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Species-specific PCR assays for *Gambierdiscus excentricus* and *Gambierdiscus silvae* (Gonyaulacales, Dinophyceae)[1]

R. Wayne Litaker

National Oceanic and Atmospheric Administration, National Ocean Service, National Centers for Coastal Ocean Science, Beaufort Laboratory, 101 Pivers Island Rd., Beaufort, North Carolina 28516, USA

Patricia A. Tester

Ocean Tester, LLC, 295 Dills Point Road, Beaufort, North Carolina, 28516, USA

and

Mark W. Vandersea

National Oceanic and Atmospheric Administration, National Ocean Service, National Centers for Coastal Ocean Science, Beaufort Laboratory, 101 Pivers Island Rd., Beaufort, North Carolina 28516, USA

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[2]Author for correspondence: e-mail Wayne.Litaker@noaa.gov

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Abstract

The two most toxic *Gambierdiscus* species identified from the Caribbean are *G. excentricus* and *G. silvae*. These species are primary causes of ciguatera fish poisoning (CFP) and likely contribute disproportionately to the toxicity of marine food webs. While *Gambierdiscus* species are difficult to distinguish using light or scanning electron microscopy, reliable species-specific molecular identification methods have been developed and used successfully to identify a number of other *Gambierdiscus* species. Corresponding species-specific assays are not yet available for *G. excentricus* and *G. silvae*, which imposes limitations on species identification and related ecological studies. The following note describes species-specific PCR assays for *G. excentricus* and *G. silvae* that can be used for these purposes.

Key index words: Caribbean; ciguatera; ciguatoxin; dinoflagellate; harmful algae; ribosomal; species-specific molecular assay

Abbreviations: CFP, ciguatera fish poisoning; CTX, ciguatoxin; ITS, internal transcribed spacer; LSU, large subunit; PCR, polymerase chain reaction

Benthic dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa* (Dinophyceae) produce ciguatoxins (CTXs), potent neurotoxins that bioaccumulate in food webs. Consumption of invertebrates or more commonly fish that have accumulated sufficient CTXs can result in ciguatera fish poisoning (CFP) (Litaker et al. 2010, Darius et al. 2017, Friedman et al. 2017).

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Ciguatera fish poisoning can cause a variety of gastrointestinal (vomiting/diarrhea), cardiovascular (bradycardia and hypotension) or neurological (asthenia/myalgia, paresthesias/dysesthesias/cold allodynia) disorders, as well as non-specific symptoms like burning/tingling sensation of the mouth and throat (Lewis 2001, Gatti et al. 2015, Lewis and Vetter 2016, Darius et al. 2017, Friedman et al. 2017). On rare occasions, the intoxication is fatal. CFP is most common in tropical or subtropical coral reefs and other warm water marine habitats (Litaker et al. 2010, Nishimura et al. 2013) but the globalization of seafood markets and increased international travel have expanded the range of populations affected by CFP. Currently, CFP is the major cause of non-bacterial seafood poisoning associated with fish consumption worldwide.

A long-standing question in ciguatera research is "Do certain *Gambierdiscus* species unequally contribute ciguatoxins to food webs?" The answer to this question is important for optimizing protocols for monitoring benthic dinoflagellate communities to assess ciguatera fish poisoning (CFP) risk. Recent research efforts have focused on establishing the relative toxicity of *Gambierdiscus* and *Fukuyoa* species (Chinain et al. 2010, Fraga et al. 2011, Litaker et al. 2017). Several of those studies have shown *G. excentricus* and *G. silvae* are the most toxic species in the Caribbean and eastern Atlantic (Fraga et al. 2011, Fraga and Rodríguez 2014, Litaker et al. 2017, Pisapia et al. 2017; Table 1). Recent work has shown *G. silvae* can be as toxic as *G. excentricus* (Robertson et al. 2018). Neither light nor scanning electron microscopy can be used to readily distinguish these species in field-collected samples (Litaker et al. 2009). To facilitate accurate species identifications, Vandersea et al. (2012) developed species-specific quantitative polymerase chain reaction (PCR) assays for identifying many of the *Gambierdiscus* and *Fukuyoa* species in the Caribbean. Others have subsequently developed equivalent assays for species occurring in the Pacific (Nishimura et al. 2016, Darius et al. 2017, Smith et al. 2017). Currently, there are no species-specific PCR

