Available online at www.sciencedirect.com
ScienceDirect
Harmful Algae 7 (2008) 614–629
HARMFUL ALGAE
www.elsevier.com/locate/hal

Phylogeography, morphological variation and taxonomy of the toxic dinoflagellate *Gambierdiscus toxicus* (Dinophyceae)

Mindy L. Richlen [a,*], Steve L. Morton [b], Paul H. Barber [a], Phillip S. Lobel [a]

[a] *Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215, USA*
[b] *NOAA/NOS Marine Biotoxin Program, 331 Ft. Johnson, Charleston, SC 29412, USA*

Received 18 August 2007; received in revised form 20 December 2007; accepted 21 December 2007

Abstract

Gambierdiscus toxicus Adachi et Fukuyo 1979 is a toxin-producing marine dinoflagellate responsible for the syndrome known as ciguatera, which sickens thousands of people every year. Despite its importance, little is known regarding the global genetic structure of this species; therefore, it is unclear whether documented variation in toxin production and outbreaks of ciguatera are the result of ecological triggers or the presence of different genetic strains across the geographic range of *G. toxicus*. We examined the molecular phylogeny and morphological characteristics of 28 globally distributed *G. toxicus* isolates by sequencing part of the large subunit (LSU) and small subunit (SSU) ribosomal DNA and analyzing the thecal architecture using scanning electron microscopy (SEM). Our analyses showed that *G. toxicus* is comprised of at least four distinct lineages (Clades A–D) separated by substantial genetic distances. One of the clades recovered in the phylogenetic analysis (Clade B) is morphologically distinct, the characteristics of which agree with the species description of *Gambierdiscus belizeanus* Faust 1995. The remaining three lineages; however, are morphologically homogeneous and may represent cryptic species. SEM analyses showed that an important morphological feature used to distinguish *Gambierdiscus* morphospecies, the apical pore (Po) plate shape, was variable among isolates of *G. toxicus* and did not reflect phylogenetic groupings; hence, its utility as a diagnostic feature is questionable. Our analyses suggest that *G. toxicus* is not a single cosmopolitan species with a worldwide circumtropical distribution but instead comprises a wide-ranging species complex. The presence of multiple cryptic species could partly explain the seemingly random patterns of ciguatera toxicity across geography.
© 2008 Elsevier B.V. All rights reserved.

Keywords: Ciguatera; Dinoflagellates; *Gambierdiscus*; Harmful algal blooms (HABs)

1. Introduction

Gambierdiscus toxicus (Dinophyceae) is an armored, toxin-producing dinoflagellate distributed circumtropically in coral reef ecosystems. It is a member of a community of dinoflagellates from several genera that are benthic, inhabiting the surfaces of macroalgae, detritus and sand. *G. toxicus* is an ecologically important species, as certain strains produce toxins that bioaccumulate in the coral reef food chain, attaining highest levels in carnivores (Anderson and Lobel, 1987; Lehane and Lewis, 2000). Consumption of toxic fish causes ciguatera fish poisoning, which sickens an estimated 50,000–500,000 people every year and is the most frequently reported marine toxin disease worldwide (Fleming et al., 1998; Ragelis, 1984). The symptoms of ciguatera have been well described and include gastrointestinal, neurological and cardiovascular disturbances (Bagnis et al., 1979; Lewis et al., 1988; Lehane and Lewis, 2000; Palafox and Buenconsejo-Lum, 2001). This syndrome significantly degrades the health and economic well being of communities dependent on reef fisheries as a food source or for export; in the United States alone, the economic impacts of ciguatera poisoning were estimated at an average of $21 million per year for the 1987–1992 period (Anderson et al., 2000). Moreover, due to increased exportation of tropical fishes to northern markets and the expansion of the tourism industry in tropical and subtropical countries, individuals living outside of ciguatera endemic regions are increasingly exposed to toxic fish (Freudenthal, 1990; Todd, 1995).

The toxin-producing capability of *G. toxicus* appears to be genetically determined (Bomber et al., 1989; Holmes et al., 1991; Babinchak et al., 1994) and is a stable temporal

* Corresponding author. Present address: Biology Department, Woods Hole Oceanographic Institution, MS 32, Woods Hole, MA 02543, USA.
Tel.: +1 508 289 2552; fax: +1 508 457 2027.
E-mail address: mrichlen@whoi.edu (M.L. Richlen).

1568-9883/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2007.12.020

characteristic that is unaffected by time in culture or culture conditions (Durand-Clement, 1986).

However, the type and quantity of toxin produced is variable among isolates and up to a 100-fold difference in the quantity of toxin produced by different genetic strains has been reported (Bomber et al., 1989; Holmes et al., 1990, 1991; Sperr and Doucette, 1996). Furthermore, differences in toxin production by *G. toxicus* have been documented among strains isolated from one geographic location, demonstrating physiological difference within a single locality (Holmes et al., 1991). Based on these observations Holmes et al. (1994) hypothesized the existence of certain ‘‘super-producing strains’’ of *G. toxicus* that greatly increase the potential for ciguatera.

In addition to genetically determined differences in toxicity, toxin production is also dependent upon a number of environmental factors. This has led several scientists to speculate that ciguatera outbreaks occur when environmental conditions favor the growth of highly toxic clones within a population (Holmes et al., 1994; Chinain et al., 1999). Morton et al. (1993) showed a 200-fold difference in *G. toxicus* potency depending on the light, salinity and temperature conditions during culture. Bomber et al. (1988) observed different relationships between light intensity and growth rate for a variety of strains, with reduced growth rates at high irradiance; furthermore, high temperatures increased toxicity in *G. toxicus* (Bomber et al., 1988). These laboratory studies suggest that individual *G. toxicus* strains may be adapted to particular specific environmental regimes. Therefore, there are two potential sources for ciguatera toxin variability in the field: (1) differences in the toxicity of the source organisms due to genetic factors (i.e., different species or strains); (2) differences in source organism toxicity that relate to environmental forcings.

Following the identification of *G. toxicus* as the toxin originator (Yasumoto et al., 1977; Adachi and Fukuyo, 1979) five additional *Gambierdiscus* species were described based on differences in cell morphology and thecal plate architecture. The first additional species reported was *G. belizeanus* Faust 1995, a sand-dwelling species that was part of a dinoflagellate assemblage observed in a shallow lagoon in Belize (Faust, 1995). *G. belizeanus* was described as a new species based on differences in fine thecal surface morphology and the shape of the posterior intercalary plate (1p). *G. yasumotoi* Holmes 1998 was identified from macroalgae in Singapore based on differences with the aforementioned species in cell size and shape, girdle displacement, shape of the apical pore plate (Po), and arrangement of the sulcal lists (Holmes, 1998). The final three species, *G. pacificus*, *G. australes* and *G. polynesiensis* were collected from red calcareous macroalgae in French Polynesia (Chinain et al., 1999). These three were described as distinct species based on differences in cell size and shapes of the 1p and Po plates. While toxin production by *G. belizeanus* has not been investigated; toxin production by the other four species is confirmed (Chinain et al., 1999).

The association between geographic patterns of fish toxicity and the preponderance of highly toxic strains of *G. toxicus* has not been explored. However, studies of *Gambierdiscus* at the molecular level may ultimately help explain the heterogeneity observed in the toxin structures present in the food chain and the differences in ciguatera symptoms. The application of molecular techniques could also foster the development of molecular markers for distinguishing toxic from non-toxic strains, a tool critical for predicting local ciguatera risk. However, surprisingly few studies have directly examined patterns of genetic variation in *G. toxicus* or sought to address the molecular basis of toxin production.

Initial research into the genetic diversity of *G. toxicus* focused on inferring genetic variation from heterogeneity in phenotypic characteristics among isolates such as morphology and physiology. Variability in cell sizes and shapes has been documented in field samples (Adachi and Fukuyo, 1979) as well as among laboratory cultures (Durand-Clement, 1987; Chinain et al., 1997), with cell size increasing during the stationary growth stage (Durand-Clement, 1986). Differences in growth rates (Bomber et al., 1989; Sperr and Doucette, 1996; Chinain et al., 1997), toxin production (Bomber et al., 1989; Holmes et al., 1990, 1991; Sperr and Doucette, 1996) and isozyme electrophoretic profiles (Chinain et al., 1997) documented substantial metabolic and biochemical heterogeneity among isolates.

Babinchak et al. (1994) directly examined genetic diversity using restriction fragment length polymorphism (RFLP) of the large subunit (LSU) ribosomal DNA gene, showing that substantial genetic variability does indeed exist among globally distributed strains. RFLP of the small subunit (SSU) rDNA recovered different ribotypes within local populations of *G. toxicus* in French Polynesia; additionally, sequence analysis of the LSU rDNA indicated the potential utility of this gene as a biogeographic marker (Chinain et al., 1998). Notwithstanding the intriguing results of these studies, a comprehensive examination of genetic variation among globally distributed strains using DNA sequencing is still lacking; furthermore, the phylogenetic relationships of the *Gambierdiscus* morphospecies are not fully understood.

The scant and fragmentary nature of the phylogenetic information that exists regarding the foremost *Gambierdiscus* species, *G. toxicus,* contributes to the confusion. Currently there is considerable uncertainty regarding phylogenetic patterns as well as the degree of morphological plasticity that exists in this species and if, in fact, *G. toxicus* is a single cosmopolitan species with worldwide circumtropical distribution or a wide-ranging species complex. Therefore, it is unclear whether documented variation in toxin production and the seemingly stochastic nature of ciguatera toxicity are strictly due to the influence of environmental triggers or also relate to the presence and relative abundance of different genetic strains across the range of *G. toxicus*.

To address the deficiency of phylogenetic information regarding *G. toxicus* we examined the genetic diversity of globally distributed strains using DNA sequencing of the LSU and SSU rDNA. These isolates were also analyzed using scanning electron microscopy (SEM) to confirm that their morphological characteristics conformed to the species

description of *G. toxicus*. Combining the morphological and molecular analysis accounts for both phenotypic and genotypic characters to provide a robust assessment of the taxonomic classification of these strains. Additionally, examining morphological variability within a phylogenetic framework distinguishes phenotypically stable from plastic characters and between ancestral and derived characters used in morphological classifications. Furthermore, this phylogenetic characterization provides a foundation for future molecular research into the evolutionary history of this important species and its association with environmental patterns of toxicity.

