
<i>Amphidinium massartii</i>	CS-259		ND (18)	ND	
<i>Amphidinium mootonorum</i>	CAWD161		ND (50)	ND	
<i>Gymnodinium aureolum</i>	SCCAP K-1561		ND (50)	ND	
<i>Gymnodinium catenatum</i>	CCMP1937	+ (52)	+ (18)	+	
<i>Karlodinium veneficum</i>	RCC2539		ND (50)	ND	
<i>Lepidodinium chlorophorum</i>	RCC2537		ND (50)	ND	
<b>Peridinales</b>					
<i>Adenoides eludens</i>	CCMP1891		ND (50)	ND	
<i>Azadinium spinosum</i>	RCC2538		ND (50)	ND	
<i>Heterocapsa triquetra</i>	RCC2540		ND (50)	ND	
<i>Pentaplastidium dalei</i>	SCCAP K-1100		ND (50)	ND	
<i>Scrippsiella trochoideae</i>	BS-46		ND (50)	ND	
<b>Prorocentrales</b>					
<i>Prorocentrum lima</i>	CS-869		ND (18)	ND	
<i>Prorocentrum micans</i>	UIO292		ND (50)	ND	
<i>Prorocentrum minimum</i>	UIO085		ND (50)	ND	
<b>Suessiales</b>					
<i>Polarella glacialis</i>	CCMP2088		ND (50)	ND	

<sup>a</sup> ND, not detected; +, amplified sequence. The group-naming system of Lilly et al. (2007) is used for *A. tamarensis* complex strains (40).

the pattern of STX production, although there may be some apparent exceptions (18). This has been attributed to the detection limits of nongenetically based methods (5, 18, 23, 24). Based on the *sxtA* data, the origin of the STX gene cluster within the dinoflagellates may have occurred through a horizontal gene transfer event between an ancestral STX<sup>+</sup> bacterium and the common ancestor of *Alexandrium* and *Pyrodinium* (18). To date, this hypothesis is based on *sxtA*, a single gene in a pathway that may consist of up to 26 proteins (14). In cyanobacteria, the product of *sxtA* is the substrate for the amidinotransferase SxtG, which is proposed to incorporate an amidino group from a second argi-

nine molecule into the STX intermediate (14). It was the aim of this study to characterize the second core gene of the STX pathway in dinoflagellates, *sxtG*, and to determine if its structure, phylogeny, and putative evolution correspond to those of *sxtA*.

## MATERIALS AND METHODS

**Culturing.** The dinoflagellate strains used in this study (Table 1) were grown in L1 media (25) or GSe media (26) at 16 to 25°C. In addition, *Polarella glacialis* CCMP2088 was grown at 5°C. All strains were grown with a 12/12-h light/dark photoperiod and a photon irradiance of ~100 mmol photons m<sup>-2</sup> s<sup>-1</sup>. Strains were not maintained axenically. The

TABLE 2 Primers specifically designed for this study or used in previous studies<sup>a</sup>

Primer name	Primer direction	Primer sequence (5'–3')	$T_m$ (°C)	Annealing site (5'–3')	Reference or source
SL	F	DCCGTAGCCATTTTGGCTCAAG	57.0	Variable	19
AUAP	R	GGCCACGCGTCTGACTAGTAC	57.9	Variable	Invitrogen
Nsf83	F	GAAACTGCGAATGGCTCATT	49.7	82–101	27
1528r	R	TGATCCTTCTGAGGTTACCTAC	57.4	1777–1800	28
18SF8	F	TTGATCCTGCCAGTAGTCATATGCTTG	58.2	8–34	5
ITSR01	R	CCTGTGTACGACTTCTCCTTCCTC	57.4	1748–1771	5
5.8S-b5'ed	F	GATGAAGAATGCGCAAMATG	50.5	2025–2045	39
5.8S-b3	R	CAAGCAHACCTTCAAGMATATCC	55.3	2112–2134	39
M13F	F	GTAACAAACGACGGCCAG	45.9		Invitrogen
M13R	R	CAGGAAAACAGCTATGA	40.8		Invitrogen
sxt001	F	TGCAGCGMTGCTACTCTACTAC	57.1	904–926	18
sxt002	R	GGTCGTGGTCYAGGAAGGAG	55.9	1429–1449	18
sxt007	F	ATGCTCAACATGGGAGTCATCC	54.8	3174–3195	18
sxt008	R	GGTCCAGTAGATGTTGACGATG	57.1	3865–3888	18
sxtG71F	F	AGGACATGGACGAKAATAGCTG	54.8	71–92	This study
sxtG127F	F	TCCGGCGACTACGAGTTC	52.6	127–144	This study
sxtG206F	F	GGGCCGTGAAGGATTACCTGA	56.3	206–226	This study
sxtG206R	R	TCAGGTAATCCTTCACGGCCC	56.3	206–226	This study
sxtG221R	R	GTTTATGCCGTCGCGCTCAGGT	58.8	221–243	This study
sxtG663F	F	CATGGAGTCGATGGTGAGCAAC	56.7	663–684	This study
sxtG663R	R	GTTGCTCACCATCGACTCCATG	56.7	663–684	This study
sxtG802F	F	CTGGACTCGMACGATAATGA	54.8	802–823	This study
sxtG1008F	F	CGAGTCCTACGGTACAAGC	55.9	1008–1027	This study
sxtG1009R	R	ATCGGCTTGTAGCCGTAGGACTC	58.8	1009–1031	This study
sxtG1042R	R	CGCAGAAGTTCATGTYGCATAT	53.0	1042–1063	This study
sxtGq559F	F	GACGGGAACGGCTACAA	49.5	559–575	This study
sxtGq605R	R	GCTCGAAGATCGGGTCTCT	52.6	605–622	This study