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assays for *Gambierdiscus excentricus* (Fraga et al. 2011) or *G. silvae*, (Fraga and Rodríguez 2014). This study developed PCR assays for both species that will prove useful for determining the relative distribution and habitat preferences of these toxicologically important benthic microalgae

To develop the assays, isolates of *G. excentricus* and *G. silvae* were established. The *G. excentricus* isolates were obtained from samples collected at Pulley Ridge reef (N 25 53.131, W 83 36.368; Pulley Ridge Gam2 = Pulleys Ridge in Litaker et al. 2017, Pisapia et al. 2017) located 160 km west of the Tortugas Ecological Reserve in the Northeastern Gulf of Mexico and from the Bahamas (N 26 38.849, W 78 04.839; Bahamas Gam5 = BahamaGam5 in Litaker et al. 2017). One isolate of *G. silvae* was obtained from Curacao (N 12 05.173, W 68 54.004; Curacao Gam11). Genomic DNA was extracted from each isolate and the ITS/5.8S and D1-D3 LSU rDNA regions were PCR amplified and sequenced using the methods described in Vandersea et al. (2012, 2017). The resulting ITS and D1-D3 LSU rDNA sequences were assembled using Vector NTI Advance 11 software and submitted to GenBank (MG981052-MG981061). These sequences were aligned with corresponding sequences in GenBank and a species-specific consensus sequence calculated for *G. excentricus* and *G. silvae*. Equivalent consensus sequences were compiled for other *Gambierdiscu*s species. All of the resulting species-specific ITS/5.8S and D1-D3 LSU rDNA consensus sequences were aligned and five to six potentially unique forward and reverse species-specific primer pairs for *G. excentricus* and *G. silvae* were identified. Selected primer pairs were located within 50 to 250 bp of each other. Potential cross reactivity with other species was evaluated by screening the primers against the National Center for Biotechnology Information Database using the Basic Local Alignment Search Tool (Altschul et al. 1990). Amplification efficiency and primer dimer formation were assessed using genomic DNA from *G. excentricus* and *G. silvae*. Candidate primer pairs were further tested for cross-

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reactivity using genomic DNA (10 ng) extracted from *Fukuyoa ruetzleri*, *Gambierdiscus australes*, *G. belizeanus*, *G. caribaeus*, *G. carpenteri*, *G. carolinianus*, *G. pacificus*, *G. polynesiensis*, *Gambierdiscus* ribotype 2 and *G. toxicus* (Vandersea et al. 2012, Darius et al. 2017). Non-specific amplification was not observed. The primer pairs chosen for final PCR assay validation are listed in Table 2.

The PCR assays were conducted as follows. All assays were performed using an Eppendorf Mastercycler® ep realplex 4 system with white Eppendorf real-time tube strips (Eppendorf North America, Inc., Westbury, NY, USA) and a total reaction volume of 10.5 $\mu L \cdot tube^{-1}$. Each PCR reaction mixture contained 4.5 µL of 5 Prime RealMasterMix SYBR ROX 2.5x (Thermo Fisher Scientific, Waltham, MA, USA; 0.05 units $\cdot \mu L^{-1}$ Taq DNA polymerase, 10 mM $Mg(CH_3COO)_2$, 1.0 mM dNTPs, 20X SYBR® Green solution) each primer at a concentration of 0.15 µM, 4.7 µL of sterile deionized water, and 1 µL of template DNA. Thermal cycling conditions included denaturation at 94°C for 2 min followed by 40 cycles at 94°C for 10 s, annealing for 15 s at 62°C with a subsequent extension at 68°C for 20 s. Following the last cycle of PCR amplification, a melting curve analysis was performed to check the specificity of the PCR reactions. The melting curve profile consisted of denaturation at 95°C for 15 s followed by an annealing step for 15 s at 60°C. Fluorescence was continuously monitored during a ten-minute temperature ramp from 60°C to 95°C. The melting curve analysis was performed by comparing the melting temperature peak of positive control DNA to negative control DNAs from non-target species. A limit of ± 0.5 C for melting temperature shifts was set as the threshold for species-specific amplifications.