2. Materials and methods

2.1. *G. toxicus* cultures

A total of 28 globally distributed *G. toxicus* isolates (16 Pacific, 12 Atlantic/Caribbean) were obtained as live or frozen (one isolate) cultures from the National Oceanic and Atmospheric Administration (NOAA) Marine Biotoxins Program, the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA), and Dr. Donald Tindall (Southern Illinois University Carbondale). Live cultures were maintained in flasks with 50 mL of ES (Provasoli, 1968) or K (Keller et al., 1987) medium at 25 °C and at a photon flux density of ca. 160 μmol photons $m^{-2} s^{-1}$, on a 14:10 light:dark cycle. A complete listing of the isolate identification numbers, known synonyms, collection location, geographic region, isolator and source is provided in Table 1, including one sample obtained from Genbank (accession no. DQ388463).

2.2. DNA extraction, PCR amplification, cloning and sequencing

DNA was extracted from live cells of each isolate using Chelex® (Bio-Rad) chelating resin (Walsh et al., 1991; Richlen and Barber, 2005) and from the frozen cell pellet (isolate BB01) using a DNeasy Tissue kit (Qiagen, Santa Clarita, CA) following the manufacturer's instructions. Because it has been used successfully in previous taxonomic and phylogenetic studies on *G. toxicus* (Chinain et al., 1998, 2001), the D8–D10 hypervariable region of the LSU rDNA was amplified from all isolates via PCR using the primers FD8 and RB (Chinain et al., 1999). To obtain additional phylogenetic resolution, 18S rDNA (SSU) was amplified from 19 selected isolates using the primers 18ScomF1 and Dino18SR1 (Lin et al., 2006). For both LSU and SSU, PCR reactions contained 1.0 μL template DNA, 14.5 μL sterile deionized water, 2.5 μL GeneAmp® 10× PCR buffer composed of 500 mM potassium chloride and 100 mM Tris–HCl (pH 8.3 at room temperature), 2.5 μL, 8 μM dNTPs (2 μM each of dATP, dCTP, dGTP and dTTP), 2.0 $MgCl_2$ (25 mM), 1.25 μL of each primer (10 mM), and 0.125 units/μL AmpliTaq® DNA polymerase. Amplifications were performed using a Biometra TGradient Thermocycler (Biometra, Germany) as follows: 94 °C for 4 min; then 35 cycles of 94 °C for 30 s, 57 °C for 1 min, 72 °C for 2 min; a final extension of 72 °C for 10 min. PCR amplification products were visualized by electrophoresis on 1% TBE low melt agarose (ISC BioExpress GenePure™) gel. If PCR products yielded more than one band, the appropriate sized band was excised from the gel and purified using a Qiagen MinElute Gel Extraction kit (Qiagen, Santa Clarita, CA) following the manufacturer's instructions. PCR products were cloned into vector pCR 2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Clones were screened for inserts by PCR amplification with plasmid primers T7 or M13F and M13R and eight positive clones from each PCR amplicon were selected for DNA sequencing.

DNA sequencing was conducted on an Applied Biosystems (ABI) 377 and ABI 3730XL capillary sequences (Applied Biosystems, Foster City, CA, USA) using BigDye© Version 3.1 (PerkinElmer) terminator chemistry. A total of 6 μL volume was used in cycle sequencing reactions, comprised of 1 μL template, 1.7 μL 5× buffer, 0.5 μL of each primer (10 mM), 1.0 μL BigDye, 1.8 μL sterile deionized water. Thermocycling parameters used in the reaction are as follows: 30 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Samples were concentrated using isopropanol precipitation, resuspended in HiDi Formamide, and stored at 4 °C until sequencing.

2.3. Phylogenetic analysis

Forward and reverse sequences were edited and aligned by eye in Sequencher™. Comparisons using the Basic Local Alignment Search Tool (BLAST) confirmed that all of the resulting LSU sequences were dinoflagellate large subunit rRNA and that SSU sequences were *G. toxicus* SSU rRNA. Modeltest V. 3.7 (Posada and Crandall, 1998) was then used to select the most appropriate model of nucleotide substitution for phylogenetic analyses.

Phylogenetic trees were constructed with PAUP* 4.0b10 (Swofford, 2002) using maximum parsimony (MP) and maximum likelihood (ML) analyses using *Prorocentrum micans* LSU (accession no. X16108) or SSU (accession no. AJ415519) rDNA as outgroups. Parsimony analyses were conducted using the heuristic search, simple addition, with gaps treated as missing data. Heuristic searches using maximum likelihood employed the following model parameters: (1) for the LSU data the Tamura–Nei plus gamma model (TrNef + G) (Tamura and Nei, 1993) was used with equal base frequencies, six variable substitution rates (AC: 1.0000, AG: 2.4526, AT: 1.0000, CG: 1.0000, CT: 3.6668 and GT: 1.0000) and a gamma distribution of 0.3029; (2) for the SSU data the TrNef + G model was employed with equal base frequencies, six variable substitution rates (AC: 1.0000, AG: 2.7241, AT: 1.0000, CG: 1.0000, CT: 3.4811 and GT: 1.0000), and a gamma distribution of 0.7001. Bootstrap support values were determined for both parsimony and maximum likelihood using 1000 replicates.

2.4. Morphological analyses

Due to the extensive variation observed in the phylogenetic analysis, morphological characters were examined to ascertain

Table 1
Isolate identification number, collection location, geographic region, isolator (if known) and source of *Gambierdiscus toxicus* isolates examined (ND: no data)

Strain number	Collection location	Region	Isolator	Source
CCMP 1649 (C103)	Grand Cayman Island	Caribbean Sea, North Atlantic	J. Babinchak	CCMP
CCMP 399 (SB03)	St. Barthelemy Island	Caribbean Sea, Atlantic Ocean	M. Durand-Clement	CCMP
CCMP 401	St. Barthelemy Island	Caribbean Sea, North Atlantic	M. Durand-Clement	CCMP, Genbank
BZ100	Belize	Caribbean Sea, North Atlantic	S. Morton	S. Morton
BZ100B	Belize	Caribbean Sea, North Atlantic	S. Morton	S. Morton
BZ100C	Belize	Caribbean Sea, North Atlantic	S. Morton	S. Morton
CCMP 1655 (MQ2)	Martinique	Caribbean Sea, North Atlantic	J. Babinchak	S. Morton
B775	Mullet Bay, Bahama	North Atlantic	D. Tindall	D. Tindall
FIT113	Knight Key, Florida	North Atlantic	D. Tindall	D. Tindall
CM515	Cancun, Mexico	Caribbean Sea, North Atlantic	D. Tindall	D. Tindall
BB01	Bermuda	North Atlantic	J. Babinchak	S. Morton
CCMP 1653 (T39)	Hawaii	North Pacific	N. Withers	CCMP
CCMP 1654 (G05)	Guam, Northern Mariana Islands	Micronesia, North Pacific	J. Babinchak	CCMP
A213	Heron Island, Australia	South Pacific	D. Tindall	D. Tindall
F106	Viti Levu, Fiji Islands	Melanesia, South Pacific	D. Tindall	D. Tindall
TT302B	Tol, Truk	Micronesia, North Pacific	D. Tindall	D. Tindall
MJ312B	Majuro, Marshall Islands	Micronesia, North Pacific	D. Tindall	D. Tindall
FP100	Gambier Islands, French Polynesia	Polynesia, South Pacific	ND	D. Tindall
177	Hawaii	North Pacific	N. Withers	D. Tindall
BIG1	Hawaii	North Pacific	M. Parsons	S. Morton
BIG2	Hawaii	North Pacific	M. Parsons	S. Morton
BIG3	Hawaii	North Pacific	M. Parsons	S. Morton
BIG5	Hawaii	North Pacific	M. Parsons	S. Morton
BIG8	Hawaii	North Pacific	M. Parsons	S. Morton
T04	Tahiti, French Polynesia	Polynesia, South Pacific	J. Babinchak	S. Morton
CCMP 1650 (MR1)	Moorea, Society Islands	Micronesia, South Pacific	J. Babinchak	CCMP
CCMP 1657 (G02)	Palau	Micronesia, North Pacific	J. Babinchak	CCMP

Sources included cultures maintained by S. Morton (NOAA Marine Biotoxins Program), the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), Dr. Donald Tindall (Southern Illinois University Carbondale) and Dr. Michael Parsons (Florida Gulf Coast University).

that the isolates examined in this study conformed to the species description of *G. toxicus*. Morphological characters important in the identification and discrimination of *Gambierdiscus* species include cell size and shape, thecal surface morphology, shape of the apical pore plate, shape and size of the posterior intercalary plate (summarized in Table 2) (Chinain et al., 1999). These characters were examined for each isolate using scanning electron microscopy. For the morphological analyses, approximately 1 mL of each culture was preserved in 4% buffered formalin and exposed to a graded series of sterile seawater/freshwater washes, beginning with 100% seawater and ending with 100% freshwater. Samples were then dehydrated using a graded series of ethanol washes, filter-mounted to a stub and then air-dried. The stubs were sputter coated with gold palladium (Tousimis Samsputter-28, Tousimis) and examined and photographed using a JOEL JSM-840 scanning electron microscope (JOEL, Tokyo, Japan). Cell dimensions were determined by examining formalin-preserved samples at 200× on a Zeiss Axioskope microscope (Ziess, Jenna, Germany). Transdiameter measurements of at least 10 cells were recorded. Morphological characters for all but two of the isolates were analyzed: BB01 was only available as a frozen cell pellet and was not suitable for SEM analysis due to the degree of fragmentation of the thecae, and the culture of isolate CCMP 1657 died before the morphology could be examined and is no longer available.

Table 2
Summary of morphological characteristics diagnostic of *Gambierdiscus* species

Species	Morphology	Cell size transdiameter (μm)	Thecal surface	Apical pore plate (Po)	Posterior intercalary plate (1p)
G. toxicus[a,d]	Flattened	42–140	Smooth	Ellipsoid	Broad plate (∼one-third of hypothecal width)
G. yasumotoi[b]	Globular	38–50	Smooth	Elongate (long-shank fishhook)	Long, narrow, pentagonal (∼20–25% of hypothecal width)
G. belizeanus[c,d]	Flattened	54–63	Rugose	Ellipsoid	Short, narrow, pentagonal (∼20–25% of hypothecal width)
G. polynesiensis[d]	Flattened	68–85	Smooth	Triangular	Broad plate (∼60% of hypothecal width)
G. australes[d]	Flattened	76–93	Smooth	Broadly ellipsoid	Long, narrow, pentagonal (∼30% of hypothecal width)
G. pacificus[d]	Flattened	67–77	Smooth	Four-sided	Short, narrow, pentagonal (∼20% of hypothecal width)

[a] Adachi and Fukuyo (1979).
[b] Holmes (1998).
[c] Faust (1995).
[d] Chinain et al. (1999).