<sup>a</sup> Melting temperature ( $T_m$ ) calculated using OligoCalc (30). F, forward; R, reverse. Annealing sites are based on the mRNA sequence of *A. fundyense* and can vary slightly between species. *sxtG* intron presence is not considered. The primer pairs and PCR annealing temperature used were as follows: 60°C for sxtG127F/sxtG1009R, sxtG206F/sxtG1009R, sxtG127F/sxtG663R, sxtG206F/sxtG663R, and sxtG663F/sxtG1009R, and 56°C for sxtG71F/sxtG663R and sxtG663F/sxtG1042R.

identity of each strain was confirmed by amplifying the 18S ribosomal DNA (rDNA) gene using the primer pair NSF83-1528R or 18sF8-ITSR01 (5, 27, 28).

**Nucleic acid isolation and cDNA synthesis.** Genomic DNA (gDNA) and total RNA were isolated from 20-ml cultures in the exponential growth phase, centrifuged for 2 min at 12,000 × *g*, washed with phosphate-buffered saline (PBS) and bead beaten on dry ice with the Fast-Prep-24 from Medinor (20 s at speed 4) using 1.4-mm beads (Medinor). The Invitrogen ChargeSwitch gDNA plant kit (Invitrogen) or the Invitrogen ChargeSwitch TotalRNA cell kit (Invitrogen) was utilized in accordance with the supplied protocol. Total RNA from *Gymnodinium catenatum* (CCMP1937) was kindly donated by Johannes Hagström, Linnaeus University, Kalmar, Sweden. First-strand cDNA was synthesized with the Invitrogen 3' rapid amplification of cDNA ends (RACE) system (Invitrogen) by following the high-GC protocol and utilizing the adapter primer (AP). DNA, RNA, and cDNA quality was checked with a NanoDrop spectrophotometer (ThermoScientific).

**Identification of *sxtG* homologs and primer design.** Putative *sxtG* homologs were identified from two previously published *Alexandrium* 454 high-throughput sequenced cDNA libraries (*A. fundyense* CCMP1719 and *A. minutum* CCMP113 [18]) deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the respective accession numbers SRX040427 and SRX040428 (18). Nine *A. fundyense* CCMP1719 and seven *A. minutum* CCMP113 putative *sxtG* contigs were identified as previously described by Stüken et al. (2011) (18). The contigs were blasted (E value, <10) against the nonredundant EST and SRA databases at NCBI (as of January 2012). Orthologous *Alexandrium* EST (accession numbers EX463008, CK786100, and CK782453) and SRA (accession number SRX111568) sequences were identified and aligned with all *sxtG* sequences before design-

ing primers. The primers used in this study have been designed using PrismaClade (29). Melting temperatures ( $T_m$ ) were calculated using OligoCalc (30).

**PCR, sequencing, and assembly.** PCRs were performed with HotStar-Taq Plus polymerase (Qiagen) or BD Advantage 2 Polymerase Mix (BD Biosciences) in the presence of 3% dimethyl sulfoxide (DMSO), 0.2 mM deoxynucleoside triphosphates (dNTPs), and 0.5 mM (each) forward and reverse primers (Table 2) in an MJ Research PTC-200 Thermo Cycler (MJ Research). *sxtG* PCRs were run under the following cycling conditions: 1 cycle of 5 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at variable temperatures (Table 2), and 1 to 2 min at 72°C; and 1 cycle of 10 min at 72°C. *sxtG* (genomic and transcript) was amplified with various primer pairs (Table 2).

The entire transcript was amplified for *A. fundyense* CCMP1719 and *A. minutum* CCMP113. The 5' end of the transcript, including the dinoflagellate spliced leader sequence, was amplified using the primers dinoSL (19) and sxtG663R. The 3' end, including the eukaryotic poly(A) tail, was amplified using the sxtG206F and the AUAP adaptor primers (Invitrogen). The 5' and 3' end products were cloned with TOPO TA (Invitrogen) before being sequenced with the M13 forward and reverse primers. Additionally, genomic amplicons of *Alexandrium affine* CCMP112 and *A. fundyense* CCMP1719 were cloned, as direct sequencing indicated the presence of divergent *sxtG* copies within these two strains. Amplification of *sxtG* was checked for all non-PSP-producing dinoflagellates listed in Table 1 (genomic and transcript) using the primer combinations as previously described (Table 2). Positive (STX<sup>+</sup> strain CCMP113 or CCMP1719) and nontemplate controls were run in all cases. PCR products were gel excised using the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced directly using an ABI3730 DNA analyzer (Applied Biosystems). Respective forward and reverse primers as well as several internal primers