The assays were used to screen single cell isolates established from macroalgal material collected from St. Croix, U.S. Virgin Islands. One *Gambierdiscus excentricus* (Saint Croix Gam7) and two *G. silvae* isolates from Saint Croix (Saint Croix Gam4 and Gam9) were identified. These results indicate the PCR primer sets will be valuable tools for identifying

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cultured cell isolates and for potentially identifying single cells selected from field samples. They will also prove useful in screening Lugol's preserved, bulk, field samples as described in Vandersea et al. (2012, 2017) and Darius et al. (2017). Broader screening of environmental samples will allow better characterization of the distribution and habitat preferences of *G. excentricus* and *G. silvae*. Adaptation of the assays to a qPCR format could also be used to verify if increased abundances of these species correlates with elevated CFP risk (Tester et al. 2010, Litaker et al. 2017).

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Table 1. Average toxicity estimates for *Gambierdiscus excentricus* and *G. silvae* determined using the N2a cytotoxicity assay. Other species occurring in the Caribbean and Gulf of Mexico include *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *Gambierdiscus* ribotype 2, and *Fukuyoa ruetzleri*. All of these species exhibited lower toxicities, ranging from 0 to 10.6 fg CTX3C equivalents cell^{-1} (Litaker et al. 2017). A recent report by Reverté et al. 2018, however, indicates *G. australes* isolates from the Canary Islands, Spain are more toxic, containing between 200 to 697 fg equivalents CTX1B · cell^{-1}.

Species	**Isolate Name**	**Locality**	**CTX-Like Toxicity**	**Reference**
Gambierdiscus excentricus	VGO790	Tenerife Island, Canary Islands' archipelago	1,100 ± 190 (fg CTX 1B eq. · cell^{-1})	Fraga et al. 2011
	VGO791	Tenerife Island, Canary Islands' archipelago	1,050 ± 180 (fg CTX 1B eq. · cell^{-1})	Fraga et al. 2011
	VGO791	Tenerife Island, Canary Islands' archipelago	1,426 ± 55 (fg CTX3C eq. · cell^{-1})	Pisapia et al. 2017
	VGO792	Tenerife Island, Canary Islands' archipelago	370 ± 170 (fg CTX 1B eq. · cell^{-1})	Fraga et al. 2011
	Pulley Ridge Gam2	Pulley Ridge reef Northeastern Gulf of Mexico	469 (fg CTX3C eq. · cell^{-1})	Litaker et al. 2017
Gambierdiscus silvae	VGO1167	Punta Hidalgo, Tenerife, Canary	10.3 ± 2.7 (fg CTX3C eq. · cell^{-1})	Pisapia et al. 2017

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		Islands		
	VGO1180	Punta Hidalgo, Tenerife, Canary Islands	12.4 ± 3.6 (fg CTX3C eq. · $cell^{-1}$)	Pisapia et al. 2017
	Curacao Gam 11	Curacao, Caribbean	19.6 (fg CTX3C eq. · $cell^{-1}$)	Litaker et al. 2017
	37 strains	US Virgin Islands, Caribbean	2,100–4,800 (fg C-CTX-1 eq. · $cell^{-1}$)	Robertson et al. 2018

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Table 2. The species-specific primer sets for *Gambierdiscus excentricus* and *Gambierdiscus silvae*.

Species	**Name of Primers**	**Tm (°C)**	**Amplicon Size (bp)**	**Sequence of PCR primers**
Gambierdiscus excentricus	ExcentF1	62	106	5' - CGAAGAGTGAAAGACTCAATTC - 3'
	ExcentR3	64		5' - CCTCTTGCAAGATGGCTCACA - 3'
Gambierdiscus silvae	Silv_ITS_F2	62	91	5' - CCACAATGCATGTGAAGCTGT - 3'
	Silv_ITS_R3	62		5' - CATACCACCGGCGCACAC - 3'

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