Short Communication

MARINE BIOTECHNOLOGY

PCR Amplification of Microsatellites from Single Cells of *Karenia brevis* Preserved in Lugol's Iodine Solution

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Abstract

A simple and effective protocol is described for multiplex polymerase chain reaction (PCR) amplification of single cells of *Karenia brevis*. The protocol requires minimum processing, avoids additions that might dilute target DNA template, and can be used on cells preserved in Lugol's iodine preservative. Destaining of Lugol's-preserved cells with sodium thiosulfate allowed successful amplification of single-copy, nuclear-encoded microsatellites in single cells of *K. brevis* that have been preserved for up to 6 years.

Keywords: dinoflagellate — HAB — *Karenia brevis* — microsatellite — single-cell PCR

Introduction

The major harmful algal bloom (HAB) species in the Gulf of Mexico is *Karenia brevis*, an unarmored dinoflagellate responsible for both fish kills and respiratory problems in humans (Steidinger et al. 1998). Factors influencing initiation, development, and dissipation of blooms of *K. brevis*, however, are not well understood. A more detailed understanding of genetic diversity within and among blooms is needed so that the dynamics and demography of this dinoflagellate can be studied in relation to environmental parameters.

Hypervariable, nuclear-encoded genetic markers such as microsatellites are powerful tools for assessment of population structure and have been developed for several dinoflagellate species (Nagai et al. 2006, 2007), including *K. brevis* (Renshaw et al. 2006). In these and other studies (Rynearson and Armbrust 2004) of genetic diversity among phytoplankton species, clonal cultures were required for extraction of sufficient quantities of DNA for genotyping. Unfortunately, in contrast to other phytoplankton species that have been studied, e.g., *Alexandrium tamarense* (Nagai et al. 2007), *Ditylum brightwellii* (Rynearson and Armbrust 2004), and *Emiliania huxleyi* (Iglesias-Rodriguez et al. 2006), there are few isolates of *K. brevis* available for genetic studies (http://ccmp.bigelow.org/). An advantage in working with dinoflagellate species (e.g., *A. tamarense*) is the use of resting-stage cysts to establish clonal cultures. The resting stage cyst for *K. brevis*, however, has not been identified or reproducibly produced in the laboratory. Consequently, clonal cultures of *K. brevis* must be established de novo from individual cells isolated from a bloom, a difficult, time consuming, and challenging task because of the high mortality of isolated single cells (B. Richardson, personal observation).

Here, we describe a simple procedure for polymerase chain reaction (PCR) amplification of nuclear-encoded microsatellites from Lugol's iodine (LI) preserved single cells of *K. brevis*. The procedure allows microsatellite genotypes to be acquired from a large number of individual cells within a bloom. Successful PCR amplification of microsatellites from cells preserved in LI solution, the preferred preservation method for marine flagellates, has the advantages that (1) external cell morphology is preserved for identification and (2) genotypes can be acquired from historical and time-course sam-

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ples, permitting tests of hypotheses linking genetic diversity and population structure of *K. brevis* with temporally varying physiological and ecological parameters.

Materials and Methods

Cell Preservation and Isolation. A 1.3-ml aliquot of cultured cells of *K. brevis* (SP1 isolate, Loret et al. 2002) was placed in a 1.5-ml Eppendorf tube, stained with 50 µl of LI solution (10 g of I_2, 20 g of KI, 20 ml of glacial acetic acid, 200 ml of dH_2O), and placed in the dark at 4°C for 3 h. Subsequently, 10 µl of 1 M sodium thiosulfate (Tittel et al. 2003) was added to destain cells. The tube was then gently inverted four times (LI coloration generally dissipated immediately) and the cells were ready to isolate once the solution was devoid of color. A 200-µl aliquot of destained cells was placed on a microscope slide and individual cells isolated using a method modified from Ki et al. (2005); individual cells were then transferred, using a Pasteur pipet, to a PCR tube (0.2 ml; VWR International, West Chester, PA) in a minimum volume (<2 µl) of sterile Optima water (Fisher Scientific, Fair Lawn, NJ). Individual PCR tubes were then observed under an Olympus SZX12 stereomicroscope to confirm presence of a single cell.

