

DNA fingerprinting reveals extensive genetic diversity in a field population of the centric diatom *Ditylum brightwellii*

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Abstract

Microsatellite markers were developed to examine the genetic diversity of field populations of the centric diatom *Ditylum brightwellii*. Polymerase chain reaction amplification of two dinucleotide microsatellite loci using DNA extracted from single cell isolates was sensitive enough to identify genetically distinct clones. Each of four culture collection isolates was genetically distinct. Moreover, 23 of 24 isolates obtained from the Hood Canal Basin in Puget Sound, Washington, displayed unique genotypes. The gene diversity of the field isolates is 0.88, indicating that the *D. brightwellii* population was composed predominantly of unrelated individuals. Maximum growth rates were measured for eight genetically distinct field isolates that were maintained at three light intensities. The growth rates of the isolates differed significantly, indicating that high levels of physiological variability also existed within the population. The results of this study indicate that both extensive genetic and physiological diversity can exist within diatom populations isolated from a single geographical locale at a single time. The observed high levels of diversity are hypothesized to result from the exposure of individual diatom cells to a constantly changing environment and thus to changing selection pressures. The extensive physiological and genetic diversity documented here may help explain how diatoms are able to bloom under a wide range of environmental conditions.

Diatoms are a remarkably versatile group of organisms, with perhaps as many as 200,000 extant species (Mann and Droop 1996) that are able to bloom under a wide variety of environmental conditions (Round et al. 1990). To quote Round and Crawford (1989), as long as there is light, diatoms seem able to exist almost anywhere that “water drips, collects, or flows.” The success of diatoms appears to derive in part from the high level of physiological diversity that exists both between and within individual species. For example, nutrient uptake rates (e.g., Carpenter and Guillard 1971; Kilham 1975), photosynthetic capabilities (Gallagher and Alberte 1985), and overall growth rates (Gallagher 1982) can vary by up to 60-fold between individual diatom cells isolated from a single species.

There is an almost intuitive sense that phenotypic variation within diatom populations reflects equally high levels of genetic variation (Brand 1990; Wood and Leatham 1992). Attempts to confirm this hypothesis, however, have remained limited, largely because of technical constraints. Gallagher (1980, 1982) and Brand et al. (1981b) were the first to confirm directly that both physiological and genetic variation existed within diatom populations. The relatively insensitive protein techniques available at the time allowed Gallagher, for example, to detect only two essentially mutually exclu-

sive summer and winter genotypes (Gallagher 1980). The physiological variability observed between individuals with the same genotype indicates that greater genetic variation within the population went undetected (Gallagher 1982). Comparably low levels of genetic diversity were observed in the diatom *Asterionella formosa* by Soudek and Robinson (1983), who also relied upon an analysis of protein variation. Quantitative genetic analysis of morphological variation only revealed small genetic differences between *Thalassiosira tumida* clones (Wood et al. 1987). In recent years, a variety of more sensitive DNA-based techniques have been used to document intraspecific genetic variation in diatoms (e.g., Medlin et al. 1991; Stabile et al. 1992; Zechman et al. 1994; Lewis et al. 1997) and other phytoplankton (e.g., Medlin et al. 1996; Bolch et al. 1999a,b). These techniques have proven useful for determining the genetic differences between diatom isolates collected during different seasons or from different geographical locales. However, no technique has been sensitive enough to describe the extent of genetic diversity present within a given species of diatom found in a single water mass at a single point in time, a critical first step for understanding diatom population dynamics.

A relatively new class of molecular markers known as microsatellites has become increasingly popular for detecting genetic variation within populations of multicellular organisms (reviewed in Bruford and Wayne 1993). Microsatellites are neutral markers that are scattered throughout the genomes of all eukaryotes examined to date, including diatoms (Glenn pers. comm.), and they consist of di-, tri-, or tetranucleotides that are tandemly repeated tens to hundreds of times (Goldstein and Pollock 1997). The number of repeat units at a given microsatellite locus can vary dramatically between individuals. This variation in length is hypothesized to result from a process known as strand slippage, which occurs when the DNA-synthesizing machinery essentially “slips” during replication of the repetitive regions (Schlöt-

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terer and Tautz 1992). Thus, the length of a repeat array (allele size) can act as a part of a DNA fingerprint. As more loci are analyzed, the DNA fingerprint for an individual becomes increasingly precise.

The goal of this study was to adapt microsatellite techniques to the study of diatom population dynamics. We chose the Hood Canal Basin in Puget Sound, Washington, as our study site because it is a highly stratified, relatively stable body of water (Ebbesmeyer et al. 1988), in which the introduction of new genotypes from the physical mixing of different water masses was expected to be at a minimum. The centric diatom *Ditylum brightwellii* was chosen as the test organism because its distinctive shape and large size allow it to be readily isolated from mixed assemblages. The development and use of microsatellites in combination with physiological studies provided enough sensitivity to allow us to quantitatively document, for the first time, the extent of genetic and phenotypic diversity in a diatom population isolated from a single body of water. The high level of diversity we observed may help to explain how diatom populations are able to respond to a wide range of environmental conditions.

Methods

Isolates and culture conditions—Single cell isolates of the centric diatom *D. brightwellii* were obtained in two ways. Culture isolates were purchased from the Provasoli–Guillard Center for Culture of Marine Phytoplankton (CCMP). CCMP 357 was originally isolated from Patricia Bay, Canada (northeast Pacific); CCMP 358 was isolated from the Gulf of Mexico; CCMP 359 was isolated from George’s Bank (northwest Atlantic); and CCMP 360 was isolated from Malborough Sound (southwest Pacific). Individual cells were isolated from each CCMP isolate to ensure that cultures were composed of genetically identical cells. Field isolates were obtained from 3-m net tows (20- μ m mesh) collected on 21 November 1997 from three sites—Pt. Whitney, Hoodspport, and Twanoh—within the Hood Canal Basin in Puget Sound, Washington (Fig. 1). Within 24 h of collection, 31, 27, and 29 *D. brightwellii* cells were isolated from Pt. Whitney, Hoodspport, and Twanoh net-tow samples, respectively, and were transferred into 3-ml sterile seawater supplemented with f/20 nutrients (Guillard 1975). Four, eight, and twenty-three isolates from each site survived the initial isolation and were transferred to sterile seawater supplemented with f/10 nutrients. All subsequent transfers were into sterile seawater supplemented with f/2 nutrients. Two of the original isolates from Pt. Whitney, five from Hoodspport, and seventeen from Twanoh were unialgal and survived long enough that DNA could be extracted. All isolates were maintained in autoclaved seawater supplemented with f/2 nutrients at 14°C on a 16:8 light:dark (LD) cycle of 66 μ mol photons $m^{-2} s^{-1}$ cool-white light. Light intensities were determined with a Licor quantum sensor with a QSL-100 4-pi sensor (Biospherical Instruments).

