

## Ecological and genetic characterization of western Gulf of Mexico *Gymnodinium breve*

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### Abstract

This report summarizes the results of the genetic characterization of three Texas isolates of *Gymnodinium breve*. Molecular characterization of clonal cultures of the toxic dinoflagellate *G. breve* from the Gulf of Mexico was conducted using sequences for the internal transcribed spacers (ITS-1 and ITS-2), 5.8S and 18S rDNA regions. Genomic DNA was extracted from three Texas coastal isolates of *G. breve* (SP-1, SP-2 and SP-3) and sequences for the 18S, 5.8S and internal transcribed spacers (ITS-1 and ITS-2) rDNA regions were obtained. The alignments of these sequences were compared to the corresponding regions for *G. breve* strains isolated from the Florida coast (the "Wilson" strain CCMP718, partial sequence of the small subunit of the ribosomal gene [GenBank, NCBI] and ITS sequence provided by W. Litaker, pers. commun.; and Piney Island strain (K. Steidinger) results provided by T. Tengs). The results of this analysis revealed that all sequences were identical. Consequently, although physiological differences may exist among clones (e.g., salinity tolerances, toxin production), ribosomal DNA sequences of both the ITS and 18S rDNA regions are useful regions to target for species-specific probes. At the intra-specific level, however, an alternative marker will be needed to assess the diversity among *G. breve* populations in the Gulf of Mexico.

### Introduction

The red tide dinoflagellate *Gymnodinium breve* Davis, first described from a massive bloom off Florida in 1935 (Davis 1948), is an important harmful algal bloom species in the Gulf of Mexico. While historical data suggests red tide blooms have occurred sporadically for over a century in Florida (Hutton 1956), blooms since the 1940's have been nearly annual. The Texas coast has historically been well buffered from the red tide problems of Florida, although red tides suspected to be *G. breve* have been reported for at least 50 years (Buskey et al. 1996). Recently, *G. breve* red tides have occurred for the first time along the Mississippi/Louisiana/Alabama coast (Dortch et al. 1998). The economic consequences of these blooms are enormous, ranging from closure of shellfish beds, fish mortalities and loss of tourism revenues (Jensen 1975, Steidinger and Vargo 1988). In addition, brevetoxins are responsible both for respiratory distress when aerosolized toxins are inhaled and neurotoxic shellfish poisoning (NSP) when ingested (see (Hemmert 1975, Steidinger and Vargo 1988)).

*Gymnodinium breve* blooms along the Texas coast have been reported infrequently until recently (Table 1). However, the past 10 years have seen 50% of the recorded or suspected *G. breve* events. Increased monitoring effort is at least a partly responsible; however, the recent two events have led to widespread public concern. In addition to the large, widely publicized event, numerous smaller shellfish bed closures due to brevetoxin have occurred since 1986 (Texas Dept. of Health cited in (Buskey et al. 1996). Low level populations were observed in residential canals

during the summer of 1998 (Villareal, unpubl. data) also suggest that *G. breve* may occur more frequently than previously suspected in coastal embayments.

Several key aspects of Texas *G. breve* red tides remain unknown: 1. Is the Texas *G. breve* genetically distinct from the Florida red tide?, and 2. Does it have the same environmental response as the Florida clones? These are important issues for understanding the dynamics of these blooms, both offshore and inshore. For example, recent work in the northern Gulf of Mexico has indicated that *G. breve* populations there can tolerate much lower salinities than previously reported for the Florida populations (Dortch et al. 1998). Our data from the Texas coast is not definitive. The original isolation of *G. breve* is a Texas clone, and it is not tolerant of salinities less than 22-24 PSU. However, this clone is anomalous in that it is homothallic whereas other known clones are heterothallic (Steidinger, per. comm.). This fundamental distinction in its sexual cycle suggests a re-evaluation of the Texas populations is required.