3. Results

3.1. LSU rDNA phylogenetic analysis

A total of 786 basepairs of the LSU rDNA gene were analyzed from each strain. Of these 178 were parsimony informative, 360 were parsimony uninformative, and the remaining were invariable. Intra-isolate sequence variability in the LSU rDNA sequences ranged from 0 to 3%. Uncorrected pair-wise sequence divergence among isolates ranged from <1 to 15%. Results of parsimony and maximum likelihood phylogenetic analyses indicate *G. toxicus* is composed of four distinct, well-supported lineages, identified as Clades A–D (Fig. 1). Clade A consisted of two isolates, the only Atlantic strain (Bermuda) used in this study and a Caribbean isolate (Cancun). Clade B was comprised of three strains from the Caribbean (St. Barthelemy (2), Martinique), two from the Pacific (Moorea, Marshall Islands), and the single Australian isolate. Clade C consisted of exclusively Pacific isolates, four of which were from Hawaii and the one from the Gambier Islands. Clade D consisted of several isolates from both the Caribbean/Atlantic (Belize, Jamaica, Bahamas, Cayman Islands and FL) and the Pacific (Hawaii, Fiji, Truk, Palau and Guam). The isolates in Clade D further split into two groups, one of which included the strains from Hawaii, Fiji and Guam, while the other included Truk, Palau, Tahiti, Hawaii and the Caribbean strains. Although these four major clades were supported by high bootstrap values (100%), the relationships among them were poorly resolved. Equivalent results were obtained from maximum parsimony (Fig. 1).

3.2. SSU rDNA phylogenetic analysis

A total of 681 characters of the SSU rDNA gene were analyzed from each strain. Of these, 228 were parsimony informative, 85 were parsimony uninformative and the remaining were invariable. Intra-isolate sequence variability in the SSU rDNA sequences ranged from 0 to 5%. Uncorrected pair-wise sequence divergence among isolates ranged from <1 to 25%. In both ML and MP analyses, the isolates again split into four major clades, the composition of which was identical in each analysis (Fig. 2). The composition of these four clades was identical to the four clades recovered in the LSU rDNA phylogeny with one exception: in the SSU rDNA analysis, BIG8 (Hawaii) clustered with Clade C, but clustered with Clade D in the LSU rDNA analysis. As was observed in the LSU rDNA phylogenetic analyses, support for the four clades was high (100% bootstrap values), but the relationships among them were poorly resolved.

3.3. Morphological analysis

The cell sizes of the 26 *G. toxicus* isolates analyzed (Table 3) fell within published values (Fukuyo, 1981; Taylor et al., 2003)

Table 3
Morphological characteristics of *G. toxicus* isolates examined, including Po plate, 1p plate, thecal surface morphology, cell shape and cell size (transdiameter)

Isolate	Apical pore plate (Po)	Posterior intercalary plate (1p)	Thecal surface	Cell shape	Cell size (μm)	Morphological group
BB01	ND	ND	ND	ND	ND	ND
CM515	Broadly ellipsoid	Broad	Smooth	Round	74–85	Group 2 (unknown)
A213	Ellipsoid	Short, narrow	Rugose	Round	58–69	Group 3 (*G. belizeanus*)
CCMP 1650	Ellipsoid	Short, narrow	Rugose	Heart-shaped	62–76	Group 3 (*G. belizeanus*)
CCMP 1655	Ellipsoid	Short, narrow	Rugose	Round	53–69	Group 3 (*G. belizeanus*)
CCMP 399	Ellipsoid	Short, narrow	Rugose	Round	51–79	Group 3 (*G. belizeanus*)
CCMP 401	Ellipsoid	Short, narrow	Rugose	Round	58–78	Group 3 (*G. belizeanus*)
MJ312B	Ellipsoid	Short, narrow	Rugose	Round	59–75	Group 3 (*G. belizeanus*)
177	Ellipsoid	Broad	Smooth	Round	65–80	Group 1 (*G. toxicus*)
BIG1	Ellipsoid	Broad	Smooth	Heart-shaped	69–83	Group 1 (*G. toxicus*)
BIG2	Ellipsoid	Broad	Smooth	Round	52–80	Group 1 (*G. toxicus*)
BIG3	Ellipsoid	Broad	Smooth	Round	50–72	Group 1 (*G. toxicus*)
CCMP 1653	Ellipsoid	Broad	Smooth	Round	71–88	Group 1 (*G. toxicus*)
FP100	Triangular	Broad	Smooth	Round	71–92	Group 2 (unknown)
BIG8	Ellipsoid	Broad	Smooth	Round	51–68	Group 1 (*G. toxicus*)
B775	Ellipsoid	Broad	Smooth	Round	75–100	Group 1 (*G. toxicus*)
BIG5	Ellipsoid	Broad	Smooth	Round	83–111	Group 1 (*G. toxicus*)
BZ100	Ellipsoid	Broad	Smooth	Round	53–73	Group 1 (*G. toxicus*)
BZ100B	Ellipsoid	Broad	Smooth	Round	64–85	Group 1 (*G. toxicus*)
BZ100C	Ellipsoid	Broad	Smooth	Round	66–82	Group 1 (*G. toxicus*)
CCMP 1649	Ellipsoid	Broad	Smooth	Round	68–91	Group 1 (*G. toxicus*)
CCMP 1654	Ellipsoid	Broad	Smooth	Heart-shaped	94–103	Group 1 (*G. toxicus*)
CCMP 1657	ND	ND	ND	ND	ND	ND
F106	Ellipsoid	Broad	Smooth	Round	75–88	Group 1 (*G. toxicus*)
FIT113	Ellipsoid	Broad	Smooth	Round	84–99	Group 1 (*G. toxicus*)
NJ920D	Broadly ellipsoid	Broad	Smooth	Round	72–99	Group 2 (unknown)
T04	Ellipsoid	Broad	Smooth	Round	84–109	Group 1 (*G. toxicus*)
TT302B	Ellipsoid	Broad	Smooth	Round	74–101	Group 1 (*G. toxicus*)

Morphological groupings are based on concordance with species descriptions (see text).

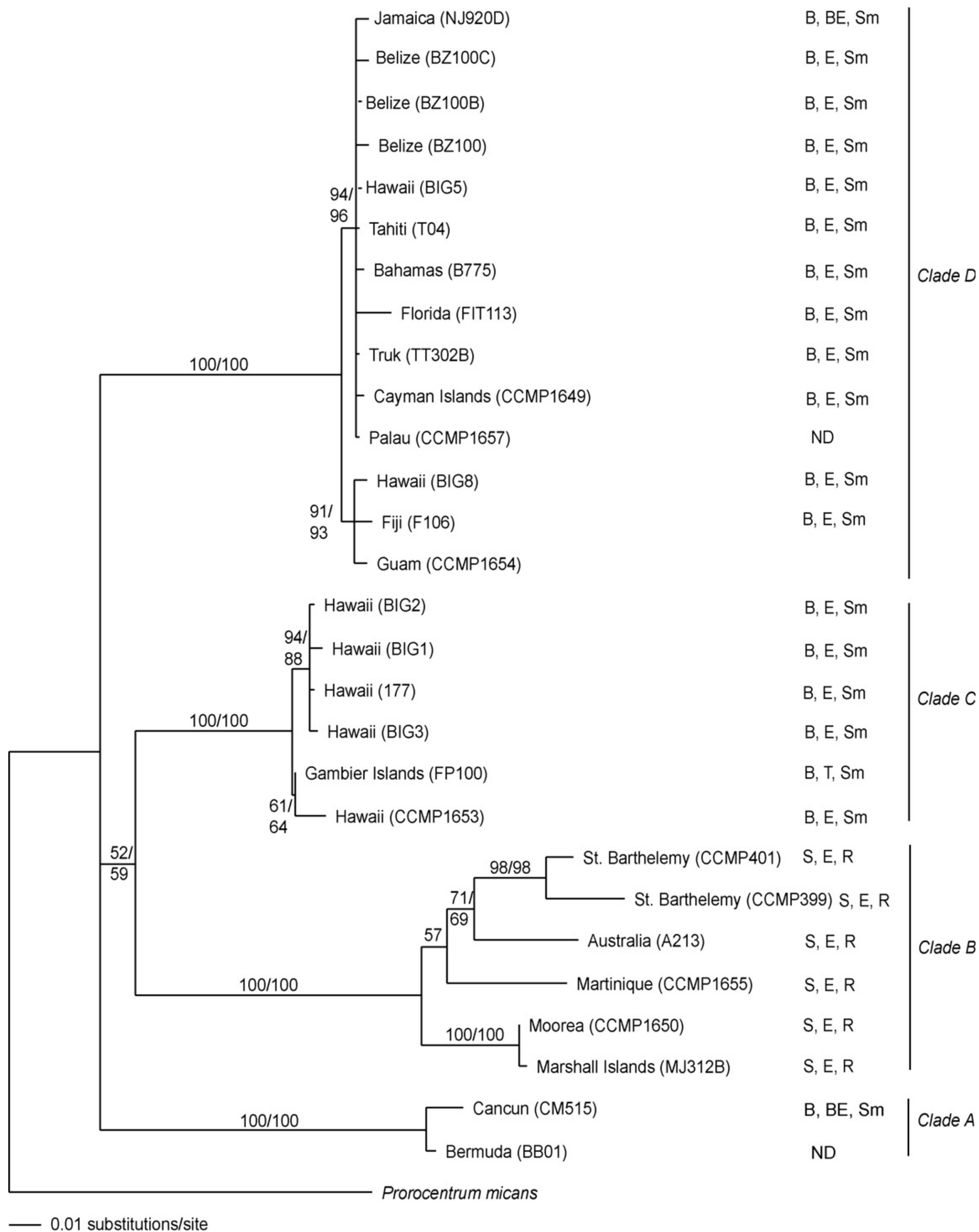

Fig. 1. Maximum likelihood tree (ML) based on analysis of the domains D8–D10 of the large subunit rDNA gene. Bootstrap values (1000 replications) greater than 50% from maximum likelihood/maximum parsimony analyses are shown above or adjacent to each node. *Prorocentrum micans* was used as an outgroup. Results of the morphological analyses of the isolates are displayed next to the corresponding branch. Morphological characters analyzed include the posterior intercalary plate (B: broad, S: short), apical pore plate (E: ellipsoid, BE: broadly ellipsoid, T: triangular) and thecal surface morphology (Sm: smooth, R: rugose). ND: no data.

and all cells were flattened anteroposteriorly. All cultures displayed the plate formula of Po, 3′, 7″, 6c, 8s, 5‴, 1p and 2⁗. Although sulcal plates were not examined in this study, all samples conformed to *Gambierdiscus* based on the above characters.

We found variability in three important features used to diagnose *Gambierdiscus* species: thecal surface morphology, Po plate shape, and 1p plate shape. The thecal surfaces of all but six of the strains were smooth and covered with numerous evenly distributed pores, which is characteristic of all but one of the *Gambierdiscus* species described thus far. However, the thecae of six isolates were rugose (see Fig. 3 and Table 3) and similar in appearance to *G. belizeanus*, which is described as having an areolate thecae surface (Faust, 1995).