DNA Extraction and Amplification. PCR tubes were centrifuged (1177 *g*) for 30 s and subjected to three cycles of freeze/thawing (-80°C for 1 min and 75°C for 1 min constituted one cycle) to lyse the cells (Sebastián and O'Ryan 2001). The lysate was then subjected to two rounds of PCR amplification. The first round was a multiplex reaction that employed five PCR primer pairs in a 20-µl reaction containing 12 µl of Go*Taq* Green Master Mix (Promega, Madison, WI), 5 µl of Optima water (Fisher Scientific), and 3 pmol of each forward and reverse primer. The microsatellites amplified were *Kbr5, Kbr7, Kbr8, Kbr9,* and *Kbr10;* details, including primer sequences, of these microsatellites be found in Renshaw et al. (2006). Amplification was carried out using a Bio-Rad PTC 100 thermal cycler (Bio-Rad, Hercules, CA) as follows: initial (one cycle) denaturation at 95°C for 180 s, followed by eight cycles of denaturation at 95°C for 80 s, annealing at 52°C for 165 s, extension at 72°C for 80 s, 50 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 105 s, extension at 72°C for 60 s, and one final extension at 72°C for 30 min. Product in each tube was diluted with 20 µl of 1× Tris-EDTA (1× TE) and used as a template for five separate reactions that used each of the five PCR primer pairs. This second round of PCR employed 10-µl reactions containing 5 µl of Go*Taq* Green Master Mix, 1.4 µl of Optima water, 5 pmol of fluorescently labeled forward primer, 5 pmol of reverse primer, and 2 µl of template. The fluorescent dyes employed were Fam, Hex (Invitrogen, Carlsbad, CA), and Ned (Applied Biosystems, Foster City, CA). Amplification was carried out using a Bio-Rad PTC 100 thermal cycler as follows: initial (one cycle) denaturation at 95°C for 180 s, annealing at 52°C for 120 s, extension at 72°C for 80 s, 40 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 75 s, extension at 72°C for 60 s, and one final extension at 72°C for 30 min. PCR products were diluted with 10 µl of 1× TE and separated and visualized on a 5% polyacrylamide gel (Long Ranger Singel Pack; Cambrex Bio Science Rockland, Rockland, ME) using an ABI Prism 377 DNA sequencer (Applied Biosystems). Gels were run for 2.5 h at 3 kV, 100 W, and a laser power of 39 mW. A size standard, 400 HD Rox (Applied Biosystems), was loaded with each sample in order to estimate fragment sizes. All gels were analyzed using Genescan Analysis 3.1.2® (Applied Biosystems); allele-calling was performed with Genotyper® software, version 2.5 (Applied Biosystems) and with STRand 2.3.48 (UC Davis-Veterinary Genetics Lab, Davis, CA). Genotypes obtained were compared to genotypes compiled previously from cetyl trimethylammonium bromide (CTAB)-extracted DNA (after Doyle and Doyle 1990) from a pellet of cultured cells of the SP1 isolate of *K. brevis*. Comparison of genotypes obtained from single cells with those from pooled cells of a culture initiated from a single cell was to confirm that products obtained from single cells were identical in size to products obtained from a cell pellet of the same culture and not a product of random amplification. The first round of PCR utilized extended denaturation, annealing, and extension times in order to maximize product from each cycle. The extended denaturation time ensured that all double-stranded DNA was denatured. The extended annealing time allowed primers from all five microsatellites to anneal to their target sequence. This step appeared especially critical in insuring equal amplification of each microsatellite and minimizing the chance that a single microsatellite would monopolize available resources. The longer extension time ensured complete synthesis of the new strands. The two rounds of PCR amplification were necessary to increase the copy number of each target sequence and allow template DNA from a single cell to be used in multiple reactions. Each reaction in the second round of PCR amplified a single microsatellite and included a fluorescently labeled forward primer.

