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AFLP Fingerprinting Shows that a Single *Prymnesium parvum* Harmful Algal Bloom Consists of Multiple Clones

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Due to slow rates of molecular evolution, DNA sequences used to identify and build phylogenies of algal species involved in harmful algal blooms (HABs) are generally invariant at the intraspecific level. This means that it is unknown whether HAB events result from the growth of a single clone, a few dominant clones, or multiple clones. This is true despite the fact that several physiological and demographic traits, as well as toxicity, are known to vary across clones. We generated AFLP fingerprints from a set of 6 clonal isolates, taken from a bloom of *Prymnesium parvum* at a striped bass mariculture facility. This new haptophyte bloom was recently implicated in fish kills at several sites in the United States. The AFLP fragments were highly reproducible and showed that all isolates were distinguishable due to abundant AFLPs unique to single isolates. These results demonstrate that blooms can be genetically diverse outbreaks and indicate that AFLP can be a powerful molecular tool for characterizing and monitoring this diversity.

Key words: *AFLP, HAB, Haptophyceae,* Prymnesium

Harmful algal bloom (HAB) events are characterized by the sudden increase in phytoplankton densities in coastal waters accompanied often by their release of toxins into the water. These outbreaks can cause severe local ecosystem and economic damage by killing coastal fauna, reducing tourism linked to aquatic activities, and destroying stocks in marine and freshwater aquaculture operations. Although they are natural phenomena, current trends indicate that HABs and their impacts have increased in frequency and intensity worldwide (Hallegraeff 1993, 2003). Anthropogenic waste (e.g., industrial and agricultural runoffs) has been proposed to trigger bloom formation in some species (reviewed in Lancelot et al. 1987; Hallegraeff 2003). The toxins of many HAB-forming species can even affect the health of marine mammals (Scholin et al. 2000) and humans (Grattan et al. 1998) when these are exposed to water, aerosol, or seafood-vectored toxins.

Despite a growing number of phytoplankton species recognized to be involved in HAB outbreaks (Hallegraeff 2003), relatively little is known about the dynamics of formation and persistence of blooms. Traditional methods of detection and quantification of toxic species (e.g., microscopy and pigment analysis) are time-consuming and require expertise, and researchers have recently started to appreciate the advantages of using molecular technologies for such purposes (Scholin et al. 2003). For instance, John et al. (2005) successfully developed specific rRNA probes to distinguish toxic North American strains from nontoxic European strains from the *Alexandrium tamarense* species complex, a task not possible using morphological features. Strain-specific DNA markers can be further used to detect the geographic origin of bloom-causing strains when human-mediated transport is suspected (Bolch et al. 1999).

Most genetic studies of HAB-forming species have focused on delineating boundaries between closely related species or between geographic populations of a species (e.g., Du et al. 2002; John et al. 2005; Lilly et al. 2005; Penna et al. 2007) instead of assessing the genetic composition of a single bloom event. Different genetic strains of a species may have differential competitive abilities, and high cell densities and growth rates during a bloom likely impose strong selection. This could potentially result in very few or even one strain being responsible for the bloom. Genetic methods to characterize clonal genetic diversity of bloom events are a reasonable approach toward evaluating this "clonal epidemic" scenario.

Attempts to detect genetic variation in single bloom events using DNA sequences have been largely uninformative. For instance, strains of *Karenia brevis* that were isolated from a bloom greatly differed in growth rate and toxin content but showed identical 18S rRNA sequences (Loret et al. 2002), whereas strains of *Alexandrium catenella* isolated from blooms at different time and places in Hong Kong showed no differences in 24S rRNA sequences (Yeung et al.