In addition to thecal surface morphology, the shape of the Po plate is also used to diagnose *Gambierdiscus* species. The Po

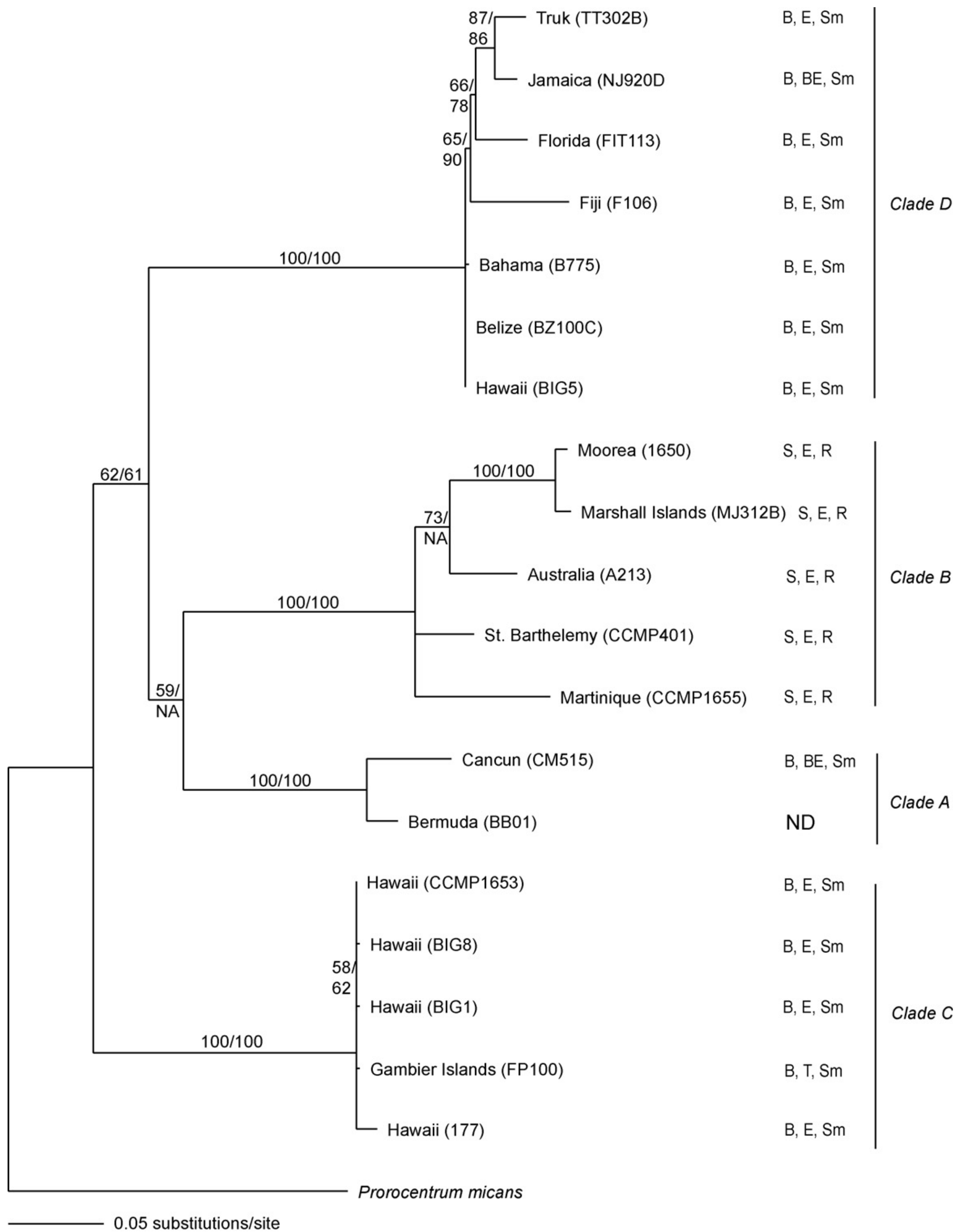

Fig. 2. Maximum likelihood tree (ML) based on analysis of the small subunit rDNA gene. Bootstrap values (1000 replications) greater than 50% from maximum likelihood/maximum parsimony analyses are shown above or adjacent to each node. *Prorocentrum micans* was used as outgroup. Results of the morphological analyses of the isolates are displayed next to the corresponding branch. Morphological characters analyzed include the posterior intercalary plate (B: broad, S: short), apical pore plate (E: ellipsoid, BE: broadly ellipsoid, T: triangular) and thecal surface morphology (Sm: smooth, R: rugose). ND: no data.

plate is ellipsoid in *G. toxicus* and *G. belizeanus*, broadly ellipsoid in *G. australes*, triangular in *G. polynesiensis*, elongated ellipsoid in *G. yasumotoi*, and four-sided in *G. pacificus* (Adachi and Fukuyo, 1979; Faust, 1995; Holmes, 1998; Chinain et al., 1999). All but three of the isolates examined had an ellipsoid-shaped Po plate featuring the fish-hook shaped opening characteristic of *G. toxicus* (see Fig. 4); however, two of the isolates featured what has been described as a ‘‘broadly ellipsoid’’ Po plate characteristic of *G. australes* and one isolate featured a ‘‘triangular’’ Po plate characteristic of *G. polynesiensis* (see Fig. 5) (Chinain et al., 1999). The pore count on the Po plate was not a stable character among the

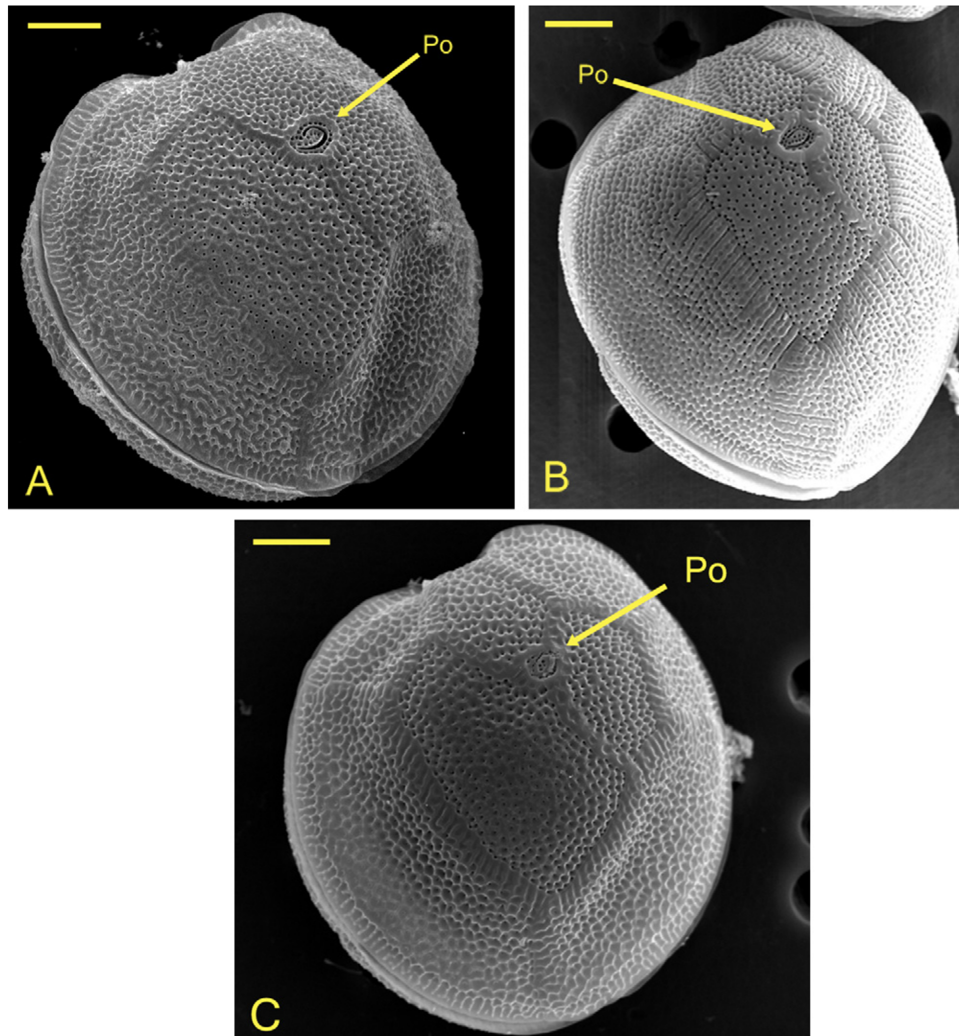

Fig. 3. SEM micrographs of isolates featuring rugose thecal surface morphology, characteristic of *G. belizeanus*. Epithecal view; Scale bars: 10 μm; subset of isolates shown: (A) A213; (B) CCMP 1650; (C) CCMP 399.

isolates examined, but varied from under 20 (CCMP 401) to 40 (B775); furthermore, variability in pore count was also observed among monoclonal cells (data not shown).

The final thecal characteristic used to distinguish *Gambierdiscus* species is the size and shape of the 1p plate. In *G. toxicus* and *G. polynesiensis* this plate is broad, occupying approximately 35 and 60% of the hypothecal width, respectively, whereas in *G. pacificus* and *G. belizeanus* this plate is short and narrow, occupying approximately 20% of the hypothecal width (Faust, 1995; Chinain et al., 1999). *G. yasumotoi* and *G. australes* both feature a long, narrow 1p plate that occupies 20–30% of the hypothecal width (Holmes, 1998; Chinain et al., 1999). The isolates we examined exhibited two of the aforementioned 1p plate morphologies. Most featured a broad plate occupying one-third or more of the hypothecal width, characteristic of *G. toxicus* (see Fig. 6). However, one group comprised of isolates from the Marshall Islands (MJ312B), St. Barthelemy (CCMP 399/CCMP 401), Moorea (CCMP 1650), Australia (A213) and Martinique (CCMP 1655) exhibited a short, narrow 1p plate and rugose thecal surface characteristic of *G. belizeanus* (see Fig. 7).

Based on the thecal plate architecture and surface morphology, the isolates are split into three morphologically distinct groups (see Table 3). The morphological features of Group 1 included a flattened cell shape, smooth thecal surface, ellipsoid Po plate and broad 1p plate, all of which are diagnostic of *G. toxicus*. Group 2 featured identical morphology to the first group, with the exception of the Po plate. While the Po plate diagnostic of *G. toxicus* is ellipsoid, two isolates in the second group featured a broadly ellipsoid Po plate characteristic of *G. australes*, and one isolate featured a triangular Po plate characteristic of *G. polynesiensis*. The morphological characteristics of Group 3 included a rugose thecal surface and ellipsoid Po plate. Furthermore, the 1p plate is short and narrow. Based on these characteristics, Group 3 agrees with the species description of *G. belizeanus*, corresponding to samples from Clade B. However, no other concordance between phylogenetic and morphological groupings was observed.