**Table 1. Historical documentation of *G. breve* red tides along the Texas coast. "?" indicates a probably *G. breve* bloom based on human respiratory distress, fish kills or both.**

Year	Location	Comments
1935?	Pt. Aransas-120 miles into Mexico	fish kill
1955?	Galveston, Texas	est. $2 \times 10^6$ lbs. fish killed
1974?	150 mi. off Brownsville, TX	fish kill in Mexico
1986	Galveston to Mexico	>22 million fish killed
1987	Corpus Christi Bay, TX	embayment bloom
1990-91	Brownsville ship channel	winter bloom
1996	coastal Texas	fish kill
1997	coastal Texas	fish kill

sources: Trebatoski 1988; Buskey et al. 1996

The goal of this project was to isolate *G. breve* from local waters and examine both the genetic and ecological characteristics of these clones. We addressed the first of these objectives:

1. Determine if the *Gymnodinium breve* isolate(s) from the western Gulf of Mexico is (are) genetically similar to the Florida isolates.

We hypothesized that western Gulf of Mexico *G. breve* populations are genetically distinct from the Florida populations. This report summarizes the results of the genetic characterization of three Texas isolates of *G. breve*.

## Materials and Methods

### *G. breve* cultures

Five clonal cultures of *Gymnodinium breve* from the Gulf of Mexico were examined in this study. Three strains were isolated during a bloom event off the coast of Brownsville, Texas in October 1999. These three clones of *G. breve* are referred to in the text as SP1, SP2, and SP3. Representative Florida strains included were the "Wilson" clone isolated from John's Pass, Florida (also available as CCMP718, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory, W. Boothbay Harbor, Maine) and the "Piney Island" B4

clone isolated by Bill Richardson from northwest Florida (Florida Marine Research Institute). Cultures were maintained in L1 medium (Guillard and Hargraves 1993) at 25°C and 70  $\mu\text{Eins m}^{-2} \text{s}^{-1}$ . Exponentially growing cultures were harvested by centrifugation (4000 rpm for 5 min).

#### *DNA extraction and amplification of the 18S and ITS regions of the rRNA gene*

Total genomic DNA was extracted using hexadecyltrimethylammonium bromide (CTAB) buffer (Doyle & Doyle 1990). PCR amplifications (Saiki et al. 1988) were performed in a 50  $\mu\text{l}$  mixture: 2 U Taq DNA polymerase, 0.1 ng total genomic DNA, 5  $\mu\text{l}$  of 10x reaction buffer, 10 mM dNTPs, 2 mM  $\text{MgCl}_2$ , 0.25  $\mu\text{M}$  of each primer. A combination of primers described by Medlin et al. (1988) and Gunderson et al. (1986) were used to amplify the 18S region. PCR conditions for 18S amplification were as follows: 30 cycles of 1 min at 94°C, 2 min at 52°C and 2 min at 72°C with a final elongation step of 6 min at 72°C. The primer combination of ITS1 and ITS4 (White et al. 1990) was used to amplify the entire ITS region using the following thermocycling profile: 30 cycles of 2 min at 94°C, 2 min at 52°C, and 4 min at 72°C with a final elongation step of 9 min at 72°C. Quality and length of PCR products were assessed by staining with ethidium bromide and visualization by UV illumination following electrophoresis through a 0.8% agarose gel. PCR products were purified (DNA Purification Kit, Bio-Rad, Hercules, CA).

#### *Sequencing*

PCR products were used directly as templates in dideoxynucleotide chain-termination sequencing reactions (BigDye sequencing kit, Applied Biosystems Inc., Foster City, CA). Sequences were obtained using an ABI 377 automated sequencer (Applied Biosystems Inc., Foster City, CA). Our results for the 18S regions in *Gymnodinium breve* from Texas and Florida were deposited in GenBank (AF352818, AF352819, AF352820, AF352821, AF352822). The sequences for the ITS regions of *G. breve* were assigned accession numbers AF352823, AF352824, AF352825, AF352826, and AF352827. Small subunit rRNA sequences used for comparison were obtained from GenBank: *Dinophysis norvegica* (AF352826), *Gymnodinium beii* (U37406), *G. fuscum* (AF022194), *G. mikimotoi* (AF022195), *G. sanguineum* (AF276818), *Gyrodinium* sp. (AB001438), *Prorocentrum mexicanum* (Y16232), and *P. micans* (M14649). Our ITS sequences from *Gymnodinium breve* were compared with ITS sequences of several dinoflagellates: *Alexandrium margalefi* (AJ251208), *Ceratium furca* (AJ276700), *Gymnodinium sanguineum* (AF131075), *Gyrodinium impudicum* (AF131074), and *Symbiodinium* sp. (AF180120).