2002). DNA fingerprinting techniques, on the other hand, were successful in detecting high levels of clonal diversity within single HAB species. Most of all isolates from the same bloom events had distinct genetic profiles based on random amplified polymorphic DNA (RAPD; Bolch et al. 1999) and microsatellites (Rynearson and Armbrust 2000; reviewed in Medlin 2007). AFLP have been shown to be sensitive at estimating genetic divergence among extremely closely related taxa at the inter- and intraspecific levels in both animals (Albertson et al. 1999; Parsons and Shaw 2001; Ogden and Thorpe 2002; Sullivan et al. 2004) and plants (Balasaravanan et al. 2003; Tremetsberger et al. 2003), and these markers are more recently becoming widely used to assess genetic diversity within and among strains of several phytoplankton species (John et al. 2004; Beszteri et al. 2007). For instance, nearly every strain assayed, whether within or between geographic locations, has exhibited unique AFLP fingerprints and diagnostic markers (Beszteri et al. 2007; Alpermann et al. 2009; Logares et al. 2009; Figueroa et al. 2010). The AFLP method exhibits several advantages over other methods for investigating DNA sequence polymorphism. It does not require prior knowledge of target sequences, it uses small amounts of DNA, it produces hundreds of dominant markers that are well dispersed throughout the genome, and it is highly reproducible (Vos et al. 1995; reviewed in Mueller and Wolfenbarger 1999). Hence, this technique is suitable for investigating clonal structure and for efficiently developing diagnostic molecular markers for strain identification in toxic algal blooms.

The toxic haptophyte *Prymnesium parvum* occurs worldwide, is highly tolerant to variations of salinity and temperature, and forms fish-killing blooms in both coastal and inland waters in Europe and the United States (Edvardsen and Paasche 1998; Lundholm and Moestrup 2006; Baker et al. 2007, 2009). In recent years, blooms of this species appeared in more than 20 US states where fish kills were observed. The state of Texas has persistent blooms in several major rivers and lakes where extensive mortality of fish was noted to accompany these blooms. The frequency and extent has been increasing (Southard et al. 2010) and has spread to aquaculture facilities and private ponds. Fish losses in that state alone were conservatively estimated to exceed 34 million fish valued at US$13 million (Southard et al. 2010). In other areas, *P. parvum* devastated miles of river in Pennsylvania and West Virginia where mining activities were common.

The only previous genetic assay of *P. parvum* blooms found no differences in DNA sequences among isolates but focused mostly on comparisons of this to a closely related species, *Prymnesium patelliferum* (Larsen and Medlin 1997). Here, we employ AFLPs to investigate the genetic composition of clonal isolates from a *P. parvum* bloom that occurred in an aquaculture facility in Elizabeth City, NC. During 2000–2001, loss of hybrid striped bass occurred in an aquaculture facility, Artesian Aquaculture, where all stock was lost in 7 of the largest ponds. In a matter of a 7-day period, fish in these ponds began behaving erratically, were found in the shallow areas, and eventually perished resulting in a total loss of stock estimated in excess of $250 000. Since the initial episode, the ponds have not been used successfully for finfish culture (Sawyer G, personal communication).

We show that this destructive *P. parvum* bloom was composed of a genetically diverse array of clones and suggest that the AFLP method can be used to find strain-specific DNA markers that can be employed to monitor and track individual genotypes potentially involved in HAB events.

Materials and Methods

Collection and Culturing of Isolates

Live samples taken from surface waters where fish-killing blooms occurred at the Artesian Aquaculture facility (Elizabeth City, NC) were sent to University of North Carolina at Wilmington's Center for Marine Science (CMS) in November 2000 and again in February 2001. Cultures of *P. parvum*, the causative agent of the fish kills, were made from single cells using pipette isolation techniques. Five single cells were selected from the sample, then placed into a Costar 96-well microtiter plate into wells holding 200 μl of filter-sterilized pond water. The plates were incubated in a growth chamber at a temperature of 22 °C, 60 mol. quanta m^2 s^{-1}, and a photoperiod of 12 light:12 dark. Once growth occurred, cells were transferred to a 24-well plate containing 2 ml of f/2 media (Anderson 2005) at a salinity of 4 psu. Once firmly established, cells were transferred to 60-ml culture tubes and maintained permanently in these tubes as part of the Toxic Algal Culture collection at CMS. An additional sample from a fish kill site at Kiawah Island, Hilton Head, SC was acquired in December 2001. The method described above was used to isolate a clone from this locality, and this was used as a "outgroup" to the North Carolina samples. Isolate designations and CMS accession numbers (CMSTAC, in parentheses) were as follows: CMS 2001 (310212), CMS 2003 (310213), CMS 2005 (310214), CMS 2009 (310217), CMS 2010 (310219), and Hilton Head (HH; 310221).