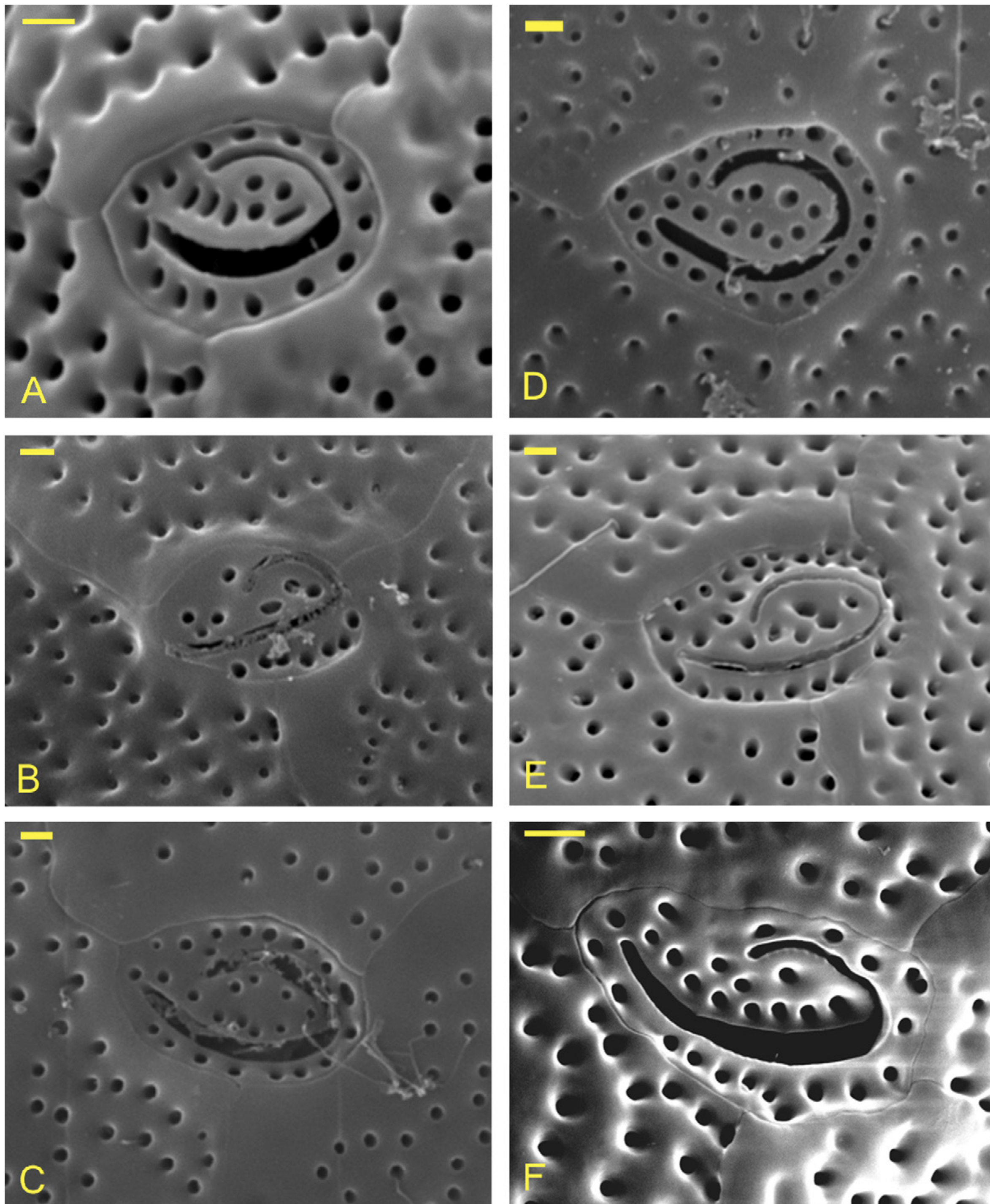

Fig. 4. SEM micrographs of isolates featuring ellipsoid apical pore plate (Po) morphology, characteristic of *G. toxicus*. Epithecal view; scale bars: 1 μm; subset of isolates shown: (A) CCMP 1650; (B) CCMP 399; (C) CCMP1649; (D) CCMP 1653; (E) FIT113; (F) 177.

4. Discussion

Our analyses indicate that *G. toxicus* is not a homogeneous taxon; instead, it is composed of genetically and morphologically distinct groups that vary across geography. Phylogenetic analyses using LSU and SSU rDNA sequences identified four, well-supported lineages separated by substantial genetic distances. Two of the clades (Clades B and D) are apparently widespread, while Clades A and C are regionally restricted to the Atlantic/Caribbean and Pacific, respectively. Our results agree with Babinchak et al. (1996), who reported limited concordance of phylogenetic patterns and biogeography using RFLP data of LSU rDNA from 20 globally distributed *G. toxicus* strains. However, these patterns contrast with Chinain et al. (1998, 2001) in which the grouping of *G. toxicus* LSU rDNA sequences corresponded to their geographic origin, a pattern that may result from restricted geographic sampling. While this study and Babinchak et al. (1996) analyzed cultures

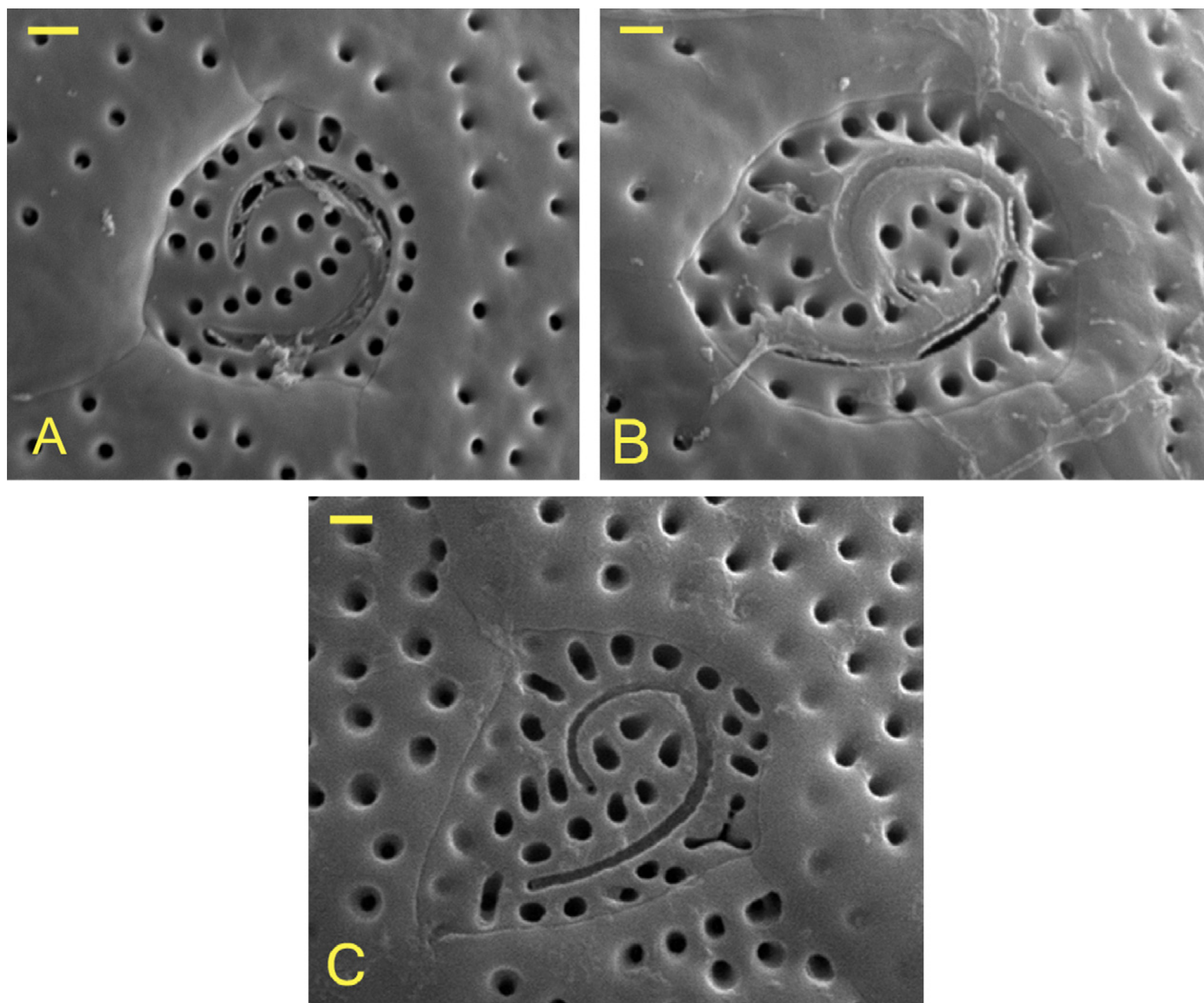

Fig. 5. SEM micrographs of isolates featuring anomalous apical pore plate (Po) morphology. Epithecal view; scale bars: 1 μm; subset of isolates shown: (A) CM515 and (B) NJ920D featured a broadly ellipsoid Po plate, characteristic of *G. australes*; (C) FP100, triangular Po plate, characteristic of *G. polynesiensis*.

isolated from the Caribbean/Western Atlantic (Bermuda) as well as the Pacific (Hawaii, Polynesia, Micronesia and Australia), studies by Chinain et al. (1998, 2001) primarily utilized isolates from the southern Pacific. Our results strongly indicate that *G. toxicus* is not a single cosmopolitan species, but instead is a species complex comprised of several distantly related groups co-occurring across geography. The presence and relative abundance of the members of this species complex among geographic regions may help explain patterns of ciguatera toxicity, particularly if differences in physiology and/or toxin-producing capabilities also exist among these groups.

While Clades A and C were regionally restricted, Clades B and D were widespread across the Pacific and Atlantic Oceans. This latter pattern may reflect recent natural dispersal events, which can occur via ocean currents, rafting on drift algae (Bomber et al., 1988) and plastic debris (Masó et al., 2003). However, this scenario would necessitate dispersal of this tropical species through temperate or subtemperate waters of Cape Horn or the Cape of Good Hope, suggesting that natural contemporary dispersal is unlikely. Natural dispersal could have occurred historically via the Panama Seaway prior to the closure of the Isthmus of Panama 3.1 million years ago (Coates and Obando, 1996). However, given that regional genetic divergence is commonplace in populations of marine species isolated by the closure of the isthmus (Bermingham and Lessios, 1993; Knowlton et al., 1993; Bermingham et al., 1997; Marko, 2002) it is extremely unlikely that Pacific and Atlantic populations would remain genetically homogeneous following 3.1 millions of years of isolation.