Microsatellite isolation and sequencing—Total genomic DNA was extracted from the isolates, essentially as described by Maass and Dalhoff (1994). Approximately $5 \times$

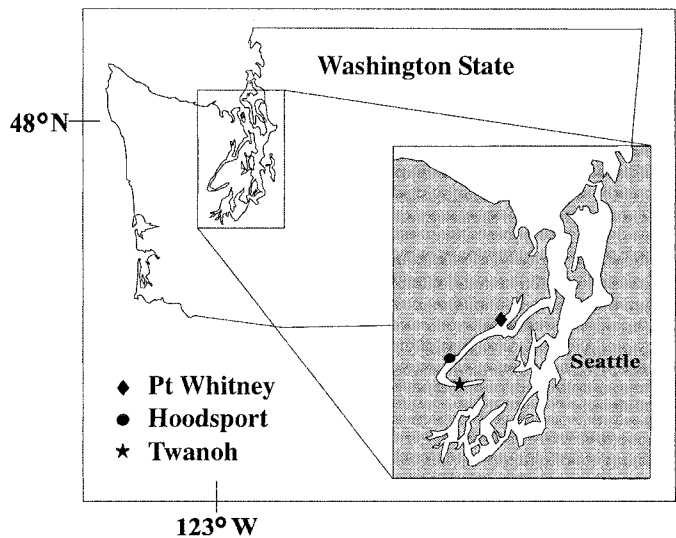


Fig. 1. Map of Washington State. Inset shows sampling sites of single cell isolates at Point Whitney, Hoodspport, and Twanoh on Hood Canal in Puget Sound.

10^6 cells were filtered onto a 0.45- μ m cellulose filter, scraped off the filter, and either processed immediately or frozen at -70°C . Fresh or frozen cells were incubated at 60°C for 1 h in 300 μ l lysis buffer (10 mM Tris–1 mM ethylenediaminetetraacetic acid [pH 7.5]; 0.5% sodium dodecyl sulfate; and 100 μ g/ml Proteinase K). Fifty microliters of 5 M NaCl and 40 μ l of 10% hexadecyltrimethylammonium bromide (CTAB) in 0.7% NaCl were then added to each tube, and the samples were incubated at 65°C for 10 min. DNA was purified using the column and wash reagents supplied with the DNeasy Plant Mini Kit (Qiagen).

Approximately 0.8 μ g genomic DNA from CCMP 358 were restriction digested to completion with *ApoI* (New England Biolabs) and electrophoresed through a 0.8% low-melting point agarose gel. Restricted DNA in the 300–600-base pair (bp) range was eluted from the agarose (Sambrook et al. 1989), ligated into the pZERO-2 vector (Invitrogen), and used to transform TOP10 *Escherichia coli* cells (Invitrogen). Colony lifts (Sambrook et al. 1989) were used to screen approximately 30,000 bacterial colonies with (GT)₁₅ and (GA)₁₅ oligonucleotides that were fluorescein labeled using the Random Prime Labeling Kit (Amersham Life Sciences). Hybridization and wash conditions were those described in Condrey and Bentzen (1998). Cross-hybridizing colonies were detected using the Signal Amplification Module (Amersham Life Sciences) and visualized with a Fluor-Imager 575 (Molecular Dynamics). False positives were eliminated by rescreening colonies with the labeled oligonucleotides. Plasmid DNA was isolated from positive clones using the Qiagen Mini Prep Kit. The cloned DNA was sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase using the T7 and SP6 primers and analyzed on an ABI 373A automated sequencer.

Microsatellite loci sequences have been deposited in GenBank under accession numbers AF263001–8.

Determination of microsatellite allele sizes—Polymerase chain reaction (PCR) primers were designed to recognize sequences that flanked the microsatellite loci. Primers 1F and 2R amplify both the Dbr4 and the Dbr9 loci. The sequence of primer 1F is TACAAAGAGACGCAATC, and the sequence of primer 2R is AATTTTCCCAAGGATAC. The Dbr4 locus alone is specifically amplified when primer 2R is used in combination with primer 3F. The sequence of primer 3F is CGCAATCTAACAAATGAA. Primers 1F and 3F were 5' end-labeled with fluorescein (Operon Technologies). PCR amplifications were performed in 10- μ l volumes containing 3–5 ng genomic DNA, 3.1 mM MgCl₂, 0.8 mM dNTPs, 0.37 units *Taq* polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, and 1.25 μ M each of the fluorescently labeled forward and unlabeled reverse primers. PCR amplifications using primers 1F and 2R consisted of an initial denaturation at 94°C for 1 min followed by 28 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for 30 s; this was followed by a final 15-min extension at 72°C. The PCR profile for amplifications using primers 2R and 3F was the same as above, except that the annealing temperature was 51°C.

The resulting PCR products were analyzed in two ways. The products were first electrophoresed through a nondenaturing 6% polyacrylamide (1:19 bis acrylamide:acrylamide) gel on a Mini-Protean II Dual Slab Cell (Biorad) to determine whether amplification was successful. The gels were stained with SYBR Green (Molecular Probes), and the products were visualized on the FluorImager 575. Product sizes were determined by comparison to a 20/100-bp size standard (Gensura) using ImageQuaNT software, version 4.2a (Molecular Dynamics).

The exact lengths of the PCR products were determined by mixing each reaction with 0.375 μ l of the fluorescein-labeled internal size standard GS500 (ABI). The samples were then heat denatured and electrophoresed through a 24-cm 6% denaturing polyacrylamide gel with 8.3 M urea and were analyzed on the 373A automated DNA sequencer (ABI). The length of each PCR product was determined using the internal size standards and 672 GeneScan software (ABI).

Microsatellite alleles from all 24 *D. brightwellii* isolates were analyzed using primers 1F and 2R. Only 23 isolates were analyzed using primers 2R and 3F because of DNA degradation of one Twanoh isolate.

Microsatellite allele sequence analysis—Primers 1F and 2R were used to amplify the Dbr4 and Dbr9 loci from CCMP 358, and the PCR products were electrophoresed on a 10% nondenaturing polyacrylamide (1:19 bis acrylamide:acrylamide) gel. Each of the three bands was eluted from the gel (Sambrook et al. 1989), ligated into the pZERO-2 vector (Invitrogen), and used to transform TOP10 *E. coli* cells (Invitrogen). Thirty-one cloned alleles were sequenced as above. DNA sequences were aligned using the clustal method on SequenceNavigator software, version 1.0.1 (ABI).

Statistical analysis of microsatellite alleles—For the single-locus data, field isolates were genotyped using primers

2R and 3F, and the number of observed heterozygotes (H_o) was determined. The data from all the field samples were pooled for statistical analyses because of the small sample size. Allele frequencies were used to calculate the gene diversity (H_e) (Weir 1996). Departures from Hardy–Weinberg equilibrium were tested in Genepop, version 3.1d (Raymond and Rousset 1995), using the Hardy–Weinberg exact test (e.g., Haldane 1954; Guo and Thompson 1992). Because more than four alleles existed for the Dbr4 locus, the Markov chain method (Guo and Thompson 1992) was used to obtain an unbiased estimate of the exact probability in the Hardy–Weinberg exact test. A *U*-test (Rousset and Raymond 1995) was performed using Genepop to test for heterozygote deficiency. F_{is} , a measure of the degree of homozygote excess (Weir and Cockerham 1984), was calculated using Genepop.