AFLP Analysis

Approximately 0.5 ml of each clonal culture, taken during logarithmic growth, were transferred to microcentrifuge tubes and centrifuged at 12 000 × *g* for 3 min. The supernatant fraction was discarded and pelleted cells were resuspended in lysis buffer (10 mM Tris–HCl pH 8.0, 100 mM EDTA pH 8.0, 0.5% sodium dodecyl sulfate). Extraction of genomic DNA followed a standard protocol (Maniatis et al. 2000). After extraction, DNA pellets were resuspended in 50 μl ddH_20 instead of TE (Tris–EDTA) buffer because the presence of EDTA would likely inhibit ligation of adaptors during the AFLP restriction–ligation reactions. The DNA samples were finally cleaned with QIAGEN Quick columns following the kit's PCR purification protocol because initial attempts to digest algal DNA without this step were very inconsistent.

For DNA restriction, endonucleases *Mse*I and *Eco*RI were used, and the entire AFLP procedure, including thermocycler conditions, closely followed the Applied Biosystems AFLP Large Plant Genome Mapping Protocol (P/N 4303186). All ligation and preselective amplification reactions were performed using the ABI AFLP Ligation and PreSelective Amplification Module (P/N 402004), and selective amplifications used primers from the ABI AFLP Selective Amplification kit (P/N 4303050). Ten randomly chosen selective primer pairs were used for amplifying fragments in all samples (Table 1). After selective amplifications, fragments were electrophoresed in the automated ABI 3100 Genetic Analyzer with ROX-500 size standard. Visualization and sizing of fragments were performed by ABI GeneScan 3.1 software, using the Local Southern Method of size calling and the lowest peak detection settings (amplitude threshold of 50 relative fluorescent units for all dyes and minimum peak half-width of 2 points). Fragments amplified in each sample were aligned manually, and the resulting binary data matrix represented the presence or absence of each fragment.

Although reproducibility of AFLPs is well documented (Mueller and Wolfenbarger 1999; John et al. 2004), even rare PCR artifacts could introduce differences between clones. We evaluated this by doing 2 parallel replicates from each clonal culture, each independently taken through DNA extraction and the entire AFLP procedure. Fragments amplified in 1 replicate but not in the other were considered PCR artifacts and were hence excluded from further analysis. The data matrix was converted to a NEXUS file using MacClade v.4.06 (Maddison DR and Maddison WP 2003) and imported into PAUP v.4.0b10 (Swofford 2003) for genetic distance analyses, where a neighbor joining (NJ) dendrogram was estimated using Nei and Li's (1979) distance index developed for restriction site polymorphisms.

Table 1 Primer pairs used in the selective amplification of AFLP markers and variation in fragments produced across 6 *Prymnesium parvum* isolates

Primers		Number of fragments		
*Eco*RI	*Mse*I	Total	Polymorphic	Unique
ACT	CAA	17	9	3
ACT	CTA	17	6	1
ACA	CAC	13	8	6
ACA	CTA	19	7	3
AAC	CTA	7	7	2
ACC	CAT	12	4	2
AAG	CTA	29	24	5
AGG	CTA	16	3	2
AGG	CTC	9	3	1
ACG	CTA	18	5	1
Total		157	76	26

Three base-pair extensions on the *Eco*RI and *Mse*I primer sequences are provided in the first 2 columns. Polymorphic fragments are defined by the presence of a fragment in at least 1 but not all isolates, whereas unique fragments are those present in only 1 isolate.

Results

A total of 168 fragments were scored when all 12 samples were included (6 isolates × 2 replicates each), with only 11 fragments detected as nonreproducible between replicates. When calculated for each pair of replicates, reproducibility ranged from 96.2% to 100%. Forty-eight percent ($n = 76$) of the reproducible 157 fragments were polymorphic among isolates, and 26 of these were unique (i.e., diagnostic) to 1 isolate (Table 1).

