The toxic dinoflagellate *Karenia brevis* historically has been a major harmful algal bloom species in the Gulf of Mexico (Steidinger and Penta, 1999). *Karenia brevis* is distributed widely in offshore waters of the Gulf, where it normally occurs at very low abundance (<100 cell l–1) (Geesey and Tester, 1993). At bloom concentration, the potent neurotoxin produced by these cells can result in fish kills, shellfish toxicity, and can cause respiratory distress in humans from inhaled sea spray. *K. brevis* blooms have been a consistent feature of the west Florida shelf for the past 20 years (Tester and Steidinger, 1997). Their frequency has increased along the Texas coast with as many reported blooms in the 1990s as in the previous four decades (Villareal et al., 2001). The origin of *K. brevis* blooms, however, remains poorly understood.

Physiological diversity may exist among isolates of *K. brevis*. Florida clones have a reported minimum and maximum salinity tolerance of 22.5 and 46 psu, respectively (Aldrich and Wilson, 1960). In recent experiments, salinity tolerance varied from 24 to 45 psu for Texas clone SP3 (Magaña, 2001). Yet, *K. brevis* was found at salinities as low as 5 psu in coastal waters off Mississippi and Louisiana, suggesting that some *K. brevis* populations can survive at much lower salinities than those previously reported (Dortch et al., 1998).

Toxin production by *K. brevis* also appears to vary...
along isolates. Baden and Tomas (1988) showed that for cultures grown under identical conditions, the toxin content varied 3-5-fold among different clones of K. brevis from Texas and Florida. It is generally assumed that toxin production is a biological process intrinsic to some toxin-producing dinoflagellates such as A. catenella (Anderson, 1990) and its genetic basis encoded by the algal genome (Ishida et al., 1993). Thus, the physiological variability observed in the field and experimental data suggested that isolates of K. brevis may be genetically distinct.

We report here the results of a comparison of several strains of K. brevis isolated from Texas and Florida based on ecophysiological parameters and genetic characteristics. We chose to target the small subunit ribosomal RNA (18S rDNA), 5.8S and internal transcribed spacer regions (ITS1 and ITS2) to discriminate these isolates. The region coding for 18S rDNA is conserved and can be used to measure both distant and close evolutionary relationships (Gunderson et al., 1986). In contrast, the nucleotide sequences of ITS1 and ITS2 are less conserved than those of the 18S region and so potentially are of great utility for identification and discrimination of inter- and intraspecific variations (van Oppen et al., 1995; Famà et al., 2000).

Five clonal cultures of K. brevis from the Gulf of Mexico were examined in this study. Three strains (SP1, SP2, and SP3) were isolated during a bloom event off the coast of Brownsville, Texas in October 1999 (S. Pargee, University of Texas Marine Science Institute, Port Aransas). Representative Florida strains included were the ‘Wilson’ clone isolated from John's Pass, Florida (also available as CCMP718, Provasoli-Guillard National Center forCulture of Marine Phytoplankton, Bigelow Laboratory, W. Boothbay Harbor, Maine) isolated by Karen Richardson from northwest Florida (Florida Marine Research Institution). Triplicate cultures used for growth experiments were maintained in 150 ml L1 medium (Guillard and Hargraves, 1993) at 25°C and 70 µEins m–2 s–1. Cells were sampled at the time of inoculation and subsequently at 3-day intervals. Growth rate was calculated using the slope of the line relating time and cell counts (Guillard, 1973). Once cultures reached late-logarithmic growth phase, an aliquot was harvested by centrifugation (4000 rpm for 5 min) and total genomic DNA was extracted using CTAB buffer (Boyle and Doyle, 1987). PCR amplifications were performed in a 50 µl mixture: 2 U Taq DNA polymerase, 0.1 mg/ml total genomic DNA, 5 µl of 10x reaction buffer, 10 mM dNTPs, 2 mM MgCl2, 0.25 µM of each primer. Primer combination of ITS1 (TCC GTA GGT GAA CCT GGG G) and ITS4 (TGC TCG CTA CTT TGA TAT CAC) designed by White et al. (1990) were used to amplify the entire ITS region.