For the two loci data, the field isolates were genotyped using primers 1F and 2R. H_e (Weir 1996) was calculated using observed allele frequencies, a method commonly employed for multilocus data sets (Lynch and Milligan 1994). The maximum number of possible allele combinations, and thus the maximum number of possible genotypes, was calculated using the binomial coefficient (Zar 1996). For the 2R and 3F primer set, the binomial coefficient was calculated for all possible heterozygote combinations, assuming two possible length alleles per individual. The total number of possible genotypes was the sum of all possible heterozygotes and homozygotes. For the multilocus primer set (1F and 2R), binomial coefficients were calculated assuming that an individual could possess one, two, three, or four different-length alleles.

Growth rate determination—Eight genetically distinct isolates were maintained in semicontinuous batch culture (Brand et al. 1981a) at 14°C on a 16:8 LD cycle of 166, 66, and 33 μ mol photons m⁻² s⁻¹. Isolate 1 originated from Pt. Whitney, isolates 2 and 3 originated from Hoodspout, and isolates 4–8 originated from Twanoh. The isolates were allowed to acclimate to each light intensity for approximately 20 generations (Brand et al. 1981a) prior to the start of growth-rate measurements. The in vivo chlorophyll fluorescence of the exponentially growing cultures was determined daily during the dark interval using a 10-AU fluorometer equipped with the in vivo Chlorophyll Optical Kit (Turner Designs). A minimum of four growth curves were determined for each isolate at each light intensity, and the specific growth rate was calculated by regressing the change of in vivo fluorescence over time. The different growth rates of an isolate at a given light intensity were compared using an *F*-test (three to four slopes) or a *t*-test (two slopes) (Zar 1996). If at least two of the four growth curves for a given isolate at a given light intensity were not statistically different from one another, these curves were used to calculate a mean growth rate for that isolate. The standard error was calculated using all data points from the curves. If the growth rates for a given isolate continued to change over the course of the four growth curves, an additional three to four growth curves were measured. At each light intensity, the variability of growth rates among isolates was estimated by calculating the coefficient of variation (CV) (Zar 1996). An analysis of variance was performed on the mean growth

Table 1. Characteristics of eight microsatellites cloned from *Ditylum brightwellii*.

Microsatellite	Repeat motif	Sequence of cloned microsatellite array*	Length (bp)
Dbr1	GA	(GA) ₈ AAGCAA(GA) ₆ T(GA) ₂ AA(GA) ₃ GTA(GA) ₉ GTGCAA(GA) ₅ (GT) ₅ (GA) ₁₀ AA(GA) ₁₈	152
Dbr2	GA	(GA) ₃₀ (A) ₅ GAGT(GA) ₃ AA(GA) ₅ AAGA	91
Dbr3	GA	(GA) ₆ TGAGCAA(GA) ₂ GTA(GA) ₂₅ (GT) ₃ (GA) ₂ TA(GA) ₂₃ CA(GA) ₁₆ C(GA) ₁₂	193
Dbr4	GT	(GT) ₉ (GC) ₅ AC(GT) ₅ CTGTTTGTCT(GT) ₃ CTGTGC (GT) ₄ CTGTGC(GT) ₅ GC(GT) ₄ (CT(GT) ₂) ₂ CT(GT) ₁₀ (CT(GT) ₂) ₂ CT(GT) ₅ CTGTAT(GT) ₂ ATGTCT(GT) ₂ (CTGTCT(GT) ₂) ₂ TT(GT) ₃ CT(GT) ₂ TT(GT) ₃	216
Dbr5	GA	(GA) ₁₈ GG(GA) ₃ (TCAAGA) ₃ TC(GA) ₁₄ TC(GA) ₁₁	116
Dbr6	GT	(GT) ₁₀ TT(GT) ₃₆	94
Dbr7	GT	((GT) ₃ TT(GT) ₂ CT) ₂ GTCT(GT) ₂ (CTGT) ₂ GTCTGTAT (GT) ₂ ATGTCT(GT) ₅ (CTGTGT) ₂ CT(GT) ₁₀ (CTGTGT) ₃ GTGC(GT) ₅ (GC(GT) ₂ CT(GT) ₃) ₂ CTGTTTGT CT(GT) ₄ GCAC(GC) ₄ (GT) ₉	214
Dbr8	GT	(GT) ₃ GATTCGTGC(GT) ₄ GATTC(GT) ₅ GATTC(GT) ₃ AT GC(GT) ₂ GACTC(GT) ₃ TAAT(GT) ₂ GATTC(GT) ₃ TTGAT TC(GT) ₆ GATTC(GT) ₂ GATTC(GT) ₇ GATTC(GT) ₁₄ GATT (GT) ₄ GATTC(GTGC) ₂ (GT) ₃ (GATTC(GT) ₄) ₂ TTC(GT) ₃ A T(GT) ₃ GATTCGTGCGTGATTC(GT) ₃ GATTC(GT) ₇ TTC (GT) ₃ AT(GT) ₂ TATTC(GT) ₄	310

* Subscripted numbers following parentheses indicate the number of repeats of the enclosed sequence.

rates of all isolates at each light intensity. Significant differences between the mean growth rates for the different isolates were determined using the Tukey multiple comparison test (Zar 1996).

A simulation of the relative proportion of any given isolate over time based on its mean growth rate was constructed using MATLAB, version 5.2 (MathWorks). The simulation began with a population composed of equal proportions of each of the eight isolates. For the duration of the simulation, exponential growth was assumed using the mean growth rate calculated for each isolate at a given light intensity. The simulation was run three separate times using the mean growth rates observed for the isolates at 166, 66, and 33 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Results

Microsatellite characterization—The most commonly observed microsatellite repeat arrays in both plants and mammals are GA and GT dinucleotide repeats (Lagercrantz et al. 1993). Therefore, two probes that recognize GA and GT repeats were used to screen a size-fractionated *D. brightwellii* genomic library. Eight genomic clones were identified that contain unique microsatellites ranging in size from 92 to 317 bp (Table 1). Microsatellites are commonly categorized by the uniformity of the repeat motifs: perfect repeats contain no intervening nonrepetitive sequence, imperfect repeats are interrupted with nonrepeat nucleotides, and compound repeats are composed of different repeat units (Weber 1990). The *D. brightwellii* microsatellites consist of all three array types: Dbr6 is characterized by an almost perfect GT repeat; Dbr2 and Dbr5 are characterized by imperfect GA repeats, and Dbr8 is characterized by imperfect GT repeats; Dbr1 and Dbr3 are characterized by interruptions of nonre-

peat nucleotides and GA/GT compound repeats; and Dbr4 and Dbr7 are characterized by GT/GC compound repeats and interruptions of nonrepeat nucleotides (Table 1).

