JOURNAL OF PLANKTON RESEARCH | VOLUME 24 | NUMBER 7 | PAGES 735–739 | 2002

SHORT COMMUNICATION

No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico

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Maximum growth rate and toxin content were significantly different among five strains of Karenia brevis isolated from Texas and Florida when grown under identical culture conditions. Sequence analysis of the 18S rRNA gene and internal transcribed spacer (ITS) regions revealed, however, that all five strains were identical. Consequently, a clear genetic basis for physiological variability among various geographical isolates of K. brevis from the Gulf of Mexico could not be assessed using these genetic markers. Both the ITS and 18S rRNA regions may be useful in species-specific probe selection. At the intra-specific level, however, an alternative marker will be needed to assess the diversity among K. brevis populations in the Gulf of Mexico.

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

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among isolates. Baden and Tomas (1988) showed that for cultures grown under identical conditions, the toxin content varied ~3-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 *Alexandrium* (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 (ITS-1 and ITS-2) 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 ITS-1 and ITS-2 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 for Culture of Marine Phytoplankton, Bigelow Laboratory, W. Boothbay Harbor, Maine) isolated by Karen Steidinger and the 'Piney Island' B4 clone isolated by Bill 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 subsampled at subsequent 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 (Doyle and Doyle, 1987). PCR amplifications were performed in a 50 μl mixture: 2 U Taq DNA polymerase, 0.1 ng total genomic DNA, 5 μl of 10× reaction buffer, 10 mM dNTPs, 2 mM $MgCl_2$, 0.25 μM of each primer. Primer combination of ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) 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 GTT GAT CCT GCC AGT) described by Medlin *et al.* (1988) as a forward primer and primer 1200R (CGG CCA TGC ACC 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 CGA 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 TTC TGC AGG TTC ACC TAC) designed by Medlin *et al.* (1988). 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 visualization 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 377 automated sequencer (Applied Biosystems Inc., Foster City, CA). Alignment of DNA fragments was accomplished using the GeneTool (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 lyses 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 2.2×150 mm 201TP 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 PbTX-2 was based on the appearance of fragment ions at 860 and

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 Palmer-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 Tomas, 1988; Anderson, 1991; Bravo *et al.*, 2001). Additionally, significantly different growth rates have been established for several isolates of the diatom *Ditylum brightwelii* 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 I: Toxin content (pg cell^{-1}), maximum growth rate (div day^{-1}) and salinity tolerance [upper and lower limits, expressed using the Practical Salinity Unit (psu)] of K. brevis strains isolated from Texas and Florida

Clone	Clone or field sample location	Isolation or sample date	Toxin content (pg cell^{-1} ± SE)		Maximum growth rate (div day^{-1} ± SE)	Salinity tolerance (psu)		References
			PbTx-2	PbTx-3		lower limit	upper limit	
SP1	Texas	Oct. 1999	13.23 ± 0.02	3.88 ± 0.03	0.94 ± 0.4			(1)
SP2	Texas	Oct. 1999	41.30 ± 0.01	17.50 ± 0.02	0.58 ± 0.06			(1)
SP3	Texas	Oct. 1999	38.20 ± 0.02	24.50 ± 0.03	0.73 ± 0.04	24	45	(1, 2)
'Wilson'	Florida	1953	11.20 ± 0.01	3.50 ± 0.02	0.2–1.0	22.5	46	(1, 3, 4)
78P5	Florida	1978	8.76	0.00				(5)
CT5D2	Florida		8.82	0.41				(5)
CT5D3	Florida		12.60	2.31				(5)
CC5	Texas		12.51	0.43				(5)
CC6	Texas		4.93	1.62				(5)
W53DB	Florida		8.70	0.42				(5)
Field sample	Louisiana, Mississippi	Oct.–Dec. 1996				5		(6)

(1) This study; (2) Magaña, 2001; (3) Aldrich and Wilson, 1960; (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 micrum*. 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 the ITS region (e.g. Bakker *et al.*, 1992; Coleman *et al.*, 1994; Pillman *et al.*, 1997; Famà *et al.*, 2000). However, the amount of ITS sequence variation among phytoplankton populations can vary significantly. High inter-individual polymorphism has been observed in the ITS region of the bloom-forming Raphidophyte *Fibrocapsa japonica* (Kooistra *et al.*, 2001). On the contrary, another recent study showed that the ITS sequence was identical in *Heterosigma akashiwo* populations collected throughout the Atlantic and Pacific coastal areas (Connell, 2000). Interestingly, *H. akashiwo* showed a wide range in toxicity between these two geographic regions. 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.

ACKNOWLEDGEMENTS

We thank J. R. Gold and H. Magaña for their helpful comments and L. Richardson for her technical assistance. This work was supported by a contract from the Texas Parks and Wildlife department to L.C and T.A.V. and an Interdisciplinary Research Faculty Development Award from Texas A&M University to L.C. and J.R.G.

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Received on October 25, 2001; accepted on March 26, 2002