TABLE 3 Comparison of *sxtG* intron sequence identity and percent identity between species and/or strains

Species and strain (intron length in bp)	Strain ID	% identity or no. of identities between intron sequences <sup>a</sup>							
		1	2	3	4	5	6	7	8
<i>A. minutum</i> CCMP113 (740)	1		99.19	98.53	27.79	38.59	42.95	43.37	39.06
<i>A. minutum</i> CS320/01 (740)	2	734		98.00	27.79	38.72	43.21	43.62	39.19
<i>A. minutum</i> CS324/16 (750)	3	739	735		27.43	38.10	42.77	43.18	38.56
<i>A. affine</i> CCMP112 (260)	4	209	209	209		41.87	33.33	33.21	39.33
<i>A. fundyense</i> CCMP1719 (368)	5	296	297	296	175		58.16	60.38	72.30
<i>A. catenella</i> CCMP1493 (487)	6	335	337	337	177	303		93.89	75.15
<i>A. catenella</i> ACCC01 (507)	7	340	342	342	181	317	476		77.71
<i>A. tamarensis</i> ATEB01 (407)	8	300	301	300	175	308	378	394	

<sup>a</sup> The upper pairwise comparison values represent percent identity between intron sequences. Unshaded cells indicate >90% identity; shaded cells indicate <80% identity. The lower pairwise comparison values represent the number of identities between intron sequences.

were utilized for sequencing (Table 2). Sequences were quality checked and assembled using the Phred/Phrap/Consed (31) package under the default settings. Further manual editing was performed in MacClade v4.07 (32).

Additional *sxtA* PCRs were performed on *Alexandrium insuetum* CCMP2082 as described in reference 18. For the *sxtA1* fragment, primers *sxt001* and *sxt002* (~550 bp) were used, and for the *sxtA4* fragment, *sxt007* and *sxt008* (~750 bp; Table 2) were used.

***sxtG* analyses and phylogenetic inference.** Dinoflagellate *sxtG* transcript structure was determined by aligning the translated mRNA sequence with that of *sxtG* from the cyanobacterium *Anabaena circinalis* AWQC131C (DQ787201). In addition, conserved domain searches (<http://pfam.sanger.ac.uk/search/sequence> [33]) were carried out. The open reading frame (ORF) was predicted using a tool available online (<http://proteomics.yzu.edu/tools/OrfPredictor.html> [34]). Catalytic and substrate-binding residues of *sxtG* from cyanobacteria have been previously determined (14). An amino acid alignment of the dinoflagellate *sxtG* coding sequences, orthologous cyanobacterial *sxtG* sequences, and a selection of closely related NCBI nr BLASTP hits (September 2012) was constructed using MAFFT v6 L-INS-I model under the default settings (35). The resulting alignment was checked manually, and poorly aligned positions were excluded using MacClade v4.07 (32). ProtTest v2.4 (36) determined LG as the optimal evolutionary model. Maximum likelihood (ML) analyses were performed with the RAxML-VI-HPC v7.2.6, PROTCATLG model with 25 rate categories (37). The most likely topology was established from 100 separate searches, and bootstrap analyses were performed with 500 pseudoreplicates.

Dinoflagellate genomic *sxtG* structure was determined by alignment against the corresponding transcript sequence using MacClade v4.07 (32). The comparison of genomic DNA to cDNA identified a splice site at nucleotide (nt) 475 (amino acid [aa] 159) for numerous species. Intron sequence identities and percentages of sequence identity were compared between and within species at the nucleotide level by pairwise alignment using CLC Main Workbench (CLC bio, Aarhus, Denmark) (Table 3). All model estimation and phylogenetic analyses were done on the freely available Biportal (38) at the University of Oslo (<http://www.biportal.uio.no/>).

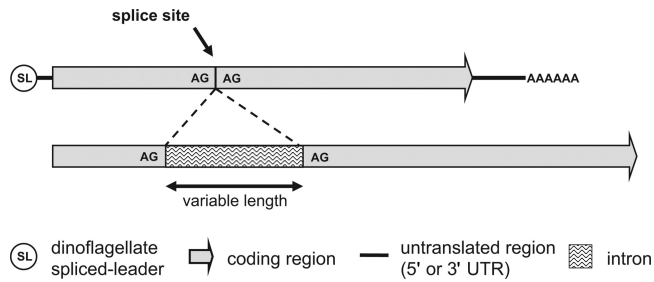
**Relative copy number determination.** Quantitative PCR (qPCR) primers were designed based on the *sxtG* gDNA sequence of *A. fundyense* CCMP1719 (JX995118) using the Universal Probe Library Design Software from Roche, version 2.45, using default parameters. Suggested primers were *in silico* tested against an alignment of all available *Alexandrium sxtG* mRNA and gDNA sequences. The chosen primers annealed to a conserved region downstream of the *sxtG* intron. The assay was optimized on strains CCMP1493 (*A. catenella*) and CCMP113 (*A. minutum*), using different annealing temperatures (55, 60, and 64°C) and primer concentrations (125 nM and 250 nM), assessing crossing point (CP) values and melting curves, and running the PCR fragments on agarose gels. The best results were obtained with the primer pair *sxtG*q559F/*sxtG*q605R