The use of a size-fractionated genomic library to screen for microsatellites meant that many of the clones (Dbr2, 3, 7, and 8) did not contain enough nonrepetitive flanking DNA to design locus-specific forward and reverse PCR primers. PCR primers were therefore developed for only four microsatellite clones—Dbr1, 4, 5, and 6—and were used to amplify either the recombinant plasmid containing the cloned microsatellite or total genomic DNA isolated from CCMP 358. When the resulting PCR products were electrophoresed on polyacrylamide gels, a characteristic pattern emerged: intensely fluorescent bands were always accompanied by less intense bands that were multiples of 2 bp smaller in length (Fig. 2). The smaller, less intense PCR products result from a common microsatellite analysis artifact known as stutter, hypothesized to result from the imperfect replication of the repeat array by the *Taq* polymerase (Luty et al. 1990). Because of their consistently smaller size and weak intensity, stutter bands do not interfere with the determination of allele sizes, and thus of genotypes. PCR amplification with the primers specific to the Dbr4 locus resulted in the least stutter and the most reproducible amplification products, and this locus was therefore chosen for further study.

***D. brightwellii* microsatellites are polymorphic**—The usefulness of any given microsatellite locus for population studies depends upon the extent of length polymorphism at that locus. To examine whether the Dbr4 locus is characterized by different-length alleles, primers 1F and 2R were used to amplify this locus from the genomic DNA of four CCMP isolates that originated from disparate areas of the world's oceans. Six different-length alleles, ranging from 243 to 281

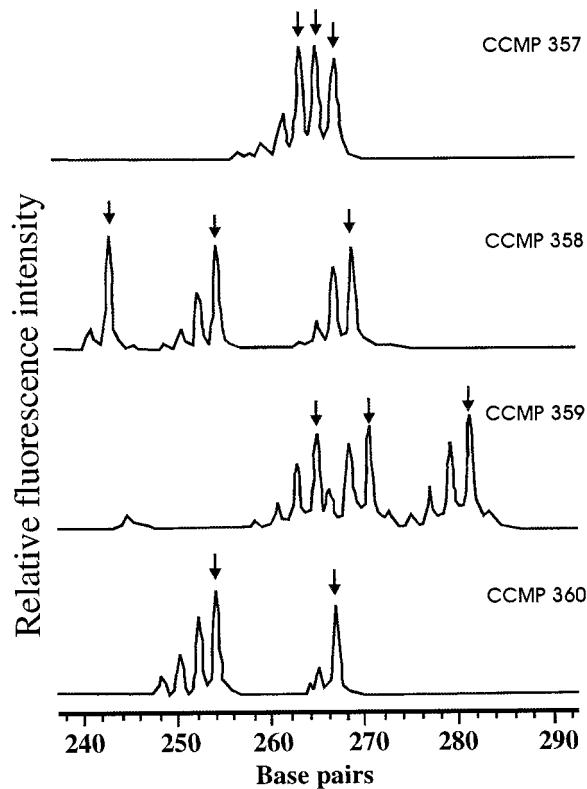


Fig. 2. Electropherograms of microsatellite alleles amplified from four CCMP cultures using primers 1F and 2R. The fluorescent intensity of the PCR products is represented by relative peak heights. Arrows indicate the allele size; the smaller peaks are stutter bands.

bp, were observed, indicating that the Dbr4 locus is polymorphic. Moreover, each of the CCMP isolates displayed a unique pattern of fluorescent products (Fig. 2), confirming that these four isolates originated from four genetically distinct individuals.

Unexpectedly, the PCR amplifications using primers 1F and 2R produced anywhere from two to three dominant bands for each CCMP isolate (Fig. 2). Since diatoms are diploid, a given individual will possess only two alleles (for each locus) that are either the same size (homozygous) or different sizes (heterozygous). Thus, no more than two PCR products per locus should be observed. The presence of more than two bands indicated either that primers 1F and 2R amplified more than the Dbr4 locus or that the Dbr4 locus itself was duplicated in the *D. brightwellii* genome. The three PCR products from CCMP 358 (Fig. 2) were therefore cloned, sequenced, and compared to the DNA sequence of the originally cloned Dbr4 microsatellite (Table 1) to determine the identity of the different PCR products.

The 269-bp band was composed of two different PCR products. The sequences of about one-half of the cloned products were identical to the originally cloned microsatellite both in the flanking region and in the repeat array (Fig. 3A). The sequences of the second 269-bp product differed at a single base pair (A to C at position 11) in the flanking sequence, displayed five insertions and six deletions within

the repeat array, and differed in the number of dinucleotide repeats present in the repetitive regions (Fig. 3A). In contrast to the heterogeneity observed within the 269-bp band, the 254- and 243-bp bands were each composed of products with a single sequence. The flanking sequence of the 254-bp PCR product was identical to that of the originally cloned Dbr4 microsatellite. The repeat array of this fragment was a different length but possessed the same overall motif as the originally cloned microsatellite. In contrast, the flanking sequence of the 243-bp PCR product displayed the same A-to-C point mutation at position 11 as was present in the newly identified 269-bp product. Moreover, the repeat array of the 243-bp product also contained the same insertions and deletions as were found in the newly identified 269-bp product. Thus, it appears that primers 1F and 2R amplify two loci, now defined as Dbr4 and Dbr9, that can be distinguished from one another by the presence of a single point mutation in the flanking sequence and by numerous insertions/deletions within the repeat array (Fig. 3A,B).

Primer 3F was therefore designed to target this single base-pair difference in the flanking sequences (Fig. 3A,B) and thus to amplify only the Dbr4 locus. When this new PCR primer was used in combination with primer 2R, only the alleles from the Dbr4 locus were amplified from each CCMP isolate (Fig. 4). The sizes of the products obtained for CCMP 358 were identical to the sizes predicted from the DNA sequences (Fig. 3A). Three CCMP isolates are heterozygous at this locus: the CCMP 357 alleles are 259 and 263 bp, the CCMP 358 alleles are 249 and 265 bp, and the CCMP 359 alleles are 267 and 277 bp. Only CCMP 360 is homozygous, with a single allele of 249 bp. Thus, analysis of this single locus is sensitive enough to define the four CCMP isolates as genetically distinct from one another. Moreover, the banding patterns obtained for each isolate were highly reproducible—repeated isolations of genomic DNA from the same isolate over the course of many months always resulted in identical banding patterns, regardless of which primer pair was used (data not shown). Primers 3F and 2R will now be referred to as the single-locus primer pair since they specifically amplify the Dbr4 locus; primers 1F and 2R will be referred to as the two-locus primer pair since they amplify both Dbr4 and Dbr9.