Alignment of DNA fragments was accomplished using the GeneTool (Bio Tools Inc.) software package. Phylogenetic analyses of aligned sequences were performed using MEGA (Kumar et al. 1993). Distances were inferred from the sequences using the Kimura two-parameter model. Evolutionary trees were constructed using the neighbor joining algorithm (Saitou & Nei 1987). Bootstrap analyses (2000 replications) were used to determine the robustness of the tree topologies (Felsenstein et al. 1985).

#### **Results**

A *G. breve* 18S rDNA sequence previously was reported for the Wilson clone (AF172714) by Tengs et al. (2000) and re-sequencing of this strain revealed some minor

sequencing errors. Using the correct sequence for the Wilson clone (AF352822 and AF352827), analysis and alignment of ITS and 18S regions of *G. breve* from Texas and Florida revealed a striking conservation of the sequences: all five isolates were identical. The size of ITS1, ITS2 and 5.8S were 227, 204 and 159 bp, respectively, and the 18S region was 1700 bp in all three isolates from Texas. The phylogenetic trees inferred from the 18S and ITS sequences both revealed an unresolved topology of *G. breve* strains since their sequences were identical (Figure 1 and 2). The tree topology inferred from the 18S sequences indicated that *Gymnodinium breve* was closely related to *G. mikimotoi*.

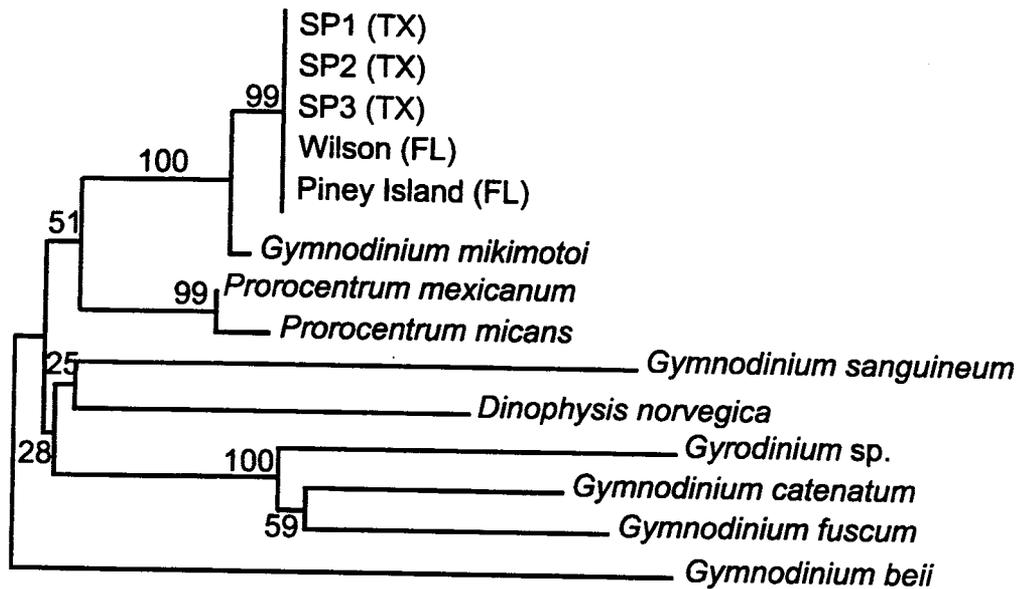
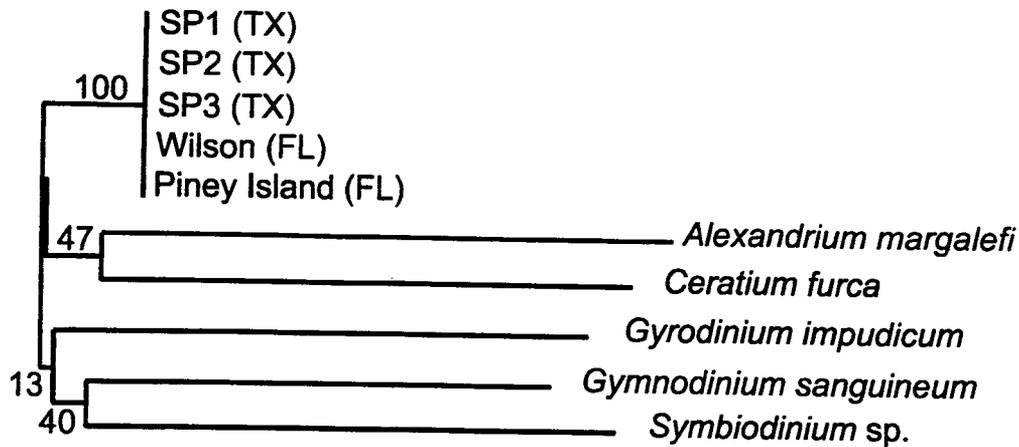