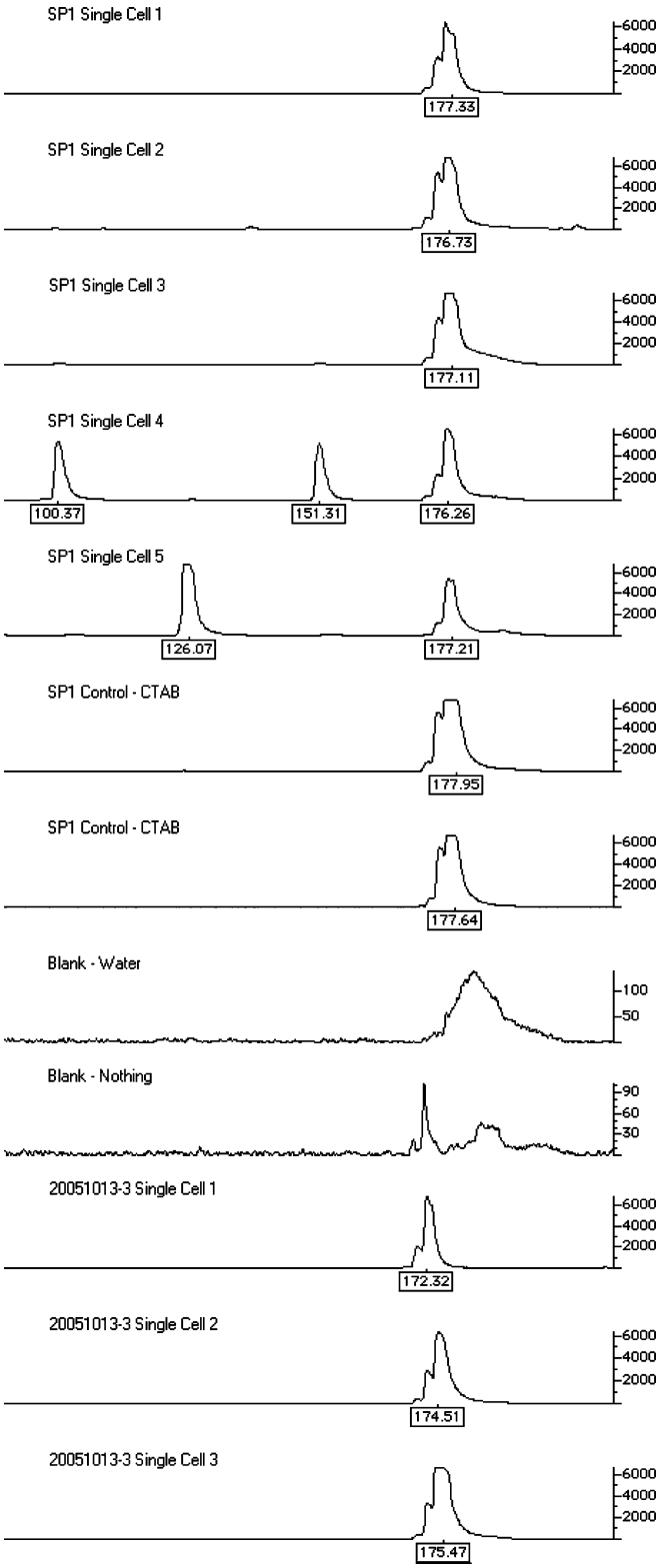

Fig. 1. PCR amplification products from microsatellite *Kbr*10. Samples 1–5 are from single-cells of *K. brevis* (SP1 isolate); samples 6 and 7 are from CTAB-extracted DNA (SP1 isolate); sample 8 is a negative control (sterile water added instead of template DNA); sample 9 is a negative control (nothing added in place of template DNA); and samples 10–12 are from single-cell samples of *K. brevis* isolated from an LI-preserved field sample (20051013-3) collected in 2005. Target band range is 169–181 bp. SP1 allele size is 177 bp. Similar results were obtained at the other four microsatellites.

Table 1. Size range of alleles detected at five microsatellites among 27 clonal cultures of *Karenia brevis*

Microsatellite	*Range (in base pairs)*
*Kbr*5	182–190
*Kbr*7	252–261
*Kbr*8	128–146
*Kbr*9	158–167
*Kbr*10	169–181

Results and Discussion

Initially, all five microsatellites were amplified successfully from single cells of *K. brevis* (SP1 isolate) fixed with LI solution. Out of ten trials, three microsatellites (*Kbr7, Kbr8,* and *Kbr9*) amplified successfully in all cases, while the two remaining microsatellites (*Kbr5* and *Kbr10*) amplified successfully in 90% of trials. Amplification also was successful with single, LI-preserved cells (n=16) sampled from a bloom occurring in Fulton Harbor near Rockport, Texas, in the fall of 2000 and that had been stored refrigerated at 4°C for 6 years. We then used the protocol to amplify the five microsatellites from single cells (n=129) isolated from a recent bloom of *K. brevis* sampled from shorelines around Corpus Christi, Texas, during the fall of 2005. *Kbr9* amplified successfully in 97% of trials; *Kbr*8 amplified successfully in 96% of trials; *Kbr5* amplified successfully in 87% of trials; *Kbr10* amplified successfully in 85% of trials; *Kbr7* amplified successfully in 71% of trials.