Microsatellites permit detection of intraspecific genetic diversity within a population from a single geographical location—To determine whether analysis of two microsatellite loci, Dbr4 and Dbr9, conveys the sensitivity necessary to distinguish between genetically distinct individuals within a single geographical location, *D. brightwellii* cells were isolated on the same day from three locations within the Hood Canal Basin in Puget Sound, Washington (Fig. 1). The DNA from each isolate was then PCR amplified using the two primer pairs. When the single-locus primer pair was used to amplify Dbr4 from 23 field isolates, 10 unique banding patterns, and thus 10 unique genotypes, were detected (Table 2), indicating that analysis of even a single microsatellite locus is sensitive enough to distinguish between genetically distinct individuals within a single population. Eight different-length alleles could be detected that ranged in size from 243 to 271 bp (Table 3). The size of the most common allele

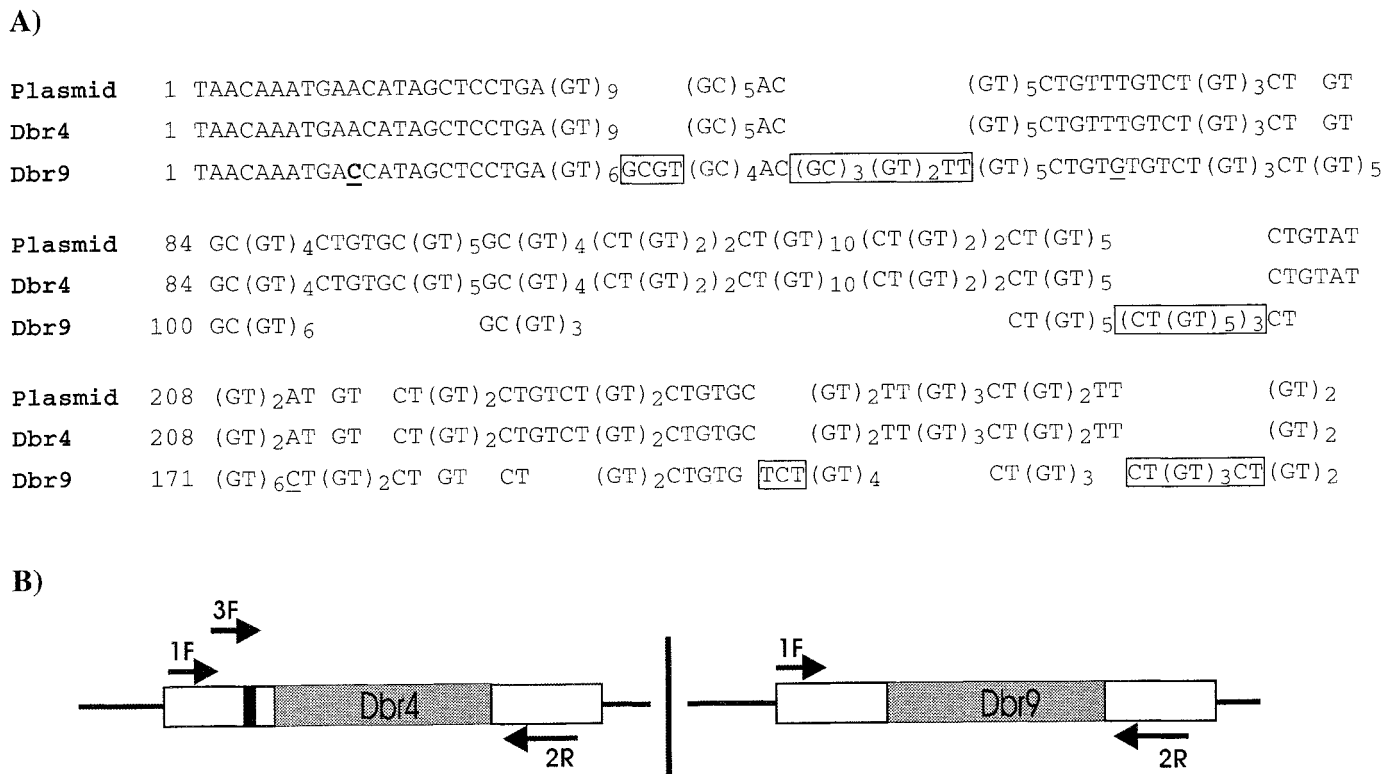


Fig. 3. Identification of two microsatellite loci using PCR primers 1F and 2R. (A) Alignment of flanking and repeat array DNA sequence of the 269-bp product amplified either from the cloned Dbr4 microsatellite (plasmid) or from CCMP 358 (Dbr4 and Dbr9). Numbers to the left of each sequence indicate base number immediately following the 1F primer sequence. Boxed regions indicate insertions, and underlined sequence indicates point mutations relative to the plasmid sequence. The single point mutation in the flanking sequence is underlined and in bold. (B) Schematic of the possible arrangement of the Dbr4 and Dbr9 loci in the *D. brightwellii* genome. A single strand of DNA is represented. The vertical line separating the two loci indicates that the distance separating the two loci is unknown. The repeat region of each locus is shaded. The flanking sequences, represented by white bars, are identical between the two loci, except for a single base-pair difference, which is represented by a darkened bar. The numbered arrows represent the PCR primers, and their placement indicates their relative annealing locations.

was 261 bp, and this allele was present within the population at a frequency of 0.478. Seven of the 23 individuals were heterozygous at this locus, representing an H_o of 0.30. The H_e of this locus was 0.69 (Table 3).

The two-locus primer pair that recognizes both Dbr4 and Dbr9 was used to analyze the field isolates to gain a better

Table 2. Number of *Ditylum brightwellii* field isolates identified for each of the 10 unique genotypes at microsatellite locus Dbr4.

Genotype*		No. of isolates
243	243	1
255	259	1
257	257	1
257	259	1
259	259	1
261	261	9
261	263	3
261	267	1
263	263	4
263	271	1

* Allele lengths in base pairs for each unique genotype.

estimate of the extent of genetic diversity within the population. The two-locus primer pair revealed 18 different alleles that ranged in size from 197 to 294 bp (Table 3). After amplification with this second primer pair, 22 of 24 isolates displayed unique banding patterns and were therefore genetically distinct from one another (Table 3). When the data from the single- and two-locus primer pairs were combined, a total of 23 of the 24 isolates were genetically distinct. The increase of unique genotypes from 10 to 23 illustrates the dramatic increase in sensitivity that resulted from the use of an additional locus. Since this primer pair amplifies two loci, it was not possible to calculate H_o . The H_e for the two loci was 0.88 (Table 3). The two-locus H_e was much larger than the single-locus H_e , indicating that Dbr9 is far more variable than Dbr4.

Hood Canal field populations display high levels of physiological diversity—To determine whether the extensive genetic variation observed in the Hood Canal population reflected comparable levels of physiological diversity, the growth rates of eight genetically distinct isolates were measured at three different light intensities. As expected, the growth rate of each isolate increased with increasing light

Table 3. Summary of population diversity data for the *Ditylum brightwellii* field samples.

Sample site	No. analyzed		No. unique genotypes			No. alleles		Allele size range		H_e	
	Single	Two*	Single	Two	Total†	Single	Two	Single	Two	Single	Two
Point Whitney	2	2	1	2	2	1	5	261	197–267	n.a.	n.a.
Hoodsport	5	5	2	5	5	2	8	261–263	220–273	n.a.	n.a.
Twanoh	16	17	10	17	17	8	17	243–271	243–294	n.a.	n.a.
All Hood Canal	23	24	10	22	23	8	18	243–271	197–293	0.69	0.88

* Single, single locus amplified with primers 3F and 2R; two, two loci amplified with primers 1F and 2R.

† Number unique genotypes achieved by combining single- and two-locus data sets.