Given that the distribution of Clades B and D cannot be easily explained by natural processes, the alternative is human aided dispersal, such as inadvertent anthropogenic transport in ships' ballast water (Hallegraeff and Bolch, 1992). Although it is not known whether *G. toxicus* is capable of encystment and survival in ballast, both cyst and non-cyst forming species of dinoflagellates have been isolated and cultured from ballast tank sediments (Hallegraeff and Bolch, 1992). The transport of *G. toxicus* strains from one region to another could serve as a mechanism by which an ecosystem is ''seeded'' with highly toxic strains and would help explain the emergence of ciguatera in an area previously regarded as non-toxic. These human-mediated dispersal events would also contribute to seemingly random nature of outbreaks and represent a latent public health issue in affected areas.

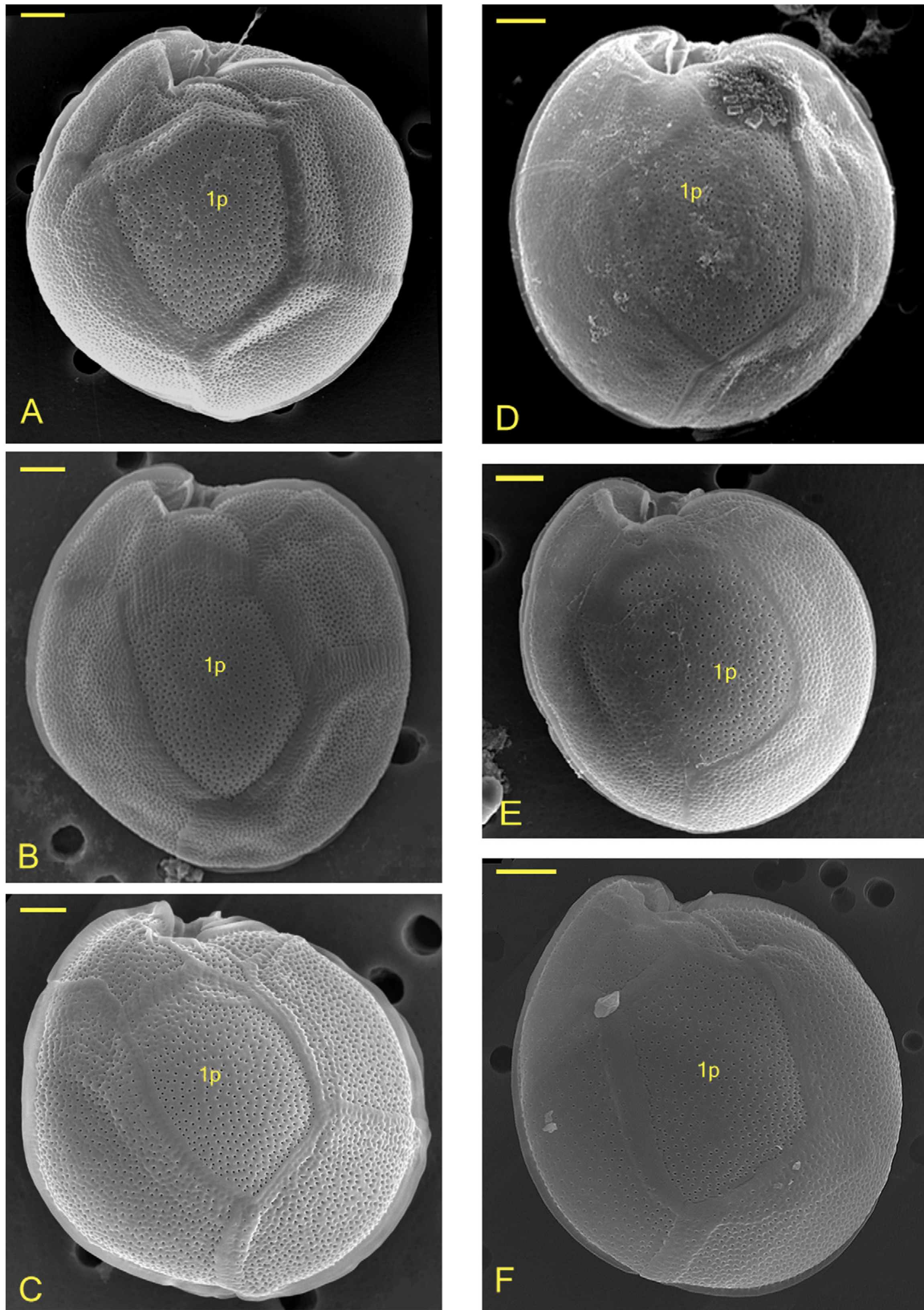

Fig. 6. SEM micrographs of isolates featuring broad posterior intercalary plate (1p) morphology, characteristic of *G. toxicus*. Hypothecal view; scale bars: 10 μm; subset of isolates shown: (A) FIT113; (B) F106; (C) 177; (D) TT302B; (E) CM515; (F) BIG2.

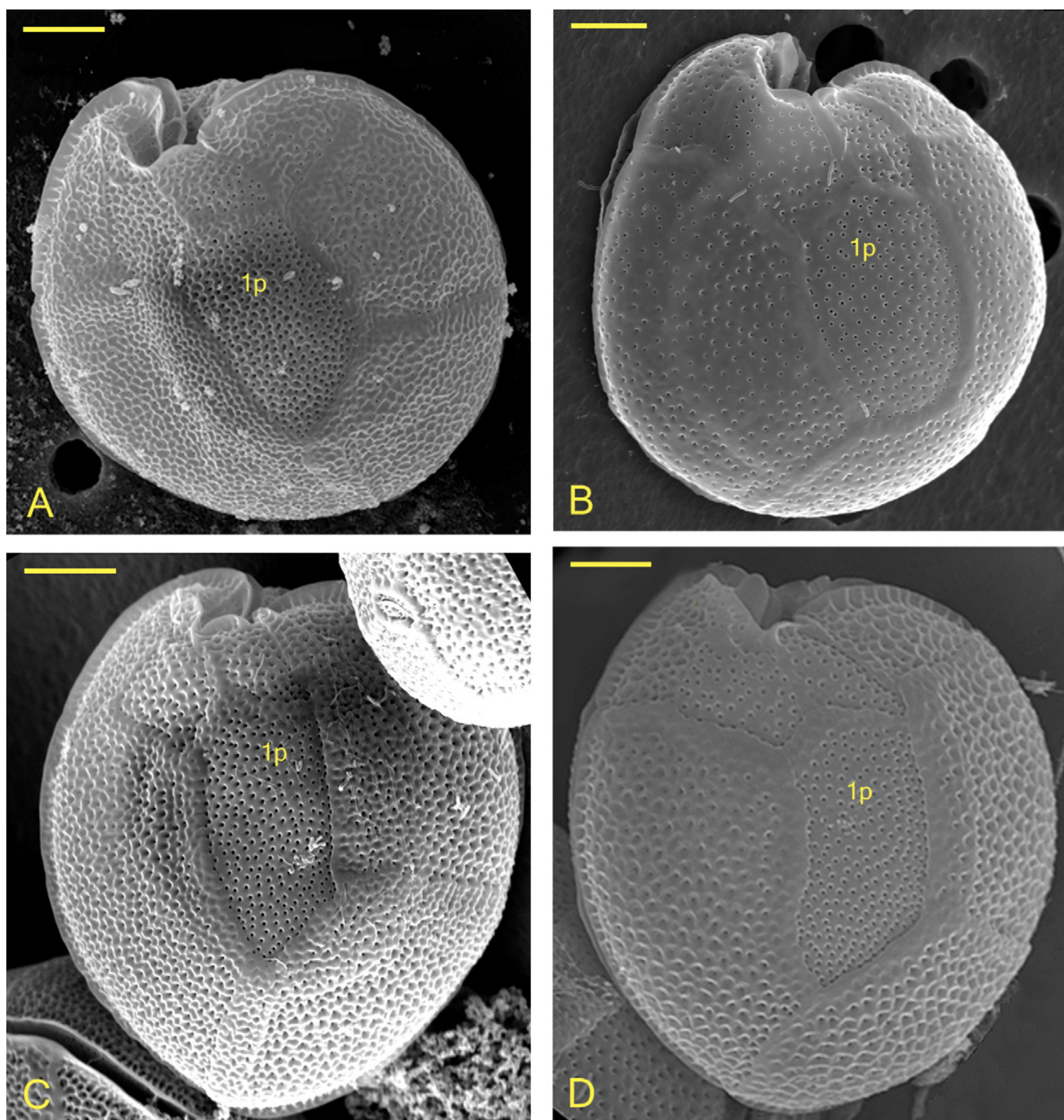

Fig. 7. SEM micrographs of isolates featuring short, narrow posterior intercalary plate (1p) morphology, characteristic of *G. belizeanus*. Hypothecal view; scale bars: 10 μm; subset of isolates shown: (A) A213; (B) CCMP 1655; (C) CCMP 1650; (D) CCMP 401.

4.1. Phylogenetic analyses

The distance values separating these lineages are in excess of what has been documented among species of dinoflagellates (Costas et al., 1995; Grzebyk et al., 1998; Edvardsen et al., 2003). Particularly notable were the distance values among the SSU rDNA sequences, which ranged from <1 to 25%. This is contrary to other studies of dinoflagellates in which these sequences are nearly identical among congeneric species (McNally et al., 1994; Costas et al., 1995). For example, in studies of symbiotic dinoflagellates isolated from coral tissue samples, sequences with only 1.6% sequence divergence were considered to potentially originate from different species (Darius et al., 2000). Thus, *G. toxicus* may have an unusually high rate of molecular evolution, or cryptic species may occur within the four divergent clades as well.

Although there was substantial concordance with respect to clade recovery, the branching order of the ancestral lineages differed between the LSU and SSU rDNA phylogenies. In the SSU rDNA phylogeny, Clade C was basal and Clades A and B form a sister group to Clade D. In contrast, the basal node in the LSU rDNA phylogeny is a trichotomy with Clades B and C forming a sister group. However, the branching order is supported by low bootstrap values, indicating uncertainty in both the branching order and placement of Clades A and C. More data from other markers may be required to resolve the evolutionary relationships among these groups.