(Table 2) with annealing temperatures of 60 or 64°C and 125 nM primer concentrations. PCR inhibitions were observed when there was >10 ng input DNA in 20- $\mu$ l reaction volumes (data not shown). DNA was quantified with a Qubit Fluorometer using the double-stranded DNA (dsDNA) HS assay (Invitrogen). All PCRs were run on a Roche LightCycler480 system in white 96-well plates with Roche SYBR green I Master chemistry. For *sxtG* qPCR, 20- $\mu$ l reaction mixtures contained 5  $\mu$ l 2 $\times$  SYBR green master mix, 125 nM each primer, and 1 to 10 ng gDNA. They were run with the following protocol: hot start, 1 cycle of 95°C for 10 min; amplification, 45 cycles of 94°C for 10 s, 64°C for 20 s, and 72°C for 10 s, with single acquisition; followed by the melting curve program, 1 cycle of 95°C for 5 s and 65°C for 1 min, with up to 97°C continuous measurements; and finally, cooling, 1 cycle of 40°C for 10 s. qPCRs (5.8S) were run according to Galluzzi et al. (2004) (39) with the slightly modified forward primer 5.8S-b5'ed and the 5.8S-b3' reverse primer (Table 2). Reaction mixtures (20  $\mu$ l) contained 5  $\mu$ l 2 $\times$  SYBR green master mix, 150 nM (each) primer, and 4 ng gDNA and were run on the following program: hot start, 1 cycle of 95°C for 10 min; amplification, 45 cycles of 95°C for 15 s and 60°C for 45 s, with single acquisition; followed by the same melting and cooling program as for *sxtG*. As a standard, DNA from strain CCMP1493 (*Alexandrium catenella*) was diluted to 10, 5, 2.5, 1.25, and 0.63 ng/DNA per reaction. The efficiency of both qPCRs was 90 to 95% using the 2nd derivative method implemented in the LightCycler 480 software release 1.5.0.

**Nucleotide sequence accession numbers.** All *sxtG* sequences generated in this study have been deposited in GenBank under the accession numbers JX995111 to JX995130 (see Table S1 in the supplemental material).

## RESULTS

**Sequence amplification and assembly.** The *sxtG* primers designed in this study (Table 2) successfully amplified *sxtG* fragments in eight *Alexandrium* species (with *A. tamarensis* group 3 and group 5 being considered separate species; see Orr et al., 2011 [5]), 11 *Alexandrium* strains (both STX<sup>+</sup> and STX<sup>-</sup>), and *Gymnodinium catenatum* (Table 1). The assembly of these fragments resulted in *sxtG* sequences ranging from 375 bp in *G. catenatum* CCMP1937 to 934 bp in *A. affine* CCMP112, with a complete ORF for *A. fundyense* CCMP1719 and *A. minutum* CCMP113 (see Table S1 in the supplemental material for a complete listing). No *sxtG* PCR products were amplified for 22 STX<sup>-</sup> dinoflagellates in five orders (Table 1), while their 18S rDNA sequences were readily amplified from the same DNA. In addition, no eukaryotic *sxtG* sequence was identified by BLAST against the NCBI nonredundant, EST, and SRA databases in any species other than those of the genus *Alexandrium* (accession numbers CK786100, CK782453, EX463008, JV310276, and SRX111568).



**FIG 1** Structure of *sxtG* in dinoflagellates. (Top) Transcript structure of *sxtG*; (bottom) genomic structure of *sxtG*.

**SxtA PCR.** *sxtA1* and *sxtA4* were not detected in *A. insuetum* CCMP2082. The 18S rDNA control was amplified, as were the *sxtA1* and *sxtA4* positive controls.

**Identification and determination of *sxtG* transcript structure.** Assembly of the nine *A. fundyense* CCMP1719 and seven *A. minutum* CCMP113 *sxtG* contigs identified by Stüken et al. (2011) (18) gave a full ORF for both species, although dinoflagellate spliced leader sequences and poly(A) tails were lacking.

The RACE experiments resulted in full-length *sxtG* transcripts for both *A. fundyense* CCMP1719 and *A. minutum* CCMP113. This included a dinoflagellate spliced leader sequence at the 5' end and a eukaryotic poly(A) tail at the 3' end. The transcripts were 1,283 bp and 1,276 bp in length, respectively, excluding the poly(A) tail (Fig. 1). Additionally, the *A. tamarensis* SRA contig (SRX111568) contained 9 bp of the 22-bp dinoSL sequence. Conserved domain searches determined that these putative *sxtG* sequences encode an amidinotransferase enzyme, as expected. The ORF was 375 amino acids (1,125 bp) long, with a GC content of 64.5% in *A. fundyense* and 63.4% in *A. minutum*. In addition, one *A. fundyense* (JX995134) and three *A. minutum* contigs (JX995131 to JX995133) identified by Stüken et al. (2011) (18) were predicted as encoding a homologous amidinotransferase.