NJ analysis revealed that clonal isolates used in this study form a genetically heterogeneous assemblage (Figure 1). Nei and Li's (1979) genetic distances between clones ranged from 0.0065 to 0.0456, but most of that variation is likely explained by a large number of unique fragments found in isolate CMS-2009. Even isolate HH, collected at a different time (nearly 12 months later) and location (~650 km to the south), was more similar to the other Elizabeth City clones than was CMS-2009 (also from Elizabeth City). Genetic distance between CMS-2009 and the other CMS isolates was over twice as large (mean ± standard error [SE]: 0.0390 ± 0.00019) as that between the CMS isolates and HH (0.0178 ± 0.0026). With the exception of CMS-2001, isolate-specific AFLP fragments were found in all cases (Table 1; Figure 1).

Discussion

Before developing methods for tracking and monitoring harmful phytoplankton blooms in coastal areas, it is crucial that we understand the genetic and demographic features of these blooms with regard to the number and source of strains involved. Due to low per-locus polymorphism, molecular methods using single DNA sequences have been largely uninformative at the level below species (Adachi et al. 1996; Medlin et al. 1998; Loret et al. 2002; Yeung et al. 2002). Most attempts using multilocus fingerprinting or genotyping techniques have overcome this limitation (Bolch et al. 1999; Rynearson and Armbrust 2000, 2004; John et al. 2004; Beszteri et al. 2007; Alpermann et al. 2009). Very few studies, however, have used such methods to assess genetic variability in isolates collected during a HAB event. In this study, we used AFLP DNA fingerprinting to show that a HAB of *P. parvum* was composed of multiple genetic strains. RAPD and microsatellites are often informative, but they suffer from inconsistent reproducibility and long development time, respectively. AFLPs are superior with respect to both of those issues, as authors of other studies on algae (Donaldson et al. 1998; John et al. 2004; Müller et al. 2005; Logares et al. 2009; Figueroa et al. 2010) have also pointed out. Each of the 6 isolates assayed in our study exhibited a distinct AFLP fingerprint when a total of 157 fragments were assayed. Given our relatively low sampling effort, both in number of isolates and in AFLP primer pairs utilized, this result suggests that the extent of genetic diversity within the bloom will be even far greater than our initial attempts have estimated.

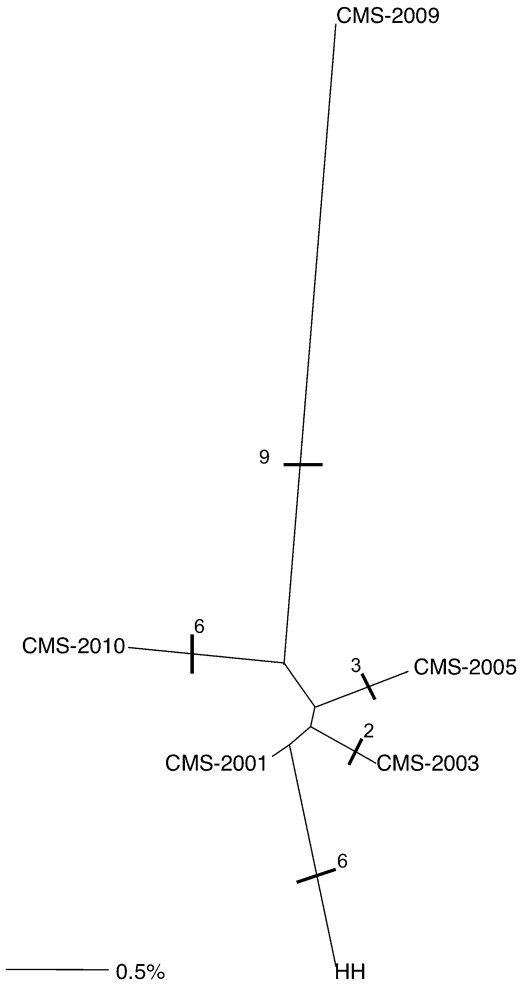

Figure 1. Unrooted NJ phylogram of *Prymnesium parvum* clonal isolates based on AFLPs. Branch lengths are Nei and Li's (1979) genetic distances estimated from 157 AFLP fragments generated using 10 selective primer combinations. Numbered hash marks on branches represent the number of diagnostic AFLP fragments unique to a clonal isolate.