Four overlapping fragments were combined to obtain the entire 18S rDNA sequence. The first fragment was obtained using primer A (AAC CTG GTG CAT CCT GCC AGT), described by Medlin et al. (1988); as a forward primer and primer 1200R (GGG CCA TCA GGC ACC) designed by Gunderson et al. (1986) as a reverse primer. The second fragment was amplified by using the combination of primer 360F (AGG GTT GTA TTC CGG AG) designed by Gunderson et al. (1986) and 1200R. The third fragment was obtained using the primer 360F and primer B (TGA TCC TTT GAC AGG TTC ACC TAC) designed by Medlin et al. (1986). The fourth product was amplified by using primer A and primer 1500R (GGG CAT CAC AGA CCT G) designed by Gunderson et al. (1986) as a reverse primer. PCR thermal cycling conditions were as described by Zechman et al. (1994). Quality and length of PCR products were assessed by staining with ethidium bromide and visualizing by UV illumination following electrophoresis through a 0.8% agarose gel. PCR products were purified (DNA Purification Kit, Bio-Rad, Hercules, CA) and then 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 3177 automated sequencer (Applied Biosystems Inc., Foster City, CA). Alignment of DNA fragments was accomplished using the GeneTools (Bio Tools Inc.) software package. Our sequences of the 18S rDNA regions in K. brevis from Texas and Florida were deposited in GenBank (AF352818, AF352819, AF352820, AF352821, AF352822). The accession number of the sequences of the ITS regions of K. brevis were AF352823, AF352824, AF352825, AF352826 and AF352827. The remaining cells were harvested using a 0.45 µm polycarbonate filter and stored at –80°C until extracted. Cells were extracted from the filters using 100% methanol and sonicated for 10 min to ensure complete lysis of the cells. The toxins in the solutions were further purified by chromatography using an Agilent (Milford, MA) Model 1100 liquid chromatography system equipped with a Vydac C18 column. The delivered gradient was 50–95% of 0.1% TFA in methanol/0.1% aqueous TFA (over 35 min) and the flow rate was 0.2 ml min–1. The eluent from the C18 chromatography was directed to the atmospheric pressure chemical ionization (APCI) source of a SCIEX (Thornhill, Ontario, Canada) API-III triple quadrupole mass spectrometer. The APCI source was operated in positive ion mode utilizing compressed nitrogen for the nebulization gas and nitrogen for the countercurrent curtain gas. The collision gas used for MS/MS analysis was a 90/10 mixture of argon/nitrogen from National Welders Supply (Charlotte, NC). Detection of PSTX-2 was based on the appearance of fragment ions at 860 and

Dubois et al., 2002
878 m/z, whereas detection of PbTX-3 was based on the appearance of fragment ions at 770 and 807 m/z. Potent fractions were quantified against standard brevetoxins PbTX-2 and PbTX-3 purchased from Calbiochem (San Diego, CA). The results are expressed as averages of 3 or 4 measurements, and are reduced to per cell averages based on cell counts obtained with a 0.1 µl Palmers-Maloney counting chamber.

The maximum growth rate was variable among the three *K. brevis* clones from Texas (Table I). Growth rates ranged almost 2-fold and the difference between SP1 and both SP2 and SP3 was significant (*t* test, *P* < 0.05). Variations in the total toxin content and ratios of the two major toxins were also evident in all strains (Table I). Total toxin content (PbTX-2 + PbTX-3) ranged almost 3-fold from 17.11 to 62.7 pg cell⁻¹ in SP1 and SP3, respectively. Toxin content of the *K. brevis* strain isolated from Florida was lower than that of the three clones from Texas (Table I). A *K. brevis* 18S rDNA sequence was reported previously for the Wilson clone (AF172714) by Tengs et al. (2000) and re-sequencing of this strain revealed some minor errors (T. Tengs, unpublished data). Using the corrected sequence for the Wilson clone (AF352822 and AF352827), analysis and alignment of the 18S rDNA and ITS regions of *K. brevis* from Texas and Florida revealed that all five isolates were identical.