***sxtG* genomic structure.** Comparisons of transspliced leader amplified cDNA and genomic amplicons did not show any signs of RNA editing. A single splice site at nt 475 (aa 159) with the dinucleotide AG 5' and 3' end boundaries was present in numerous species (Table 1; Fig. 1). The intron sequence has several short palindromic repeats and stop codons in all forward reading frames (standard codon table). The removal of the intron resulted in the restoration of the ORF. Intron length varied from 260 bp in *A. affine* CCMP112 to 750 bp in *A. minutum* CS 324/16 (Table 1). Intron intraspecies pairwise percent identity was >90%, while interspecies percent identity was <80% (Table 3). No variation in intron sequence was observed within a strain, despite multiple amplifications and sequencing. We were unable to determine intron presence for *A. andersoni* CCMP222. Likewise, only cDNA for *G. catenatum* CCMP1937 was amplified in this study.

The cloning of *sxtG* for *A. affine* CCMP112 and *A. fundyense* CCMP1719 revealed a second gene, most likely a pseudogene, not present in the cDNA. For *A. fundyense* CCMP1719 (JX995130), the pseudogene sequence had a 369-bp intron at nt 475 that diverged from the functional copy at nt 658. The divergent sequence had stop codons in the reading frame (standard code). In the case of *A. affine* CCMP112 (JX995129), the pseudogene sequence had a 260-bp intron and, in congruence with that of *A. fundyense* CCMP1719, diverged at nt 658. However, no stop codons were observed (standard code).

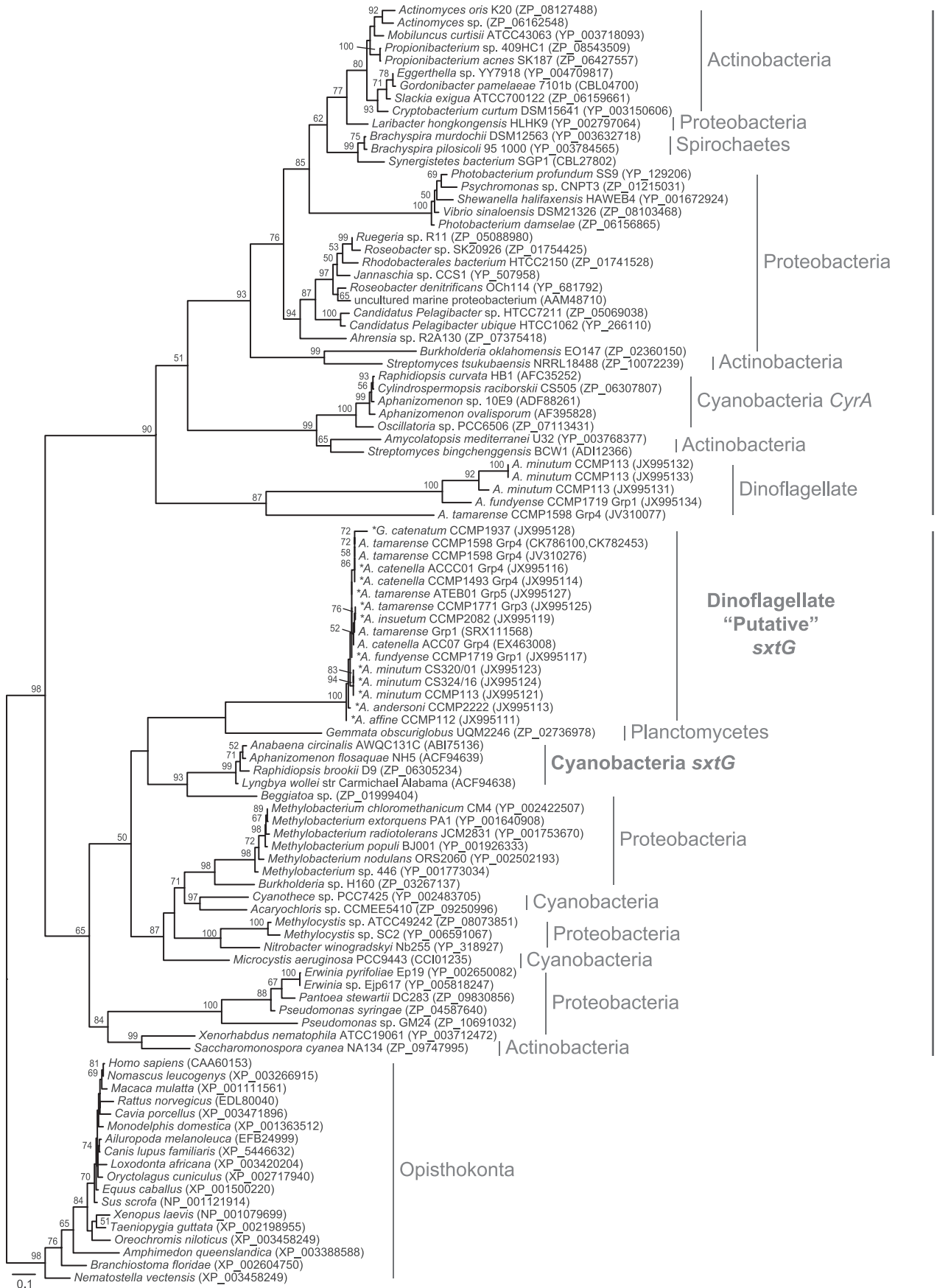
**The phylogeny of *sxtG*.** For interpretation of the phylogenetic inference (Fig. 2), statistical support is defined as follows: full, 100 bootstrap value (BP); high, >90 BP; moderate, >65 BP; and low, >50 BP. The phylogenetic inference of *sxtG*-encoded amino acid sequences shows that dinoflagellate *sxtG* sequences form a fully supported clade (Fig. 2). The branching pattern within the dinoflagellate clade is unclear, with minimal support between species and strains. The *A. minutum* strains form a highly supported monophyletic clade (94 BP), while species and strains within the *A. tamarensis* complex place paraphyletic *G. catenatum* branches with moderate support (86 BP) within a clade formed by group 4 *A. tamarensis* complex strains, as defined by Lilly et al. (2007) (40). The STX<sup>-</sup> species *A. affine* was basal, with *A. andersoni* next to diverge; however, this placement was not supported. The STX<sup>-</sup> *A. insuetum* formed a moderately supported (76 BP) clade with a group 3 *A. tamarensis*. The planctomycete *Gemmata obscuriglobus* is the sister group to the dinoflagellate *sxtG* clade; however, this grouping lacked support. The proteobacterium *Beggiatoa* further excludes the dinoflagellate sequences from a sister relationship with the highly supported cyanobacterial *sxtG* clade (99 BP). The four previous clades form a low-support (50 BP) group with a cluster of cyanobacterium and proteobacterium species. This is further included in a low-support (65 BP) monophyly with an actinobacterium and proteobacterium clade, constituting the cluster defined as amidinotransferase 1. The amidinotransferase 1 clade is excluded from opisthokonta and the amidinotransferase 2 clades with high support, 98 BP and 90 BP, respectively. The amidinotransferase 2 clade harbors the additional homologous dinoflagellate amidinotransferase sequences identified by Stüken et al. (2011) (18). This fully supported clade forms a moderately supported (87 BP) grouping with an additional *A. tamarensis* amidinotransferase (JV310077). The sister grouping to this dinoflagellate clade consists of actinobacteria and the cyanobacterial *aoaA* and *cyrA* genes, involved in the synthesis of cylindrospermopsin (41).