‡ n.a., not applicable because of small sample size.

intensity (Fig. 5). At any given light intensity, however, the different isolates displayed a range of mean growth rates. At $166 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, the mean growth rate of isolates 2 and 8 was significantly slower than that of the rest of the isolates ($P < 0.01$). These two isolates grew at 1.09 d^{-1} , and the rest of the isolates grew at 1.37 d^{-1} , a difference of about 20%. At $66 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, isolate 1 grew the fastest, with a mean growth rate of 0.97 d^{-1} , significantly faster than the slowest growing isolate, isolate 2, with a mean growth rate of 0.72 d^{-1} ($P < 0.001$), a difference of about 26%. The other six isolates at this light intensity displayed a statistically indistinguishable continuum of growth rates between these two extremes. At $33 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, three significantly distinct growth rates were apparent ($P < 0.01$). Isolates 1, 6, and 8 grew the fastest, with a mean growth rate of 0.46 d^{-1} . Isolates 3, 4, and 7 grew at an intermediate rate of 0.38 d^{-1} , and isolates 2 and 5 grew most slowly, with

a mean growth rate of 0.31 d^{-1} . The two extreme growth rates at this light intensity differed from one another by as much as 33%. The CV ranged from 10.45% at $166 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ to 15.21% at $33 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Interestingly, the rates at which the isolates grew relative to one another at one light intensity did not predict how the isolates would grow relative to one another at a different light intensity. This indicates that the observed physiological variation is not simply the result of differences in cell diameter among the isolates (see, for example, Paasche 1973) but rather has a genetic basis. Furthermore, isolates originating from a single location within Hood Canal displayed significantly different growth rates from one another at a given light intensity. The only consistent feature among the isolates was that isolate 2 grew at the slowest rate for each light intensity (Fig. 5).

Since diatoms can reproduce rapidly via asexual reproduction, under stable environmental conditions the clone with the fastest growth rate in a population is expected to become numerically dominant in a relatively short period of time. To determine the amount of time during which an initially diverse population could be maintained, a simulation

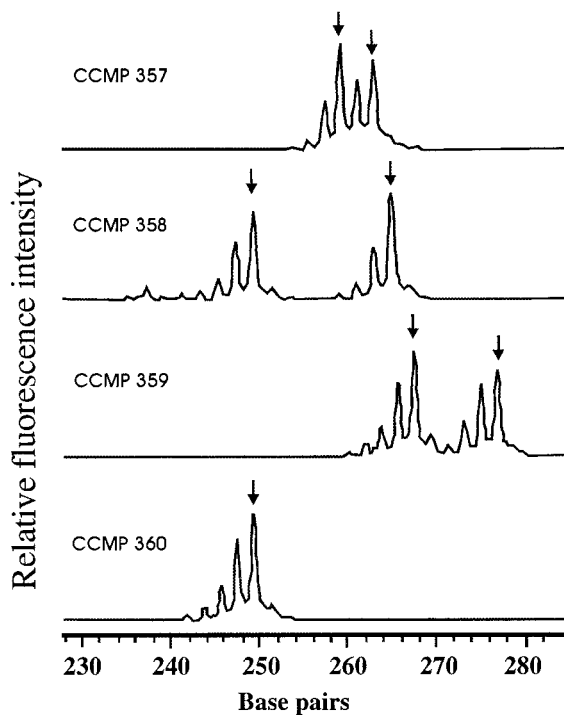


Fig. 4. Electropherograms of Dbr4 alleles amplified using primers 2R and 3F. The fluorescent intensity of the PCR products is represented by the relative peak heights. Arrows indicate the allele size; the smaller peaks are stutter bands.

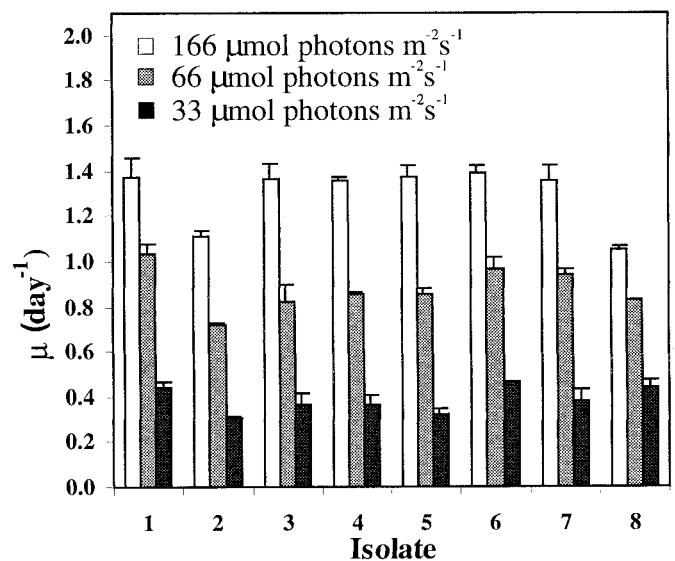


Fig. 5. Maximum growth rates (μ) of genetically distinct isolates from Hood Canal, maintained at 166 , 66 , or $33 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Error bars represent the standard error of the mean growth rate.

was performed that assumed that the eight genetically distinct *D. brightwellii* isolates were initially present within a population in equal proportions and that each was growing exponentially at its mean growth rate (Fig. 5). For each light intensity, an immediate change in population composition was observed within the first few days (Fig. 6). As expected, the isolate with the fastest growth rate did come to numerically dominate the population, but the speed at which this occurred varied depending on the light intensity examined. Surprisingly, the amount of time necessary for one isolate to represent 50% of the population was not correlated with light intensity. For example, at $66 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, the fastest growing isolate represented 50% of the population within 15 d. At $166 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, the highest light intensity, it took 69 d for the fastest growing isolate to represent 50% of the population.

Discussion

Extensive genetic and phenotypic diversity exists within a D. brightwellii population from a stratified environment—We have shown that analysis of just two microsatellite loci can provide the analytic sensitivity necessary to distinguish between individual diatom clones within a field population sampled at a single time. This level of sensitivity far surpasses that provided by other commonly utilized DNA-based markers, such as ribosomal DNA sequences (Medlin et al. 1991; Zechman et al. 1994) or restriction fragment-length polymorphism patterns (Stabile et al. 1992). For example, eight alleles were observed for the Dbr4 locus, which means that this locus provides the sensitivity to detect 36 possible genotypes. The two-locus data set contained 18 different alleles, which means that as many as 4,065 different genotypes could potentially be resolved. These numbers are minimum estimates, because further sampling would likely reveal even more alleles. Furthermore, in contrast to randomly amplified DNA polymorphism (RAPD) markers, which are increasingly being used in phytoplankton studies (Medlin et al. 1996; Lewis et al. 1997; Bolch et al. 1999a,b), the determination of microsatellite allele lengths is highly reproducible, and the resulting codominant alleles are inherited in a Mendelian fashion (Jarne and Lagoda 1996). Microsatellites thus provide a sensitive tool for measuring the extent of genetic variation present within field populations of diatoms.

The level of diversity detected within the *D. brightwellii* population can be estimated using different methods. One is to count the number of unique genotypes. Twenty-three of the 24 isolates analyzed in this study were genetically distinct. This number is remarkable given the fact that diatoms can clonally reproduce at least once, and sometimes twice, per day to create genetically identical individuals. Only two isolates, which came from different sampling sites, were found that displayed the same banding pattern at the two loci examined (analysis of more loci would be required to confirm that these two isolates are truly clonal). Comparably high levels of genetic diversity have been detected in populations of the freshwater diatom *Fragilaria capucina* (Lewis et al. 1997), which indicates that genetic diversity may be common to both marine and freshwater diatom populations.