That phytoplankton blooms are not dominated by single or few rapidly dividing strains is becoming increasingly appreciated, but the precise mechanisms that promote this pattern are not yet understood. High genetic diversity at this spatial scale can be generated by several nonmutually exclusive processes. Meiotic recombination is an obvious pathway in sexual organisms, but sexual reproduction has not been directly documented in the genus *Prymnesium* (Moestrup and Thomsen 2003). This explanation cannot be ruled out, however, because a haplodiploid life cycle (Larsen and Edvarsen 1998) is consistent with sexual recombination. A likely explanation in a predominantly asexual taxon, if this were a more appropriate description of the reproductive biology of this species, is the creation of new genotypes by mutation and their differentiation in frequency driven by evolutionary forces. In fact, geographic subdivision has been detected in several phytoplankton species (*Gymnodinium catenatum*, Bolch et al. 1999; *Ditylum brightwellii*, Rynearson and Armbrust 2004; *Pseudo-nitzschia multiseries*, Evans et al. 2004), including *P. parvum* (Larsen and Medlin 1997). The genetic composition of a population may be influenced by movement of water masses among geographic sources of genotypes and by the stochastic assemblage of benthic resting cysts (known to exist in *Prymnesium*; Moestrup and Thomsen 2003).

Finally, the possibility that some genetic differences between isolates arose through artificial selection, and genetic drift during prolonged culturing in laboratory settings cannot be excluded. The 6 clones used in this study were cultured independently for 16–20 months. Therefore, it is not unlikely that different mutations have occurred in each culture during this period, which would reduce genetic similarity among cultures. Unfortunately, we did not maintain parallel duplicates of each isolate. Even though the effect of long-term culturing on genetic divergence cannot be directly estimated for our *P. parvum* isolates, a previous study of AFLPs in a different species provides a useful insight. Müller et al. (2005) compared more than 400 AFLP fragments between duplicate cultures in 10 strains of *Chlorella vulgaris* that had been maintained by different laboratories, and under slightly different conditions, for several decades. They found that AFLP fingerprints were identical between duplicates of a strain in all cases, even though many differences among strains were found (Müller et al. 2005). This result, coupled with the shorter culturing time and fewer AFLP markers used in our study, suggests a minor role for differences induced by culturing.

Besides providing genome-wide estimates of genetic divergence, AFLP often detects individual- or strain-specific fingerprints that contain diagnostic markers. Although we sampled a small number of clones (and therefore have probably overestimated the uniqueness of individual fragments), we collected a total of 26 markers restricted to single clones. This suggests that diagnostic markers can be developed into strain-specific DNA probes through relatively little effort and following standard molecular genetic methods. A fragment of interest can be excised from a high-resolution AFLP gel, the DNA purified and then amplified and sequenced with the same primers used in the AFLP procedure. The sequences may then be used to design high-specificity PCR primers or to develop SNP markers (see, e.g., Bensch et al. 2002) that can quantify a target clone, even in low concentration nonbloom conditions. In a full-scale monitoring project, researchers could increase the sampling of both AFLP loci and number of isolates from a monotypic bloom until the number of new diagnostic fragments is saturated. Probes developed from these markers could then be used to monitor, track, and manage condition and transport of water samples in aquaculture ponds, for instance.

Conclusions

The frequency and impact of harmful phytoplankton blooms in many coastal areas of the world has apparently increased over the last decades, and this trend is often linked

to anthropogenic factors (Hallegraeff 2003). As a result, recent research efforts have focused on describing the genetics, physiology, and demographics of blooms, with the intention of developing predictive and preventative protocols. Here, we contributed to the small but growing body of literature that suggests that HABs can be genetically heterogeneous and not composed of a few dominant clones. There is a real possibility that clones vary in meaningful ways, such as with respect to toxicity or growth rate. Monitoring genetic diversity through the use clone-specific molecular markers seems to be a worthwhile approach, and our work shows that AFLP can be a promising method for marker development.

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