**Relative copy number determination.** All strains tested were positive in the 5.8S qPCR (Fig. 3). However, only the strains of the STX<sup>+</sup> species *A. tamarensis*, *A. fundyense*, *A. catenella*, and *A. minutum* had positive amplification in the *sxtG* qPCR (Fig. 3). The remaining STX<sup>-</sup> strains (*A. affine*, *A. andersoni*, and *A. insuetum*) had repeatedly unspecific melting curves. *sxtG* is present in considerably fewer copies in the genome than rDNA, though variance of copy number between the strains was relatively constant per ng DNA (Fig. 3).

## DISCUSSION

We have previously identified putative dinoflagellate ESTs homologous to cyanobacterial *sxtG* genes (14, 18). Building on this work, we characterized the entire mRNA transcript sequences as well as partial genomic gene fragments of *sxtG* in dinoflagellates in the present work. Our results show that the putative *sxtG* ESTs originally identified by Stüken et al. (2011) (18) have a typical dinoflagellate structure and are the most likely candidates to be the dinoflagellate homologs of the cyanobacterial *sxtG* genes.

**The transcriptomic and genomic structure of *sxtG*.** We have amplified the entire *sxtG* mRNA transcripts from *A. fundyense* CCMP1719 and *A. minutum* CCMP113 and partial transcripts from five strains. In contrast to the bacterial transcripts, the dinoflagellate *sxtG* transcripts were monocistronic and possessed poly(A) tails and dinoflagellate spliced leader sequences (Fig. 1).



**FIG 2** *sxtG* phylogenetic tree. Single-gene phylogeny inferred from 363 amino acid characters. The tree is reconstructed with maximum likelihood inference (RAxML). Numbers on the internal nodes represent bootstrap (BP) values of >50%. \*, taxon sequences generated from this study. Group 1 to group 5 (Grp 1 to Grp 5) denotations are from the *A. tamarensis* complex group naming system (40).