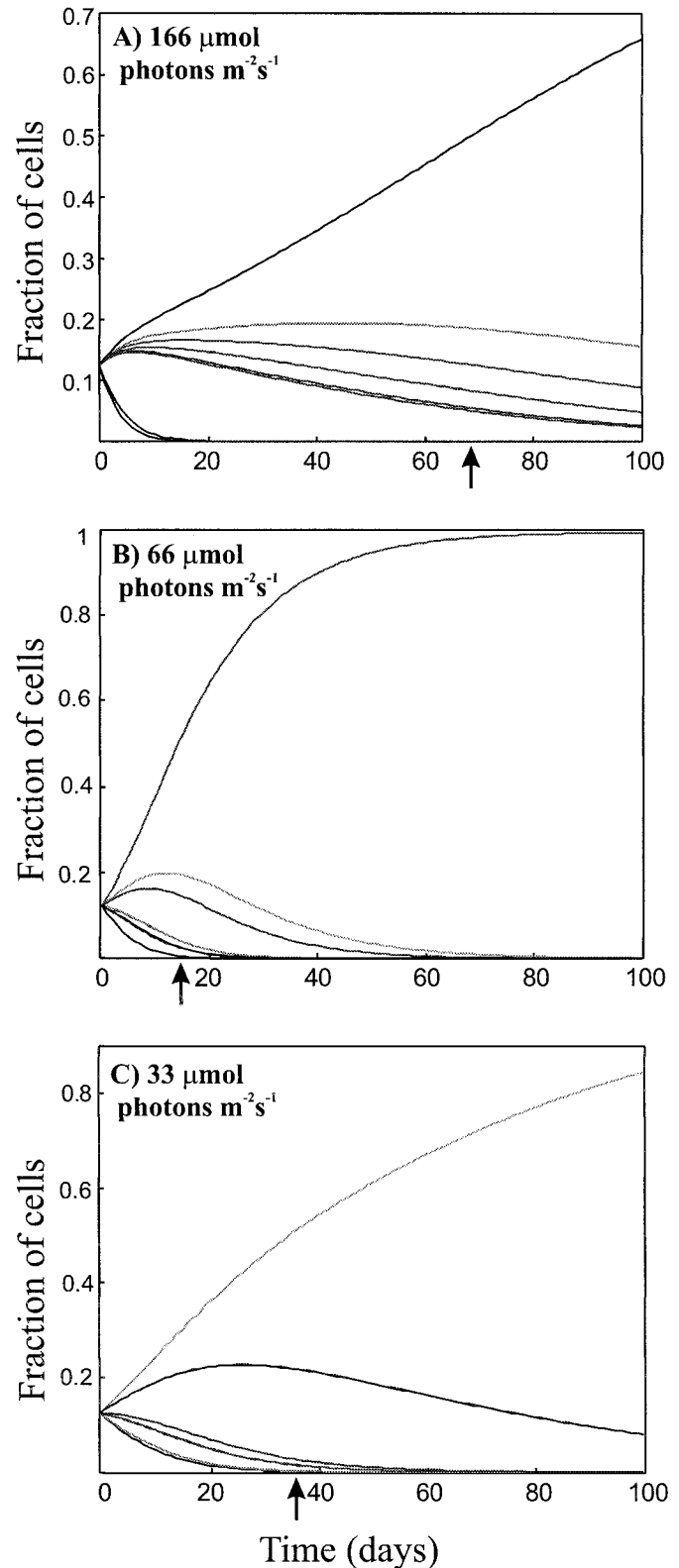


Fig. 6. Simulation of changes in population diversity over time based on the mean growth rates of isolates maintained at (A) $166 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, (B) $66 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, or (C) $33 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Individual curves represent the relative abundance of each isolate. Arrows indicate time when the dominant clone represents 50% of the population.

Table 4. Comparison of gene diversity levels using different variable markers.

Marker type	Organism	Reference	n^*	$H_e \dagger$
Allozyme	<i>Skeletonema costatum</i> (diatom)	Gallagher 1980	457	0.027 ± 0.029
RAPD	<i>Emiliania huxleyi</i> (coccolithophorid)	Medlin et al. 1996	17	0.2429 ± 0.015
Microsatellite	<i>Ditylum brightwellii</i> (diatom)	This study	24	0.88
Microsatellite	<i>Gracilaria gracilis</i> (rhodophyte)	Wattier et al. 1998	71	0.986

* Number of isolates analyzed.

† Standard error of H_e calculated when >1 locus analyzed.

Furthermore, as has been seen in other diatoms (e.g., Gallagher 1982), physiological studies revealed that the subset of eight isolates examined displayed different relative growth rates both within and between the different light intensities. Thus, the measured genetic diversity reflected an underlying physiological diversity. It appears likely that even greater levels of genetic (and physiological) variation existed within this population. The number of unique genotypes (G) relative to sample size (N), or the $G:N$ ratio, is the probability of detecting new genotypes with increased sampling (Pleasants and Wendel 1989). The $G:N$ ratio for the *D. brightwellii* population is 0.96, indicating that there is a high probability that even more unique genotypes are present within the population. Ultimately, the rate of increase in the number of new genotypes with increased sample size will plateau and eventually begin to decrease, especially during periods of rapid growth, as different clonal lines expand.

This simple quantification of the number of unique genotypes within a diatom population, however, provides no information about the relatedness of the individuals. The estimate of relatedness used here is the level of heterozygosity (H_e) expected for the population given the measured allele frequencies. H_e reflects the extent of diversity at individual loci and is frequently referred to as a gene diversity index (Weir 1996). The H_e for a population of closely related individuals will be near 0, because there should be relatively few alleles within the population, each at a high frequency. In contrast, the H_e for a population of unrelated individuals should be closer to 1, because this population is expected to contain many alleles, each at a low frequency. The overall H_e for the two loci examined in this study was 0.88, which indicates that not only is the *D. brightwellii* population composed of genetically distinct individuals but also that a high proportion of these individuals are unrelated to one another. The H_e calculated here is a conservative estimate of overall relatedness, since increased sample sizes would likely reveal additional alleles in the population. Unlike the $G:N$ ratio, H_e can potentially remain unchanged, even during blooms, if the relative abundance of genotypes does not change.

Such a high value of gene diversity has not been observed before in phytoplankton populations, presumably because of limitations inherent in the previously used techniques (Table 4). For example, Gallagher's now-classic study of genetic diversity within populations of *S. costatum* relied on an analysis of allozymes and thus was able to detect an H_e of only 0.027 ± 0.029 (Gallagher 1980). This low H_e translated into an ability to identify only two dominant genotypes within the populations. In retrospect, detection of only two dominant genotypes was likely a vast underestimate of the true

number of genotypes within these populations. The recent use of RAPD markers by Medlin et al. (1996) to analyze populations of the coccolithophorid *Emiliania huxleyi* provided a much better estimate of diversity within a phytoplankton community. Medlin et al. (1996) also found that each isolate analyzed was genetically distinct. The overall H_e for the population was 0.243 ± 0.015 , an order of magnitude higher than had been previously observed. However, this level of gene diversity in the phytoplankton may actually be relatively low, perhaps because of the limitation of RAPDs, which are able to detect only two allele states—presence or absence—per locus. Thus, RAPDs will likely underestimate the true range of variation at any given locus and consequently the true range of variation within the population.