Figure 1. Phylogenetic tree inferred from the 18S region sequence in *Gymnodinium breve* from Texas (SP1, SP2 and SP3) and Florida (Wilson and Piney Island clones) and other dinoflagellates using the neighbor-joining method.



**Figure 2. Phylogenetic tree inferred from the ITS region sequence in *Gymnodinium breve* from Texas (SP1, SP2 and SP3) and Florida (Wilson and Piney Island clones) and other dinoflagellates using the neighbor-joining method.**

## Discussion

Our finding that the 18S region is identical in all strains of *G. breve* examined from Texas and Florida is not surprising since this region of the rDNA is known to be highly conserved (White et al. 1990). The ITS region, however, is thought to evolve faster than the 18S ribosomal RNA gene (Goff et al. 1994, Zechman et al. 1994) and so has been used to examine systematic relationships among closely related species or to identify genetically distinct populations within a species (Coleman et al. 1994). Recently, the ITS region was proposed as a tool to identify strains at the intraspecific level (Coleman and Mai 1997; van Hannen et al. 2000). Only a few studies have shown a lack of ITS divergence among isolates of the same species (e.g., Connell 2000). However, the amount of ITS sequence variation among populations can vary significantly (Litaker et al. 2000). In the case of *G. breve* from Texas and Florida, ITS and 18S regions should be considered as a valuable species-specific marker since they are identical. A number of additional *G. breve* isolates from each of seven Florida locations (Apalachicola, Charlotte Harbor, Florida Keys, Jacksonville, Mexico Beach, Piney Island, and Sarasota) will be sequenced by the Florida Marine Research Institute to confirm our results for the 18S and ITS regions. At the intra-specific level, however, an alternative marker will be needed to assess the diversity among *G. breve* populations in the Gulf of Mexico. Future studies dealing with the molecular identification of *Gymnodinium breve* isolates should focus on DNA regions that have a higher evolutionary rate than the 18S, 5.8S, or ITS regions.