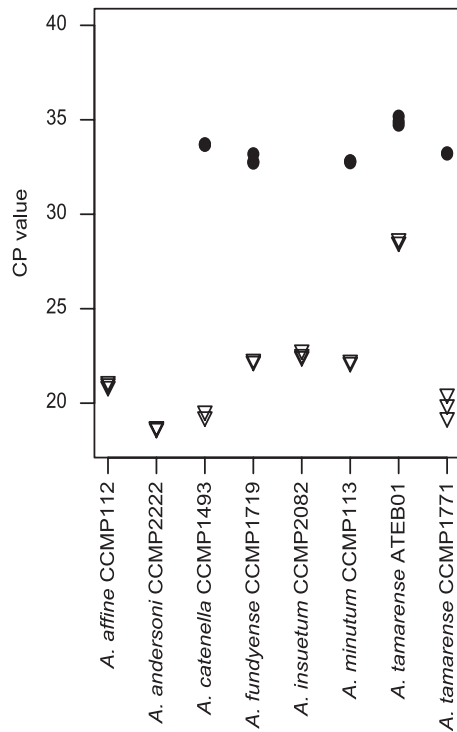


FIG 3 Relative copy number results, showing crossing point (CP) values obtained with 4 ng of input gDNA. Triangles represent 5.8S rDNA, and circles represent *sxtG*.

The result demonstrates that both the first gene and the second gene of the STX pathway are borne in the nuclear genome of dinoflagellates (18). In congruence with *sxtA*, the GC content of *sxtG* was ~20% higher in dinoflagellates (with a GC of ~64%) than for the STX<sup>+</sup> cyanobacterium species, suggesting that the gene has been modified extensively since its introduction into the dinoflagellates (18).

The genomic sequences of *sxtG* contain noncanonical introns that lack a GT/AG splicing site and vary in length and sequence between various *Alexandrium* species (Table 1; Fig. 1). The introns show high interspecies and low intraspecies variability, with no observed variation within a strain (Table 3). This may prove useful when trying to identify strains within species or to differentiate between species of *Alexandrium*. For example, the low percent identity between the *A. tamarensis* complex group 4 strains (ACCC01 and CCMP1493) and group 5 strain (ATEB01) confirms that these two groups are different enough to be considered separate species (5, 40). The confirmed presence of introns in *sxt* genes may also explain the amplification problems of the full-length genomic *sxtA* sequence (A. Stüken, unpublished data).

It is thought that introns are rare in dinoflagellate genomes, as a gene should be purged of introns with every passage through the DNA-RNA cycle (42). However, to date, when introns have been found, most lack a GT/AG splice site and are present only in a selection of species (43–47). Considering the variation in sequence length and identity (Table 3) and that the intron is not present for all species, multiple independent losses and acquisitions of the *sxtG* intron are likely. This “birth and death” pattern of gene evolution has also been observed for actin genes in *Dinophysis* species (48) and is supported by the presence of nonfunctional *sxtG* copies at least in the case of *A. fundyense* CCMP1719.

**Occurrence and distribution of *sxtG*.** Unlike with *sxtA*, the presence of *sxtG* is not specific to species known to produce STX. The *sxtG* amidinotransferase was present and transcribed in all tested *Alexandrium* species, including those for which *sxtA* and STX synthesis have not been detected (5, 18). However, some inconsistencies to this pattern were observed. First, it is uncertain if the divergent *sxtG* genomic copy amplified from *A. affine* CCMP112 is functional, as no additional copy was detected. No stop codons were observed in the sequence. We cannot exclude the possibility that the gene would be extensively modified post-transcriptionally, for example, by mRNA editing, though this may seem unlikely, as all other strains appeared free from editing.

Further, while qPCR results showed the *sxtG* copy number to be relatively constant between the known STX<sup>+</sup> species, no or unspecific amplification was observed for the STX<sup>-</sup> species (Fig. 3). It is unclear why qPCR and conventional PCR results for the STX<sup>-</sup> species are discrepant. It is possible that low genomic *sxtG* copy numbers, large genome sizes, or a combination of these factors may account for this. For example, the predicted DNA content per cell of *A. affine* CCMP112 (A. Stüken and Rosa I. Figueroa, unpublished data) is higher than that of *A. tamarensis* CCMP1598, which is estimated at 103.5 pg DNA cell<sup>-1</sup> (49). A low-copy-number gene in such a large genome may be difficult to detect. However, the results for *A. andersoni* CCMP2222, with 21.8 pg DNA cell<sup>-1</sup>, and *A. insuetum* CCMP2082, with 30.8 pg DNA cell<sup>-1</sup> (49), are not in line with such an explanation.

Consistent with *sxtA* distribution (18, 50), we did not detect *sxtG* in non-PSP dinoflagellate genera other than the *Alexandrium* genus and *Gymnodinium catenatum* (Table 1). The presence of a divergent, nonhomologous *sxtG* sequence cannot be discounted for non-PSP dinoflagellate genera. However, searches of multiple GenBank databases gave *sxtG* hits to *Alexandrium* only, suggesting that it was not present in the other organisms. The fact that STX<sup>-</sup> *Alexandrium* spp. carry and transcribe *sxtG* may suggest that this gene is also involved in other biochemical pathways or that the capacity for STX synthesis has been comparatively recently lost in these species.