The composition of the four clades recovered in our analyses was nearly identical between the LSU and SSU rDNA phylogenies with one exception: the Hawaiian isolate BIG8 branched with Clade C in the SSU rDNA phylogeny and Clade D in the LSU rDNA phylogeny. There are two potential explanations for this pattern. First, either the LSU or SSU sequence for this isolate may be a pseudogene. Pseudogenes have been reported in both the LSU and SSU rDNA in dinoflagellates (Scholin et al., 1993; Yeung et al., 1996; Rehnstam-Holm et al., 2002; Santos et al., 2003); furthermore, our previous analyses documented the potential for pseudogenes in *G. toxicus* LSU rDNA (Richlen and Barber, 2005). However, the SSU rDNA and LSU rDNA phylogenies were completely concordant for the remaining strains, indicating that the results are robust and not impacted by pseudogenes with the potential exception of the BIG8 Hawaii strain. A less likely alternative explanation is that BIG8 is not a monoclonal culture, but is composed of more than one isolate. Regardless of the cause, we cannot be confident in the placement of the Hawaii isolate given the discrepancy between analyses. However, the placement of the BIG8 Hawaii strain does not impact the overall results of the study.

4.2. Morphological analyses

Although taxonomic classifications of dinoflagellates are primarily based on external morphological features such as the thecal plate architecture, the relationship between the phenotypic characteristics such as morphology and physiology, and the genotype is poorly understood. In most instances the morphological features closely correspond with the molecular phylogeny; however, sometimes there is a marked lack of concordance (Scholin, 1998; Wilcox, 1998).

In our analyses, certain morphological characters used to distinguish *Gambierdiscus* species were homogeneous among distantly related isolates. For example, with the exception of Clade B (below), 1p plate architecture and fine thecal surface morphology remained conserved among distantly related groups. In contrast, other features were heterogeneous among closely related isolates. For example, all but three of the isolates featured ellipsoid Po plate architecture diagnostic of *G. toxicus*. However, NJ920D (Clade D) and CM515 (Clade A) featured a broadly ellipsoid Po plate and FP100 (Clade C) featured a triangular Po plate, while the remaining isolates in each of these clades featured the typical ellipsoid Po plates. Hence, our analyses showed no phylogenetic concordance with respect to this particular morphological feature.

In only one case did a morphologically unique group correspond to a monophyletic clade (Clade B) in the molecular analysis. Clade B isolates from the Caribbean Sea and the Pacific Ocean were distinguished by a short, narrow 1p and rugose thecal surface, a morphology that agrees with the species description of *G. belizeanus*, the only *Gambierdiscus* species with this particular thecal surface morphology (Faust, 1995). The morphology of Clade B also contrasts with published descriptions of *G. toxicus* and the other isolates used in our analyses, all of which exhibit a broad 1p plate and smooth thecal surface. Based upon our morphological and rDNA analyses, results suggest that *G. toxicus* isolates from Clade B are likely *G. belizeanus*. However, neither Genbank sequences nor live cultures of this species are available for comparison.

4.3. Taxonomic implications

Although substantial genetic distances separate the four main clades recovered in the SSU and LSU rDNA analyses of *G. toxicus* isolates, Clades A, C and D are morphologically homogenous, exhibiting the features diagnostic of *G. toxicus*. Despite the absence of phenotypic information that demonstrates true functional diversity among Clades A, C and D, the depth of divergence among these clades is comparable to that of Clade B, which is clearly morphologically unique. Thus, we suggest that *G. toxicus* is not a single cosmopolitan species with a worldwide circumtropical distribution but instead comprises a wide-ranging species complex. Additional studies examining the characteristics of these isolates within a phylogenetic framework may uncover physiological or ecological differences among these groups that support their classification as distinct species.

The limitations of and problems with using morphological characters to define species often presents challenges in dinoflagellate systematics, prompting the discussion of phytoplankton species concepts and how molecular, physiological and morphological criteria should be used to delineate species boundaries (Taylor, 1985, 1993; Manhart and McCourt, 1992; Wood and Leatham, 1992; Anderson et al., 1994; Gallagher, 1998; Scholin, 1998). Although morphospecies designations frequently provide an accurate representation of evolutionary relationships, in certain phytoplankton a disconnect has been observed between morphospecies classifications, physiological characteristics and genetic relatedness, calling into question the utility of using certain characters to diagnose and identify new species. The degree and nature of phenotypic disconnect documented thus far is highly variable among taxa. For example, both morphospecies and toxin production agree with phylogenetic classifications in *Pseudo-nitzchia* diatoms (Scholin et al., 1994a). In contrast, previous morphospecies classifications were not supported by phylogenetic analyses in both the *Alexandrium tamarense* complex, yet toxin production is strictly concordant with phylogenetic lineages (Scholin et al., 1994b; Scholin, 1998). These examples illustrate the problems associated with evaluating morphospecies classifications that are not necessarily reflective of ecological and/or physiological differences and emphasize the need for quantitative studies that test the utility of species boundaries (Anderson et al., 1994). McNally et al. (1994) suggested that the morphological characters used in the determination of systematic affiliations among some dinoflagellate groups need to be re-evaluated, particularly in the context of which structures may be appropriately regarded as primitive and which are derived.

Although the association between phylogenetic relationships and phenotypic characteristics is sometimes tenuous, evaluating phenotypic characters within a phylogenetic frame-

work is an effective method for distinguishing plastic from stable characteristics. Combining morphological data with phylogenetic analyses is a useful approach that is increasingly being used to assess taxonomic classifications based on morphospecies and to evaluate evolutionary relationships among taxa. This method has an added advantage in that it also provides a framework for distinguishing between ancestral and derived characters used in morphological classifications and allows testing of whether a morphological feature used in taxonomic classifications is truly diagnostic. Our analyses showed that an important morphological feature used to distinguish *Gambierdiscus* morphospecies, Po plate shape, was variable among isolates of *G. toxicus* and did not reflect phylogenetic groupings; hence its utility as a diagnostic feature is questionable. However, the size of the 1p plate appears to be a stable and useful morphological feature for taxonomic purposes. This is not surprising, as hypothecal characters have been shown to be more conservative than epithecal ones (Taylor, 1987). We found that epithecal features are limited in their taxonomic utility for discriminating *Gambierdiscus* species and their use should be restricted or at least carefully evaluated if they are to be used for taxonomic purposes. Furthermore, the plastic nature of the Po plate warrants further quantitative review of the *Gambierdiscus* genus utilizing a geographically broad dataset to determine if the species described using certain epithecal features should be synonymized.

Given the substantial physiological differences that have been observed among *G. toxicus* strains (Bomber et al., 1989; Sperr and Doucette, 1996; Chinain et al., 1997), the identification of multiple cryptic species raises the possibility that variation in characteristics such as growth rates and toxin production could have a phylogenetic basis. If so, differential distribution of these phylogroups across tropical marine ecosystems could help explain the seemingly random distribution of ciguatera outbreaks. Conversely, a lack of concordance would indicate that the causes underlying variability in these characteristics are more complex, suggesting that physiological difference could be strain specific or environmentally driven. This research provides a framework within which physiological characters may be examined to determine if these features involve a phylogenetic component or are derived, which may foster the development of new hypotheses regarding the molecular and ecological determinants of toxicity. Such work will be greatly facilitated by further detailed physiological and morphological analyses that help distinguish among the cryptic lineages of *G. toxicus*.

Acknowledgements

We are grateful to Dr. Donald Tindall and Dr. Michael Parsons for providing several of the *Gambierdiscus* cultures used in this research. We wish to thank Dr. Donald Anderson of the Woods Hole Oceanographic Institution for their support. We would also like to thank Dave Kulis and Linda McCauley of the Woods Hole Oceanographic Institution and Elizabeth Jones of Boston University for laboratory advice and assistance. Funding for this research was provided by the Palmer-McLeod Fellowship and also by research grants from the Phycological Society of America and the American Museum of Natural History's Lerner-Gray Fund for Marine Research. This project stems from work begun in BI 536, the Molecular Ecology and Evolution course at Boston University. Additional funding was provided by the DoD Legacy Resource Management Program (DACA87-01-H-00013) and the Army Research Office (DAAD19-02-1-0218).[SES]

References

Adachi, R., Fukuyo, Y, 1979. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera endemic area. Bulletin of the Japanese Society of Scientific Fisheries 45, 67–71.

Anderson, D.M., Hoagland, P., Kaoru, Y., White, A.W., 2000. Estimated Annual Economic Impacts from Harmful Algal Blooms (HABs) in the United States. Woods Hole Oceanographic Institution WHOI-2000-11, Woods Hole, MA.

Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallagher, J.C., Balech, E., 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Marine Biology 120, 467–478.

Anderson, D.M., Lobel, P.S., 1987. The continuing enigma of ciguatera. Biological Bulletin 172, 89–107.

Babinchak, J.A., Moeller, P.D.R., Van Dolah, F.M., Eyo, P.B., Ramsdell, J.S., 1994. Production of ciguatoxins in cultured *Gambierdiscus toxicus*. Memoirs of the Queensland Museum 34, 447–453.

Babinchak, J.A., Doucette, G.J., Ball, R.M., 1996. Partial characterization of the LSU rRNA gene from the ciguatoxic dinoflagellate *Gambierdiscus toxicus*. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. UNESCO, Sendai, Japan, pp. 459–462.

Bagnis, R., Kuberski, T., Laugier, S., 1979. Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. American Journal of Tropical Medicine and Hygiene 28, 1067–1073.

Bermingham, E., Lessios, H.A., 1993. Rate variation of protein and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panama. Proceedings of the National Academy of Sciences of the United States of America 90, 2734–2738.

Bermingham, E., McCafferty, S.S., Martin, A.P., 1997. Fish biogeography and molecular clocks perspectives from the Panamanian Isthmus. In: Kocher, T.D., Stepien, C.A. (Eds.), Molecular Systematics of Fishes. Academic Press, San Diego, pp. 113–128.

Bomber, J.W., Morton, S.L., Babinchak, J.A., Norris, D.R., Morton, J.G., 1988. Epiphytic dinoflagellates of drift algae-another toxigenic community in the ciguatera food chain. Bulletin of Marine Science 43, 204–214.

Bomber, J.W., Tindall, D.R., Miller, D.M., 1989. Genetic variability in toxin potencies among seventeen clones of *Gambierdiscus toxicus* (Dinophyceae). Journal of Phycology 25, 617–625.

Chinain, M., Germain, M., Sako, Y., Pauillac, S., Legrand, A., 1997. Intraspecific variation in the dinoflagellate *Gambierdiscus toxicus* (Dinophyceae). I. Isozyme analysis. Journal of Phycology 33, 36–43.

Chinain, M., Germain, M., Sako, Y., Pauillac, S., Legrand, A., 1998. Genetic diversity in French Polynesian strains of the ciguatera-causing dinoflagellate *Gambierdiscus toxicus*: RFLP and sequence analyses on the SSU and LSU rRNA genes. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. United Nations Educational, Scientific, and Cultural Organization, Paris, France, pp. 287–290.