## References:

- Buskey, E. J., Stewart, S., Peterson, J. & Collumb, C. 1996. Current status and historical trends of brown tide and red tide phytoplankton blooms in the Corpus Christi Bay National Estuary program study area. *Corpus Christi Bay National Estuary Program, CCBNEP-07* 85 pp.
- Coleman, A. W., & Mai, J. C. 1997. Ribosomal DNA ITS-1 and ITS-2 sequence comparisons as a tool for predicting genetic relatedness. *J. Mol. Evol.* 45:168-177.
- Coleman, A. W., Suarez, A., & Goff, L. J. 1994. Molecular delineation of species and syngens in volvocacean green algae (chlorophyta). *J. Phycol.* 30:80-90.
- Connell, L.B. 2000. Nuclear ITS region of the alga *Heterosigma akashiwo* (Chromophyta: Raphidophyceae) is identical in isolates from Atlantic and Pacific basins. *Mar. Biol.* 136:953-960
- Davis, C. C. 1948. *Gymnodinium brevis* sp nov., a cause of discolored water and animal mortality in the Gulf of Mexico. *Botanical Gazette* 109:358-360.
- Dortch, Q., Moncreiff, C. A., Menderhall, W. M., Parsons, M., Franks, J. S. & Hemphill, K. W. 1998. Spread of *Gymnodinium breve* into the northern Gulf of Mexico. In Reguera, B., Blanco, J. & Fernández, M. L. [Ed.] *Harmful Algae*. Xunta de Galicia and intergovernmental Oceanographi Commission of UNESCO, Santiago de Compostela, Spain, pp. 143-144.
- Doyle J.J., Doyle J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791
- Goff, L.J., Moon, D.A., & Coleman, A.W. 1994. Molecular delineation of species and species relationships in the red algal agarophytes *Gracilariopsis* and *Gracilaria* (Gracilariales). *J. Phycol.* 30:521-537
- Guillard R.R.L. and P.E Hargraves. 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia* 32: 234-236.
- Gunderson, J.H., McCutchan, T.F. & Sogin, M.L. 1986. Sequence of the small subunit ribosomal RNA gene expressed in the bloodstream stages of *Plasmodium berghei*: evolutionary implications. *Protozoology* 33(4):525-529
- Hemmert, W. H. 1975. The public health implications of *Gymnodinium breve* red tides, a review of the literature and recent events. In LoCicero, V. R. [Ed.] *Proc. First. Internat. Conf. on Toxic Dinoflag.* MIT Science and Technology Foundation, Wakefield, MA, pp. 489-497.
- Hutton, R. F. 1956. An annotated bibliography of red tides occurring in the marine waters of Florida. *Quarterly Journal of the Florida Academy of Science* 19:123-146.
- Jensen, A. C. 1975. The economic halo of a red tide. In LoCicero, V. R. [Ed.] *Proc. First. Internat. Conf. on Toxic Dinoflag.* MIT Science and Technology Foundation, Wakefield, MA, pp. 507-516.
- Kumar, S., Tamura, K. & Nei, M. 1993. MEGA: molecular evolutionary genetics analysis, version 1.02, *Inst. Mol. Evol. Gen.*, Pennsylvania State, University Park, PA.
- Litaker, W., Reece, K.S., Stokes, N.A., Steidinger K. & Tester, P. 2000. Polymorphisms in the ITS regions of dinoflagellates: implications for phylogeny and probe development. *Symposium on Harmful Marine Algae in the U.S.*, Dec 4-9, 2000, Woods Hole, p143.
- Medlin L., Elwood H. J., Stickel S. and Sogin M. L. (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491-499
- Saiki R., Gelfand D. H., Stoffel S., Scharf S. J., Higuchi R., Horn G. T., Mullis K. B. and Erlich H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
- Saitou, N. & Nei, M. 1987. The neighbor-joining method. A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406--25

- Steidinger, K. A. & Vargo, G. A. 1988. Marine dinoflagellate blooms: dynamics and impacts. In Lembi, C. A. & Waaland, J. R. [Ed.] *Algae and Human Affairs*. Cambridge University Press, Cambridge, pp. 373-401.
- Tengs, T., Dahlberg, O.J., Shalchian-Tabrizi, K., Klaveness, D., Rudi, K., Delwiche, C.F. & Jakobsen, K.S. 2000. Phylogenetic analyses indicate that the 19'hexanoyloxy-fucoanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Mol. Biol. Evol.* 17(5):718-29
- Trebatoski, B. 1988. Observations on the 1986-87 Texas red tide (*Ptychodiscus brevis*). *Texas Water Commission Rep.* 88-02
- Van Hannen, E.J., Lurling, M. and Van Donk, E. 2000. Sequence analysis of the ITS-2 region: a tool to identify strains of *Scenedesmus* (Chlorophyceae). *J. Phycol.* 36: 605-607
- White T.J., T. Bruns, S. Lee, J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ & White TJ eds). pp. 315-322. Academic Press, San Diego.
- Zechman, F.W., Zimmer, E.A. & Theriot, E.C. 1994. Use of ribosomal DNA internal transcribed spacers for phylogenetic studies in diatoms. *J. Phycol.* 30:507-12