PCR amplifications from single cells frequently generated multiple, extraneous bands for all five microsatellites; an example is shown in Figure 1 (SP1 Single Cells 4 and 5). CTAB-extracted DNA from cell pellets of the same culture did not produce multiple bands due, presumably, to a higher initial copy number of template DNA. The target-band range for each of the five microsatellites (Table 1) was determined based on observed, single-band genotypes (phenotypes) of CTAB-extracted DNA from 27 different cultures (Table 2). The extraneous bands were observed in about 40% of the amplifications from single cells and invariably fell outside of the target band range (Figure 1). In addition, the target band was the brightest band in the target range and almost always the brightest band observed. Finally, in amplifications of single cells from the same culture, the same target band was observed, whereas the extraneous bands would be of different sizes.

Table 2. Cultures genotyped to obtain a size range of alleles at five microsatellite loci in *Karenia brevis*

Collection number	*Collection location*	*Collection date*
CCFWC250	Neptune Beach, FL	October 1999
CCFWC251	Neptune Beach, FL	October 1999
CCFWC252	Neptune Beach, FL	October 1999
CCFWC253	Duck Key, FL	February 1995
CCFWC254	New Pass, FL	October 1999
CCFWC256	Charlotte, FL	May 1996
CCFWC257	Charlotte, FL	May 1996
CCFWC258	Mexico Beach, FL	June 1998
CCFWC259	Mexico Beach, FL	June 1998
CCFWC260	Mexico Beach, FL	June 1998
CCFWC261	Apalachicola Bay, FL	June 1998
CCFWC262	Apalachicola Bay, FL	June 1998
CCFWC263	Panacea, FL	May 1996
CCFWC265	Panacea, FL	May 1996
CCFWC266	South Padre Island, TX	October 1999
CCFWC267	South Padre Island, TX	October 1999
CCFWC268	John's Pass, FL	1953
CCFWC269	Corpus Christi Bay, TX	1986
CCMP2228	Sarasota, FL	August 2001
CCMP2229	Manasota Key, FL	August 2001
CCMP2281	Navarre, FL	September 1999
CCMP718	John's Pass, FL	1953
SP1	South Padre Island, TX	October 1999
SP2	South Padre Island, TX	October 1999
TSP3	South Padre Island, TX	October 1999
NTSP3	South Padre Island, TX	October 1999
NBK	Nueces Bay, TX	February 2002

Different approaches to PCR amplification of microsatellites from single cells of *K. brevis* were also evaluated: 95% ethanol preservation and pre-extraction precipitation, ethanol precipitation after destaining of LI-fixed cells, Chelex (Bio-Rad, Hercules, CA) extraction as described by Richlen and Barber (2005), CTAB extraction (Doyle and Doyle 1990), and a freeze/thaw, buffer-incubation method as described by Kai et al. (2006). Ethanol preservation and precipitation after destaining of LI-fixed cells yielded cells that were difficult to ascertain visually, precluding species identification and confirmation of single cells inside PCR tubes. Chelex extraction required a small volume (~10 µl) of Chelex solution to be added to the PCR tube containing the single cell. Once Chelex extraction is complete, the entire supernatant (minus beads) must be transferred to another PCR tube and used as template. This significantly reduced DNA template concentration for the initial PCR amplification and resulted in inconsistent amplification. The CTAB extraction method contained several steps that involved addition/removal of solutions to the tube containing the cellular DNA, and similar to Chelex extraction significantly reduced DNA template concentration. The freeze-thaw buffer-incubation (Kai et al. 2006) also involved addition of buffer, again reducing initial DNA template concentration.

We also tried the whole-genome-amplification (WGA) method, using Genomiphi (GE Healthcare, UK) and the phi29 polymerase (Raghunathan et al. 2005). Results using WGA produced gels that were difficult to score because of apparent (and extensive) nonspecific amplification, presumably artifacts of background synthesis (Hutchison et al. 2005; Raghunathan et al. 2005). Successful amplifications were achieved using destained, LI-fixed cells and the lysis buffers (SDS-Proteinase K and TritonX-100-Proteinase K) as described in Kai et al. (2006). However, resulting gels contained numerous additional bands relative to those observed using the freeze-thaw extraction method.

The method reported here permits successful microsatellite genotyping of single cells of *K. brevis* and bypasses the need to establish cultures. The method is straightforward and relatively rapid, and it significantly reduces the amount of time needed to obtain multiple genotypes from a bloom. Work to obtain genotypes from multiple individuals from different blooms is now in progress.

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