Chinain, M., Faust, M.A., Pauillac, S., 1999. Morphology and molecular analysis of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. Journal of Phycology 35, 1282–1296.

Chinain, M., Revel, T., Holmes, M.J., Ung, A., Pauillac, S., 2001. Molecular characterization and classification of the ciguatera dinoflagellate Gambierdiscus. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J., Lewis, R.J. (Eds.), Harmful Algal Blooms 2000. Intergovernmental Oceanographic Commission of UNESCO, Paris, France.

Coates, A.G., Obando, J.A., 1996. The geological evolution of the Central American isthmus. In: Jackson, J.B.C., Budd, A.F., Coates, A.G. (Eds.), Evolution and Environment in Tropical America. University of Chicago Press, Chicago, pp. 21–56.

Costas, E., Zardoya, R., Bautista, J., Garrido, A., Rojo, C., Lopez-Rodas, V., 1995. Morphospecies vs. genospecies in toxic marine dinoflagellates: an analysis of *Gymnodinium catenatum/Gyrodinium impudicum* and *Alexandrium minutum/A. lusitanicum* using antibodies, lectins and gene sequences. Journal of Phycology 31, 801–807.

Darius, H.T., Martin, P.M.V., Grimont, P.A.D., Dauga, C., 2000. Small subunit rDNA sequence analysis of symbiotic dinoflagellates from seven scleractinian corals in a Tahitian lagoon. Journal of Phycology 36, 951–959.

Durand-Clement, M., 1986. A study of toxin production by *Gambierdiscus toxicus* in culture. Toxicon 24, 1153–1157.

Durand-Clement, M., 1987. Study of production and toxicity of cultured *Gambierdiscus toxicus*. Biological Bulletin 172, 108–121.

Edvardsen, B., Shalchian-Tabrizi, K., Jakobsen, K.S., Medlin, L.K., Dahl, E., Brubak, S., Paasche, E., 2003. Genetic variability and molecular phylogeny of *Dinophysis* species (Dinophyceae) from Norwegian waters inferred from single cell analyses or rDNA. Journal of Phycology 39, 395–408.

Faust, M.A., 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. Journal of Phycology 31, 996–1003.

Fleming, L.E., Baden, D.G., Bean, J.A., Weisman, R., Blythe, D.G., 1998. Seafood toxin diseases: issues in epidemiology and community outreach. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 245–248.

Freudenthal, A.R., 1990. Public health aspects of ciguatera poisoning contracted on tropical vacations by North American tourists. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier Science Publishing Inc., New York, pp. 463–468.

Fukuyo, Y., 1981. Taxonomical study on benthic dinoflagellates collected in coral reefs. Bulletin of the Japanese Society of Scientific Fisheries 47, 967–978.

Gallagher, J.C., 1998. Genetic variation in harmful algal bloom species: an evolutionary ecology approach. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin.

Grzebyk, D., Sako, Y., Berland, B., 1998. Phylogenetic analysis of nine species of Prorocentrum (Dinophyceae) inferred from 18S ribosomal sequences, morphological comparisons, and description of *Prorocentrum panamensis*, sp. nov. Journal of Phycology 34, 1055–1068.

Hallegraeff, G.M., Bolch, C.J., 1992. Transport of diatom and dinoflagellate resting spores in ships' ballast water: implications for plankton biogeography and aquaculture. Journal of Plankton Research 14, 1067–1084.

Holmes, M.J., 1998. *Gambierdiscus yasumotoi* sp. nov. (Dinophyceae), a toxic benthic dinoflagellate from southeastern Asia. Journal of Phycology 34, 661–668.

Holmes, M.J., Lewis, R.J., Gillespie, N.C., 1990. Toxicity of Australian and French Polynesian strains of *Gambierdiscus toxicus* (Dinophyceae) grown in culture: characterization of a new type of maitotoxin. Toxicon 28, 1159–1172.

Holmes, M.J., Lewis, R.J., Poli, M.A., Gillespie, N.C., 1991. Strain dependent production of ciguatoxin precursors (gambiertoxins) by *Gambierdiscus toxicus* (Dinophyceae) in culture. Toxicon 29, 761–765.

Holmes, M.J., Lewis, R.J., Sellin, M., Street, R., 1994. The origin of ciguatera in Platypus Bay. Memoirs of the Queensland Museum 34, 505–512.

Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton. Journal of Phycology 23.

Knowlton, N., Weigt, L.A., Solorzano, L.A., Mills, D.K., Bermingham, E., 1993. Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. Science 260.

Lehane, L., Lewis, R., 2000. Ciguatera: recent advances but the risk remains. International Journal of Food Microbiology 61, 91–125.

Lewis, R.J., Chaloupka, M.Y., Gillespie, N.C., Holmes, M.J., 1988. An analysis of the human response to ciguatera in Australia. In: Choat, J.H., Barnes, D., Borowitzka, M.A., Coll, J.C., Davies, P.J., Flood, P., Hatcher, B.G., Hopley, D., Hutchings, P.A., Kinsey, D., Orme, G.R., Pichon, M., Sale, P.F., Sammarco, P., Wallace, C.C., Wilkinson, C., Wolanski, E., Bellwood, O. (Eds.), Proceedings of the 6th International Coral Reef Symposium, Townsville, Australia. 6th International Coral Reef Symposium Executive Committee, pp. 67–72.

Lin, S., Zhang, H., Hou, Y., Miranda, L., Bhattacharya, D., 2006. Development of a dinoflagellate-oriented PCR primer set leads to detection of picoplanktonic dinoflagellates from Long Island Sound. Applied and Environmental Microbiology 72, 5626–5630.

Manhart, J.R., McCourt, R.M., 1992. Molecular data and species concepts in the algae. Journal of Phycology 28, 730–737.

Marko, P.B., 2002. Fossil calibration of molecular clocks and the divergence times of geminate species pairs separated by the Isthmus of Panama. Molecular Biology and Evolution 19, 2005–2021.

Masó, M., Garcés, E., Pagès, F., Camp, J., 2003. Drifting plastic debris as a potential vector for dispersing harmful algal bloom (HAB) species. Scientia Marina 67, 107–111.

McNally, K.L., Govind, N.S., Thome, P.E., Trench, R.K., 1994. Small-subunit ribosomal DNA sequence analyses and a reconstruction of the inferred phylogeny among symbiotic dinoflagellates (Pyrrophyta). Journal of Phycology 30, 316–329.

Morton, S.L., Bomber, J.W., Tindall, D.R., Aikman, K.E., 1993. Response of *Gambierdiscus toxicus* to light: cell physiology and toxicity in. In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, pp. 541–546.

Palafox, N.A., Buenconsejo-Lum, L.E., 2001. Ciguatera fish poisoning: review of clinical manifestations. Journal of Toxicology-Toxin Reviews 20, 141–160.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Provasoli, L., 1968. Media and prospects for the cultivation of marine algae. In: Watanabe, A., Hattori, A. (Eds.), Cultures and Collections of Algae (Proc Jpn Conf Hakone). Japanese Society of Plant Physiology, Tokyo, pp. 63–75.

Ragelis, E.P., 1984. Ciguatera seafood poisoning: overview. In: Ragelis, E.P. (Ed.), Seafood Toxins. American Chemical Society, Washington, D.C., pp. 25–36.

Rehnstam-Holm, A.-S., Godhe, A., Anderson, D.M., 2002. Molecular studies of *Dinophysis* (Dinophyceae) species from Sweden and North America. Phycologia 41, 348–357.

Richlen, M.L., Barber, P.H., 2005. A technique for the rapid extraction of microalgal DNA from single live and preserved cells. Molecular Ecology Notes 5, 688–691.

Santos, S.R., Kinzie III, R.A., Sakai, K., Coffroth, M.A., 2003. Molecular characterization of nuclear small subunit (18S)-rDNA pseudogenes in a symbiotic dinoflagellate (Symbiodinium, Dinophyta). The Journal of Eukaryotic Microbiology 50, 417–421.

Scholin, C.A., 1998. Morphological, genetic, and biogeographic relationships of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella*, and *A. fundyense*. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin.

Scholin, C.A., Anderson, D.M., Sogin, M.L., 1993. Two distinct small-subunit ribosomal RNA genes in the North American toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae). Journal of Phycology 30, 744–754.

Scholin, C.A., Villac, M.C., Buck, J.M., Powers, D.A., Fryxell, G.A., Chavez, F.P., 1994a. Ribosomal DNA sequences discriminate among toxic and non-toxic *Pseudonitzschia* species. Natural Toxins 2, 152–165.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994b. Identification of group and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. Journal of Phycology 30, 999–1011.

Sperr, A.E., Doucette, G.J., 1996. Variation in growth rate and ciguatera toxin production among geographically distinct isolates of *Gambierdiscus toxicus*. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. United Nations Educational, Scientific and Cultural Organization, Paris, France, pp. 309–312.

Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer, Sunderland, MA.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10, 512–526.

Taylor, F.J.R., 1985. The taxonomy and relationships of red tide flagellates. In: Anderson, D.M., White, A.W., Baden, D.G. (Eds.), Toxic Dinoflagellates. Elsevier, New York, pp. 11–26.

Taylor, F.J.R., 1987. Dinoflagellate morphology. In: Taylor, F.J.R. (Ed.), The Biology of Dinoflagellates. Blackwell/University of California Press.

Taylor, F.J.R., 1993. The species problem and its impact on harmful phytoplankton species. In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier Science Publishers, New York.

Taylor, F.J.R., Fukuyo, Y., Larsen, J., Hallegraeff, G.M., 2003. Taxonomy of harmful dinoflagellates. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. United Nations Educational, Scientific and Cultural Organization, Paris, France.

Todd, E.C.D., 1995. Estimated costs of paralytic shellfish, diarrhetic shellfish and ciguatera poisoning in Canada. In: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, Paris.

Walsh, S.P., Metzger, D.A., Higuchi, R., 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10, 506–513.

Wilcox, T.R., 1998. Large-subunit ribosomal RNA systematics of symbiotic dinoflagellates: morphology does not recapitulate phylogeny. Molecular Phylogenetics and Evolution 10, 436–448.

Wood, A.M., Leatham, T., 1992. The species concept in phytoplankton ecology. Journal of Phycology 28, 723–729.

Yasumoto, T., Nakajima, I., Bagnis, R.A., Adachi, R., 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bulletin of the Japanese Society of Scientific Fisheries 46, 1397–1404.

Yeung, P.K.K., Dong, K.F., Wong, F.T.W., Wong, J.T.Y., 1996. Sequence data for two large-subunit rRNA genes from an Asian strain of *Alexandium catenella*. Applied and Environmental Microbiology 62, 4199–4201.