The gene diversity detected in the *D. brightwellii* population is over three times that detected using RAPDs (Medlin et al. 1996) and is similar in magnitude to the diversity detected in another study that used microsatellites to estimate macroalgal gene diversity (Wattier et al. 1998) (Table 4). The high H_e values confirm that microsatellites provide an extremely sensitive means of detecting variation within diatom populations. Moreover, their use now makes it feasible to monitor changes in the genetic diversity of a population during the intervals of rapid cell division that characterize blooms. Thus, despite the fact that the identification of microsatellite markers can initially be more labor intensive than the identification of RAPD markers, the increased sensitivity, reproducibility, and the ability to identify all alleles within a population helps to explain why microsatellites have become the markers of choice for population studies (Jarne and Lagoda 1996).

Maintenance of genetic diversity within diatom populations—The extremely high level of genetic diversity within the *D. brightwellii* population is even more remarkable given the fact that the diatom life cycle is characterized by asexual reproduction that is only infrequently interrupted by either sexual reproduction (e.g., Jewson 1992a,b) or resting spore formation (reviewed in McQuoid and Hobson 1996). Since accurate measurements of the onset of either of these events in field populations is difficult (e.g., Waite and Harrison 1992; Crawford 1995), it remains unclear how these life cycle stages affect genetic variation within diatoms. We looked for evidence of a recent sexual event by determining whether the *D. brightwellii* population was in Hardy-Weinberg equilibrium. If H_o is not significantly different from H_e , then the population, by definition, conforms to the Hardy-Weinberg principle, by which an idealized population of diploid indi-

viduals is reproducing sexually. Only the single-locus data could be used for this analysis, because H_o could not be calculated for the two-locus data set. The H_o for the single locus is 0.30 and is significantly different from the single-locus H_e of 0.69 ($P < 0.0001$). This departure translates into a 57.3% excess of homozygotes. Although homozygote excess could arise either through the presence of null alleles (Wattier et al. 1998) or through mixing of isolated populations (Wahlund effect) (Hartl and Clark 1997), a more fundamental process may be leading to the extreme departure from Hardy–Weinberg equilibrium. Since Hardy–Weinberg equilibrium is most dependent on the assumption that a population is sexual (Hartl and Clark 1997), it appears likely that the *D. brightwellii* population was reproducing asexually at the time of sampling. In this regard, it is interesting to that *D. brightwellii* has been reported to display asexual (von Stosch 1965) as well as sexual (Steele 1965; Waite and Harrison 1992) cell enlargement. Thus, sexual events may be particularly rare in this species.

Until recently, it was generally assumed that clonally reproducing populations, like the *D. brightwellii* population, would display relatively little genetic or physiological variation (Hughes 1989). These populations were expected to be dominated by only a few rapidly reproducing clones (c.f., Fig. 6). With the advent of DNA-based markers, it appears, however, that phytoplankton populations commonly harbor high levels of diversity. Several recent studies with different groups of phytoplankton have found that each isolate examined was genetically distinct (*Peridinium volzii*, Hayhome et al. 1987; *E. huxleyi*, Medlin et al. 1996; *Gambierdiscus toxicus*, Chinain et al. 1997; *Pseudo-nitzschia pseudodelicatissima*, Skov et al. 1997; and *Gymnodinium catenatum*, Bolch et al. 1999a). In each study, the number of isolates examined was fairly low (<50), which indicates that the extent of clonal diversity within phytoplankton has not yet been fully revealed.

The most commonly evoked mechanism for maintaining diversity within asexual populations is the influx of new genotypes into a population through either immigration (Pleasants and Wendel 1989; Turner et al. 1992) or mutation (Brookfield 1992; Turner et al. 1992). The Hood Canal Basin was chosen as the study site because a shallow sill crosses the entrance to the basin and thus restricts water flow. The Hood Canal Basin is stratified year round, and approximately 277 d are required for water entering the canal to make a complete transit (Cokelet et al. 1991). Thus, immigration due to either horizontal or vertical mixing of water masses is expected to be at a relative minimum (although it is not entirely eliminated) at this site. Mutation undoubtedly plays a role in generating the diversity within microsatellite allele lengths (Schlötterer and Tautz 1992). However, it is unlikely that mutation alone could lead to such wide variations in the observed growth rates of the different isolates.

Theoretical (e.g., Tilman 1982) and recent experimental evidence (Bell 1997) indicates that genetic and physiological diversity will be maintained within asexually reproducing populations if the environment is heterogeneous over either temporal or spatial scales. This environmental variability is hypothesized to prevent prolonged directional selection (Bell 1997). Even within a relatively stable body of water like

Hood Canal, the environment experienced by individual diatom cells will be in constant flux as the cells move passively through the water column. The most likely explanation for the observed diversity within Hood Canal, therefore, is that selection pressure for particular phenotypes (and thus particular genotypes) constantly changes. For example, the relative growth rates of the *D. brightwellii* isolates at one light intensity appeared unrelated to the relative growth rates of these same isolates at a different light intensity. Thus, as these cells are mixed vertically through the water column, the relative success of any given clone will constantly shift. It remains unclear whether the relatively small-scale heterogeneity expected to exist within Hood Canal results in a maximum level of diversity or whether even higher levels of diversity would be observed in populations from more strongly mixed environments. Moreover, we are currently examining whether the directional selection pressures that presumably exist during the spring bloom are of sufficient strength to result in the dominance of a relatively few fast-growing clones.

Ecological implications of genetic variation within diatom populations—The high levels of genetic and physiological diversity observed within the *D. brightwellii* population may help to explain how diatoms are able to thrive in highly dynamic environments. First, the presence of a broad range of physiological attributes within a species means that at least a portion of the population will likely be suited to changing environmental conditions. For example, the presence of different ecotypes within *Prochlorococcus* populations is hypothesized to explain the broad depth distribution of these organisms (Moore et al. 1998). Moreover, unless an individual genotype is completely eliminated from a population, even clones that are initially rare can become numerically important as the environment changes. In a sense, then, diatom populations may have a vast genetic and phenotypic reservoir that allows a continual shifting in population structure in response to environmental conditions. Such a reservoir may help explain the cosmopolitan distribution of many diatom species (Round et al. 1990).

The constant realignment of the genetic composition of a population may also explain why diatom bloom dynamics remain so unpredictable despite years of study. Diatom community dynamics likely reflect a complex interaction between changing environmental conditions and the extent of genetic and behavioral diversity present within and between individual species. Thus, over short time intervals (within a season), the response of an individual species may be defined by the relative proportion of a few genotypes (and thus phenotypes) that are best suited to the conditions present at any given time. Over the longer time frame (interannually), the response of a species may instead be defined by the entire suite of genotypes present within a given body of water. Thus, predictions of bloom dynamics will likely require a greater understanding not only of the dynamics of individual species within a community but also of the behavior of different genotypes within a species.

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