The analysis of *sxtG* in *Pyrodinium bahamense* would be desirable, as it produces saxitoxin. However, cultures of this species are currently not publicly available. The presence of both *sxtA* and *sxtG* in this species has recently been reported, though the sequences are yet to be made available in GenBank (51). The *P. bahamense* *sxtG* sequences presented by Hackett et al. (2013), however, group phylogenetically with the *A. tamarensis* group 4 strain CCMP1598, contig 87049 (51), JV310276 in Fig. 2, indicating that the amino acid sequences are similar.

**The phylogeny of *sxtG*.** The phylogenetic inference of *sxtG* (Fig. 2) shows that dinoflagellate *sxtG* sequences form a fully supported clade. The branching pattern within the clade is unclear, a result of high sequence conservation of the coding sequence between species. Even at the nucleotide level (results not shown), sequences are highly conserved, limiting *sxtG* as a phylogenetic marker. Thus, it was not possible to determine if the evolution of *sxtG* mirrors that of *sxtA* or even the *Alexandrium* genus (5, 18). As the entire ORF was not amplified from all species, lower conservation cannot be completely rejected, with putative degenerate binding sites being an explanation.

The planctomycete *Gemmata obscuriglobus* was found to be the sister group to the dinoflagellate *sxtG* clade. However, it is difficult to make conclusions regarding the relationships, as the clades

were poorly supported. It appears that this dinoflagellate amidinotransferase protein, congruent with *sxtA*, was not acquired directly from cyanobacteria (18). It is difficult to conclude if *sxtG* was attained independently of *sxtA*, with the inferred phylogenies lacking resolution (18). Considering the inference in Fig. 2, this common ancestor may be, as previously suggested for the cyanobacterial homolog, a proteobacterium (13). A second dinoflagellate amidinotransferase that groups more distantly to *sxtG* with homologous actinobacterial and cyanobacterial cylindrospermopsin *aoaA* and *cyrA* sequences was additionally identified. This may suggest that multiple amidinotransferases have been acquired by HGT in parallel or separate events. It is not clear what pathway, if any, these genes are involved in. As dinoflagellates have been found to synthesize a multitude of different compounds, it is possible that they are involved in the synthesis of an unrelated compound.

**The origin of STX in dinoflagellates.** The origin of the biosynthetic pathway and genes responsible for STX synthesis in the dinoflagellates has been proposed through an HGT event with an ancestral STX<sup>+</sup> bacterium (18). The hypothesis, at present based on the starting gene of STX synthesis, *sxtA*, proposes that the HGT probably occurred before *Alexandrium* and *Pyrodinium* diverged within the order Gonyaulales. Thus, STX synthesis may have been secondarily lost for some descendant species. *Gymnodinium catenatum*, order Gymnodiniales, which has an *sxtA* sequence that is conserved to and branches within the *Alexandrium* genus, probably independently acquired STX from a later dinoflagellate-dinoflagellate transfer (18). This is further supported by recent improvements in the resolution of dinoflagellate order relationships and the nondetection of *sxtA* for multiple non-PSP-producing dinoflagellate species (50).

The results presented here add support to this hypothesis. However, *sxtG*, in contrast to *sxtA*, is not exclusive to STX<sup>+</sup> species but is present in all *Alexandrium* species. As far as we can deduce from our results, *sxtG* is absent from non-PSP-producing dinoflagellate genera and may have so few genomic copies in STX<sup>-</sup> *Alexandrium* species that it is difficult to detect via qPCR. This would suggest that some *Alexandrium* species might have had the capacity to synthesize STX until the secondary loss of essential genes, possibly in unison with *sxtA*. Considering the rDNA phylogeny of *Alexandrium*, it appears that *sxtA* has been lost within the genus on multiple occasions independently, rather than from a single event (5). If an HGT occurred prior to the split of *Alexandrium* and *Pyrodinium*, we may expect other possible descendant genera to also produce *sxtG*. Considering the broadly sampled dinoflagellate phylogenies presented by Orr et al. (2012), *Ceratium*, *Gambardiscus*, and *Pyrocystis* may all have had this capacity (50). The apparent lack of *sxtG* for these genera may indicate an ancient HGT event, with non-PSP-producing *Alexandrium* having only recently lost the ability to synthesize STX with the loss of *sxtA*. Multiple separate events cannot be discounted, though this seems unlikely.

Congruent with phylogenies based on *sxtA1* and *sxtA4* (18), the *G. catenatum* *sxtG* sequence branches within the *Alexandrium* genus (Fig. 2). This further strengthens a secondary dinoflagellate-dinoflagellate transfer theory for the origin of STX in this species (18), although additional *sxtG* sequences for *G. catenatum* are needed to assess this fully. In addition, the lack of both a homologous *sxtA* gene and a homologous *sxtG* gene for *Lepidodinium*

*chlorophorum*, the possible sister taxon to *G. catenatum*, may add more support to this scenario (50).

Despite these advances, it is difficult to conclude fully on the evolutionary origin of the STX pathway in dinoflagellates at present. The phylogenetic relationships within both the Gonyaulales order and the *Alexandrium* genus need to be more robustly resolved (5, 50). In addition, previously identified *sxt* homologs need to be characterized in detail to resolve the origin of STX in dinoflagellates (18).

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