Until recently, it was generally assumed that clonally reproducing populations, such as *K. brevis*, would display relatively little physiological or genetic variation (Hughes, 1989). Our findings of high variability in the maximum growth rate and the toxin content among three clones of *K. brevis* isolated from the same bloom in Texas is inconsistent with this assumption. Intra-strain variability in toxin production has been observed within natural populations of many toxic microalgal species, including cyanobacteria (Skulberg et al., 1993), prymnesiophytes (Edvardsen and Paasche, 1998) and dinoflagellates (Baden and Thomas, 1988; Anderson, 1991; Bravo et al., 2001). Additionally, significantly different growth rates have been established for several isolates of the diatom *Ditylum brightwellii* originating from a single location in the Hood Canal (Rynearson and Armbrust, 2000). In our case, however, the physiological variation observed in the population of *K. brevis* from the Gulf of Mexico did not reflect comparable levels of genetic diversity. Based on 18S rDNA and ITS sequences, all five isolates of *K. brevis* from Texas and Florida were identical. The 18S region is a coding region that is highly conserved. Combined with 28S rDNA sequence data (Daugbjerg et al., 2000; Hansen et al., 2000), it has been used to redefine the phylogeny of the major genera of dinoflagellates (Saunders et al., 1997). A close relationship between *Gymnodinium* and *Gyrodinium* emerged from these studies (Hansen et al., 2000). It also resulted in the splitting of the genus *Gymnodinium* into four genera. The two fish-killing species *Gymnodinium breve* and *G. mikimotoi* have been grouped in the genus *Karenia* and

<table>
<thead>
<tr>
<th>Clone or field sample location</th>
<th>Isolation or sample date</th>
<th>Toxin content (pg cell⁻¹ ± SE)</th>
<th>Maximum growth rate (div day⁻¹ ± SE)</th>
<th>Salinity tolerance (psu)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1 Texas Oct. 1999</td>
<td>13.23 ± 0.02</td>
<td>3.88 ± 0.03</td>
<td>0.94 ± 0.4</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>SP2 Texas Oct. 1999</td>
<td>41.30 ± 0.01</td>
<td>17.50 ± 0.02</td>
<td>0.58 ± 0.06</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>SP3 Texas Oct. 1999</td>
<td>38.20 ± 0.02</td>
<td>24.50 ± 0.03</td>
<td>0.73 ± 0.04</td>
<td>24 45 (1, 2)</td>
<td></td>
</tr>
<tr>
<td>Wilson Florida 1953</td>
<td>11.20 ± 0.01</td>
<td>3.50 ± 0.02</td>
<td>0.2–1.0</td>
<td>22.5 46 (1, 3, 4)</td>
<td></td>
</tr>
<tr>
<td>78P5 Florida 1978</td>
<td>8.76 ± 0.03</td>
<td>0.00</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT5D2 Florida</td>
<td>8.62 ± 0.41</td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT5D3 Florida</td>
<td>12.60 ± 2.31</td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC5 Texas</td>
<td>12.51 ± 0.43</td>
<td>1.56</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC6 Texas</td>
<td>4.93 ± 1.62</td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W53D8 Florida</td>
<td>8.70 ± 0.42</td>
<td></td>
<td>5 (6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) This study; (2) Magaña, 2001; (3) Aldrich and Wilson, 1992; (4) Shanley and Vargo, 1993; (5) Baden and Thomas, 1988; (6) Dortch et al., 1998.
have been renamed as *K. brevis* and *K. mikimotoi*, respectively. *Karenia brevis* and *K. mikimotoi* appeared to be closely related and formed a strongly supported sister group with the other bloom-forming genus, *Karlodinium*. In our case, the lack of variability in the 18S rDNA region yields no opportunity for identification of isolate-specific probes. This is the reason why the ITS region, a faster evolving marker, has been targeted. Most studies have reported intra-specific and/or intra-individual polymorphism in this region. As we observed in *K. brevis*, variation in toxin production and ecophysiological response were higher than ITS variation in this species. Future studies dealing with the genetic variability of natural populations of *K. brevis* should focus on DNA regions that evolve faster than the ribosomal ITS region.

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